# Minimally oxidized LDL is a potent inhibitor of lecithin:cholesterol acyltransferase activity

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**Abstract** The oxidation of low density lipoproteins (LDL) has been implicated in the development of atherosclerosis. As a variety of highly reactive lipid peroxidation products can transfer from oxidized LDL to HDL, we evaluated the potential deleterious effects of LDL oxidation on HDL-cholesterol metabolism. To address this issue, we exposed the HDL-containing d > 1.063 g/ml fraction of human plasma to copper-oxidized LDL and assessed lecithin:cholesterol acyltransferase (LCAT) activity and apolipoproteinA-I (apoA-I) structure. To determine whether LCAT was directly affected by oxidized LDL, independent of crosslinking of apoA-I, we used an exogenous, [$^{14}$C]cholesterol-labeled proteoliposome substrate to measure plasma LCAT activity. We observed an inhibition of LCAT activity where copper-oxidized LDL possessing only $2.3 \pm 0.1$ and $7.3 \pm 1.4$ TBARS produced $24 \pm 3\%$ and $47 \pm 10\%$ reductions in [$^{14}$C]cholesterol esterification by 1 h, respectively. Copper-oxidized LDL that had been passed through a GF-5 desalting column, while retaining only one-third of its original TBARS, possessed nearly all of its LCAT inhibitory capacity suggesting that the LCAT inhibitory factor(s) was a lipophilic oxidation product. Analysis of polar lipids isolated from copper-oxidized LDL indicated that phospholipid and sterol fractions effectively inhibited LCAT. Copper-oxidized LDL, with as little as 6.3 TBARS, also produced intermolecular crosslinking of apoA-I molecules. ▩ Taken together, these data suggest that products of LDL oxidation may adversely affect HDL-cholesterol metabolism by two separate mechanisms: *1)* a direct inhibitory effect on LCAT activity and *2)* through crosslinking of apoA-I. If occurring in vivo, minimally oxidized LDL may impair cholesteryl ester formation on HDL thereby limiting the ability of HDL to function efficiently in the putative antiatherogenic reverse cholesterol transport pathway.—**Bielicki, J. K., T. M. Forte, and M. R. McCall.** Minimally oxidized LDL is a potent inhibitor of lecithin:cholesterol acyltransferase activity. *J. Lipid Res.* 1996. **37:** 1012–1021.

**Supplementary key words** lipid peroxidation • inhibition of LCAT activity • crosslinking of apoA-I • reverse cholesterol transport • early atherogenesis

Epidemiological studies indicate that elevated plasma concentrations of high density lipoprotein (HDL) are protective against the development of atherosclerosis (1–3). The beneficial effects of HDL may, in part, be related to its ability to promote the efflux of cholesterol from cholesterol-laden cells of the arterial wall and to transport this cholesterol to the liver for catabolism (4–7). This process has been termed reverse cholesterol transport. ApolipoproteinA-I is the major structural protein of HDL and the physiological activator of LCAT (8, 9). This enzyme catalyzes the formation of cholesteryl esters on HDL and, in so doing, promotes the maturation of HDL particles in plasma and facilitates reverse cholesterol transport by maintaining a concentration gradient for the diffusion of cellular unesterified cholesterol to HDL (4, 10). In transgenic mice, expression of the human LCAT gene is associated with increased plasma cholesteryl ester synthesis and elevated concentrations of HDL-cholesterol, suggesting that LCAT may play an important role in modulating HDL concentrations in vivo (11).

In contrast to the beneficial effects of HDL, elevated levels of low density lipoproteins (LDL) are associated with an increased risk of atherosclerosis. The atherogenic effects of LDL may be related to its susceptibility to oxidation and its subsequent effects on the arterial wall (12, 13). The oxidation of LDL polyunsaturated fatty acids yields a variety of highly reactive lipid peroxidation products that promote atherosclerotic lesion formation. These products include oxidized phospholipids and oxysterols which can promote monocyte/endothelial cell interactions and induce cytotoxicity (14–18). Aldehydic decomposition products can also modify the protein component (apoB) of LDL (19, 20). Extensive chemical modifications of apoB lead to foam cell formation via mechanisms that involve the unregu-

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lated uptake of oxidized LDL via scavenger and/or oxidized LDL receptors on macrophages (20–23).

Recent experimental evidence suggests that products of LDL oxidation can transfer from minimally oxidized LDL to HDL (24–26). Thus, it has been proposed that by accepting and transporting such oxidation products, HDL may protect against some of the atherogenic effects of oxidized LDL. In support of this hypothesis, Bowry, Stanley, and Stocker (27) demonstrated that, in humans, a majority of plasma lipid hydroperoxides are carried on HDL, suggesting that HDL may transport oxidized lipid products in vivo. The potential deleterious effects of these oxidized lipids on HDL-cholesterol metabolism have not yet been considered.

