# Effects of cellular cholesterol loading on macrophage foam cell lysosome acidification[S]

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**Abstract** **Macrophages incubated with mildly oxidized low density lipoprotein (OxLDL), aggregated low density lipoprotein (AggLDL), or cholesteryl ester-rich lipid dispersions (DISPs) accumulate cholesterol in lysosomes followed by an inhibition of lysosomal cholesteryl ester (CE) hydrolysis. The variety of cholesterol-containing particles producing inhibition of hydrolysis suggests that inhibition may relate to general changes in lysosomes. Lysosome pH is a key mediator of activity and thus is a potential mechanism for lipid-induced inhibition. We investigated the effects of cholesterol accumulation on THP-1 macrophage lysosome pH. Treatment with OxLDL, AggLDL, and DISPs resulted in inhibition of the lysosome's ability to maintain an active pH and concomitant decreases in CE hydrolysis. Consistent with an overall disruption of lysosome function, exposure to OxLDL or AggLDL reduced lysosomal apolipoprotein B degradation. The lysosomal cholesterol sequestration and inactivation are not observed in cholesterol-equivalent cells loaded using acetylated low density lipoprotein (AcLDL). However, AcLDL-derived cholesterol in the presence of progesterone (to block cholesterol egression from lysosomes) inhibited lysosome acidification. Lysosome inhibition was not attributable to a decrease in the overall levels of vacuolar ATPase. However, augmentation of membrane cholesterol in isolated lysosomes inhibited vacuolar ATPase-dependent pumping of $H^+$ ions into lysosomes. These data indicate that lysosomal cholesterol accumulation alters lysosomes in ways that could exacerbate foam cell formation and influence atherosclerotic lesion development.—**Cox, B. E., E. E. Griffin, J. C. Ullery, and W. G. Jerome. **Effects of cellular cholesterol loading on macrophage foam cell lysosome acidification.** ***J. Lipid Res.*** **2007.** 48: **1012–1021.**

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Atherosclerosis is the leading cause of death in the Western world and accounts for more than half of all mortalities in developed countries. Thus, it is crucial to understand all aspects of this disease. One hallmark of an atherosclerotic lesion is the presence of macrophage foam cells. In advanced lesions, many foam cells contain large, cholesterol-enriched lysosomes. This led deDuve and Peters (1, 2) to suggest that atherosclerosis may be a lysosomal storage disorder. Recently, Du and colleagues (3) showed that introduction of lysosomal acid lipase, the enzyme responsible for the hydrolysis of cholesteryl esters (CEs), into mice could significantly reduce atherosclerosis. This highlights even further that lysosomes are important in atherogenesis.

We have modeled lysosomal cholesterol accumulation using macrophages in culture and have shown that the lysosomal accumulation can be mimicked by treating human macrophages with mildly oxidized low density lipoprotein (OxLDL) as well as with particles that lack oxidized lipids, such as small aggregated low density lipoprotein (AggLDL) or cholesteryl ester-rich lipid dispersions (DISPs) (4–6). Importantly, lysosomal hydrolysis of the CE in the particles initially proceeds at a normal rate, but egression of the resultant free cholesterol (FC) from the lysosome is inhibited. This produces an increase in the lysosomal FC concentration. However, as time goes by, there is a dramatic inhibition of lysosomal CE hydrolysis, resulting in a lysosomal accumulation of CE (7). This shift usually occurs after ~48 h of incubation with mildly OxLDL or AggLDL and slightly faster with DISP (6). The lysosomal cholesterol accumulation and subsequent decrease in lysosomal CE hydrolysis are not observed in acetylated low density lipoprotein (AcLDL)-treated cells, even when they accumulate equivalent amounts of cholesterol (8). With AcLDL, CE accumulates within cytoplasmic droplets (7). With regard to OxLDL, the difference between OxLDL and AcLDL that leads to different metabolic fates

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Abbreviations: AcLDL, acetylated low density lipoprotein; AggLDL, aggregated low density lipoprotein; apoB, apolipoprotein B; CD, methyl-β-cyclodextrin; CE, cholesteryl ester; DISP, cholesteryl ester-rich lipid dispersion; FC, free cholesterol; LAMP-1, lysosome-associated membrane protein-1; OxLDL, oxidized low density lipoprotein; TBARS, thiobarbituric acid-reactive substances; v-ATPase, vacuolar $H^+$-ATPase.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of four figures.

is not clear, but the differences is not in the delivery of cholesterol because particle uptake and delivery to lysosomes are equivalent for mildly OxLDL and AcLDL (9).

