LDL Modified by Hypochlorous Acid Is a Potent Inhibitor of Lecithin-Cholesterol Acyltransferase Activity

Mark R. McCall,* Anitra C. Carr,* Trudy M. Forte, Balz Frei

Abstract—Modification of low density lipoprotein (LDL) by myeloperoxidase-generated HOCl has been implicated in human atherosclerosis. Incubation of LDL with HOCl generates several reactive intermediates, primarily N-chloramines, which may react with other biomolecules. In this study, we investigated the effects of HOCl-modified LDL on the activity of lecithin-cholesterol acyltransferase (LCAT), an enzyme essential for high density lipoprotein maturation and the antiatherogenic reverse cholesterol transport pathway. We exposed human LDL (0.5 mg protein/mL) to physiological concentrations of HOCl (25 to 200 μmol/L) and characterized the resulting LDL modifications to apolipoprotein B and lipids; the modified LDL was subsequently incubated with apolipoprotein B–depleted plasma (density >1.063 g/mL fraction), which contains functional LCAT. Increasing concentrations of HOCl caused various modifications to LDL, primarily, loss of lysine residues and increases in N-chloramines and electrophoretic mobility, whereas lipid hydroperoxides were only minor products. LCAT activity was extremely sensitive to HOCl-modified LDL and was reduced by 23% and 93% by LDL preincubated with 25 and 100 μmol/L HOCl, respectively. Addition of 200 μmol/L ascorbate or N-acetyl derivatives of cysteine or methionine completely prevented LCAT inactivation by LDL preincubated with ≤200 μmol/L HOCl. Protecting the free thiol groups of LCAT with 5,5'-dithio-bis-(2-nitrobenzoic acid) before exposure to HOCl-modified LDL, which inhibits lipid hydroperoxide–mediated inactivation of LCAT, failed to prevent the loss of enzyme activity. Our data indicate that N-chloramines from HOCl-modified LDL mediate the loss of plasma LCAT activity and provide a novel mechanism by which myeloperoxidase-generated HOCl may promote atherogenesis. (Arterioscler Thromb Vasc Biol. 2001;21:1040-1045.)

Key Words: chloramines ▪ HDL ▪ LDL ▪ lecithin-cholesterol acyltransferase ▪ hypochlorous acid

Oxidative stress is thought to play a causal role in the pathogenesis of atherosclerosis.1 Although there are many determinants in the development of an atherosclerotic lesion, substantial in vitro evidence links oxidized forms of LDL to molecular processes relevant to atherogenesis.1 Recent in vitro and in vivo evidence suggests that myeloperoxidase (MPO) can oxidize LDL to an atherogenic form. Immuno-reactive and catalytically active MPO has been found in human atherosclerotic lesions.2 Moreover, a specific marker of MPO-catalyzed oxidation, 3-chlorotyrosine, is elevated 30-fold in LDL isolated from human lesions compared with plasma-derived LDL.3 In addition, immunohistochemical studies have revealed the presence of HOCl-modified proteins in human atherosclerotic lesions4 and the colocalization of MPO and HOCl-modified proteins with monocyte/macrophages, endothelial cells, and the extracellular matrix.5 A number of studies have also shown that HOCl-modified LDL exerts various pathophysiological effects on leukocytes and vascular cells.6–9 Thus, there is strong evidence for a role of MPO in LDL oxidation and human atherogenesis.

MPO is a heme-containing enzyme, released from activated neutrophils and monocytes, that catalyzes the production of strong oxidants.10 The predominant product of this enzyme at physiological chloride ion concentrations is HOCl,11 an oxidant that readily reacts with a variety of biomolecules, such as thiols, thioethers, ascorbate, and amines, including amino acids.12 Because of the high reactivity of HOCl, its reactions are dependent on the relative concentrations and reactivities of compounds in the immediate vicinity. Thiols and methionine residues are manyfold more reactive with HOCl than are other amino acids and amines.12 At neutral pH, reagent or MPO-generated HOCl preferentially oxidizes the apoB moiety of LDL.13,14 Neither LDL-associated lipids nor antioxidants (eg, α-tocopherol and β-carotene) appear to be major targets of HOCl.14,15 Of the various amino acid residues in apoB modified by HOCl, lysine residues quantitatively represent the major target.13,14

