Inhibitory Effects of Oxidized Low-density Lipoprotein on the Activity of Plasminogen: Cholesterol Acyltransferase

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Lechitin:cholesterol acyltransferase (LCAT) is an enzyme that is important in the cholesterol reverse-transport system by converting the surface cholesterol of high-density lipoprotein (HDL) to cholesterol ester inside HDL. We have investigated changes in plasma LCAT activity induced by oxidized low-density lipoprotein (ox-LDL). (i) LCAT activity was inhibited by the addition of even a small amount of ox-LDL. (ii) This inhibitory effect was dose-dependent and was related to the degree of oxidation of LDL. (iii) As this inhibitory effect was not prevented by antioxidants, it is suggested that it does not occur via oxidation of substrate HDL. These results suggest that cholesterol reverse-transport via LDL may be affected by ox-LDL and that they are noteworthy in suggesting a new physiological function for ox-LDL.

Key words: lechitin-cholesterol acyltransferase activity; oxidized low-density lipoprotein; high-density lipoprotein; cholesterol reverse-transport

In plasma, lechitin:cholesterol acyltransferase (LCAT) [EC 2.3.1.43] mostly associates with substrate high-density lipoprotein (HDL) and produces cholesterol ester by transferring a fatty acid of phosphatidylcholine to the 3-OH group of unesterified cholesterol. This reaction is important in cholesterol reverse-transport by HDL and therefore the properties of this enzyme have been much studied.

It is well known that unesterified cholesterol molecules can be exchanged between cells and extracellular lipoproteins, the efflux process being thought to be mediated by HDL. This clearance of cholesterol from peripheral cells is the first step in the reverse transport of excess cholesterol to he liver for excretion from the body.

On the other hand, low-density lipoprotein (LDL) particles are small enough to penetrate between endothelial cells and therefore they can accumulate within the intima.

LDL in the intima is oxidized by various species of active oxygen released by macrophages and other cells in the arterial wall. Oxidized LDL (ox-LDL) may be recognized by the scavenger receptors of macrophages; its uptake by these receptors may promote the formation of foam cells and thus increase cholesterol accumulation in the arterial wall. Moreover, several studies have revealed that ox-LDL may exist in plasma and a fraction of human plasma LDL has been identified that has characteristics resembling ox-LDL. Recent studies have further shown that antibodies circulate in human plasma that can recognize ox-LDL, but not native LDL.

It has been reported that the activity of LCAT is affected by various factors in the body (e.g., disease, dietary treatment). This study was designed to investigate whether changes in plasma LCAT activity result from the presence of ox-LDL.

Materials and Methods

Materials. Cholesterol (99+%), crystallized lyophilized bovine serum albumin, and t-phosphatidylcholine (egg yolk lecithin, Type III-E) were all obtained from Sigma Chemicals. TLC plates, silic acid-impregnated plastic sheets, was purchased from KODAK. [7(N)-3H]-cholesterol (specific activity: 263 (GBq mmol) were purchased from Amersham Corp. All other chemicals were of reagent grade or better.

LDL preparation. Human plasma LDL (1.006-1.063 g ml) was prepared, from fresh human plasma derived from fasting nonobese subjects, by sequential ultracentrifugation using a Beckman TL-100 at 100,000 rpm for 4 h at 4 C. The isolated lipoprotein were dialyzed against 0.02 M phosphate buffered saline, 0.15 M NaCl, pH 7.4 and stored under N2 at 4 C until use.

LDL modification. The dialyzed LDL was diluted to 0.2 mg protein ml and oxidative modification done by treatment with 5 μm copper sulfate at 37 C for various incubation times. The oxidation was stopped by the addition of 1 mm EDTA and 0.02 mm butylated hydroxytoluene and the ox-LDL dialyzed against 0.02 M phosphate buffered saline, 0.15 M NaCl, pH 7.4.

