Lysosomal sequestration of free and esterified cholesterol from oxidized low density lipoprotein in macrophages of different species

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Abstract **Macrophage foam cells of atherosclerotic lesions store lipid in lysosomes and cytoplasmic inclusions. Oxidized low density lipoprotein (oxLDL) has been proposed to be the atherogenic particle responsible for the free and esterified cholesterol stores in macrophages. Currently, however, there is a paucity of data showing that oxLDL can induce much cholesterol accumulation in cells. The present studies compare the ability of mildly oxLDL (TBARS = 5 to 10 nmols/mg LDL protein) with acetylated LDL to induce free cholesterol (FC) and esterified cholesterol (EC) accumulation in pigeon, THP-1, and mouse macrophages. Mildly oxLDL stimulated high levels of loading comparable to acLDL where the cellular cholesterol concentrations ranged from 160 to 420 μg/mg cell protein with EC accounting for 52–80% of the cholesterol. Pigeon and THP-1 macrophages stored most (60–90%) of oxLDL cholesterol (both FC and EC) in lysosomes, and the bulk (64–88%) of acLDL cholesterol in cytoplasmic inclusions. Consistent with lysosomal accumulation, cholesterol esterification was 75% less in THP-1 macrophages enriched with oxLDL cholesterol compared with acLDL. Furthermore, addition of an acyl-CoA:cholesterol acyltransferase inhibitor did not significantly affect either cholesterol loading or the percent distribution of FC and EC in THP-1 and pigeon cells incubated with oxLDL. Surprisingly, mouse macrophages stored most of oxLDL (71%) and acLDL (83%) cholesterol within cytoplasmic inclusions. Also, in mouse macrophages, esterification paralleled cholesterol loading, and was 3-fold more in oxLDL treated cells compared with acLDL treated cells. Inhibition of ACAT led to a 62% and 90% reduction in the %EC in oxLDL and acLDL treated mouse macrophages, respectively. The results demonstrate that mildly oxidized low density lipoprotein (oxLDL) stimulates macrophage foam cell formation and lipid engorgement of lysosomes. However, the fate of oxLDL cholesterol markedly differs in macrophages of different species.—**Yancey, P. G., and W. G. Jerome. **Lysosomal sequestration of free and esterified cholesterol from oxidized low density lipoprotein in macrophages of different species. *J. Lipid Res.* 1998.** 39: **1349–1361.**

Supplementary key words mildly oxidized LDL • macrophage foam cells • lipid-engorged lysosomes

A hallmark feature of atherosclerotic lesions is the presence of lipid-filled cells in the arterial intima. In the early disease stages, foam cells originate as monocyte-derived macrophages while in later stages vascular smooth muscle cells also become lipid loaded (1). During the early fatty streak phase of the disease, cholesteryl ester accumulates in cytoplasmic inclusions of macrophages (1). However, as the disease advances from a fatty streak to a fibrous plaque, lipid accumulates in the lysosomes of macrophages, and as the plaque becomes complicated the smooth muscle cells also store lipid lysosomally (1–3). Lysosomal lipid accumulation has been demonstrated in the lesions of humans and many animal models including primates, pigeons, and rabbits (1–3). Several factors indicate that lysosomal lipid buildup may be particularly important in atherogenesis. First, it coincides with the transition point of a fatty streak into a fibrous plaque (4). Second, studies on the regression of atherosclerotic lesions suggest that lysosomal lipid is not as easily cleared as that in cytoplasmic inclusions even when excess cholesterol acceptors are infused (5–7).

Under normal conditions, receptor-mediated uptake of lipoproteins trafficks the particles to lysosomes where at an acidic pH, the protein and lipid components are degraded to products that can readily transverse the lysosomal membrane (8). The cholesteryl ester is hydrolyzed by acid cholesterol esterase, and the resulting excess free cholesterol is then transported to the endoplasmic reticulum where it is esterified by the enzyme, acyl-CoA:cholesterol acyltransferase (ACAT). This cholesteryl ester is then stored in cytoplasmic inclusions where it can be continu-

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase, acLDL, acetylated low density lipoprotein, BSA, bovine serum albumin, CE, cholesteryl ester, EM, electron microscopy, EC, esterified cholesterol, FBS, fetal bovine serum, FC, free cholesterol, LDL, low density lipoprotein, MEM, Eagle's minimum essential medium, oxLDL, oxidized low density lipoprotein, PBS, phosphate-buffered saline, TBARS, thiobarbituric acid-reactive substances, TC, total cholesterol, and TPA, phorbol ester.

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ally hydrolyzed by a neutral cholesterol esterase and re-esterified by the enzyme ACAT (8).

Most of the lipid within foam cells is derived from plasma low density lipoprotein (LDL) cholesterol (8). However, macrophages in culture do not load with significant amounts of cholesterol when incubated with LDL. The LDL must first be modified so it can stimulate cholesterol accumulation (8). These chemically modified LDLs include acetylated LDL (acLDL) and oxidized LDL (oxLDL) (8, 9). The binding and internalization of both acLDL and oxLDL are mediated by receptors, mainly scavenger receptors, on macrophages (8, 10). Of the two particles, only the oxLDL has potential to be found in arteries, and indeed, in vivo studies suggest that oxidative modification of LDL may occur in atherosclerotic lesions (11). Unlike the LDL receptor, scavenger receptors are not down-regulated by cholesterol buildup in cells (8, 10). Thus, both acLDL and oxLDL should be able to induce free and esterified cholesterol accumulation in macrophages comparable to that in foam cells of lesions.

Many studies have shown that acLDL does stimulate significant (100–600 μg/mg cell protein) cholesteryl ester accumulation in macrophages from many species and that most of this cholesteryl ester is derived from ACAT (8, 12–14). However, comparative studies of acLDL and oxLDL with macrophages have demonstrated notable differences in the metabolism of the two particles. First, unlike acLDL, oxLDL does not induce either significant accumulation of cholesterol or stimulation in ACAT activity (15, 16). It was recently proposed that this could be due to heavy oxidation resulting in the LDL containing almost no free cholesterol or cholesteryl ester (17). One goal of the present studies was to compare the ability of mildly oxLDL with acLDL to stimulate both cholesterol accumulation and ACAT activity in macrophages from several species.

Another reported difference in metabolism of the two particles is that the protein moiety of oxLDL is hydrolyzed at a much slower rate than that of acLDL (18–20). In addition, immunolocalization and ultrastructural studies suggest that oxLDL accumulates in the lysosomes of both cultured macrophages and human atherosclerotic tissues (20, 21), and more recent studies have demonstrated a buildup of oxLDL free cholesterol in the lysosomes of J774 macrophages. From this and other data, it has been postulated that degradation of the entire oxLDL particle could be arrested in the lysosome (18). Taken together, except for the failure of studies to show significant cholesterol accumulation, oxLDL could explain the lysosomal buildup of cholesterol in foam cells of atherosclerotic lesions. In the present studies, we use a combined quantitative ultrastructural and biochemical approach to examine the potential of mildly oxLDL to produce lysosomal lipid accumulation. We also compare the metabolism of acLDL and oxLDL in cultured macrophages from atherosclerosis-susceptible species (human and pigeon) and a more resistant species (mouse) (22). We show that mildly oxidized LDL can produce significant accumulation of free and esterified cholesterol in lysosomes of human and pigeon but not mouse macrophages. In contrast, acLDL produces primarily cholesteryl ester accumulation in cytoplasmic droplets regardless of the species of origin of the macrophages.

MATERIALS AND METHODS

Materials

Male $B_6C_3F_1$ mice were purchased from Charles River Laboratories, Raleigh, NC. Stigmasterol and cholesterol were obtained from Steraloids, Wilton, NH. Bovine serum albumin (BSA; fatty acid-free from fraction V), EDTA, triolein, and cholesteryl ester were purchased from Sigma Chemical Company, St. Louis, MO. The radioisotopes, [^{3}H]oleate (9.2 Ci/mmol) and cholesteryl [^{14}C]oleate (59.5 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Heat-inactivated fetal bovine serum (FBS) was obtained from Atlanta Biologicals, Norcross, GA. Chick serum was purchased from JRH Biosciences, Lenexa, KS, and heat-inactivated by incubation at 56°C for 1 h. Eagle's minimum essential medium (MEM), RPMI, Eagle's vitamins, l-glutamine, penicillin, and streptomycin were obtained from Mediatech, Washington, DC. All tissue-culture plastic ware was obtained from Falcon, Lincoln Park, NJ. All chemical solvents and other chemical reagents were purchased from Fisher Scientific, Pittsburgh, PA. The ACAT inhibitor, compound CP113,818 was a generous gift from Pfizer Central Research, Groton, CT.

