Differences in the metabolism of oxidatively modified low density lipoprotein and acetylated low density lipoprotein by human endothelial cells: inhibition of cholesterol esterification by oxidatively modified low density lipoprotein[1]

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Abstract The rate of degradation of oxidatively modified low density lipoprotein (Ox-LDL) by human endothelial cells was similar to that of unmodified low density lipoprotein (LDL), and was approximately 2-fold greater than the rate of degradation of acetylated LDL (Ac-LDL). While LDL and Ac-LDL both stimulated cholesterol esterification in endothelial cells, Ox-LDL inhibited cholesterol esterification by 34%, demonstrating a dissociation between the degradation of Ox-LDL and its ability to stimulate cholesterol esterification. Further, while LDL and Ac-LDL resulted in a 5- and 15-fold increase in cholesteryl ester accumulation, respectively, Ox-LDL caused only a 1.3-fold increase in cholesteryl ester mass. These differences could be accounted for, in part, by the reduced cholesteryl ester content of Ox-LDL. However, when endothelial cells were incubated with Ac-LDL in the presence and absence of Ox-LDL, Ox-LDL led to a dose-dependent inhibition of cholesterol esterification without affecting the degradation of Ac-LDL. This inhibitory effect of Ox-LDL on cholesteryl ester synthesis was also manifest in normal human skin fibroblasts incubated with LDL and in LDL-receptor-negative fibroblasts incubated with unesterified cholesterol to stimulate cholesterol esterification. Further, the lipid extract from Ox-LDL inhibited cholesterol esterification in LDL-receptor negative fibroblasts. These findings suggest that the inhibition of cholesterol esterification by oxidized LDL is independent of the LDL and scavenger receptors and may be a result of translocation of a lipid component of oxidatively modified LDL across the cell membrane. **—Jialal, I., and A. Chait.** Differences in the metabolism of oxidatively modified low density lipoprotein and acetylated low density lipoprotein by human endothelial cells: inhibition of cholesterol esterification by oxidatively modified low density lipoprotein. *J. Lipid Res.* 1989. **30:** 1561–1568.

Supplementary key words ACAT • scavenger receptor • oxysterols • LDL receptor

Lipoprotein endothelial cell interactions have been implicated in the initiation of atherosclerosis (1,2). Recently, much attention has focused on the role of modified lipoproteins, especially low density lipoprotein (LDL), in the initiation and propagation of atherosclerosis (3). Certain modified forms of LDL, e.g., acetylated LDL (Ac-LDL) and malondialdehyde-modified LDL, are taken up by scavenger receptors that are present on macrophages and endothelial cells (3–5). Recently, it has been shown that oxidatively modified LDL (Ox-LDL) also appears to be recognized by scavenger receptors (6,7), but may, in addition, be taken up by other receptor-dependent mechanisms (8). Biological effects that have been attributed to Ox-LDL include cytotoxicity, inhibition of chemotaxis of resident macrophages, stimulation of monocytes chemotaxis, and inhibition of platelet-derived growth factor-like protein production by endothelial cells (7,9–12). Havekes et al. (13) have shown that the uptake and degradation of Ac-LDL by human endothelial cells was only 25% that of LDL and that Ac-LDL failed to cause an increase in the cholesterol content of the cells, although it previously has been reported that Ac-LDL promotes cholesterol mass accumulation in bovine aortic-endothelial cells (5,14). The present study was undertaken to determine whether Ox-LDL, which can be generated by endothelial cells (6) and which has been implicated in the pathogenesis of atherosclerosis (7), can influence cholesterol metabolism in human endothelial cells. Results indicate that while both Ox-LDL and Ac-LDL are processed by human

Abbreviations: LDL, low density lipoprotein; Ox-LDL, oxidatively modified low density lipoprotein; Ac-LDL, acetylated low density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; TBARS, thiobarbituric acid reacting substances; ACAT, acyl CoA:cholesterol acyltransferase; LPDS, lipoprotein-deficient serum; TLC, thin-layer chromatography.

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endothelial cells, Ox-LDL, in contrast to Ac-LDL, inhibited cholesterol esterification and failed to stimulate cholesteryl ester mass accumulation.

MATERIALS AND METHODS

Materials

2-Thiobarbituric acid, bovine serum albumin, L-cysteine, and butylated hydroxytoluene were from Sigma Chemical Co. (St. Louis, MO); $CuSO_4$ was from Mallinckrodt, Inc., (Paris, KY); Ficoll-Paque was from Pharmacia Fine Chemicals (Piscataway, NJ); and acetic anhydride was from BDH Chemicals (Toronto, Ontario). Dulbecco's modified Eagle's Medium (DMEM), fetal calf serum, penicillin-streptomycin, and L-glutamine were obtained from Gibco Laboratories (Grand Island, NY). Carrier-free Na[^{125}I] and [^{14}C]oleate were obtained from New England Nuclear (Boston, MA). All chemicals were reagent grade.