Purification of human LCAT and apo A-I. Human plasma LCAT was purified with the method developed by Doi and Nissula. The final preparation of human LCAT was purified approximately 5900-fold with a 14% yield. Human plasma apolipoprotein A-I (apo A-I) was prepared in a similar way to that described previously. This preparation of human apo A-I gave a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Enzyme assay. The activity of the enzyme LCAT was measured using one of two methods. LDL or oxidized LDL were added to the medium before the LCAT reaction had begun. The first, Stokke and Norum's method, involved the addition to 100 μl of whole human plasma and 30 μl of [7(N)-3H]-cholesterol (533 Bq) in 50 mg ml bovine serum albumin emulsion; this was incubated for 4 h at 37 C under N2 with 20 μl of 10.4 mM DTNB, which is an LCAT inhibitor. The reaction was started by the addition of 20 μl of 100 mM 2-mercaptoethanol at 37 C. Following incubation, the lipids were extracted and separated by TLC, using hexane diethylether acetic acid (70:30:1, v/v). The radioactivity in cholesterol and cholesterol ester fractions was measured using a liquid scintillation

Abbreviations: apo A-I, apolipoprotein A-I; DTNB, 5,5-dithiobis (2-nitrobenzoic acid); HDL, high-density lipoprotein; LCAT, lecithin: cholesterol acyltransferase; LDL, low-density lipoprotein; ox-LDL, oxidized low density lipoprotein; VIDL, very-low density lipoprotein.

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counter. In the second method, the enzyme activity was measured in a manner similar to that described previously.24,25* Lecehin-cholesterol vesicles prepared by the method of Batzri and Korn26* was used as substrate for the enzyme assay. A typical preparation contained 900 nmol egg yolk phosphatidylcholine and 150 nmol of [7N–3H]cholesterol per ml. The assay mixture consisted of 100 μl of vesicle solution, 15 μg of human apo A1, 4 mm 2-mercaptoethanol, 0.7 mm EDTA, 2.5 mg of bovine serum albumin, and the enzyme in a final volume of 250 μl of 39.2 mm sodium phosphate buffer, pH 7.4. Test-tubes containing assay mixture were reacted for 30 min at 37 °C under N2. The rest of the procedure was the same as in the first method.

Characteristics of oxidized LDL. The degree of oxidative modification of LDL was measured using one of two methods. The first, the relative fluorescence spectrum was measured for each sample by scanning the emission spectra at an excitation wavelength of 365 nm. In the second method, ion-exchange chromatography was done using a Pharmacia FPLC system consisting of an LCC-500 gradient programmer, two P-500 pumps, and a mono Q HR 5/5 column.27* Protein was estimated by the method of Lowry.28* Cholesterol in lipoprotein was measured using an enzymatic kit purchased from Wako Chemicals, Japan.

Results

The inhibitory effects of ox-LDL on LCAT activity

The effects of ox-LDL on plasma LCAT activity are shown in Figs. 1 and 2. Figure 1 shows the inhibitory effect of ox-LDL on LCAT activity measured using Stokke and Norum's method. The activity of LCAT was progressively inhibited by ox-LDL in a dose-dependent manner; with 20 μg of added ox-LDL protein, a considerable (40%) inhibition of LCAT activity was observed. The inhibitory action on LCAT activity of various ox-LDL samples which had undergone different degrees of oxidation (depending on their incubation times with Cu2+) is shown in Fig. 2. The LCAT activity was inhibited as a function of the degree of oxidative modification up to that induced by 5 h (40 μg) or 12 h (20 μg) incubation time. These results suggest that the plasma LCAT activity is influenced both by the amount and the degree of oxidation of LDL in plasma.

The inhibitory effects of ox-LDL on artificial substrate with purified enzyme

The inhibitory effects of ox-LDL on LCAT activity was examined using purified enzyme and cofactor in a vesicle solution. As shown in Fig. 3, the inhibitory effect was observed with very small amounts of ox-LDL. With a 1-hour reaction time, the addition of even 10 μg ox-LDL to the medium greatly reduced the LCAT activity (to about 40% of that seen when native LDL was added).

