Cholesterol delivered to macrophages by oxidized low density lipoprotein is sequestered in lysosomes and fails to efflux normally

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Abstract **Oxidized low density lipoprotein (LDL) has been found to exhibit numerous potentially atherogenic properties, including transformation of macrophages to foam cells. It is believed that high density lipoprotein (HDL) protects against atherosclerosis by removing excess cholesterol from cells of the artery wall, thereby retarding lipid accumulation by macrophages. In the present study, the relative rates of HDL-mediated cholesterol efflux were measured in murine resident peritoneal macrophages that had been loaded with acetylated LDL or oxidized LDL. Total cholesterol content of macrophages incubated for 24 h with either oxidized LDL or acetylated LDL was increased by 3-fold. However, there was no release of cholesterol to HDL from cells loaded with oxidized LDL under conditions in which cells loaded with acetylated LDL released about one-third of their total cholesterol to HDL. Even mild degrees of oxidation were associated with impairment of cholesterol efflux. Macrophages incubated with vortex-aggregated LDL also displayed impaired cholesterol efflux, but aggregation could not account for the entire effect of oxidized LDL. Resistance of apolipoprotein B (apoB) in oxidized LDL to lysosomal hydrolases and inactivation of hydrolases by aldehydes in oxidized LDL were also implicated. The subcellular distribution of cholesterol in oxidized LDL-loaded cells and acetylated LDL-loaded cells was investigated by density gradient fractionation, and this indicated that cholesterol derived from oxidized LDL accumulates within lysosomes. Thus impairment of cholesterol efflux in oxidized LDL-loaded macrophages appears to be due to lysosomal accumulation of oxidized LDL rather than to impaired transport of cholesterol from a cytosolic compartment to the plasma membrane.**—Dhaliwal, B. S., and U. P. Steinbrecher. **Cholesterol delivered to macrophages by oxidized low density lipoprotein is sequestered in lysosomes and fails to efflux normally.** ***J. Lipid Res.*** **2000.** 41: **1658–1665.**

Supplementary key words macrophages • oxidized LDL • foam cells • cholesterol efflux • lysosomes

Oxidation of low density lipoprotein (LDL) has been implicated as a causal factor in the pathogenesis of atherosclerosis. This is based on several lines of evidence, including the demonstration that oxidatively modified LDL exists in atherosclerotic lesions in experimental animals as well as in humans (1–4), and that inhibition of oxidation by several antioxidants can slow the progression of the disease in some animal models (5–13). It has also been reported that the susceptibility of LDL to oxidation correlates with the severity of coronary atherosclerosis, as evaluated by angiography (14). These observations prompted a number of investigators to explore the possible mechanisms by which oxidized LDL might contribute to atherogenesis. In vitro studies have revealed that extensively oxidized LDL has many potentially atherogenic actions, which may account for the progression of atherosclerotic lesions (12). Among these is the ability of oxidized LDL to interact with scavenger receptors on the surface of macrophages and lead to foam cell formation (15, 16).

Ultrastructural studies of macrophage-derived foam cells within atherosclerotic lesions in animals and humans suggest that a significant proportion of the lipid in these cells is in lysosomes rather than cytosolic lipid droplets (17–22). Several studies in J774 cells (a murine macrophage cell line) have reported accumulation of free cholesterol in lysosomes after incubation of cells with oxidized LDL (23, 24). In contrast, Kritharides et al. (25) examined the subcellular distribution of sterols in mouse peritoneal macrophages incubated with oxidized LDL and concluded that there was equilibration of cholesterol to all membrane pools. However, their data show a significant increase in the proportion of cholesterol in an endosomal compartment rather than the lysosomal compartment. This discrepancy may be due to species differences in the processing of oxidized LDL by macrophages in that

Abbreviations: AOP LDL, arachidonic acid-oxidation product LDL; apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HDL, high density lipoprotein; LDL, low density lipoprotein; PBS, phosphate-buffered saline.

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murine macrophages store 71% of cholesterol derived from mildly oxidized LDL within cytoplasmic inclusions, whereas pigeon macrophages and THP-1 cells store 60–90% of it within lysosomes (26).

It has been reported that the protein moiety of oxidized LDL is degraded by macrophages at a much slower rate than that of acetylated LDL (27–29), and that there is much less stimulation of acyl-coenzyme A:cholesterol acyltransferase activity and cholesteryl ester formation by oxidized LDL than acetylated LDL (30). However, there is limited information about the relative rates of release of cholesterol from macrophages loaded with oxidized LDL or acetylated LDL. It has been demonstrated that extensively oxidized LDL is resistant to lysosomal enzymes (27), and oxidized LDL may also inactivate lysosomal acid hydrolases (31). It has also been established that apolipoprotein (apo) B from oxidized LDL accumulates within the lysosomal compartment (27, 28, 32). The only direct study of cholesterol release from macrophages loaded with oxidized LDL found that apoA-I-mediated cholesterol efflux was about 40% lower in macrophages loaded with oxidized LDL compared with macrophages loaded with acetylated LDL (25).

