3-Chlorotyrosine, a Specific Marker of Myeloperoxidase-catalyzed Oxidation, Is Markedly Elevated in Low Density Lipoprotein Isolated from Human Atherosclerotic Intima

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Abstract

Oxidation of LDL may be of pivotal importance in atherogenesis, but the mechanisms that promote oxidation in vivo remain poorly understood. We have explored the possibility that one pathway involves myeloperoxidase, a heme protein secreted by phagocytes. Myeloperoxidase is the only human enzyme known to generate hypochlorous acid (HOCl), a potent oxidizing agent, at physiological halide concentrations. LDL exposed to the complete myeloperoxidase-H_2O_2-Cl^- system underwent chlorination of its protein tyrosyl residues. Treatment of LDL with reagent HOCl resulted in 3-chlorotyrosine formation, implicating HOCl as an intermediate in the enzymatic reaction pathway. In contrast, 3-chlorotyrosine was undetectable in LDL oxidized by hydroxyl radical, copper, iron, hemin, glucose, peroxynitrite, horseradish peroxidase, lactoperoxidase, or lipoxygenase. These results indicate that 3-chlorotyrosine is a specific marker for LDL oxidation by myeloperoxidase. To address the role of myeloperoxidase in promoting LDL oxidation in vivo, we used stable isotope dilution gas chromatography-mass spectrometry to quantify 3-chlorotyrosine in human aortic tissue and in LDL isolated from atherosclerotic lesions. The level of 3-chlorotyrosine in atherosclerotic tissue obtained during vascular surgery was sixfold higher than that of normal aortic intima. Moreover, the level of 3-chlorotyrosine was 30-fold higher in LDL isolated from atherosclerotic intima compared with circulating LDL. The detection of 3-chlorotyrosine in human atherosclerotic lesions indicates that halogenation reactions catalyzed by the myeloperoxidase system of phagocytes constitute one pathway for protein oxidation in vivo. These findings raise the possibility that the myeloperoxidase-H_2O_2-Cl^- system plays a critical role in converting LDL into an atherogenic form. (*J. Clin. Invest.* 1997. 99:2075–2081.) Key words: atherosclerosis • inflammation • vascular disease • halogenation • phagocyte

Introduction

An increased level of LDL is a major risk factor for 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, 3). The pathways for oxidation in vivo have not yet been defined. One potential mechanism involves myeloperoxidase (4), a heme protein secreted by activated phagocytes (5, 6). Myeloperoxidase uses hydrogen peroxide (H_2O_2) generated by phagocytes to generate potent microbicidal oxidants (6–8) and tyrosyl radical (9), which triggers LDL lipid peroxidation (10). Catalytically active myeloperoxidase is present in human atherosclerotic lesions, where it colocalizes with lipid-laden macrophages, the cellular hallmark of the early atherosclerotic lesion (4). Patterns of immunostaining for the enzyme at different stages of atherosclerosis (4) are remarkably similar to those for protein-bound lipid oxidation products (11), suggesting that myeloperoxidase promotes lipoprotein oxidation in vivo.

The best characterized product of myeloperoxidase is hypochlorous acid (HOCl;[1] references 6 and 7):

$$Cl^- + H_2O_2 + H^+ \rightarrow HOCl + H_2O. \qquad (1)$$
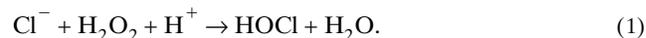
This potent oxidant chlorinates electron-rich substrates and oxidatively bleaches heme groups (5–8, 12–14). LDL exposed to reagent HOCl at neutral pH becomes aggregated and is rapidly taken up and degraded by macrophages (15). Lipoproteins with similar properties that are rich in apolipoprotein B100, the major protein of LDL, have been isolated from atherosclerotic lesions (16–18). The unregulated uptake of modified LDL may be of critical importance in converting macrophages into foam cells (1–3). A monoclonal antibody which selectively reacts with HOCl-modified proteins recognizes unknown epitopes in human atheroma and in LDL isolated from atherosclerotic tissue (19). These results suggest that myeloperoxidase may contribute to atherogenesis by catalyzing oxidative reactions within the artery wall.

