Inhibitory Effects of Lipid Oxidation on the Activity of Plasma Lecithin-Cholesterol Acyltransferase

Shin KAMIYAMA,† Tokuhisa YAMATO, and Yuji FURUKAWA

Laboratory of Nutrition, Department of Applied Biological Chemistry, Faculty of Agriculture, Tohoku University, Sendai 981-8555, Japan

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We investigated the effects of free radical generation on the esterification of cholesterol by lecithin-cholesterol acyltransferase (LCAT). A water-soluble free radical initiator, 2,2'-azobis-amidinopropane dihydrochloride (AAPH), inhibited the activity of plasma LCAT as a function of the incubation time after its addition. When a small amount of oxidized HDL was added to plasma, LCAT activity was dose-dependently inhibited. To identify the effects of HDL oxidation on LCAT activity, a purified enzyme and cofactor in a vesicle solution (an artificial substrate) were used. i) LCAT activity was inhibited by the oxidation of substrate vesicles, this inhibition being related to the degree of oxidation. ii) This inhibition was observed even if apolipoprotein A-I was not oxidized. iii) Oxidized phosphatidylcholine, but not oxidized cholesterol, in the vesicles affected LCAT activity. iv) The addition of 0–40% of oxidized vesicles to normal substrate vesicles resulted in the activity of LCAT being inhibited in a dose-dependent manner. These results suggest that the esterification of cholesterol by LCAT may be affected by the oxidation of substrate phosphatidylcholine via free radical generation in the plasma.

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

In plasma, lecithin-cholesterol acyltransferase (LCAT) [EC 2.3.1.43] mostly associates with substrate high-density lipoprotein (HDL) and produces a cholesterol ester by transferring a fatty acid of phosphatidylcholine to the 3-OH group of unesterified cholesterol.1–3) This reaction plays an important role in cholesterol reverse-transport by HDL, and therefore the properties of this enzyme have been extensively studied.4–6) 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.7) This clearance of cholesterol from peripheral cells is the first step in the reverse transport of excess cholesterol to the liver for excretion from the body.8) It has been demonstrated that LDI9,10) and HDL11) undergo oxidative modification, a process which results in perturbation of both the physicochemical and biological properties of the particles. Lipid peroxidation of HDL results in a loss of the ability of these lipoproteins to incorporate cholesterol,12–14) and may explain the possible involvement of oxidized HDL in the pathogenesis of atherosclerosis. Furthermore, it has been shown that oxidized HDL caused an intracellular accumulation of unesterified cholesterol in macrophages.15) Anantharamaiah et al. have reported that the oxidation of apo A-I altered its secondary structure and its lipid affinity.16) Moreover, Mazière et al. have reported copper- and malondialdehyde-induced modification of HDL and a parallel loss of LCAT activation.17)

Most of these reports have considered the effect of the denaturation of apo A-I to be important. However, as the activity of LCAT is sensitive to the kind of phosphatidylcholine molecular species in the substrate HDL or lecithin-cholesterol vesicle,18) the possibility exists that the oxidation of substrate phosphatidylcholine or cholesterol on the HDL surface may affect the activity of plasma LCAT. The present study was designed to assess how lipid peroxidation and free radical generation in the plasma might affect LCAT activity.

Materials and Methods

Materials. Cholesterol (99+%), crystallized lyophilized bovine serum albumin, and L-phosphatidylcholine (egg-yolk lecithin, type III-E) were all obtained from Sigma Chemicals. TLC plates (silic acid-impregnated plastic sheets) were purchased from Kodak and [4-14C]cholesterol (specific activity of 1.85–2.29 GBq/mmol) was purchased from Amersham Corp. All other chemicals were of reagent grade or better.

Purification of human LCAT and apo A-I. Human plasma LCAT was purified by the method developed by Doi and Nishida.19) 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 way similar to that described previously.20,21) This preparation of human apo A-I gave a single band with sodium dodecyl sulfate polyacrylamide gel electrophoresis.

† To whom correspondence should be addressed.

