

Oxidized low density lipoprotein leads to macrophage accumulation of unesterified cholesterol as a result of lysosomal trapping of the lipoprotein hydrolyzed cholesteryl ester

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Abstract The early atherosclerotic lesion is comprised of foam cell macrophages filled with cholesteryl ester (CE), unesterified cholesterol (UC), and cholesterol oxides. Upon incubation of macrophages with oxidized low density lipoprotein (Ox-LDL), they accumulate UC rather than CE, which was shown to accumulate after incubation of cells with acetylated LDL (Ac-LDL). Using lipoproteins that were doubly labeled in their CE as well as in their protein moieties, we have demonstrated that lysosomal hydrolysis of the Ox-LDL CE was similar to the hydrolysis of the CE in Ac-LDL or native LDL whereas, as shown previously, a markedly impaired degradation of the protein moiety of Ox-LDL was observed. Cell fractionation revealed that the UC was derived from the hydrolyzed CE in Ox-LDL and was trapped in the macrophage lysosomal fraction, whereas in cells incubated with Ac-LDL, the lipoprotein UC was rapidly transported to the microsomal and cytosolic compartments. Lysosomal accumulation of Ox-LDL-derived UC could be related to the effect of the oxysterols in Ox-LDL, as oxidation of Ac-LDL or incubation of macrophages with Ac-LDL in the presence of oxysterols, in comparison to cell incubation with Ac-LDL, resulted in lysosomal accumulation of unesterified cholesterol. As a consequence of lysosomal trapping of Ox-LDL-derived UC, its availability to esterification was markedly impaired (by 6-fold), in comparison to the cholesterol esterification rate of Ac-LDL-derived UC. However, when the cholesterol esterification was expressed per lysosomal released UC, cellular cholesterol esterification rate of Ox-LDL-derived UC was found to be similar to that of Ac-LDL-derived UC. High density lipoprotein (HDL)-mediated efflux of the Ox-LDL-derived cholesterol from macrophages was similar to that found for Ac-LDL-derived cholesterol after 24 h of cell incubation with HDL₃. Major defects in the cellular metabolism of Ox-LDL-derived 7-ketocholesterol were also found and could be related to its lysosomal trapping (together with the UC), its limited capacity to be esterified, and a 40% reduction in its HDL-mediated efflux from macrophages, in comparison to the efflux of the Ox-LDL-derived UC. **■** We conclude that upon incubation of macrophages with Ox-LDL, lysosomal hydrolysis of the lipoprotein CE is not impaired but cellular accumulation of the Ox-LDL-derived UC occurs as a result of trapping of the hydrolyzed CE in the macrophage lysosomal compartment which may be related to the effect of oxysterols in Ox-LDL.—**Maor, I., and M. Aviram.** Oxidized low density lipoprotein

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Cholesterol accumulates in arterial wall cells in areas of the atherosclerotic lesions not only as cholesteryl ester (CE) but also as unesterified cholesterol (UC) and as oxidized forms of cholesterol such as 7-ketocholesterol, epoxide, 3,5-diene, and 7 β hydroxy cholesterol (1–3). Whereas cellular cytosolic CE accumulates in relatively inert droplets, the accumulation of UC can lead to its crystallization and precipitation in the cells with harmful effects on various cellular functions. In the early atherosclerotic lesion, monocyte-derived macrophages are filled with cholesterol, which is mainly derived from plasma lipoproteins (4–7). Although most of the cellular cholesterol in macrophages is derived from plasma low density lipoprotein (LDL) (8), native LDL has to undergo some modifications, including lipid peroxidation (9–12) which converts it to oxidized LDL (Ox-LDL), in order to form foam cells. Ox-LDL, like acetylated LDL (Ac-LDL), is taken up by macrophages via the scavenger receptor (8), which, unlike the LDL receptor, is not regulated by the cellular cholesterol content and thus can lead to cellular cholesterol accumulation (13). Whereas the up-

Abbreviations: Ox-LDL, oxidized low density lipoprotein(s); Ac-LDL, acetylated LDL; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; apoB-100, apolipoprotein B-100; CE, cholesteryl ester; UC, unesterified cholesterol; HDL, high density lipoprotein(s); FCS, fetal calf serum; TLC, thin-layer chromatography.

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take of Ac-LDL by macrophages was shown to cause cellular accumulation of CE, the uptake of Ox-LDL caused the accumulation of UC and an impaired cholesterol esterification (14, 15).

Ox-LDL, unlike Ac-LDL, was shown, in ultrastructural and immunological studies, to accumulate in lysosomes (16, 17), and macrophage lysosomal degradation of the Ox-LDL protein moiety was shown to be reduced in comparison to the cellular degradation of the Ac-LDL apoB-100 (18–21). The resistance of the Ox-LDL CE moiety (rather than its protein constituent) to lysosomal hydrolases, however, was not directly assessed and the availability of the Ox-LDL-derived UC to esterification and efflux was not analyzed.

EXPERIMENTAL PROCEDURE

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin, streptomycin, and L-glutamine were obtained from Gibco Laboratories (Grand Island, NY). [³H]cholesteryl linoleate and carrier-free Na [¹²⁵I] were obtained from DuPont-New England Nuclear (Boston, MA). Compound 58-035 [3-(decyldimethylsilyl)-N (2-(4-methyl phenyl)-2-phenylethyl) propanamide] was generously provided by Sandoz (East Hanover, NJ).

Cells

J-774 A.1 murine macrophage-like cell line was purchased from ATCC (Rockville, MD). This strain demonstrates a high activity of acyl-CoA:cholesterol acyltransferase (ACAT). The cells were plated at 1×10^6 cells/16-mm dish in DMEM supplemented with 10% FCS. The cells were fed every 3 days and were used for experiments within 7 days of plating (22).

Lipoproteins

LDL was prepared from human plasma that was derived from fasted normolipidemic volunteers. LDL was prepared by discontinuous density gradient ultracentrifugation as described previously (23). The LDL 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 μ m) and used within 2 weeks. HDL₃ was prepared by ultracentrifugation using the fraction obtained between $d 1.125$ and 1.210 g/ml.

LDL was iodinated by the method of McFarlane as modified for lipoproteins (24). LDL was also radiolabeled with 1 mCi/ml of [³H]cholesteryl linoleate labeled in its cholesterol moiety (25). Specifically, 2×10^8 cpm of [³H]cholesteryl linoleate (1 mCi) plus 100 μ l dimethyl sulfoxide were warmed to 40°C for 5 min, followed by addition of 5 mg LDL and 0.9 ml 20 mM Tris-HCl contain-

ing 150 mM NaCl and 1 mM EDTA, pH 7.4. The mixture was incubated for 3 h at 40°C and the labeled LDL was then dialyzed against 150 mM NaCl, 1 mM EDTA, pH 7.4. The final LDL preparation contained 98% of the radioactivity in CE and had a specific radioactivity of 315 cpm/ μ g CE. LDL was acetylated by repeated additions of acetic anhydride (26) to 4 mg LDL protein/ml, diluted 1:1 (v/v) with saturated ammonium acetate, at 4°C. Acetic anhydride was added at 40-fold molar excess with regard to total lysine residues in LDL, and the modification was confirmed by electrophoresis on cellulose acetate at pH 8.6 in barbital buffer (27).

LDL oxidation and characterization

Lipoprotein oxidation was performed by incubation of pre-dialyzed LDL (1 mg of protein/ml in EDTA-free PBS) with copper sulfate (10 μ M) for 24 h at 37°C. Lipoprotein oxidation was confirmed by analysis of thiobarbituric acid-reactive substances (TBARS) which measures malondialdehyde (MDA) equivalents (28) by the lipid peroxidation test (29) and also by analysis of the conjugated diene content of the lipoprotein (30).

TLC analysis of the labeled cholesterol and cholesterol oxide derivatives in the lipoproteins was performed using a developing system of hexane-acetone-acetic acid 80:20:1 (v/v/v). The R_f values for 7-ketocholesterol, 5,6-epoxy cholesterol, unesterified cholesterol, 7-ketocholesteryl ester, 3,5 cholestendien 7-one, and cholesteryl ester were found to be 0.19, 0.22, 0.41, 0.56, 0.63, and 0.96, respectively.

Metabolism of lipoprotein by cells

[³H]CE-labeled LDL was acetylated, oxidized, or labeled with Na¹²⁵I (double labeling) as described in this section (for the nonlabeled LDL). The changes in cellular [³H]CE and [³H]UC were analyzed after incubation of macrophages with the labeled lipoproteins under the indicated conditions. Cells were incubated in DMEM containing 0.2% bovine serum albumin, in the absence of serum, to prevent cholesterol efflux during the incubation. Cellular hydrolysis of the lipoprotein CE was analyzed in the presence of an ACAT inhibitor (10 μ g/ml Sandoz 58-035) in order to prevent esterification of the lipoprotein-derived UC released from the hydrolyzed CE and which could interfere with the analysis of net CE hydrolysis.

Esterification of the lipoprotein-labeled cholesterol was analyzed at the end of the incubation. The cells were washed twice with PBS at 4°C, and incubated for 30 min with 1 ml hexane-isopropyl alcohol 3:2 (v/v) in a 16-mm dish at room temperature to extract cellular lipids. After two more extractions with these solvents, the pooled lipid extract was dried under nitrogen and dissolved in chloroform. The labeled cholesteryl ester (CE) was isolated by TLC on silica gel plates using hexane-diethyl ether-acetic acid 130:40:1.5 (v/v/v). The CE spots were visualized with

iodine vapors, scraped into vials containing 4 ml scintillation fluid, and counted in a β scintillation counter.

Cholesterol esterification of both cellular and lipoprotein-derived cholesterol was estimated by measurement of the stimulation of [^3H]oleate incorporation into cholesteryl ester (31). The cells were incubated for 24 h with unlabeled lipoproteins, followed by medium removal and a further incubation of the cells with radiolabeled oleate (0.2 mM, 10 $\mu\text{Ci/ml}$ of [^3H]oleate in the presence of 0.07 mM fatty acid-free albumin) for 2 h at 37°C.