Lipoprotein isolation and modification

Pigeon LDL was isolated from the plasma of White Carneau (WC) pigeons fed a diet of commercial pigeon pellets containing 0.5% cholesterol and 5% corn oil for at least 1 month. Human LDL was isolated from plasma given by normocholesterolemic human volunteers. Both human and pigeon LDLs ($1.006 \leq d \leq 1.063$) were isolated by sequential ultracentrifugation as previously described (14). Before use, the LDL was dialyzed extensively against saline containing 1 mm EDTA and sterilized by filtration through a Millipore filter (0.45 μm). Lipoproteins were used within a month of isolation. Pigeon and human LDL were acetylated following the procedure of Basu et al. (23). To oxidize LDL, the native lipoproteins (2 mg LDL protein/ml) were first dialyzed 2 times against 1 L of 0.9% NaCl to remove the EDTA. To oxidize human LDL, the lipoproteins were dialyzed at 37°C for 2 h against 0.9% NaCl containing 20 μm $CuSO_4$. Pigeon LDL was oxidized by dialysis at 4°C for 18 h against 0.9% NaCl containing 20 μm $CuSO_4$. To end the oxidation, human and pigeon LDL were extensively dialyzed against saline containing 1 mm EDTA, and then sterilized by filtration through a 0.45-μm Millipore filter. Oxidation of LDLs was assayed by measuring the thiobarbituric acid-reactive substances (TBARS) using malonaldehyde bis(dimethyl acetal) (MDA) as a standard. Briefly, LDL, oxLDL, or acLDL (100–200 μg protein) were incubated for 15 min at 4°C in saline containing 1 mm EDTA and 10% trichloroacetic acid. Then thiobarbituric acid (.335%, w/v, final concentration of assay) was added and the samples were incubated for 15 min at 100°C. After centrifugation for 15 min at 2000 rpm, the absorbance of the supernatant was read at 535 nm. Oxidation conditions were set to insure that for both pigeon and human oxLDL, the thiobarbituric acid reactivity ranged from 5 to 10 nmols MDA/mg LDL protein. Oxidized LDL within this range of TBARS was not cytotoxic as measured by cell numbers and total protein. The oxidation or acetylation of LDL was also confirmed by agarose gel electrophoresis as described (24). In addition, there was no aggregation of the particles as judged by negative stain electron microscopy (EM) with phosphotungstic acid.

Cell culture and isolation of pigeon and mouse macrophages

Mouse peritoneal macrophages were isolated from $B_6C_3F_1$ mice by peritoneal lavage with phosphate-buffered saline (PBS) 4 days after injection intraperitoneally of 0.5 ml of 10% thioglycollate as described previously (14). The cells were plated onto 35-mm wells at a density of 1.5×10^6 cells and incubated for 4 h at 37°C in MEM containing 10% FBS so that the macrophages could adhere. After removal of non-adherent cells by washing with PBS, the experiments were initiated. Pigeon monocytes were obtained from random-bred WC pigeons fed cholesterol-free pigeon pellets. Briefly, 25 ml to 27 ml of blood was collected into 3.8% sodium citrate (pH 7.3) from each pigeon by cardiac puncture after a lethal dose of sodium pentobarbital (0.45 ml i.v. per bird). The blood was then centrifuged at 150 *g* for 10 min, and the resulting buffy coat at the red cell/plasma interface was swirled into the plasma with a transfer pipette. The leukocyte-rich plasma was layered over Isolymph (Gallard-Schlenshinger), and centrifuged at 400 *g* for 40 min. The mononuclear-rich cell fraction was then removed from the plasma/Isolymph interface. To isolate the monocytes from this cell mixture, the cells were resuspended in Puck's saline and plated in 35-mm wells at a density of 4×10^6 cells and incubated for 30 min at 37°C. The non-adherent cells were then removed by washing two times with PBS, and the monocytes were incubated for 2 days in MEM containing 5% chick serum. After which, the cells were maintained in culture in MEM containing 10% FBS and 1% chick serum to minimize the amount of lipid loading that occurs with high concentrations of chick serum. Pigeon monocytes were maintained in culture for at least 7 days before initiating experiments so that differentiation into macrophages could occur. In some experiments, WC pigeon peritoneal macrophages were isolated as described (14) and used for comparison to the pigeon monocyte-derived macrophages. The experiments with the pigeon peritoneal macrophages were initiated at the end of a 4 h adherence incubation. For experiments with the THP-1 human monocyte/macrophage cell line, the cells were plated onto 35-mm wells at a density of 1.5×10^6 cells and incubated for 3 days at 37°C in RPMI medium containing 10% FBS and phorbol ester (TPA, 50 ng/ml of medium) to allow for differentiation into macrophages (25). TPA was included in the tissue culture medium during the entire time of the experiments. Culture media for all cell incubations was supplemented with 20 mm HEPES, 23 mm bicarbonate, Eagle's vitamins, 200 mm l-glutamine, 1.5 mg/ml glucose, 100 μg/ml streptomycin, and 100 IU/ml penicillin. All cells were incubated in a humid atmosphere at 37°C with 95% air and 5% CO_2.

Cholesterol loading of cells with modified lipoproteins and cholesterol quantitation

In these studies, cholesterol accumulation from oxLDL or acLDL was compared among THP-1, mouse, and pigeon macrophages. The different cells were loaded with the modified lipoprotein cholesterol by incubation for 1–7 days at 37°C in culture media containing 1% FBS alone or with 100–200 μg of oxLDL or acLDL protein/ml (as indicated in the figure legends). When the cholesterol loading incubations were longer than 3 days, the media was changed every 3–4 days to fresh media containing the modified lipoproteins. For some experiments (as indicated in the figure legends), the cellular pools of cholesterol were allowed to equilibrate after cholesterol loading by incubating the cells for 12–24 h in tissue culture medium containing 1% BSA. In some experiments, parallel incubations were done where the ACAT inhibitor, compound CP113,818 (1.5 μg/ml of media, 0.1% dimethyl sulfoxide final concentration), was included in the cholesterol loading and equilibration medium. In THP-1, pigeon, and mouse macrophages, significant differences were not observed in the ultrastructural distribution of oxLDL lipid when equilibration incubations ranged from 0 h to 24 h. In addition, continued inhibition of ACAT during equilibration up to 96 h did not affect the distribution of oxLDL derived free and esterified cholesterol in THP-1 cells (data not shown). For these reasons, an equilibration incubation was not included in all of the experiments.

At the end of the incubations, the cells were washed 3 times with 2 ml of PBS. To quantitate cellular cholesterol, the cell lipids were extracted by the addition of 2 ml of isopropanol (13) containing 10–20 μg of stigmasterol as an internal standard (14). After incubation at room temperature overnight, the cholesterol content of the lipid extract was quantified by the procedure of Ishikawa et al. (26) as modified by Klansek and colleagues (27). The cell proteins were then solubilized by the addition of 1 N NaOH to the wells, and the protein content was measured using the method of Lowry et al. (28).

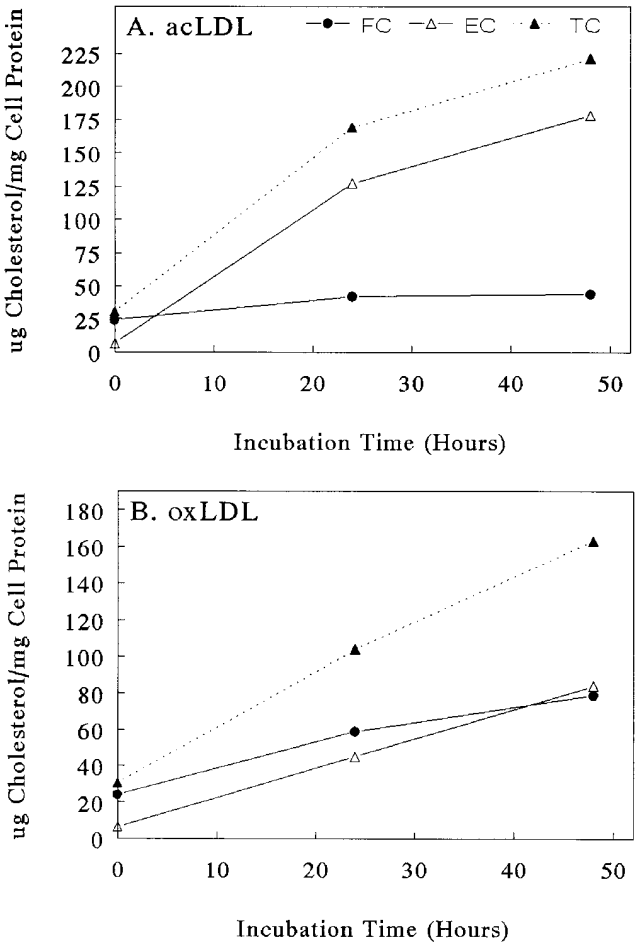


Fig. 1. Time courses of free cholesterol (FC), esterified cholesterol (EC), and total cholesterol (TC) accumulation in pigeon monocyte-derived macrophages incubated with pigeon acLDL or oxLDL. Pigeon macrophages were incubated for up to 48 h at 37°C in MEM containing 1% FBS alone or with 100 μg of protein/ml of either acLDL (panel A) or oxLDL (panel B). After cholesterol loading, the macrophages were equilibrated by incubation for 12 h at 37°C in MEM containing 1% BSA. After equilibration, the cells were harvested and the cell cholesterol contents were measured by gas–liquid chromatography as described under Materials and Methods. Values are the mean for duplicate dishes.