Effects of antioxidants on inhibition of LCAT activity by oxidized LDL

In this experiment, to protect lipid against oxidation of substrate phosphatidylcholine on the HDL surface, one of a number of antioxidants, ascorbate, α-tocopherol, and probucol were added before the LCAT reaction had begun. Table shows the effects of antioxidants on inhibition of LCAT activity by oxidized LDL. The dose of these antioxidants was efficient in protecting lipid against oxidation.

![Graph 1](image1)

**Fig. 1.** The Effects of Oxidized LDL on LCAT Activity. Oxidized LDL samples were prepared by incubation with 5 μg CuSO4 for 12 h at 37 °C. All LDL activity was measured using Stokke and Norum's method. LDL or oxidized LDL were added to the medium which was then incubated under standard assay conditions. Activity was calculated as the ratio of cholesterol counts to total radioactivity. Values shown are mean ± SEM of triplicate observations. Open circles, native LDL added; closed circles, 12 h-oxidized LDL added.

![Graph 2](image2)

**Fig. 2.** The Relationship of LDL Oxidation Time to LCAT Activity. Oxidized LDL samples were prepared by incubation with 5 μg CuSO4 for various times at 37 °C. LCAT activity was measured using Stokke and Norum's method. Oxidized LDL (20 μg or 40 μg) was added to the medium which was then incubated under standard assay conditions. Values shown are mean ± SEM of triplicate observations. Circles, 20 μg of oxidized LDL added; squares, 40 μg of oxidized LDL added.

![Graph 3](image3)

**Fig. 3.** The Inhibitory Effect of Oxidized LDL on Artificial Substrate with Purified Enzyme. Oxidized LDL samples were prepared by incubation with 5 μg CuSO4 for 12 h at 37 °C. Each 100 μl of vesicle solution contained 90 nmol egg yolk phosphatidylcholine and 15 nmol of [7N–3H]cholesterol (specific activity, 263 GBq nmol). Purified human enzyme (0 units) and 15 μg of apo A1 were added to the medium containing vesicle and either LDL or oxidized LDL added, which was then incubated under the standard assay conditions. Values shown are mean ± SEM of triplicate observations. Open circles, native LDL added; closed circles, oxidized LDL added.
Characteristics of oxidized LDL

The degree of oxidative modification of LDL was measured using one of two methods for ox-LDL. Figures 4(a) and (b) show, respectively, the fluorescence spectrum and FPLC elution pattern of ox-LDL. As the time of incubation with CuSO₄ increased, LDL was detected as a rising fluorescence intensity resulting from lipid peroxidation and protein modification (Fig. 4(a)) and as an increase in the fraction number reflecting a net increment in negative charge (Fig. 4(b)). These data indicate that it was both lipid and protein in the LDL that underwent oxidation.

Discussion

These experiments indicated that ox-LDL added to plasma inhibits the esterification of cholesterol by LCAT. As shown in Fig. 1, plasma LCAT activity was reduced to about half the value in intact plasma by the addition of a small amount (one to five with plasma cholesterol) of ox-LDL. Further, the degree of this inhibition increased both with the amount of added ox-LDL (Fig. 1) and with the degree of oxidative modification (Fig. 2). This inhibition was also observed when using purified enzyme and cofactor in vesicle solution. As shown in Fig. 3, the addition of even 10 μg of ox-LDL to the medium greatly reduced the LCAT activity. In this case, the amount of added lipoprotein was less than one to two with the free cholesterol in the medium.