The reaction of HOCl with the ε-amino group of lysine residues results in the formation of N-chloramines (reaction 1). Lysine chloramines can decompose to form aldehydes (reaction 2) and/or react directly with free thiols and/or

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methionine residues. The reactions are as follows: reaction 1, R-CH₂-NH₂+HOCl→R-CH₂-NHCl+H₂O; reaction 2, R-CH₂-NHCl+H₂O→R-CH=O+N³H₄⁺+Cl⁻. It has been suggested that lysine chloramine–derived aldehydes participate in HOCl-induced cross-linking of apoB and aggregation of LDL, which result in the conversion of LDL to a ligand for the scavenger receptors of macrophages. However, it is important to note that lysine chloramines are more likely to react with free thiols and/or methionine residues than to decompose to aldehydes. The specificity of the former reactions suggests that biomolecules possessing biologically active cysteine and/or methionine residues may be inactivated by HOCl-modified LDL. Thus, unlike lipoygenase-dependent or metal ion–dependent modifications of LDL, which involve the derivatization of lysine residues by lipid hydroperoxide breakdown products, HOCl directly modifies apoB lysine residues to N-chloramines, which may enhance the atherogenicity of LDL in a number of ways.

Lecithin-cholesterol acyltransferase (LCAT), an enzyme essential for HDL maturation and the antiatherogenic reverse cholesterol transport pathway, has 2 free cysteine residues that can modulate enzymatic activity. It has been demonstrated that LCAT loses activity when exposed to copper-oxidized LDL or lipoygenase-generated hydroperoxides. The mechanism of this inactivation is thought to involve adduct formation between aldehydic lipid hydroperoxide breakdown products and the free cysteine residues of LCAT.

Methods

Materials

ApoA-I was isolated from human plasma and purified as previously described. Egg 1-α-phosphatidylcholine was purchased from Avanti Polar Lipids, and [4-¹⁴C]cholesterol was obtained from NEN Products. Human leukocyte MPO and HOCl were obtained from Calbiochem and Aldrich, respectively. 7-Fluorobenzo-2-oxa-1,3-diazole-4-sulfonamide (ABD-F) was from Molecular Probes. PD-10 gel filtration columns were from Pharmacia Biotech. All other reagents were from Sigma Chemical Co. All solvents were high-performance liquid chromatography (HPLC) grade. PBS was composed of 10 mmol/L sodium phosphate buffer, 140 mmol/L NaCl, and 100 μmol/L diethylenetriamine pentaacetic acid, pH 7.4.

Lipoprotein Isolation

Blood was obtained with informed consent from fasted volunteers. Heparin was used to prevent coagulation, and plasma was separated from cellular blood components by low-speed centrifugation (1200g, 4°C, 20 minutes). Lipoproteins (LDL, density [d]=1.019 to 1.063 g/mL; HDL, d=1.063 to 1.21 g/mL) were rapidly isolated from plasma by preparative sequential ultracentrifugation with use of an Optima-TL ultracentrifuge and a TLA-100.4 rotor (Beckman Instruments). Standard methods were used, but corrections were made for the higher gravitational forces generated by the Optima ultracentrifuge and the shorter path length of the TLA-100.4 rotor. Isolated lipoproteins were desalted by gel filtration with the use of PD-10 columns equilibrated with PBS. The protein content of isolated lipoproteins was determined by using the Lowry Micro Method Kit (Sigma P5656). The d=1.063 g/mL fraction of plasma (containing LCAT, HDL, and non-apoB plasma proteins) and lipoprotein-depleted plasma were isolated after a single ultracentrifugation step and dialyzed into PBS.

LDL Incubations With HOCl

HOCl was standardized at 292 nm [ε=350 (mol/L)⁻¹·cm⁻¹] as previously described. Bolus HOCl was added with gentle mixing to LDL (0.5 mg protein/mL) in PBS; incubations were carried out for 30 minutes at 37°C. Final HOCl concentrations ranged from 25 to 200 μmol/L, corresponding to HOCl:apoB molar ratios from 25:1 to 200:1. Modified LDL was kept on ice (for <1 hour) until characterized and used in incubations containing LCAT.