Myeloperoxidase is the only human enzyme known to generate HOCl at physiological concentrations of halides (6, 20). However, most oxidation products generated by HOCl are either nonspecific or decompose to yield uninformative compounds (8, 21, 22). Recent studies demonstrate that the myeloperoxidase-H_2O_2-Cl^- system oxidizes L-tyrosine to yield

3-chlorotyrosine (Scheme I; references 23–25). This electrophilic addition compound is stable and therefore may serve as a molecular fingerprint of myeloperoxidase-catalyzed oxidation reactions.
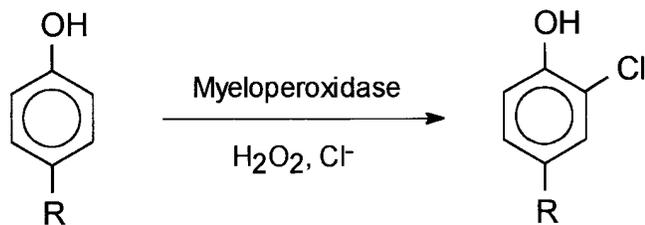
Scheme 1. 3-Chlorotyrosine formation by the myeloperoxidase-H_2O_2-Cl^- system.

We have developed a quantitative assay for measuring tissue levels of 3-chlorotyrosine (Hazen, S.L., J.R. Crowley, D.M. Mueller, and J.W. Heinecke, manuscript submitted for publication). This method combines gas chromatography (GC)with stable isotope dilution mass spectrometry (MS). We have used the method to demonstrate that levels of 3-chlorotyrosine are elevated in human atherosclerotic tissue obtained during surgery and in LDL isolated from vascular lesions. These results indicate that myeloperoxidase constitutes one pathway for protein oxidation in human atherosclerosis. Moreover, they provide the first direct demonstration that myeloperoxidase-catalyzed halogenation reactions occur in vivo.

Methods

Rabbit anti–human apolipoprotein B100 antiserum was a gift from Dr. Gustav Schonfeld (Washington University, St. Louis, MO). Peroxynitrite was provided by Monsanto Corp. (St. Louis, MO). All other reagents were purchased from either Sigma Chemical Co. (St. Louis, MO) or the indicated sources.

General procedures. All glassware was rendered chlorine-demand free (< 3% consumption of 1 mM HOCl in a 10-min incubation at 37°C within a gas-tight vial as monitored by the oxidation of iodide to triiodide) before use (12, 25, 26). Buffers were treated with Chelex-100 resin to remove transition metal ions and demonstrated to be chlorine-demand free. Myeloperoxidase (donor: hydrogen peroxide, oxidoreductase, EC 1.11.1.7) was isolated (final A_{430}/A_{280} ratio of 0.6) as previously described (27, 28). Enzyme concentration was determined spectrophotometrically (ϵ_{430} = 170 $mM^{-1}cm^{-1}$; 29). Human neutrophils were isolated by buoyant density centrifugation (30). Medium A (HBSS (magnesium-, calcium-, phenol-, and bicarbonate-free; GIBCO-BRL, Gaithersburg, MD); pH 7.2) supplemented with 100 μM diethylenetriamine pentaacetic acid (DTPA) was used for neutrophil isolation and cell experiments. L-3-[$^{13}C_6$]Chlorotyrosine was synthesized from L-[$^{13}C_6$]tyrosine using reagent HOCl and quantified by reverse phase HPLC analysis (25). Protein concentration was determined using the Markwell-modified Lowry protein assay (31) with BSA as standard. H_2O_2 concentration was determined spectrophotometrically (ϵ_{240} = 39.4 $M^{-1}cm^{-1}$; 32). LDL (d = 1.02–1.07 g/ml) was isolated from nonfasted healthy male and female subjects by sequential density ultracentrifugation (33). LDL oxidation by lipoxygenase was performed as described (34).

Tissue collection. Fresh surgical specimens of human aortic tissue were obtained from transplant donors and vascular surgery patients. Tissue was immediately rinsed in ice-cold normal saline, placed in buffer A (65 mM sodium phosphate, pH 7.4, 100 μM DTPA, 100 μM butylated hydroxy toluene), and frozen under N_2 at −80°C until anal-

ysis. LDL was isolated from the intima of thoracic aortas obtained at necropsy within 10 h of death. Autopsy tissue was rinsed in ice-cold phosphate-buffered saline supplemented with 100 μM DTPA and immediately frozen in buffer A under N_2 at −80°C until analysis. Control studies demonstrated no detectable change in 3-chlorotyrosine content of normal or atherosclerotic tissue stored for up to 6 mo under these conditions.