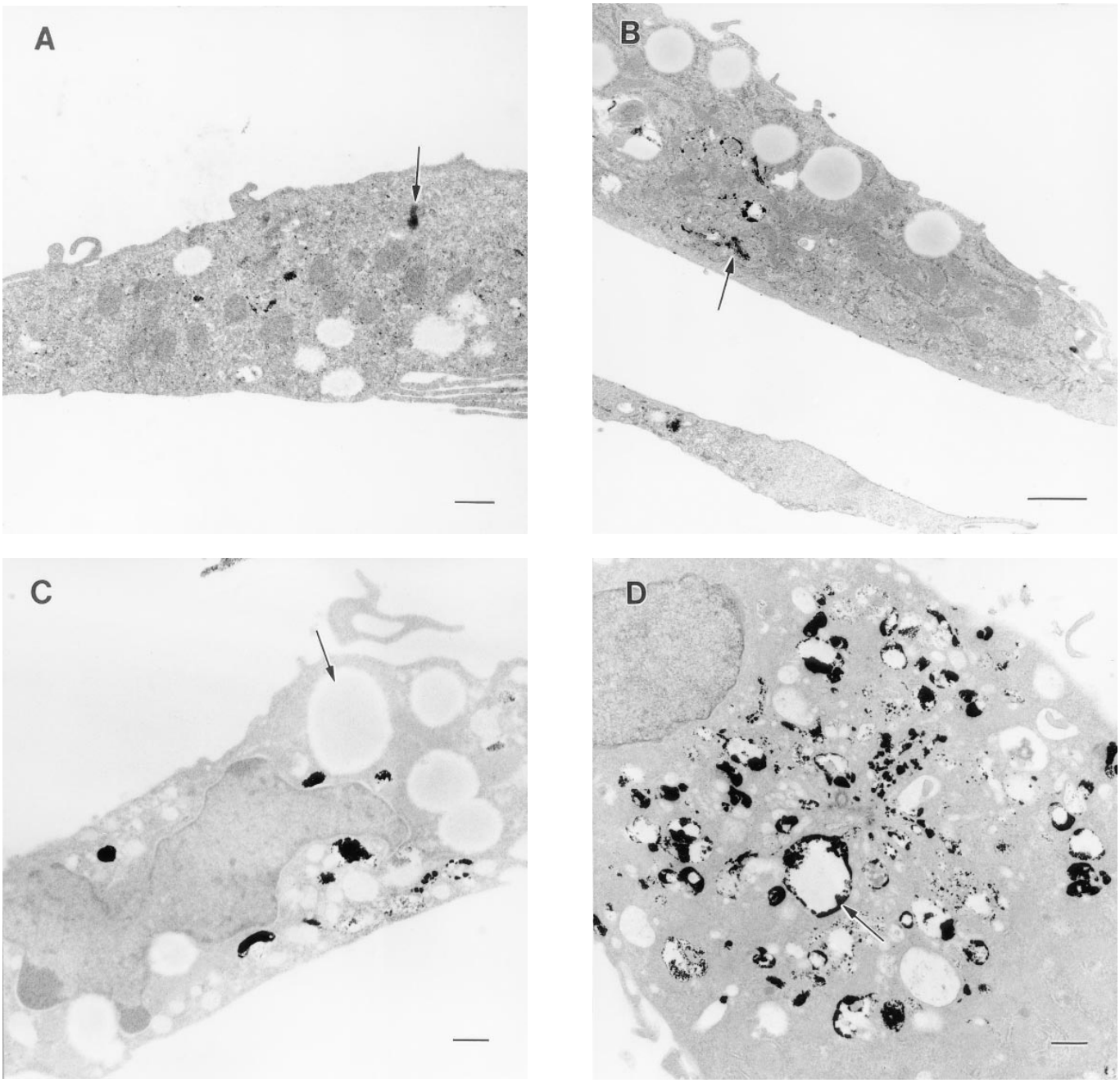


Fig. 2. Electron micrographs of pigeon monocyte-derived macrophages incubated with acLDL or oxLDL and stained to demonstrate acid phosphatase. Pigeon macrophages were incubated for either 6 h (panels A and B) or 48 h (panels C and D) at 37°C in MEM containing 1% FBS alone or with 100 μg of protein / ml of either acLDL or oxLDL followed by a 12-h equilibration phase in MEM containing 1% BSA. A: Pigeon macrophage incubated with acLDL for 6 h shows lipid accumulation primarily as cytoplasmic lipid droplets that lack acid phosphatase reaction product. Limited acid phosphatase activity is seen associated with a few lysosomes (arrow). Magnification = 14,800×, bar = 0.5 μm. B: Pigeon macrophage incubated with oxLDL for 6 h shows similar cell distribution of lipid to acLDL-treated cells. Limited acid phosphatase activity is seen associated with a few lysosomes (arrow). Magnification = 9,900×, bar = 1 μm. C: Pigeon macrophage incubated with acLDL for 48 h. Although some lipid is associated with acid phophatase-positive lysosomes, most of the lipid is within cytoplasmic droplets (arrow). Magnification = 11,000×, bar = 0.5 μm. D: Pigeon macrophage incubated with oxLDL for 48 h. Lipid in this cell is primarily within engorged lysosomes, identified by the dark reaction product (arrow). Magnification = 11,500×, bar = 0.5 μm.

Measurement of oleic acid incorporation into cell cholesteryl ester

The esterification of lipoprotein and cell cholesterol was measured by including a complex of BSA with sodium [9,10-^{3}H]oleate (9.2 Ci / mmol) (29) in the tissue culture media during cholesterol loading and equilibration. Each well received 31 nmol of [^{3}H]oleate / BSA complex (molar ratio of oleate / BSA, 6:1) containing 20,000–25,000 dpm / nmol. The cell incubations were extracted with isopropanol containing cholesteryl [1-^{14}C]oleate as an internal standard. After drying under N_2, the cell lipids

were dissolved in 25 μl of chloroform–methanol 2:1, (v/v), and plated onto LK6D Silica gel 60 thin-layer chromatography plates (Whatman Ltd. Clifton, NJ). Cholesteryl esters were separated from other lipids using hexane–ethyl ether–acetic acid 80:20:1, (v/v/v) as the solvent system, and visualized with iodine. After evaporation of the iodine stain, the cholesteryl ester bands were cut and the radioactivity was measured by liquid scintillation counting. For some experiments, the incorporation of [^{3}H]oleate in cell triglyceride was also measured. No significant differences were observed between oxLDL- and acLDL-treated macrophages in the availability of fatty acids for triglyceride synthesis (data not shown).

Electron microscopy and cytochemistry using acid phosphatase as a lysosomal marker

Lysosomes and related organelles of macrophages loaded with either oxLDL or acLDL cholesterol were localized microscopically by the presence of acid phosphatase. At the end of the incubations with the modified lipoproteins, the cells were first washed 4 times in 0.1 m cacodylate buffer (pH 7.4) containing 0.1 m sucrose at 4°C, and then washed 2 times in the same buffer at room temperature. The cells were then fixed for 2 min at 22°C and 8 min at 4°C in 4% glutaraldehyde in 0.1 m cacodylate/sucrose buffer, washed, and stained to show the presence of acid phosphatase using our standard modification (1) of the Gomori lead precipitation method (30). The reaction medium contained β-glycerophosphate (0.25%, w/v) as the substrate and lead nitrate (0.08%) as the capture reagent. Cells incubated in the same reaction medium without the β-glycerophosphate were used as a reaction control. After the 1 h reaction incubation, the cells were washed in the cacodylate/sucrose buffer, post-fixed in OsO_4, and then washed a third time in the cacodylate/sucrose buffer. The macrophages were scraped from the bottom of the wells using a rubber policeman, enbloc stained in 25% ethanol saturated with uranyl acetate, and embedded in epoxy resin. A Philips EM400 operated at 80 keV was used to view thin sections (80 nm) of the embedded cells. All EM sections were viewed without further staining to verify the enzymatic reaction and then stained with the uranyl acetate and lead citrate.

Quantitative estimates of the partitioning of lipid between inclusions and lysosomes in the different macrophages loaded with acLDL or oxLDL cholesterol were critical for making an integrated interpretation of the biochemical and EM data. The volume of the different cell compartments including lysosomes and inclusions was estimated using point counting sterologic techniques (31), as routinely done in our laboratory (1). Five thin sections from different levels of the embedded block were cut for each sample. Ten to fifteen areas from each thin section were randomly chosen by shifting the region of view a predetermined distance in both the x and y directions. The distances used were based on a random number generator algorithm. From these random areas, the average volume density (volume per volume of cell cytoplasm) was computed for both lysosomal (acid phosphatase positive) and cytoplasmic inclusion (acid phosphatase negative) lipid stores.

Statistical analysis

Means and standard deviations of measures of triplicate samples were computed for each condition. Comparison of means was done by analysis of variance followed by the multiple comparison test of Tukey. Percentage data were corrected to a normal distribution using the arcsin transform before analysis.