Characterization of LDL Protein Modifications

Unmodified lysine residues were assessed by fluorescence and density (e.g., total protein, cholesterol, and triglycerides) of LDL. LDLs exhibited no change in protein, triglycerides, or cholesterol content under incubation conditions (data not shown). LDLs were first incubated with HOCl for 30 minutes at 37°C, and excess HOCl was removed by dialysis in PBS. LDL protein content was assessed by fluorescence spectrophotometry. LDL was then incubated with apoA-I:egg yolk phosphatidylcholine:cholesterol at a molar ratio of 0.8:250:1. The assay is dependent on the amount of active LCAT and independent of endogenous plasma substrates and cofactors. In addition to the proteoliposome substrate, LCAT reaction mixtures contained 20 mmol/L Tris HCl (pH 8.0), 0.15 mol/L NaCl, 0.27 mmol/L EDTA, 0.5% human albumin, and 5 mmol/L β-mercaptoethanol. Aliquots (40 μL) of reconstituted plasma (i.e., mixtures of control or HOCl-modified LDL with the d=1.063 g/mL fraction) were added to start the reaction; incubations were carried out for 30 minutes at 37°C. The reaction was terminated by the addition of ethanol (final concentration 50%). After hexane extraction, the labeled reaction product ([¹³C]cholesterol ester) was separated from the reaction substrate ([¹³C]cholesterol) by thin-layer chromatography. The radioactivity associated with the labeled substrate and product was quantified by liquid scintillation counting. Results are expressed either as percent esterification of [¹³C]cholesterol per 30 minutes or as a percentage of control LCAT activity.

Assessment of LCAT Activity

LCAT activity was measured by the exogenous “common substrate” (i.e., proteoliposome) method of Chen and Albers. This method uses an excess of [¹³C]cholesterol-labeled proteoliposome substrate composed of human apoA-I:egg-yolk phosphatidylcholine:unesterified cholesterol at a molar ratio of 0.8:250:12.5. The assay is dependent on the amount of active LCAT and independent of endogenous plasma substrates and cofactors. In addition to the proteoliposome substrate, LCAT reaction mixtures contained 20 mmol/L Tris HCl (pH 8.0), 0.15 mol/L NaCl, 0.27 mmol/L EDTA, 0.5% human albumin, and 5 mmol/L β-mercaptoethanol. Aliquots (40 μL) of reconstituted plasma (i.e., mixtures of control or HOCl-modified LDL with the d=1.063 g/mL fraction) were added to start the reaction; incubations were carried out for 30 minutes at 37°C. The reaction was terminated by the addition of ethanol (final concentration 50%). After hexane extraction, the labeled reaction product ([¹³C]cholesterol ester) was separated from the reaction substrate ([¹³C]cholesterol) by thin-layer chromatography. The radioactivity associated with the labeled substrate and product was quantified by liquid scintillation counting. Results are expressed either as percent esterification of [¹³C]cholesterol per 30 minutes or as a percentage of control LCAT activity.
**Characterization of HOCl-Modified LDL**

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<th>Cys, μmol/L</th>
<th>Trp, μmol/L</th>
<th>Lys, μmol/L</th>
<th>RNHCl, μmol/L</th>
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</table>

Values are mean±SD (n=4).

Ultracentrifugally isolated human LDL was incubated with varying concentrations of HOCl ranging from 25 to 200 μmol/L, corresponding to HOCl:apoB molar ratios of 25:1 to 200:1. Decreases in LDL-associated cysteine (Cys), tryptophan (Trp), and lysine (Lys) residues were assessed fluorometrically and were determined by assuming 4, 37, and 356 Cys, Trp, and Lys residues per LDL particle, respectively. Increases in LDL-associated N-chloramines (RNHCl) were calculated by the thionitrobenzoic acid assay, and percentage increases in the net negative charge of LDL was preincubated for 30 minutes at 37°C with the d>1.063 g/mL fraction of human plasma (containing HDL, LCAT, and plasma proteins). The reconstituted plasma incubations contained 50 μg of LDL protein and represented a 3-fold dilution of plasma with an LDL protein concentration of 1 mg/mL. Aliquots of this incubation mixture were subsequently assayed for LCAT activity as described in Methods. Results are expressed as percentage of control LCAT activity (15±1% esterification of [14C]cholesterol over 30 minutes). Values are mean±SD (n=4).