Lipoproteins

Human LDL (d 1.019–1.063 g/ml) was prepared by discontinuous density gradient ultracentrifugation (15) as previously described (16). The isolated LDL was dialyzed against 150 mM NaCl, 1 mM EDTA, pH 7.4, stored at 4°C under N_2 in the dark, and used within 2 weeks. LDL was acetylated by the method of Basu et al. (17) and radio-iodinated by the iodine monochloride method as modified for lipoproteins (18). LDL or ^{125}I-labeled LDL was oxidatively modified in a cell-free system as previously described (19). Briefly, LDL (300 μg/ml) was incubated in Earle's salts (NaCl, 116 mM; KCl, 5.36 mM; $CaCl_2$, 1.8 mM; $MgSO_4$, 0.81 mM; NaH_2PO_4, 1 mM) buffered at pH 7.4 with 25 mM HEPES with 3 μM Cu(II) and 3 mM L-cysteine for 24 h at 37°C in air in borosilicate glass tubes. Oxidative modification was confirmed by measurement of the lipid peroxide content of Ox-LDL by a modification of the thiobarbituric acid reacting substances (TBARS) assay of Buege and Aust (20) as described (16) and by demonstration of increased electrophoretic mobility on 0.5% agarose gel relative to native LDL. For some experiments LDL was also oxidized in the presence of 5 μM Cu(II) in phosphate-buffered saline (PBS) as described by Steinbrecher et al. (21).

Cell culture

Human endothelial cells were isolated from umbilical cord vein as described by Jaffe et al. (22) and cultured in 16- or 35-mm multiwell dishes. The cells were grown in DMEM containing 15% calf serum supplemented with 5% fetal bovine serum, 2% endothelial cell growth supplement prepared from bovine brain, and pituitary according to Gospodarowicz, Cheung, and Lirette (23), 50 μg/ml heparin, 100 μg/ml streptomycin, 100 U/ml penicillin, and fungizone 0.25 μg/ml at 37°C in a humidified atmosphere containing 5% CO_2. All experiments were performed in confluent cultures between the 5th and 10th passages. Prior to all experiments the cells were washed with DMEM (serum-free) and all incubations were performed in this medium. The endothelial nature of the cells was established by their typical cobblestone morphology at confluency and the presence of factor VIII R:Antigen.

Human skin fibroblasts were cultured from punch biopsies of the medial thigh from normal volunteers as described previously (24). Subcultures were used between passages 4 and 12. The cells were plated at 2.5×10^4 cells/16-mm dish in DMEM supplemented with 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 5 days in culture, the medium was changed to DMEM supplemented with 10% lipoprotein-deficient human serum (LPDS; prepared from $d > 1.25$ g/ml infranatant of serum) and used after 48 h of incubation.

LDL receptor-negative human skin fibroblasts (GM2000E) derived from a patient with homozygous familial hypercholesterolemia were obtained from N. I. G. M. S. Human Genetic Mutant Cell Repository. The cells were maintained in culture essentially as described above for normal human skin fibroblasts except that calf serum was used.

Assays

The uptake and degradation of ^{125}I-labeled LDL, ^{125}I-labeled Ac-LDL, and ^{125}I-labeled Ox-LDL by human endothelial cells was measured by the appearance of trichloracetic acid-soluble radioactivity (non-iodide) formed by the cells and excreted into the medium (serum-free) following a 5-h preincubation of the cells with the respective radiolabeled lipoproteins (25). Degradation rates were corrected for cell-free controls incubated in parallel. Proteins were measured by the method of Lowry et al. (26) using bovine serum albumin as standard.

The rate of esterification of cholesterol was estimated following preincubation of cells for 24 h with the respective lipoproteins, washing the cells with serum-free medium (DMEM) and then pulsing the cells for 2 h with [^{14}C]oleate (0.5 μCi/ml) in DMEM. The cells were then chilled on ice, washed three times with PBS, and the sterols were extracted in isopropanol–hexane and subjected to thin-layer chromatography. The band corresponding to a cholesteryl ester standard was cut out for quantitation by scintillation counting as described (27,28).

Esterified and unesterified cholesterol that accumulated in cells following incubation with lipoproteins were extracted and separated by thin-layer chromatography as described previously (28). The bands corresponding to cholesteryl ester and unesterified cholesterol standards were scraped and hydrolyzed in 1 N ethanolic KOH. The cholesterol content was then determined enzymatically by the

method of Heider and Boyett (29). In Ox-LDL numerous additional bands more polar than cholesteryl ester were visualized on the thin-layer plates. These presumably represent oxysterol esters, which were not quantitated. In addition, the lipid composition of the various lipoproteins was also determined. Experiments were performed to determine whether the presence of oxidized lipid in Ox-LDL affected the hydrolysis and/or enzymatic determination of cholesteryl esters. When LDL and Ox-LDL were mixed, the observed cholesteryl ester content (determined both enzymatically and colorimetrically) was the same as that predicted from the cholesteryl ester content of the individual lipoproteins prior to mixing (data not shown). All results are expressed as the mean ± SEM of triplicate determinations unless stated otherwise. All experiments were repeated on at least two occasions with similar results.

RESULTS

Compared to LDL, Ac-LDL and both forms of Ox-LDL demonstrated increased electrophoretic mobility on agarose gel electrophoresis, suggesting an increased net negative charge (**Fig. 1**). However, while the TBARS content of LDL and Ac-LDL was low (<1.7 nmol MDA/mg protein), LDL oxidized either in the presence of L-cysteine and Cu(II) or PBS and Cu(II) showed a significant increase in TBARS content (27.2 ± 0.9 and 38.9 ± 1.2 nmol malondialdehyde equivalents/mg protein respectively), consistent with lipid peroxidation.

Oxidation of LDL in the presence of Cu(II) and L-cysteine resulted in substantial changes in lipid composition (**Table 1**). There was a 57% reduction in the cholesteryl ester content and a 55% reduction in the triglyceride content of Ox-LDL relative to LDL, while the content of unesterified cholesterol and phospholipid remained essentially unchanged. Similar changes in lipoprotein composition were observed with LDL oxidized by 5 μM Cu(II) in PBS (data not shown). Presumably some cholesteryl esters were converted to oxysterol esters, which would migrate differently on TLC and hence not be detected by the methods used.