Previously, we observed a rapid inhibition of plasma LCAT activity upon initiating lipid peroxidation with copper ions (28). As these studies were performed with whole plasma, the underlying mechanisms contributing to LCAT inhibition were not fully explored. More recently, we demonstrated that aldehydes (e.g., 4-hydroxynonenal and malondialdehyde) known to be generated by the oxidation of LDL are able to inhibit plasma LCAT activity and to crosslink HDL-apolipoproteins (29). These observations suggested that LDL oxidation may adversely affect HDL-cholesterol metabolism. As a variety of highly reactive lipid peroxidation products can be generated in vivo by the oxidation of LDL, we evaluated the LCAT inhibitory capabilities of oxidized LDL. We found that minimally oxidized LDL was a potent inhibitor of LCAT activity and crosslinker of apoA-I molecules on HDL. In addition, polar lipid fractions accounted for a majority of the LCAT inhibitory capacity of oxidized LDL. These new findings suggest that minimally oxidized LDL may contribute to the development of atherosclerosis by impairing HDL-mediated reverse cholesterol transport.

## METHODS

### Isolation of low density lipoproteins (LDL) from human plasma

Blood was collected from nonsmoking, healthy individuals into tubes containing EDTA and samples were chilled to 4°C. Plasma was obtained after low speed centrifugation to remove blood cells, and pools of plasma were prepared. Plasma lipid concentrations were 200 ± 18, 44 ± 6, and 127 ± 27 mg/dl for total cholesterol, HDL-cholesterol, and triglycerides, respectively (n = 15 pools). Low density lipoproteins (LDL, d 1.019–1.063 g/ml) were isolated by preparative, sequential ultracentrifugation (30) and dialyzed (4°C) against phosphate (10 mM)-buffered saline (PBS, pH 7.4). At the end of dialysis, gentamicin sulfate (50 µg/ml) was added. The HDL-containing d > 1.063 g/ml fraction of human plasma was also dialyzed against PBS, and after dialysis, EDTA (2.7 mM), BHT (20 µM), and gentamicin were added.

### LDL oxidation

LDL was incubated (37°C) with copper (20 nmol · mg LDL protein$^{-1}$ · ml$^{-1}$) for 0.5–6 h to produce various degrees of oxidation. Control LDL was either incubated without copper or with copper plus EDTA (2.7 mM). At the end of incubations, samples were cooled to 4°C; BHT and EDTA were added. In some experiments, control and copper-oxidized LDLs were passed through GF-5 desalting columns (supplied by Pierce; exclusion limit of 5,000 mol wt) to remove copper and water-soluble oxidation products (i.e., malondialdehyde). Routinely, 0.5 ml of LDL (3 mg protein/ml) was passed through columns with predetermined aliquots of PBS-EDTA (4°C). Recoveries of LDLs ranged from 88 to 97% and were independent of the levels of oxidation obtained in this study.

Oxidation of LDL with the water-soluble free radical initiator 2,2'-azobis-2-amidinopropane hydrochloride (AAPH) was performed at 37°C for 0.5 h with 16 µmol AAPH · mg LDL protein$^{-1}$ · ml$^{-1}$; to stop these incubations and quench free radicals, samples were cooled to 4°C and reduced glutathione (5 mM), EDTA, and BHT were added. AAPH was removed from incubations by passing samples (4°C) through GF-5 desalting columns using PBS/EDTA as the elution buffer. The oxidized LDL recovered from columns was dialyzed against PBS/EDTA to ensure complete removal of AAPH. Control LDL was handled similarly except AAPH was omitted from treatments.

### Assessment of LDL oxidation

Thiobarbituric acid reactive substances (TBARS) were measured by the method of Kosugi, Kojima, and Kikugawa (31), and conjugated dienes were monitored by ultraviolet absorption spectroscopy at 234 nm. Protein was measured by the method of Markwell et al. (32). To assess increases in LDL net negative charge, agarose gel electrophoresis was performed using Paragon Lipogels following the manufacturers (Beckman) instructions. Aggregation of LDL particles was assessed by nondenaturing gradient gel electrophoresis (33).

### Incubation of oxidized LDL with the d > 1.063 g/ml fraction of human plasma

Control and copper-oxidized LDLs were separately added-back to the d > 1.063 g/ml fraction of human plasma that contains HDL, LCAT, and plasma proteins. Final concentration of LDL was 1 mg protein/ml. These reconstituted plasma samples were incubated at 37°C

for 0.5–6 h, and plasma LCAT activity was assessed as described below. Preliminary experiments using copper-oxidized LDLs that ranged in TBARS from $2.8 \pm 0.8$ to $35.6 \pm 4.3$ (means $\pm$ SD, n = 3) demonstrated that for each TBARS level maximal LCAT inhibition was achieved by 1 h. As a result, this time point was used for subsequent incubations.