The objective of the present study was to investigate the mechanisms by which oxidized LDL causes impaired efflux of cholesterol from macrophages.

MATERIALS AND METHODS

Amplex Red reagent was purchased from Molecular Probes (Eugene, OR). Butylated hydroxytoluene (was from J. T. Baker (Toronto, ON, Canada). Percoll, Folin and Ciocalteu's phenol reagent, fatty acid-free bovine serum albumin, horseradish peroxidase, cholesterol esterase, cholesterol oxidase, arachidonic acid, 4-methylumbelliferyl α-D-glucoside, and 4-methylumbelliferyl *N*-acetyl-β-D-glucosaminide were purchased from Sigma (St. Louis, MO). Lipopolysaccharide-free sterile water was purchased from Baxter (Toronto, ON, Canada). Centriflo CF25 membrane cones were obtained from Amicon (Beverly, MA). Carrier-free $Na^{125}I$ was purchased from New England Nuclear (Guelph, ON, Canada). [^{3}H]cholesteryl linoleate was purchased from Amersham (Piscataway, NJ). Dulbecco's modified Eagle's medium (DMEM) and gentamicin were purchased from GIBCO (Mississauga, ON, Canada). HyClone defined fetal bovine serum was supplied by Professional Diagnostics (Edmonton, AB, Canada). Tissue culture plates were purchased from Canlab (Vancouver, BC, Canada). Solvents were purchased from Fisher Scientific (Vancouver, BC, Canada). Intralipid was obtained from Pharmacia (Dorval, PQ, Canada). Cholesterol assay kits and standards were obtained from Boehringer Mannheim (Mannheim, Germany). All other chemicals were the highest grade available from Sigma, BDH Chemical (Toronto, ON, Canada), Fisher Scientific (Vancouver, BC, Canada), or VWR Canlab (Vancouver, BC, Canada).

LDL isolation and labeling

LDL (d = 1.019–1.063 g/ml) and HDL_3 (d = 1.125–1.210 g/ml) were isolated by sequential ultracentrifugation of ethylenediaminetetraacetic acid (EDTA)-anticoagulated fasting plasma obtained from normolipidemic volunteers (33). Ethical approval for blood withdrawal was obtained from the University of British Columbia Clinical Research Ethics Board. LDL was radio-iodinated by a modification of the iodine monochloride method (34). Specific radioactivities ranged from 80 to 140 cpm/ng. When iodinated modified LDL was used, the iodination was performed before oxidation or acetylation of the LDL. Cholesteryl esters in LDL were labeled by exchange with a microemulsion containing [^{3}H]cholesteryl linoleate, as previously described (30).

LDL modification

LDL stock solutions had protein concentrations ranging between 6 and 10 mg/ml, and were dialyzed against Dulbecco's phosphate-buffered saline (PBS) containing EDTA at 10 μmol/liter. LDL was oxidized by incubating LDL (200 μg/ml) in PBS containing 5 μM $CuSO_4$ at 37°C for 24 h (15, 35). Acetylation of LDL was performed by addition of four aliquots each of 1 μl of acetic anhydride at 10-min intervals to 2 mg of LDL in 600 μl of ice-cold 50% saturated sodium acetate (36). LDL was aggregated by vortexing a 1-mg/ml solution at low speed for 15 sec (37). To produce $NaBH_4$-treated oxidized LDL, 500 μg of oxidized LDL was incubated with 150 μl of a freshly prepared solution of 1 M $NaBH_4$ in 0.1 M NaOH (38). Arachidonic acid-oxidation product LDL (AOP LDL) was generated by incubating LDL with thermally autoxidized arachidonic acid, as previously described (27).

Cell culture

Female CD-1 Swiss mice were supplied by Charles River (Montreal, PC, Canada). Resident peritoneal macrophages were obtained by peritoneal lavage with ice-cold Dulbecco's PBS (15, 36). All procedures involving animals complied with guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Univerisity of British Columbia. The peritoneal cells were pelleted by centrifugation, and then resuspended in DMEM containing 10% (v/v) fetal bovine serum (FBS) and gentamicin (50 μg/ml). Next, the cells were plated either in 35-mm-diameter (Falcon; Becton Dickinson, Lincoln Park, NJ) or 100-mm-diameter (Corning, Acton, MA) tissue culture plates at a density of 6.0×10^6 or 2.0×10^7 cells/plate, respectively. The larger plates were used for the subcellular fractionation experiments as well as all experiments in which the colorimetric cholesterol assay was used. The smaller plates were used in all experiments in which the fluorimetric cholesterol assay was used. The freshly plated cells were incubated at 37°C for 1 h, and then washed four times with prewarmed Dulbecco's PBS to remove nonadherent cells. After washing, cells were cultured overnight in DMEM containing 10% FBS and gentamicin (50 μg/ml) prior to use in experiments. Cells were washed with serum-free DMEM and then, unless otherwise specified, modified LDL (50 μg/ml) was added in DMEM containing gentamicin (50 μg/ml) and fatty acid-free bovine serum albumin (tissue culture grade, 0.5 mg/ml) to minimize toxicity. After 24 h of incubation with modified LDL, cells were washed with warm Dulbecco's PBS three times to remove LDL-containing medium. One set of plates was harvested at this point, and the remainder were incubated for a further 12 h in DMEM containing HDL_3 at 75 μg/ml before harvesting. Cells were lysed by incubation in 1 ml of cold 0.1 M NaOH for 15 min at 4°C. The cells were then scraped off the plate and aliquoted and assayed for protein and cholesterol content. Lipid extraction was performed according to the method of Bligh and Dyer (39). Radioactivity of the cells was counted on either an LKB (Bromma, Sweden) 1282 gamma spectrometer or a Beckman (Fullerton, CA) LS5000CE scintillation spectrometer, depending on the isotope used. Cell viability was established by trypan blue staining at the end of either the 24-h "loading" incubation or the 12-h "efflux" incubation. Both acetylated LDL-loaded cells and oxidized LDL-loaded cells showed a viability of ≥90%.