TABLE 1. Lipid composition of LDL and Ox-LDL

	LDL	Ox-LDL[a]
Total cholesterol (μg/mg protein)	1477 ± 30	891 ± 25
Cholesteryl ester (μg/mg protein)	1026 ± 31	439 ± 20
Unesterified cholesterol (μg/mg protein)	451 ± 7	453 ± 11
Triglycerides (μg/mg protein)	112 ± 3	51 ± 3
Phospholipids (nmol P/mg protein)	1.1 ± 0.03	1.08 ± 0.01

[a]LDL was oxidatively modified in the presence of L-cysteine and Cu(II) as described in Methods. Values are the mean ± standard deviation of three determinations.

The rates of degradation of ^{125}I-labeled Ox-LDL and LDL by human endothelial cells were similar, while the rate of degradation of Ox-LDL was approximately twice that of Ac-LDL, especially at higher ligand concentrations (**Fig. 2**). Degradation of both ^{125}I-labeled Ox-LDL and Ac-LDL was competed for by Ac-LDL, fucoidin, and polyinosinic acid, but not by LDL or polycytidylic acid (competitor concentrations up to 20-fold excess), consistent with uptake of Ox-LDL and Ac-LDL via the scavenger receptor (data not shown). Also, the degradation of both Ox-LDL and Ac-LDL was inhibited (72 ± 4 and

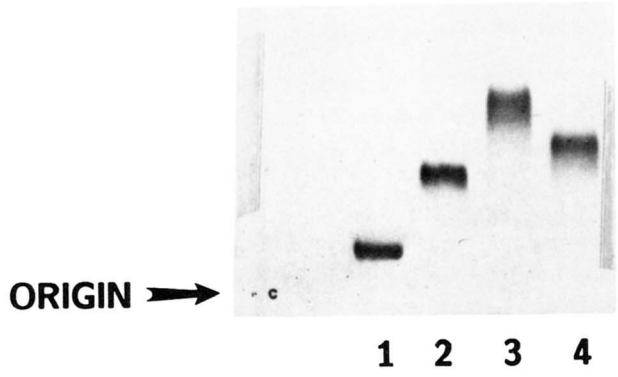

Fig. 1. Electrophoretic mobility on agarose gels of LDL, Ox-LDL, and Ac-LDL. LDL was modified as described in Methods. Lane I, unmodified LDL; lane 2, Ox-LDL [modified in the presence of L-cysteine and Cu(II)]; lane 3, Ox-LDL [modified in the presence of PBS and Cu(II)]; and lane 4, Ac-LDL.

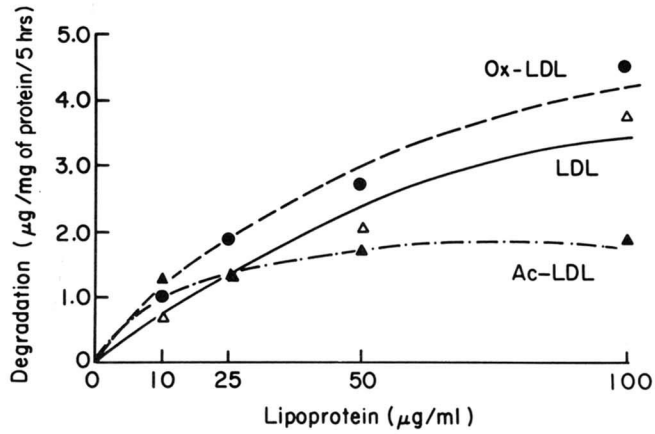

Fig. 2. Degradation of LDL, Ox-LDL, and Ac-LDL by human endothelial cells. Confluent endothelial cells were washed twice in serum-free medium prior to addition of medium containing ^{125}I-labeled LDL (△-△), Ox-LDL (●--●), or Ac-LDL (▲- - - ▲) at the concentrations indicated. After 5 h incubation at 37°C, the media were removed for analysis of lipoprotein degradation by measurement of the TCA-Soluble ^{125}I radioactivity (noniodide) as described in Methods. Results are presented as μg of protein degraded per mg of cell protein per 5 h. Each point represents the mean of triplicate determinations.

85 ± 2%, respectively) by the lysosomotrophic agent chloroquine at a concentration of 50 μM, suggesting that both these modified LDL forms of LDL are processed lysosomally.

Since Ox-LDL at concentrations of ≥75 μg/ml was found to be cytotoxic to human endothelial cells, all experiments designed to examine the effects of Ox-LDL on cholesterol esterification and mass accumulation were performed using Ox-LDL concentrations ≤50 μg/ml. To obtain an estimate of the delivery of cholesterol to endothelial cells and its subsequent esterification by acyl CoA-cholesterol acyltransferase (ACAT), the incorporation of exogenously added [^{14}C]oleate into cholesteryl esters was measured. Preincubation of cells with LDL caused a 50% increase in cholesterol esterification, while Ac-LDL caused a 280% increase. However, Ox-LDL inhibited cholesterol esterification by 34% compared to cells incubated in media alone (**Fig. 3**). LDL oxidized in the presence of 5 μM Cu(II) in PBS inhibited cholesterol esterification by 11%. No significant inhibition of [^{14}C]oleate incorporation into either triglycerides or phospholipids was observed with Ox-LDL (data not shown).