In this report, we investigate one possible mechanism by which lysosomal cholesterol accumulation could influence lysosomal hydrolysis of CE by lysosomal acid lipase and show that long-term incubation of cells with mildly OxLDL, AggLDL, and DISP inhibits the lysosome's ability to maintain an active pH. The timing of the pH increase correlates with the inhibition of CE hydrolysis and a reduction in lysosomal proteolysis, indicated by lysosomal accumulation of apolipoprotein B (apoB) and apoB fragments. The reduction in lysosomal activity is not attributable to a reduction in cellular vacuolar $H^+$-ATPase (v-ATPase) levels and, thus, appears to indicate a more direct effect on lysosome function. Inhibition of FC egression from lysosomes by progesterone treatment and augmentation of isolated lysosomes with FC using cyclodextrins inhibited lysosome acidification, suggesting that increases in lysosomal unesterified cholesterol may mediate the inactivation of lysosomes. Modulation of lysosome activity by FC concentration could have profound influences on certain stages of atherosclerotic lesion progression.

## EXPERIMENTAL PROCEDURES

### Materials

THP-1 monocytes were obtained from the American Type Culture Collection (Manassas, VA). Phospholipids for the production of lipid dispersions were obtained from Avanti Polar Lipids (Birmingham, AL). All other chemicals were obtained from Sigma (St. Louis, MO). Tissue culture supplies were obtained through VWR International (St. Louis, MO).

### Cell culture

THP-1 monocytes were cultured and differentiated to macrophages as described previously (9). Briefly, monocytes were plated at a density of $0.75 \times 10^6$ cells/ml. After differentiation into macrophages with phorbol 12-myristate 13-acetate (50 μg/ml) for 3 days, cells were cleared of residual triglyceride by incubation with 4 mg/ml fatty acid-free BSA in medium containing 1% FBS. Cells were loaded with modified lipoproteins or DISP at concentrations of 75–100 μg/ml in medium containing 1% FBS. Cholesterol loading of cells was confirmed by extracting cellular lipids into isopropanol and quantifying cholesterol and CE content by gas-liquid chromatography, as described previously (6). We tested for loss of cells during culture by daily microscopic analysis of cell numbers and by protein levels per dish. In the time frame of the experiments, significant cell or protein loss was not observed.

### LDL isolation and modification

LDL was isolated from plasma of healthy, fasted, normolipidemic subjects using a standard NaCl density isolation reported previously (10). Blood was obtained using procedures approved by the Human Subjects Institutional Review Board. LDL was taken at a density of $1.006 < d < 1.063$. After isolation, the LDL was dialyzed extensively against 0.9% NaCl with 0.3 mM EDTA for 72 h (three changes), stored under nitrogen, and maintained at 4°C. Protein was determined by the method of Lowry et al. (11). LDL acetylation was carried out using a standard acetic anhydride-sodium acetate acetylation on ice (12). To oxidize LDL, EDTA was first removed by dialysis in 0.9% NaCl for 36 h (three changes) at 4°C, and then the LDL was dialyzed for an additional 1 h at room temperature in 1 liter of 0.9% NaCl. After this, 1 ml of 20 mM $CuSO_4$ was added (final $Cu^{2+}$ concentration, 10 μM) and allowed to equilibrate without stirring for the first hour and dialyzed for an additional 3–5 h with stirring. Oxidation was terminated by placing the LDL in a solution of 0.9% NaCl with 1 mg/ml EDTA (pH 7.4). After 1 h at room temperature, the solution was placed at 4°C for 12 h. EDTA was equilibrated to 0.3 mM by dialysis in 0.9% NaCl and 0.3 mM EDTA for 36 h with three changes. Only mildly OxLDL was used, as determined by thiobarbituric acid-reactive substances (TBARS) between 5 and 10 ng/mol and minimal conjugated diene formation (13). LDL aggregation was performed by first vortexing the LDL, under nitrogen, for 1 min or until the LDL became cloudy. The vortexed LDL was then placed on ice and sonicated, using a probe-type sonicator (Branson Digital Sonicator, Danbury, CT) at 50% power for 20 min using 10 s pulses. The aggregates were then passed through a 0.45 μm filter to remove any remaining large or chain aggregates. The small size of the AggLDL was confirmed by negative stain transmission electron microscopy. Particle size ranged from 40 to 250 nm. All modified LDL was analyzed further for uniformity by agarose gel electrophoresis and checked for degree of oxidation by TBARS and conjugated dienes as described (13). AggLDL and AcLDL were used only if no oxidation was detected.