Cells were also labeled in their plasma membrane with [^3H]UC [by a 1-h incubation at 37°C with 1 $\mu\text{Ci/ml}$, 55 Ci/mmol of [^3H]UC added as an ethanolic solution (32)] and macrophage esterification rate of the cellular labeled cholesterol was assessed by TLC analysis, after cell incubation with the various unlabeled lipoproteins.

For studies of the cellular metabolism of 7-keto cholesterol, the spot corresponding to 7-keto cholesterol from the TLC of [^3H]CE-Ox-LDL was scraped, solubilized in chloroform, and used in the esterification and efflux studies of this cholesterol oxide derivative.

LDL protein degradation was measured after a 5-h incubation of ^{125}I -labeled LDL (180–300 cpm/ng of protein) with cells at 37°C. The hydrolysis of the LDL protein was assayed by determination of trichloroacetic acid (TCA)-soluble, non-iodide radioactivity in the incubation medium (33). Cell-free LDL degradation was minimal and was subtracted from the 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. (34) and for the determination of cell-associated lipoprotein. Cellular content of UC and CE mass was determined after lipid extraction of the washed cells with hexane-isopropyl alcohol, using the ferric chloride procedure (35).

Cholesterol efflux from cells after their incubation with [^3H]CE-labeled lipoproteins was measured after a cell wash in ice-cold PBS ($\times 3$). Cells were then incubated with DMEM in the absence or presence of 100 μg of HDL₃ protein/ml for 1–24 h at 37°C. Cellular and medium [^3H]UC were analyzed by TLC and quantitated by scraping off the TLC spots corresponding to UC, CE, or 7-keto cholesterol into scintillation vials and counting the radioactivity. HDL₃-mediated cholesterol efflux was calculated as the ratio of [^3H]UC in the medium/[^3H]UC in the medium + the radioactivity in cells.

Cell subfractionation

J-774 A.1 macrophages were incubated with [^3H]CE-labeled lipoproteins under the conditions specified for each experiment. At the end of the incubation, cells ($1 \times 10^6/35\text{-mm}$ dish) were washed with cold PBS ($\times 3$), harvested, and suspended in 2 ml 250 mM sucrose containing 5 mM Tris-HCl buffer (pH 7.4). The cells were

then sonicated at 20 W for 20 sec ($\times 2$), homogenized in a Teflon/glass homogenizer (15 strokes), and centrifuged at 500 g for 10 min to remove cell debris. The supernatant was centrifuged at 10,000 g for 45 min in order to precipitate the lysosome-rich fraction. The supernatant was centrifuged again at 100,000 g for 60 min to sediment the microsome-rich fraction. The final supernatant was used as the cytosol-rich fraction (36). The lysosome- and microsome-rich pellets were resuspended in saline. The lysosomal-rich fraction (identified by β -glucuronidase activity analysis) was freeze-thawed 4 times in order to disrupt these organelles. The lysosomal extract activity was measured by using citrate-buffered DMEM at pH 4.5 (the control buffered medium was at pH 9.0, where lysosomal activity is blocked).

Statistical analysis

Results are given as the mean \pm SD. Significance of the results was assayed by the Student's *t*-test.

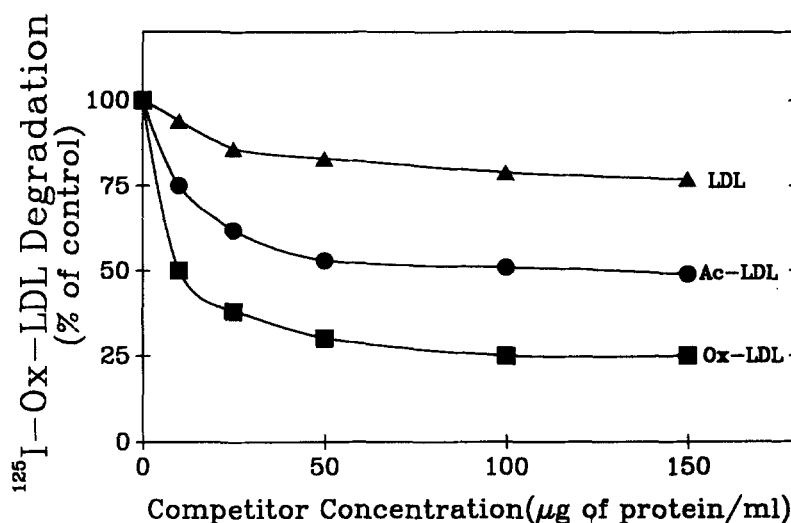
RESULTS

In order to directly follow cellular cholesterol metabolism, J-774 A.1 macrophages were incubated with cholesteryl ester-labeled lipoproteins (Ox-LDL, Ac-LDL, or native LDL) and changes in the cellular content of [^3H]CE, in ^3H -labeled unesterified cholesterol and in [^3H]cholesterol oxides, were determined. The major events in cellular cholesterol metabolism that were studied were lipoprotein cholesterol uptake, lysosomal hydrolysis of the lipoprotein CE, cholesterol esterification of the lysosomal released lipoprotein-derived UC, and cholesterol efflux of the lipoprotein-derived UC.

Characterization of the Ox-LDL

LDL was oxidized by incubation (1 mg of LDL protein/ml of EDTA-free PBS) with 10 μM CuSO₄ for 24 h at 37°C. LDL content of MDA equivalents, peroxides, and conjugated dienes increased after 24 h of incubation in the presence of copper ions from 0.4 ± 0.1 , 9 ± 2 , and 10 ± 3 to 36 ± 4 , 94 ± 12 , and 195 ± 22 nmol/mg LDL protein, respectively. No LDL aggregation occurred during its oxidation as no lipoprotein bands with increased molecular weights, could be detected in nondenatured gradient polyacrylamide gel electrophoresis of the Ox-LDL, in comparison to native LDL, and as no radioactivity precipitated by high speed centrifugation (10,000 g for 10 min) of the labeled Ox-LDL (data not shown). As the centrifugation procedure may not remove small aggregates, we also performed column chromatography analysis of the labeled Ox-LDL and of the native labeled LDL (1 mg of protein/ml) on Bio-Gel A-15m (4% agarose, 200–400 mesh beads). Both preparations were eluted as a single peak and no radioactivity was present at higher or

Fig. 1. Competition for macrophage degradation of ^{125}I -labeled Ox-LDL by unlabeled LDL, Ac-LDL, and Ox-LDL. Competition studies were performed using $5\ \mu\text{g}$ ^{125}I -labeled Ox-LDL protein/ml that was incubated with J-774 A.1 macrophages in the presence of the indicated concentrations of unlabeled lipoproteins, for 5 h at 37°C prior to analysis of lipoprotein degradation. Each point is the mean of duplicate determinations (each performed in triplicate).



lower particle size. Native LDL eluted at a volume of 35 ± 5 ml and the Ox-LDL elution peak was at 37 ± 6 ml ($n = 3$). The ability of Ox-LDL to bind to the scavenger receptor was determined by competition studies. Upon incubation of J-774 A.1 macrophages with ^{125}I -labeled Ox-LDL ($5\ \mu\text{g}$ of protein/ml) for 5 h at 37°C in the presence of a 50-fold excess concentration of unlabeled Ox-LDL, Ac-LDL, or native LDL, cellular degradation of the ^{125}I -labeled Ox-LDL was reduced by up to 75%, 51%, or by only 23%, respectively (Fig. 1). Binding of ^{125}I -labeled Ox-LDL ($10\ \mu\text{g}$ of protein/ml) to J-774 A.1 cells was also studied by incubation of macrophage with the lipoproteins for 4 h at 4°C . The addition of 50-fold-excess unlabeled Ox-LDL or native LDL to the labeled Ox-LDL reduced the binding of ^{125}I -labeled Ox-LDL to the cells from 835 ± 22 to 176 ± 10 , or only to 795 ± 19 ng of protein/mg cell protein, respectively, demonstrating again that Ox-LDL did not bind to the LDL receptor.

Cholesterol oxide derivatives in Ox-LDL

Thin-layer chromatography analysis of the radioactivity distribution among the various cholesterol derivatives of Ox- ^{3}H CE-LDL in comparison to ^{3}H CE-labeled Ac-LDL or native LDL was performed (Fig. 2). In both native LDL and Ac-LDL, the radioactivity in ^{3}H CE accounted for 97% of the total lipoprotein radioactivity. In Ox-LDL, a 17% reduction in the ^{3}H CE radioactivity was noted after 24 h of LDL oxidation. This reduction resulted from the formation of oxidized forms of cholesterol, mainly 7-keto cholesterol (6% of total lipoprotein radioactivity), 7-keto cholesteryl ester (3%), 5,6 epoxy cholesterol (1.5%), and 3,5-cholestendien-7-one (1.6%). Similarly, in nonlabeled Ox-LDL, the formation of these cholesterol oxide derivatives was noted (data not shown). The formation of 7-keto-CE in Ox-LDL was further confirmed by LDL oxidation (with copper ions), us-

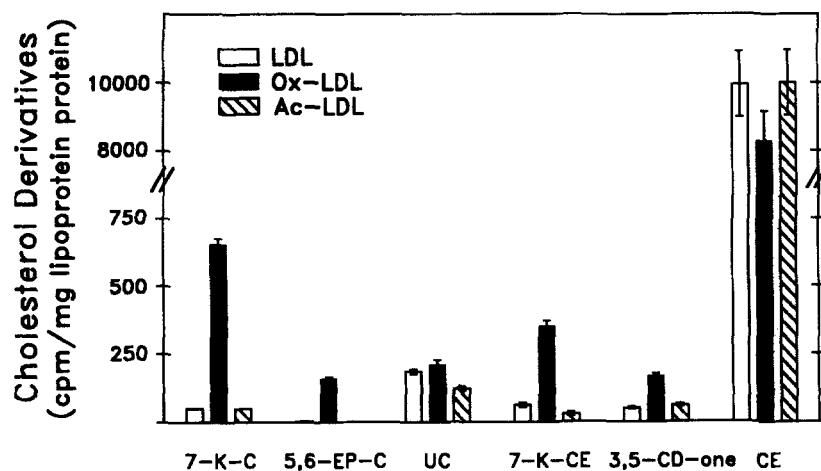
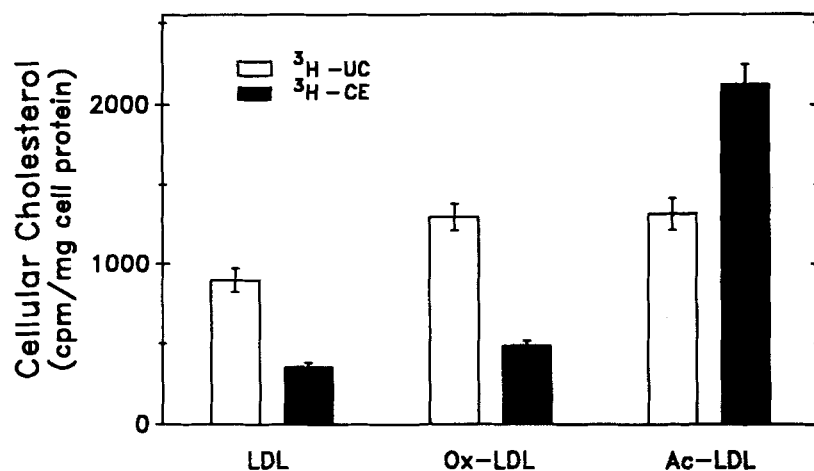


