

## Low Density Lipoprotein Modification by Cholesterol Oxidase Induces Enhanced Uptake and Cholesterol Accumulation in Cells\*

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Oxidation of low density lipoprotein (LDL) by cells of the arterial wall or in the presence of copper ions was shown to result in the peroxidation of its fatty acids as well as its cholesterol moiety. LDL incubation with cholesterol oxidase (CO) resulted in the conversion of up to 85% of the lipoprotein unesterified cholesterol (cholest-5-en-3-ol) to cholestenone (cholest-4-en-3-one) in a dose- and time-dependent pattern. Plasma very low density lipoprotein (VLDL) and high density lipoprotein (HDL) could be similarly modified by CO. In cholesterol oxidase-modified LDL (CO-LDL), unlike copper ion-induced oxidized LDL (Cu-Ox-LDL), there was no fatty acids peroxidation, and lipoprotein size or charge as well as LDL cholesteryl ester, phospholipids, and triglycerides content were not affected. CO-LDL, however, demonstrated enhanced susceptibility to oxidation by copper ions in comparison to native LDL. Upon incubation of CO-LDL with J-774 A.1 macrophage-like cell line, cellular uptake and degradation of the lipoprotein was increased by up to 62% in comparison to native LDL but was 15% lower than that of Cu-Ox-LDL.

Similarly, the binding of CO-LDL to macrophages increased by up to 80%, and cellular cholesterol mass was increased 51% more than the mass obtained with native LDL.

Several lines of evidence indicate that CO-LDL was taken up via the LDL receptor: 1) Excess amounts of unlabeled LDL, but not acetyl-LDL (Ac-LDL), effectively competed with <sup>125</sup>I-CO-LDL for the uptake by cells. 2) The degradation of CO-LDL by various types of macrophages and by fibroblasts could be dissociated from that of Ac-LDL and was always higher than that of native LDL. 3) A monoclonal antibody to the LDL receptor (IgG-C7) and a monoclonal antibody to the LDL receptor binding domains on apoB-100 (B1B6) inhibited macrophage degradation of CO-LDL. The receptor for Cu-Ox-LDL, which is not shared with Ac-LDL, was also partially involved in macrophage uptake of CO-LDL, since Cu-Ox-LDL demonstrated some competition capability with CO-<sup>125</sup>I-LDL for its cellular degradation.

CO-LDL cellular degradation was inhibited by chloroquine, thus implying lysosomal involvement in the cellular processing of the lipoprotein. Incubation of macrophages with LDL in the presence of increasing concentrations of cholestenone resulted in up to 52% enhanced lipoprotein cellular degradation suggesting that the cholestenone in CO-LDL might be involved in the enhanced cellular uptake of the modified lipopro-

tein. Oxidation of LDL by incubation with macrophages resulted in the oxidation of both fatty acids and cholesterol including the formation of cholestenone. Thus, if CO-LDL is produced under certain pathological conditions, it can potentially lead to foam cell formation and to accelerated atherosclerosis.

Unlike the uptake of native low density lipoprotein (LDL)<sup>1</sup> by macrophages which is regulated by the LDL receptor and thus cannot cause massive cellular cholesterol accumulation, the uptake of oxidized LDL can lead to foam cell formation (1-3). LDL oxidation induced by either cells of the arterial wall (such as endothelial cells, smooth muscle cells, and macrophages) or by metal ions (copper or iron) involves the peroxidation of LDL polyunsaturated fatty acids as well as its cholesterol moiety, resulting in the formation of several cholesterol oxidation derivatives (3-6). Fatty acids and cholesterol peroxidation products were shown to affect LDL composition as well as its interaction with cells (1, 3, 6-11).

Since both fatty acids and cholesterol are oxidized in oxidized LDL (which is taken up by macrophages at enhanced rate), the question arises whether LDL cholesterol oxidation affects lipoprotein characteristics and hence might be responsible for some of the properties of oxidized LDL. Cholesterol oxidase (CO, EC 1.1.3.6) is a flavoprotein with molecular mass of 32 kDa that demonstrates thermal stability and possesses a broad pH optimum around 7.5 (12, 13). Cholesterol oxidase specifically converts cholesterol (5-cholesten-3-ol) to cholestenone (4-cholesten-3-one) by an oxidation step which is followed by isomerization of the C5-C6 double bond to the C4-C5 position. The action of CO on cholesterol generates both cholestenone and hydrogen peroxide (12, 13). LDL unesterified cholesterol, unlike cell membrane unesterified cholesterol, is readily susceptible to oxidation by cholesterol oxidase (14, 15). CO modification of LDL allows us to study the effect of one cholesterol oxidation product, rather than a mixture of oxidized cholesterol derivatives which are formed during LDL oxidation, on the changes which occur in the lipoprotein characteristics. Cholesterol oxidase is a product of a number of bacteria which occur in the large intestine and among human pharyngeal flora (16). Thus, absorption of CO through the gut could gain access of the enzyme to the bloodstream and there convert LDL cholesterol to cholestenone. Choles-

<sup>1</sup> The abbreviations used are: LDL, low density lipoprotein; CO-LDL, cholesterol oxidase-modified LDL; Cu-Ox-LDL, copper ion-induced oxidized LDL; DMEM, Dulbecco's modified Eagle's medium; apoB-100, apolipoprotein B-100; CE, cholesteryl ester; UC, unesterified cholesterol; MDA, malondialdehyde; PBS, phosphate-buffered saline; BSA, bovine serum albumin; mAb, monoclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography.

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tenone can also possibly be carried by the chylomicrons to the liver for VLDL synthesis where it can substitute for some of the cholesterol in VLDL. Cholesterol oxidase is also apparently present in liver where it produces cholestenone as an intermediate in the conversion of cholesterol to cholesterol (17). Intravenous injection of CO into hyperlipidemic, but not into normolipidemic rabbits, was shown to be lethal within hours, whereas injection of CO-LDL even into normolipidemic rabbits was demonstrated to be lethal (18). The present study was carried out to analyze changes in the physicochemical properties and interaction with cells of CO-LDL in comparison to native LDL and copper ion-induced oxidized LDL.

## EXPERIMENTAL PROCEDURES

### Materials

Cholesterol oxidase (EC 1.1.3.6) of bacterial origin (*Proactinomyces erythropolis*, Nocardia) was purchased from Boehringer Mannheim. Ficoll-Paque and Sephadex G-100 were purchased from Pharmacia LKB Biotechnology Inc. Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 culture medium, fetal calf serum, penicillin streptomycin, and L-glutamine were obtained from GIBCO. Carrier-free  $^{125}\text{I}$  was obtained from Du Pont-New England Nuclear. A monoclonal antibody that reacts with the human LDL receptor (IgG C7) was derived from the supernatant of a hybridoma purchased from American Type Culture Collection (Rockville, MD). Monoclonal antibody toward the apolipoprotein B-100 epitopes which are located at the LDL receptor binding domain (mapped to amino acid residues 3214–3506) was a generous gift from Drs. G. Schonfeld and E. Krul (Washington University, St. Louis, MO).

**Cells**—J-774 A.1 murine macrophage-like cell line was purchased from ATCC. J-774 A.1 cells were plated at  $2.5 \times 10^5$  cells/16-mm dish in DMEM supplemented with 10% fetal calf serum. The cells were fed every 3 days and were used for experiments within 7 days of plating (19).