The phenomenon of foam-cell formation via the uptake of ox-LDL by macrophage-scavenger receptors is well known. Cholesterol from various tissues is thought to be transferred to the liver by the cholesterol reverse-transports system. This process, LCAT in plasma mostly associates with HDL and is important in cholesterol esterification. Nevertheless, no study has yet identified the effect of ox-LDL on this system. This paper reports the effect of the ox-LDL on plasma cholesterol esterification and suggests a new physiological role for ox-LDL. Having established that the inhibition occurred, we then tried in two ways to identify the mechanism underlying it.

First, the activity of LCAT is sensitive to the kind of phosphatidylcholine molecular species in the substrate HDL or lecithin-cholesterol vesicle. Lipid peroxides can oxidize other lipids via a chain reaction, and the possibility exists that the inhibitory effect of ox-LDL on LCAT activity was caused by oxidation of substrate phosphatidylcholine on the HDL surface via such a chain reaction. In this experiment, one of a number of antioxidants was added before the LCAT reaction had begun. Ascorbate (3,3-dimethyl-1-cyano-2-propenyl) (major water phase antioxidant), α-tocopherol, and probucol (chain-breaking lipid phase antioxidants) were selected as they are reported to be efficient in protecting lipid against oxidation. However, as shown in Table, none had any effect on the inhibition by ox-LDL.

Second, in the elution profile from FPLC chromatography, we could detect some interaction between ox-LDL

Table

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Concentration</th>
<th>Esterified cholesterol (nmol h⁻¹)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>LDL</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>77.2 ± 5.0</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>100 μM</td>
<td>96.5 ± 5.3</td>
</tr>
<tr>
<td></td>
<td>200 μM</td>
<td>100.5 ± 4.8</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>100 μM</td>
<td>72.6 ± 2.2</td>
</tr>
<tr>
<td></td>
<td>200 μM</td>
<td>64.4 ± 0.3</td>
</tr>
<tr>
<td>Probucol</td>
<td>10 μM</td>
<td>78.8 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>20 μM</td>
<td>77.2 ± 5.0</td>
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</table>

Oxidized LDL samples were prepared by incubation with 5 μM CuSO₄ for 12 h at 37 C. After addition of ascorbate in water or of α-tocopherol or probucol in ethanol to the medium, 20 μg of native LDL or oxidized LDL was added and enzyme activity measured by Stokke and Norn's method. Values shown are mean ± SEM of triplicate observations.

Fig. 4. The Fluorescent Spectra (a) and Chromatographic Patterns Obtained by FPLC (b) of Oxidized LDL.

Oxidized LDL samples were prepared by incubation with 5 μM CuSO₄ for various times at 37 C. (a). The relative fluorescence spectrum was measured for each sample by scanning the emission spectra at an excitation wavelength of 365 nm. A Pharmacia FPLC system was used for anion exchange chromatography using a mono Q HR 5.5 column. The multistep gradient elution was done as shown in the figure with two buffers, A, 0.01 M Tris-HCl containing 1 mM EDTA, pH 7.4; B, 1 M NaCl in buffer A, at 1 ml/min flow rate. The effluent was monitored by 280 nm absorbance using a single-path ultraviolet monitor and 1 ml fractions were collected.
and HDL on the other hand, and LCAT (data not shown). This suggests the possibility that the enzyme transferred from HDL to ox-LDL and/or that a direct interaction between ox-LDL and HDL affected LCAT activity as a result of degeneration of substrate HDL.

In recent years, new properties (e.g., minimally-modified LDL) and functions of ox-LDL have been reported. There are several reports that can be taken to suggest that a significant amount of ox-LDL exists in human plasma. It is tempting to speculate that this ox-LDL or that in the endothelial cells of an early atherosclerotic region might inhibit HDL-associated LCAT and have an influence over cholesterol reverse-transport involving HDL. It has been reported that changes in LCAT activity may be a factor in determining the risk of coronary heart disease.

Considering the importance of LCAT in cholesterol metabolism, the identification of mechanisms influencing its activity represents an interesting and enlightening study.

References