## Assessment of plasma LCAT activity

LCAT activity was measured by the exogenous substrate method described by Chen and Albers (34). As this method utilizes an excess of [$^{14}$C]cholesterol-labeled proteoliposome substrate composed of human apoA-I: egg-yolk phosphatidylcholine: unesterified cholesterol (0.8:250:12.5 molar ratios) for assessment of LCAT activity, changes in enzymatic activity reflect alterations in LCAT and are independent of modifications of endogenous substrates and crosslinking of apoA-I (LCAT cofactor). In addition to this proteoliposome substrate, reaction mixtures (0.25 ml final volume) contained 20 mM Tris-HCl (pH 8.0), 0.15 mM NaCl, 0.27 mM EDTA, 0.5% human serum albumin, and 5 mM β-mercaptoethanol. To assess LCAT activity, 7.5-μl aliquots of reconstituted plasma samples were added to reaction mixtures and samples were incubated for 0.5 h at 37°C. Reactions were stopped by cooling samples (4°C) and by adding ethanol (0.25 ml). Lipids were extracted with hexane, and unesterified cholesterol and cholesteryl esters were separated by instant thin-layer chromatography (Gelman Sciences) using toluene as a mobile carrier phase. Radioactivity associated with cholesterol and cholesteryl ester bands was quantified by liquid scintillation counting. Initial rates of [$^{14}$C]cholesterol esterification were evaluated. Results are expressed as a percentage of control LCAT activity in reconstituted plasma samples (i.e., d > 1.063 g/ml fraction plus control LDL) prior to 37°C incubations (t = 0). The average LCAT activity at t = 0 was $10 \pm 2\%$ esterification of [$^{14}$C]cholesterol per 0.5 h (n = 10 pools of plasma).

The LCAT assay system normally contains excess substrate (proteoliposome) to ensure full activation of the LCAT enzyme. Thus, maximal rates of cholesterol esterification are evaluated. In order to insure that reductions in LCAT activity were not due to modification of proteoliposomes by oxidized LDL, we evaluated LCAT activity using up to an 8-fold increase in proteoliposome concentration. We found that even at the highest proteoliposome concentration, LCAT activity was inhibited to the same extent as in our standard assay conditions. This observation provided strong evidence that reductions in LCAT activity induced by minimally oxidized LDL were largely independent of our exogenous substrate and were most likely the consequence of a direct inactivation of the LCAT enzyme.

## Evaluation of apolipoproteinA-I crosslinking

High density lipoproteins (HDL, d 1.063–1.21 g/ml) were isolated from reconstituted plasma samples by sequential, preparative ultracentrifugation (30). Crosslinking of apoA-I was evaluated by Western blots from SDS-PAGE (4–20% gels). Blots were developed using monoclonal antibodies specific for human apoA-I.

## Isolation of polar lipid fractions from control and copper-oxidized LDL and their incubation with the d > 1.063 g/ml fraction of human plasma

Lipids were extracted from control and copper-oxidized LDL by an acidified Bligh and Dyer procedure (35). Polar lipid fractions were separated from neutral lipids by thin-layer chromatography using silica-gel H plates and a solvent system composed of petroleum ether–ethyl ether–acetic acid 160:40:2. The $R_f$ values of standards were as follows; phospholipid, 0.00 (origin); unesterified cholesterol, 0.13; free fatty acid, 0.33; and cholesteryl esters, 0.81. After reisolation from the silica-gel (36), phospholipid and sterol fractions (at amounts equivalent to those used for LDL incubations, i.e., 1 mg phospholipid/ml and 0.4 mg unesterified cholesterol/ml) were separately dried (under a gentle stream of nitrogen) to the surface of glass vials, and the d > 1.063 g/ml fraction of human plasma was added. Samples were incubated at 37°C for up to 6 h in the presence of EDTA, BHT, and gentamicin. As a majority (> 80%) of the LCAT inhibitory capacity of oxidized LDL was associated with polar lipid fractions co-migrating with phospholipid and unesterified cholesterol standards, no other lipid fractions were analyzed for LCAT inhibitory capabilities.

## RESULTS

### Characterizations of control and copper-oxidized LDL

LDL exposed to copper ions (20 nmol · mg LDL protein$^{-1}$ · ml$^{-1}$) for 0.5 h possessed low TBARS ($2.3 \pm 0.1$ nmol MDA eq./mg LDL protein) that were only slightly higher than controls ($2.0 \pm 0.5$). After an initial lag period, TBARS rose rapidly to 50 nmol MDA eq./mg LDL protein by 6 h (**Fig. 1A**). Conjugated dienes followed a similar pattern (Fig. 1B). Changes in both TBARS and conjugated dienes were completely inhibited by chelating copper ions with EDTA.

Oxidation of LDL was accompanied by changes in net negative charge where at 2.4 TBARS (**Fig. 2A,** lane 6) only a subtle increase was observed compared to controls; with increasing TBARS (6.3, 13, and 40), LDL migration increased substantially (18, 70, and 130% over controls, respectively). Aggregation of LDL particles, as
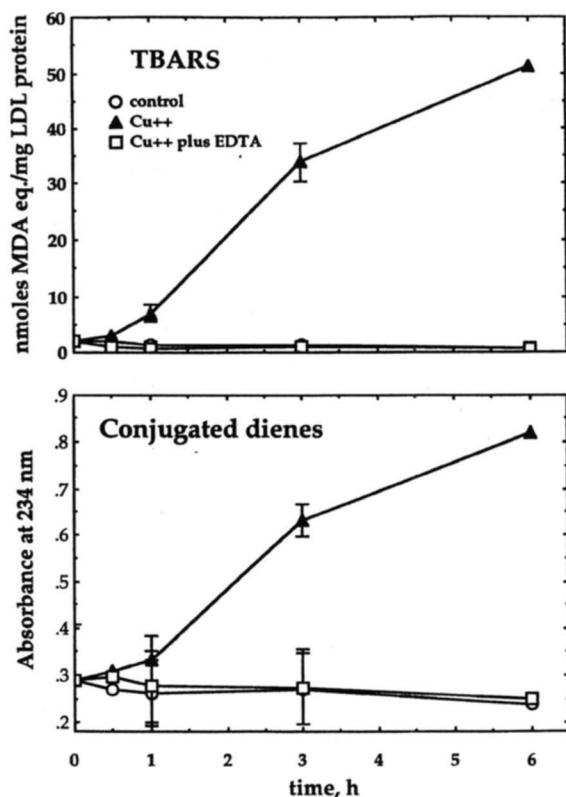
**Fig. 1.** Time-course of TBARS and conjugated diene formation upon copper oxidation of LDL. Low density lipoproteins were incubated (37°C) with copper (▲) for 0.5 to 6 h. Control samples were either incubated in the absence of copper (○) or with copper plus EDTA (□). Concentration of copper was 20 nmol · mg LDL protein$^{-1}$ · ml$^{-1}$. At the times indicated, samples were removed and EDTA and BHT were added as described in Methods section. Means ± SD, n = 3, are shown.