Human monocytes were isolated by density gradient centrifugation from blood derived from fasting normolipidemic subjects (20). 20 ml of blood (anticoagulated with 10 units/ml heparin) was layered over 15 ml of Ficoll-Paque and centrifuged at  $500 \times g$  for 30 min at 23 °C. The mixed mononuclear cell band was removed by aspiration, and the cells were washed twice in RPMI-1640 culture medium containing 100 units/ml of penicillin, 100  $\mu\text{g}/\text{ml}$  of streptomycin, and 2 mM glutamine. The cells were plated at  $3 \times 10^5$  monocytes per 16-mm dish (Primaria; Falcon Labware, Becton Dickinson, Oxnard, CA) in the same medium. Human monocyte-derived macrophages were used within 7–10 days of plating.

Mouse peritoneal macrophages were harvested from the peritoneal fluid of female BALB/c mice (15–25 g) 4 days after intraperitoneal injection into each mouse of 3 ml of 24 g/liter thioglycolate in saline (19). The cells ( $10\text{--}20 \times 10^6/\text{mouse}$ ) were washed and centrifuged three times with phosphate-buffered saline (PBS) at  $1000 \times g$  for 10 min, then resuspended to  $10^8/1$  in DMEM containing 10% horse serum (heat-inactivated at 56 °C for 30 min), 100 units/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 2 mM glutamine. The cell suspension was dispensed into 60-mm plastic Petri dishes and incubated in a humidified incubator (5%  $\text{CO}_2$ , 95% air) for 2 h. The dishes were washed once with 5 ml of DMEM to remove nonadherent cells, and the monolayer was then incubated under similar conditions for 18 h.

Human skin fibroblasts were cultured from punch biopsies of the skin of the anterior thigh from normal volunteers (21). Subcultures were used between passages 4–12. The cells were plated at  $5 \times 10^5$  cells per 35-mm dishes in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin, and 2 mM glutamine. After 5 days in culture the medium was changed to DMEM supplemented with 10% human-lipoprotein-deficient serum ( $d > 1.25$  g/ml, prepared by ultracentrifugation) for 48 h to up-regulate cellular LDL receptors.

**Lipoproteins**—LDL was prepared from human plasma derived from fasted normolipidemic volunteers. LDL was prepared by discontinuous density gradient ultracentrifugation as described previously (22). The lipoprotein was washed at  $d$  1.063 g/ml and dialyzed against 150 mM NaCl, 1 mM EDTA (pH 7.4) under nitrogen in the dark at 4 °C. LDL was then sterilized by filtration (0.22  $\mu\text{m}$ ) and used within 2 weeks. LDL was iodinated by the method of McFarlane (23) as modified for lipoproteins. LDL was acetylated by repeated additions

of acetic anhydride to 4 mg/ml LDL diluted 1:1 (v/v) with saturated ammonium acetate at 4 °C (24). Acetic anhydride was added at 40-fold molar excess with regard to total amino acid lysine residues in LDL, and the modification was confirmed by electrophoresis on cellulose acetate at pH 8.6 in barbital buffer.

**Cholesterol Oxidase-modified LDL (CO-LDL)**—Cholesterol oxidase-modified LDL was prepared by incubation of LDL (1 mg of protein/ml) with 1 unit/ml of cholesterol oxidase for 1–24 h at 37 °C. The enzyme reaction was terminated by the addition of 2 ml of Dole reagent (2-propanol/heptane/water, 40:10:1, v/v/v) to 1 ml of reaction mixture followed by the addition of 1 ml of heptane. The mixture was extracted by vortexing and centrifuged at  $1500 \times g$  for 10 min at 4 °C. The upper phase (heptane) was removed and monitored for cholestenone concentration at 235 nm using appropriate standards (25). Similarly, cholestenone was measured in the lipid extracts of macrophages. The production of hydrogen peroxides was analyzed in the lipid extract of the lipoproteins (26). For studies of lipoprotein-cell interaction, the modified lipoprotein was separated from excess cholesterol oxidase and hydrogen peroxide by passage over a Sephadex G-100 minicolumn (10  $\times$  2 cm), eluted with 50 mM Saline-EDTA (1 mM) buffer (pH 7.4), and used immediately for the experiments.

**Copper Ion-induced Oxidized LDL**—Copper ion-induced oxidized LDL (Cu-Ox-LDL) was produced by incubation of LDL (1 mg of protein/ml in EDTA free PBS) with copper sulfate (20  $\mu\text{M}$ ) for 24 h at 37 °C.

LDL oxidation was assayed by the thiobarbituric acid-reactive substances assay which measures LDL malondialdehyde (MDA) equivalents (27) by lipid peroxidation test (26) and by conjugated dienes determination, the first step in fatty acid peroxidation (26).

Lipoproteins were analyzed for their composition by determining their protein (28), unesterified and esterified cholesterol (29), triglycerides (30), and phospholipids (31) content. Lipoproteins were also analyzed for possible changes in their charge by lipoprotein electrophoresis on agarose (32). Vitamin E content of the lipoproteins was analyzed with the bathophenanthroline-ferric chloride assay (33). The concentration of total carotenoids was determined in the lipoprotein fractions using trifluoroacetic acid, which reacts with the conjugated double bond of the carotenoids (34). LDL fatty acids distribution was analyzed by gas-liquid chromatography as described elsewhere (35).

### Metabolism of Lipoprotein by Cells

LDL degradation was measured following incubation of  $^{125}\text{I}$ -LDL (180–300 cpm/ng of protein) with cells for 5 h at 37 °C using the conditions described for each specific experiment. The hydrolysis of LDL protein was assayed in the incubation medium by measurement of trichloroacetic acid-soluble, noniodide radioactivity (36).

Cell-free LDL degradation was minimal and was subtracted from total degradation. The cell layer was washed three times with PBS and extracted by a 1-h incubation at room temperature with 0.5 ml of 0.1 N NaOH for measurement of protein by the method of Lowry *et al.* (28). IgG C7, a mouse monoclonal antibody that binds to the LDL receptor of human and bovine cells and B1B6 a monoclonal antibody that binds to the LDL receptor binding domains on LDL-apoB-100 were used to assess binding of lipoproteins to the LDL receptor (37–39). High affinity binding of lipoproteins to cells was studied by incubation of  $^{125}\text{I}$ -labeled lipoproteins with or without 50-fold excess of unlabeled lipoprotein, for 4 h at 4 °C (40). After extensive washing (four times) with PBS, cells were extracted by incubation with 0.1 N NaOH for 1 h at room temperature, and the bound radiolabeled LDL was counted. LDL cholesterol uptake by cells was estimated by measurement of the stimulation of [ $^3\text{H}$ ]oleate incorporation into cholesteryl ester (41). The cells were incubated for 18 h with the lipoproteins, followed by medium removal and further incubation of the cells with the radiolabeled oleate (0.2 mM, 10  $\mu\text{Ci}/\text{ml}$  [ $^3\text{H}$ ]oleate in the presence of 0.07 mM fatty acid-free albumin) for 2 h at 37 °C. The cells were then washed twice with PBS at 4 °C and incubated for 30 min with 1 ml of hexane/isopropyl alcohol (3/2, v/v) in a 16-mm dish at room temperature to extract cellular lipids. After two more washes with these solvents the pooled lipid extract was dried under nitrogen and resolubilized in chloroform. The labeled cholesteryl ester (CE) was isolated by thin-layer chromatography (TLC) on silica gel plates using hexane/diethyl ether/acetic acid solution (130:40:1.5, v/v/v). Cellular content of unesterified cholesterol (UC) and CE was determined after lipid extraction (29).