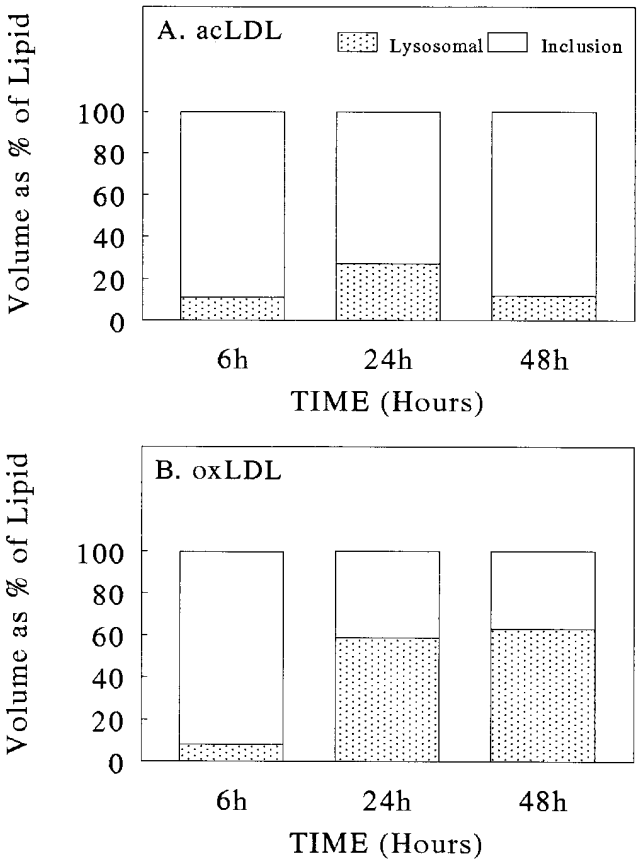
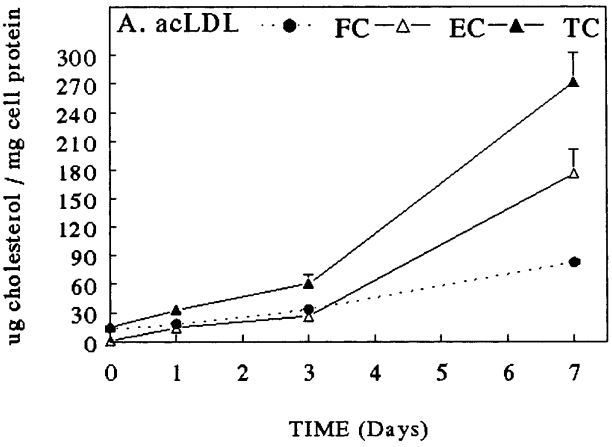


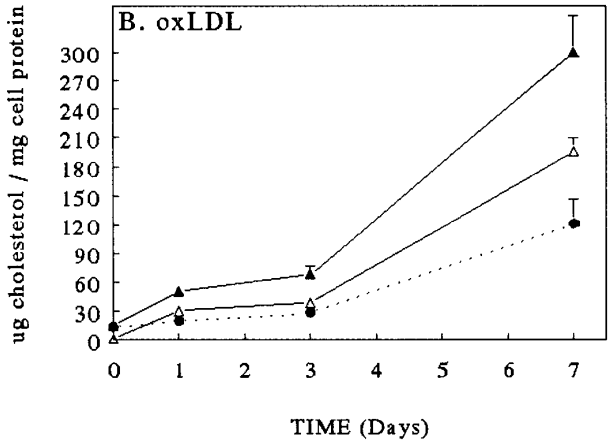
Fig. 3. Quantitation of lysosomal and cytoplasmic lipid accumulation in pigeon monocyte-derived macrophages incubated with pigeon acLDL or oxLDL. Pigeon macrophages were incubated for up to 48 h at 37°C in MEM containing 1% FBS alone or with 100 μg of protein/ml of either acLDL (panel A) or oxLDL (panel B). After cholesterol loading, the cells were equilibrated by incubation for 12 h at 37°C in MEM containing 1% BSA. At the end of each incubation, the cells were fixed, stained with acid phosphatase, and the distribution of total cellular lipid within lysosomes and inclusions was quantitated as described under Materials and Methods. The results are expressed as volume as percentage of lipid.

RESULTS

The accumulation of acLDL or oxLDL cholesterol in macrophages and the detection of lysosomal and inclusion lipid

The time courses of free, esterified, and total cholesterol accumulation in pigeon monocyte-derived macrophages incubated with acLDL and oxLDL are shown in **Figs. 1A** and **1B**. The cells were incubated for up to 2 days with either 100 μg protein/ml of pigeon acLDL (Fig. 1A) or pigeon oxLDL followed by a 12-h equilibration phase in MEM containing 1% BSA. By the end of the incubation with acLDL, the pigeon macrophages accumulated a significant amount of total cholesterol (210 μgTC /mg cell protein). Typical of pigeon macrophages incubated with acLDL, during the first 24 h of incubation, there was a modest increase in the free cholesterol content of the cells. However, the free cholesterol still only accounted for





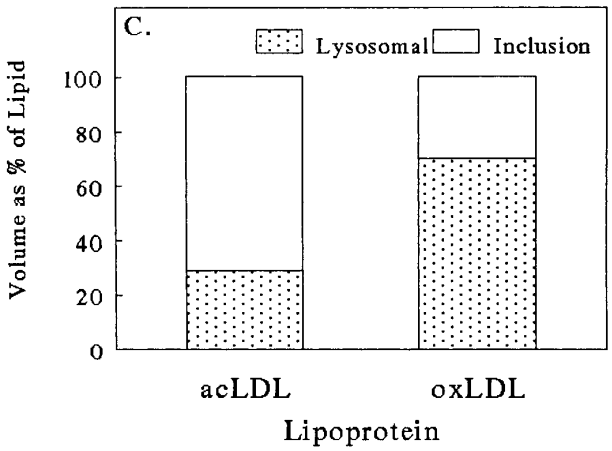


Fig. 4. Time courses of cellular cholesterol accumulation and quantitation of the distribution of lysosomal and inclusion lipid in THP-1 macrophages incubated with pigeon acLDL or oxLDL. THP-1 macrophages were incubated for up to 7 days at 37°C in RPMI containing 1% FBS and 50 ng TPA/ml alone or with 125 μg of protein/ml of either pigeon acLDL or oxLDL. The macrophages were then equilibrated by incubation for 24 h at 37°C in RPMI containing 1% BSA and 50 ng TPA/ml. The cells were harvested and the cell cholesterol contents were measured by gas–liquid chromatography as described under Materials and Methods. Shown are the time courses of free cholesterol (FC), esterified cholesterol (EC), and total cholesterol (TC) accumulation in cells incubated with either acLDL (panel A) or oxLDL (panel B). Values are the mean ± SD for triplicate dishes. Also shown is the quantitation of lysosomal and cytoplasmic lipid accumulation in the THP-1 macrophages after 7 days of incubation with acLDL or oxLDL (panel C). After equilibration, the cells were fixed, stained with acid phosphatase, and the distribution of total cellular lipid within lysosomes and inclusions was quantitated as described under Materials and Methods. The results are expressed as volume as percentage of lipid. Representative electron micrographs are shown in Fig. 5.

18% of the cellular cholesterol. After incubation with the oxLDL (Fig. 1B), the cells accumulated 167 μg total cholesterol/mg cell protein. In contrast to incubation with acLDL, however, 48% of the total cellular cholesterol was free cholesterol and 52% was esterified. In addition, the free cholesterol content with oxLDL increased during the entire length of incubation. Thin sections of pigeon macrophages incubated for 6 h or 48 h with either pigeon acLDL or oxLDL and stained with acid phosphatase are shown in **Fig. 2**. Quantitation of the distribution of cellular lipid in lysosomes and cytoplasmic inclusions after 6 h, 24 h, and 48 h of loading is depicted in **Fig. 3**. After 6 h of incubation with either acLDL (Figs. 2A and 3A) or oxLDL (Figs. 2B and 3B), the initial lipid accumulation was mostly seen in cytoplasmic inclusions (≥88%) and not significantly associated with acid phosphatase. With acLDL, even after 48 h of loading, the majority of the cell lipid was still in cytoplasmic inclusions (Figs. 2C and 3A) despite the cells accumulating a large amount of cholesterol (Fig. 1). In contrast, as early as 24 h of loading with oxLDL, there was a major buildup of lysosomal lipid (59%, Fig. 3B), and by the end of 48 h (Figs. 2D and 3B), most of the cell lipid (63%) was associated with acid phosphatase rather than cytoplasmic inclusions. Similar results were obtained with pigeon-elicited peritoneal macrophages loaded by incubation with pigeon acLDL and oxLDL, and when pigeon macrophages were incubated with human modified LDL (data not shown). In addition, when pigeon macrophages were loaded with very high levels of cholesterol (total cholesterol = 384 ± 19 μg/mg cell protein) by prolonged incubation with pigeon oxLDL, an even greater percentage (90%) of the cell lipid was associated with lysosomes (data not shown).