Exposure of endothelial cells to LDL caused a 5-fold increase in cholesteryl ester mass over control, while Ac-LDL caused a 15-fold increase. However, Ox-LDL caused only 1.3-fold increase in cholesteryl ester mass (**Fig. 4A**). Unesterified cholesterol content of endothelial cells also failed to increase after exposure to Ox-LDL (data not shown). When the cholesteryl ester mass was expressed as a percentage of the total cholesterol accumulation, cholesteryl ester comprised a significantly greater percentage following preincubation with LDL and Ac-LDL than with Ox-LDL (Fig. 4B).

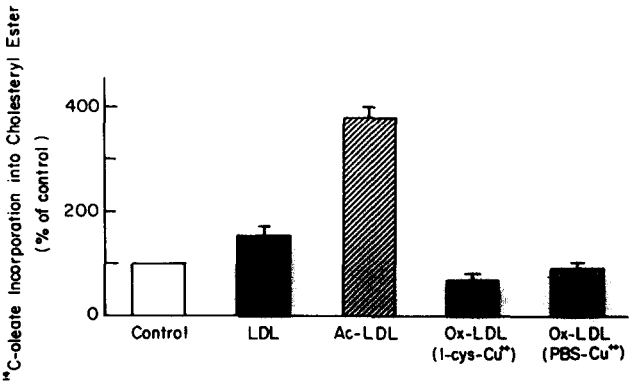

Fig. 3. Effect of the lipoproteins on [^{14}C]oleate incorporation into cholesteryl esters. Human endothelial cells were incubated with the various lipoproteins (50 μg protein/ml). Thereafter, the cells were washed twice in serum-free medium and pulsed with [^{14}C]oleate (0.5 μCi/ml) for 2 h at 37°C. The cells were then chilled on ice, washed three times with PBS, and the lipids were extracted for analysis of [^{14}C]cholesteryl esters and protein as described in Methods. Results are expressed as % of control dishes to which no lipoprotein was added.

To test whether Ox-LDL inhibited cholesterol esterification, endothelial cells were cholesterol-loaded for 24 h with Ac-LDL (100 μg protein/ml) in the presence and absence of Ox-LDL prior to the measurement of [^{14}C]oleate incorporation into cholesteryl esters. Ox-LDL caused a marked inhibition of Ac-LDL-mediated cholesterol esterification (**Fig. 5A**). Studies performed in parallel that exa-

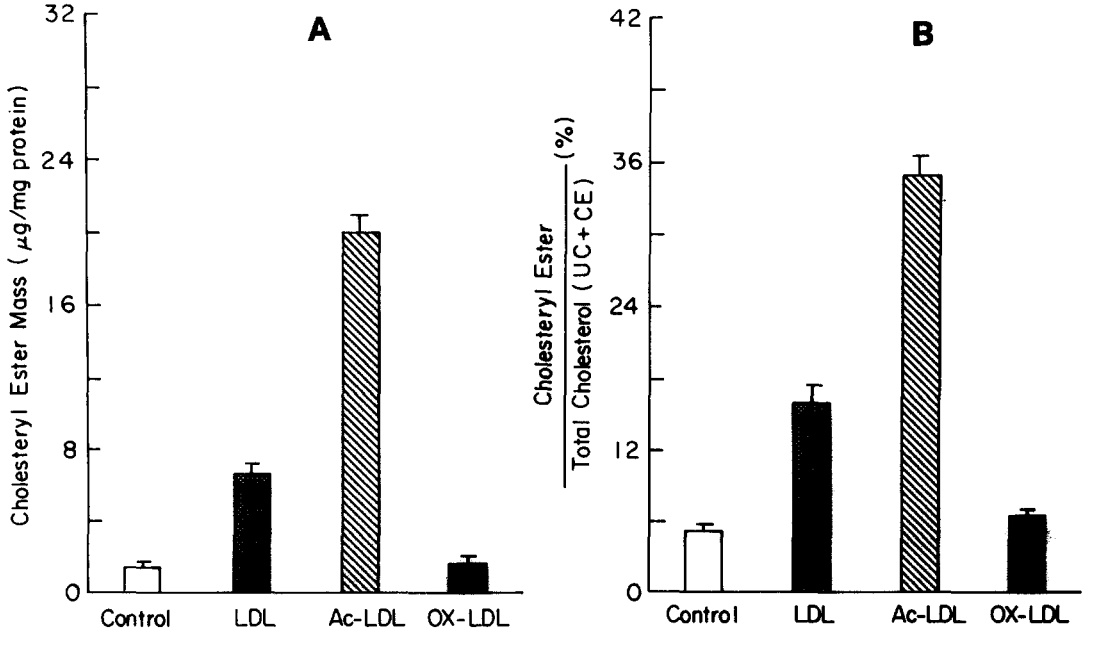

Fig. 4. Effect of LDL, Ac-LDL, and Ox-LDL on cholesterol accumulation in human endothelial cells. Cells were incubated with the lipoproteins (50 μg protein/ml) for 24 h at 37°C. Thereafter the cells were chilled on ice, washed three times with PBS, and the sterols were extracted for measurements of free and ester cholesterol mass as described in Methods. Fig. 4A shows the cholesteryl ester mass accumulation per mg of cell protein. Fig. 4B depicts cholesteryl ester as a percentage of total cellular cholesterol.