**Effects of HOCl-Modified LDL on LCAT Activity**

Incubation of HOCl-modified LDL with the d>1.063 g/mL fraction of plasma with 0.5 mg/mL BSA treated with 25 to 200 μmol/L HOCl resulted in a maximal decrease in LCAT activity by only 13%. These data suggest that the reactive component(s) responsible for inactivation of LCAT is specific to lipoproteins and/or that there is a specific interaction between LCAT and the lipoproteins that does not occur with BSA.

**Results**

Characterization of HOCl-Modified LDL

As previously reported by us, treatment of LDL with increasing concentrations of HOCl (25 to 200 μmol/L) resulted in dose-dependent oxidation of apoB cysteine, tryptophan, and lysine residues (Table). Because apoB contains approximately 4 cysteine, 37 tryptophan, and 356 lysine residues, these data suggest that thiols are more sensitive to modification by HOCl than are tryptophan residues and that tryptophan residues are more sensitive than are lysine residues. A small increase in the electrophoretic mobility of LDL was also observed (1.5-fold at 200 μmol/L HOCl, Table) and is likely the result of HOCl-induced lysine modification. As expected, the decrease in apoB lysine residues and the increase in LDL electrophoretic mobility reflect the incremental increases in formation of N-chloramines (Table). Similar modifications were observed by using MPO/HOCl/Cl− to generate HOCl instead of adding reagent HOCl (data not shown).

HOCI-modified BSA had very little effect on LCAT activity. In 2 independent experiments, incubation of the d>1.063 g/mL fraction of plasma with 0.5 mg/mL BSA treated with 25 to 200 μmol/L HOCl resulted in a maximal decrease in LCAT activity by only 13%. These data suggest that the reactive component(s) responsible for inactivation of LCAT is specific to lipoproteins and/or that there is a specific interaction between LCAT and the lipoproteins that does not occur with BSA.

Role of N-Chloramines in LCAT Inactivation by HOCl-Modified LDL

To determine the reactive species associated with HOCl-modified LDL and the possible amino acid target(s) on LCAT, ascorbate and several N-acetylated amino acids were incubated with HOCl-modified LDL before its addition to the LCAT-containing samples (Figure 2). The sulfur-containing amino acids cysteine and methionine (200 μmol/L each) had very little effect on LCAT activity.
significantly inhibited inactivation of LCAT by HOCl-modified LDL (Figure 2A). In contrast, the N-acetyl derivatives of tryptophan, lysine, histidine, and tyrosine (200 μmol/L each) did not exert any significant effect on inactivation of LCAT by HOCl-modified LDL (Figure 2B). These data mimic the reactivity of model N-chloramines, such as N-acetyl-lysine chloramine, toward these amino acids.16 Pre-incubation of HOCl-modified LDL with 200 μmol/L ascorbate, which we have previously shown to reduce N-chloramines back to their parent amines,16 also significantly inhibited the inactivation of LCAT (Figure 2A). Higher concentrations of ascorbate (400 μmol/L) completely prevented enzyme inactivation (data not shown). These data suggest that LDL-associated N-chloramines are the reactive species involved in inactivation of LCAT and that the likely target(s) on LCAT are cysteine and/or methionine residues.