Shown in **Figs. 4A** and **4B** are the time courses of cholesterol accumulation in THP-1 macrophages incubated with pigeon acLDL and oxLDL. After 7 days of loading with either acLDL or oxLDL, the cells accumulated similar amounts of cholesterol (~285 μg/mg cell protein). In both oxLDL- and acLDL-treated THP-1 cells, esterified cholesterol accounted for most of the cell cholesterol (62% with oxLDL and 70% with acLDL). Despite similarities in the cell free and esterified cholesterol contents, most of the lipid (71%) in THP-1 cells was associated with cytoplasmic inclusions (Figs. 4C and **Fig. 5A**) after loading with acLDL cholesterol, while incubation with oxLDL induced a substantial buildup (70%) of lipid in lysosomes (Figs. 4C and 5B). This difference in distribution pattern mimics what was seen with pigeon macrophages. Similar

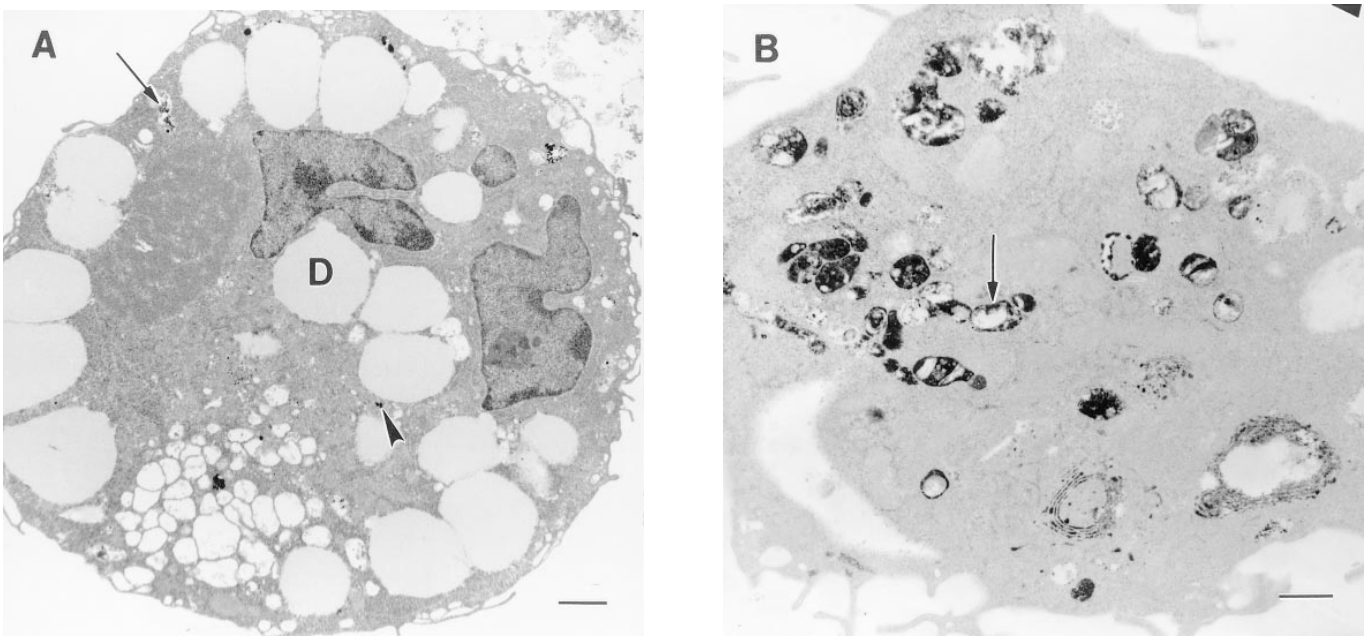


Fig. 5. Electron micrographs of THP-1 macrophages incubated with pigeon acLDL or oxLDL for 7 days and stained to demonstrate acid phosphatase. THP-1 macrophages were incubated for 7 days at 37°C in RPMI containing 1% FBS and 50 ng TPA/ml alone or with 125 μg protein/ml of acLDL (panel A) or oxLDL (panel B). The cells were then equilibrated by incubation for 24 h at 37°C in RPMI containing 1% BSA and 50 ng TPA/ml. A: After 7 days of incubation with acLDL, the lipid is predominantly within cytoplasmic droplets (D) but some acid phosphatase activity can be seen associated with lipid (arrow) as well as within small lysosomes (arrowhead). Magnification = 10,700×, bar = 0.5 μm. B: In contrast to acLDL treated cells, 7 days incubation with oxLDL produced significant lysosomal lipid accumulation as evidenced by the acid phosphatase staining associated with lipid-containing vacuoles (arrow). Magnification = 13,300×, bar = 0.5 μm.

cholesterol accumulation was observed when THP-1 macrophages were incubated with human oxLDL or acLDL and, again, oxLDL but not acLDL induced lysosomal lipid engorgement (data not shown). This suggests that with oxLDL, both free and esterified cholesterol accumulated in lysosomes.

Similar to pigeon and human macrophages, the mouse peritoneal macrophages accumulated substantial amounts of total cholesterol when incubated with either acLDL (246 ± 17 μg cholesterol/mg cell protein) or mildly oxLDL (274 ± 9 μg cholesterol/mg cell protein) (**Figs. 6A** and **6B**). In both cases, the free cholesterol only increased during the first day of loading, and after that most of the accumulated cholesterol (~75%) was esterified. Shown in Fig. 6C is the ultrastructural distribution of lysosomal and cytoplasmic inclusion lipid quantitated from thin sections of the mouse macrophages incubated for 2 days with either pigeon acLDL or oxLDL. The acLDL cholesterol, as with pigeon and THP-1 macrophages, accumulated within cytoplasmic inclusions of the mouse peritoneal macrophages. However, in striking contrast to pigeon and THP-1 cells, most of the oxLDL lipid (71%) was also localized in cytoplasmic inclusions. Thus, while cells derived from pigeon and human showed substantial lysosomal lipid accumulation with oxLDL, mouse cells appeared to clear lysosomal cholesterol efficiently and to store excess cholesterol as cytoplasmic cholesteryl ester droplets. Acetylated LDL cholesterol, on the other hand, was efficiently cleared from lysosomes in all types of macrophages.

Cell cholesteryl ester synthesis and the effects of ACAT inhibition on the cellular distribution of free and esterified cholesterol in macrophages incubated with acLDL and oxLDL

The ultrastructural and cell cholesterol mass data combine to suggest that most of the oxLDL derived free and esterified cholesterol in pigeon and THP-1 macrophages is localized in lysosomes. To substantiate this, we assessed cholesterol mobilization out of lysosomes by measuring the incorporation of [^{3}H]oleate into cell cholesteryl esters. Shown in **Figs. 7A** and **7B** are the time courses of cell cholesteryl ester synthesis and accumulation in THP-1 macrophages incubated for 7 days with pigeon acLDL or oxLDL. Cell cholesteryl ester synthesis was over 2-fold higher in cells incubated with acLDL throughout the 7-day incubation when compared with cells incubated with oxLDL, although the esterified cholesterol contents during loading were similar.

We next studied the effects of an ACAT inhibitor, CP113,818, on both the distribution of cellular free and esterified cholesterol (**Fig. 8A**) and cholesteryl ester synthesis (Fig. 8B) during incubation of THP-1 macrophages with human acLDL or oxLDL. The hypothesis being that if oxLDL cholesteryl ester accumulated in lysosomes, then inhibition of ACAT during loading with oxLDL should have little effect on the cell esterified cholesterol content. As predicted, inhibition of ACAT did not significantly affect loading or the percent distribution of free and esterified cholesterol when THP-1 macrophages were loaded