Tissue processing. Surgical aortic specimens were thawed in buffer A supplemented with 10 mM 3-aminotriazole, an inhibitor of myeloperoxidase (9, 14). All subsequent steps were performed at 4°C unless otherwise indicated. Atherosclerotic lesions were classified morphologically using the criteria of the Pathobiological Determinants of Atherosclerosis in Youth Study (35). Aortic intima was resected from the media, frozen in liquid N_2, and pulverized under liquid N_2 with a stainless steel mortar and pestle. Tissue powder ($\sim$ 70 mg wet wt) was suspended in 1 ml of buffer B (H_2O supplemented with 100 μM DTPA and 10 mM 3-aminotriazole), and then delipidated three successive times with H_2O/methanol/water-washed diethyl ether (final ratios 1:3:7, vol/vol/vol). The single-phase extraction mixture was vortexed, incubated for 30 min at 0°C, and the protein was recovered by centrifugation (5,000 g for 10 min). To remove Cl^- potentially able to catalyze chlorination during acid hydrolysis, the protein pellet was suspended and dialyzed against 50 mM sodium phosphate (pH 7.0), 100 μM DTPA, and 10 mM 3-aminotriazole, and then dialyzed against buffer B.

Protein hydrolysis. HBr was used for protein hydrolysis because preliminary experiments demonstrated that 3-chlorotyrosine was generated in L-tyrosine exposed to HCl. After the addition of 10 pmol 3-[$^{13}C_6$]chlorotyrosine and 100 nmol L-[$^{13}C_6$]tyrosine, delipidated and dialyzed protein ($\sim$ 2–4 mg) suspended in buffer B was dried under vacuum in a glass reaction vial. Each vial then received 0.5 ml of 6 N HBr containing 1% phenol. The reaction vials were alternately degassed under vacuum and purged with argon five times, and incubated at 120°C for 24 h. After hydrolysis, 1.5 ml of 0.1% trifluoroacetic acid (TFA) was added, and the mixture was passed over a solid-phase C18 extraction column (Supelclean LC-18 SPE tubes, 3 ml; Supelco Inc., Bellefonte, PA) equilibrated with 0.1% TFA. The column was washed with 2 ml of the same buffer, eluted with 2 ml of 50% methanol in 0.1% TFA, and the recovered amino acids were dried under vacuum.

Several control experiments indicated that postmortem changes were unlikely to be contributing to protein chlorination. First, normal and atherosclerotic aortic intima were each pulverized in liquid nitrogen. Intimal powder was then thawed, homogenized in PBS (5:1 vol/g) using a Potter Elvejehiem Homogenizer (Fisher, St. Louis, MO), and incubated with 100 nmol L-[^{13}C]tyrosine overnight at 25°C. Reaction mixtures were centrifuged at 100,000 g and amino acids in the supernatant were isolated using a solid-phase C18 extraction column after addition of phenol (1%) and TFA (0.1%) as described above. No L-[^{13}C]chlorotyrosine formation was observed in homogenates of either normal or atherosclerotic intima by GC/MS analysis. Second, normal and lesion aortic intima were each pulverized in liquid nitrogen. Half of the tissue powder from each specimen was analyzed by the routine method; the other half from each specimen was incubated for 10 h at room temperature before analysis. No differences in 3-chlorotyrosine content were observed. Third, the levels of 3-chlorotyrosine were comparable in aortic tissue obtained at autopsy and fresh at the time of vascular surgery. Fourth, normal and lesion aortic intima were each pulverized in liquid nitrogen. Half of the tissue powder was analyzed by the routine method; the other half from each specimen was incubated in PBS (1:5 wt/vol) for 10 h at room temperature in the presence of exogenous purified myeloperoxidase (40 nM) before analysis. No significant increases in the content of 3-chlorotyrosine were observed in samples supplemented with myeloperoxidase.

LDL isolation from human atherosclerotic intima. Lesion LDL was isolated by sequential density ultracentrifugation (d = 1.02–1.07 g/ml; 33) from fatty streaks and intermediate lesions of human thoracic aortae using a modification of the method of Steinbrecher and

Lougheed (18) as described (36). A metal chelator (100 μM DTPA) and myeloperoxidase inhibitor (10 mM 3-aminotriazole) were included in all solutions used for lipoprotein isolation.

Lesion LDL was subjected to SDS-PAGE (37) with Western blot analysis using a rabbit anti–human apolipoprotein B100 antiserum (38). The polyclonal antibody detected a 500-kD protein in lesion LDL, the mass of intact apolipoprotein B100. As previously noted by other investigators (16–19), both aggregated and lower molecular mass forms of immunoreactive protein were also present in LDL isolated from lesions. Analysis of lesion LDL preparations by high resolution size exclusion FPLC (tandem Superose 6 and 12 columns; Pharmacia LKB, Piscataway, NJ) demonstrated that immunoreactive apolipoprotein B100, total cholesterol, and the majority of protein mass exhibited an apparent M_r identical to that of LDL isolated from plasma.

