Cholesterol Chlorohydrin Synthesis by the Myeloperoxidase–Hydrogen Peroxide–Chloride System: Potential Markers for Lipoproteins Oxidatively Damaged by Phagocytes†

Jay W. Heinecke,*,‖,‡ Wei Li,‖ Dianne M. Mueller,‖ Alan Bohrer,‖ and John Turk‖,§

Department of Medicine, Department of Molecular Biology and Pharmacology, and Mass Spectrometry Resource, Washington University School of Medicine, St. Louis, Missouri 63110

Received March 14, 1994; Revised Manuscript Received June 8, 1994®

ABSTRACT: Myeloperoxidase, a heme protein secreted by activated phagocytes, uses hydrogen peroxide to produce potent cytotoxins. One important substrate is chloride, which is converted to hypochlorous acid (HOCl). This diffusible oxidant plays a critical role in the destruction of invading pathogens. Under pathological conditions, HOCl may also injure normal tissue. Recent studies have shown that myeloperoxidase is a component of human atherosclerotic lesions. Because oxidized lipoproteins may play a central role in atherogenesis, we have explored the possibility that cholesterol is a target for damage by myeloperoxidase. Three major classes of sterol oxidation products were apparent when cholesterol–phosphatidylcholine multilamellar vesicles which had been exposed to a myeloperoxidase–hydrogen peroxide–chloride system were subsequently analyzed by normal-phase thin layer chromatography. The products were identified by gas chromatography–mass spectrometry as cholesterol α- and β-chlorohydrins (6β-chlorocholestane-3β,5α-diol and 5α-chlorocholestane-3β,6β-diol), cholesterol α- and β-epoxides (cholesterol 5α,6α-epoxide and cholesterol 5β,6β-epoxide), and a novel cholesterol chlorohydrin. Conversion of cholesterol to the oxidation products required active myeloperoxidase, hydrogen peroxide, and halide and could be blocked by catalase or by scavengers of HOCl. Moreover, in the absence of the enzymatic system, reagent HOCl generated the same distribution of products. These results indicate that myeloperoxidase can convert cholesterol to chlorohdyrins and epoxides by a reaction involving HOCl. Other oxygenated sterols are cytotoxic and mutagenic and are potent regulators of cholesterol homeostasis in cultured mammalian cells. Cholesterol chlorohydrins might similarly mediate powerful biological effects in the artery wall. Because chlorohydrins are stable under our experimental conditions, chlorinated sterols may prove useful as markers for lipoproteins oxidatively damaged by activated phagocytes.

Phagocytic white blood cells employ the myeloperoxidase–hydrogen peroxide (H_2O_2)[1] system to kill invading bacteria, viruses, and tumor cells (Klebanoff & Clark, 1978; Klebanoff, 1980; Nauseef, 1988; Weiss, 1989; Segal, 1989). Myeloperoxidase reacts with H_2O_2 to form a ferryl π-cation radical complex that is reduced to the native state by the oxidation of halide and other substrates (Hurst & Barette, 1989). The best-characterized product of this system is hypochlorous acid (HOCl; Harrison & Schultz, 1976).

$$H_2O_2 + Cl^- + H^+ \rightarrow HOCl + H_2O$$

This potent cytotoxin chlorinates protein amines, inactivates sulfhydryl groups, and oxidatively bleaches heme groups and iron–sulfur centers (Zgliczynski et al., 1968; Zgliczynski et al., 1971; Pereira et al., 1973; Stelmaszynska & Zgliczynski, 1978; Albrich et al., 1981; Thomas et al., 1982). Another substrate for myeloperoxidase is tyrosine, which is converted to the tyrosyl radical by removal of a hydrogen atom (Heinecke et al., 1993a,b). Tyrosyl radical reacts with protein-bound tyrosyl residues to yield dityrosine (Heinecke et al., 1993b; Francis et al., 1993). The crosslinking reaction takes place at plasma concentrations of chloride (Cl^-) and tyrosine, suggesting that it may act as a marker for proteins oxidatively modified by phagocytes. Myeloperoxidase may generate other oxidizing species, including singlet oxygen (Steinbeck et al., 1992) and hydroxyl radical (Ramos et al., 1992), but a physiological role for these species is not yet established.

Reactive intermediates produced by activated phagocytes may injure normal tissue under pathological conditions, and such species have been implicated in reperfusion injury, aging, and atherogenesis (Klebanoff, 1980; Weiss, 1989; Steinberg et al., 1989; Stadtman, 1992). The mechanisms for cellular damage are poorly understood because the toxic intermediates are short-lived and difficult to measure directly. An alternative approach is to detect stable end products of such oxidizing reactions. Chlorinated compounds represent attractive candidates for monitoring phagocyte-mediated tissue damage because myeloperoxidase (Harrison & Schultz, 1976) and

† Gas chromatography–mass spectrometry experiments were performed at the Washington University School of Medicine Mass Spectrometry Resource (supported by National Institutes of Health Grant RR00954). This research was supported by a Grant-in-Aid from the American Heart Association and award R01 HD29920 from the National Institutes of Health. J.W.H. is an Established Investigator of the American Heart Association.

* Address correspondence to this author at the Division of Atherosclerosis, Nutrition and Lipid Research, Box 8046, 660 South Euclid Ave., St. Louis, MO 63110. Tel (314)-362-6923; Fax (314)-362-3513.

‖ Department of Medicine.

‡ Department of Molecular Biology and Pharmacology.

§ Mass Spectrometry Resource.

® Abstract published in *Advance ACS Abstracts,* August 1, 1994.

[1] Abbreviations: HOCl, hypochlorous acid; H_2O_2, hydrogen peroxide; Cl^-, chloride; cholesterol α-chlorohydrin, 6β-chlorocholestane-3β,5α-diol; cholesterol β-chlorohydrin, 5α-chlorocholestane-3β,6β-diol; cholesterol α-epoxide, cholesterol 5α,6α-epoxide; cholesterol β-epoxide, cholesterol 5β,6β-epoxide; GC, gas chromatography; MS, mass spectrometry; TLC, thin layer chromatography; BSTFA, *N,O*-bis(trimethylsilyl)trifluoroacetamide; HFB, heptafluorobutyric anhydride derivative; TMS, trimethylsilyl derivative; TMSOH, trimethylsilanol; LDL, low-density lipoprotein.

0006-2960/94/0433-10127$04.50/0

eosinophil peroxidase (Weaver et al., 1981) are the only human enzymes known to produce HOCl. The protein products generated by HOCl, however, are either nonspecific (Albrich et al., 1981) or rapidly decompose to yield uninformative compounds (Zgliczynski et al., 1971; Pereira et al., 1973; Stelmaszynska & Zgliczynski, 1978). HOCl also reacts with unsaturated fatty acids and phospholipids to form fatty acyl chlorohydrins (Winterbourn et al., 1992). These electrophilic addition products are apparently stable and may therefore represent specific markers for myeloperoxidase-mediated injury. Activated neutrophils lyse phospholipid liposomes by a process that requires both halide and myeloperoxidase (Sepe & Clark, 1985a,b), raising the possibility that polar chlorohydrins can disrupt membrane structure (Winterbourn et al., 1992). Another target for damage may be cholesterol, which has a double bond in its steroid nucleus and is a major component of plasma membranes and circulating lipoproteins. Indeed, oxidized lipoproteins are thought to play a pivotal role in atherogenesis (Steinberg et al., 1989; Zhang et al., 1993), and oxygenated sterols have been isolated from vascular lesions of hypercholesterolemic animals (Hodis et al., 1992). Recent studies have demonstrated that myeloperoxidase is a component of human atherosclerotic tissue (Daugherty et al., 1994). The patterns of immunostaining in vascular lesions for the enzyme and protein-bound lipid oxidation products are remarkably similar, raising the possibility that myeloperoxidase is a physiological catalyst for lipoprotein oxidation in vivo.

In the current studies we explore the ability of myeloperoxidase-generated HOCl to react with cholesterol. We have characterized the reaction and demonstrate that it forms chlorohydrins, one of which appears to be a novel compound.

EXPERIMENTAL PROCEDURES

Materials

Fisher Chemical supplied H_2O_2 (30%, ACS grade), sodium phosphate, and diethyl ether. Sterols, diethylenetriamine pentaacetic acid (twice recrystallized), superoxide dismutase, heptane (HPLC grade), and 2-propanol (HPLC grade) were obtained from Sigma Chemical Co. [^{14}C]Cholesterol was provided by Du Pont NEN Research Products. Chelex 100 resin and catalase were from Bio-Rad and Boehringer Mannheim, respectively. Other reagents were from the indicated sources (Heinecke et al., 1993a,b).