Abbreviations: AAPH, 2,2'-azobis-amidinopropane dihydrochloride; apo A-I, apolipoprotein A-I; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); HDL, high-density lipoprotein; LCAT, lecithin-cholesterol acyltransferase; LDI, low-density lipoprotein; ox-HDL, oxidized high-density lipoprotein; VLDL, very-low-density lipoprotein.
Experiment 1. A free radical initiator, namely, 2,2'-azobis-amidinopropane dihydrochloride (AAPH), was used to generate free radicals in plasma. Plasma collected from normolipidemic subjects was sterilized by means of a 0.22 μm millipore filter and treated with 2 mM AAPH at 37°C for various incubation times. The oxidation was stopped by adding 0.02 mM butylated hydroxy toluene. The method of Stokke and Norum was used to determine the LCAT activity. To 100 μl of whole human plasma was added 30 μl of [4,14C]-cholesterol (333 Bq) in 25 mg/ml of a bovine serum albumin emulsion; this was incubated for 4 hr at 37°C under N2, with 20 μl of 10.4 mM DTNB, which is an LCAT inhibitor. Either HDL or oxidized HDL dialyzed against 0.02 M phosphate-buffered saline at pH 7.4 was added to the medium before the LCAT reaction had begun. The reaction was started by adding 20 μl of 100 mM 2-mercaptoethanol at 37°C. Following the incubation, the lipids were extracted and separated by TLC, using hexane-diethyl ether-acetic acid (70:30:1, v/v). The radioactivity in the cholesterol and cholesterol ester fractions was measured with a liquid scintillation counter.

Experiment 2. The effects of oxidized HDL on plasma LCAT activity were measured. Human plasma HDL (d, 1.063-1.21 g/ml) was prepared from fresh human plasma derived from fasting normolipidemic subjects by sequential ultracentrifugation with a Beckman TL-100 at 100,000 rpm for 4 hr at 4°C. Oxidative modification was achieved by treating with 2 mM AAPH at 37°C for 24 hr. Plasma LCAT activity was measured by the Stokke and Norum method as already described. Either HDL or oxidized HDL dialyzed against 0.02 M phosphate-buffered saline at pH 7.4 was added to the medium before the LCAT reaction had begun.

Experiment 3. The inhibitory effect of the oxidation of substrate vesicles on LCAT activity was examined. The enzyme activity was measured in a manner similar to that described previously. Lecithin-cholesterol vesicles (vesicles) prepared by the method of Batzri and Korn were used as a substrate for the enzyme assay. A typical preparation contained 900 nmol egg-yolk phosphatidylcholine and 150 nmol of [4,14C]-cholesterol per ml. Oxidative modification of the vesicles was carried out by treating with 2 mM AAPH at 37°C for various incubation times. The oxidation was stopped by adding 0.02 mM butylated hydroxy toluene, and the vesicles were dialyzed against 39.2 mM phosphate-buffered saline at pH 7.4. The assay mixture consisted of 100 μl of a vesicle solution, 15 μg of human apo A-I, 4 mM 2-mercaptoethanol, 0.7 mM EDTA, 2.5 mg of bovine serum albumin, and the enzyme, all in a final volume of 250 μl of a 39.2 mM sodium phosphate buffer at pH 7.4. Test tubes containing the assay mixture were reacted for 60 min at 37°C under N2, the remaining the procedure being the same as that in the first method.

Experiment 4. The effect of vesicles containing either oxidized phosphatidylcholine or oxidized cholesterol on LCAT activity was examined. A vesicle solution was prepared as just described and oxidized by incubating with 2 mM AAPH at 37°C for 5 hr. Lipids in the vesicles or oxidized vesicles prepared as already described were extracted by the method of Bligh and Dyer. Phosphatidylcholine and cholesterol were separated on TLC plates by using hexane-acetone-acetic acid (80:20:1, v/v). Four kinds of vesicle solution were reconstituted as a substrate for the enzyme assay by using the four possible combinations of these lipids and the corresponding native lipids (see Fig. 5).

Experiment 5. A vesicle solution was added to vesicles that had been prepared by using [4,14C]-cholesterol. Various quantities of vesicles that had been oxidized with AAPH for 24 hr were added so that the final solution contained 0-40% of oxidized vesicles. The ratio of added vesicles to labeled vesicles was 1:1. 100 μl of these mixed-vesicle solutions was used to provide substrate vesicles, and LCAT activity was measured as already described.