Role of Lipid Hydroperoxides in LCAT Inactivation by HOCl-Modified LDL

Recent studies have reported that oxidized lipids,20 in particular, lipid hydroperoxides,21,22 can inhibit LCAT activity. Thus, it is possible that lipid hydroperoxides rather than, or in addition to, N-chloramines are responsible for the inactivation of LCAT by HOCl-modified LDL. Treatment of LDL with increasing concentrations of HOCl resulted in a small dose-dependent increase in lipid hydroperoxides, which was inhibited by the lipid-soluble antioxidant butylated hydroxytoluene (BHT), as shown in Figure 3A. In contrast, treatment of LDL with HOCl did not cause an increase in TBARS from background levels (~2 nmol/mg LDL protein, data not shown). Furthermore, no significant loss of vitamin E was observed in LDL exposed to concentrations of HOCl of up to 200 μmol/L (data not shown), consistent with previous reports.13,14 HOCl-modified LDL containing BHT, and thus containing decreased levels of lipid hydroperoxides (Figure 3A), was equally potent in inhibiting LCAT activity as was HOCl-modified LDL not containing BHT (Figure 3B). These data suggest that LDL-associated lipid hydroperoxides are not involved in the inactivation of LCAT by HOCl-modified LDL.

Discussion

The present study demonstrates that HOCl-modified LDL inhibits plasma LCAT activity. The HOCl concentrations required for LDL modification to completely inhibit LCAT activity (ie, ~100 to 150 μmol/L) are well within the range considered physiologically relevant (ie, the levels generated extracellularly over a 30-minute period by 5×10⁶ neutrophils/mL).33,34 Inactivation of LCAT by HOCl-modified LDL appears to be independent of lipid hydroperoxides and, at least in part, dependent on N-chloramines, inasmuch as ascorbate completely inhibited inactivation of the enzyme by HOCl-modified LDL. Although HOCl could potentially inactivate LCAT directly, its reactivity is less selective than that of N-chloramines, which, as we have previously shown, react predominantly with thiol(s), methionine, and ascorbate.16 Also, the association of LCAT with oxidatively modified lipoproteins may enhance inactivation of the enzyme by LDL-associated N-chloramines (see below).

Interestingly, inactivation of LCAT by LDL-associated chloramines does not appear to involve thiol modification but, rather, may involve oxidation of methionine residues. There are several lines of evidence in support of this notion: (1) reversible protection of the thiol groups of LCAT with DTNB did not
inhibit inactivation of the enzyme by HOCl-modified LDL; (2) preincubation of HOCl-modified LDL with N-acetyl-methionine protected against subsequent inactivation of LCAT; (3) methionine residues are the only other major protein-associated targets, apart from thiol residues, that are readily oxidized by N-chloramines; and (4) preliminary experiments have shown that HOCl-modified LDL mediates inactivation of the methionine-dependent protein α1-antiproteinase by an N-chloramine–dependent mechanism (see below).

Our data suggest that HOCl-modified LDL inhibits LCAT activity by a mechanism different from that proposed for LCAT inactivation by copper-oxidized LDL. In contrast to copper-induced oxidation of LDL, the HOCl concentrations used in the present study did not affect LDL-associated TBARS or vitamin E levels. Use of a sensitive HPLC-postcolumn chemiluminescence method demonstrated that HOCl induced the formation of small amounts of lipid hydroperoxides. However, the levels of lipid hydroperoxides formed were not associated with a measurable increase in LCAT inactivation. It should be noted that copper-oxidized LDL containing 4 to 7 nmol TBARS/mg protein also contains substantial levels of lipid hydroperoxides (9 to 25 nmol/mg LDL protein; M.R. McCall, B. Frei, unpublished data, 2000) but reduces LCAT activity by only 50%. The mechanism by which HOCl-modified LDL inhibits LCAT activity likely involves N-chloramines, which account for 30% of the HOCl added to LDL. The N-acetyl derivatives of cysteine and methionine, as well as ascorbate, effectively protected against inactivation of LCAT by HOCl-modified LDL. We have shown previously that both ascorbate and these sulfur-containing amino acids are effective scavengers of N-chloramines. In contrast, the N-acetyl derivatives of tryptophan, lysine, histidine, and tyrosine, which cannot scavenge N-chloramines, did not protect against LCAT inactivation.