Mass spectrometric analysis. *n*-Propyl, per heptafluorobutyryl (HFB), and *n*-propyl, per pentafluoropropionyl derivatives of amino acids, were prepared as described (25, 27, 39) and analyzed on a Hewlett Packard 5890 Gas Chromatograph interfaced with a Hewlett Packard 5988A Mass Spectrometer equipped with extended mass range (Hewlett Packard Co., Palo Alto, CA). Chromatographic separations were performed with a 30-m DB-17 capillary column (0.25 mm i.d., 0.25 μm film thickness; J&W Scientific Inc., Folsom, CA) with He as the carrier gas. An aliquot of derivatized sample was diluted 1:50 (vol/vol) with ethyl acetate and then 1 μl was analyzed with a 1:50 split before mass analysis. The column was run with the following temperature gradient: 150–250°C at 20°C/min. The injector, transfer line, and source temperatures were set at 250, 250, and 130°C, respectively.

The negative-ion chemical ionization mass spectrum of the *n*-propyl, per-HFB derivative of 3-chlorotyrosine demonstrated a low abundance molecular ion (M·⁻) at mass-to-charge ratio (m/z) 649. Major ions were also observed at m/z 629 (M-HF)⁻ and 451 (M-HFB)⁻. The mass spectrum demonstrated the isotopic clusters expected of a monochlorinated molecule, with ions of relative abundance of 3:1 (for ³⁵Cl and ³⁷Cl) at m/z 649 and 651, m/z 629 and 631, and m/z 451 and 453.

Amino acids were quantified using stable isotope dilution GC/MS analysis in the negative-ion chemical ionization mode (25). 3-Chlorotyrosine was monitored as its *n*-propyl-per-HFB derivative using selected ion monitoring of the base peak at m/z 451 (M-HFB)⁻, another prominent ion at m/z 629 (M-HF)⁻, and their corresponding isotopically labeled internal standard ions at m/z 457 and 635, respectively. L-Tyrosine was monitored as its *n*-propyl-per-HFB derivative using the base peak at m/z 417 (M-HFB)⁻, another major ion at m/z 595 (M-HF)⁻, and their corresponding isotopically labeled internal standard ions at m/z 423 and 601, respectively. The ratio of ion currents of the two characteristic ions of each compound and its corresponding internal standard were monitored in all analyses to ensure that interfering ions were not coeluting with the analyte. The limit of detection in the assay was < 1 fmol of 3-chlorotyrosine.

Results

3-Chlorotyrosine is a specific marker for LDL oxidation mediated by the myeloperoxidase–H_2O_2-Cl^- system of activated phagocytes. We used human neutrophils, a well-characterized source of H_2O_2 and myeloperoxidase, as a model system to explore the role of HOCl in promoting chlorination of LDL. LDL isolated from human plasma contained very low levels of 3-chlorotyrosine as monitored by stable isotope dilution GC/MS. A dramatic increase in 3-chlorotyrosine content was apparent in LDL incubated with activated neutrophils (Fig. 1). Formation of 3-chlorotyrosine required activation of the cells with phorbol ester. Addition of peroxidase inhibitors (3-aminotriazole, azide, or cyanide) or a peroxide scavenger (catalase)
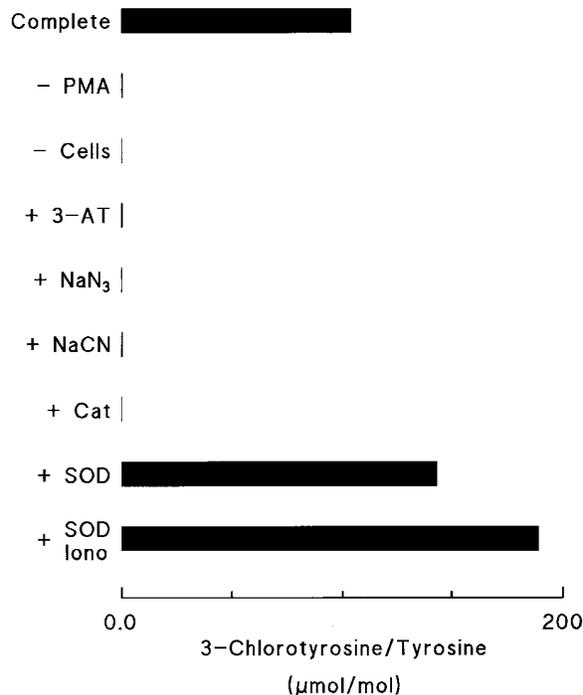