determined by nondenaturing gradient gel electrophoresis, was observed at 40 TBARS (Fig. 2B, lane 9). At this relatively high level of oxidation, extensive fragmentation of apoB was seen by SDS-PAGE; no fragmentation was obtained at 2.4 TBARS and only a very little was seen at 6.3 TBARS (data not shown).

## Inhibition of plasma LCAT activity by copper-oxidized LDL

**Figure 3** summarizes the dependence of LCAT inhibition on the extent of LDL oxidation. Copper-oxidized LDL exhibiting as little as 2.3 ± 0.1 and 7.3 ± 1.4 TBARS produced 24 ± 3% and 47 ± 10% reduction in plasma LCAT activity, respectively. LDL incubated with copper plus EDTA, a condition that completely prevented the oxidation of LDL (Fig. 1A and B), failed to inhibit LCAT activity suggesting that LCAT inhibition was dependent on the oxidation of LDL (data not shown). In order to establish that the inhibition of LCAT activity was not the result of residual copper bound to apoB of oxidized LDL, we oxidized LDL with the water-soluble free radical initiator, 2,2'-azobis-2-amidinopropane hydrochlo-

ride (AAPH) and investigated whether oxidized LDL that had been separated from AAPH using desalting columns inhibited LCAT activity. We observed 21% and 45% reductions in LCAT activity with oxidized LDL at 2.5 and 5.8 TBARS, respectively. Thus, the inhibition of LCAT activity was not contingent upon the presence of either copper or AAPH; but rather, LCAT activity was inhibited by products of LDL oxidation.

## Crosslinking of apolipoproteinA-I molecules by copper-oxidized LDL

In addition to inhibiting LCAT activity, oxidized LDL produced intermolecular crosslinking of apoA-I molecules as seen by Western blot analysis (**Fig. 4**). Crosslinked forms of apoA-I were detected with oxidized LDL possessing as little as 6.3 TBARS (Fig. 4, lane 7) indicating that a change in apoA-I structure was a sensitive indicator (yet not as sensitive as plasma LCAT activity) of LDL oxidation.
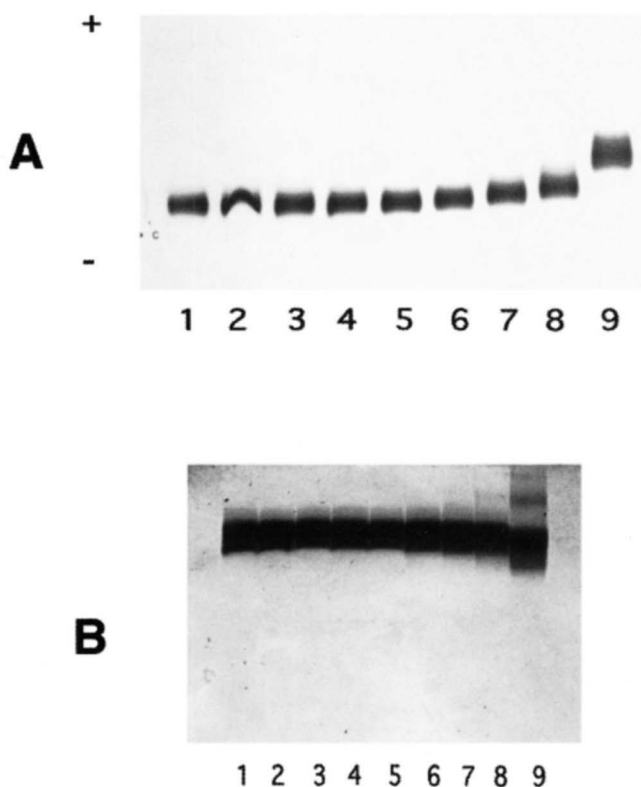