Fig. 2. TLC analysis of cholesterol derivatives in ^{3}H CE-labeled native LDL, Ox-LDL, and Ac-LDL. Lipid extraction of ^{3}H CE-labeled lipoproteins ($0.1\ \text{mg}$ of protein/ml) was carried out using 5 volumes of chloroform-methanol 2:1 (v/v) to 1 volume of lipoprotein. Thin-layer chromatographic analysis was performed on silica gel G using hexane-acetone-acetic acid 80:20:1 (v/v/v). The whole TLC line was assayed for radioactive cholesterol derivatives. The lipid spots, identified according to their R_f values (by using the various standards of cholesterol derivatives), were scraped off the plates, and the radioactivity was counted in a β -scintillation counter. 7-Keto cholesterol was also identified by high performance liquid chromatographic analysis. The oxidized CE (7-keto-CE) was identified by saponification of the TLC area between UC and CE, using 1 N KOH (incubation at 60°C for 30 min) and reparation of the hydrolyzed cholesterol (7-keto cholesterol) derivative on TLC. Results represent mean \pm SD of three experiments. 7-K-C, 7-keto cholesterol; 5,6-EP-C, 5,6-epoxycholesterol; UC, unesterified cholesterol; 7-K-CE, 7-ketocholesteryl ester; 3,5-CD-one, 3,5-cholestendien-7-one; CE, cholesteryl ester.

Fig. 3. Cellular uptake of lipoprotein cholesterol by J-774 A.1 macrophages. J-774 A.1 macrophages ($1 \times 10^6/16$ -mm dish) were incubated with [^3H]CE-labeled-LDL, Ox-LDL, or Ac-LDL (25 μg protein/ml) for 24 h at 37°C. The cells were then washed ($\times 3$) with ice-cold phosphate-buffered saline and cellular lipids were extracted ($\times 3$) with 1 ml hexane-isopropanol 3:1 (v/v). TLC analysis of the cellular lipid extracts was performed; the TLC spots corresponding to UC and CE were scraped off and the radioactivity was counted in a β scintillation counter. Results represent mean \pm SD ($n = 3$). UC, unesterified cholesterol; CE, cholesteryl ester.



ing lipoprotein that was labeled in the fatty acid of the CE moiety, rather than in the cholesterol moiety. The appearance of radioactivity in the same TLC spot as that found for 7-keto-CE that was labeled in its cholesterol moiety confirmed the formation of 7-keto-CE in Ox-LDL.

Macrophage uptake of Ox-LDL-derived cholesterol

Upon incubation of the [^3H]CE-labeled lipoproteins (25 μg protein/ml) with J-774 A.1 macrophage-like cell line for 24 h at 37°C, the cellular uptake rates of Ox-LDL and Ac-LDL, in comparison to native LDL (measured as total radioactivity in the cells), were 1.8- and 2.7-fold higher, respectively (Fig. 3). In macrophages that were incubated with labeled Ox-LDL, the [^3H]UC accounted for 78% of the total radioactivity whereas in cells that were incubated with Ac-LDL, the [^3H]UC accounted for only 38% of total radioactivity. The CE/UC radioactivity ratio was 0.36 ± 0.04 in cells that were incubated with Ox-LDL, whereas in Ac-LDL-treated cells, this ratio was 3.5-fold higher (1.30 ± 0.11) with higher contents of both cellular [^3H]UC and [^3H]CE (Fig. 3). Heavily oxidized LDL was prepared by increasing the time of LDL incubation with 10 μM CuSO_4 for up to 48 h. This incubation resulted in a $41 \pm 6\%$ ($n = 3$) reduction in the [^3H]CE radioactivity content of this heavily oxidized LDL. On using this heavily oxidized LDL (56 nmol MDA equivalents/mg LDL protein), the ratio of cellular labeled CE/UC was found to be similar to that obtained with Ox-LDL (0.37 ± 0.03 , $n = 3$). These results suggest that heavily oxidized LDL causes similar cellular UC accumulation as the Ox-LDL that was used throughout this study. Of interest is that, although native LDL caused substantially less cellular uptake of labeled cholesterol than Ac-LDL or Ox-LDL, there was also some retention of the labeled native LDL-derived UC in comparison to Ac-LDL. In cells incubated with CE-labeled native LDL, the CE/UC radioactivity ratio was 0.39 ± 0.05 . Upon incubation of [^3H]CE-labeled Ox-LDL, native LDL, or Ac-LDL with J-774 A.1 macrophages in the presence of [^{14}C]oleic acid, TLC analysis of the cellular lipid extracts

revealed that the CE spot contained both the lipoprotein-derived tritiated cholesterol and the fatty acid-derived ^{14}C (data not shown). These results indicate cellular reesterification of the lipoprotein-derived cholesterol after lysosomal hydrolysis of the lipoprotein CE constituent. Macrophage cholesterol mass analysis after 24 h of cell incubation with 25 μg protein/ml of Ox-LDL, native LDL, or Ac-LDL revealed 2.2-, 1.5-, and 2.5-fold increments in the total cellular cholesterol, respectively, in comparison to control cells incubated in the absence of lipoproteins (Table 1). In cells incubated with Ox-LDL, however, the increment in cellular UC accounted for 70% of the increment in total cellular cholesterol, whereas in cells incubated with Ac-LDL, most of the increment was in cellular content of the CE (Table 1).

The fate of the lipoprotein-derived cholesterol in macrophages over time was assessed by measuring the cellular content of UC and CE in a pulse-chase experiment. Cells were preincubated with 25 μg protein/ml of the various lipoproteins for 24 h at 37°C (pulse). The cells were then washed and further incubated in a lipoprotein-free medium for up to 3 days (chase incubation). Fig. 4 demonstrates that even after as long as 3 days of chase incubation, the cellular CE/UC mass ratio in macrophages that were preincubated with Ox-LDL was not changed in

TABLE 1. Effect of modified LDL on macrophage cholesterol mass

Lipoprotein	Total Cholesterol	$\mu\text{g}/\text{mg}$ cell protein		
		UC	CE	7-K-C
None	33 ± 3	20 ± 2	13 ± 1	0.3 ± 0.1
Ox-LDL	70 ± 5	45 ± 4	24 ± 3	4.5 ± 0.4
LDL	51 ± 4	27 ± 3	23 ± 3	0.6 ± 0.2
Ac-LDL	81 ± 5	37 ± 4	44 ± 4	0.7 ± 0.3

J-774 A.1 macrophages (2×10^6 cells/35-mm dish) were incubated without (none) or with 25 μg of protein/ml of Ox-LDL, native LDL, or Ac-LDL for 24 h at 37°C. At the end of the incubation, cellular cholesterol content was determined. Results are given as the mean \pm SD ($n = 3$). UC, unesterified cholesterol; CE, cholesteryl ester; 7-K-C, 7-keto cholesterol.

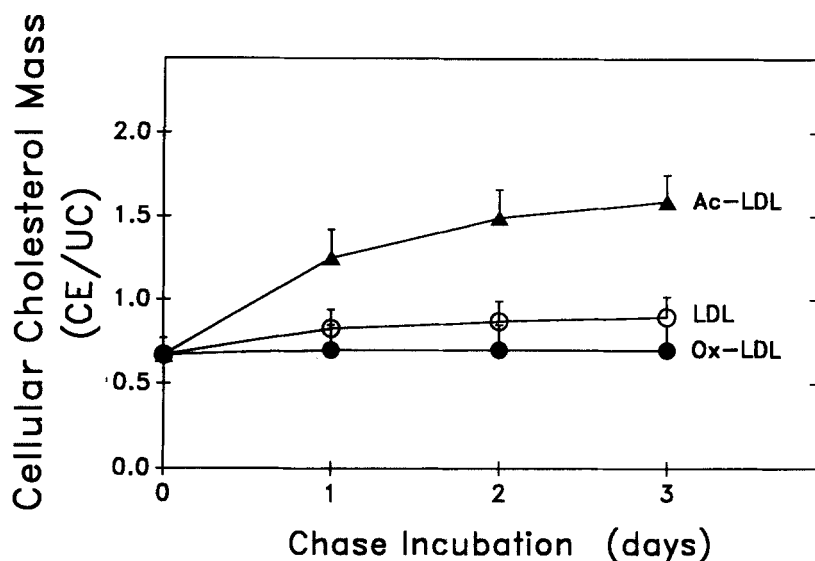


Fig. 4. Time course study of the cellular uptake of modified LDL by J-774 A.1 macrophages. Cells ($2 \times 10^6/35$ -mm dish) were incubated with LDL, Ox-LDL, or Ac-LDL ($25 \mu\text{g protein/ml}$) for 24 h at 37°C (pulse). The cells were then washed and further incubated for up to 3 days in a lipoprotein-free medium (chase incubation). At the indicated time points, cells were treated with hexane-isopropanol 3:2 (v/v) and the lipid extracts of the cells were analyzed (by TLC) for the content of unesterified cholesterol (UC) and cholesteryl ester (CE) mass. Results are given as the mean of three experiments \pm SD.

comparison to the pre-chase ratio, and these cells still contained the major fraction of their cholesterol as UC (Fig. 4), whereas in cells that were incubated with Ac-LDL, the CE/UC ratio increased from a pre-chase value of 0.67 to a value of 1.67 after 3 days of chase incubation.