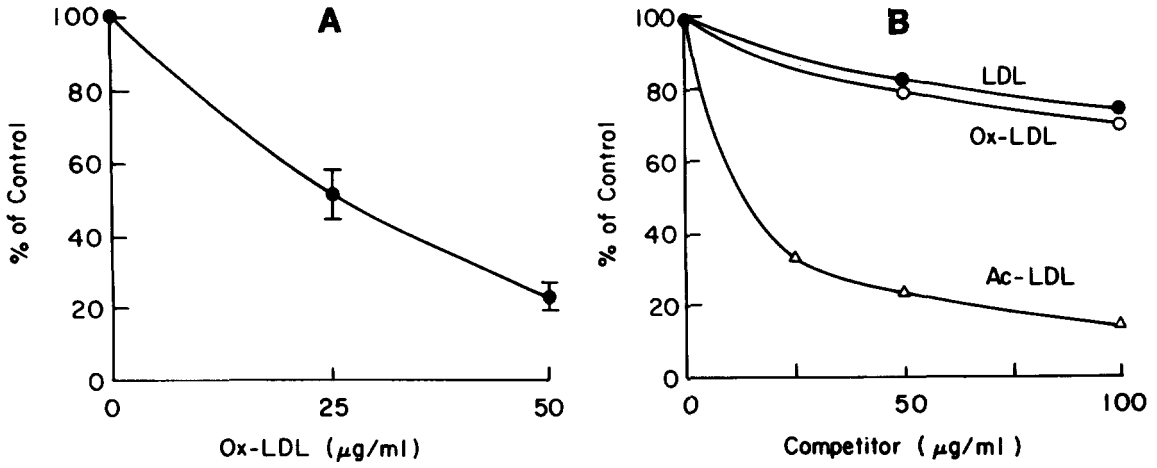

Fig. 5. Effect of Ox-LDL on Ac-LDL-stimulated cholesterol esterification (A) and on ^{125}I-labeled Ac-LDL degradation (B). A: Human endothelial cells were loaded with Ac-LDL (100 μg protein/ml) for 24 h in the presence and absence of Ox-LDL at the concentrations indicated. Thereafter the cells were washed, pulsed with [^{14}C]oleate for 2 h at 37°C for measurement of [^{14}C]oleate incorporation into cholesteryl esters as described in Methods; 100% value = 1.85 nmol/mg protein. B: Human endothelial cells were incubated with ^{125}I-labeled Ac-LDL (5 μg/ml) in the presence and absence of the indicated concentrations of competing unlabeled lipoprotein (LDL, Ox-LDL, or Ac-LDL). Medium was removed after 5 h incubation at 37°C for determination of ^{125}I-labeled degradation products as described in Methods; 100% value = 1.3 μg LDL protein degraded/mg protein.

mined the effect of Ox-LDL on the degradation of ^{125}I-labeled Ac-LDL failed to show any inhibition of ^{125}I-labeled Ac-LDL degradation by Ox-LDL (Fig. 5B), suggesting that Ox-LDL did not interfere with the uptake of degradation of Ac-LDL. LDL oxidized in PBS in the presence of 5 μM Cu(II) in PBS also caused an inhibition of cholesterol esterification (76 ± 4% at 50 μg protein/ml) stimulated by Ac-LDL (100 μg protein/ml).

To determine whether the scavenger receptor was required for Ox-LDL to mediate this inhibitory effect on the incorporation of [^{14}C]oleate into cholesteryl esters, the effect of Ox-LDL on cholesterol esterification was studied in human fibroblasts which are devoid of scavenger receptors (4). Skin fibroblasts in which the LDL receptor had previously been up-regulated with LPDS were cholesterol-loaded by exposure to LDL (50 μg protein/ml). Ox-LDL caused a dose-dependent inhibition of cholesterol esterification stimulated by LDL in normal skin fibroblasts (**Fig. 6**). To further determine whether this inhibitory effect of Ox-LDL was independent of receptor-mediated processing, similar studies were undertaken in LDL receptor-negative fibroblasts that were loaded with cholesterol by exposure to unesterified cholesterol (50 μg/ml in ethanol) and the effect of Ox-LDL on cholesterol esterification was examined. Both types of Ox-LDL [modified by exposure to either L-cysteine-Cu(II) or PBS-Cu(II)] caused a dose-dependent inhibition of cholesterol esterification (**Fig. 7**). To determine whether the inhibitory component of Ox-LDL was in the lipid phase, the lipids were extracted with chloroform–methanol (30), dried under N_2, dissolved in ethanol, and added to receptor-negative fibroblasts that had previously been loaded with cholesterol in ethanol. The lipid extract of both types of Ox-LDL [(L-cysteine-Cu(II) and PBS-Cu(II)] had an inhibitory effect on cholesterol-mediated cholesterol esterification (**Fig. 8**), consistent with a lipid component of Ox-LDL being inhibitory to cellular cholesterol esterification.

DISCUSSION

Ox-LDL and Ac-LDL are both modified forms of LDL that are believed to be processed by scavenger receptors that are present on both macrophages and endothelial cells (4). In the present study, the degradation of both modified lipoproteins by human endothelial cells was inhibited by fucoidin, polyinosinic acid, and Ac-LDL, but not LDL, consistent with scavenger receptor uptake.