### Heparin-Sepharose Affinity Chromatography

Subfractionation of LDL, Cu-Ox-LDL, and CO-LDL by heparin-Sepharose was carried out as previously described (38). Sepharose complexed with heparin (Affi-Gel Heparin, Bio-Rad) was packed into a small column (10 × 2 cm). The column was saturated with bovine serum albumin (BSA) and equilibrated to 0.05 M NaCl, 2 mM phosphate buffer (pH 7.4). 1 mg of lipoprotein protein was applied to the column, and elution was begun at a flow rate of 30 ml per h. After the unbound fraction was eluted (and the absorbance at 280 nm had decreased to baseline), the retained fraction (bound) was eluted with 0.8 M NaCl. Recovery of the lipoproteins protein ranged between 80 and 85%. The capacity of the column for LDL binding was more than double the loads used.

### Solid Phase Competitive Binding Radioimmunoassay

Radioimmunoassay of CO-LDL, Cu-Ox-LDL, and LDL was performed in microtiter plates (38). The plates were coated with 150  $\mu$ l of purified mAb B1B6 (10  $\mu$ g/ml) overnight, and then wells were blocked with 3% BSA-PBS. Serial dilutions of the lipoproteins in 1% BSA-PBS were added followed by the addition of a constant amount of  $^{125}$ I-LDL (500 ng). After incubation for 4 h at room temperature, the wells were washed three times with PBS and binding (*B*) was determined. The maximal binding (*B*<sub>0</sub>) was determined in wells where competing lipoprotein was not added, and the results were expressed as a ratio (*B*/*B*<sub>0</sub>).

### Other Assays

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 3–10% gradient gel using mercaptoethanol as the reducing agent (42). Electrophoresis was performed at constant current (5 mA) for 16 h. The gels were stained with 0.1% Coomassie Brilliant Blue R and destained with 10% acetic acid. Nondenaturing polyacrylamide gradient gel electrophoresis of the lipoprotein preparations was performed on 3–10% gels to compare their relative sizes (43).

Free lysine amino groups in LDL were estimated with trinitrobenzenesulfonic acid. LDL (50  $\mu$ g of protein) was mixed with 1 ml of 4% NaHCO<sub>3</sub>, pH 8.4, and 50  $\mu$ l of 0.1% TNBS and heated for 1 h at 37 °C, after which the absorbance at 340 nm was measured (44). LDL free fatty acid content was analyzed colorimetrically using a NEFA C kit (Wako Pure Chemicals, Osaka, Japan), based on fatty acid acylation with coenzyme A. The acyl-CoA is then oxidized to yield hydrogen peroxide which is measured by an enzymatic colorimetric assay. Statistical analysis was performed using nonpaired Student's *t* test. Results are given as mean  $\pm$  S. D.

## RESULTS

LDL incubation with 1 unit/ml of CO resulted in the formation of CO-LDL. CO-LDL demonstrated a significant reduction (by 85%) in its UC content which was the result of its conversion to cholestenone (Table I). No significant effect on LDL cholesteryl ester and phospholipid content could be found in CO-LDL (Table I). In contrast, Cu-Ox-LDL demonstrated only 26% reduction in its UC content, but in Cu-Ox-LDL there was also a reduction in all other lipid constituents and an elevation in its free fatty acids content (Table

TABLE I

Chemical composition of LDL, CO-LDL, and Cu-Ox-LDL

CL, cholestenone; PL, phospholipids; TG, triglycerides; FFA, free fatty acids. Results are the mean  $\pm$  S.D. (in parentheses) of three different determinations.

	UC	CL	CE	PL	TG	FFA
	mg/mg lipoprotein protein					mol/mol apoB
LDL	0.39 (0.04)	0.02 (0.01)	1.28 (0.14)	0.85 (0.09)	0.14 (0.03)	50 (9)
CO-LDL	0.06 <sup>a</sup> (0.02)	0.37 <sup>a</sup> (0.03)	1.28 (0.17)	0.82 (0.07)	0.07 <sup>a</sup> (0.03)	67 (7)
Cu-Ox-LDL	0.29 <sup>a</sup> (0.03)	0.03 (0.01)	0.65 <sup>a</sup> (0.09)	0.75 (0.07)	0.05 <sup>a</sup> (0.02)	230 <sup>a</sup> (25)

<sup>a</sup> *p* < 0.01 (versus LDL).

I). The effect of CO on LDL UC was time- and dose-dependent and resulted in the production of cholestenone as well as hydrogen peroxide (Fig. 1, A and B). Treatment of VLDL or HDL with CO also resulted in the formation of modified lipoproteins with similar effect on cholestenone production to that shown for LDL (data not shown). LDL that was treated with CO demonstrated the formation of conjugated dienes in the cholesterol moiety, and this process was also time- and dose-dependent (Fig. 2). A similar pattern was demonstrated for the increase in Cu-Ox-LDL-conjugated dienes which derived mainly from fatty acid peroxidation (Fig. 2).

The addition of antioxidants such as catalase, superoxide dismutase, butyl hydroxytoluene, and vitamin E to LDL prior to its incubation with CO did not affect cholestenone production (data not shown). Incubation of LDL (1 mg of protein/ml) with CO (1 unit/ml) for 24 h at 4 °C resulted in the formation of only 0.15 mg of cholestenone/mg of LDL protein, whereas similar incubations at 32 or 37 °C resulted in the formation of 0.35 or 0.39 mg of cholestenone/mg of LDL protein, respectively (*n* = 3). Table II demonstrates that in Cu-Ox-LDL a substantial reduction in linoleic (C-18:2) and arachidonic (C-20:4) fatty acids was noted, as well as the consumption of the LDL antioxidants vitamin E and carotenoids. In CO-LDL, however, none of these constituents was significantly affected (Table II). Analysis of LDL fatty acids oxidation by the thiobarbituric acid-reactive substances assay revealed that unlike Cu-Ox-LDL, which contained 61 mol of MDA equivalents per mol of LDL, there was no MDA production in CO-LDL (Table II). When CO-LDL was oxidized in the presence of copper ions however, the content of lipoprotein-associated MDA doubled in comparison to that found in Cu-Ox-LDL (125  $\pm$  23 vs. 61  $\pm$  7 mol of MDA/mol of lipoprotein, *n* = 3). Trinitrobenzenesulfonic acid reactivity, which measures free lysine groups on LDL apoB-100, was not affected in CO-LDL, whereas 52% reduction was found in Cu-Ox-LDL in comparison to native LDL (data not shown). On agarose gel electrophoresis, CO-LDL migrated similar to native LDL, suggesting that there was no change in the charge of the modified lipoprotein. Unlike Cu-Ox-LDL, which demonstrated fragmentation of its apoB-100 and the loss of its ability to bind to heparin, CO-LDL remained intact (as analyzed by SDS-PAGE) and bind to heparin similarly to native LDL. The size of CO-LDL also did not change as analyzed by nondenaturing gradient gel electrophoresis (data not shown). The immunoreactivity of Cu-Ox-LDL with monoclonal antibody (mAb) B1B6 (which is directed toward the LDL receptor binding domains on apoB-100) was substantially reduced, whereas that of CO-LDL was only minimally affected. The ED<sub>50</sub> values for LDL, Cu-Ox-LDL, and CO-LDL toward mAb B1B6 were 4.3  $\pm$  0.8, 9.4  $\pm$  2.6, and 4.0  $\pm$  0.9  $\mu$ g of LDL protein/ml, respectively, *n* = 4). TLC analysis of the lipoprotein lipid extracts demonstrated the production of cholestenone only in CO-LDL (data not shown). In Cu-Ox-LDL, multiple spots appeared and could be related to various cholesterol oxidation derivatives as well as to fatty acids hydroperoxides as was also shown elsewhere (6). The TLC spots in the cholestenone area were analyzed in all three types of lipoproteins. In LDL and Cu-Ox-LDL, however, we could not detect any cholestenone by either direct cholestenone determination or by high pressure liquid chromatography (HPLC) analysis (data not shown).