Analytical procedures

Protein was assayed by the method of Lowry et al. (40) in presence of 0.05% sodium deoxycholate to minimize turbidity. Bovine serum albumin was used as the standard. Initial experiments were done using a colorimetric cholesterol assay. Lipid extracts were dissolved in isopropanol and 50-μl aliquots were assayed for total cholesterol and free cholesterol, using colorimetric enzymatic kits with a final reaction volume of 1 ml. Absorbances of the colored product were read at 500 nm by an LKB 4054 Ultrospec Plus spectrophotometer. To enhance sensitivity, later experiments were done with a fluorimetric cholesterol assay. Lipid extracts were dissolved in dimethyl sulfoxide and 50-μl aliquots were assayed for total cholesterol and free cholesterol using the Amplex Red cholesterol assay (Molecular Probes) with a final reaction volume of 2 ml (41). Lipoprotein electrophoresis was performed with the Titan Gel lipoprotein kit (Helena Laboratories, Beaumont, TX) in 50 mM barbital buffer (pH 8.6) according to the manufacturer instructions. Lipoprotein bands were visualized by staining with Fat Red 7B. Acetylated or oxidized LDL had electrophoretic mobility greater than 3-fold that of native LDL. To measure the degradation of various modified LDLs, the LDL-containing medium was harvested at the end of the 24-h "loading" incubation. LDL degradation was estimated as the amount of trichloroacetic acid-soluble noniodide radioactivity in the medium (42).

Subcellular fractionation

Macrophages were washed three times with ice-cold Dulbecco's PBS containing 0.02% EDTA. Cells were removed from the plates with cell lifters and sedimented at 1,100 *g* for 10 min at 4°C. Between 18 and 20 million cells were resuspended in 2 ml of cold 10 mM Tris, 0.25 M sucrose, 1 mM EDTA (pH 7.5) and disrupted by nitrogen cavitation at 445 kPa for 15 min in a precooled Kontes minicell disruption chamber (Mandel Scientific, Edmonton, AB, Canada). Nuclei, mitochondria, and intact cells were removed by centrifugation at 100 *g* for 5 min at 4°C. An aliquot of the resultant postnuclear supernatant was reserved for latency determination and the remainder was mixed with 10 mg of bovine serum albumin and layered over 10 ml of 40% Percoll in 10 mM Tris, 0.25 M sucrose, 1 mM EDTA (density 1.070 g/ml) on a cushion of 0.5 ml of 2 M sucrose. Self-forming gradients were generated by centrifugation at 20,000 rpm for 3 h at 4°C in a Beckman SW-40 Ti rotor. Fractions of 0.5 ml were collected from the bottom of the gradient and assayed for radioactivity, using a Beckman LS5000CE scintillation spectrometer. Individual fractions were frozen at −20°C and assayed the following day for neutral α-glucosidase and *N*-acetylglucosaminidase. Density profiles of blank gradients spun in parallel were measured with a digital densitometer (Paar, Graz, Austria).

RESULTS AND DISCUSSION

HDL is considered to be the preferential physiological acceptor for cholesterol from extrahepatic cells (43, 44). Compared with other HDL density fractions density, HDL_3 has been proposed to be the major promoter of cholesterol efflux, and for this reason HDL_3 was used in all the experiments presented here (43, 45). To establish conditions under which HDL concentration would not be rate limiting for efflux, a concentration curve for HDL-mediated efflux was created. **Figure 1** shows that cholesterol efflux increases as a function of HDL_3 concentration up to a maximum HDL_3 concentration of 60 μg/ml. The