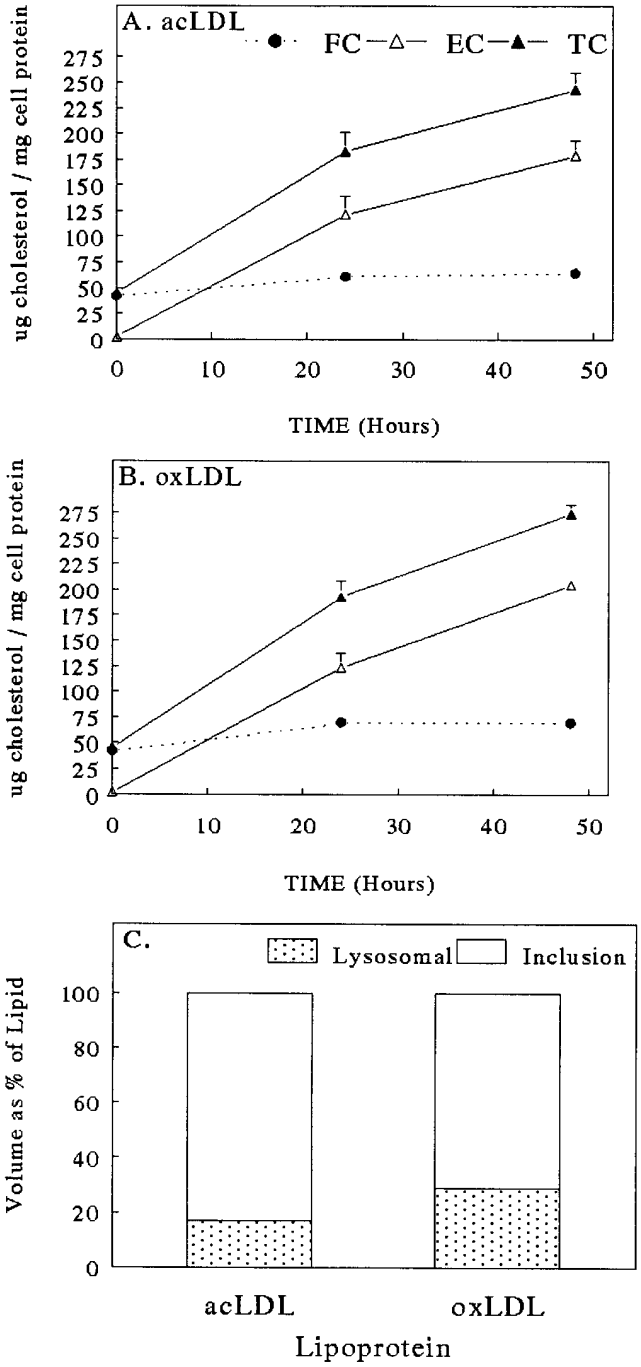


Fig. 6. Cellular cholesterol accumulation and distribution of lysosomal and inclusion lipid in mouse peritoneal macrophages incubated with pigeon acLDL or oxLDL. Mouse peritoneal macrophages were incubated for up to 48 h at 37°C in MEM containing 1% FBS alone or with 100 μg of protein/ml of either acLDL or oxLDL. The macrophages were then equilibrated by incubation for 24 h at 37°C in MEM containing 1% BSA. After equilibration, the cells were harvested and the cholesterol contents were measured by gas–liquid chromatography as described under Materials and Methods. Shown are the time courses of free cholesterol (FC), esterified cholesterol (EC), and total cholesterol (TC) accumulation in cells incubated with either acLDL (panel A) or oxLDL (panel B). Values are the mean ± SD for triplicate dishes. Also shown is the ultrastructural quantitation of lysosomal and cytoplasmic lipid accumulation in the mouse cells after 2 days of incubation with acLDL or oxLDL (panel C). After equilibration, the cells were fixed, stained with acid phosphatase, and the distribution of total cellular lipid within lysosomes and inclusions was quantitated as described under Materials and Methods. The results are expressed as volume as percentage of lipid.

with oxLDL cholesterol (Fig. 8A) although CP113,818 was effective in inhibiting cholesteryl ester synthesis (Fig. 8B). When the cells were incubated with acLDL in the presence of the ACAT inhibitor, the percent esterified cholesterol decreased by 60% in comparison to incubation with acLDL alone. Measurement of the nmols of [^{3}H]oleate incorporated into cholesteryl ester showed that synthesis was 4-fold higher when the cells were incubated with acLDL relative to incubation with oxLDL (Fig. 8B). This was true although the oxLDL-treated cells were more cholesterol-enriched compared with acLDL-treated cells (146 ± 8 and 259 ± 32 μg cholesterol/mg protein for acLDL- and oxLDL-treated cells, respectively) (Fig. 8B). Taken together, the data indicate that the cholesteryl ester accumulation seen with oxLDL is primarily unhydrolyzed lipoprotein cholesteryl ester, remaining in the lysosomes. Similarly, when pigeon macrophages were enriched with oxLDL cholesterol (442 μg cholesterol/mg cell protein, % esterified cholesterol = 67% ± 1%), inhibition of ACAT only reduced the percent esterified cholesterol content by 11 ± 1% compared with cells where ACAT was not inhibited (data not shown). This suggests that like THP-1 macrophages, pigeon macrophages store a substantial amount of oxLDL cholesterol in lysosomes as unhydrolyzed cholesteryl ester.

In contrast to THP-1 and pigeon macrophages, incubation of mouse peritoneal macrophages with oxLDL and the ACAT inhibitor produced a 62% decrease in the percent esterified cholesterol when compared with incubation of the cells with oxLDL alone (**Fig. 9A**). Similarly, addition of the ACAT inhibitor to acLDL-treated cells caused a 90% reduction in the percent esterified cholesterol compared with cells where ACAT was not inhibited (Fig. 9A). Cholesteryl ester synthesis in oxLDL-treated cells was 3-fold higher than that in the acLDL-treated cells (Fig. 9B). Consistent with this, the oxLDL-treated cells were more enriched with cholesterol compared with those incubated with acLDL (155 ± 1 and 309 ± 5 μg cholesterol/mg protein for acLDL and oxLDL treated cells, respectively). Thus, in contrast to pigeon and THP-1 macrophages, mouse cells appear to hydrolyze oxLDL cholesteryl esters efficiently, and readily make the resulting free cholesterol available to ACAT for esterification. The addition of the ACAT inhibitor also affected the total cholesterol accumulation in mouse macrophages incubated with acLDL and oxLDL (92 ± 0 and 201 ± 6 μg total cholesterol/mg protein for acLDL- and oxLDL-treated cells, respectively) suggesting that the excess free cholesterol generated was made available for release from the cell.

DISCUSSION

The accumulation of cholesterol in macrophages is an important feature of atherosclerosis (1, 3, 32). Addition-

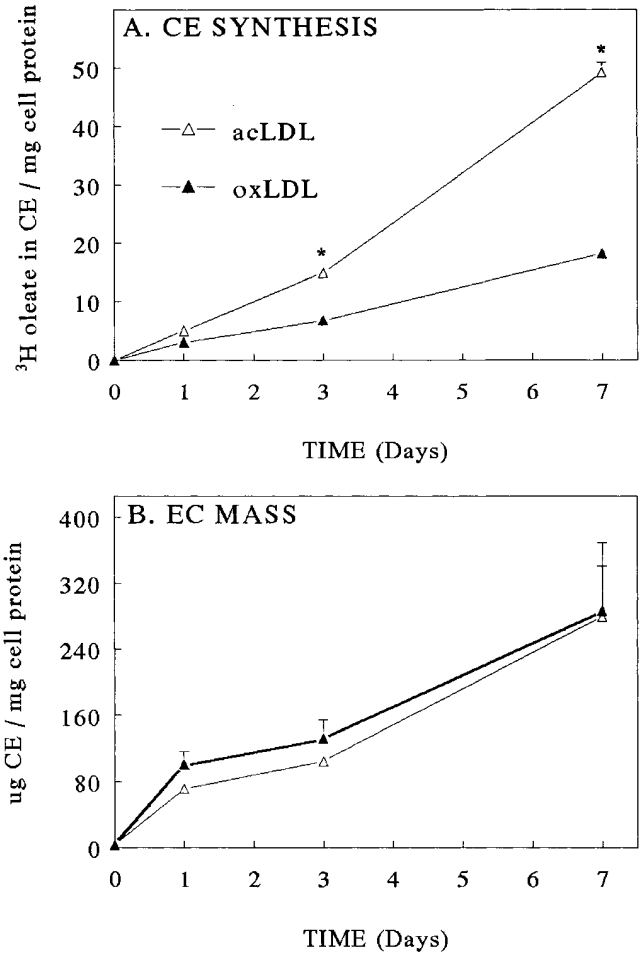


Fig. 7. Time courses of cholesteryl ester synthesis and cholesteryl ester accumulation THP-1 macrophages in the presence of pigeon acLDL or oxLDL. THP-1 macrophages were incubated for up to 7 days at 37°C in RPMI containing 1% BSA, 50 ng TPA/ml, and 31 nmol [^{3}H]oleate alone or with 150 μg protein/ml of acLDL or oxLDL. After cholesterol loading, the cells were equilibrated by incubation for 24 h in RPMI containing 1% FBS, 50 ng TPA/ml, and 31 nmol [^{3}H]oleate. At the end of each incubation, the cells were harvested and the incorporation of [^{3}H]oleate into cell cholesteryl ester (CE) and the cell esterified cholesterol (EC) content were determined as described under Materials and Methods. Shown are cell CE synthesis (panel A) and EC content (panel B). The results are expressed as nmols of [^{3}H]oleate in cell CE/mg cell protein, and as μg EC/mg cell protein for CE synthesis and EC content, respectively. The values are mean ± SD for triplicate dishes. *$P < 0.05$ (acLDL vs oxLDL).