When macrophages were incubated with 100  $\mu$ g of protein/ml of LDL, CO-LDL, and Cu-Ox-LDL for 24 h at 37 °C followed by cell wash and extraction of cellular lipids, cholestenone appeared on TLC analysis only in cells that were incubated with CO-LDL (data not shown). Furthermore,

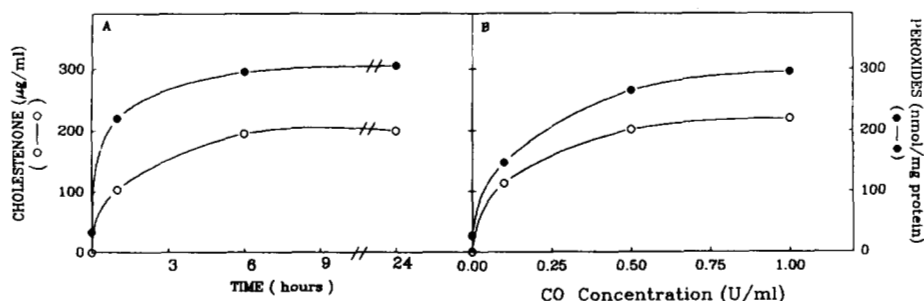


FIG. 1. The effect of cholesterol oxidase incubation with LDL on the production of cholesterolone and hydrogen peroxide: time (A) and concentration (B) studies. A, LDL (1 mg of protein/ml) was incubated with 1 unit/ml of CO at 37 °C for an increasing period of time. At the indicating time points, samples were taken for analysis of LDL-associated cholesterolone (○) and peroxides (●). B, LDL (1 mg of protein/ml) was incubated with increasing CO concentrations for 3 h at 37 °C prior to analysis of cholesterolone and peroxides.

FIG. 2. Conjugated dienes formation in the production of CO-LDL and Cu-Ox-LDL: time (A) and oxidant concentration (B) studies. A, LDL (1 mg of protein/ml) was incubated at 37 °C with 1 unit/ml of CO (○) or 20 µM CuSO<sub>4</sub> (●), and samples were taken at various time points for analysis of LDL-associated conjugated dienes. B, LDL (1 mg of protein/ml) was incubated with increasing concentrations of CO for 3 h or with increasing concentrations of CuSO<sub>4</sub> for 24 h at 37 °C prior to analysis of LDL-associated conjugated dienes.

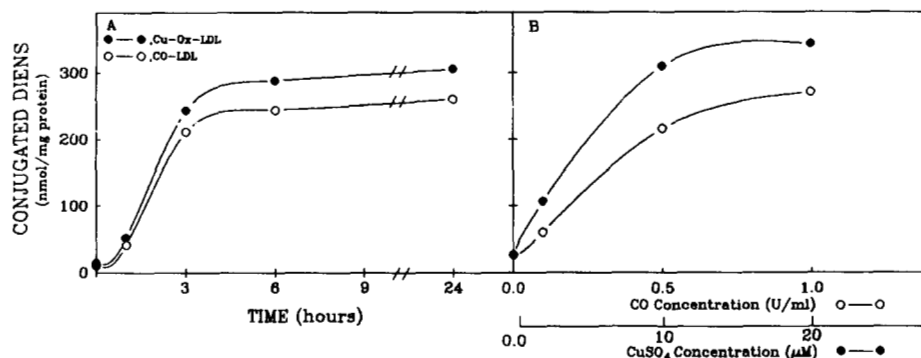


TABLE II

Effect of LDL oxidation on its fatty acids, antioxidants, and oxidation products

Results are given as mean ± S.D. (in parentheses) of three separate determinations.

	LDL	CO-LDL	Cu-Ox-LDL
	<i>mol/mol LDL</i>		
Fatty acids			
18:2	1000 ± 104	915 ± 111	145 ± 15 <sup>a</sup>
20:4	140 ± 14	128 ± 12	7 ± 3 <sup>a</sup>
Antioxidants			
Vitamin E	7.10 ± 0.81	6.90 ± 0.65	0.05 ± 0.02 <sup>a</sup>
Carotenoids	0.55 ± 0.07	0.49 ± 0.05	0.02 ± 0.02 <sup>a</sup>
Oxidation products			
MDA equivalents	2.5 ± 0.3	3.5 ± 0.7	61 ± 7 <sup>a</sup>

<sup>a</sup> *p* < 0.01 (versus LDL).

analysis of cellular cholesterolone revealed that macrophage cholesterolone was increased from  $0.2 \pm 0.1$  to  $3.8 \pm 0.4$  µg/mg cell protein ( $n = 3$ ) following incubation with CO-LDL. Spectral analysis between 200 and 600 nm revealed the appearance of cholesterolone at 235 nm in CO-LDL, whereas Cu-Ox-LDL demonstrated several peaks between 212 and 268 nm which could be attributed to 7-ketocholesterol, cholest-3,5-dien-7-one, 7-hydroxycholesterol, 25-hydroxycholesterol, and cholesterol 5,6-epoxide. Cholesterolone could not be detected in Cu-Ox-LDL. Incubation of J-774 A.1 macrophages with LDL in Ham's F-10 medium (which contain copper ions), however, resulted in LDL cholesterol and fatty acids oxidation and in the appearance (as detected by HPLC analysis) of cholest-5-en-3,7-diol, hydroxyoctadecadienoic acids, 7-ketocholesterol, and also cholesterolone (data not shown).

LDL that had been incubated with increasing concentrations of cholesterol oxidase was taken up and degraded by the

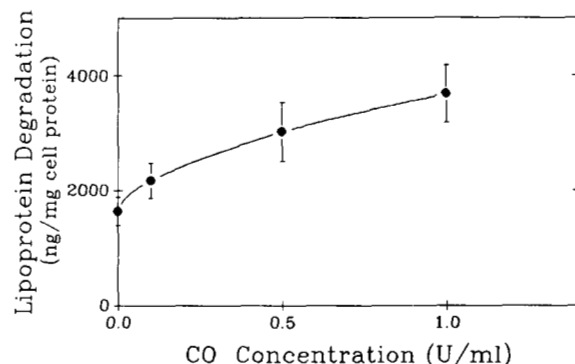


FIG. 3. The effect of LDL incubation with increasing cholesterol oxidase concentrations on lipoprotein degradation by macrophages. <sup>125</sup>I-labeled LDL (1 mg of protein/ml) was incubated for 3 h at 37 °C with increasing concentrations of CO, and the lipoprotein was applied onto a Sephadex G-100 minicolumn. J-774 A.1 macrophages were then incubated with the CO-LDL (25 µg of protein/ml) for 5 h at 37 °C prior to analysis of cellular degradation of the lipoprotein.