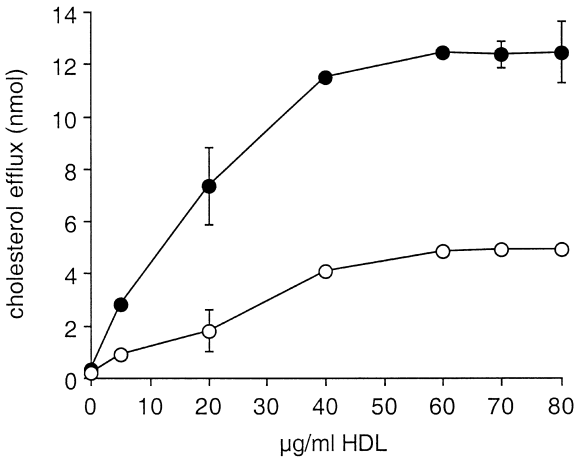

Fig. 1. Concentration dependence of HDL-mediated cholesterol efflux. LDL was radiolabeled with [^{3}H]cholesteryl linoleate and then acetylated or oxidized. Macrophages were incubated for 24 h with a 50-μg/ml concentration of acetylated LDL (solid circles) or oxidized LDL (open circles). Cells were then washed and exposed to varying concentrations of HDL_3 for 12 h, after which time the radioactivity in the medium was measured. Values shown are means ± SD for triplicate plates from one of three similar experiments. Error bars not visible are within the plot symbols.

release of [^{3}H]cholesterol into the medium was accompanied by an equivalent loss of cholesterol from cells (data not shown). HDL_3 concentrations of 40 to 60 μg/ml were required to achieve maximal cholesterol efflux from lipid-loaded cells. In contrast, Kritharides et al. (25) reported that near-maximal efflux of cholesterol from lipid-loaded cells occurred at an apoA-I concentration of 20 μg/ml. This difference may be due, in part, to the higher total cholesterol content of our cells prior to treatment with HDL_3. We used HDL_3 at 75 μg/ml in all subsequent experiments. While this is more than an order of magnitude lower than the concentration of HDL in human plasma, it approximates HDL concentrations in interstitial fluid and is well above the estimated K_m for HDL-mediated cholesterol efflux in cultured murine macrophages (25).

Figure 2 shows a time course for appearance of [^{3}H]cholesterol in medium containing HDL_3 at 75 μg/ml. Incubation for 12 h resulted in a substantial efflux of cholesterol from acetylated LDL-loaded cells, whereas an incubation time of 24 h was associated with a decrease in viability (data not shown). Therefore, 12 h was deemed to be the optimal duration to be used in determining the efficiency of cholesterol efflux from these lipid-loaded cells. **Figure 3** shows that macrophages incubated for 24 h with acetylated LDL (50 μg/ml) or oxidized LDL (50 μg/ml) accumulated free and esterified cholesterol. On subsequent exposure to HDL_3 at 75 μg/ml for 12 h, there was a significant decrease in total cholesterol content of acetylated LDL-loaded cells compared with the oxidized LDL-loaded cells, which exhibited a much smaller change in total cholesterol. Pooled data from 10 experiments with nearly identical degrees of cholesterol loading showed that oxidized LDL-loaded cells released approximately 10% of their total cholesterol content whereas acetylated

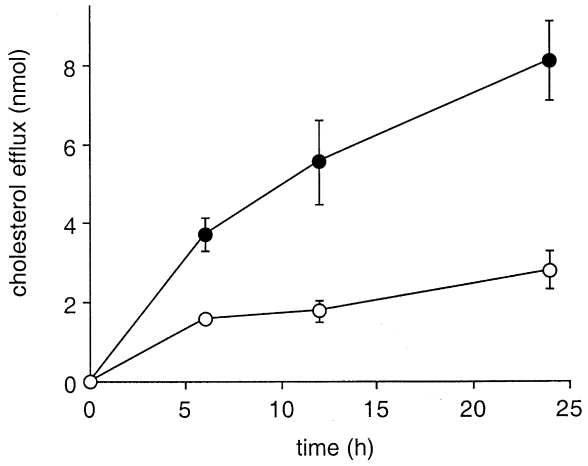

Fig. 2. Effect of HDL incubation time on cholesterol efflux. Conditions for this experiment were the same as described in the legend to Fig. 1, except that the HDL concentration was constant at 75 μg of HDL_3 per ml and the incubation time with HDL varied between 6 and 24 h. Again, solid circles represent acetylated LDL-derived cholesterol and open circles represent oxidized LDL-derived cholesterol. The values are means ± SD for triplicate plates. A replicate experiment yielded similar results. Error bars not visible are within the plot symbols.

LDL-loaded cells released 30% of their total cholesterol. These results are qualitatively similar to those reported previously by Kritharides et al. (25).