Lysosomal involvement in the hydrolysis of the lipoprotein cholesteryl ester

Lysosomal involvement in the hydrolysis of the CE moiety of Ox-LDL was demonstrated by using chloroquine, an inhibitor of lysosomal hydrolases. The chloroquine was not cytotoxic and did not affect lipoprotein uptake by the macrophages (data not shown). J-774 A.1 macrophages were incubated with $25 \mu\text{g protein/ml}$ of [^3H]CE-labeled lipoprotein for 24 h at 37°C in the presence of $10 \mu\text{g/ml}$ Sandoz 58-035 (to prevent reesterification of the cholesterol). Sandoz 58-035 was found in preliminary experiments to inhibit cellular cholesterol esterification rate in J-774 A.1 macrophages by 90–95% with no effect on the cellular uptake and degradation of all three lipoproteins. Under inhibition of macrophage cholesterol esterification, the [^3H]UC, derived from lysosomal hydrolysis of CE, was not further metabolized and thus this experimental procedure allows for analysis of lysosomal CE hydrolysis. Under these conditions, cellular [^3H]UC contents were 4924 ± 150 , 2688 ± 125 , and 5418 ± 235 cpm/mg cell protein after cell incubation with CE-labeled Ox-LDL, native LDL, or Ac-LDL, respectively. The addition of chloroquine ($40 \mu\text{M}$) to the incubation system resulted in about 80% reduction in the [^3H]UC cellular radioactivity (to values of 1034 ± 111 , 537 ± 43 , and 1246 ± 103 cpm/mg cell protein, respectively), suggesting that the macrophage lysosomes were involved in the hydrolysis of the core CE of all three lipoproteins.

The hydrolysis of the lipoprotein [^3H]CE and its conversion to [^3H]UC were also studied in the presence of $10 \mu\text{g/ml}$ of Sandoz 58-035 and were compared with the hydrolysis of the lipoprotein apoB-100 by using lipoproteins (LDL, Ox-LDL, and Ac-LDL) that were doubly labeled with [^3H]CE and with ^{125}I in their CE and protein moieties, respectively). Double labeling of the lipoproteins did not affect the electrophoretic mobility of the lipoproteins and did not cause lipoprotein aggregation. Cellular degradation of the protein moiety of Ox-LDL was 1.4-fold higher than that of native LDL but 2.8-fold lower than that of Ac-LDL (Fig. 5A). Cellular association of the Ox-LDL protein, however, was 2.1-fold higher than that of Ac-LDL and 5.4-fold higher than that of native LDL (Fig. 5B). These results thus demonstrated that only 32% of the Ox-LDL protein that was taken up by the cells was degraded in comparison to degradation values of 65% and 75% for native LDL and Ac-LDL, respectively (Fig. 5C). In contrast to the impaired degradation of the Ox-LDL protein moiety, its CE moiety was hydrolyzed almost similarly to that of LDL or Ac-LDL (Fig. 5 D–F). Cellular content of [^3H]UC was 1.2- or 2.2-fold higher in cells that were incubated with [^3H]CE-labeled Ox-LDL or Ac-LDL, in comparison to cells incubated with native LDL (Fig. 5D). A similar pattern was obtained in cellular [^3H]CE content (Fig. 5E). Thus, unlike the impaired macrophage degradation of the Ox-LDL apoB-100 (Fig. 5C), the hydrolysis of Ox-LDL-derived CE (to form UC) was almost similar to that of native LDL or Ac-LDL (Fig. 5F), suggesting that the protein moiety but not the CE constituent of Ox-LDL is resistant to lysosomal hydrolases.

To directly analyze the involvement of the macrophage lysosomes in this phenomenon, we studied lipoprotein protein degradation and lipoprotein [^3H]CE hydrolysis in

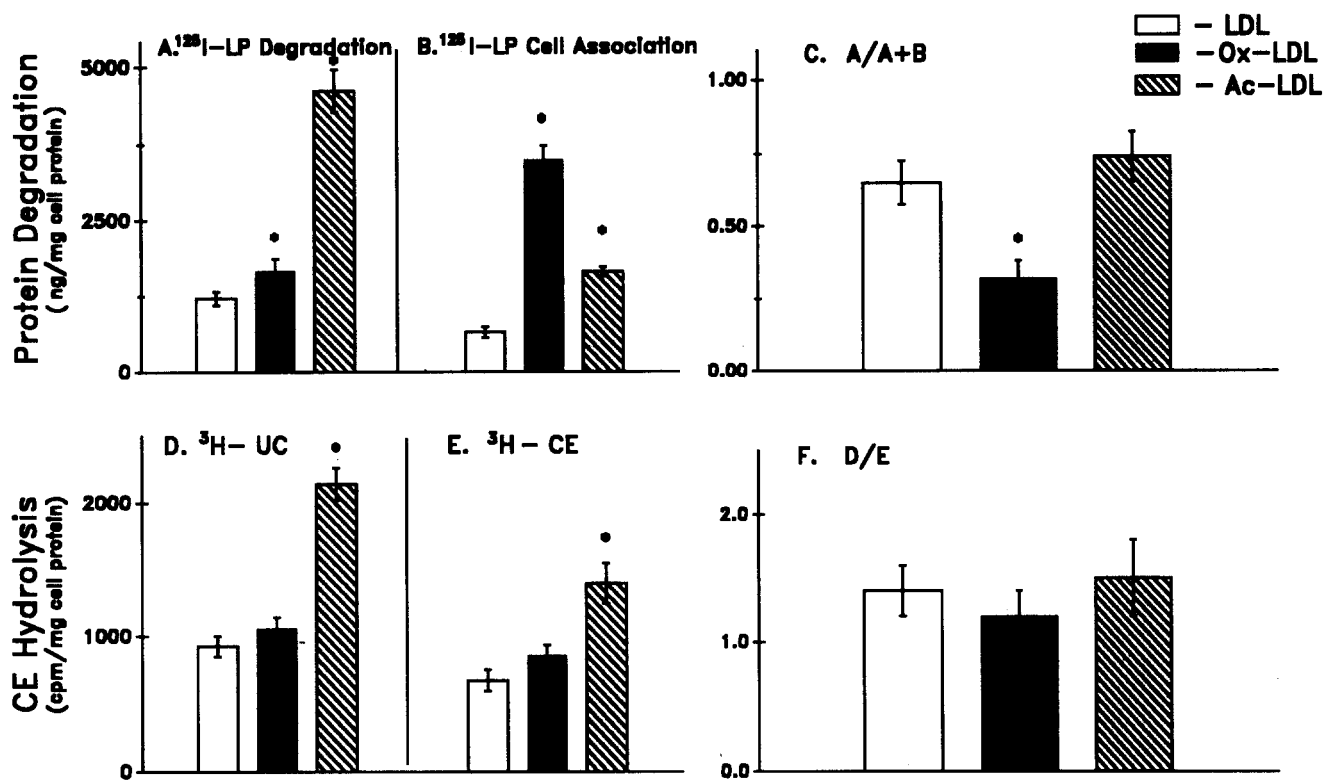


Fig. 5. Lipoprotein protein degradation and cholesteryl ester (CE) hydrolysis by J-774 A.1 macrophages. Cells (1×10^6 /16-mm dish) were incubated with lipoproteins [$25 \mu\text{g}$ lipoprotein (LDL, Ox-LDL, Ac-LDL) protein/ml] that were doubly labeled in the protein moiety (with ^{125}I) and in their core cholesteryl ester constituent (with ^3H CE). Incubation was performed for 5 h at 37°C in the presence of $10 \mu\text{g}/\text{ml}$ of Sandoz 58-035 (in order to prevent cellular cholesterol esterification and thus to be able to follow only the hydrolysis of the lipoprotein ^3H CE). At the end of the incubation, lipoprotein (LP) protein degradation was measured in the medium (A) and LP-cell association was determined in the cells (B). The cells were also analyzed (after lipid extraction and TLC analysis) for their content of ^3H UC (D) and ^3H CE (E). Results represent mean \pm SD ($n = 3$). * $P < 0.01$ (vs. native LDL); UC, unesterified cholesterol; CE, cholesteryl ester.

a cell-free system by using lysosomal extract that was prepared from J-774 A.1 macrophages. Doubly labeled lipoproteins ($10 \mu\text{g}$ protein/ml, labeled in the protein moiety with ^{125}I and in the cholesterol moiety with ^3H CE) were incubated with $10 \mu\text{g}$ of lysosomal extract protein/ml for 5 h at 37°C at pH 4.5. Protein degradation (measured as trichloroacetic acid-soluble, non-iodide radioactivity) of the Ox-LDL was 2-fold or 7-fold lower than the protein

degradation of LDL or Ac-LDL, respectively, (Table 2) as was also shown in previous studies (17, 21). In contrast, the lipoprotein ^3H UC/ ^3H CE + ^3H UC ratios were similar, with values of 0.49, 0.45, and 0.53 for Ox-LDL, native LDL, and Ac-LDL, respectively (Table 2), suggesting again that the Ox-LDL protein moiety, but not the CE, was resistant to the macrophage lysosomal hydrolases. In similar control experiments carried out at pH 9.0, no

TABLE 2. Lipoprotein protein degradation and CE hydrolysis by lysosomal extract from J-774 A.1 macrophages

Lipoprotein	Protein Degradation $\mu\text{g}/\mu\text{g}$ extract protein	Lipoprotein Cholesterol Hydrolysis	
		^3H UC	^3H CE
Ox-LDL	0.124 ± 0.01	57 ± 3	59 ± 4
LDL	0.235 ± 0.02	64 ± 5	77 ± 6
Ac-LDL	0.879 ± 0.03	75 ± 7	66 ± 5

Lysosomal extract ($10 \mu\text{g}$ of protein/ml) from J-774 A.1 macrophages was incubated with $10 \mu\text{g}$ of protein/ml of lipoproteins doubly labeled in the protein (^{125}I) and in the cholesteryl ester (^3H CE) moieties for 5 h at 37°C , pH 4.5. At the end of the incubation, lipoprotein protein degradation and lipoprotein cholesterol hydrolysis were determined. Results represent mean \pm SD ($n = 3$).

lipoprotein protein degradation or CE hydrolysis was obtained (data not shown).