**Fig. 2.** Electrophoretic characteristics of control and copper-oxidized LDL. Low density lipoproteins were either incubated (37°C) in the absence or presence of copper (20 nmol · mg LDL protein$^{-1}$ · ml$^{-1}$) for 0.5 to 6 h. Increases in LDL net negative charge were assessed by agarose gel electrophoresis (panel A). Aggregation of LDL particles was evaluated by nondenaturing gradient gel electrophoresis (panel B); protein load for nondenaturing gradient gel was 4 μg per well. Lane 1 contains 4°C control (unincubated) LDL; lanes 2, 3, 4, and 5 are control LDL incubated at 37°C for 0.5, 1, 3, and 6 h, respectively (average TBARS of these control LDLs = 2.1 ± 0.2 nmol MDA eq./mg LDL protein); lanes 6, 7, 8, and 9 are oxidized LDLs with 2.4, 6.3, 13 and 40 TBARS, respectively.
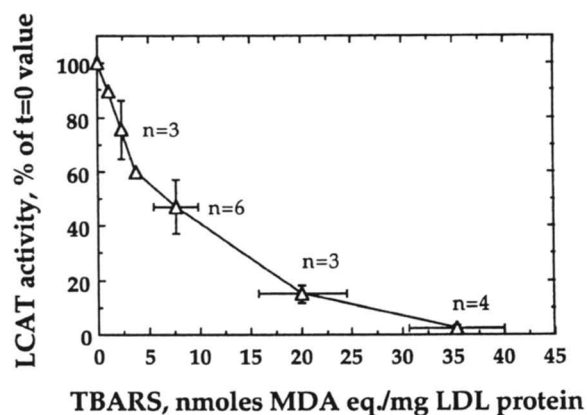
**Fig. 3.** Dependence of LCAT inhibition on the extent of LDL oxidation. Copper-oxidized LDLs with known TBARS were separately added to the d > 1.063 g/ml fraction of human plasma to yield final LDL concentrations of 1 mg protein/ml. After 1 h incubation (37°C), aliquots were removed and LCAT activity was assessed by the exogenous substrate procedure. Results are expressed as percentage of t = 0 LCAT activity which was $9.2 \pm 1.7\%$ (n = 7) esterification of [$^{14}$C]cholesterol per 0.5 h. Values with no error bars are from a single experiment in which LCAT activity was measured in duplicate.

## Inhibition of plasma LCAT activity by copper-oxidized LDL that had been passed through a GF-5 desalting column

To evaluate whether the LCAT inhibitory factor was associated with oxidized LDL or whether it was readily released into the aqueous phase, we passed our copper-
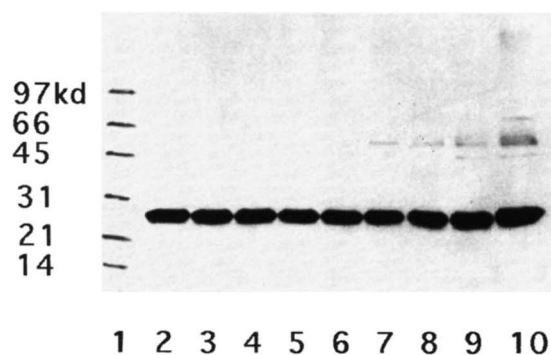


**Fig. 4.** Crosslinking of apolipoproteinA-I by copper-oxidized LDL. Control (without copper) and copper-oxidized LDLs were separately added to the d > 1.063 g/ml fraction of human plasma to yield final LDL concentrations of 1 mg protein/ml, and samples were incubated (37°C) for 3 h. After incubations, HDL were isolated by sequential, preparative ultracentrifugation. HDL-apolipoproteins were separated by SDS-PAGE electrophoresis (protein load was 5 μg per well) and then transferred to nitrocellulose sheets for Western blot analysis using monoclonal antibodies specific for human apoA-I. Lane 1 contains molecular weight standards; lane 2 shows immunoreactivity of apoA-I from reconstituted plasma incubations with 4°C control LDL; lanes 3, 4, 5, and 6 show apoA-I from incubations with control LDLs that had been incubated at 37°C for 0.5, 1, 3, and 6 h as described in Fig. 2 (average TBARS of control LDLs = $2.1 \pm 0.2$ nmol MDA eq./mg LDL protein); lanes 7, 8, 9, and 10 show apoA-I from incubations with oxidized LDLs possessing 6.3, 13, 18, and 40 TBARS, respectively.
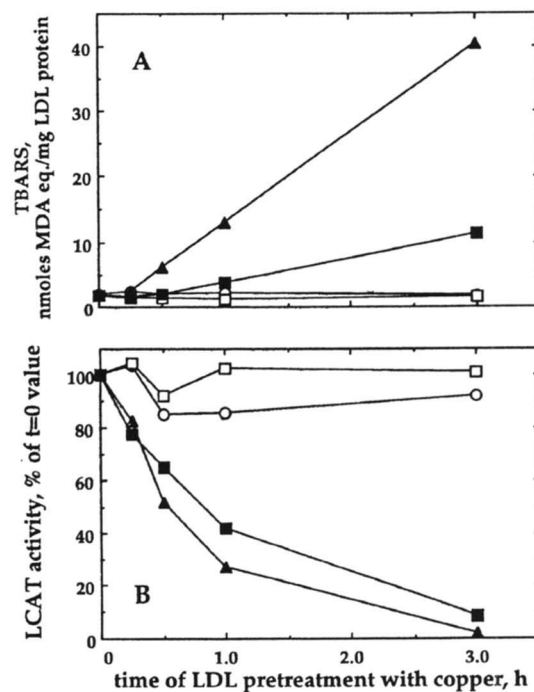