Other methods. Protein was estimated by the method of Lowry. Lipid peroxides were measured as thiobarbituric acid reactive substances (TBARS) by using 1,1,3,3-tetraethoxypropane, which produces malondialdehyde (MDA), as a standard according to Yagi’s method. The cholesterol in lipoprotein was determined with an enzymatic kit purchased from Wako Chemicals (Japan).

Results

Experiment 1. Effect of a free radical initiator on the activity of plasma LCAT
In this experiment, 2,2'-azobis-amidinopropane-dihydrochloride (AAPH), a water-soluble free radical initiator, was added to plasma and incubated before the LCAT reaction had begun. As shown as Fig. 1-a, the LCAT activity in the plasma was inhibited as a function of the incubation time. After 24 hr of incubation, the activity had been reduced to about 40% of that existing when plasma was incubated without AAPH. The TBARS value in plasma that had been incubated with AAPH for 24 hr was about 1.5 times that in plasma incubated without AAPH, as shown in Fig. 1-b.

Next, to protect against oxidation of the plasma lipids, one of a number of antioxidants (ascorbate, α-tocopherol, or probucol) was added before the addition of AAPH. Fig. 2 shows the effects of these antioxidants on the inhibition of LCAT activity induced by adding AAPH. Ascorbate was effective at a concentration of 100 μM or more, but α-tocopherol and probucol had no such effect on the inhibition.

Experiment 2. Inhibitory effects of oxidized HDL on plasma LCAT activity
As shown in Fig. 3, LCAT activity (measured by the Stokke and Norum method) was inhibited by the addition of oxidized HDL in a dose-dependent manner; with 20 μg of added oxidized HDL protein, a considerable (40%) inhibition of LCAT activity was observed.

Experiment 3. Inhibitory effect of the oxidation of
Inhibitory Effects of the Oxidation of Either Substrate Vesicles or HDL on LCAT Activity

Fig. 1. Effect of the Oxidation of Plasma on LCAT Activity (a) and TBARS Value (b).

Plasma collected from normal lipidemic subjects was treated with 2 mM AAPH at 37°C for various incubation times (see Materials and Methods). (a) LCAT activity was measured by the Stokke and Norum method. (b) TBARS value was measured by using 1,1,3,3-tetraethoxypropane as a standard according to Yagi’s method. Each value shown is the mean ± SEM of triplicate observations. Activity was calculated as nmol of esterified cholesterol in 1 ml of plasma for every 1 hr of reaction time. Open circles, plasma incubated without AAPH; closed circles, plasma incubated with AAPH.

Fig. 2. Effects of Antioxidants on the Inhibition of LCAT Activity Induced by the AAPH Treatment.

Various concentrations of ascorbate (a), α-tocopherol (b), or probucol (c) were added to plasma which was then treated with 2 mM AAPH at 37°C for 24 hr. LCAT activity was measured by the Stokke and Norum method. Activity was calculated as nmol of esterified cholesterol in 1 ml of plasma for every 1 hr of reaction time. Each value shown is the mean ± SEM of triplicate observations.

substrate vesicles on LCAT activity

The inhibitory effect of the oxidation of substrate vesicles on LCAT activity was examined by using a purified enzyme and cofactor in a vesicle solution. As shown in Figure 4a, LCAT activity was progressively inhibited as a function of the oxidation time of the substrate vesicles. The TBARS value of the vesicle solution was measured as an index of the degree of oxidation as shown in Fig. 4B; after 5 hr of oxidation, the TBARS value had increased to about three times its value before oxidation. The reduction in LCAT activity was related to the degree of oxidation, and after 12 hr of oxidation, LCAT activity had been completely inhibited. There was no detectable effect on LCAT activity or TBARS value when the vesicle solution was incubated at 37°C for 12 hr without AAPH.

Experiment 4. Effect of oxidized phosphatidylcholine and oxidized cholesterol in substrate vesicles on LCAT activity

Fig. 3. Inhibitory Effects of Oxidized HDL on Plasma LCAT Activity.

Oxidized HDL samples were prepared by incubating with 2 mM AAPH for 12 hr at 37°C (see Materials and Methods). LCAT activity was measured by the Stokke and Norum method. Either HDL or oxidized HDL was added to the medium, and it was then incubated under standard assay conditions. Activity was calculated as nmol of esterified cholesterol in 1 ml of plasma for every 1 hr of reaction time. Each value shown is the mean ± SEM of triplicate observations. Open circles, native HDL added; closed circles, oxidized HDL added.
Fig. 5. Effect of Oxidized Phosphatidylcholine and Oxidized Cholesterol on LCAT Activity.