We further assessed Ox-LDL CE hydrolysis in J-774 A.1 macrophages (in ACAT-inhibited cells) by analyzing the cellular processing of the labeled CE in a pulse-chase experiment. Cells were preincubated with 25 μg protein/ml of each of the [^3H]CE-labeled lipoproteins (LDL, Ox-LDL, Ac-LDL) for 3 h at 37°C in the presence of 10 $\mu\text{g}/\text{ml}$ Sandoz 58-035. The cells were then washed and further incubated in lipoprotein-free medium (in the presence of 58-035) for a period of up to 24 h at 37°C, during which cell samples were analyzed for their [^3H]CE and [^3H]UC content. As shown in Fig. 6, at the end of the pulse and after up to 1 h of chase incubation, the CE/UC radioactivity ratio was highest for Ox-LDL pretreated cells and lowest for Ac-LDL pretreated cells, suggesting some impaired hydrolysis of Ox-LDL-derived CE, but these limited differences (up to only 17%), completely disappeared after 24 h of chase incubation (Fig. 6). These results strengthen the previous data that there is only minimal reduction in Ox-LDL CE hydrolysis by macrophages, in comparison to the hydrolysis of CE from native LDL or from Ac-LDL.

To directly study the possible accumulation of Ox-LDL-derived UC in lysosomes, the rates of lipoprotein CE lysosomal hydrolysis and the removal of the lipoprotein-derived UC from the lysosomes were analyzed in the lysosomal-rich fraction of the macrophages. Macrophages were incubated with [^3H]CE-labeled lipoproteins for 2 h at 4°C to allow for substantial lipoprotein binding to the cells. This was followed by a cell wash and a further chase incubation at 37°C up to 24 h to allow cellular processing of the lipoprotein. Cells were fractionated after 3 and 24 h of chase incubation. The recovery of the lysosomal marker, β -glucuronidase, was similar (74–79%) for all three lysosomal preparations (obtained from cells

that were incubated with Ox-LDL, LDL, or Ac-LDL). This high recovery may be the result of the presence of prelysosomal endosomes together with the more dense lysosomes.

After 3 h of chase incubation, the lysosomal-rich fraction from cells that were preincubated with Ox-LDL, native LDL, or Ac-LDL contained 4300, 750, and 3000 cpm of [^3H]cholesteryl ester/ 10^6 cells, respectively. After 24 h of chase incubation, a substantial CE hydrolysis occurred and the lysosomal extract of the cells treated by all three types of lipoproteins contained only 30–50 cpm of [^3H]CE/ 10^6 cells (Fig. 7A). At this time point (after 24 h of chase incubation) the lysosomal-rich fraction of the Ox-LDL-treated macrophages contained a substantial amount of [^3H]UC, which was 5.5- and 25-fold higher than the amount of [^3H]UC found in native LDL- or Ac-LDL-treated cells, respectively (Fig. 7B). This trapping of Ox-LDL-derived [^3H]UC in macrophage lysosomes was further assessed by analysis of the distribution of lipoprotein-derived [^3H]cholesterol among the lysosomal, microsomal, and cytosolic fractions (Table 3). Prior to the chase incubation, the cellular contents of radiolabeled cholesterol were 4684, 2280, or 5160 cpm/ 10^6 cells for cells treated with Ox-LDL, native LDL, or Ac-LDL, respectively. After 3 h of chase incubation, the lysosomal fraction of Ox-LDL-treated cells contained 93% of the total cellular labeled cholesterol, whereas in LDL- or Ac-LDL-treated cells, the lysosomes contained only 65 or 60% of the total cellular [^3H]cholesterol. This was paralleled by a substantial reduced content of [^3H]cholesterol in the microsomal and cytosolic fractions of the Ox-LDL-treated macrophages in comparison to these cellular fractions in LDL- or Ac-LDL-treated cells (Table 3). After 24 h of chase incubation, 78% of the total cellular [^3H]cholesterol was still found in the macrophage lysosomal fraction of Ox-LDL-treated cells in comparison

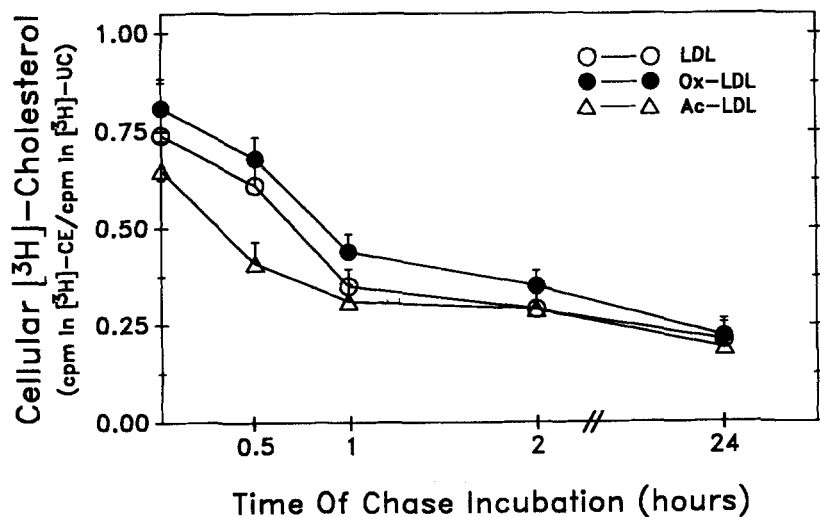


Fig. 6. Time course analysis of [^3H]CE hydrolysis by J-774 A.1 macrophages: a pulse-chase study. Cells ($1 \times 10^6/16\text{-mm}$ dish) were incubated with 25 μg [^3H]CE-labeled lipoprotein protein/ml (pulse) in the presence of 10 $\mu\text{g}/\text{ml}$ of Sandoz 58-035 for 3 h at 37°C. The cells were then washed and further incubated for up to 24 h in lipoprotein-free DMEM, in the presence of the ACAT inhibitor (chase incubation). Cells were analyzed at the indicated time points for their [^3H]CE and [^3H]UC content. Results represent mean \pm SD ($n = 3$).

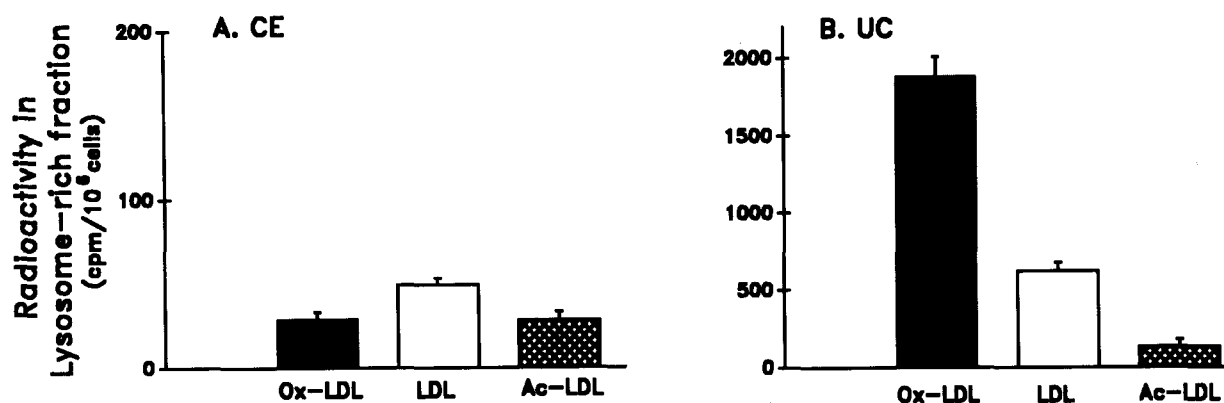


Fig. 7. Cellular lysosomal content of labeled CE and UC in macrophages that were preincubated with [³H]CE-labeled Ox-LDL, LDL, or Ac-LDL. J-774 A.1 macrophages ($1 \times 10^6/16$ -mm dish) were incubated with [³H]CE-labeled lipoprotein (25 μ g protein/ml) for 2 h at 4°C (pulse) followed by a cell wash and a chase incubation for 24 h at 37°C. Cells were collected after 24 h of chase incubation, the contents of six dishes were combined, and the lysosomal-rich fraction was prepared and analyzed for its content of [³H]CE (A) and [³H]UC (B) by TLC. Results are given as the mean \pm SD ($n = 3$).

to values of 55% and only 8% of the total radioactivity in lysosomes derived from native LDL- or Ac-LDL-treated cells (Table 3). At this time point, the macrophage cytosolic fraction derived from Ox-LDL-treated cells contained only 7% of the total cellular [³H]cholesterol in comparison to values of 28% and 38% that were found for native LDL- or Ac-LDL-treated cells, respectively (Table 3).