### Lipid dispersion production and experimentation

DISPs were produced as described previously (14) by mixing phosphatidyl choline, phosphatidyl serine, and cholesteryl oleate (1:0.1:30 by weight) in RPMI 1640 medium. The resulting mixture was sonicated for 20 min at 50% amplitude, and the size and uniformity of the DISPs were determined by negative stain transmission electron microscopy. We confirmed that the DISPs were not oxidized by measuring TBARS and conjugated diene formation.

### Cellular staining

The locations of neutral lipid and FC accumulation in cells were determined by staining with Nile Red (15) and filipin (16), respectively. Anti-human lysosome-associated membrane protein-1 (LAMP-1) antibody (BD Biosciences, San Jose, CA) staining was used as a marker for lysosomes/late endosomes. The method for immunofluorescent LAMP-1 staining has been described previously (6). Controls included replacing the primary antibody with preimmune serum and leaving the primary antibody out of the protocol. LysoSensor Yellow/Blue DND-160 staining (Molecular Probes, Eugene, OR) was used to determine changes in lysosomal pH (17, 18). Cells were washed two times in PBS, and the dye was added to cells at a concentration of 5 μM in medium containing 1% FBS. All images were collected within 10 min after the placement of dye on the cells to avoid artifacts produced by the alkaline properties of the dye. As a positive control, we stained macrophages in which the number of active lysosomes/late endosomes was increased by incubation with polystyrene beads rather than lipoprotein. For simplicity, in the following descriptions, we use the generic term "lysosome" to refer to LAMP-1-positive hydrolytic compartments (lysosomes/late endosomes), because both appear to be equally affected.

### Microscopy

A Zeiss Axioplan Imaging E fluorescence/bright-field microscope was used for all light microscopy. Images were collected using a Photometrics Coolsnap HQ digital camera with a cooled charge-coupled device chip (Roper Scientific, Tucson, AZ). Image analysis was conducted using MetaMorph imaging software

(Universal Imaging, Downingtown, PA). To determine changes in the number of active lysosomes, a grid was placed on each image as an unbiased selector of cells to evaluate. The number of vesicles with pH < 4.8 (active lysosomes) and pH > 4.8 (inactive lysosomes) was counted based on intensity of the blue and green channels compared with known pH standards. This pH value was chosen because the lysosomal acid lipase should have no activity above pH 4.8, and this value is greater than the $Pk_a$ of LysoSensor, such that there is a significant blue shift in the fluorescence of the probe. Vesicles in at least 20 cells per condition from three experiments were counted. The data from the different experiments were combined to obtain means and SEM. To test for significance, ANOVA with Tukey's posttest was performed using GraphPad Prism software (San Diego, CA). To quantify the percentage of vesicles in macrophages that were lysosomes/late endosomes, we used Nomarski differential interference microscopy to identify vesicles. A grid of points overlaid on the image was used to arbitrarily select vesicles for analysis, and fluorescence microscopy was used to determine whether the selected vesicles were LAMP-1-positive. One hundred to 200 vesicles were assayed for each condition.

### Western blotting

After incubation with loading vehicle for the indicated times, cells were washed three times in PBS. Cells were lysed in 2× loading buffer (0.1 M DTT, 20% glycerol, 0.1 M Tris, pH 6.8, and 1% SDS), loaded on a 4–12% gradient Bis-Tris SDS-PAGE gel (Invitrogen, Carlsbad, CA), run for 15 min at 100 V and for 90 min at 120 V, and transferred to nitrocellulose for 90 min at 25 V. Blots were stained using primary antibodies to apoB (dilution, 1:20,000) (a gift from Dr. Larry Swift, Vanderbilt University), v-ATPase H-subunit (1:1,000) (Santa Cruz), or β-actin (1:5,000) (Sigma). Secondary HRP-conjugated antibodies were obtained from Promega (Madison, WI) and detected using ECL (Perkin-Elmer Life Sciences, Boston, MA) and Kodak Biomax film.