Methods

Isolation of Myeloperoxidase (Donor: Hydrogen Peroxide, Oxidoreductase, EC 1.11.1.7). The starting material was Nonidet P-40 treated leukocytes obtained by leukopheresis from a patient with chronic myelogenous leukemia (Hickstein et al., 1987). Myeloperoxidase was extracted from the leukocyte pellet with cetyltrimethylammonium bromide and subjected to lectin-affinity chromatography, ammonium sulfate precipitation, and gel filtration chromatography (Rakita et al., 1990). Myeloperoxidase prepared by this method had an A_{430}/A_{280} ratio of 0.71 (Heinecke et al., 1993a). Enzyme concentration was determined spectrophotometrically (E_{430} = 170 mM^{-1} cm^{-1}; Morita et al., 1986).

Oxidation of Multilamellar Lipid Vesicles by Myeloperoxidase. Reactions were carried out in buffer A (140 mM NaCl, 20 mM sodium citrate, 100 μM diethylenetriamine pentaacetic acid, pH 4.5). For experiments with chloride-free buffer A, sodium citrate was substituted for NaCl in buffer A. Buffer A was prepared with glass-distilled deionized water and passed over Chelex 100 resin to remove transition metal ions potentially able to catalyze cholesterol oxidation. The reaction mixture contained a final concentration of 400 nM myeloperoxidase, 0.45 mM [^{14}C]cholesterol, 1.05 mM dipalmitoyl phosphatidylcholine (Avanti Polar Lipids) and 600 μM H_2O_2 in a final volume of 40 μL. After a 30-min incubation at 37 °C in air, the reaction mixture was subjected to analysis. Multilamellar lipid vesicles (cholesterol:phospholipid, 3:7, mol:mol) were prepared using the method of Sepe and Clark (1985a) in modified form. Lipids in ethanol were added to a borosilicate glass test tube, and the solvent was evaporated under N_2. Following the addition of buffer A, the test tube was briefly vortexed, subjected to bath sonication (Branson 1200) under air at room temperature for 5 min, and used immediately. The concentration of H_2O_2 was determined spectrophotometrically (E_{240} = 39.4 M^{-1} cm^{-1}; Nelson & Kiesow, 1972).

Normal-Phase Thin Layer Chromatography. Reaction mixtures were dried under N_2 and extracted twice with 2-propanol, and the organic phases were combined and concentrated to dryness under N_2. The residue was redissolved in 2-propanol and analyzed by thin layer chromatography (TLC) on silicic acid (Whatman; silica gel 60A linear-K pre absorbent strips; 250-μm layer) with heptane:diethyl ether:acetic acid (30:70:2; v:v:v). Reaction products were quantified by phosphor imaging (Molecular Dynamics Phosphorimager) under conditions where radioactivity was a linear function of [^{14}C]cholesterol.

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 (Research Plus Inc., Bayonne, NJ) with concentrated HCl as described (Maerker et al., 1988) except that buffer (100 mM sodium phosphate, pH 7) was added to the reaction mixture (buffer:reaction mixture, 5:14, v:v) prior to neutralization.

Derivatization Reactions. Trimethylsilyl (TMS) ether derivatives of hydroxylated compounds were formed with excess *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA, Pierce, Rockford, IL) in pyridine (1:1; v:v) at 60 °C for 15 min as described (Turk et al., 1983). Heptafluorobutyrate (HFB) derivatives of hydroxylated compounds were formed with excess heptafluorobutyric anhydride (Pierce) in acetonitrile (1:3; v:v) for 1 h at room temperature as described (Heinecke et al., 1993a). Products of derivatization reactions were concentrated to dryness under N_2 and reconstituted in heptane for analysis. Base-catalyzed dehydrohalogenation of chlorohydrin compounds to the corresponding epoxides was performed by treating the compounds with NaOH in tetrahydrofuran (Turk et al., 1983).

Gas Chromatography–Mass Spectrometry. Aliquots of the reaction products were analyzed on an 8-m capillary column (Hewlett-Packard Ultraperformance, 0.17-μm dimethylsilicone film, i.d. 0.31 mm) by gas chromatography–mass spectrometry (GC–MS; Hewlett-Packard 5988) in methane (source pressure 1 Torr) in either the positive-ion or negative-ion chemical ionization mode (source temperature 200 °C) as previously described (Ramanadham, 1993a,b). The injector and interface temperatures were both 280 °C. The initial GC oven temperature was 85 °C for 0.5 min, followed by a 20 °C/min increasing ramp to 300 °C. Under these GC conditions, standard saturated fatty acid methyl esters exhibited the following retention times: C14:0, 2.88 min; C16:0, 3.88 min; C18:0, 4.80 min; C20:0, 5.66 min; C22:0, 6.45 min; C24:0, 7.19 min; C26:0, 7.88 min; C28:0, 8.52 min; C30:0, 9.13 min; C31:0, 9.53 min.

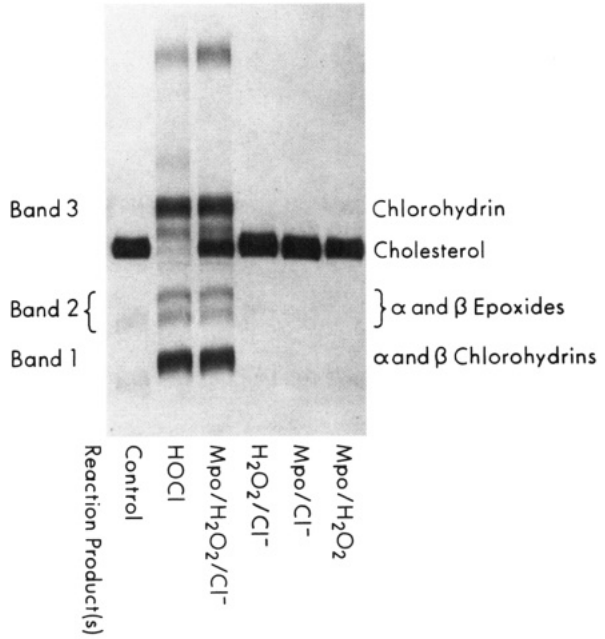

FIGURE 1: Normal-phase TLC analysis of [^{14}C]cholesterol oxidation products formed by HOCl and the myeloperoxidase–H_2O_2–Cl^- system. The complete system (Mpo/H_2O_2/Cl^-) consisted of 400 nM myeloperoxidase (Mpo), 0.45 mM [^{14}C]cholesterol, 1.05 mM dipalmitoyl phosphatidylcholine, and 0.6 mM H_2O_2 in buffer A (140 mM NaCl, 20 mM sodium citrate, 100 μM diethylenetriamine pentaacetic acid, pH 4.5). Where indicated, either HOCl (0.6 mM) was substituted for the Mpo/H_2O/Cl^- system (HOCl) or enzyme (H_2O_2/Cl^-), oxidant (Mpo/Cl^-) or halide (Mpo/H_2O_2) was omitted from the reaction mixture. After a 30-min incubation at 37 °C, the reaction mixture was subjected to TLC on silicic acid using heptane:diethyl ether:acetic acid (30:70:2, v:v:v) as described in the Methods. Radiolabeled sterols were detected by autoradiography.

Standard cholesterol adducts were found to yield much more informative mass spectra in positive-ion and negative-ion chemical ionization modes because of the paucity of high mass ions, including molecular ions, observed in electron impact mass spectra. Similar experience has previously been reported with other lipid epoxides and halohydrins (Turk et al., 1983; Winterbourn et al., 1992). Mass spectra of standard cholesterol α- and β-chlorohydrins and cholesterol α- and β-epoxides are presented in the supplementary material.

RESULTS

Synthesis of Cholesterol Oxidation Products by Myeloperoxidase. Three major radiolabeled reaction products (termed bands 1–3) were apparent by autoradiography when [^{14}C]cholesterol–phosphatidylcholine multilamellar vesicles were oxidized by the complete myeloperoxidase–H_2O_2–Cl^- system and subjected to analysis by normal-phase TLC on silicic acid (Figure 1). The principal products were found in band 1 and band 3 which each constituted ~40% of the oxidized sterol. The yield of the various oxidation products was little affected by the incorporation of either positively (sterylamine) or negatively (dicetyl phosphate) charged lipids into the vesicles (Sepe & Clark, 1985a). The reaction required the complete myeloperoxidase system: when either enzyme, H_2O_2, or Cl^- was omitted from the reaction, little sterol oxidation was apparent (Figure 1). When [^{14}C]cholesterol was treated with reagent HOCl alone, the product yield was essentially identical to that observed with the myeloperoxidase system (Figure 1), strongly implicating HOCl in the reaction pathway.