Vesicle solutions were prepared and oxidized by incubating with 2 mM AAPH for various times at 37°C. Phosphatidylcholine and cholesterol were extracted from the vesicle or oxidized vesicle solutions and reconstituted vesicle solutions prepared from these lipids. Four kinds of vesicle solution were prepared (see the figure) and then used as a substrate for the LCAT reaction. LCAT activity was measured by using a purified enzyme and cofactor in the vesicle solution (see Materials and Methods). Each value shown is the mean ± SEM of triplicate observations. nPc, native phosphatidylcholine; nCh, native cholesterol; oXPc, oxidized phosphatidylcholine; oxCh, oxidized cholesterol.

Fig. 6. Inhibitory Effect of an Oxidized Vesicle Solution on LCAT Activity.

A vesicle solution was prepared and oxidized by incubating with 2 mM AAPH for 24 hr at 37°C. Oxidized vesicles and normal vesicles were mixed in various proportions (to give solutions containing 0–40% of oxidized vesicles), and then LCAT activity was measured by using a purified enzyme and cofactor in the vesicle solution (see Materials and Methods). Each value shown is the mean ± SEM of triplicate observations.

In this experiment, four kinds of vesicle solution were prepared and used as substrates for the LCAT reaction. These contained (i) native phosphatidylcholine and native cholesterol, (ii) native phosphatidylcholine and oxidized cholesterol, (iii) oxidized phosphatidylcholine and native cholesterol, and (iv) oxidized phosphatidylcholine and oxidized cholesterol. As shown in Fig. 5, low activity was associated with oxidized phosphatidylcholine (whether or not oxidized cholesterol was present).

Experiment 5. Inhibitory effect of the oxidized vesicle solution on LCAT activity

As shown in Fig. 6, when 10% of the oxidized vesicle solution was present in the substrate vesicle solution used for the assay, LCAT activity was decreased to about 40% of the level existing with the native substrate vesicle solution.

Discussion

These experiments indicate that the oxidation of plasma HDL or lecithin-cholesterol vesicles (which act as an artificial substrate) inhibited the esterification of cholesterol by LCAT. AAPH was used to generate free radicals in the plasma, and the effects of various maneuvers on the LCAT activity in the plasma were assessed. AAPH is a free radical initiator that is frequently used in biological studies of lipid peroxidation when radical generation at a constant known rate is needed. In plasma samples that had undergone different degrees of oxidation (depending on the incubation time with AAPH), LCAT activity was inhibited as a function of the degree of oxidation (Fig. 1). After 24 hr of incubation, the activity had been reduced to about 40% of that existing when plasma was incubated without AAPH. The reason for this decrease in LCAT activity that occurred with control plasma is not clear, but this effect may have been due to deactivation of the LCAT enzyme in the plasma as a result of the incubation medium containing DTNB (an LCAT inhibitor).

To confirm that the inhibition occurring with AAPH reduction was due to the peroxidation of plasma lipids, we added one of three antioxidants (ascorbate, α-tocopherol, or probucol) before adding AAPH (Fig. 2). A dose of 100 μM or more of ascorbate was effective for attenuating the inhibition induced by AAPH, and at 1 mM, the LCAT activity had almost completely recovered (i.e., to 80% of that existing in native plasma, and to 100% of that when plasma was incubated without AAPH). In contrast, neither α-tocopherol nor probucol had any effect on the inhibition of LCAT. At present, we do not know the reason for this difference in action. Since ascorbate acts as a radical-trapping antioxidant in water, and as AAPH is a water-soluble radical initiator, it is possible that ascorbate in the plasma reduced radicals derived from AAPH to stable compounds. It is also possible that α-tocopherol and probucol could not be taken up by the plasma lipoproteins because these agents were added to the plasma in an ethanol solution. If so, our observations should not be taken to indicate that these antioxidants are any less effective than ascorbate. No inhibitory effect was found by adding ethanol as a vehicle (1% of the total volume).