### Lysosomal isolation and modification

THP-1 macrophages were incubated with MyOne magnetic beads (Invitrogen) for 72 h at a concentration of 0.03 mg/ml. After 72 h, the cells were rinsed, and medium containing 1% FBS was added for an additional 72 h to allow for bead incorporation into lysosomes. The cells were then rinsed two times in cold STE buffer (0.25 M sucrose, 0.01 M Tris-HCl, 1 mM EDTA, and 0.1% ethanol). The cells were scraped into 1 ml/dish of STE buffer containing protease inhibitors (Sigma), and six 100 mm dishes were used for each condition. The cell suspension was then placed in a cell disruption chamber (Kontes) and disrupted using three passes of 20 min each at 150 p.s.i. This method consistently resulted in disruption of >95% of cells but left lysosomes intact. The homogenate was then placed in the presence of a magnet to isolate a pure population of intact lysosomes. Western blotting of both the lysosome isolate and the supernatant for LAMP-1 was conducted to assess purity and yield by comparison with whole cell extracts. The isolated lysosomes were resuspended in buffer containing 150 mM KCl to generate high $K^+$ levels inside the lysosome, providing a membrane potential during stimulation of the v-ATPase with ATP. To enrich the lysosomes with unesterified cholesterol, isolated lysosomes were incubated with varied concentrations of methyl-β-cyclodextrins (CDs), which were enriched with FC (19). CDs have been used extensively as a means of modulating membrane cholesterol levels (20–22). After a 15 min incubation, the lysosomes were reisolated and washed in buffer to remove the CDs. Aliquots were taken for cholesterol (via gas-liquid chromatography) and protein (via the Lowry method) analysis to confirm the increase in lysosomal unesterified cholesterol. As controls, lysosomes were incubated with α-CD, which does not mobilize FC, and then subjected to the activation protocol.

### Lysosomal activation

Lysosomal activation was carried out using a modification of a procedure described previously (23), and proton pumping was measured as the quenching of acridine orange fluorescence as hydrogen ions are pumped into the lysosomal lumen. Briefly, the Tris-HCl activation buffer was replaced with one containing HEPES. The isolated lysosomes were placed in a cuvette containing activation buffer and 6.7 μM acridine orange. A steady baseline was obtained, and then the v-ATPases were primed with $MgCl_2$. As soon as the baseline was reestablished, the v-ATPases were activated by the addition of ATP (1.4 μM final concentration) and valinomycin (to promote the movement of $K^+$ from inside to out for membrane potential generation). v-ATPase-driven pumping of hydrogen ions into the lysosome lumen was determined as the quenching of acridine orange fluorescence when excited at 495 nm and recorded at 530 nm using an SLM Aminco 8100 dual-wavelength spectrophotometer. As controls, beads that had not been incubated with cells were subjected to activation, and lysosomes were activated with ATP in the absence of valinomycin.

## RESULTS

We recently demonstrated that mildly OxLDL, AggLDL, and DISP appear to produce a general inhibition of CE hydrolysis (6). One alteration that could explain these data would be an unfavorable change in the lysosome/late endosome environment. Therefore, we sought to determine whether there was a change in lysosomal pH as cellular and/or lysosomal cholesterol levels increased. We used LysoSensor Yellow/Blue DND-160, which fluoresces yellow in an active lysosomal environment and has a significant blue shift as pH approaches neutrality (17, 18). We classified intracellular vesicles as active if they had pH < 4.8 and inactive if they had pH > 4.8. This pH was chosen because it is greater than that usually associated with lysosomes and well above the narrow pH range of human lysosomal acid lipase. Lysosomal acid lipase has a narrow pH range, with a peak at pH 3.8–4.0 and little activity above pH 4.5 (24). In untreated cells, the majority of vesicles in macrophages exhibited an active pH. Cells treated with mildly OxLDL for 24 h also still contained predominantly active lysosomes (**Fig. 1A**). However, after 7 days of mildly OxLDL treatment (and cellular cholesterol levels increasing to >150 μg/mg cell protein), the majority of vesicles in the cells had pH > 4.8 (Fig. 1B). To determine whether the lysosomal neutralization was dependent upon oxidized lipids, we investigated the effect of cholesterol loading from AggLDL or DISP, both of which lacked oxidized lipids, on lysosomal pH. Cells loaded with small AggLDL (Fig. 1C) and DISP (Fig. 1D) (both of which reach cholesterol levels in excess of 250 μg/mg cell protein) showed similar uptake-dependent increases in lysosomal pH, such that the majority of vesicles had pH > 4.8 by day 7. The lysosomal neutralization did not occur in cells treated with AcLDL (Fig. 1E), even though these cells also obtained