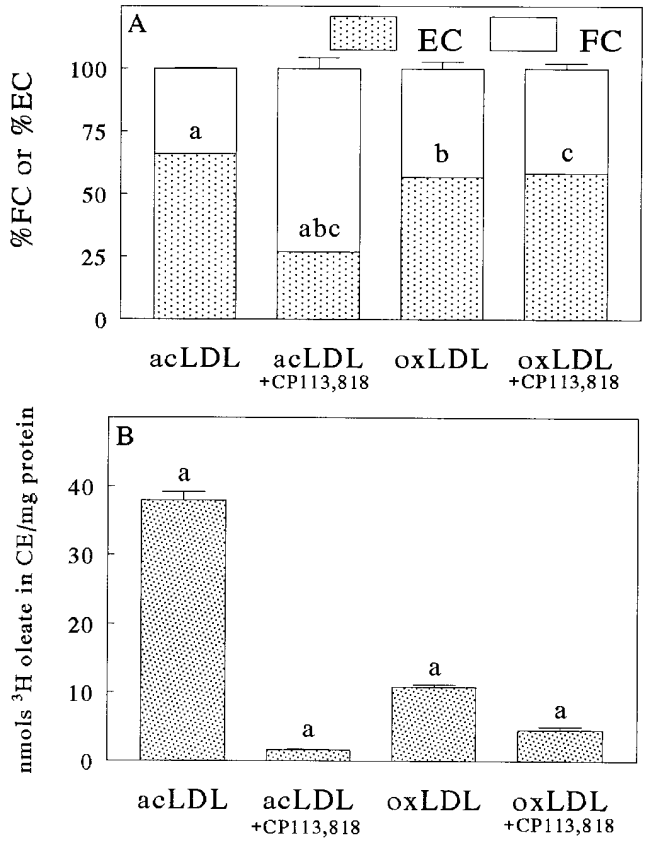


Fig. 8. The effects of ACAT inhibition on the distribution of cell free (FC) and esterified cholesterol (EC) and cell cholesteryl ester (CE) synthesis in THP-1 macrophages incubated with human acLDL or oxLDL. THP-1 macrophages were incubated for 7 days at 37°C in RPMI containing 1% FBS, 50 ng TPA/ml, and 31 nmols of [^{3}H]oleate alone or with one of the following: 175 μg acLDL protein/ml, 175 μg acLDL protein/ml + 1.5 μg CP113,818/ml, 175 μg oxLDL protein/ml, or 175 μg oxLDL protein/ml + 1.5 μg CP113,818/ml. At the end of each incubation, the cells were harvested, and the incorporation of [^{3}H]oleate into cell CE and the cell FC and EC contents were determined as described under Materials and Methods. The results are expressed as percentage of total cellular cholesterol present as FC or EC or as nmols of [^{3}H]oleate in cell CE for the distribution of cellular FC and EC (panel A) or cell CE synthesis (panel B), respectively. The cellular total cholesterol concentrations for the different incubations are as follows: *1*) acLDL alone = 146 ± 8 μg TC/mg cell protein; *2*) acLDL + CP113,818 = 90 ± 8 μg TC/mg cell protein; *3*) oxLDL alone = 259 ± 32 μg TC/mg cell protein; and *4*) oxLDL + CP113,818 = 247 ± 15 μg TC/mg cell protein. The values are the mean ± SD for triplicate determinations. Panel A, $P < 0.001$ (bars with same letters). Panel B, $P < 0.05$ (bars with same letters).

ally, the macrophage foam cells of atherosclerotic lesions are enriched in both free and esterified cholesterol with total levels ranging from 100 μg to 1000 μg/mg cell protein (33, 34). It has been proposed that oxLDL is the atherogenic particle responsible for the buildup of cholesterol in lesions (9). Consistent with this concept, oxLDL has not only been found in atherosclerotic lesions, but is avidly taken up via nonregulated scavenger receptors (9, 11). In the studies reported in this manuscript, we compared the abilities of mildly oxLDL and a more widely studied, but nonphysiological particle, acLDL, to induce free and esterified cholesterol accumulation in macrophages from several species. These studies demonstrate that mildly oxLDL is effective at inducing foam cell formation in THP-1, pigeon, and mouse macrophages. The cellular cholesterol concentrations produced by oxLDL ranged from ~160 to ~420 μg/mg cell protein with cholesteryl esters accounting for 52–80% of the cholesterol. These results are contrary to those published by other investigators where oxLDL was shown to be ineffective at stimulating cholesterol accumulation, in particular cholesteryl esters, in various macrophages including J774, mouse peritoneal, and human monocyte derived macrophages

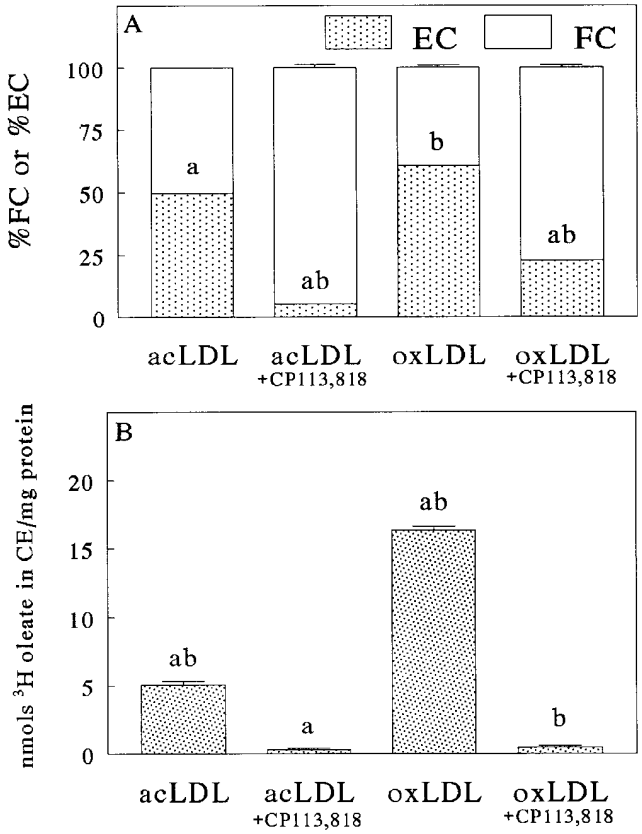


Fig. 9. The effects of ACAT inhibition on the distribution of cell free (FC) and esterified cholesterol (EC) and cell cholesteryl ester (CE) synthesis in mouse macrophages incubated with pigeon acLDL or oxLDL. Mouse macrophages were incubated for 7 days at 37°C in MEM containing 1% FBS and 31 nmols of [3H]oleate alone or with one of the following: 150 μg acLDL protein/ml, 150 μg acLDL protein/ml + 1.5 μg CP113,818/ml, 150 μg oxLDL protein/ml, or 150 μg oxLDL protein/ml + 1.5 μg CP113,818/ml. After each incubation, the cells were harvested, and the incorporation of [^{3}H]oleate into cell CE and the cell FC and EC contents were measured as described under Materials and Methods. The results are expressed as percentage of total cellular cholesterol present as FC or EC or as nmols of [^{3}H]oleate in cell CE for the distribution of cellular FC and EC (panel A) or cell CE synthesis (panel B), respectively. The cellular total cholesterol concentrations for the different incubations are as follows: *1*) acLDL alone = 155 ± 8 μg TC/mg cell protein, and *2*) acLDL + CP113,818 = 92 ± 0 μg TC/mg cell protein, *3*) oxLDL alone = 309 ± 5 μg TC/mg cell protein, and *4*) oxLDL + CP113,818 = 201 ± 6 μg TC/mg cell protein. The values are the mean ± SD for triplicate determinations. Panel A, $P < 0.001$ (bars with same letters). Panel B, $P < 0.05$ (bars with same letters).

(16, 20, 35–40). In these published studies, the cellular cholesterol levels ranged from 41 to 140 μg/mg cell protein with cholesteryl esters only accounting for 7–31% of the total cholesterol.

These discrepancies may be attributed to many factors. First, the LDL used in the present studies was mildly oxidized (TBARS = 5 to 10 nmol/mg LDL protein) while the LDL in many other studies was heavily oxidized (16, 20, 35, 41). Heavy oxidation results in LDL particles that contain almost no free or esterified cholesterol (35, 41) and as a result heavily oxLDL would not necessarily be expected to induce significant cholesterol accumulation. However, in our studies mildly oxLDL contained only 10–20% less cholesterol than either the original or acLDL. This suggests that the high cholesterol content of mildly oxLDL contributes to its ability to induce significant cholesterol accumulation. Consistent with this possibility, recent studies of Greenspan, Mao, and Gutman (17) showed that heavily oxLDL enriched with free cholesterol promoted greater accumulation of cholesterol in mouse macrophages compared with cells incubated with oxLDL not enriched in free cholesterol.

A second factor mediating the differences between ours and other investigators' results could be the length of the incubation with oxLDL. We incubated the different macrophage types with either oxLDL or acLDL for times up to 7 days while in most other studies shorter incubation periods (24 h) were used. Our longer loading period may more accurately model what occurs in arteries, where LDL is retained for a significant time (42). Previous studies from this laboratory have shown that the lifetime of [^{3}H]thymidine-labeled monocytes that enter the arterial wall and differentiate into macrophages is greater than 11 weeks (43) and, the degree of lipid loading in macrophages of early atherosclerotic lesions increases over time (1). From this, it follows that the process to load macrophages to the high levels observed in atherosclerotic foam cells (33, 34) could require at the very least several days. Furthermore, while it remains to be demonstrated that mildly oxLDL is metabolized via scavenger receptors, the rate and degree of cholesterol loading with oxLDL compared with acLDL were similar in pigeon, mouse, and THP-1 macrophages. Along this line, it should be noted that many studies comparing acLDL and oxLDL metabolism in macrophages do not report high degrees of lipid accumulation even with acLDL when short incubation times were used (16, 38, 40). This suggests that acLDL, although exclusively metabolized via macrophage scavenger receptors, also requires a matter of days to induce cholesterol accumulation comparable to that in foam cells of lesions (33, 34).