**Fig. 5.** Inhibition of LCAT activity by copper-oxidized LDL recovered from GF-5 desalting columns. Control LDL (without copper) and copper-oxidized LDL were applied (0.5 ml of 3 mg LDL protein/ml) to GF-5 desalting columns and samples eluted with predetermined volumes of PBS-EDTA (4°C). LDL fractions were concentrated to 3 mg protein/ml and added to the d > 1.063 g/ml fraction of human plasma to yield final LDL concentrations of 1 mg protein/ml. Panel A shows TBARS values of pre- and post-column LDLs, and panel B shows LCAT activity. Control LDLs are denoted by open symbols, pre-column (○) and post-column (□); copper-oxidized LDLs are closed symbols, pre-column (▲) and post-column (■). Results for LCAT are expressed as a percentage of t = 0 activity which was 7.0% esterification of [$^{14}$C]cholesterol per 0.5 h. Values shown are from a single, representative experiment, and LCAT activity was measured in duplicate.

oxidized LDLs through GF-5 desalting columns. Data shown in **Fig. 5A** indicate that oxidized LDL that had been passed through a desalting column possessed only one-third of the original TBARS. Regardless of this loss of TBARS, however, post-column oxidized LDL retained full LCAT inhibitory capacity (Fig. 5B). These data suggest that water-soluble oxidation products (e.g., malondialdehyde) which readily dissociate from oxidized LDL were not likely involved in LCAT inhibition; rather, lipophilic oxidation product(s) which are carried on oxidized LDL were probably responsible.

## Inhibition of plasma LCAT activity by polar lipid fractions isolated from copper-oxidized LDL

To establish whether the LCAT inhibitory factor was a lipid, we isolated lipid fractions from control and copper-oxidized LDLs by thin-layer chromatography. When incubated with the d > 1.063 g/ml fraction of human plasma, phospholipid and sterol fractions from
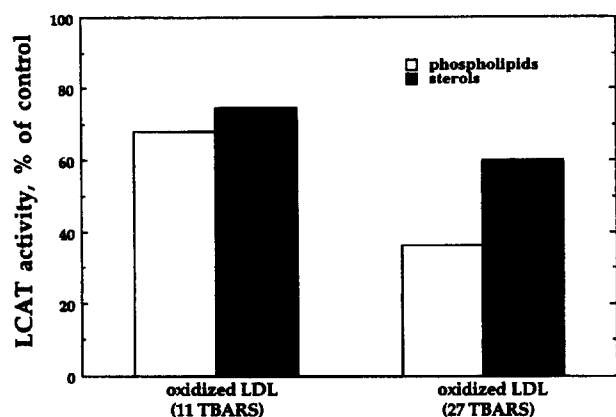
**Fig. 6.** Inhibition of LCAT activity by polar lipid fractions isolated from copper-oxidized LDL. Phospholipid and sterol fractions were isolated from control and copper-oxidized LDL by TLC. Lipid fractions were added to glass vials at amounts equivalent to those used for LDL incubations (i.e., 1 mg phospholipid/ml and 0.4 mg unesterified cholesterol/ml), and the solvent was evaporated under a gentle stream of nitrogen. Immediately thereafter, the d > 1.063 g/ml fraction of human plasma (containing BHT, EDTA, and gentamicin) was added; samples were incubated (37°C) for 6 h. Results are expressed as a percentage of control LCAT activity obtained from incubations (6 h) with phospholipid and sterol fractions from unoxidized LDL. The LCAT activity of these controls were 13.1 and 13.0% esterification of [$^{14}$C]cholesterol per 0.5 h, respectively, and did not differ compared to the original LCAT activity in the d > 1.063 g/ml fraction of human plasma at t = 0. Each set of bars represents lipids isolated from different batches of oxidized LDL; LCAT activity was measured in duplicate.

oxidized LDL possessing 11 TBARS produced 13% and 28% reductions, respectively, in LCAT activity by 1 h. Maximal reductions of about 30% were obtained with both fractions by 6 h (**Fig. 6**). Nearly twice this level of inhibition was obtained with lipids isolated from LDL having 27 TBARS (Fig. 6). When both phospholipid and sterol fractions were combined, an additive inhibition was obtained that nearly equaled the LCAT inhibitory capacity of the original oxidized-LDLs (data not shown).

## DISCUSSION

In the present study, we found that minimally oxidized LDL inhibits LCAT activity and produces intermolecular crosslinking of apoA-I molecules on HDL. Through the use of an exogenous substrate procedure, we determined that minimally oxidized LDL had a direct inhibitory effect on the LCAT enzyme. As these experiments were performed in a plasma background, our findings indicate that LCAT as well as apoA-I on HDL exhibited an extreme susceptibility to modification(s) by products of LDL lipid peroxidation.