J-774 A.1 macrophage-like cell line at increased rate, which was CO concentration-dependent (Fig. 3). High affinity and saturable degradation of CO-LDL was observed similarly to the pattern shown for native LDL and Cu-Ox-LDL (Fig. 4). CO-LDL at a concentration of 75 µg of lipoprotein protein/ml, however, was degraded 62% more than native LDL but 15% less than Cu-Ox LDL. Lipoprotein binding to J-774 A.1 macrophages (performed at 4 °C) revealed that CO-LDL cellular binding was increased in comparison to native LDL by up to 80% at the highest concentration (15 µg of protein/ml) used (data not shown). Upon incubation of CO-LDL (100 µg of protein/ml) with J-774 A.1 macrophages for 48 h at 37 °C, cellular cholesterol mass was elevated by 51% in comparison

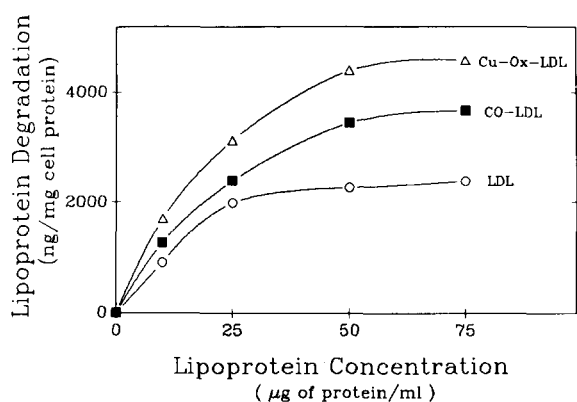


FIG. 4. The effect of lipoprotein concentration on macrophage degradation of LDL (○), CO-LDL (■), and Cu-Ox-LDL (△). J-774 A.1 macrophages were incubated for 5 h at 37 °C with increasing concentrations of lipoproteins prior to analysis of lipoprotein degradation. Results shown are the means of duplicate dishes from one experiment representative of three experiments which varied by less than 6%.

TABLE III

## Effect of CO-LDL on macrophage cholesterol mass

J-774 macrophages ( $10^6/35$ -mm dish) were incubated with no additions (None) or with 100 µg of protein/ml of LDL, CO-LDL or Cu-Ox-LDL for 48 h at 37 °C prior to analysis of cellular total cholesterol (TC) mass as well as UC and CE content. Results are given as mean  $\pm$  S.D. ( $n = 3$ ).

Addition	TC	$\mu\text{g}/\text{mg cell protein}$	
		UC	CE
None	35 $\pm$ 3	10 $\pm$ 2	25 $\pm$ 3
LDL	44 $\pm$ 4 <sup>a</sup>	13 $\pm$ 2	31 $\pm$ 3 <sup>a</sup>
CO-LDL	53 $\pm$ 4 <sup>a</sup>	13 $\pm$ 3	40 $\pm$ 3 <sup>a</sup>
Cu-Ox-LDL	65 $\pm$ 6 <sup>a</sup>	24 $\pm$ 3 <sup>a</sup>	41 $\pm$ 4 <sup>a</sup>

<sup>a</sup>  $p < 0.01$  (versus none).

to cells incubated without lipoproteins, and this could be attributed to elevation in cellular cholesteryl ester content (Table III). Native LDL and Cu-Ox-LDL increased cellular cholesterol mass by 26 and 85%, respectively (Table III).

A possible effect of any remaining LDL-associated CO on the cells was analyzed by preincubation of J-774 A.1 macrophages with increasing concentrations of CO, followed by the addition of 25 µg of protein/ml of  $^{125}\text{I}$ -LDL for degradation assay. Cellular cholestenone content was not significantly affected and LDL degradation was also not changed by this treatment (data not shown).

Thus, the increased cellular uptake of CO-LDL could not be related to a possible direct effect of CO on the cells. Cellular uptake of CO-LDL was followed by lysosomal degradation of the lipoprotein since chloroquine (100 µM) inhibited CO-LDL macrophage degradation (by 74%) to a similar extent as found for LDL, Ac-LDL, and Cu-Ox-LDL (by 81, 92, and 74%, respectively). Cellular processing of the lipoproteins was studied by incubation of J-774 A.1 macrophages with  $^{125}\text{I}$ -labeled lipoproteins (25 µg of protein/ml) for 3 h at 4 °C followed by cell wash with DMEM (to remove nonbound lipoproteins) and analysis of the reduction in cell-associated lipoproteins during 10 h of incubation at 37 °C (Fig. 5). CO-LDL demonstrated similar reduction in its cell-association to that of native LDL (Fig. 5). The reduction in cell-associated Ac-LDL was the most rapid one, whereas Cu-Ox-LDL demonstrated impaired removal rate. A 50% reduction in cell association of LDL, CO-LDL, Cu-Ox-LDL, and Ac-LDL was noted after 6.5, 7.5, 10.5, and 2.5 h of incubation, respectively (Fig. 5).

Analysis of lipoprotein degradation by various cells which

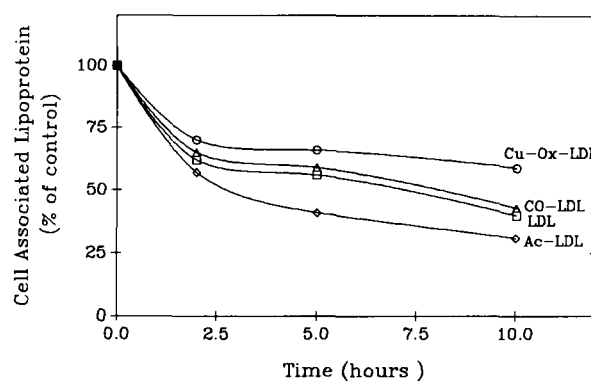


FIG. 5. Time study of macrophage processing of CO-LDL, LDL, Cu-Ox-LDL, and Ac-LDL. J-774 A.1 macrophages were incubated with  $^{125}\text{I}$ -labeled lipoproteins (25 µg of protein/ml) for 3 h at 4 °C followed by extensive cell wash with DMEM. Cell-associated  $^{125}\text{I}$ -labeled lipoproteins determined at this time point (zero time) represent the 100% control values and were 914, 413, 1384, and 2414 ng of protein/mg of cell protein for CO-LDL, LDL, Cu-Ox-LDL, and Ac-LDL, respectively. The cells were then incubated for up to 10 h at 37 °C, and samples were removed at the indicated time points for analysis of cell-associated  $^{125}\text{I}$ -lipoproteins. Results shown are the means of duplicate dishes from one experiment representative of three experiments which varied by less than 9%.

TABLE IV

## Cellular degradation of CO-LDL, LDL, Cu-Ox-LDL, and Ac-LDL by different cells

Cells were incubated with 25 µg of protein/ml of  $^{125}\text{I}$ -labeled lipoproteins for 5 h at 37 °C prior to analysis of lipoprotein degradation. Each value represents the mean of two experiments performed in triplicates. Numbers in parentheses refer to changes (in percentage) in comparison to LDL. MPM, mouse peritoneal macrophages; HMDM, human monocyte-derived macrophages; HSF, human skin fibroblasts.