One plausible explanation for the impaired cholesterol efflux with oxidized LDL was that cholesterol in oxidized LDL was trapped in the endosomal or lysosomal compartment because of altered internalization pathways or degradation of oxidized LDL. If that is the case, then there should be a relationship between the efficiency of LDL degradation and the amount of cholesterol efflux from cells. Results in **Fig. 4** show that there is indeed such a relationship. It seems reasonable to postulate that if apoB in oxidized LDL was not degraded, the cholesterol associated with that apoB would also remain trapped in lysosomes. However, it has been reported that the lysosomal hydrolysis of cholesteryl ester in oxidized LDL is normal (23). Hence, the association between lysosomal retention of apoB and impairment of efflux may not be causal. To address this further, we wanted to define the extent of oxidation of LDL that was required to have an effect on cholesterol efflux and that was required to affect lysosomal degradation of apoB. It was not possible simply to compare cells loaded with mildly oxidized versus extensively oxidized LDL because mildly oxidized LDL is not a ligand for scavenger receptors and does not lead to cholesterol accumulation. Therefore, we tested the effect of varying degrees of oxidation of acetylated LDL on subsequent cholesterol efflux. Acetylation renders apoB hypersensitive to cathepsin-mediated degradation, whereas oxidization of apoB has the opposite effect (27). Results in **Fig. 5** indicate that increasing the degree of oxidation of acetylated LDL from 5 h of oxidation to 24 h of oxidation resulted in poorer degradation and greater accumulation within the cells and this was as-

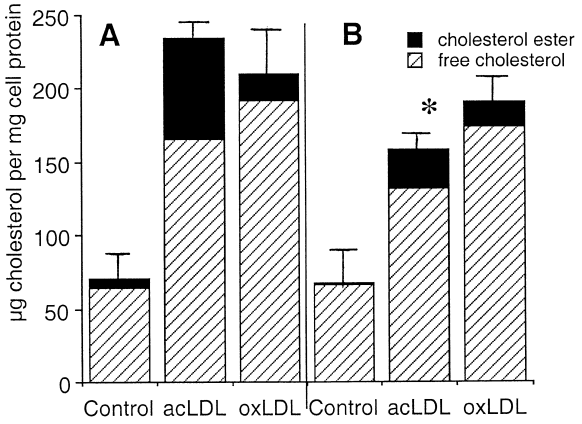

Fig. 3. Cholesterol content of cells after lipid-loading and exposure to HDL_3. Macrophages were incubated for 24 h with a 50-μg/ml concentration of acetylated LDL or oxidized LDL, and harvested (A). Parallel plates were incubated a further 12 h with HDL_3 at 75 μg/ml (B) and then both sets were assayed for total and free cholesterol content. Each column represents total cholesterol; within each column the hatched area represents the free cholesterol content, and the solid area represents the cholesteryl ester content of the cells. Each value represents the mean ± SD of triplicate determinations from one of 10 similar experiments. * $P <$ 0.0001.

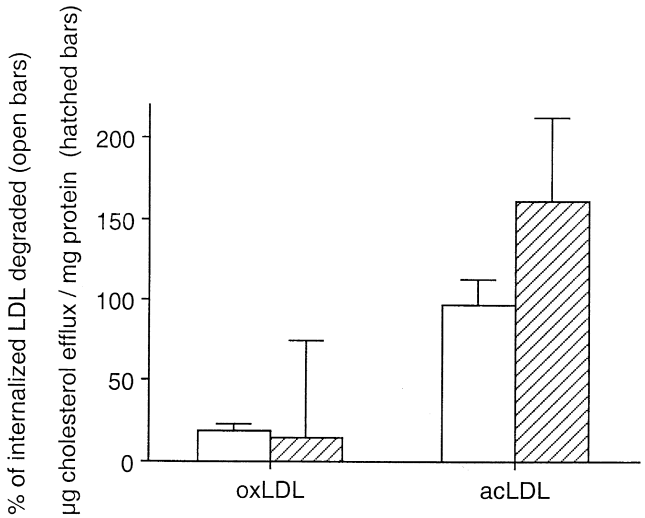

Fig. 4. Correlation between LDL degradation and cholesterol efflux. Macrophages were incubated with ^{125}I-labeled acetylated or oxidized LDL as indicated in the legend for Fig. 3. After 24 h, cells were assayed for cholesterol and radioactivity, and media from the same cells were assayed for LDL degradation products. Parallel dishes were then incubated for 12 h with HDL_3 and cholesterol content was determined. Open columns represent LDL degradation as a percentage of the sum of cell-associated and degraded LDL. Hatched columns represent micrograms of cholesterol efflux per milligram of cell protein. Total cholesterol content of cells at the end of loading was 374 μg/mg for oxidized LDL and 330 μg/mg for acetylated LDL, whereas that of control cells incubated without LDL was to 75 μg/mg. The values are means ± SD for triplicate plates from one of two experiments with similar results. Large error bars for cholesterol efflux are due to summation, as this value is derived by subtraction of two measurements.