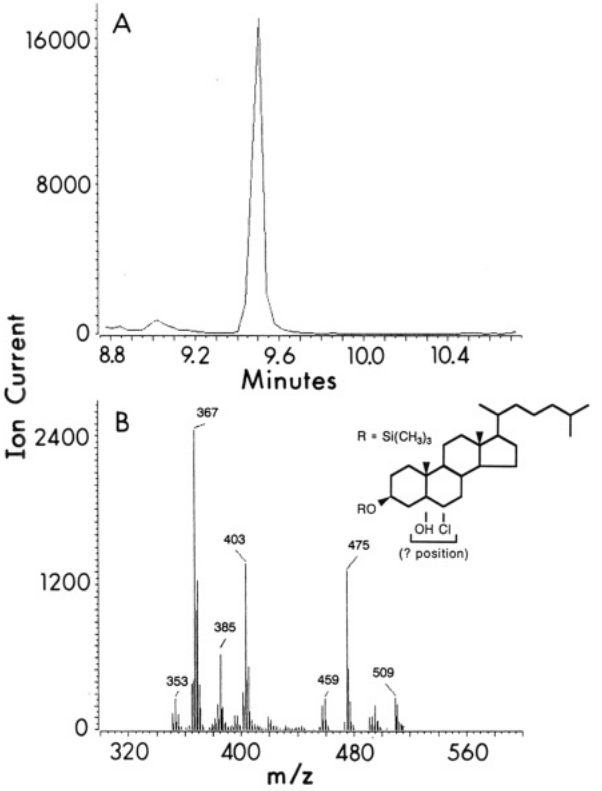

FIGURE 2: Total ion chromatogram (A) and mass spectrum (B) of the mono-TMS derivative of cholesterol chlorohydrin(s) in TLC band 3. Cholesterol–phospholipid vesicles were oxidized with the complete-myeloperoxidase–H_2O_2–Cl^- system. The reaction product(s) in band 3 was isolated by TLC, derivatized with BSTFA, and analyzed by positive-ion chemical ionization GC–MS as described in the Methods. The mass spectrum of the trimethylsilylated band 3 sterol (B) strongly resembles that of the mono-TMS derivatives of α- and β-chlorohydrins (Appendix, Figure 8A). Ions observed in the mass spectrum consistent with a mono-TMS derivative of cholesterol chlorohydrin (B, inset) include m/z 509 ($M^+ - 1$), 475 ($M^+ - Cl$), 459 ($M^+ - 1 - CH_3 - Cl$), 403 ($M^+ + 1 - TMSOH - H_2O$), 385 ($M^+ - TMSOH - Cl$), and 367 ($M^+ - TMSOH - Cl - H_2O$).

Formation of Cholesterol α- and β-Chlorohydrins and α- and β-Epoxides. To determine whether the myeloperoxidase–H_2O_2–Cl^- system converted cholesterol into chlorohydrins, we first established the GC retention times and mass spectra of authentic cholesterol α- and β-chlorohydrins and then analyzed the oxidation products in bands 1–3 by derivatization and GC–MS. This approach demonstrated that the material in band 1 is a mixture of cholesterol α- and β-chlorohydrins (Appendix, Figures 7–9). The material in band 2 was similarly shown by comparison with authentic standards to be a mixture of cholesterol α- and β-epoxides (Appendix, Figure 10). The fast moving band in Figure 1 that migrated more rapidly than band 3 proved difficult to characterize and was not further investigated.

Formation of a Novel Cholesterol Chlorohydrin. The oxidized sterol(s) in band 3 was a major product of the myeloperoxidase system (Figure 1). When band 3 was isolated by TLC from myeloperoxidase-oxidized [^{14}C]cholesterol–phospholipid vesicles and subjected to trimethysilylation and positive-ion chemical ionization GC–MS, both its GC retention time (Figure 2A) and positive-ion chemical ionization mass spectrum (Figure 2B) were similar to that of the α- and β-chlorohydrins of cholesterol (Appendix, Figure 8). As predicted for a cholesterol chlorohydrin, the negative-ion chemical ionization mass spectrum (Figure 3A) of the band 3 HFB derivative contained prominent ions at m/z 634 (M^- for ^{35}Cl) and 636 (M^- for ^{37}Cl). HFB derivatives generally

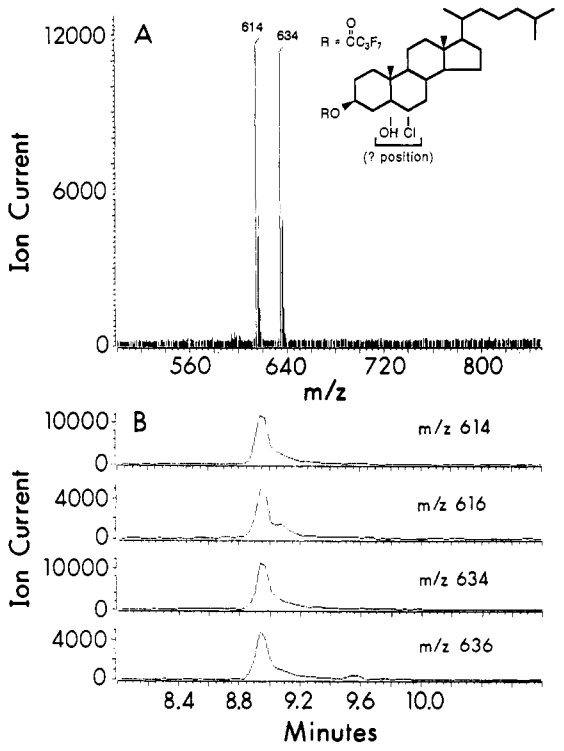

FIGURE 3: Negative-ion chemical ionization mass spectrum of the mono-HFB derivative of the cholesterol chlorohydrin in TLC band 3. The sterol oxidation product(s) in band 3 was isolated by TLC from the complete myeloperoxidase–H_2O_2–Cl^- system, derivatized with heptafluorobutyric anhydride, and analyzed by negative-ion chemical ionization GC–MS. The predicted m/z of a mono-HFB–cholesterol chlorohydrin derivative is 634 (inset). Loss of HF from M^- would yield an ion with m/z 614. The reconstructed ion chromatogram with monitoring at m/z 634 and 636, as well as at m/z 614 and 616, reveals the isotopic ratios expected for a chlorinated compound (B).

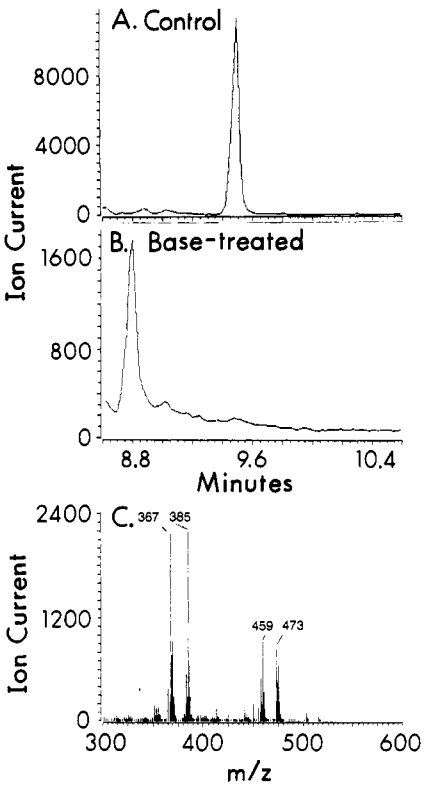

FIGURE 4: Base-catalyzed dehydrohalogenation of the cholesterol chlorohydrin in band 3 to its corresponding epoxide. The sterol oxidation product in band 3 was isolated by TLC and exposed to NaOH as described in the Methods. Control (A) and base-treated (B) TLC band 3 oxidation products were then derivatized with BSTFA and analyzed by positive-ion chemical ionization GC–MS. The mass spectrum of the base-treated band 3 sterol is shown (C). Ions observed in the mass spectrum consistent with the TMS derivative of a cholesterol epoxide include m/z 473 ($M^+ - 1$), 459 ($M^+ - CH_3$), 385 (M^+ – TMSOH), and 367 ($M^+ + 1$ – TMSOH – H_2O). Ions at m/z 503 and 515 represent electrophilic addition products of cholesterol epoxide with $C_2H_5^+$ and $C_3H_5^+$, respectively, as is often observed in methane positive-ion chemical ionization (Harrison, 1983).

fragment with loss of HF; the expected ions were also present at m/z 614 and 616 (Figure 3A). Because the natural isotope abundance of ^{35}Cl to ^{37}Cl is 3:1, monochlorinated compounds characteristically demonstrate an ion cluster at M^- and $M^- + 2$. Reconstructed ion chromatograms confirmed that the ions m/z 636, 634, 616, and 614 all coeluted and exhibited the expected isotope abundance (Figure 3B).