Figure 1. Formation of 3-chlorotyrosine in LDL exposed to activated human phagocytes. Human neutrophils (10⁶/ml) were incubated at 37°C in medium A (pH 7.2) supplemented with DTPA (100 μM) and LDL (512 μg protein/ml). Neutrophils were activated with phorbol ester (*PMA*; 200 nM) and maintained in suspensions by intermittent inversion (*Complete*). After a 60-min incubation, neutrophils were removed by centrifugation. The supernatant was delipidated and dialyzed, ¹³C-labeled internal standards were added, and the samples were subjected to acid hydrolysis. The content of L-tyrosine and 3-chlorotyrosine in acid hydrolysates was determined by stable isotope dilution GC/MS analysis as described in Methods. Additions or deletions to the complete system were as indicated. The final concentrations of additions were: 3-aminotriazole (*3-AT*), 10 mM; NaN₃, 1 mM; NaCN, 1 mM; catalase (*Cat*), 10 μg/ml; superoxide dismutase (*SOD*), 10 μg/ml; and ionomycin (*Iono*), 1 μM. 3-Chlorotyrosine content of LDL is normalized to the content of the precursor amino acid L-tyrosine. Values are the mean of duplicate determinations. Similar results were observed in three independent experiments.

inhibited 3-chlorotyrosine production, implicating myeloperoxidase and H_2O_2 in the cell-mediated reaction. Addition of superoxide dismutase (which catalyzes the conversion of O_2^- into H_2O_2) with and without the calcium ionophore ionomycin (which both enhances NADPH oxidase activation and stimulates myeloperoxidase secretion) resulted in further generation of the chlorinated amino acid. Collectively, these results implicate the myeloperoxidase–H_2O_2-Cl^- system of phagocytes in the chlorination of tyrosyl residues of apolipoprotein B100.

To investigate whether enzymatically generated HOCl was oxidizing LDL, we determined the reaction requirements for chlorination of apolipoprotein B100 by myeloperoxidase. The presence of the complete myeloperoxidase–H_2O_2-Cl^- system resulted in 3-chlorotyrosine production at neutral pH (Table I). Chlorination required enzyme, H_2O_2, and Cl^-, and was blocked by peroxidase inhibitors and catalase, implicating HOCl in the reaction pathway. Incubation of LDL with re-

Table I. 3-Chlorotyrosine Formation in LDL Oxidized by the Myeloperoxidase-H_2O_2-Cl^- System

Condition	3-Chlorotyrosine/L-tyrosine
	μmol/mol
Complete system	
LDL + MPO + H_2O_2 + Cl^-	230*
Complete system minus	
MPO	< 1
H_2O_2	< 1
Cl^-	< 1
Complete system plus	
Catalase (10 μg/ml)	< 1
NaN_3 (1 mM)	< 1
NaCN (1 mM)	< 1
3-Aminotriazole (10 mM)	< 1
LDL + HOCl (100 μM)	310*

The complete system consisted of buffer C (100 mM NaCl, 20 mM sodium phosphate, pH 7.0), supplemented with LDL (512 μg protein/ml), myeloperoxidase (*MPO*; 40 nM), and H_2O_2 (100 μM). After a 1-h incubation at 37°C, the 3-chlorotyrosine content of LDL protein was determined by stable isotope dilution GC/MS as described in Methods. Values are the mean of duplicate determinations. Similar results were observed in three independent experiments. *The yield of 3-chlorotyrosine formation was: complete system, 350 μmol/mol of H_2O_2; LDL + HOCl, 470 μmol/mol of reagent HOCl.

Figure 2. Production of 3-chlorotyrosine in LDL modified by different oxidation systems. LDL (512 μg protein/ml) was incubated at 37°C in phosphate-buffered saline (10 mM sodium phosphate, 150 mM NaCl, pH 7.4) alone (*Control*) or with the indicated oxidation system. The complete myeloperoxidase-H_2O_2-Cl^- system consisted of 20 nM myeloperoxidase and 100 μM H_2O_2. HOCl was used at 100 μM. Horseradish peroxidase (10 μg/ml) and lactoperoxidase (10 μg/ml) were used with 100 μM H_2O_2. LDL was exposed to myeloperoxidase (*MPO*), HOCl, horseradish peroxidase (*HRP*), and lactoperoxidase for 60 min at 37°C. All other oxidation reactions were carried out for 24 h at 37°C. Final concentrations of oxidants were: peroxynitrite (*ONOO$^-$*), 100 μM; $CuSO_4$, 10 μM; $CuSO_4$ and H_2O_2, 100 μM and 2 mM, respectively; $FeSO_4$, 10 μM; hemin, 10 μM; and glucose, 100 mM. LDL oxidation by lipoxygenase was performed in 50 mM borate, 150 mM NaCl supplemented with phospholipase A_2 as described (34). Reaction products were delipidated, hydrolyzed, and the 3-chlorotyrosine content of the amino acid hydrolysate was determined by stable isotope dilution GC/MS analysis. Values are the mean of duplicate determinations. Similar results were observed in three independent experiments.

agent HOCl resulted in 3-chlorotyrosine formation, confirming that HOCl generated by myeloperoxidase could serve as an intermediate in the halogenation reaction.