Cell type	Lipoprotein degradation			
	LDL	CO-LDL	Cu-Ox-LDL	Ac-LDL
	<i>ng protein/mg cell protein</i>			
J-774 A.1	1699	2791(64%)	3511(107%)	4733(178%)
MPM	847	1111(31%)	1311(55%)	3098(265%)
HMDM	906	1306(44%)	1679(85%)	2655(193%)
U-937	2441	3091(27%)	855(-65%)	839(-65%)
HSF	1933	2601(35%)	155(-91%)	83(-96%)

possess the LDL receptor, the Ac-LDL receptor, or both of them was carried out. Table IV demonstrated that CO- $^{125}\text{I}$ -LDL was more rapidly degraded by all cells as compared to  $^{125}\text{I}$ -LDL. In J-774 A.1 cells, mouse peritoneal macrophages, and human monocyte-derived macrophages the degradation of Ac-LDL and Cu-Ox-LDL was higher than that of CO-LDL and LDL, but CO-LDL degraded by these three types of cells 64, 31, and 44% more than native LDL, respectively.

In U-937 macrophages, which possess only small number of scavenger receptors, the degradation rates of Ac-LDL and Cu-Ox-LDL were 65% lower than that of native LDL, but CO-LDL still demonstrated 27% enhanced degradation in comparison to native LDL (Table IV). In human skin fibroblasts which do not possess the scavenger receptor, CO-LDL still degraded 35% more than native LDL, whereas cellular degradation of Ac-LDL and Cu-Ox-LDL were minimal (Table IV). To further analyze the cellular receptor involved in the uptake of CO-LDL, competition studies were carried out. The degradation of 10 µg/ml CO- $^{125}\text{I}$ -LDL by J-774 A.1 macrophages was inhibited by 86, 76, 46, and 17%, when using 20-fold concentration of nonlabeled CO-LDL, LDL, Cu-Ox-LDL, and Ac-LDL, respectively (Table V). Furthermore, both mAb

TABLE V

Involvement of LDL receptor, Ac-LDL receptor, and Ox-LDL receptor in cellular uptake of CO-LDL

J-774.A.1 macrophages were incubated with  $^{125}\text{I}$ -labeled lipoproteins (10  $\mu\text{g}$  of protein/ml) in the absence (Control) or presence of 20-fold concentration of nonlabeled LDL, CO-LDL, Cu-Ox-LDL, or Ac-LDL, or with monoclonal antibody (mAb) to the LDL receptor (IgG C7, 10% volume concentration), or with mAb B1B6 (20  $\mu\text{g}/\text{ml}$ , directed against the LDL receptor binding domains on apoB-100) or with polyinosinic acid (10  $\mu\text{M}$ , the inhibitors of the scavenger receptor). Cellular degradation was measured after 5 h of incubation at 37 °C. Numbers in parenthesis refer to changes (in percentage) in comparison to Control. Three separate experiments were performed for each competitor, and the results varied between 3 and 11%.

Additions	Lipoprotein degradation			
	$^{125}\text{I}$ -CO-LDL	$^{125}\text{I}$ -LDL	$^{125}\text{I}$ -Cu-Ox-LDL	$^{125}\text{I}$ -Ac-LDL
	ng/protein/mg cell protein			
None (Control)	1469	908	1955	2588
Nonlabeled LDL	353(-76%)	139(-86%)	1703(-13%)	2495(-4%)
Nonlabeled CO-LDL	201(-86%)	200(-78%)	1003(-49%)	2411(-7%)
Nonlabeled Cu-Ox-LDL	795(-46%)	711(-21%)	115(-94%)	811(-85%)
Nonlabeled Ac-LDL	1219(-17%)	855(-6%)	789(-60%)	391(-85%)
IgC C7	401(-73%)	215(-76%)	1788(-9%)	2403(-7%)
mAb B1B6	403(-73%)	235(-74%)	1759(-10%)	2555(-1%)
Polyinosinic acid	1204(-18%)	861(-5%)	585(-70%)	515(-80%)

IgG C7 and mAb B1B6 inhibited cellular degradation of CO- $^{125}\text{I}$ -LDL by 73%, whereas polyinosinic acid did not affect CO-LDL degradation (Table V). Similar studies were carried out with  $^{125}\text{I}$ -labeled LDL, Cu-Ox-LDL, and Ac-LDL (Table V). The results of these studies demonstrated the involvement of the LDL receptor but not the Ac-LDL receptor in the cellular uptake of CO-LDL. However, the macrophage receptor for Ox-LDL (45) was also partially involved in the uptake of CO-LDL as demonstrated by these competition experiments (Table V).

Furthermore, the addition of 20-fold excess concentration of nonlabeled LDL + Ac-LDL to macrophages that were incubated with  $^{125}\text{I}$ -Cu-Ox-LDL, reduced the cellular degradation of Cu-Ox-LDL by 66%, whereas 94% inhibition was obtained by the addition of 20-fold excess nonlabeled Cu Ox-LDL. The addition of 20-fold excess nonlabeled CO-LDL to cells incubated with  $^{125}\text{I}$ -Cu-Ox-LDL in the presence of excess LDL + Ac-LDL, resulted in a further inhibition of macrophage degradation of  $^{125}\text{I}$ -Cu-Ox LDL (up to 88%), and this may also suggest the involvement of the macrophage receptor for Ox-LDL (but not the Ac-LDL receptor) in the uptake of CO-LDL. Apolipoprotein E was previously shown to inhibit cellular uptake of Ac-LDL but not that of native LDL (46). Upon incubation of apoE (5  $\mu\text{g}/\text{ml}$ ) with 1 mg of protein/ml of CO-LDL, LDL, Cu-Ox-LDL, and Ac-LDL for 1 h at 37 °C, apoE was found to be associated with all lipoproteins as determined by SDS-PAGE (data not shown). Upon incubation of apoE-treated lipoproteins with J-774 A.1 macrophages, cellular degradation rates of both LDL and CO-LDL were not significantly affected, whereas those of apoE-treated Ac-LDL and apoE-treated Cu-Ox-LDL were inhibited by 38 and 48%, respectively, in comparison to the nontreated lipoproteins (Table VI). The increased cellular degradation of CO-LDL could have resulted from the effect of CO-LDL cholestenone or of any remaining CO-LDL-associated hydrogen peroxides.

Both hydrogen peroxide (up to 0.01%) and cholestenone

TABLE VI

Effect of apoE on macrophage uptake of lipoproteins

$^{125}\text{I}$ -Labeled LDL, CO-LDL, Cu-Ox-LDL, and Ac-LDL (1 mg of protein/ml) were incubated with apoE (5  $\mu\text{g}/\text{ml}$ ) for 1 h at 37 °C. The lipoprotein was then reisolated by passage over a Sephadex G-100 minicolumn (10  $\times$  2 cm) and used immediately for experiments. ApoE-treated lipoproteins (25  $\mu\text{g}$  of protein/ml) were incubated with J-774 A.1 macrophages for 5 h at 37 °C prior to lipoprotein degradation analysis.

	Lipoprotein degradation			
	LDL	CO-LDL	Cu-Ox-LDL	Ac-LDL
	ng/mg cell protein			
Control lipoproteins	1179 $\pm$ 189	1901 $\pm$ 178	2859 $\pm$ 239	4331 $\pm$ 359
ApoE-treated lipoproteins	1320 $\pm$ 175	2261 $\pm$ 171	1495 $\pm$ 157 <sup>a</sup>	2677 $\pm$ 291 <sup>a</sup>

<sup>a</sup>  $p < 0.01$  versus control ( $n = 3$ ).