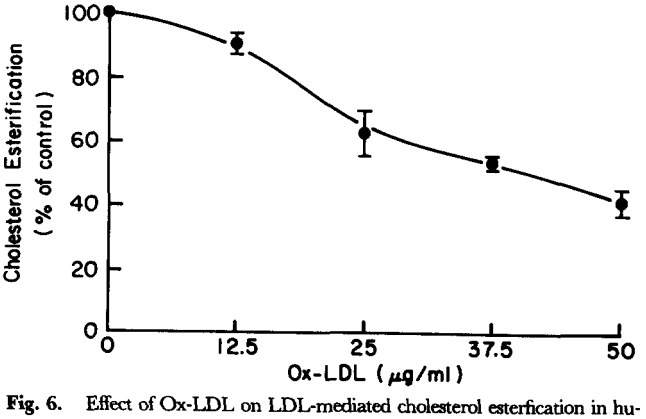

Fig. 6. Effect of Ox-LDL on LDL-mediated cholesterol esterfication in human fibroblasts. Confluent human skin fibroblasts were preincubated in 10% lipoprotein deficient serum for 48 h at 37°C to induce maximum expression of LDL receptors. Cells were washed in serum-free medium and then incubated with LDL (50 μg/ml) in the presence and absence of Ox-LDL at the indicated concentrations for 24 h at 37°C. Thereafter, the cells were washed, pulsed with [^{14}C]oleate for 2 h and [^{14}C]oleate incorporation into cholesteryl ester was determined as described in Methods; 100% value = 3.16 nmol/mg protein.

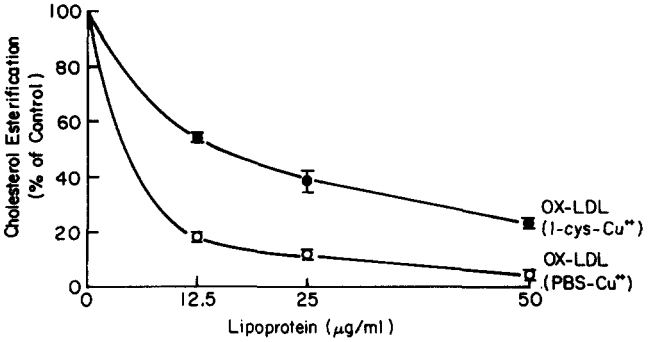

Fig. 7. The effect of Ox-LDL on cholesterol esterification in LDL receptor negative fibroblasts. LDL-receptor negative fibroblasts were grown to confluency as described in Methods. Thereafter the cells were washed twice and preincubated with cholesterol (50 μg/ml) in DMEM supplemented with albumin (2 mg/ml) derived from a stock (10 mg/ml cholesterol in 95% ethanol) for 24 h at 37°C. The cells were washed three times with PBS and then incubated in serum-free medium or Ox-LDL [modified by incubation with L-cysteine and Cu(II) or PBS and Cu(II)] at the indicated concentrations for 24 h. The cells then were washed and pulsed for 2 h at 37°C with [^{14}C]oleate for measurement of its incorporation into cholesteryl esters as described in Methods; 100% value = 5.1 nmol/mg protein.

However, the observations that Ox-LDL was more avidly processed by these cells than was Ac-LDL, and the failure of Ox-LDL to compete for the degradation of Ac-LDL, are consistent with alternate receptor-mediated uptake mechanisms for Ox-LDL as has been suggested to occur in macrophages (8). In accord with the only other group that has reported Ac-LDL processing in human endothelial cells, the present study concurs with the observation of lesser degradation of Ac-LDL than LDL (13). However, while Ac-LDL led to an increase in the cholesteryl ester mass of endothelial cells in the present study, it failed to result in intracellular accumulation in that study (13). The reason for this discrepancy is unclear, but possibly relates to differences in cell culture conditions.

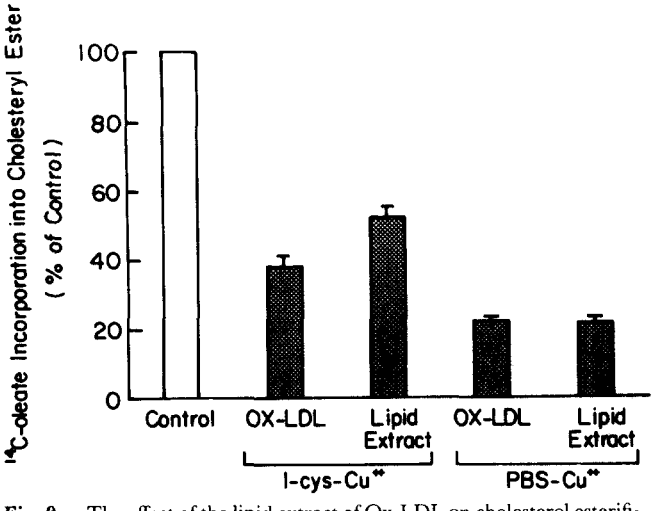

Fig. 8. The effect of the lipid extract of Ox-LDL on cholesterol esterification in LDL receptor-negative fibroblasts. LDL receptor-negative fibroblasts were grown to confluency and then incubated with cholesterol (50 μg/ml) for 24 h at 37°C as described in the legend to Fig. 7. The lipid moiety of Ox-LDL [modified by incubation with L-cysteine and Cu(II) or PBS and Cu(II)] was extracted with chloroform–methanol 2:1, dried under N_2, dissolved in ethanol, and then added to the cells that had previously been exposed to cholesterol for 24 h at 37°C. The lipid extract added to cells was equivalent to that present in Ox-LDL added to cells at a concentration of 50 μg protein/ml. The cells were then washed and pulsed with [^{14}C]oleate for 2 h at 37°C for measurement of its incorporation into cholesteryl esters as described in Methods. The final concentration of ethanol to which the cells were exposed was 0.5% (v/v); an ethanol control run in parallel did not inhibit cholesterol esterification; 100% value = 1.2 nmol/mg protein.