### Inhibition of LCAT activity by products of LDL oxidation

Several lines of experimental evidence indicate that

LCAT inhibition was produced by a lipophilic oxidation product. First, oxidized LDL that had been passed through a GF-5 desalting column retained nearly all its LCAT inhibitory capability despite the loss of a majority of TBARS. This observation suggests that water-soluble lipid decomposition products (e.g., malondialdehyde) that are readily released from LDL particles were not likely involved in LCAT inhibition; but rather, the LCAT inhibitory factor(s) was carried on the oxidized LDL. Second, we found that HDL isolated from oxidized-LDL plus d > 1.063 g/ml incubations effectively inhibited LCAT activity when added to a freshly prepared d > 1.21 g/ml fraction of human plasma (unpublished observation). This suggests that the LCAT inhibitory factor(s) was transferable and could associate with HDL. Third, lipid fractions isolated from oxidized LDL inhibited LCAT activity and, when combined, these fractions accounted for nearly all the LCAT inhibitory capacity of oxidized LDL. These results establish that the LCAT inhibitory factor(s) was, most likely, a lipid rather than a water-soluble oxidation product and that several inhibitory compounds were generated as a consequence of LDL oxidation.

Previously, we reported that aldehydic lipid decomposition end-products known to be generated by the oxidation of LDL were capable of inhibiting LCAT activity. The $\alpha,\beta$-unsaturated aldehydes, 4-hydroxynonenal and acrolein, were found to be potent inhibitors of LCAT where micromolar concentrations produced a rapid inhibition of enzymatic activity (29). In contrast, much higher concentrations (millimolar) of malondialdehyde, acetylaldehyde, and hexanal were required to inhibit LCAT. In the present study, substantial reductions (24%) of LCAT activity were observed with minimally oxidized LDL possessing only 2.3 nmol MDA equivalents/mg LDL protein. Using this TBARS value, we estimate that the minimal aldehyde concentration in our reconstituted plasma system was approximately 2.3 $\mu$M (this is a minimal estimate as the colored thiobarbituric acid end-product is not formed by all aldehyde species; as a result, the actual aldehyde concentration may be somewhat higher). This estimate of 2.3 $\mu$M, however, is much lower than the concentration (160 $\mu$M) of 4-hydroxynonenal required to achieve comparable levels of LCAT inhibition (29). Thus, our new findings suggest that, in addition to aldehydes, there may be other oxidation products associated with minimally oxidized LDL that are capable of inhibiting LCAT activity. These inhibitory compounds may very well include oxidized phospholipids and oxysterols, as well as a variety of other oxidized lipid products that may have been present in the lipid fractions we isolated by TLC. At this time, we cannot rule out the possibility that oxidized phospholipids possessing aldehydic functional groups may

have participated in the inhibition of LCAT activity.

LCAT utilizes phosphatidylcholine and unesterified cholesterol for cholesteryl ester formation on HDL. The active site of LCAT is bounded by two cysteine residues (37) and contains a catalytic triad of aspartic acid, histidine, and serine residues (9). The reaction mechanism involves transfer of the sn-2 fatty acid of phosphatidylcholine to the active site serine to form a transient fatty acyl intermediate (38); cholesteryl esters are generated upon the transfer of the serine-bound fatty acid to the 3-hydroxyl of cholesterol. As LCAT activity was rapidly inhibited by minimally oxidized LDL, it seems reasonable to speculate that oxidation of LCAT substrates yields products that modify the active site to produce inhibition of enzymatic activity. As stated above, we observed an inhibition of LCAT activity with both phospholipid and sterol fractions isolated from oxidized LDL. The oxidation of LDL yields a variety of fragmented phospholipids (25) with sn-2 short-chain aldehydic and carboxyl groups which could conceivably react with LCAT's active site residues to produce an inhibition of enzymatic activity. In addition, modification of non-catalytic amino acids could produce conformational changes in the enzyme and/or sterically block the active site to produce an inhibition of enzymatic activity. With regard to sterols obtained from oxidized LDL, it has been previously shown that numerous oxysterols that are generated by copper oxidation of LDL (i.e., 25-hydroxycholesterol, 27-hydroxycholesterol, 7-keto-cholesterol, and α- and β-epoxycholesterols) can be esterified by LCAT (39, 40). The fact that we observed an inhibition of LCAT activity with sterol fractions isolated from oxidized LDL suggests that a unique oxysterol may have been, in part, responsible for the inhibition of LCAT activity. The identification of specific polar lipid species that are effective inhibitors of LCAT activity and the quantification of these compounds in human plasma and/or atherosclerotic plaques may help establish whether the inhibition of LCAT activity that we observed can occur in vivo as a consequence of LDL oxidation.

### Crosslinking of apoA-I molecules by oxidized LDL

In addition to inhibiting LCAT activity, we found that oxidized LDL possessing as few as 6.3 TBARS induced crosslinking of apoA-I molecules on HDL. As BHT and EDTA were present, it is unlikely that the crosslinking resulted from direct, copper-mediated free radical formation during reconstituted plasma incubations. It is far more likely that apoA-I crosslinking was induced by reactive lipid- and/or protein-breakdown products generated as a result of LDL oxidation. Because in the present study we did not identify specific lipid- or protein-breakdown products from our copper-oxidized LDL, we can only speculate as to the chemical species involved in these crosslinking reactions. In this regard, it has been demonstrated that apoA-I on HDL can be crosslinked by specific aldehydes known to be generated during the oxidation of LDL (29). Whether other lipid peroxidation products generated as a consequence of LDL oxidation induce crosslinking of HDL-apolipoproteins remains to be determined.