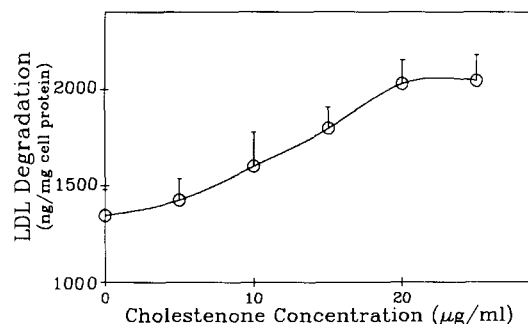


FIG. 6. The effect of cholestenone on LDL degradation by macrophages. J-774 A.1 macrophages were incubated for 5 h at 37 °C with 10  $\mu\text{g}$  of protein/ml of  $^{125}\text{I}$ -LDL, in the presence of increasing concentrations of cholestenone. Cellular degradation of the lipoprotein was then measured.

(up to 50  $\mu\text{g}/\text{ml}$ ) were not cytotoxic to the cells as determined by viability assays. Upon incubation of J-774 A.1 macrophages with hydrogen peroxides (0–0.01%) together with  $^{125}\text{I}$ -LDL (25  $\mu\text{g}$  of protein/ml), no effect on LDL degradation could be found (data not shown). However, when J-774 A.1 macrophages were incubated with 10  $\mu\text{g}$  of protein/ml of  $^{125}\text{I}$ -LDL in the presence of increasing concentrations of cholestenone, cellular degradation of LDL was increased in a dose-dependent pattern by up to 52% (Fig. 6). Similar results were found when lipoprotein uptake was measured by analysis of macrophage cholesterol esterification rate (data not shown).

## DISCUSSION

The present study demonstrates that CO modification of LDL (which leads to the conversion of the lipoprotein unesterified cholesterol to cholestenone), results in the formation of modified lipoprotein. This modified lipoprotein (CO-LDL) demonstrated increased binding to macrophages, was taken up by cells at enhanced rates, and delivered more cholesterol into the cells than native LDL. This effect could be related to the cholestenone present in CO-LDL since cholestenone was shown to increase cellular degradation of LDL. The ratio of UC to CE in CO-LDL was only 17% of the ratio found in native LDL. Recently, we have shown that in cholesterol esterase-modified LDL, the elevation in the UC/CE ratio resulted in the formation of a modified lipoprotein that was taken up by the LDL receptor on cells at a reduced rate (47). Thus, in CO-LDL the reduced UC/CE ratio in comparison to native LDL could possibly contribute to the enhanced cellular uptake of CO-LDL. CO-LDL was shown to be taken up mainly

via the LDL receptor and this was supported by the observation that CO-LDL was taken up by cells that do not possess the Ac-LDL receptor but only the LDL receptor such as fibroblasts. Furthermore, cellular uptake of CO-LDL was inhibited by mAbs directed against the LDL receptor (IgG C7) or against the LDL receptor binding domains on apoB-100 (B1B6).

Competition experiments also supported the involvement of the LDL receptor in the cellular uptake of CO-LDL.

Macrophages were shown to possess a receptor which is specific for Ox-LDL and is different from the Ac-LDL receptor (45). The present study demonstrates that cellular uptake of CO-LDL was partially mediated via these receptors. It is possible that the macrophage-specific receptor for Ox-LDL recognizes epitopes which are associated with LDL cholesterol oxidation rather than epitopes which appeared during fatty acid peroxidation. Fatty acids peroxidation and aldehyde formation in Cu-Ox-LDL contribute to derivatization of lysine groups on LDL apoB-100 which results in its recognition by the Ac-LDL receptor (1, 44). These changes did not occur in CO-LDL which was also not recognized by the Ac-LDL receptor. Conjugated diens are formed during the production of Cu-Ox-LDL as a result of the formation of conjugated hydroperoxides from C-18:2 and C-20:4 fatty acids. The content of conjugated diens correlates with the levels of MDA and lipid hydroperoxides during the lag phase of fatty acid peroxidation but as the oxidation progresses, diens conjugation decreases for some time indicating the onset of the decomposition phase (4, 5).

Unlike Cu-Ox-LDL, in CO-LDL, MDA was not formed since fatty acids were not oxidized. However, CO-LDL production is associated with conjugated diens formation as a result of the isomerization of the C5-C6 double bond to the C4-C5 position (12, 13). Thus, the large differences in MDA equivalents between CO-LDL and Cu-Ox-LDL are associated with a much smaller differences in the content of conjugated diens. Even though the LDL receptor is regulated by cellular cholesterol content CO-LDL (which was shown to be taken up mainly by the LDL receptor pathway), when incubated with cells, resulted in cellular cholesterol accumulation. Similarly, the uptake of other modified forms of LDL such as platelet-modified LDL (48), lipoprotein lipase and hepatic triglyceride lipase-modified LDL (39, 49), as well as phospholipase C-modified LDL (50) occur via the LDL receptor and resulted in cellular cholesterol accumulation. These modifications increased macrophage LDL uptake by the LDL receptor via different mechanisms such as a change in LDL cholesterol/protein ratio (48), a reduction in LDL triglycerides that was associated with changes in apoB-100 epitopes expression (39, 40) and a phagocytosis of aggregated LDL via the LDL receptor (50). CO-LDL, however, was not aggregated as evident by electrophoresis analysis on cellulose acetate as well as on nondenatured gradient gels, and its size on these gels was similar to that of native LDL. The enhanced cellular uptake of CO-LDL was not associated with the action of CO on the macrophages, since CO incubation with the cells did not affect LDL uptake by the cells. Unlike the lipoprotein UC the cholesterol of erythrocyte membranes (14), and probably that of other cells, is not susceptible to CO activity unless large amounts of cholesterol are present in the cell membrane (14). This may explain the ineffectiveness of CO on the ability of macrophages to take up LDL.

The action of CO on LDL resulted in the formation of both cholestenone and hydrogen peroxide, and either of these substances could possibly affect cellular uptake of the lipoprotein. *In vivo*, oxygen, which is a metabolic product of

hydrogen peroxide, can produce gas embolism, but this occurs only at a very high hydrogen peroxide concentration. In our study hydrogen peroxide at a nontoxic concentration did not affect cellular uptake of LDL. Cholestenone however, which is present in small amounts in plasma (51) may have an important role in the increased cellular uptake of CO-LDL.

The effect of cholestenone to increase cellular uptake of LDL might be related to the increased binding affinity of CO-LDL to macrophages. Furthermore, cholestenone internalization into the cells could have suppressed cellular cholesterol synthesis which in turn could result in increased production of LDL receptors (41) and enhanced uptake of the lipoprotein by the macrophages. Indeed, oxygenated derivatives of cholesterol were shown to be potent inhibitors of sterol biosynthesis in mammalian cells (52). LDL cholesterol oxidation by endothelial cells produce mainly epoxycholesterol, whereas in Cu-Ox-LDL, 7-ketocholesterol, and cholestendien-7-one were the major cholesterol oxidation products (53). Macrophages were also shown to be able to oxidize cholesterol to several cholesterol oxidation products including cholestenone (54), and following their incubation with oxidized LDL they accumulate ceroid (55). Thus, arterial wall macrophages can potentially lead to the formation of oxidized lipoproteins with increased cellular uptake. The presence of cholestenone in plasma together with the finding that macrophage oxidation of LDL cholesterol also produces cholestenone, may suggest that CO-LDL if formed under certain pathological conditions can contribute to macrophage foam cell formation and to accelerated atherosclerosis.

