LDL Modified by Hypochlorous Acid Is a Potent Inhibitor of Lecithin-Cholesterol A cyltransferase 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. Immunoreactive 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...
methionine residues. The reactions are as follows: reaction 1, \( R-CH_2-NH_2 + HOCl \rightarrow R-CH_2-NHCl + H_2O \), reaction 2, \( R-CH_2-NHCl + H_2O \rightarrow R-CH=O + NH_3 + Cl^- \). 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 lipoxynase-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 lipoxygenase-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. Considering the lability of N-chloramines with free thiols.

**Methods**

**Materials**

ApoA-I was isolated from human plasma and purified as previously described. Egg 1-\( \alpha \)-phosphatidylcholine was purchased from Avanti Polar Lipids, and [\( ^4 \)C]cholesterol was obtained from NEN Products. Human leukocyte MPO and HOCl were obtained from Calbiochem and Aldrich, respectively. Thiobarbituric acid–reactive substances (TBARS) in LDL were determined, as previously described, by use of a specific and sensitive method with HPLC separation and post-column chemiluminescence detection. Vitamin E was assessed by HPLC with electrochemical detection as previously described.

**Incubations of HOCl-Modified LDL With LCAT**

Control and HOCl-modified LDLs were added to the d\( > \)1.063 g/mL fraction of human plasma (containing HDL, LCAT, and plasma proteins) and incubated at 37°C for 30 minutes. Preliminary studies demonstrated that LCAT inactivation was complete within 15 minutes, and incubations lasting >30 minutes did not result in additional loss of LCAT activity. The reconstituted plasma incubations contained 50 \( \mu \)g of LDL protein and represented a 3-fold dilution of plasma with an LDL protein concentration of 1 mg/mL. In some experiments, ascorbate or the \( \alpha \)-acetyl derivatives of cysteine, histidine, lysine, methionine, tryptophan, and tyrosine (final concentration 200 \( \mu \)mol/L) were preincubated at 37°C for 30 minutes with HOCl-modified LDL. In other experiments, the d\( > \)1.063 g/mL fraction of plasma was incubated at 37°C for 30 minutes in the presence or absence of the reversible thiol-blocking reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB, 1.7 mmol/L); excess DTNB was subsequently removed by dialysis in PBS. LCAT activity was completely inhibited after this procedure and could be restored by the addition of 5 mmol/L \( \beta \)-mercaptoethanol.

**Assessment of LCAT Activity**

LCAT activity was measured by the exogenous “common substrate” (ie, proteoliposome) method of Chen and Albers. This method uses an excess of [\( ^{14} \)C]cholesterol–labeled proteoliposome substrate composed of human apoA-Egg-gylk 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 \( \mu \)mol/L Tris HCl (pH 8.0), 0.15 mmol/L NaCl, 0.27 mmol/L EDTA, 0.5% human albumin, and 5 mmol/L \( \beta \)-mercaptoethanol. Aliquots (40 \( \mu \)L) of reconstituted plasma (ie, 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 ([\( ^{14} \)C]cholesterol ester) was separated from the reaction substrate ([\( ^{14} \)C]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 [\( ^{14} \)C]cholesterol per 30 minutes or as a percentage of control LCAT activity.
Characterization of HOCl-Modified LDL

<table>
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<th>HOCl, μmol/L</th>
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<th>Trp, μmol/L</th>
<th>Lys, μmol/L</th>
<th>RNHCl, μmol/L</th>
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<td>68±7</td>
<td>80±27</td>
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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.31,32 Increases in LDL-associated N-chloramines (RNHCl) were calculated by the thionitrobenzoic acid assay, and percentage increases in the net negative charge of LDL (ie, increases in anodic mobility) were assessed by agarose gel electrophoresis with Beckman Paragon Lipo-gels. REM indicates relative electrophoretic mobility.

Results

Characterization of HOCl-Modified LDL

As previously reported by us,16 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,32 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/H₂O₂/Cl⁻ to generate HOCl instead of adding reagent HOCl (data not shown).

Effects of HOCl-Modified LDL on LCAT Activity

Incubation of HOCl-Modified LDL with the d>1.063 g/mL fraction of human plasma (containing HDL, LCAT, and plasma proteins) resulted in dose-dependent inactivation of LCAT activity (Figure 1), as determined by the exogenous substrate assay observed. In contrast to HOCl-modified lipoproteins, HDL-associated apoA-I can account for the loss of enzymatic activity observed. In contrast to HOCl-modified lipoproteins, HOCl-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 little effect on LCAT activity.

HOCI-modified LDL effectively inhibits LCAT activity. LDL modified with the indicated concentrations of HOCl was incubated at 37°C for 30 minutes 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).

Figure 1. HOCl-modified LDL effectively inhibits LCAT activity. LDL modified with the indicated concentrations of HOCl was incubated at 37°C for 30 minutes 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).

Note: Cys, cysteine; Trp, tryptophan; Lys, lysine; RNHCl, N-chloramines; REM, relative electrophoretic mobility.
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.

Involvement of Thiols in LCAT Inactivation by HOCl-Modified LDL

Because LCAT contains 2 free cysteine residues (Cys31 and Cys184) located in proximity to its active site,19 it is possible that LDL-associated N-chloramines are inactivating the enzyme by oxidizing the thiols to sulfenic or sulfinic acids. Therefore, the LCAT-containing samples were preincubated with the reversible thiol-specific reagent DTNB to sterically block the active site of LCAT and prevent its free cysteine residues from reacting with HOCl-modified LDL. However, treatment of LCAT with DTNB failed to inhibit inactivation of the enzyme by HOCl-modified LDL (Figure 4). These data suggest that inactivation of LCAT by HOCl-modified LDL is thiol independent and may, therefore, involve oxidation of the methionine residues of LCAT.

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. 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 thiols, methionine, and ascorbate.15 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 chemilumines-
cence 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 measur-
able 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 pro-
tected 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 trypto-
phan, 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 sulfonic acids, may
not cause sufficient steric hindrance to inhibit the enzyme
compared with derivatization of the thiole with DTNB.

Thus, we hypothesize that LDL-associated N-chloramines alter the enzymatic activity of LCAT by oxidizing 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 lipox-
genase 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 resi-
dues to methionine sulfoxide. In plasma, these include the pro-
tease 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 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
case of 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, Kosek et al estimated that ≈20% of LCAT is
bound to LDL in plasma. Indirect evidence for an association
of LCAT with HOCl-modified LDL was obtained when reagent
HOCl-mediated was compared with HOCl-modified LDL-
mediated inactivation of LCAT in whole plasma. Direct addition
of 100 and 200 µmol/L HOCl to plasma did not cause measur-
able inhibition of LCAT activity, whereas addition of LCAT
modified with 100 or 200 µmol/L HOCl caused activity, respectively (M.R. McCall, A.C.
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 trans-
port of foam cell–derived cholesterol to the liver for reutilization
or catabolism. HCl-modified HDL impairs cholesterol efflux
from macrophages, and we have now shown that HOCl-
modified LDL is a potent inhibitor of LCAT activity. Thus,
HCl-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. Fur-
thermore, 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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