The addition of oxidized HDL to plasma (Fig. 3) resulted in the plasma LCAT activity decreasing in a dose-dependent manner, much as it did when we used the oxidized substrate vesicle solution. In the present experiment, the oxidized or native HDL that was added did not contain labeled cholesterol, which is the tracer for the LCAT reaction, so the possibility must be considered that this inhibitory effect of oxidized HDL on LCAT activity was due to direct inhibition by oxidized HDL of the reaction involving native HDL, which did.
contain labeled cholesterol. We have previously reported the inhibitory effect of oxidized LDL on plasma LCAT activity,29 and the inhibitory effect of oxidized HDL observed in the present experiment may have involved the same mechanism.

We tried in several ways to identify the mechanism underlying the inhibitory effects of the oxidation of HDL on LCAT activity by using a purified enzyme and cofactor in a vesicle solution. As shown in Fig. 4, the decrease in LCAT activity induced by the oxidation of substrate vesicles with 2 mM AAPH was related to the degree of oxidation; after 12 hr of oxidation, the LCAT activity had been completely inhibited. Since the vesicle solution used in this experiment was oxidized without apo A-I or LCAT, this inhibition was presumably caused by oxidation of the lipid, and not by oxidation of apo A-I. This idea is supported by the result of an additional experiment: three kinds of vesicle solution (vesicle only, vesicles with LCAT, and vesicles with apo A-I) were prepared and oxidized with AAPH. After 1 hr of oxidation, the inhibition of LCAT activity we detected was by 25.5%, 28.9%, and 16.0%, respectively. Mazière et al. have reported that copper- and malondialdehyde-induced modification of HDL resulted in a dose-dependent loss of LCAT activity.7,10 They suggested that this inhibition may have been caused by a modification of apo A-I. However, the current observations suggest that the oxidation of lipids in HDL that acts as a substrate for LCAT had a rather important influence on LCAT activity.

LCAT catalyzes the transfer of the phosphatidylcholine acyl group to cholesterol.4-9 Many studies have dealt with the specificity of LCAT towards acyl donor phospholipids,10,20-22 and there have been some studies on the use of cholesterol derivatives as acyl acceptors.33,34 In the present experiment, we investigated whether it was the oxidation of phosphatidylcholine or of cholesterol that affected LCAT activity. As shown in Fig. 5, vesicle solutions containing oxidized phosphatidylcholine showed lower LCAT activity, whereas the activity was actually increased in a vesicle solution containing oxidized cholesterol and native phosphatidylcholine. It has been reported that LCAT activity was sensitive to the kind of phosphatidylcholine molecular species present in the substrate HDL or lecithin-cholesterol vesicle,15,16 but that LCAT catalyzed the esterification of oxyesters as well as the esterification of native cholesterol.17 This is consistent with the results of the present experiment, showing that the oxidation of phosphatidylcholine had more affect on the LCAT reaction than the oxidation of cholesterol did.

As shown in Fig. 6, LCAT activity was decreased to 40% of the control value by adding even 10% of oxidized vesicles to normal vesicles. This reduction is greater than would be expected from the ratio of oxidized vesicles to native vesicles. This inhibitory effect of oxidized vesicles could be explained by the same mechanism as that involved in the inhibition of plasma LCAT activity by oxidized HDL (see experiment 2), although a further detailed study is necessary.

It is known that HDL undergoes oxidative modifications in vitro,10 resulting in the loss of ability of these lipoproteins to incorporate cholesterol,12-16 and this could explain the possible involvement of oxidized HDL in the pathogenesis of atherosclerosis. The results of the present study also suggest that the oxidation of HDL can affect the reverse-transport system via inhibition of the esterification produced by LCAT, without the need for a more fundamental change (such as denaturation of apo A-I). In the cholesterol reverse-transport system, LCAT plays an important role by virtue of its esterification of the cholesterol that is to be transferred to the liver from various tissues. It is tempting to speculate that the oxidation of HDL in the endothelial cells of an early atherosclerotic region might inhibit HDL-associated LCAT, and thus influence the cholesterol reverse-transport that involves HDL. It has been reported that changes in LCAT activity may be a factor helping to determine the risk of coronary heart disease.35,36 Further clarification of the effects of free radical generation in the plasma and of the influence of the oxidation of HDL on cholesterol reverse-transport will be important, but must await a future investigation.

References