Experiments in which band 3 was treated with base provided additional evidence that a chlorohydrin was present. Under these conditions, vicinal hydroxyl and chlorine groups undergo dehydrohalogenation to the corresponding epoxide (Turk et al., 1983). Exposure of band 3 to NaOH caused the disappearance of the chlorohydrin and the appearance of a material (Figure 4A,B) which exhibited a GC retention time identical to the TMS derivatives of cholesterol epoxides (8.8 min; compare Figure 4B with 10A in the Appendix). The base-treated material also exhibited a positive-ion chemical ionization mass spectrum of the TMS derivatives of a cholesterol epoxide (compare Figure 4C with 10B in Appendix). These results provide direct evidence for the presence of a chlorinated sterol in band 3. This oxidized sterol may represent a diastereomer of the α- and/or β-chlorohydrin or a regionally isomeric chlorohydrin formed after migration of the double bond in cholesterol following hydroxylation and dehydration.

Reaction Conditions for Cholesterol Chlorohydrin Synthesis. We used normal-phase TLC and quantitative phosphor imaging of the α- and β-chlorohydrins in band 1 to establish the optimum reaction conditions for [^{14}C]cholesterol oxidation by myeloperoxidase. Similar results were observed when the reaction was characterized using either the cholesterol epoxides in band 2 or the chlorohydrin(s) in band 3 (data not shown). The addition of myeloperoxidase to buffer A containing cholesterol–phosphatidylcholine liposomes, H_2O_2, and Cl^- led to rapid formation of the chlorohydrins (Figure 5A). Chloramines derived from amino acids rapidly decompose (Zgliczynski et al. 1971). In contrast, the concentration of the cholesterol chlorohydrins remained constant over the next 100 min, indicating that the compounds were stable under these conditions. Chlorohydrin generation required the complete myeloperoxidase–H_2O_2–Cl^- system and was inhibited by catalase, a scavenger of H_2O_2 (Table 1). Heat-inactivation of catalase blocked most of its effects. Aminotriazole, a suicide substrate that irreversibly inactivates the heme protein catalase by derivatizing an active site histidine (Margoliash et al., 1960), and azide, a competitive inhibitor of heme proteins, inhibited chlorohydrin synthesis by myeloperoxidase (Table 1). It is important to note that aminotriazole also reacts with HOCl (Zgliczynski & Stelmaszynska, 1975). These results demonstrate that myeloperoxidase chlorinates cholesterol by a reaction that requires active enzyme, H_2O_2, and Cl^-.

The oxidation of cholesterol to α- and β-chlorohydrins was maximal under acidic conditions (Figure 5B). The bell-shaped pH-dependence profile, with a rapid increase at pH 3–4 and a decrease at neutral pH, together with the known stability

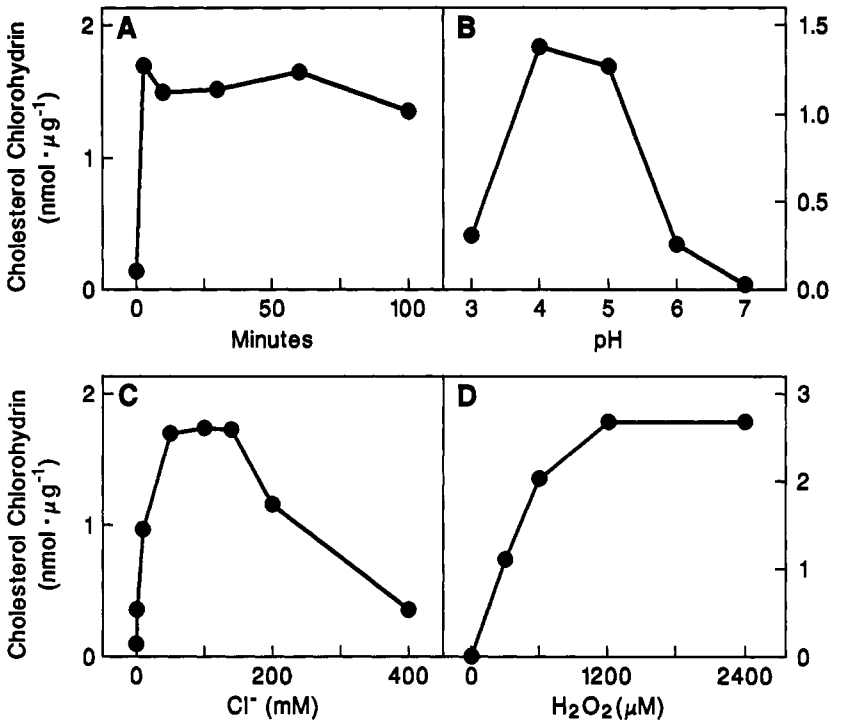

FIGURE 5: Oxidation of [^{14}C]cholesterol by the myeloperoxidase–H_2O_2–Cl^- system. The reaction was initiated by the addition of myeloperoxidase (16 pmol) to 40 μL of buffer A (pH 4.5) containing 0.45 mM [^{14}C]cholesterol, 1.05 mM phosphatidylcholine, 0.6 mM H_2O_2, and 140 mM Cl^-. At the indicated times (A) or after a 30-min incubation (B–D) at 37 °C, the reaction products in band 1 were analyzed by normal-phase TLC and quantified by phosphor imaging as described in the Methods. Conditions were varied by assaying the reaction mixture with the indicated final concentrations of hydrogen ion (B), chloride ion (C), and H_2O_2 (D). Cholesterol oxidation was terminated in the time course experiment by adding 200 nM catalase to the reaction mixture.

Table 1: Requirements of the Myeloperoxidase–H_2O_2–Chloride System for Conversion of [^{14}C]Cholesterol into Chlorohydrins[a]

conditions	[^{14}C]cholesterol chlorohydrins (nmol·μg^{-1})
complete system	2.00
complete system *minus*	
myeloperoxidase	0.06
chloride	0.10
H_2O_2	0.06
complete system *plus*	
azide (20 mM)	0.17
aminotriazole (50 mM)	0.05
catalase (400 nM)	0.05
heat-inactivated catalase	1.70

[a] The complete system consisted of 400 nM myeloperoxidase, 0.45 mM [^{14}C]cholesterol, 1.05 mM dipalmitoyl phosphatidylcholine, and 0.6 mM H_2O_2 in buffer A (140 mM NaCl, 20 mM sodium citrate, 100 μM diethylenetriamine pentaacetic acid, pH 4.5). After a 30-min incubation at 37 °C, the reaction mixture was subjected to analysis by TLC on silicic acid using heptane:diethyl ether:acetic acid (30:70:2, v:v:v) as described in the Methods. Radiolabeled sterols in band 1 were quantified by phosphor imaging. Sodium citrate was substituted for NaCl to prepare chloride-free buffer A. Catalase was heat-inactivated by boiling for 10 min. Values are means of duplicate determinations and are representative of the results found in three independent experiments.

Table 2: Effect of Scavengers of Reactive Oxygen Intermediates on [^{14}C]Cholesterol Chlorohydrin Synthesis by Myeloperoxidase[a]

conditions	[^{14}C]cholesterol chlorohydrin (nmol·μg^{-1})
complete system	1.40
complete system *plus*	
vitamin E (0.6 mM)	0.04
L-methionine (0.6 mM)	0.05
ascorbic acid (0.6 mM)	0.19
L-lysine (0.6 mM)	0.19
glycine (0.6 mM)	0.45
mannitol (10 mM)	0.98
superoxide dismutase (1.6 μM)	1.14

[a] Reactions were carried out as described in the legend to Table 1. Methionine, glycine, lysine, ascorbic acid, and vitamin E were present in the reaction mixture at the same final concentration as H_2O_2.

of myeloperoxidase at low pH (Naskalski, 1977), suggests that enzymatic activity is influenced by ionization of multiple amino acid residues. A similar dependence on pH has been observed previously for HOCl generation by myeloperoxidase (Stelmaszynska & Zgliczynski, 1974; Zgliczynski et al. 1977; Naskalski, 1977). Chlorohydrin synthesis was chloride-dependent; it was optimal at physiological [Cl^-] (50–150 mM) but decreased at higher concentrations of halide (Figure 5C). Previous studies have shown that high concentrations of Cl^- inhibit the enzyme (Stelmaszynska & Zgliczynski, 1974; Zgliczynski et al. 1977). Chlorohydrin synthesis was directly proportional to the concentration of H_2O_2 up to 1.2 mM and then remained constant up to 2.4 mM (Figure 5D).