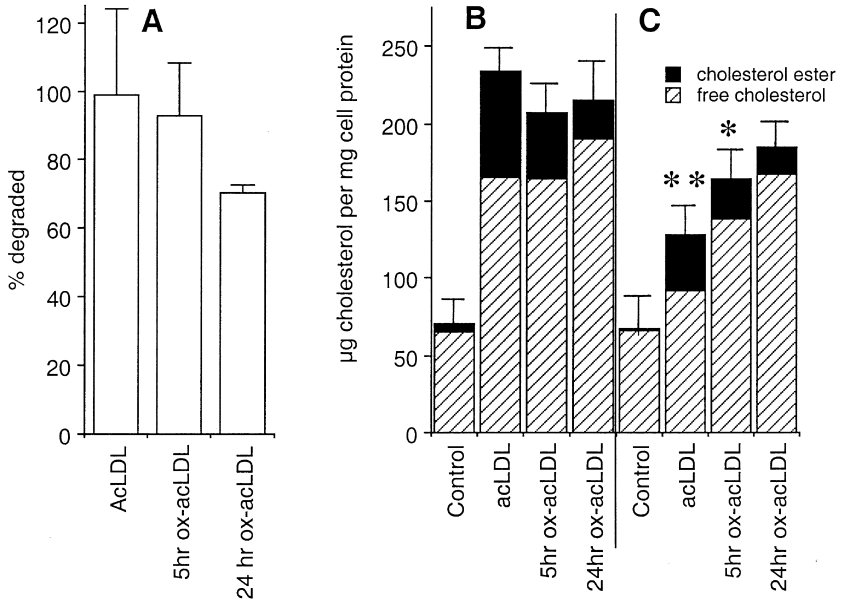

Fig. 5. Effect of varying degrees of oxidation in cholesterol efflux from macrophages. Cells were incubated for 24 h with ^{125}I-labeled acetylated LDL (acLDL), acetylated LDL that had been oxidized for 5 h (5 h ox-acLDL), or acetylated LDL that had been oxidized for 24 h (24 h ox-acLDL). Cells were processed as indicated in the legend to Fig. 4. (A) Values for LDL uptake and degradation (LDL degradation as a percentage of the sum of cell-associated and degraded LDL); (B) total and free cell cholesterol content after loading; (C) total and free cell cholesterol content after incubation with HDL_3. Values shown are means ± SD for triplicate plates from one of two experiments with similar results. A statistically significant decrease in total cholesterol occurred in only two of the conditions: the plates loaded with acetylated LDL and then exposed to HDL_3 (** $P < 0.001$), and plates loaded with 5 h ox-acLDL and then exposed to HDL_3 (* $P < 0.01$). The values are means ± SD for triplicate plates from one of two experiments with similar results.

sociated with decreased efflux of cholesterol to HDL_3. Even mildly oxidized (5 h) acetylated LDL resulted in impaired apoB degradation and impaired efflux of cholesterol compared with unoxidized acetylated LDL, and the degree of impairment increased with more extensive oxidation. Overall, these findings support the hypothesis that altered lysosomal degradation is responsible for the observed impairment of cholesterol efflux.

Oxidized LDL is known to be susceptible to aggregation (46), and it seemed possible that aggregation of LDL might alter its intracellular metabolism. To determine whether aggregation of LDL could impair cholesterol efflux from macrophages, the efflux of cholesterol from macrophages loaded with vortex-aggregated LDL was measured. As shown in **Fig. 6**, the efflux of cholesterol from the aggregated LDL-loaded cells was intermediate (31% of total) between that from acetylated LDL-loaded cells (51%) and from oxidized LDL-loaded cells (5%). It should be noted that the degree of aggregation in vortexed LDL is much greater than that in our oxidized LDL preparations. This experiment suggests that although aggregation of LDL is not the sole or predominant cause of the impaired cholesterol efflux with oxidized LDL, aggregation and oxidation could have additive effects.

To investigate the role of the modified protein component in the impairment of cholesterol efflux, cells were loaded with LDL modified by arachidonic acid-oxidation products (AOP LDL). This modification selectively derivatizes amino groups without causing oxidation of lipid components of LDL and leads to scavenger receptor-mediated uptake and impaired degradation of LDL (27). As shown in **Fig. 7**, AOP LDL-loaded cells exhibited a profound impairment of cholesterol efflux to HDL_3, which supports the hypothesis that resistance of oxidized apoB to lysosomal degradation is an important factor in lysosomal trapping of cholesterol derived from oxidized LDL. To determine whether inactivation of cathepsins by aldehydes in oxidized LDL also played a role in impaired degradation and cholesterol efflux with oxidized LDL, macrophages were loaded with oxidized LDL that had been treated with sodium borohydride. As indicated in Fig. 7, elimination of aldehydes from oxidized LDL increased the rate of cholesterol efflux, suggesting that inactivation of lysosomal enzymes by aldehydes is a contributing factor in impairment of cholesterol efflux from oxidized LDL-loaded macrophages, consistent with previous reports (47).

Although it has previously been reported that the apoB component of oxidized LDL accumulates in the lysosomes of macrophages (32), there are conflicting reports regarding the lysosomal accumulation of cholesterol in oxidized LDL-loaded cells (23, 24, 26, 29, 48–50). Therefore, we investigated the subcellular distribution of cholesterol in oxidized LDL-loaded cells both before and after incubation