To establish the specificity of 3-chlorotyrosine as a marker for protein damage by myeloperoxidase, we examined the ability of a variety of in vitro oxidation systems to generate the chlorinated amino acid in apolipoprotein B100 of LDL. Significant levels of 3-chlorotyrosine were detected in LDL exposed to the complete myeloperoxidase–H_2O_2-Cl^- system and reagent HOCl (Fig. 2). In contrast, there was little change in the 3-chlorotyrosine content of LDL oxidized by copper, iron, a hydroxyl radical generating system (H_2O_2 plus copper), lactoperoxidase, horseradish peroxidase, peroxynitrite, glucose, or lipoxygenase (Fig. 2). All of the systems resulted in the expected levels of LDL oxidation as monitored by the appearance of lipid oxidation products (thiobarbituric reacting substances assay and lipid hydroxyperoxides; references 26 and 33). Collectively, these results demonstrate that 3-chlorotyrosine production is a highly specific marker for LDL oxidation by the myeloperoxidase–H_2O_2-Cl^- system.

3-Chlorotyrosine is present at increased levels in human atherosclerotic tissue obtained at surgery. To determine whether 3-chlorotyrosine might be formed in vivo by myeloperoxidase, we searched for the chlorinated amino acid in human aortic tissue harvested at surgery. An inhibitor of myeloperoxidase was included in the buffers used for tissue processing to prevent ex vivo protein oxidation. When amino acids isolated from acid hydrolysates of atherosclerotic tissue were derivatized and analyzed by GC/MS, we detected an amino acid that exhibited major ions and a retention time identical to that of authentic 3-chlorotyrosine. The identity of the compound was
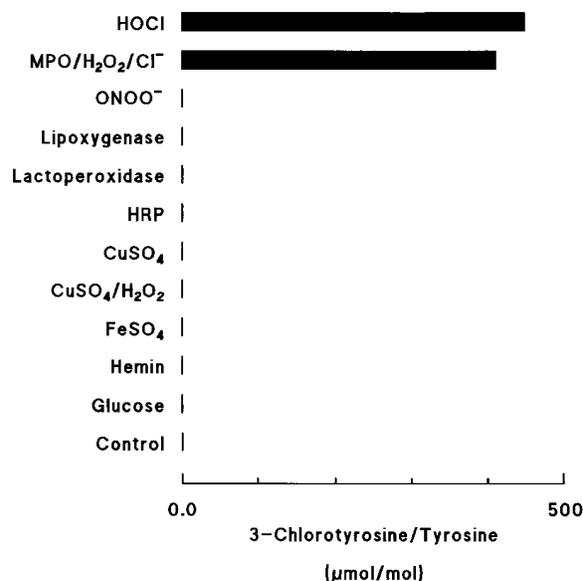
confirmed by comparison with authentic standards using both heptafluorobutyryl and pentafluoropropionyl derivatives of 3-chlorotyrosine. Selected ion monitoring demonstrated that ions derived from the amino acid coeluted with the ions derived from 3-[$^{13}C_6$]chlorotyrosine for both derivatives. The isotopic distribution between ^{35}Cl and ^{37}Cl was exploited to confirm further the identity of 3-chlorotyrosine. Selected ion monitoring of the ions at m/z 451 and 453 ((M-HFB)$^-$ for ^{35}Cl and ^{37}Cl, respectively) demonstrated the expected ratio of relative ion currents (∼ 3:1) for a monochlorinated compound. Collectively, these results indicate that protein-bound 3-chlorotyrosine is present in amino acid hydrolysates prepared from human atherosclerotic tissue.

To determine whether HOCl generated by myeloperoxidase might play a role in damaging proteins in atherosclerotic lesions, we quantified the levels of 3-chlorotyrosine in normal and atherosclerotic aortic tissue removed at surgery. Advanced atherosclerotic lesions exhibited a sixfold increase in 3-chlorotyrosine content when compared with normal aortic tissue (Fig. 3).
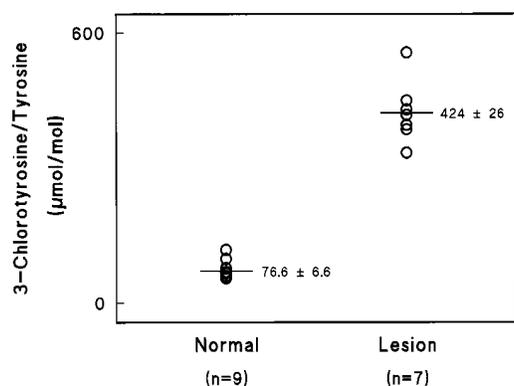
Figure 3. 3-Chlorotyrosine content of normal and atherosclerotic human aortic tissue harvested at surgery. Human thoracic aortic tissue obtained at surgery was immediately placed in ice-cold antioxidant buffer and frozen at −80°C until analysis. Intima from normal and advanced atherosclerotic lesions was pulverized in liquid N_2, delipidated, and dialyzed under N_2. ^{13}C-Labeled internal standards were added, and the protein was subjected to HBr hydrolysis. Amino acids were isolated by solid-phase extraction on a C18 column, derivatized, and subjected to stable isotope dilution GC/MS analysis as described in Materials and Methods. Results represent the mean±SEM.