Additional factors should be taken into account regarding mildly oxLDL. First, oxidation of LDL is believed to occur in the arterial intima outside the vascular system and away from antioxidants (44). Second, very little extracellular lipid is present during the fatty streak and the early fibrous plaque phase (1). Taken together, LDL is probably not oxidized until it is in the arterial wall, and then the level of oxidation may only proceed to a point at which the LDL can be taken up and internalized by macrophages. Thus, it is possible that the LDL that enters the arterial wall needs only to be mildly oxidized. Regardless of the reasons, the present studies have shown that mildly oxLDL at least in vitro can stimulate foam cell formation including significant free and esterified cholesterol accumulation, suggesting it may be more atherogenic than more heavily oxLDL.

Another important finding in the present studies was that mildly oxLDL cholesterol enrichment of macrophages mimics an important feature of atherosclerotic foam cells; lysosomal lipid engorgement. Such free and es-

terified cholesterol-filled lysosomes have been described in both macrophage and smooth muscle foam cells in the atherosclerotic lesions of humans and many animal models. (1–3, 45). In the present studies, lysosomal lipid engorgement in pigeon macrophages was not as prominent at early times of incubation with oxLDL as it was during longer incubation times where the majority of the cellular lipid volume (65%) was in lysosomes. This suggests some time is required before lysosomal accumulation occurs, and is consistent with studies of very early atherosclerotic lesions, where most foam cell lipid is found within cytoplasmic droplets rather than lysosomes (1, 5). THP-1 cells also stored the majority (70%) of their cholesterol derived from oxLDL in lysosomes.

The lysosomal engorgement of oxLDL lipid in pigeon and THP-1 macrophages is similar to published reports describing the mild enrichment of lysosomes with free cholesterol from oxLDL in J774 and human monocyte-derived macrophages (38). However, there are notable differences. For example, both the substantial degree of lipid loading and lysosomal lipid engorgement obtainable with mildly oxLDL suggest that not only free cholesterol from oxLDL but cholesteryl esters as well are trapped in lysosomes. If it were only free cholesterol building up in the lysosomes which store 60–70% of oxLDL cholesterol, then virtually all the cell free cholesterol would have to be in lysosomes rather than in other organelles. Such a possibility is highly unlikely. This conclusion is further substantiated by the observation that the addition of an ACAT inhibitor to the oxLDL cholesterol loading medium did not significantly affect either: *1*) the percent distribution of cholesteryl ester and free cholesterol in pigeon and THP-1 cells or *2*) the degree of cholesterol loading with oxLDL. In addition, we found that when THP-1 macrophages were loaded to high levels with oxLDL cholesterol ($\geq$200 μg TC/mg cell protein), cholesteryl ester synthesis was low. Surprisingly, cholesteryl ester synthesis was lower in oxLDL-loaded cells compared with acLDL-loaded cells even when the oxLDL-treated cells were ~2-fold more enriched with cholesterol. When the ultrastructure, ACAT inhibition, and cholesteryl ester synthesis data are considered together, it is clear that the majority of oxLDL free and esterified cholesterol is sequestered in lysosomes.

There were two other interesting findings in the present studies. First, although both THP-1 and pigeon macrophages store the bulk of oxLDL lipid in lysosomes, they can efficiently metabolize acLDL cholesterol. Second, and in contrast, mouse peritoneal macrophages do not store oxLDL cholesterol lysosomally but metabolize oxLDL lipid almost as efficiently as acLDL. Consistent with this, inhibition of ACAT affected both the amount and distribution of free cholesterol and cholesteryl ester in oxLDL-loaded mouse cells. In addition, the levels of cholesteryl ester synthesis paralleled the degrees of loading with oxLDL and acLDL in mouse macrophages. This similar metabolism of mildly oxLDL and acLDL cholesterol in mouse peritoneal macrophages suggests that the lysosomal engorgement of oxLDL cholesterol in pigeon and THP-1 macrophages is not solely the result of a property of the oxLDL. Likewise, because the two types of cells can efficiently metabolize acLDL, the build up of oxLDL cholesterol in their lysosomes is not due exclusively to a property of these cells. It follows that species specific cellular factors and properties unique to oxLDL work in concert to exacerbate the lysosomal buildup of oxLDL cholesterol observed in pigeon and THP-1 macrophages. In turn, properties unique to mouse macrophages are able to overcome any oxLDL factor resulting in the mildly oxLDL cholesterol metabolism being only slightly aberrant in these cells.

The possible oxLDL and cell specific factors that influence cholesterol metabolism are too many to cover in detail in this discussion. However, some prominent possibilities for differences in lipoproteins include: *1*) The presence of oxidized lipids and proteins in oxLDL; *2*) differences in intracellular trafficking of oxLDL and acLDL; and *3*) structural modifications altering substrate presentation to lysosomal enzymes. Among the more obvious possibilities for cell specific factors include: *1*) rates of normal egression and trafficking of lipids from the lysosome, *2*) genetic differences in basal lysosomal enzymatic activity and, *3*) differences in rates of trafficking of hydrolases to and from the lysosome.

Support exists in the literature for several of these possibilities. First, the recent cloning of the gene for Nieman-Pick Type C disease (46) underscores that genetic factors can influence the movement and subsequent metabolism of cholesterol out of the cell. Cells from Nieman-Pick Type C patients show a marked increase in lysosomal cholesterol accumulation. Second, mouse macrophages, which in this study showed little difference in response to mildly oxLDL compared to acLDL, in some studies have shown decreases in the degradation of the protein component of heavily oxLDL relative to acLDL (18, 19). Thus, for some forms of oxLDL, differences in metabolism of oxLDL and acLDL exist even in mouse peritoneal macrophages. A similar resistance of oxLDL protein to degradation by lysosomal cathepsins has also been observed in J774 macrophages (20). From these studies, it was proposed that the hydrolysis of the entire oxLDL particle, including the cholesteryl ester, may be arrested in the lysosome (18). This is clearly not so when mouse peritoneal macrophages are incubated with mildly oxLDL, and similarly, other studies have shown no difference in the hydrolysis of oxLDL and acLDL cholesteryl ester in J774 macrophages (38). However, from the current studies, an arrestment in degradation of the entire oxLDL particle in THP-1 and pigeon macrophages remains a distinct possibility.

Finally, other possible cell specific factors affecting oxLDL metabolism include acid sphingomyelinase activity and lysosomal sphingomyelin content. Studies of Maor and Aviram (38) have shown that the free cholesterol generated from the hydrolysis of oxLDL cholesteryl esters is trapped in the lysosomes of J774 macrophages. These investigators later showed that the lysosomal cholesterol enrichment was coincident with a reduction in acid sphingomyelinase activity. It was proposed that 7-ketocholesterol present in oxLDL inhibits the enzyme leading to a lysosomal accumulation of sphingomyelin which traps the free choles-

terol (39). Similarly, when Niemann-Pick Type C cells are incubated with LDL, there is a concurrent accumulation of lysosomal cholesterol and sphingomyelin that is associated with a decrease in acid sphingomyelinase activity (47).

In the current studies, only small differences were observed in lysosomal cholesterol accumulation between oxLDL and acLDL in mouse peritoneal macrophages. These differences, however, were in mouse macrophages heavily loaded with oxLDL cholesterol, and were not nearly as dramatic as those in THP-1 and pigeon macrophages. Similarly, other studies (40) using mouse peritoneal macrophages showed no enrichment of lysosomes with oxLDL-derived free cholesterol. It remains to be determined whether differences in lysosomal sphingomyelin hydrolysis can explain the differences in lysosomal accumulation of mildly oxLDL cholesterol between THP-1, pigeon, and mouse macrophages. Nonetheless, our studies underscore that metabolism of oxLDL cholesterol is vastly different among types of macrophages. Thus, conflicting reports in the literature may not only be attributed to differences in the oxLDL used but to differences in the type of macrophage as well. Indeed, when all aspects are considered, it is conceivable that the basal lysosomal function of a particular macrophage has a major influence on how that cell metabolizes some forms of oxLDL.

In summary, these studies have demonstrated that mildly oxLDL can induce high levels of cholesterol accumulation in THP-1, pigeon, and mouse macrophages. In pigeon and human macrophages, the oxLDL cholesterol is largely stored within lysosomes while in mouse macrophages it is stored in cytoplasmic inclusions. These features of mildly oxLDL mimic important events in the development of the atherosclerotic lesion, and make mildly oxLDL a strong candidate as an atherogenic particle. In particular, its ability to stimulate massive lipid engorgement of lysosomes may be important, as lysosomal cholesterol in atherosclerotic foam cells is not readily regressible (5). In contrast, mildly oxLDL does not stimulate significant lysosomal lipid accumulation in macrophages from mice, a species generally regarded as resistant to the development of atherosclerosis (22).

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