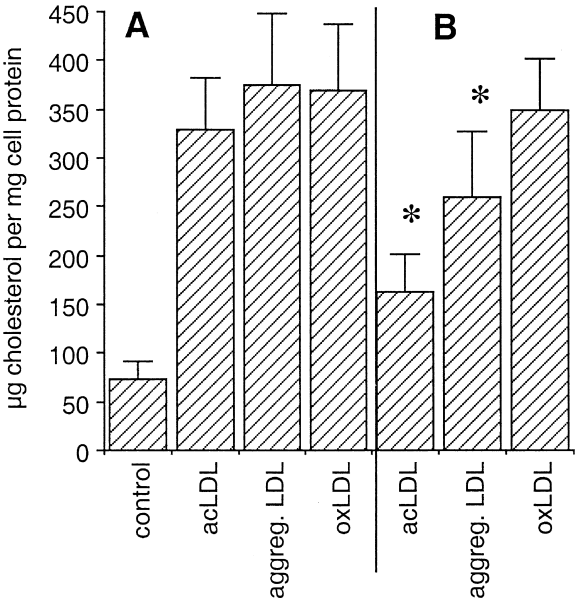

Fig. 6. Accumulation and efflux of aggregated LDL-derived cholesterol. Each column represents the micrograms of total cholesterol per milligram of cell protein. (A) Macrophages that were loaded for 24 h with no lipoprotein, acetylated LDL (acLDL, 50 µg/ml), oxidized LDL (oxLDL, 50 µg/ml), or vortex-aggregated LDL (aggreg. LDL, 50 µg/ml). (B) Macrophages that were treated in the same manner as the cells in (A), only they underwent a further 12-h incubation in the presence of HDL_3. There was a significant accumulation of cholesterol in the macrophages loaded with modified LDL compared with the control cells. Both the acetylated LDL-loaded macrophages and the vortex-aggregated LDL-loaded macrophages exhibited a significant (* $P < 0.05$) decrease in total cholesterol on exposure to HDL_3. Each value represents the mean ± SD of four determinations from a single experiment.

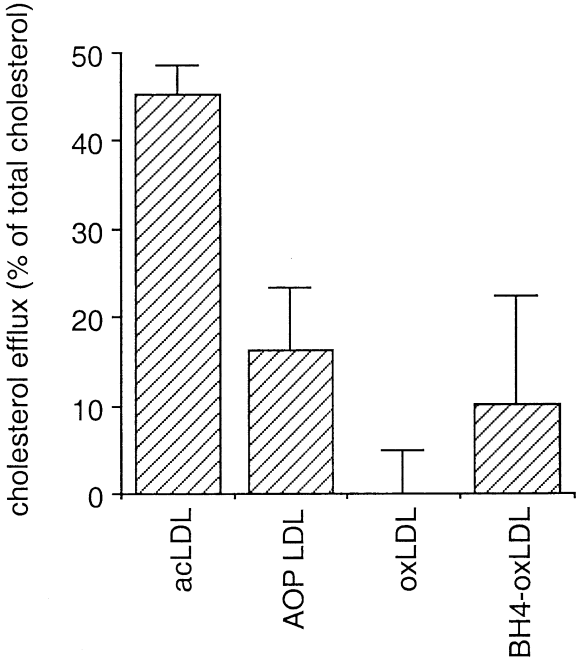

Fig. 7. Effect of apoB derivatization and reactive aldehydes in oxidized LDL on cholesterol efflux. Macrophages were loaded with acetylated LDL (acLDL), arachidonic acid oxidation product LDL (AOP LDL), oxidized LDL (oxLDL), or borohydride-treated oxidized LDL (BH_4-oxLDL). Each value is expressed as the mean percentage decrease ± SD from triplicate plates from one of two experiments with similar results.

with HDL_3. After loading cells with [^{3}H]cholesterol-labeled oxidized LDL, cells were disrupted and fractionated on a density gradient. Before loading the cells with oxidized LDL, we determined the relative distribution of the marker enzymes in control (nonloaded) macrophages. The results from one experiment are presented in **Fig. 8**. Loading cells with oxidized LDL resulted in a marked lowering of the median density of the lysosomal fraction. There was no appreciable change in density of fractions associated with the lysosomal marker after 12 h of incubation with HDL_3, but less than one quarter of the radiolabeled cholesterol was effluxed during this incubation. These findings differ from those reported by Kritharides et al. (25). This group found that there was equilibration of cholesterol to all membrane pools in murine macrophages loaded with oxidized LDL. The contrast between this and our observations may be based on differences in the analytical methods used to fractionate the cells and detect cholesterol. Kritharides et al. (25) combined the collected fractions into five major fractions representing large ranges in density, in which the density range of 1.035 to 1.050 g/ml was labeled the endosomal fraction and the density range of 1.050 to 1.095 g/ml was labeled the lysosomal fraction. In our experience (51), the fractions containing lysosomal enzyme activity in mouse macrophages loaded with oxidized LDL are located in the density range of 1.045 to 1.055 g/ml. Kritharides et al. (25) reported a large proportion of free cholesterol and 7-ketocholesterol in both their endosomal and lysosomal fractions; this is in agreement with our data, which demonstrate that most of the oxidized LDL-derived cholesterol is found in the 1.050-g/ml density fraction. Unlike Kritharides et al. (25), we did not find any accumulation of cholesterol in the density range 1.020 to 1.035 g/ml. This may be explained by the fact that they measured the distribution of all cholesterol in the cell, whereas we measured only the radiolabeled cholesterol delivered by oxidized LDL. Our conclusions also differ from those of Yancey et al. (26, 49), who reported that mouse peritoneal macrophages stored most of their oxidized LDL-derived cholesterol (71%) within cytoplasmic inclusions. However, the LDL used by this group was only mildly oxidized, and would therefore be more susceptible to lysosomal degradation.