Scavengers of Reactive Oxygen Species. Vitamin E is a hydrophobic antioxidant that terminates lipid peroxidation by repairing peroxyl radicals (Niki et al., 1984). Incorporation of vitamin E into the [^{14}C]cholesterol–phospholipid liposomes strongly inhibited chlorohydrin synthesis (Table 2). Ascorbate, a water-soluble vitamin which rapidly reacts with HOCl/$^-$OCl (Hu et al., 1993), also inhibited cholesterol oxidation. Methionine, glycine, and lysine scavenge HOCl (Zgliczynski et al., 1971; Stelmaszynska & Zgliczynski, 1978; Weiss et al., 1982; Winterbourn, 1985). Methionine also directly reacts with H_2O_2 (Weiss et al., 1982). Inclusion of methionine (at a concentration equal to that of H_2O_2) in the reaction mixture almost completely blocked the oxidation of cholesterol by myeloperoxidase (Table 2). Glycine and lysine also inhibited myeloperoxidase-mediated chlorination of cholesterol. High concentrations of mannitol (which scavenges hydroxyl radical)

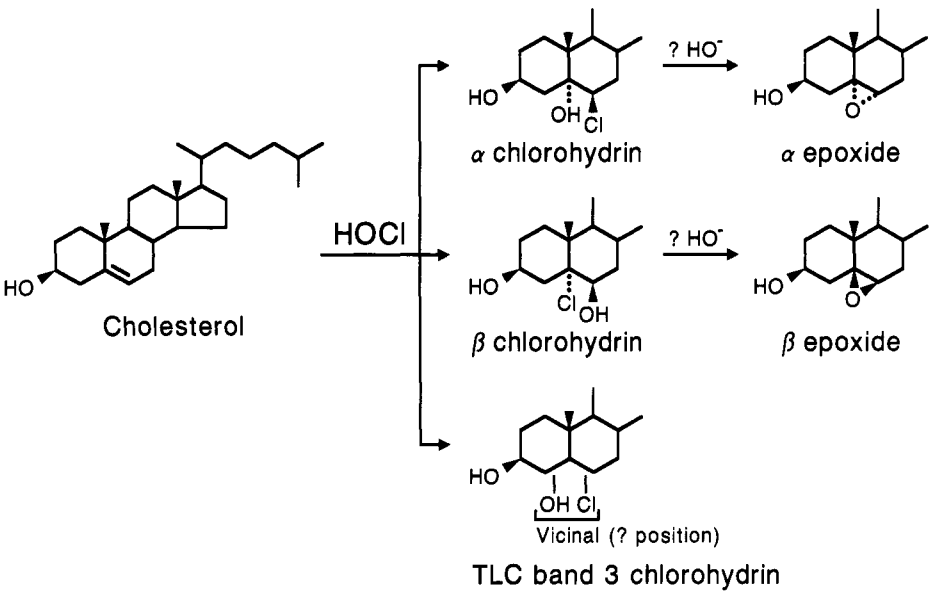

FIGURE 6: Proposed reaction pathway for the synthesis of cholesterol chlorohydrins and epoxides by the myeloperoxidase–H_2O_2–Cl^- system.

and superoxide dismutase exerted very modest inhibitory effects on the reaction. Thus, chlorohydrin synthesis by myeloperoxidase was substantially blocked by compounds known to react with HOCl, as well as by heme protein inhibitors, but was minimally affected by scavengers of hydroxyl radical or superoxide.

DISCUSSION

Several lines of evidence demonstrate that HOCl generated by the myeloperoxidase–H_2O_2–Cl^- system converts cholesterol into chlorohydrins. First, the retention times on gas chromatography, the mass spectra, and the retention factors on silicic acid chromatography of the oxidized sterols in band 1 were indistinguishable from those of authentic cholesterol α- and β-chlorohydrins. Second, the negative-ion chemical ionization mass spectra of the band 1 and band 3 sterols showed the isotopic cluster expected for a chlorinated compound, providing direct evidence for covalent incorporation of chlorine. Third, the optimal reaction conditions for cholesterol chlorohydrin synthesis by myeloperoxidase were virtually identical to those previously reported for HOCl synthesis. Finally, cholesterol chlorohydrin formation was inhibited by HOCl scavengers. These results, taken together, indicate that cholesterol is converted into its chlorohydrins by myeloperoxidase. Van den Berg et al. (1993) recently reported that reagent HOCl fails to chlorinate cholesterol. The failure of these workers to detect chlorohydrins may reflect differences in the reaction conditions or the analytical procedures they employed.[2]

Chlorohydrin synthesis by myeloperoxidase required both Cl^- and H_2O_2, and it was suppressed by catalase, a scavenger of H_2O_2. Other inhibitors included vitamin E, ascorbic acid, and various amino acids which are known to react with HOCl, but superoxide dismutase and the hydroxyl radical scavenger mannitol had little effect. These results strongly implicate a diffusible oxidant in chlorohydrin formation. It is significant that reagent HOCl generated the same family of products as myeloperoxidase, confirming that HOCl was the chlorinating intermediate.

These observations suggest that several factors may be critical in chlorohydrin generation by phagocytes. One influence may be the agonist for leukocyte activation. Phagocytosis is a potent stimulus for the secretion of myeloperoxidase into the phagocytic vacuole, and it results in high local concentrations of the enzyme (Klebanoff & Clark, 1978). The phagolysosome that results from fusion of this vacuole with lysosomes undergoes acidification which provides the optimal conditions for cholesterol chlorohydrin synthesis. Depletion of antioxidants in such an microenvironment might also favor the reaction of HOCl with lipids and proteins.

On the basis of these results and the chemistry of HOCl (Wade, 1978), the reaction pathway for the production of cholesterol chlorohydrins by myeloperoxidase (Figure 6) likely involves Cl^+ derived from HOCl, which electrophilically attacks the double bond in cholesterol to form a chloronium ion. This intermediate then undergoes backsided nucleophilic attack by HO^- to yield the chlorohydrin. Cholesterol epoxides also accounted for a significant portion of the oxidation products. The yield of epoxides in cholesterol exposed to either reagent HOCl or the myeloperoxidase–H_2O_2–Cl^- system was similar, implicating HOCl in the reaction mechanism. A potential pathway for cholesterol epoxide formation involves the base-catalyzed dehydrohalogenation of chlorohydrins, but this reaction should be slow under the acidic conditions used in our experiments. Myeloperoxidase can directly oxygenate butadiene although epoxidation is apparently independent of Cl^- (Duescher & Elfarra, 1992). It is therefore possible that the synthesis of cholesterol epoxide by myeloperoxidase involves reactions other than dehydrohalogenation of chlorohydrins.

Our studies have also revealed a chlorinated sterol that differs in several respects from the α- and β-chlorohydrins of cholesterol. This cholesterol chlorohydrin migrates more rapidly than cholesterol on normal-phase TLC, possibly because the steric properties of the molecule cause it to interact with silicic acid in an anomalous manner. The compound also appeared to be more stable than either the α- or

[2] The investigations of van den Berg et al. (1993) were carried out at neutral pH, whereas we found that chlorohydrin synthesis by myeloperoxidase was optimal at acid pH. Preliminary studies suggest that the reaction of reagent HOCl with cholesterol at pH 7.0 yields 10-fold less chlorohydrins than at pH 4.5. The analytical methods employed by van den Berg et al. may also have resulted in breakdown of the chlorohydrins. Indeed, even under the gentle conditions we used for derivatization, partial conversion of cholesterol chlorohydrins into epoxides and other compounds was observed in our studies.

β-cholesterol chlorohydrin when it was subjected to derivatization and GC analysis in that little evidence of dehydrohalogenation to the corresponding epoxide was observed. Despite these differences, the sterol was clearly a chlorohydrin because its positive-ion and negative-ion chemical ionization mass spectra, its retention times on GC–MS, and its isotopic clusters were quite similar to those of cholesterol α- and β-chlorohydrin. We are currently investigating the structure of this compound, which may represent a diastereoisomer of one of the known cholesterol chlorohydrins. Alternatively, the double bond in cholesterol may have first migrated to a new position by sequential hydroxylation and dehydration and then reacted with HOCl to form the novel chlorohydrin.

Elevated levels of low-density lipoprotein (LDL), the major carrier of blood cholesterol, are an important risk factor for coronary artery disease. Convincing evidence shows that oxidized LDL rather than native LDL triggers the pathological events of atherosclerosis (Steinberg et al., 1989). We have recently shown that myeloperoxidase is expressed in human atherosclerotic lesions where it colocalizes with tissue macrophages (Daugherty et al., 1994). LDL exposed to myeloperoxidase-generated tyrosyl radical undergoes lipid peroxidation (Savenkova et al., 1994), and LDL modified by HOCl promotes cholesteryl ester formation by cultured macrophages (Hazell & Stocker, 1993). Lipoproteins with similar properties have been isolated from atherosclerotic tissue (Daugherty et al., 1988; Yla-Herttula et al., 1989). Oxidatively damaged lipids mediate many of the atherogenic effects of modified LDL (Berliner et al., 1990; Zhang et al., 1993). Oxygenated sterols have cytotoxic and mutagenic properties and are powerful regulators of cholesterol homeostasis (Smith, 1981; Hwang, 1991). It is possible that cholesterol chlorohydrins generated by myeloperoxidase exert potent biological effects in vascular lesions or other sites of inflammation.