Apolipoprotein B100 containing lipoproteins isolated from human atherosclerotic lesions demonstrate a marked increase in 3-chlorotyrosine. To assess directly the possible role of myeloperoxidase in catalyzing LDL oxidation in vivo, we isolated LDL ($d = 1.02–1.07$ g/ml) from human atherosclerotic tissue obtained at autopsy. Mass spectrometric analysis of lesion LDL demonstrated levels of 3-chlorotyrosine that were 30-fold higher than those observed in circulating LDL (Fig. 4). These results indicate that one pathway for LDL oxidation in the human artery wall involves halogenation of the aromatic ring of L-tyrosine by myeloperoxidase.
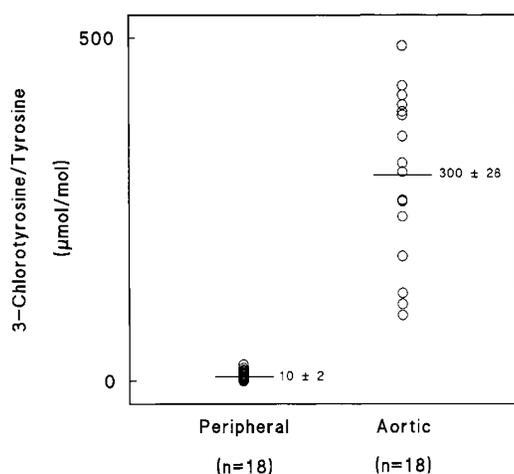

Figure 4. Level of 3-chlorotyrosine in LDL recovered from human atherosclerotic tissue and from plasma. LDL was isolated by sequential ultracentrifugation from plasma and atherosclerotic aorta, respectively, as described in Methods. 3-Chlorotyrosine content of the lipoproteins was determined by stable isotope dilution GC/MS analysis. Results represent the mean±SEM.

Discussion

Myeloperoxidase is the only human enzyme known to produce HOCl at physiological concentrations of halide ions (6, 20). The detection of chlorinated compounds in tissues would therefore constitute strong support for the hypothesis that myeloperoxidase is a physiological catalyst for oxidative damage.

In vitro studies demonstrated that 3-chlorotyrosine was indeed a specific marker for LDL oxidation by the myeloperoxidase–H_2O_2-Cl$^-$ system, and that HOCl was an intermediate in the reaction. Activated phagocytes likewise used myeloperoxidase to chlorinate the aromatic ring of tyrosine residues on apolipoprotein B100 of LDL. Analysis of human atherosclerotic lesions obtained during vascular surgery revealed a sixfold increase in the content of protein-bound 3-chlorotyrosine in comparison with normal aortic intima. Moreover, the level of 3-chlorotyrosine was 30-fold higher in lesion LDL compared with circulating LDL. The detection of 3-chlorotyrosine in human atherosclerotic aortae and in LDL isolated from atherosclerotic lesions strongly supports the hypothesis that myeloperoxidase is one important pathway for the oxidative modification of lipoproteins. This finding raises the possibility that myeloperoxidase is a pivotal agent in the development of vascular disease.

Previous studies have shown that tyrosyl residues of peptides and BSA are substrates for halogenation by HOCl at neutral pH (23, 24). Chlorination was proposed to involve intramolecular attack by a chloramine intermediate (23). Chlorination of LDL and model proteins with the myeloperoxidase system is optimal under acidic conditions (pH 5–6) (Hazen, S.L., and J.W. Heinecke, unpublished observations). We demonstrated recently that phagocytes use molecular chlorine (Cl_2), which is in equilibrium with myeloperoxidase-generated HOCl, to chlorinate the aromatic ring of free L-tyrosine (25). This reaction is also optimal at acidic pH and takes place in the phagolysosomal compartment (25). In contrast, the α-amino moiety of free L-tyrosine is preferentially chlorinated by HOCl at neutral pH, generating an unstable intermediate which subsequently decomposes to yield the reactive aldehyde *p*-hydroxyphenylacetaldehyde (30). The detection of 3-chlorotyrosine in human atherosclerotic tissues suggests that HOCl, Cl_2, or other HOCl-derived species serve as reactive intermediates for LDL oxidation in vivo (23, 25, 40).