Inactivation of LCAT by lipid hydroperoxides isolated from copper-oxidized LDL is thought to involve the free cysteine residues of LCAT. Although N-chloramines react readily with thiol groups, reversible blocking of the free cysteine residues of LCAT with DTNB did not protect against loss of enzyme activity by HOCl-modified LDL. Oxidation of the thiol groups to higher oxidation states, such as sulfenic or sulfinic acids, may alter the enzymatic activity of LCAT by oxidizing methionine residues. The latter may involve oxidation of methionine residues and thereby modifying protein conformation. Although N-acetyl histidine did not protect against inactivation of LCAT by HOCl-modified LDL, we cannot rule out the possibility that N-chloramines directly affect the amino acids in the catalytic site of LCAT (ie, aspartate, histidine, and serine). However, it is of particular interest to note that lipid hydroperoxides and N-chloramines can oxidize methionine residues. This suggests that LDL-associated protein modifications induced by HOCl and LDL-associated lipid modifications induced by copper or lipooxygenase ultimately contribute to LCAT inactivation via this common mechanism.

A number of proteins have been shown to be inactivated via N-chloramine–dependent oxidation of essential methionine residues to methionine sulfone. In plasma, these include the protease inhibitors α1-antiproteinase and α2-macroglobulin as well as the fifth component of human complement. Oxidation of the methionine residues was associated with conformational changes in some of the proteins. We have found that HOCl-modified LDL also inactivates α1-antiproteinase with an IC50 of 58 μmol/L HOCl used to modify LDL (0.5 mg protein/mL). This inactivation is presumably due to oxidation of the reactive site methionine residue in α1-antiproteinase by LDL-associated N-chloramines, inasmuch as preincubation of the HOCl-modified LDL with ascorbate almost completely abrogated inactivation of the protease inhibitor (A.C. Carr, B. Frei, unpublished data, 2001).

The data of the present study indicate that the reaction of HOCl-modified LDL with LCAT is specific to lipoproteins. For example, HOCl-modified albumin did not inhibit LCAT, in contrast to HOCl-modified HDL. Because it is unlikely that N-chloramines formed on LDL transfer to HDL, where they react with LCAT, our data suggest that LCAT and LDL interact directly in the reconstituted plasma system. On the basis of binding affinities, Kosel et al. estimated that 20% of LCAT is bound to LDL in plasma. Indirect evidence for an association of LCAT with HDL has been suggested by Carr, B. Frei, unpublished data, 2000). The degree of LCAT inactivation by HOCl-modified LDL in whole plasma was less than that in reconstituted plasma, suggesting some protection by low molecular weight antioxidants, such as ascorbate. Thus, it would appear that a localized microenvironment within the artery wall in which antioxidant defenses have been depleted is the most likely site for LCAT inactivation by HOCl-modified LDL to occur in vivo.

HDL has antiatherogenic properties that are due to multiple functions in the reverse cholesterol transport pathway. It facilitates the efflux and net transfer of excess cholesterol from atherosclerotic foam cells; it provides the activator (ie, apoA-I) and substrates (ie, cholesterol and phospholipids) for LCAT, the enzyme that maintains the concentration gradient along which foam cell–derived cholesterol flows; and it facilitates the transport of foam cell–derived cholesterol to the liver for reutilization or catabolism. HOCl-modified HDL impair cholesterol efflux from macrophages, and we have now shown that HOCl-modified LDL is a potent inhibitor of LCAT activity. Thus, HOCl-modified lipoproteins (HDL and LDL) may enhance atherosclerosis in part by impairing the antiatherogenic reverse cholesterol transport pathway.

Although the exact role of LCAT in human atherosclerosis and coronary artery disease remains to be established, a few studies have shown that LCAT activity is significantly reduced (24% to 50% of control individuals) in patients with coronary artery disease and in patients after myocardial infarction. Furthermore, there is evidence that some cases of human LCAT deficiency are associated with premature atherosclerosis and coronary artery disease.

In summary, we have shown that inactivation of LCAT by HOCl-modified LDL is independent of lipid hydroperoxides but is, at least in part, dependent on N-chloramines. The small molecule antioxidant ascorbate, which eliminates N-chloramines, completely inhibited inactivation of the enzyme. Inhibition of LCAT activity by HOCl-modified LDL does not appear to involve thiol modification but may involve oxidation of methionine residues. The latter may
result in conformational changes and subsequent LCAT inactivation, suggesting a novel additional mechanism by which MPO-derived HOCl may accelerate atherosclerosis.

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References
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