Our observation that apoA-I on HDL can be crosslinked by oxidation products generated on minimally oxidized LDL may have some far reaching metabolic consequences. It has been demonstrated that aldehyde-induced crosslinking of apoA-I impairs the ability of HDL to promote the efflux of cholesterol from macrophage-derived foam cells (41). In addition, crosslinked apoA-I on HDL appears to impair the ability of HDL to interact with LCAT (42) and to increase the rate of clearance of HDL from the plasma compartment (43, 44). These experimental observations suggest that conditions, in vivo, that predispose individuals to LDL oxidation may also negatively affect HDL-mediated cholesterol transport.

### Physiological significance

LDL is thought to be sequestered in the vascular subendothelial space. During the early stages of atherosclerosis, oxidative bursts from inflammatory cells present in the developing atherosclerotic lesion can promote the oxidation of LDL resulting in the formation of lipid hydroperoxides and their aldehydic breakdown products (12, 13). Evidence for the presence of oxidized LDL has been obtained from both humans and experimental animals (19, 20, 45, 46). While the retention of LDL in the arterial wall and its subsequent oxidation may contribute to the development of atherosclerosis (47), HDL and its associated LCAT are thought to more readily enter and exit subendothelial spaces and protect against the development of atherosclerosis (48). This protection is often attributed to HDL-mediated reverse cholesterol transport. Recent experimental evidence suggests that, in addition to its reverse cholesterol transport function, HDL may specifically transport oxidized lipid products that arise from the oxidation of LDL (24–27). Thus, HDL may protect against the accumulation of oxidized lipids in the arterial wall by acting as a sink for lipid peroxidation products. Although the transport of oxidation products by HDL is thought to be a protective and beneficial process, the present study suggests that this process may actually compromise HDL-mediated reverse cholesterol transport.

The noteworthy feature of the present study is that minimally oxidized LDL produced substantial reductions in LCAT activity and crosslinking of HDL-apolipo-

TABLE 1. Comparisons between the present study and other published studies regarding extent of LDL oxidation and associated atherogenic response

| Atherogenic Response | Method(s) of Oxidation | TBARS[a] | Reference |
|---|---|---|---|
| Inhibition of LCAT activity | copper (or AAPH) | $2.3 \pm 0.1$ | present study |
| Monocyte migration | iron | 4.2 to 10.5[b] | 50 |
| Monocyte/endothelial cell interactions | iron | 10.5[b] | 14 |
| | iron (or storage at 4°C) | 4.2 to 7.8[b] | 51 |
| | endothelial cells and human monocytes | 4.0 | 52 |
| Cytotoxicity | dialysis in absence of EDTA | 5.6[b] | 16 |
| | iron | 10.1[b] | 17 |
| | UV irradiation plus copper | 4.0 to 6.0 | 18 |
| Crosslinking of apoA-I | copper | 6.3 | present study |
| Enhanced uptake and degradation of oxidized LDL by macrophages | rabbit aortic macrophages | 40 | 20 |
| | endothelial cells | 45 | 53 |

[a]TBARS values are nmol MDA eq./mg LDL protein.

[b]TBARS were originally reported as nmol MDA eq./mg LDL cholesterol; for comparisons, these values were normalized to LDL protein by multiplying by 2.1. This conversion factor was used as the cholesterol content of LDL is approximately twice the protein mass (i.e., total cholesterol = 45% and protein = 21% of LDL particle weight, ref. 54).

proteins. These alterations to key components of the putative antiatherogenic reverse cholesterol transport system have not yet been considered in relationship to the degree of LDL oxidation nor have they been placed in context with other molecular and cellular events known to be affected by oxidized LDL. **Table 1** relates responses of LCAT activity and apoA-I structure to the degree of LDL oxidation and compares it with responses of other proatherogenic parameters such as monocyte migration and cytotoxicity. Minimally modified LDL, generally in the range of 2 to 5 nmol MDA eq./mg LDL cholesterol, is a chemoattractant of circulating human monocytes (13, 49, 50), stimulates endothelial cells to bind monocytes (14, 51, 52), and is cytotoxic towards fibroblasts, endothelial cells, and vascular smooth muscle cells (15–18). To facilitate comparisons between reported studies and the present one, the TBARS values originally reported were normalized to LDL protein instead of cholesterol. As a result, these TBARS are approximately 2-fold higher than the originally reported values (Table 1). In our system, inhibition of LCAT activity and changes in apoA-I structure were obtained at 2 and 6 nmol MDA eq./mg LDL protein, respectively, suggesting that reductions in LCAT activity and changes in HDL-structure are sensitive indices of LDL oxidation and that modifications in these parameters may reflect some of the earliest responses to LDL oxidation (Table 1). As discussed above, crosslinking of HDL-apolipoproteins is associated with a hypercatabolism of such particles, suggesting that oxidative modifications of HDL may result in decreased concentrations of circulating HDL. Moreover, inhibition of LCAT activity and crosslinking of apoA-I may limit the ability of HDL to promote cholesterol efflux from arterial wall cells.

Taken together, these observations suggest that, in addition to other atherogenic mechanisms (Table 1), the oxidation of LDL in the arterial wall may predispose to the development of atherosclerosis by reducing HDL-cholesterol concentrations and impairing HDL-mediated reverse cholesterol transport.

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