In contrast to Ac-LDL, Ox-LDL did not lead to appreciable cholesterol accumulation in human endothelial cells in the present study. Although Ox-LDL was taken up and degraded at twice the rate of Ac-LDL by endothelial cells, the cholesterol content of Ox-LDL was approximately half that of Ac-LDL. Therefore, the net amount of cholesterol delivered to endothelial cells by these two modified lipoproteins should be roughly equivalent. However, the findings reported here reveal that while Ac-LDL caused a 280% increase in cholesterol esterification (a measure of cholesterol delivery and esterification by ACAT), Ox-LDL inhibited esterification by 34%. These findings demonstrate a clear dissociation between Ox-LDL degradation and cholesterol esterification. When the effect of these lipoproteins on the accumulation of cholesteryl ester in endothelial cells was investigated, it was evident that while both Ac-LDL and LDL caused a significant increment in cholesteryl ester mass, Ox-LDL failed to elicit a similar effect, with cholesteryl ester accounting for only 5% of the total cholesterol mass after exposure of cells to Ox-LDL, while the percentage of cholesteryl ester was higher with LDL (16%) and Ac-LDL (35%).

A possible explanation for the inability of Ox-LDL to stimulate cholesteryl ester accumulation in endothelial cells is that uptake and degradation of Ox-LDL could result in the intracellular accumulation of oxysterol esters, which might migrate differently than cholesteryl esters on thin-layer chromatography and hence not be detected by the method used for measurement of cholesteryl esters. However, it is likely that the failure of Ox-LDL to lead to cholesteryl ester accumulation in endothelial cells is also due in part to inhibition of cholesterol esterification by Ox-LDL. The finding that Ox-LDL inhibited Ac-LDL-mediated cholesterol esterification without interfering with the degradation of Ac-LDL suggests that Ox-LDL may directly inhibit ACAT. Alternatively, Ox-LDL might reduce the availability of cholesterol for ACAT, possibly by stimulating the translocation or efflux of cholesterol from cytoplasmic pools. The inhibition by Ox-LDL of LDL-mediated cholesterol esterification in cultured human fibroblasts suggests that the scavenger receptor is not essential for Ox-LDL to exert this inhibitory effect on cholesterol esterification since fibroblasts are devoid of scavenger receptors (4). The observation that Ox-LDL also inhibited cholesterol esterification stimulated by free cholesterol exposure in LDL receptor-negative

fibroblasts indicates that the LDL receptor also is not involved and raises the possibility that the inhibitory factor in Ox-LDL may be an oxysterol that can translocate across the cell membrane independent of receptor-mediated processing. Further evidence that the inhibitory moiety of Ox-LDL resides in the lipid phase was obtained when the lipid extract of Ox-LDL was shown to inhibit cholesterol esterification. It previously has been reported that oxysterols such as 7-keto 20-oxycholesterol, 22-hydroxycholesterol, and progesterone can inhibit ACAT activity (4, 31). Direct assessment of ACAT activity in microsomes obtained from cells preincubated with Ox-LDL should provide insights as to whether components of Ox-LDL can directly inhibit ACAT. However, Ox-LDL did not cause net accumulation of unesterified cholesterol in human endothelial cells, raising the possibility that Ox-LDL might stimulate cholesterol efflux. The lipid phase of Ox-LDL has also been shown to be toxic to cells (10,32), and was cytotoxic to endothelial cells in the present study when used at high concentrations. Indeed, it is conceivable that certain oxysterols might be responsible for both the cytotoxic properties of Ox-LDL and for the inhibition of cholesterol esterification observed.

Macrophages are precursors of arterial wall foam cells in early atherosclerotic lesions (33) and Ox-LDL has been suggested to play an important early role in atherogenesis (7). However, although LDL that has been oxidatively modified by exposure to aortic smooth muscle or endothelial cells, or that has been oxidatively modified in a cell-free system, has been shown to stimulate the incorporation of [^{14}C]oleate into cholesteryl esters in macrophages (34–36), there is little information concerning cholesteryl ester mass accumulation following exposure of this cell type to Ox-LDL. The total cholesterol content of macrophages has been reported to increase slightly (36) to moderately (37) after exposure of mouse macrophages to endothelial cell-modified LDL. Therefore, future studies should determine whether Ox-LDL also inhibits cholesterol esterification in macrophages and leads to less cholesteryl ester accumulation than other modified forms of LDL such as Ac-LDL or malondialdehyde-modified LDL in this cell type.