The links between lipoprotein oxidation and myeloperoxidase suggest that the enzyme may participate in atherogenesis. Because the only plausible source of HOCl in human atherosclerotic lesions is myeloperoxidase, chlorinated compounds may represent specific markers for lipoproteins damaged by phagocytes. The detection of cholesterol chlorohydrins in vascular lesions would strongly support the hypothesis that myeloperoxidase is one important pathway for the oxidative modification of lipoproteins and therefore potentially a pivotal agent in the development of vascular disease.

ACKNOWLEDGMENT

We thank Dr. L. Sage for critical reading of the manuscript.

APPENDIX

Formation of Cholesterol α- and β-Chlorohydrins. Standard cholesterol α-chlorohydrin treated with the trimethylsilylating reagent BSTFA in pyridine demonstrated early (peak a) and late (peak c) eluting components upon positive-ion chemical ionization GC–MS analysis (Figure 7A). Peak c contained the mono-TMS derivative of cholesterol α-chlorohydrin (supplementary material, Figure 11A). It exhibited a GC retention time of 9.6 min and a positive-ion chemical ionization mass spectrum which contained 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 ($M^+ - TMSOH - Cl - H_2O$). The GC retention time (8.9 min) and positive-ion chemical ionization mass spectrum (data not shown) of the material in peak a (Figure 7A) was identical to that of the trimethysilylated epimeric cholesterol 5,6-epoxides (supplementary material, Figure 13). Halohydrins exposed to base have been shown to undergo dehydrohalogenation to the corresponding epoxide (Turk et al., 1983).

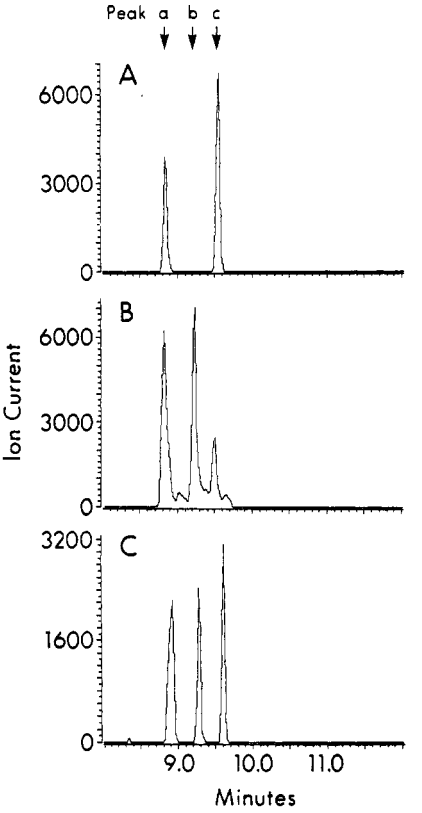

FIGURE 7: Total ion chromatogram of trimethylsilylated cholesterol α-chlorohydrin (A), trimethylsilylated cholesterol β-chlorohydrin (B), and the trimethylsilylated cholesterol oxidation products in TLC band 1 (C). Standard cholesterol α-chlorohydrin (A) and β-chlorohydrin (B) were derivatized with BSTFA and analyzed by positive-ion chemical ionization GC–MS as described in the Methods. Band 1 sterols were isolated by normal-phase TLC from cholesterol–phospholipid vesicles oxidized with the complete-myeloperoxidase–H_2O_2–Cl^- system, derivatized with BSTFA, and analyzed by GC–MS (C). The retention times and mass spectra of the late eluting component (peak c), middle eluting component (peak b), and early eluting component (peak a) in the oxidized band 1 sterols (C) were essentially identical to those of trimethysilylated cholesterol α-cholesterol, the unknown compound found in cholesterol β-chlorohydrin (but not the α-chlorohydrin) treated with BSTFA, and the trimethylsilylated epimeric cholesterol 5,6-epoxides, respectively.

The TMS derivative of standard cholesterol β-chlorohydrin showed three major components (Figure 7B). The GC retention time and positive-ion chemical ionization mass spectrum of the late eluting compound (peak c) was similar to that of trimethylsilylated cholesterol α-chlorohydrin except for greater prominence of the ions at m/z 509 and 511 and a lesser abundance of the ions at 491 and 493. In addition, a prominent ion was observed at m/z 459 ($M^+ - 1 - Cl - CH_3$). The GC retention time and mass spectrum of the early eluting compound (peak a) was identical to that of the trimethysilylated epimeric cholesterol 5,6-epoxides. Thus, both the α- and the β-chlorohydrins appeared to undergo substantial conversion to epoxides upon derivatization with BSTFA in pyridine.

Treatment of authentic cholesterol β-chlorohydrin (Figure 7B) but not the α-chlorohydrin (Figure 7A) with BSTFA yielded an additional compound (peak b) with a GC retention time of 9.3 min. The mass spectrum of the unknown compound included prominent ions at m/z 473, 459, 385, and 369

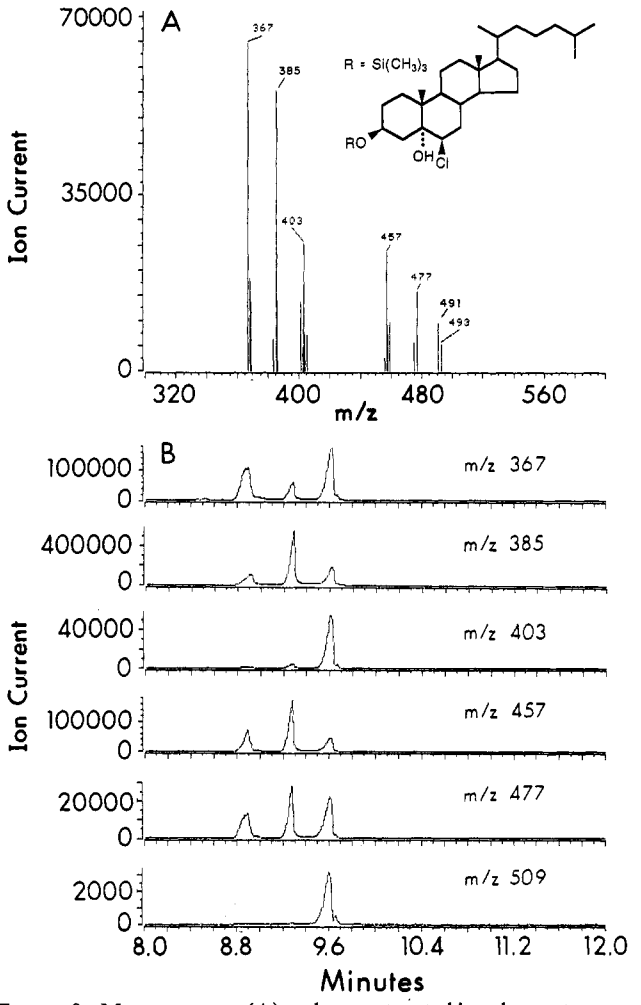

FIGURE 8: Mass spectrum (A) and reconstructed ion chromatogram (B) of trimethylsilylated cholesterol chlorohydrins in TLC band 1. The reaction products in band 1 were isolated by TLC, derivatized with BSTFA, and analyzed by positive-ion chemical ionization GC–MS. The proposed structure for the mono-TMS derivative of cholesterol α-chlorohydrin in band 1 is shown in the inset. Ions observed in the mass spectrum (A) consistent with the mono-TMS derivatives of a cholesterol chlorohydrin include m/z 493 ($M^+ + 1 - H_2O$), 491 ($M^+ - 1 - H_2O$), 477 ($M^+ - CH_3 - H_2O$), 457 ($M^+ - Cl - H_2O$), 403 ($M^+ + 1 - TMSOH - H_2O$), 385 ($M^+ - TMSOH - Cl$), and 367 ($M^+ - TMSOH - Cl - H_2O$). The molecular ion, which for the authentic α- and β-chlorohydrins has a calculated m/z of 510, is poorly visualized in the mass spectrum (A). The reconstructed ion chromatogram (B) confirms that the late eluting compound (peak c) seen in Figure 7C exhibits all of the major ions expected for a cholesterol chlorohydrin including $M^+ - 1$ (m/z 509).

(supplementary material, Figure 11B) and could be rationalized on the basis either of a cholesterol epoxide or a ketocholesterol derivative. The GC retention time of this material clearly distinguishes it from both the α- and β-chlorohydrins and α- and β-epoxides of cholesterol, but its identity has not been established.