Availability of the lipoprotein [³H]UC to cellular esterification

The trapping of Ox-LDL-derived UC in macrophage lysosomes would provide less substrate for the cholesterol esterification process and could thus be responsible for the reduced cellular cholesterol esterification after cell incubation with Ox-LDL, in comparison to Ac-LDL. Cellular cholesterol esterification rate was studied and was found to increase from 0.40 ± 0.03 in cells that were incubated

without lipoproteins to 0.98 ± 0.06 , 0.93 ± 0.05 , or to 1.62 ± 0.13 nmol esterified cholesterol/mg cell protein in cells that were incubated with Ox-LDL, native LDL, or Ac-LDL, respectively ($n = 3$). Cellular esterification rate of lipoprotein-derived [³H]UC was also analyzed in J-774 A.1 macrophages after 24 h of cell incubation with [³H]CE-labeled lipoproteins (LDL, Ox-LDL, and Ac-LDL) in the absence or presence of 10 μ g/ml of the ACAT inhibitor, Sandoz 58-035 (Fig. 8). As the lipoprotein was labeled with [³H]CE, the difference between the cellular [³H]CE content in the absence (Fig. 8A) or presence (Fig. 8B) of Sandoz 58-035 represents the net cellular cholesterol esterification of the lipoprotein-labeled unesterified cholesterol. Fig. 8C demonstrates a 6-fold reduction in the cholesterol esterification rate of Ox-LDL-derived cholesterol, (which was similar to that of native LDL-derived UC), in comparison to Ac-LDL-

TABLE 3. Cellular distribution of lipoprotein-derived cholesterol in macrophages after their incubation with Ox-LDL, LDL, or Ac-LDL

Lipoprotein	Time of Incubation	[³ H]Cholesterol (% of total)		
		Lysosome-rich Fraction	Microsome-rich Fraction	Cytosol-rich Fraction
	<i>h</i>			
Ox-LDL	3	93 \pm 8	3 \pm 1	4 \pm 1
LDL	3	65 \pm 5	14 \pm 2	21 \pm 3
Ac-LDL	3	60 \pm 5	17 \pm 2	23 \pm 3
Ox-LDL	24	78 \pm 5	15 \pm 2	7 \pm 2
LDL	24	55 \pm 5	17 \pm 2	28 \pm 3
Ac-LDL	24	8 \pm 2	54 \pm 5	38 \pm 3

J-774 A.1 macrophages were incubated with [³H]CE-labeled Ox-LDL, LDL, and Ac-LDL for 2 h at 4°C followed by cell wash and a further chase incubation. After 3 and 24 h of chase incubation, cells were collected and fractionated. The radioactivity in the lysosome-, microsome-, and cytosol-rich fractions was determined and expressed as percentage of the total cellular radioactivity. Results represent mean \pm SD ($n = 3$).

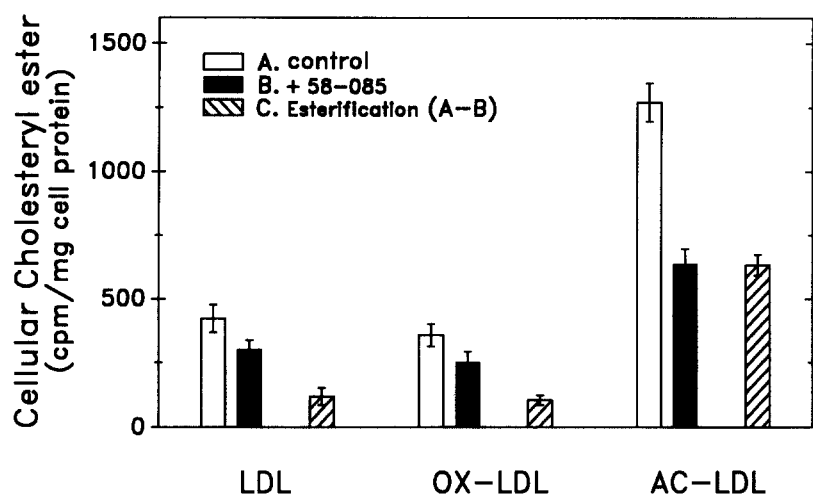


Fig. 8. Cholesterol esterification rate of lipoprotein-derived cholesterol by J-774 A.1 macrophages. Cells ($1 \times 10^6/16$ -mm dish) were incubated with $25 \mu\text{g}$ [^3H]CE-labeled lipoprotein protein/ml in the absence or presence of $10 \mu\text{g/ml}$ of Sandoz 58-035 for 24 h at 37°C . The cells were washed, cellular lipids were extracted and separated by TLC and analyzed for their [^3H]CE content in the scraped CE spots. Results represent mean \pm SD ($n = 3$).

derived cholesterol (Fig. 8). The reduction in the cellular CE/UC radioactivity ratio in the presence of Sandoz 58-035 was less in cells that were incubated with Ox-LDL or native LDL (from 0.32 to 0.19 or from 0.39 to 0.20, respectively) in comparison to cells that were incubated with Ac-LDL (from 0.96 to 0.34). As Ox-LDL-derived UC was shown in this study to be trapped in macrophage lysosomes, the reduced cellular cholesterol esterification rate of this cholesterol may reflect a reduced availability of Ox-LDL-derived UC to the cellular ACAT compartment. The availability of the lipoprotein-derived [^3H]UC to esterification is dependent on both lipoprotein cholesterol uptake and on the amount of lysosomal trapped Ox-LDL-derived UC. Zhang, Basra, and Steinbrecher (21) were the first to demonstrate that calculation of the fate of the lipoprotein-derived cholesterol under an impaired processing should consider the amount of cholesterol released from lysosomes. Using the specific radioactivity of the labeled CE in LDL ($315 \text{ cpm}/\mu\text{g}$ of CE), it could be calculated from Table 3 that the contents of the unesterified cholesterol released from lysosomes after 24 h of cell incubation with the lipoproteins were 10, 9, and $44 \mu\text{g/mg}$ cell protein for the experiments that used Ox-LDL, LDL, and Ac-LDL, respectively. For example, prior to the chase incubation, in macrophages that were incubated with Ox-LDL, there were 4684 cpm of labeled cholesterol/ 10^6 cells, which corresponds to $44 \mu\text{g}$ of cholesterol/mg cell protein. As only 22% of the labeled cholesterol was released from the lysosomes after 24 h of incubation (Table 3), this can be calculated (0.22×44) to be equal to $10 \mu\text{g}$ of cholesterol released from lysosomes/mg cell protein. Using this calculated value for released lysosomal cholesterol, the cellular cholesterol esterification rates in cells treated with labeled Ox-LDL, LDL, or Ac-LDL were found to be similar, i.e., 12.9, 13.8, or 14.3 cpm/mg cell protein per μg cholesterol released from lysosomes, respectively.

The effect of the lipoprotein-derived UC was further assessed by analysis of the effect of Ox-LDL-, LDL-, and Ac-LDL-derived cholesterol on the esterification rate of ^3H -labeled cellular cholesterol (obtained by 1 h of cell incubation with $1 \mu\text{Ci/ml}$ of [^3H]UC at 37°C), rather than the lipoprotein-derived cholesterol. The assumption was made that the different amounts of UC released from lysosomes after cell incubation with the different lipoproteins can affect the esterification rate of the ^3H -labeled cellular cholesterol. [^3H]cholesterol-labeled J-774 A.1 macrophages were incubated with LDL, Ox-LDL, or Ac-LDL ($25 \mu\text{g}$ of protein/ml) for 24 h at 37°C (Fig. 9). In the presence of these lipoproteins, the esterification rate of cellular [^3H]cholesterol increased, in comparison to cells incubated without lipoproteins (control), by 26%, 58%, and 694%, respectively (Fig. 9A). However, when these results were corrected for the different amounts of lysosomal-released cholesterol (Table 3), macrophage cholesterol esterification rates of the cellular labeled cholesterol were found to be similar for cells treated with any of the three lipoproteins (Fig. 9B). Similar results were found when cellular cholesterol was labeled using $1 \mu\text{Ci/ml}$ sodium [^3H]acetate as the precursor (data not shown).

Cholesterol efflux from macrophages preincubated with cholesteryl ester-labeled lipoproteins

Incubation of J-774 A.1 macrophages with the [^3H]CE-labeled lipoproteins for 24 h at 37°C was followed by cell wash and a second incubation in the presence of $100 \mu\text{g}$ HDL₃ protein/ml for 1–24 h at 37°C , prior to the analysis of the cellular efflux of unesterified cholesterol. No measurable amount of cellular released CE could be found in the medium (data not shown). HDL-mediated efflux of the lipoprotein-derived unesterified cholesterol was found to be similar for cells that were incubated with Ox-LDL, native LDL, or Ac-LDL after 24 h of cell incubation with the HDL₃ (Fig. 10). Cell fractionation at this time point

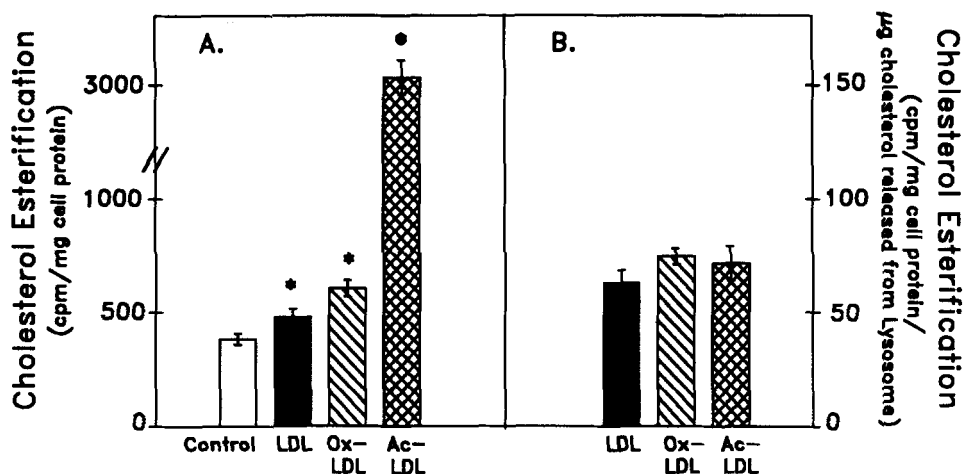


Fig. 9. The effect of Ox-LDL, LDL, and Ac-LDL on the cholesterol esterification rate of cellular cholesterol. J-774 A.1 macrophages were prelabeled with cholesterol by 1 h of cell incubation with 1 μ Ci/ml of 3 H-labeled cholesterol, followed by a cell wash. These cholesterol-labeled cells ($1 \times 10^6/16$ -mm dish) were incubated for 24 h at 37°C with 10 μ g protein/ml of unlabeled Ox-LDL, LDL, or Ac-LDL in the presence of 0.2 mM unlabeled oleic acid complexed with 0.07 mM fatty acid-free albumin. The cholesterol esterification rate of the labeled cellular cholesterol was assayed by TLC analysis of the CE spot (A). The availability of the cellular 3 H]UC to esterification was then calculated as described in the text (B). Results are given as mean \pm SD ($n = 3$). * $P < 0.01$ (vs. Control).

revealed that, in cells that were preincubated with Ox-LDL where 3 H]UC was shown to be trapped, HDL₃ induced a 23% reduction in the 3 H]UC content of the lysosomal-rich fraction in comparison to cells that were incubated in the absence of HDL₃ (from 2521 ± 111 to 1912 ± 95 cpm/ 10^6 cells, $n = 3$) suggesting that cellular cholesterol efflux was associated with removal of UC from lysosomes. After 1 h of cell incubation with HDL, however, the efflux of Ac-LDL-derived cholesterol was about 30% lower than that of Ox-LDL- or native LDL-derived UC (Fig. 10). This reduced efflux resulted from the reduced availability of Ac-LDL-derived CE to efflux as shown by cellular cholesterol efflux analysis in the absence or presence of the ACAT inhibitor, Sandoz 58-035 (Table 4). In the absence of Sandoz 58-035, cellular

cholesterol efflux from Ac-LDL-treated cells was about 30% lower than that obtained from LDL- or Ox-LDL-treated cells (Table 4). Under conditions that prevented the esterification of the lipoprotein-derived 3 H]UC (in the presence of Sandoz 58-035), however, cellular cholesterol efflux from cells treated with any of the lipoproteins was similar (Table 4).