A key question is whether myeloperoxidase catalyzed halogenation reactions are of importance in the development of vascular disease. The presence of 3-chlorotyrosine in atherosclerotic lesions does not necessarily prove that protein chlorination by myeloperoxidase is pathogenic. The relatively low yield of 3-chlorotyrosine formation suggests that alternative targets are oxidized preferentially by HOCl. One important moiety may be reactive amino groups of proteins (12, 13, 22). Oxidation of LDL by HOCl at neutral pH consumes apolipoprotein B100 lysine ε-amino groups, and the resultant aggregated lipoproteins induce foam cell formation (15, 41), which may be of critical importance in atherogenesis (1–3).

The lipid component of LDL represents another potential substrate for oxidation by HOCl. We have demonstrated recently that LDL oxidation by the myeloperoxidase–H_2O_2-Cl$^-$ system results in the generation of a family of chlorinated and oxygenated sterols (14, 42). At acidic pH and plasma concentrations of Cl$^-$, nearly 50% of the H_2O_2 in the reaction mixture is used for sterol chlorination, suggesting that LDL cholesterol

may be a major target for oxidation under these conditions (42). Oxygenated sterols are cytotoxic, mutagenic, and potent regulators of sterol metabolism (43–46). Chlorinated and oxygenated sterols generated by myeloperoxidase may similarly exert potent biological effects in the artery wall. We have demonstrated that the oxidizing intermediate in the formation of chlorinated sterols is Cl_2 derived from HOCl (42). Detection of chlorinated sterols in human atherosclerotic tissue would thus strongly support the notion that Cl_2-mediated halogenation reactions take place in vascular disease, and raise the possibility that chlorinated lipids play a role in atherogenesis.

The level of 3-chlorotyrosine varied considerably in atherosclerotic intima as well as in LDL isolated from atherosclerotic tissue. The content of an amino acid oxidation product in a tissue will be dependent upon both its rate of formation and degradation. The influence of genetic and environmental factors on such pathways is unknown. Differences in the absolute level of myeloperoxidase, perhaps related in part to polymorphisms in the promoter region of the enzyme (47, 48), may be one important factor controlling the rate of chlorination within the artery wall. Differences in H_2O_2 production, tissue or plasma antioxidant levels, or a host of other factors may also be significant (2, 3, 49). Yet another important factor may be differences in the rates of proteolytic breakdown of oxidized proteins (50, 51).

The detection of low levels of 3-chlorotyrosine in apparently normal aortic intima suggests that some degree of phagocyte activation is occurring in nonatherosclerotic arterial tissue. Indeed, phagocytes are present in normal aortic intima, and it has been suggested that certain oxidation reactions may exert antiatherogenic effects (52). In addition, HOCl is a relatively long-lived, selectively reactive oxidant that could potentially diffuse away from its site of initial generation (53). We also cannot exclude the possibility that residual chloride in tissue samples contributed to chlorination during acid hydrolysis of proteins. A background level of chlorination would lead us to underestimate the true difference in the 3-chlorotyrosine contents of normal and atherosclerotic intima. Finally, truly normal intima may not be present in aortae obtained from middle-aged and elderly men with known atherosclerosis or multiple risk factors for coronary artery disease; in this context, it is worth mentioning that a Veteran's Administration hospital was our primary source of tissue.

It is interesting to note that the 3-chlorotyrosine level of plasma LDL recovered from different donors also varied. We have observed the highest levels in two donors with known coronary artery disease. In contrast, 3-chlorotyrosine was generally undetectable in LDL isolated from young, healthy donors (Hazen, S.L., and J.W. Heinecke, unpublished observations). These preliminary observations suggest that the 3-chlorotyrosine content of plasma LDL may provide information regarding the risk for atherosclerotic vascular disease.

The oxidation hypothesis of atherosclerosis has focused considerable interest on the potential role of antioxidants in treating vascular disease. The demonstration of elevated levels of 3-chlorotyrosine in atherosclerotic aortic tissue establishes myeloperoxidase as one pathway for protein oxidation in the artery wall. Moreover, these observations provide the first direct evidence that myeloperoxidase executes halogenation reactions in vivo. The links between lipoprotein oxidation and reactive intermediates generated by myeloperoxidase implicate the enzyme in LDL oxidation in vivo, and suggest that myeloperoxidase represents a rational target for the development of specific interventions designed to prevent human atherosclerotic vascular disease.

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