Chemically synthesized α and β chlorohydrins comigrated with the sterol oxidation products in band 1 on normal-phase TLC, suggesting that myeloperoxidase was chlorinating cholesterol. Treatment of the products in band 1 with BSTFA followed by positive-ion chemical ionization GC–MS analysis revealed three major components (Figure 7C). The latest eluting of the three components exhibited a GC retention time identical to that of peak c of trimethylsilylated cholesterol α- and β-chlorohydrin. The positive-ion chemical ionization mass spectrum of this peak (Figure 8A) was essentially identical to that of mono-TMS cholesterol α-chlorohydrin (supplementary material, Figure 11A). Although the $M^+ -$ 1 ion was poorly visualized in the spectrum, reconstructed ion chromatograms indicated that the m/z 509 ion was present and coeluted with the other major ions in the spectrum (Figure 8B).

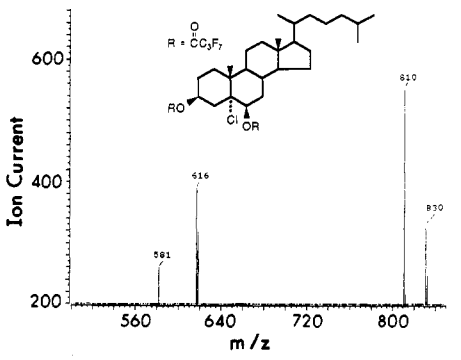

FIGURE 9: Negative-ion chemical ionization mass spectrum of the bis(heptafluorobutyrate) derivative of cholesterol β-chlorohydrin in TLC band 1. The sterol oxidation products in band 1 were isolated by TLC, converted to the HFB derivatives, and analyzed by negative-ion chemical ionization GC–MS as described in the Methods. The calculated m/z for the bis(heptafluorobutyrate) derivative of a cholesterol chlorohydrin is 830. The proposed structure for the derivatized β-chlorohydrin is shown in the inset. Ions observed in the mass spectrum consistent with the bis-HFB derivative of a cholesterol chlorohydrin include m/z 830 (M^-), 810 ($M^- - HF$), 616 ($M^- - C_3F_7CO_2H - H_2O$), and 581 ($M^- - C_3F_7CO_2H - Cl$).

The first eluting of the three products in band 1 (peak a) exhibited a GC retention time and a mass spectrum identical to that of a cholesterol epoxide (data not shown). The second eluting product in band 1 demonstrated a GC retention time (Figure 7C) and positive-ion chemical ionization mass spectrum (data not shown) identical to that of the unidentified product found in peak b of cholesterol β-chlorohydrin treated with BSTFA. The total ion chromatogram of band 1 subjected to derivatization and analysis by GC–MS, as well as the mass spectrum of each component, is that expected for a mixture of cholesterol α- and β-chlorohydrins (compare Figure 7C with Figure 7A and 7B). The decomposition products seen on mass spectrometry are presumably formed upon treatment of the compounds in band 1 with derivatizing reagents since they are also observed upon derivatization of authentic chlorohydrin standards.

To search for further evidence that cholesterol chlorohydrins are present in band 1, the material was derivatized with heptafluorobutyric anhydride and analyzed with both negative-ion and positive-ion chemical ionization GC–MS. As shown for negative-ion chemical ionization in Figure 9, both modes of ionization resulted in the visualization of a material with the GC retention time and mass spectral properties of the bis-HFB derivative of cholesterol β-chlorohydrin. Ions observed in the negative-ion chemical ionization spectrum displayed in Figure 9 included m/z 830 (M^- for ^{35}Cl), 832 (M^- for ^{37}Cl), 616 ($M^- - CF_3(CF_2)_2CO_2H$) and 581 ($M^- - CF_3(CF_2)_2CO_2H - Cl$). Based upon the TLC behavior, the GC retention times and mass spectra of the TMS derivatives, and the GC retention times and mass spectra of the HFB derivatives, we conclude that the sterol oxidation products generated by myeloperoxidase which migrate in band 1 contain both cholesterol α- and β-chlorohydrins.

Formation of Cholesterol α- and β-Epoxides. The standard cholesterol epoxides and the oxidized sterols in band 2 comigrated on normal-phase TLC. No such material was observed in standard cholesterol chlorohydrins subjected to chromatography, indicating that the analytical procedure itself was not generating the sterols in band 2. Derivatization of

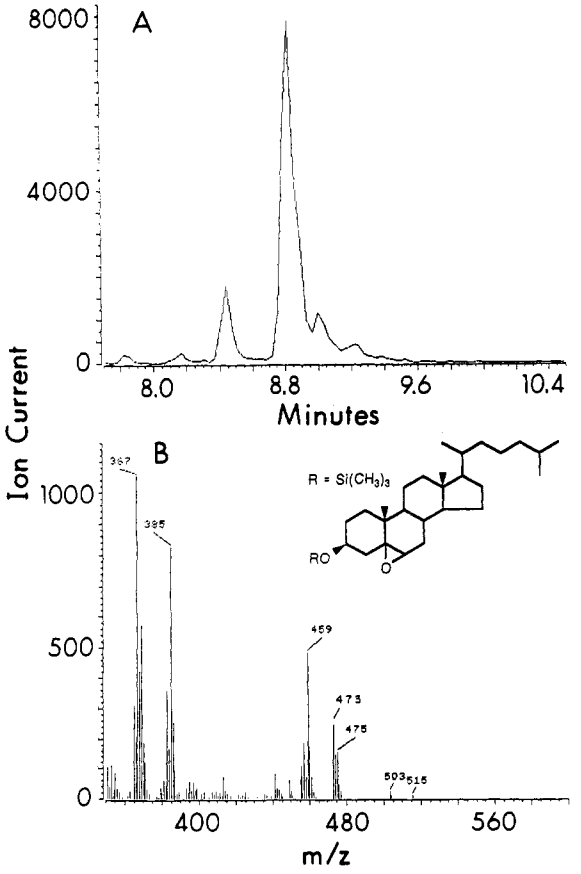

FIGURE 10: Total ion chromatogram (A) and mass spectrum (B) of the TMS derivatives of cholesterol epoxides in TLC band 2. Cholesterol oxidation products synthesized with the complete myeloperoxidase–H_2O_2–Cl system were isolated by TLC and trimethylsilylated with BSTFA. The reaction products in band 2 were then analyzed by positive-ion chemical ionization GC–MS (A). The retention time of the major peak observed at 8.82 min was identical to that of the TMS derivative of authentic cholesterol α- and β-epoxides (supplementary material, Figure 13). The mass spectrum (B) of the major peak is that expected for the TMS derivatives of a mixture of cholesterol α- and β-epoxides. The calculated m/z of a trimethylsilylated cholesterol epoxide is 474 (B, inset). Ions observed in the mass spectrum consistent with the proposed structure include m/z 475 ($M^+ + 1$), 473 ($M^+ - 1$), 459 ($M^+ - CH_3$), 385 ($M^+ -$ TMSOH), and 367 ($M^+ + 1 -$ TMSOH $- H_2O$). Ions at m/z 503 and 515 represent electrophilic addition products of cholesterol epoxide with $C_2H_5^+$ and $C_3H_5^+$, respectively, as is often observed in methane positive-ion chemical ionization (Harrison, 1983).

band 2 with BSTFA followed by positive-ion chemical ionization GC–MS analysis revealed one prominent peak with a GC retention time (Figure 10A) identical to that of the TMS derivatives of authentic cholesterol α- and β-epoxides. The positive-ion chemical ionization mass spectrum of the peak (Figure 10B) was that expected for a cholesterol epoxide (supplementary material, Figure 13A) with prominent ions at m/z 475 ($M^+ + 1$), 473 ($M^+ - 1$), 459 ($M^+ - CH_3$), 385 ($M^+ -$ TMSOH), and 367 ($M^+ -$ TMSOH $- H_2O$). Trace amounts of cholesterol (retention time 8.5 min) were also observed in this band. These results indicate that HOCl generated by the myeloperoxidase–H_2O_2–Cl^- system can convert cholesterol to an epoxide. The principal products of cholesterol autoxidation are the α- and β-epoxides, 7α-hydroxycholesterol (cholest-5-ene-3β,7α-diol), 7β-hydroxycholesterol (cholest-5-ene-3β,7β-diol), 7-ketocholesterol (3β-hydroxycholest-5-ene-7-one), and cholesterol triol (5α-cholestane-3β,5,6β-triol) (Smith, 1981). With the exception of the epoxides, none of these well-known cholesterol autoxidation products was a prominent component of the reaction mixture by GC–MS analysis, and none of them comigrated on normal-phase TLC with cholesterol oxidation products generated by myeloperoxidase.