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#### REFERENCES

- Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989) *N. Engl. J. Med.* **320**, 915-924
- Aviram, M. (1991) in *Blood Cell Biochemistry: Vol. 2. Megakaryocytes, Platelets, Macrophages and Eosinophils* (Harris, J. R., ed) pp. 179-208, Plenum Publishing Corporation, New York
- Heinecke, J. W. (1987) *Free Rad. Biol. Med.* **3**, 65-73
- Jurgens, G., Hoff, H. F., Chisolm, G. M., and Esterbauer, H. (1987) *Chem. Phys. Lipids* **45**, 315-339
- Esterbauer, H., Rotheneder, M., Striegl, G., Waeg, G., Ashy, A., Sattler, W., and Jurgens, G. (1989) *Fat Sci. Technol.* **91**, 316-324
- Zhang, H., Basra, H. J. K., and Steinbrecher, U. P. (1990) *J. Lipid Res.* **31**, 1361-1369
- Aviram, M. (1990) *Atherosclerosis* **84**, 141-143
- Aviram, M. (1991) *Biochem. Biophys. Res. Commun.* **179**, 359-365
- Aviram, M. (1989) *Thromb. Res.* **53**, 561-568
- Aviram, M., Dankner, G., and Brook, J. G. (1990) *Arteriosclerosis* **10**, 559-563
- Fuhrman, B., Brook, J. G., and Aviram, M. (1991) *J. Lipid Res.* **32**, 1113-1123
- Smith, A. G., and Brooks, C. J. W. (1976) *J. Steroid. Biochem.* **7**, 705-713
- Richmond, W. (1973) *Clin. Chem.* **19**, 1350-1356
- Lange, Y., Matthies, H., and Steck, T. L. (1984) *Biochim. Biophys. Acta* **769**, 551-562
- Slotte, J. P., and Gronberg, L. (1990) *J. Lipid Res.* **31**, 2235-2242
- Linder, R. (1984) *Biochim. Biophys. Acta* **779**, 423-435
- Salen, G., Shefer, S., and Berginer, V. M. (1983) in *The Metabolic Basis of Inherited Diseases* (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L., and Brown, M. S., eds) 5th Ed., pp. 713-730, McGraw-Hill, New York
- Bernheimer, A. W., Robinson, W. G., Linder, R., Mullins, D., Yip, Y. K., Cooper, N. S., Seidman, I., and Uwajima, T. (1987) *Biochem. Biophys. Res. Commun.* **148**, 260-266
- Aviram, M., Williams, K. J., McIntosh, R. A., Carpentier, Y. A.,

- Tall, A. R., and Deckelbaum, R. J. (1989) *Arteriosclerosis* **9**, 67-75
20. Boyum, A. (1968) *Scand J. Clin. Invest.* **21**, (suppl.) 77-99
21. Chait, A., Bierman, E. L., and Albers, J. J. (1979) *J. Clin. Invest.* **64**, 1309-1319
22. Aviram, M. (1983) *Biochem. Med.* **30**, 111-118
23. Bilheimer, D. W., Eisenberg, S., and Levy, R. I. (1972) *Biochim. Biophys. Acta* **260**, 212-221
24. Basu, S. K., Goldstein, J. L., Anderson, R. G., and Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U. S. A.* **73**, 3178-3182
25. Moore, N. F., Patzer, E. J., Barenholz, Y., and Wagner, R. R. (1977) *Biochemistry* **16**, 4708-4715
26. El-Saadani, M., Esterbauer, M., El-Sayed, M., Goher, M., Nassar, A. Y., and Jurgens, G. (1989) *J. Lipid Res.* **30**, 627-630
27. Buege, J. A., and Aust, S. D. (1978) *Methods Enzymol.* **52**, 302-310
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
29. Chiamori, N., and Henry, R. J. (1959) *Am. J. Clin. Pathol.* **31**, 305-309
30. Wieland, O. (1957) *Biochem. J.* **324**, 313-317
31. Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466-468
32. Chin, H. P., and Blankenhorn, P. H. (1968) *Clin. Chim. Acta* **20**, 305-308
33. Hashim, S. A., and Schutringer, G. R. (1966) *Am. J. Clin. Nutr.* **19**, 137-144
34. Ben Amotz, A., Mokady, S., Edelstein, S., and Avron, M. (1989) *J. Nutr.* **199**, 1013-1019
35. Aviram, M., Brox, J., and Nordoy, A. (1986) *Ann. Nutr. Metabol.* **30**, 143-148
36. Bierman, E. L., Stein, O., and Stein, Y. (1974) *Circ. Res.* **35**, 136-154
37. Tolleshaug, H., Holgood, K. K., Brown, M. S., and Goldstein, J. L. (1983) *Cell* **32**, 941-951
38. Keidar, S., Goldberg, A. C., Cook, K., Bateman, J., and Schonfeld, G. (1990) *Metabolism* **39**, 281-288
39. Aviram, M., Lund-Katz, S., Phillips, M. C., and Chait, A. (1988) *J. Biol. Chem.* **263**, 16842-16848
40. Innerarity, J. G., Pitas, R. E., and Mahley, R. H. (1986) *Methods Enzymol.* **124**, 542-565
41. Brown, M. S., Ho, Y. K., and Goldstein, J. L. (1980) *J. Biol. Chem.* **255**, 9344-9352
42. Marzetta, C. A., and Rudel, L. L. (1986) *J. Lipid Res.* **27**, 753-762
43. Musliner, T. A., McVicker, K. M., Iosefa, J. F., and Krauss, R. M. (1987) *Biochim. Biophys. Acta* **919**, 97-110
44. Steinbrecher, U. P., Witztum, J. L., Parthasarathy, S., and Steinberg, D. (1987) *Arteriosclerosis* **7**, 135-143
45. Sparrow, C. P., Parthasarathy, S., and Steinberg, D. (1989) *J. Biol. Chem.* **264**, 2599-2604
46. Maor, I., Brook, J. G., and Aviram, M. (1991) *Atherosclerosis* **88**, 163-174
47. Aviram, M., Keidar, S., Rosenblat, M., and Brook, J. G. (1991) *J. Biol. Chem.* **266**, 11567-11574
48. Aviram, M., Fuhrman, B., Keidar, S., Maor, I., Rosenblat, M., Dankner, G., and Brook, J. G. (1989) *J. Clin. Chem. Clin. Biochem.* **27**, 3-12
49. Aviram, M., Bierman, E. L., and Chait, A. (1988) *J. Biol. Chem.* **263**, 15416-15422
50. Suits, A. G., Cait, A., Aviram, M., and Heinecke, J. W. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86**, 2713-2717
51. Gray, M. F., Lawrie, T. D. V., and Brooks, C. J. W. (1971) *Lipids* **6**, 836-843
52. Kandutsch, A. A., Chen, H. W., and Heiniger, H. J. (1978) *Science*, **201**, 498-501
53. Bhadra, S., Arshad, M. A. Q., Rymaszewski, Z., Norman, E., Wherley, R., and Subbiah, M. T. R. (1991) *Biochem. Biophys. Res. Commun.* **176**, 431-440
54. Carpenter, K. L. H., Ballantine, J. A., Fussell, B., Enright, J. H., and Mitchinson, M. J. (1990) *Atherosclerosis* **83**, 217-229
55. Mitchinson, M. J., Ball, R. Y., Carpenter, K. L. H., and Enright, J. H. (1990) *Eur. Heart J.* **11**, Suppl. E, 116-121