This study demonstrated that 50% of the Ac-LDL-derived cholesterol was not available for efflux from the cells (as it was in the form of CE), whereas for LDL or Ox-LDL, only 21% and 31% of their labeled cholesterol was in the form of CE (and thus not available for efflux), respectively (Table 4). In order to demonstrate that our results with the labeled cholesterol indeed represent efflux of cholesterol and not just exchange of labeled cholesterol

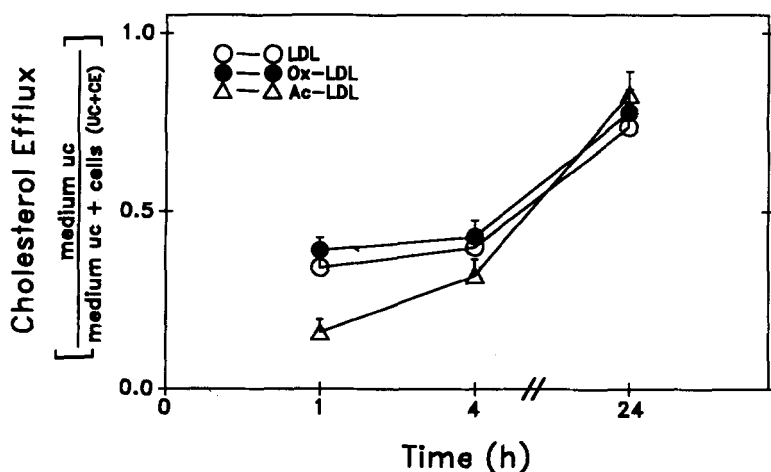


Fig. 10. Cholesterol efflux from macrophages that were preincubated with 3 H]CE-labeled lipoproteins. J-774 A.1 macrophages ($1 \times 10^6/16$ -mm dish) were incubated with 25 μ g 3 H]CE-labeled lipoprotein protein/ml for 24 h at 37°C. Cells were washed with PBS ($\times 3$) followed by a second incubation in the presence of 100 μ g HDL₃ protein/ml for 1-24 h at 37°C. At the end of the incubation, cell-labeled cholesterol and medium-labeled cholesterol were analyzed by TLC and the efflux was calculated.

TABLE 4. HDL-mediated cholesterol efflux of lipoprotein-derived cholesteryl ester from J-774 A.1 macrophages

Lipoproteins	Cholesterol Efflux		
	Medium UC		HDL-mediated Efflux from CE pool (C)
	Control (A)	+ 58-085 (B)	
	Medium UC + cells (UC + CE)		
Ox-LDL	0.38 ± 0.03	0.50 ± 0.05	0.12 ± 0.02
LDL	0.41 ± 0.04	0.52 ± 0.05	0.11 ± 0.02
Ac-LDL	0.29 ± 0.03	0.58 ± 0.05	0.29 ± 0.03

J-774 A.1 macrophages (1×10^6 /16-mm dish) were incubated with 25 μ g [3 H]CE-labeled lipoprotein protein/ml in the absence (Control, A) or presence of 10 μ g/ml of Sandoz 58-035 (B) for 24 h at 37°C. Cells were washed with PBS ($\times 3$) and further incubated in the absence or presence of 100 μ g HDL₃ protein/ml for 4 h at 37°C. At the end of the incubation, medium labeled UC and cellular labeled UC and CE were analyzed by TLC. HDL-mediated UC efflux from the cellular CE pool (C) was calculated by subtraction of A from B. Results represent mean \pm SD (n = 3).

between the lipoproteins and the cell membrane, we have also measured cellular cholesterol mass. After 24 h of cell incubation with Ox-LDL, native LDL, or Ac-LDL, HDL₃ (100 μ g of protein/ml)-mediated cholesterol efflux resulted in a similar reduction in cellular cholesterol mass (by 29%, 30%, and 25%, respectively) in comparison to cells that were incubated in the absence of HDL₃ (data not shown).

Macrophage metabolism of 7-keto cholesterol

Cellular analysis of 7-keto cholesterol revealed that this major cholesterol oxide derivative of Ox-LDL (Fig. 1) was taken up by the cells (as part of the Ox-LDL) and represents $7 \pm 2\%$ of the total cellular cholesterol radioactivity (data not shown). Accumulation of 7-keto cholesterol in macrophages was shown after cell incubation with Ox-LDL but not with native LDL or Ac-LDL (Table 1). 7-Keto cholesterol contributed $6.5 \pm 0.5\%$ of the total cellular cholesterol mass after 24 h of J-774 A.1 macrophage incubation with 10 μ g of Ox-LDL protein/ml (Table 1).

Analysis of the labeled 7-keto cholesterol in the cells in the chase incubation study (Fig. 6) revealed a subtle increment in its cellular content from 458 ± 15 at the end of the pulse to 499 ± 19 or to 517 ± 17 cpm/mg cell protein after 1 h or 24 h of chase incubation, respectively (n = 3), suggesting some lysosomal hydrolysis of 7-keto CE. This was also confirmed in chloroquine (40 μ M)-treated cells where a 13% reduction in the cellular content of 7-keto cholesterol was noted (374 ± 15 vs. 428 ± 12 cpm/mg cell protein, obtained in the presence or absence of chloroquine, respectively, n = 3).

Macrophage content of 7-keto cholesterol only minimally increased in the presence of 10 μ g/ml of Sandoz 58-035 (data not shown), suggesting the very limited availability of 7-keto cholesterol to undergo the esterifica-

tion reaction. Upon incubation of 10 μ g/ml of purified [3 H]UC or [3 H]7-keto cholesterol in ethanolic solutions with J-774 A.1 macrophages for 24 h at 37°C, the ratio of cellular CE/UC radioactivity was 0.18 ± 0.03 in cells incubated with labeled UC, in comparison to a ratio of only 0.03 ± 0.02 for 7-keto cholesteryl ester/7-keto cholesterol in cells that were incubated with labeled 7-keto cholesterol, suggesting again a minimal ability of this oxidized cholesterol to be esterified. The HDL-mediated efflux of the Ox-LDL-derived 7-keto cholesterol and of UC from cells that were preincubated with Ox-LDL, expressed as cpm in medium sterols/cpm in medium sterols + cpm in cell sterols, was only 0.10 ± 0.02 for 7-keto cholesterol in comparison to a ratio of 0.25 ± 0.04 for unesterified cholesterol (n = 3). On using purified [3 H]7-keto cholesterol or [3 H]UC (10 μ g/ml), under similar experimental conditions, similar results were found. Whereas $45 \pm 5\%$ of HDL-mediated cholesterol efflux was obtained for cells that were incubated with [3 H]UC, only $21 \pm 4\%$ HDL-mediated efflux of 7-keto cholesterol was found for macrophages that were incubated with purified labeled 7-keto cholesterol (n = 3).

Effect of oxysterols on lysosomal UC accumulation

Accumulation of UC in macrophages after their incubation with Ox-LDL could have resulted from an inhibition in cholesterol esterification by the oxidized lipid of Ox-LDL (15). This inhibition can be secondary to the effect of oxysterols on UC transport out of the lysosome. The possible effect of oxysterols on lysosomal trapping of the unesterified cholesterol was analyzed by macrophage incubation with [3 H]CE-labeled Ac-LDL in the presence of oxysterols (derived from Ox-LDL), or by cell incubation with [3 H]CE-labeled oxidized Ac-LDL (prepared from Ac-LDL, similar to the preparation of Ox-LDL from native LDL).

The incubation of [^3H]CE-labeled Ac-LDL in the presence of oxysterols (10 $\mu\text{g}/\text{ml}$) caused the accumulation of unesterified cholesterol in the lysosomes (40% of the total cellular labeled cholesterol was found in the lysosomal-rich fraction in comparison to only 8% found in lysosomes obtained from cells that were incubated with Ac-LDL alone) (Fig. 11). Similarly, upon macrophage incubation with oxidized Ac-LDL (Ox-Ac-LDL), 46% of the total cellular labeled cholesterol was found as [^3H]UC in the macrophage lysosomal fraction (Fig. 11). These results suggest again that oxysterols may be responsible for the trapping of the lipoprotein-hydrolyzed CE in the lysosomes as unesterified cholesterol.