Three lines of evidence suggested that myeloperoxidase generates both the α- and β-epoxides. First, band 2 was clearly composed of two compounds which had slightly different mobilities on normal-phase TLC (Figure 1). The rapidly and slowly migrating compounds comigrated with authentic cholesterol α- and β-epoxide, respectively. Second, the ratio of the peak heights for the ions at m/z 459 and 367 seen on positive-ion chemical ionization GC–MS analysis of the standard trimethylsilylated α- and β-epoxides were 0.9 and 0.3, respectively (supplementary material, Figure 13). The ratio for band 2 was 0.45 (Figure 10B) consistent with a mixture of the two epoxides. Finally, when the mass spectra of the HFB derivatives were examined by positive-ion chemical ionization GC–MS, the α-epoxide demonstrated a prominent ion at m/z 581 ($M^+ + 1 - H_2O$) that was much less abundant in the β-epoxide. The molecular ion clusters were also different: the major ion was $M^+ - 1$ for the α-epoxide and $M^+ + 1$ for the β-epoxide. The mass spectrum of the HFB derivatives of band 2 was that expected for a mixture of the isomers (data not shown). These results collectively indicate that the predominant products in band 2 are the epimeric 5,6-epoxides of cholesterol.

REFERENCES

Albrich, J. M., McCarthy, C. A., & Hurst, J. K. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 210–214.

Berliner, J. A., Territo, M. C., Sevanian, A., Ramin, S., Kim, J. A., Bamshad, B., Esterson, M., & Fogelman, A. M. (1990) *J. Clin. Invest. 85*, 1260–1266.

Daugherty, A., Zweifel, B. S., Sobel, B. E., & Schonfeld, G. (1988) *Arteriosclerosis 8*, 768–777.

Daugherty, A., Rateri, D. L., Dunn, J. L., & Heinecke, J. W. (1994) *J. Clin. Invest. 94*, 437–444.

Duescher, R. J., & Elfarra, A. A. (1992) *J. Biol. Chem. 267*, 19859–19865.

Francis, G. A., Mendez, A. J., Bierman, E. L., & Heinecke, J. W. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 6631–6635.

Harrison, A. G. (1983) *Chemical Ionization Mass Spectrometry*, p 63, CRC Press, Boca Raton.

Harrison, J. E., & Schultz, J. (1976) *J. Biol. Chem. 251*, 1371–1374.

Hazell, L. J., & Stocker, R. (1993) *Biochem. J. 290*, 165–172.

Heinecke, J. W., Li, W., Daenke, H. L., & Goldstein, J. A. (1993a) *J. Biol. Chem. 268*, 4069–4077.

Heinecke, J. W., Li, W., Francis, G. A., & Goldstein, J. A. (1993b) *J. Clin. Invest. 91*, 2866–2872.

Hickstein, D. D., Ozols, J., Williams, S. A., Baenager, J. V., Locksley, R. M., & Roth, G. J. (1987) *J. Biol. Chem. 262*, 5576–5580.

Hodis, H. N., Chauhan, A., Hashimoto, S., Crawford, D. W., & Sevanian, A. (1992) *Atherosclerosis 96*, 125–134.

Hu, M. L., Louie, S., Cross, C. E., Motchnik, P., & Halliwell, B. (1993) *J. Lab. Clin. Med. 121*, 257–262.

Hurst, J. K., & Barette, W. C., Jr. (1989) *Crit. Rev. Biochem. Mol. Biol. 24*, 271–328.

Hwang, P. L. (1991) *BioEssays 13*, 583–589.

Klebanoff, S. J. (1980) *Ann. Intern. Med. 93*, 480–489.

Klebanoff, S. J., & Clark, R. A. (1978) *The Neutrophil*, North Holland Publishing Co., Amsterdam.

Maerker, G., Nungesster, E. H., & Bunick, F. J. (1988) *Lipids 23*, 761–765.

Margoliash, E., Novograodsky, A., & Schejter, A. (1960) *Biochem. J. 74*, 339–348.

Morita, Y., Iwamoto, H., Albara, S., Kobayash, T., & Hasegawa, E. (1986) *J. Biochem. (Tokyo) 99*, 761–770.

Naskalski, J. W. (1977) *Biochim. Biophys. Acta 485*, 291–300.

Nauseef, W. M. (1988) *Hematol./Oncol. Clinics North Am. 2*, 135–158.
Nelson, D. P., & Kiesow, L. A. (1972) *Anal. Biochem. 49*, 474–478.
Niki, E., Saito, T., Kawakami, A., & Kamiya Y. (1984) *J. Biol. Chem. 259*, 4177–4182.
Pereira, W. E., Hoyano Y., Summons, R. E., Bacon, V. A., Duffield, A. M. (1973) *Biochim. Biophys. Acta 313*, 170–190.
Rakita, R. M., Michel, B. R., & Rosen, H. (1990) *Biochemistry 29*, 1075–80.
Ramanadham, S., Bohrer, A., Mueller, M., Jett, P., Gross, R. W., & Turk, J. (1993a) *Biochemistry 32*, 5339–5351.
Ramanadham, S., Bohrer, A., Gross, R. W., & Turk, J. (1993b) *Biochemistry 32*, 13499–13509.
Ramos, C. L., Pou, S., Britigan, B. E., Cohen, M. S., & Rosen, G. M. (1992) *J. Biol. Chem. 267*, 8307–8312.
Savenkova, M. I., Muller, D. M., & Heinecke, J. W. (1994) *J. Biol. Chem.* (in press).
Segal, A. W. (1989) *J. Clin. Invest. 83*, 1785–1793.
Sepe, S. M., & Clark, R. A. (1985a) *J. Immunol. 134*, 1888–1895.
Sepe, S. M., & Clark, R. A. (1985b) *J. Immunol. 134*, 1896–1901.
Smith, L. L. (1981) *Cholesterol Autoxidation*, pp 49–62, Plenum Press, New York.
Stadtman, E. R. (1992) *Science 257*, 1220–1224.
Steinbeck, M. J., Khan, A. U., & Karnovsky, M. J. (1992) *J. Biol. Chem. 267*, 13425–13433.
Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., & Witztum, J. L. (1989) *N. Engl. J. Med. 320*, 915–924.
Stelmaszynska, T., & Zgliczynski, J. M. (1974) *Eur. J. Biochem. 45*, 305–312.
Stelmaszynska, T., & Zgliczynski, J. M. (1978) *Eur. J. Biochem. 92*, 301–308.
Thomas, E. L., Jefferson, M. M., & Grisham, M. B. (1982) *Biochemistry 21*, 6299–6308.
Turk, J., Henderson, W. R., Klebanoff, S. J., & Hubbard, W. C. (1983) *Biochim. Biophys. Acta 751*, 189–200.
Van den Berg, J. J. M., Winterbourn, C. C., & Kuypers, F. A. (1993) *J. Lipid Res. 34*, 2005–2012.
Wade, L. C. (1987) *Organic Chemistry*, pp 306–309, Prentice-Hall, Englewood Cliffs, NJ.
Weaver, R., Plat, H., & Hamers, M. N. (1981) *FEBS Lett. 123*, 327–331.
Weiss, S. J. (1989) *N. Engl. J. Med. 320*, 365–376.
Weiss, S. J., Klein, R., Slivka, A., & Wei, M. (1982) *J. Clin. Invest. 70*, 598–607.
Winterbourn, C. C. (1985) *Biochim. Biophys. Acta 840*, 204–210.
Winterbourn, C. C., Van den Berg, J. M., Roitman, E., & Kuypers, F. A. (1992) *Arch. Biochem. Biophys. 296*, 547–555.
Yla-Herttula, S., Palinski, W., Rosenfeld, M. E., Parthasarathy, S., Carew, T. E., Butler, S., Witztum, J. L., & Steinberg, D. (1989) *J. Clin. Invest. 84*, 1086–1095.
Zgliczynski, J. M., & Stelmaszynska, T. (1975) *Eur. J. Biochem. 56*, 157–162.
Zgliczynski, J. M., Stelmaszynska, T., Ostrowski, W., Naskalski, J., & Sznajd, T. (1968) *Eur. J. Biochem. 4*, 540–547.
Zgliczynski, J. M., Stelmaszynska, T., Domanski, J., & Ostrowski, W. (1971) *Biochim. Biophys. Acta 235*, 419–424.
Zgliczynski, J. M., Selvaraj, R. J., Paul, B. B., Stelmaszynska, T., Poskitt, P. K. F., Sbarra, A. J. (1977) *Proc. Soc. Exp. Bio. Med. 154*, 418–422.
Zhang, H., Yang, Y., & Steinbrecher, U. P. (1993) *J. Biol. Chem. 268*, 5535–5542.