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REFERENCES

1. Goode, T. B., P. E. Davies, M. A. Reidy, and D. E. Bowyer. 1977. Aortic endothelial cell morphology observed in situ by scanning electron microscopy during atherogenesis. *Atherosclerosis.* **27:** 235–251.
2. Gerrity, R. G., H. K. Naito, M. Richardson, and C. J. Schwartz. 1979. Dietary induced atherogenesis in swine. Morphology of the intima in prelesion stages. *Am. J. Pathol.* **95:** 775–792.
3. Haberland, M. E., and A. M. Fogelman. 1987. The role of altered lipoproteins in the pathogenesis of atherosclerosis. *Am. Heart J.* **113:** 573–577.
4. Brown, M. S., and J. L. Goldstein. 1983. Lipoprotein metabolism in the macrophage. Implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52:** 223–261.
5. Stein, O., and Y. Stein. 1980. Bovine aortic endothelial cells display macrophage-like properties towards acetylated ^{125}I-labelled low density lipoprotein. *Biochim. Biophys. Acta.* **620:** 631–635.
6. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **78:** 6499–6503.
7. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. C. Witztum. 1989. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *New Engl. J. Med.* **320:** 915–924.
8. Sparrow, C. P., S. Parthasarathy, and D. Steinberg. 1989. A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein. *J. Biol. Chem.* **264:** 2599–2609.
9. Jurgens, G., H. P. Hoff, G. M. Chisolm, and H. Esterbauer. 1987. Modification of human serum low density lipoprotein by oxidation—characterization and pathophysiological implications. *Chem. Phys. Lipids.* **45:** 315–336.
10. Morel, D. W., J. W. Hessler, and G. M. Chisolm. 1983. Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J. Lipid Res.* **24:** 1070–1076.
11. Fox, P. L., G. M. Chisolm, and P. E. DiCorletto. 1987. Lipoprotein-mediated inhibition of endothelial cell production of platelet-derived growth factor-like protein depends on free radical lipid peroxidation. *J. Biol. Chem.* **262:** 6046–6054.
12. Quinn, M. T., S. Parthasarathy, L. G. Fong, and D. Steinberg. 1987. Oxidatively modified low density lipoproteins: a potential role in recruitment and retention of monocyte macrophages during atherogenesis. *Proc. Natl. Acad. Sci. USA.* **84:** 2995–2998.
13. Havekes, L., A. Mommaas-Klenhuis, D. Schouten, E. DeWit, M. Scheffler, and V. Van Hinsbergh. 1985. High affinity uptake and degradation of acetylated low density lipoprotein by confluent human vascular endothelial cells. *Atherosclerosis.* **56:** 81–92.
14. Baker, D. P., B. Van Lenten, A. M. Fogelman, P. Edwards, C. Kean, and J. A. Berliner. 1984. LDL scavenger and β-VLDL receptors on aortic endothelial cells. *Arteriosclerosis.* **4:** 248–254.
15. Chung, B. H., T. Wilkinson, J. Geer, and J. Segrest. 1980. Preparative and quantitative isolation of plasma lipoproteins: rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J. Lipid Res.* **21:** 284–291.
16. Heinecke, J. W., H. Rosen, L. Suzuki, and A. Chait. 1987. The role of sulphur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *J. Biol. Chem.* **262:** 10098– 10103.
17. Basu, S. K., J. L. Goldstein, R. G. Anderson, and M. S. Brown. 1976. Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibrobalsts. *Proc. Natl. Acad. Sci. USA.* **73:** 3178–3182.

18. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoproteins. Preliminary in vitro and in vivo observations. *Biochim. Biophys. Acta.* **260:** 212–222.
19. Heinecke, J. W. and A. Chait. 1987. Role of thiol-generated reactive oxygen species in modification of low density lipoproteins. *Arteriosclerosis.* **7:** 503a.
20. Buege, J. A., and S. D. Aust. 1978. Microsomal lipid peroxidation. *Methods Enzymol.* **52:** 302–310.
21. Steinbrecher, U. P., J. Witztum, S. Parthasarathy, and D. Steinberg. 1987. Decrease in reactive amino groups during oxidation or endothelial cell modification of LDL. *Arteriosclerosis.* **7:** 135–143.
22. Jaffe, E. A., R. L. Nachman, C. G. Becker, and C. R. Minick. 1974. Culture of human endothelial cells derived from unbilical veins: identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52:** 2745–2756.
23. Gospodarowicz, D., J. Cheung, and M. Lirette. 1983. Bovine brain and pituitary growth factors: comparison of their abilities to support the proliferation of human and bovine vascular endothelial cells. *J. Cell Biol.* **97:** 1677–1685.
24. Chait, A., E. L. Bierman, and J. J. Albers. 1979. Low density lipoprotein receptor activity in cultured human skin fibroblasts. Mechanisms of insulin-induced stimulation. *J. Clin. Invest.* **64:** 1309–1319.
25. Bierman, E. L., O. Stein, and Y. Stein. 1974. Lipoprotein uptake and metabolism by rat aortic smooth muscle cells in tissue culture. *Circ. Res.* **35:** 136–150.
26. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
27. Brown, M. S., Y. K. Ho, and J. L. Goldstein. 1980. The cholesteryl ester cycle in macrophage foam cells. *J. Biol. Chem.* **255:** 9344–9352.
28. Brinton, E. A., R. Kenagy, J. F. Oram, and E. L. Bierman. 1985. Regulation of high density lipoprotein binding activity of aortic endothelial cells by treatment with acetylated low density lipoprotein. *Arteriosclerosis.* **5:** 329–335.
29. Heider, J. G., and R. L. Boyett. 1978. The picomole determination of free and total cholesterol in cells in culture. *J. Lipid Res.* **19:** 514–518.
30. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
31. Bates, S. R., C. Jett, and J. E. Miller. 1983. Prevention of the hyperlipidemic serum or LDL-induced cellular cholesterol ester accumulation by 22-hydroxycholesterol and its analogue. *Biochim. Biophys. Acta.* **753:** 281–293.
32. Hessler, J. R., D. W. Morel, C. J. Lewis, and G. M. Chisolm. 1983. Lipoprotein oxidation and lipoprotein-induced cytotoxicity. *Atherosclerosis.* **3:** 215–272.
33. Ross, R. 1986. The pathogenesis of atherosclerosis. An update. *New Engl. J. Med.* **314:** 488–500.
34. Heinecke, J. W., H. Rosen, and A. Chait. 1984. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest.* **74:** 1890–1894.
35. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1985. Superoxide-mediated modification of low density lipoproteins by arterial smooth muscle cells. *J. Clin. Invest.* **77:** 757–761.
36. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1983. Enhanced macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis.* **3:** 149–159.
37. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1982. Interactions of plasma lipoproteins with endothelial cells. *Ann. NY Acad. Sci.* **401:** 102–116.