DISCUSSION

The present study has demonstrated that the accumulation of unesterified cholesterol (UC) in macrophages after their incubation with Ox-LDL is the result of a reduced esterification rate of the Ox-LDL-derived cholesterol, secondary to reduced availability of the Ox-LDL-derived UC to the ACAT-accessible compartment(s). The study has demonstrated directly that the CE in Ox-LDL is hydrolyzed normally in the lysosomes but the resulting Ox-LDL-derived UC is trapped in the lysosomes (as a result of its association with oxysterols) and thus is not available for the esterification reaction. Aortic foam cells have been shown to accumulate cholesterol in lysosomes (37, 38). Phagocytic uptake of cholesteryl ester droplets by either vascular smooth muscle cells (39) or by J-774 A.1 macrophages (40) resulted in lysosomal accumulation of unesterified cholesterol, similar to the results obtained when macrophages were incubated with Ox-LDL. However, the mechanisms for lysosomal accumulation of UC by the phagocytic uptake of CE

droplets and by receptor-mediated uptake of Ox-LDL are different. The lysosomal trapped UC was not available for HDL-mediated cholesterol efflux only when it resulted from the phagocytic uptake of CE droplets (40), whereas the Ox-LDL-derived UC was available for HDL-mediated efflux. On comparison of the three lipoproteins used in the present study, only Ac-LDL cholesterol was rapidly processed and not retained in the lysosomes. Upon using CE-labeled native LDL, retention of its labeled unesterified cholesterol in the cellular lysosomal fraction was also observed (though not as much as Ox-LDL-UC), in comparison to Ac-LDL. Native LDL, however, unlike Ox-LDL, does not cause macrophage cholesterol accumulation and it binds and internalizes to the cells via the LDL receptor which is regulated by the cellular levels of unesterified cholesterol. Ox-LDL, in contrast, is taken up by the cells via scavenger receptor(s) (8, 41-44). In native LDL, unlike Ox-LDL, the cellular degradation of its protein was not impaired. Although the Ox-LDL used in the present study was not aggregated and, like Ac-LDL, it was endocytized via scavenger receptor(s), the fate of the hydrolyzed CE from these two lipoproteins was different, with a rapid transfer of the Ac-LDL-derived UC out of the lysosomes in comparison to an impaired transport of the Ox-LDL-derived UC out of the lysosomes. Previous studies did not analyze the lipoprotein CE processing in the cells but only demonstrated the impaired lysosomal degradation of the protein moiety of Ox-LDL that led to intracellular accumulation of nondegraded apoB-100 (17-21). The resistance of the Ox-LDL protein to macrophage lysosomal hydrolysis may be related to the fact that the Ox-LDL apoB-100 is fragmented (7, 8), as was also shown for oxidized VLDL (45). It is also possible that the oxidative modification, which involves binding of aldehydic derivatives of the lipoprotein peroxidized polyunsaturated fatty acids to the

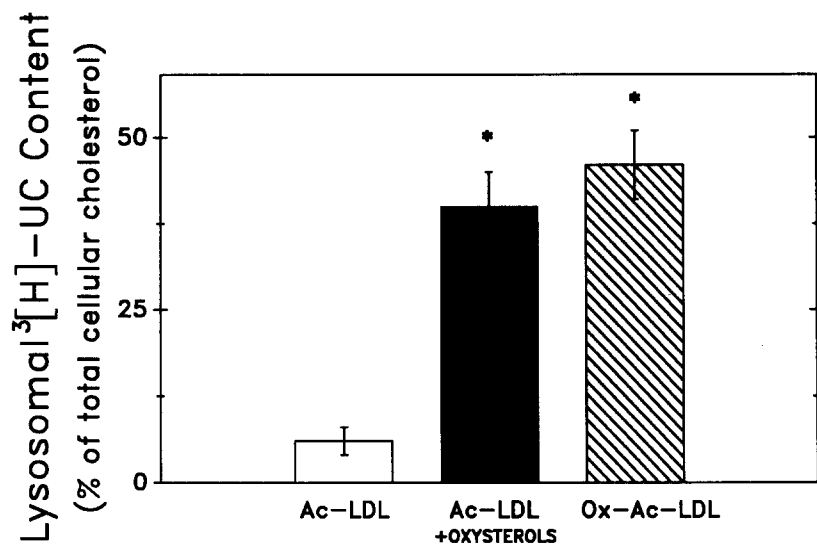


Fig. 11. Effect of oxysterols on Ac-LDL-mediated lysosomal cholesterol accumulation. J-774 A.1 macrophages were incubated for 2 h at 4°C with [^3H]CE-labeled Ac-LDL, or with labeled Ac-LDL in the presence of 10 $\mu\text{g}/\text{ml}$ of oxysterols (separated from Ox-LDL by TLC and scraped from the area between the phospholipids and UC spots as described in ref. 21) or with oxidized labeled Ac-LDL (Ox-Ac-LDL). This incubation was followed by a cell wash and a chase incubation for 24 h at 37°C, at the end of which cells were collected and fractionated. The [^3H]UC in the lysosomal-rich fraction was determined and expressed as the percentage of total cellular labeled cholesterol. Results represent mean \pm SD ($n = 3$).

LDL apoB-100 amino lysine residues (30, 46), can render the apolipoprotein more resistant to intracellular proteolysis. Using cell fractionation techniques and fluorescence microscopy analysis, it was shown (16, 17) that Ox-LDL accumulated in organelles with density properties similar to those of endo/lysosome, but lipolysis of the lipoprotein-derived CE was not studied. The possibility was raised that the hydrolysis of the entire lipoprotein particle may be arrested by the lipoprotein oxidized lipids (19). The selective resistance of the Ox-LDL protein moiety to lysosomal degradation in comparison to the almost normal hydrolysis of the Ox-LDL-derived CE probably resulted from the action of different lysosomal hydrolases on the protein and on the CE components of the lipoprotein. Lysosomal trapping of Ox-LDL-derived UC may be related to its association with other components of the lipoprotein, such as the fragmented apoB-100, individual phospholipids in Ox-LDL, or the oxidized phospholipids. Possible association of the Ox-LDL-derived UC (in association with some other lipoprotein constituents) to lysosomal membrane phospholipids can also contribute to this phenomenon. It has been previously shown by Zhang et al. (21) that the addition of oxysterols (from oxidized LDL) to macrophages led to a substantial inhibition in Ac-LDL-mediated cellular cholesterol esterification. Similarly, Jialal and Chait (15) have demonstrated that Ox-LDL markedly inhibited Ac-LDL-mediated cellular cholesterol esterification with no effect on the cellular degradation of the Ac-LDL protein moiety. Based on our results, which demonstrated UC accumulation in lysosomes after cell incubation with Ox-LDL, a possible explanation for their findings of reduced cellular cholesterol esterification might be that oxysterols in Ox-LDL can cause the accumulation of UC (derived from either Ox-LDL or from Ac-LDL) in the lysosomes and thus prevent its esterification. In the present study we have demonstrated, indeed, that both the addition of oxysterols to Ac-LDL or the oxidation of Ac-LDL resulted in lysosomal trapping of UC. These results thus suggest that the reduced cellular cholesterol esterification observed in the previous studies (15, 21) can be the result of oxysterol-induced lysosomal trapping of the lipoprotein-derived UC.

Accumulation of UC in macrophages after their incubation with Ox-LDL could have resulted (in addition to its lysosomal trapping) from an inhibitory effect of Ox-LDL on ACAT-mediated cholesterol esterification (15). As the cholesterol sources for the ACAT reaction include both lipoprotein-derived cholesterol and cellular-derived cholesterol, we studied the effect of Ox-LDL (in comparison to Ac-LDL and native LDL) on cholesterol esterification rate by using both sources of cholesterol. In both studies, when the stimulation of cholesterol esterification was corrected for the amount of cholesterol released from lysosomes, it was shown that the effects of Ox-LDL, Ac-LDL, and native LDL on cholesterol esterification were

similar. Although both Ox-LDL and Ac-LDL share a common receptor (the Ac-LDL receptor), a substantial amount of Ox-LDL is taken up by macrophages via different receptors (41–44, 47, 48). Binding of different lipoprotein ligands to the same cellular receptor can result in different metabolic routes, as was shown for several modified forms of lipoproteins (49–58). It is possible that some of the modified lipoproteins shared intracellular routes with Ox-LDL and it is thus of interest that phospholipase D-modified LDL, which is highly susceptible to oxidation (58), can induce macrophage UC accumulation similar to that of Ox-LDL which resulted in cellular UC/CE mass of 1.5 ± 0.3 (M. Aviram, unpublished observation).

The finding of a similar HDL-mediated cholesterol efflux in cells that were preincubated with the various lipoproteins suggests that reduced cholesterol efflux was not responsible for the cellular UC accumulation. In addition, the efflux data showed that the lysosomal trapped UC was available for efflux when an appropriate cholesterol acceptor such as HDL was present in the medium.

Oxidized sterols are present in macrophage foam cells in the early atherosclerotic lesion (2). In macrophage-oxidized LDL, 7-keto cholesterol was shown to be the major oxidized cholesterol derivative (59, 60), whereas in endothelial cell-oxidized LDL, 5,6-epoxycholesterol was shown to be the major cholesterol oxide derivative (61). The present study demonstrated that Ox-LDL-derived 7-keto cholesterol was also trapped in the macrophage lysosomes (like Ox-LDL-derived UC, possibly as a result of their association in the same particle) and hence its availability to esterification was limited. In addition, 7-keto cholesterol esterification rate was inhibited in comparison to the esterification rate of unesterified cholesterol, when using Ox-LDL or purified sterols. This limited esterification rate of 7-keto cholesterol may have resulted from the unavailability of oxidized cholesterol to the ACAT cellular compartment, or it may be that 7-keto cholesterol is not a good substrate for the ACAT reaction. Finally, the efflux of Ox-LDL-derived 7-keto cholesterol from macrophages was significantly reduced in comparison to Ox-LDL-derived UC. Possible explanations for these results are that the 7-keto cholesterol and the UC are located in different cellular compartments; that 7-keto cholesterol may be bound to intracellular membranes more tightly than UC; or that its intracellular translocation to the plasma membrane is impaired.

The present study showed the accumulation of unesterified cholesterol (and 7-keto cholesterol) in macrophages that were incubated with Ox-LDL, and demonstrated trapping of these lipids in the lysosomal compartment. Lysosomal accumulation of Ox-LDL-derived UC was shown to be related to the effect of the oxysterols that are present in Ox-LDL. As CE accumulation in macrophage foam cells is the hallmark of the early atherosclerotic lesion and as Ox-LDL was demonstrated

in areas of the atherosclerotic plaque, it is surprising that Ox-LDL leads to UC rather than to CE accumulation in macrophages.

It is still possible that, at longer time of incubation (more than the several days studied), the Ox-LDL-derived UC can be converted into CE. It may also be speculated that cellular accumulation of Ox-LDL-derived UC (rather than the relatively inert CE droplets) by macrophage subpopulation can result in its crystallization and precipitation on cell membranes (62–64) with possible cytotoxic effects, leading to cell death and to the release of their cholesterol extracellularly. Such a process will result in the enrichment of the atherosclerotic lesion with CE-loaded macrophages and also with extracellular unesterified cholesterol (derived from UC-loaded dead macrophages). Extracellular accumulation of unesterified cholesterol-rich particles were indeed demonstrated in areas of the atherosclerotic lesion (65, 66). ■■

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