The results of this study confirm that macrophages loaded with extensively oxidized LDL have a reduced ability to release cholesterol to HDL compared with cells loaded with acetylated LDL. The results are consistent with the notion that this impairment of efflux is due to sequestration of oxidized LDL within lysosomes.

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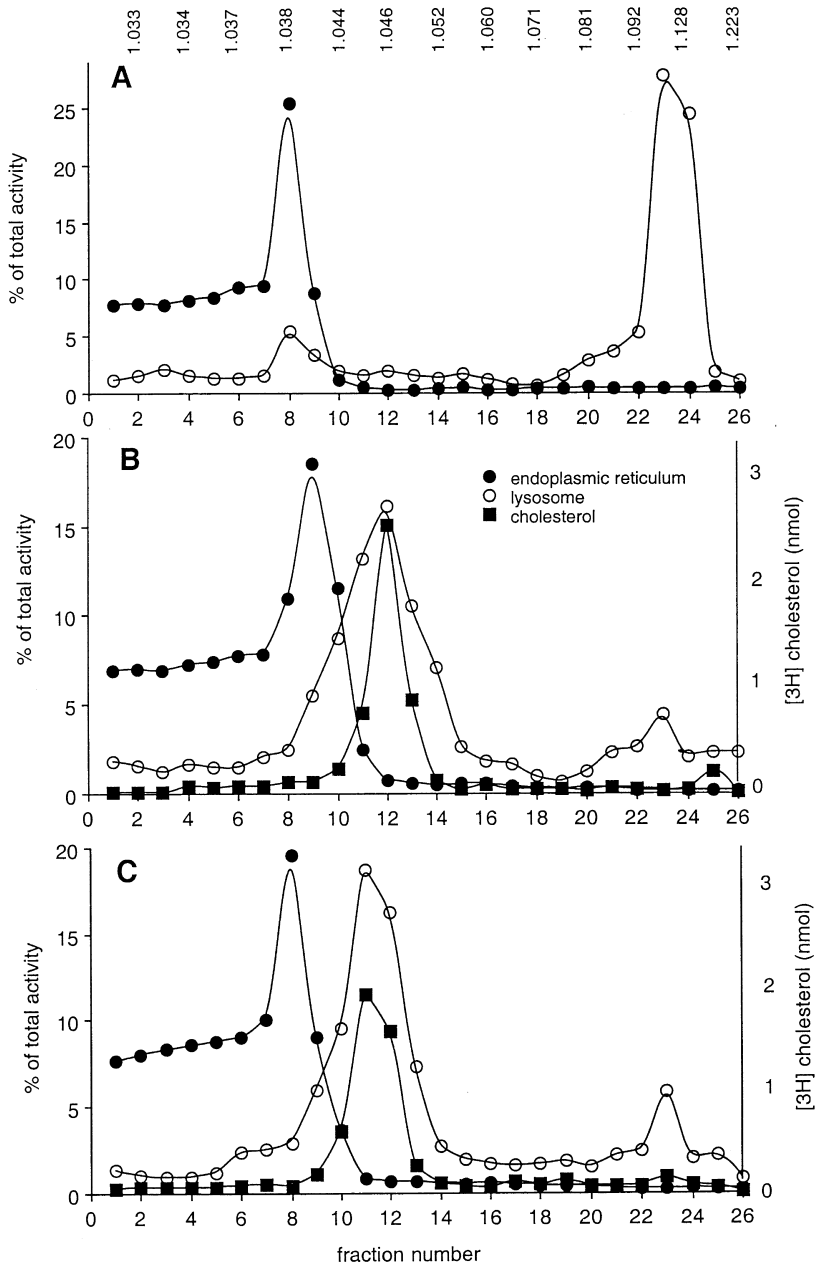

Fig. 8. Subcellular fractionation of macrophages. Macrophages were incubated for 24 h in the absence of LDL (A), or with oxidized LDL (50 μg/ml) labeled with [^{3}H]cholesteryl linoleate (B). An additional set of cells was incubated for 24 h with oxidized LDL (50 μg/ml) followed by a 12-h incubation with HDL_3 at 75 μg/ml (C). Cells were then disrupted by nitrogen cavitation, and fractionated by density gradient centrifugation. Activity of the lysosomal marker *N*-acetylglucosaminidase (open circles), the endoplasmic reticulum marker neutral α-glucosidase (solid circles), and cholesterol radioactivity (solid squares) was then determined in individual density fractions. Each point represents the percentage of total enzyme activity recovered or the number of nanomoles of [^{3}H]cholesterol in each fraction. The density of individual fractions is shown at the top of the graph.

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