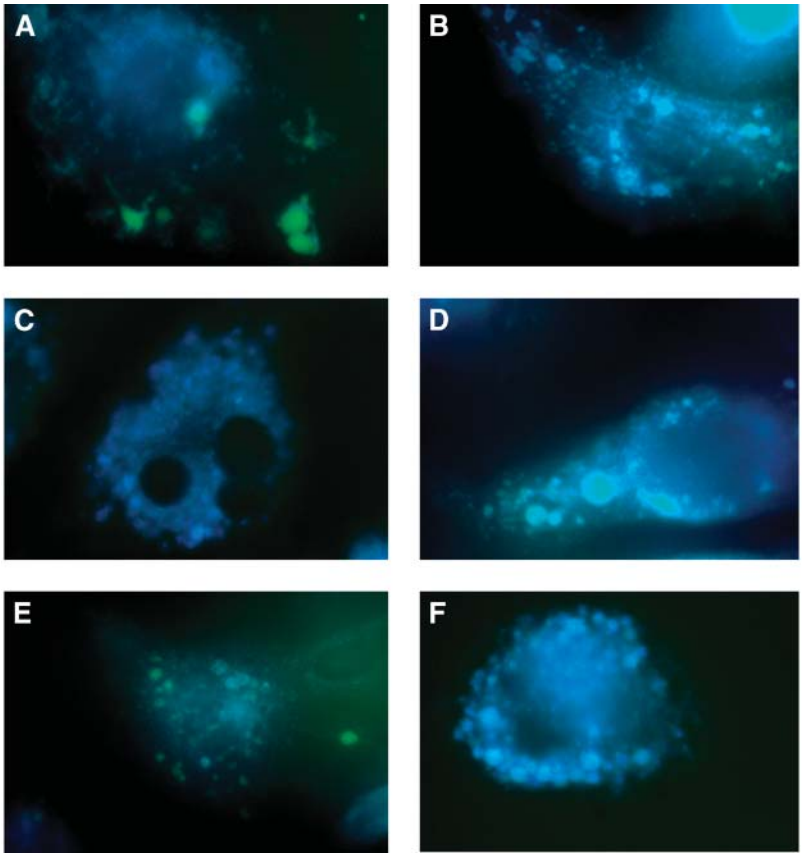


**Fig. 1.** LysoSensor Yellow/Blue DND-160 staining of macrophages. A: After 24 h of treatment with 75 μg/ml mildly oxidized low density lipoprotein (OxLDL), lysosomes remained active (pH < 4.8), as indicated by the yellow fluorescence pattern. B: After 7 days of incubation with mildly OxLDL, most lysosomes showed a predominantly blue fluorescence pattern, indicating an increase in pH to near neutrality. C: Similar lysosome pH increases were observed in THP-1 macrophages incubated for 7 days with 75 μg/ml aggregated low density lipoprotein (AggLDL). D: Treatment of macrophages for 7 days with 60 μg/ml cholesteryl ester-rich lipid dispersion (DISP) also resulted in an increase in lysosome pH. E: The increase in pH was not observed in cells treated with 75 μg/ml acetylated low density lipoprotein (AcLDL) for 7 days. F: To verify our ability to detect lysosomal neutralization in the presence of AcLDL, macrophages were incubated with 75 μg/ml AcLDL for 24 h in the presence of 50 μM chloroquine, a lysomotrophic agent that increases lysosomal pH. Magnification = 500×.

total cellular cholesterol levels >150 μg/mg cell protein. The major difference between AcLDL-treated cells and those treated with other lipid particles was that AcLDL-treated cells accumulated cholesterol in cytoplasmic CE droplets and lacked lysosomal cholesterol (FC and CE) accumulation. This observation concurs with our previous studies showing that cholesterol does not accumulate in lysosomes when AcLDL is the loading vehicle; rather, cholesterol is stored as cytoplasmic CE droplets (7). To verify that our methods could detect an increase in pH in the presence of AcLDL, we incubated cells in the presence of AcLDL and 50 μM chloroquine, a lysomotrophic agent that increases lysosomal pH. These cells demonstrated large lysosomes, indicative of chloroquine inhibition of lysosome function, and the LysoSensor Yellow/Blue fluorescence indicated a pH > 4.8 (Fig. 1F), confirming that AcLDL does not interfere with our ability to detect increases in lysosome pH.

To expand our analysis, we quantified changes in the number of active or inactive vesicles in cells with the various treatments over time. Within the initial 24 h, lipoprotein treatment did not change the number of active lysosomes compared with controls, and >85% of the intracellular vesicles showed pH < 4.8 (**Fig. 2**). After 72 h of treatment, however, there was a blue shift in fluorescence such that, depending upon the treatment, only 45–60% of the vesicles now showed an active pH. The number of inactive lysosomes continued to increase with time, and after 7 days of treatment with mildly OxLDL, DISP, or AggLDL the majority (>85%) of the vesicles were inactive (Fig. 2). Importantly, the timing of lysosomal neutralization coincided with the timing (previously reported) of the inhibition of CE hydrolysis (8).

From these data, it appears that most of the cholesterol-engorged lysosomes lost the ability to maintain an active pH. Control cells treated with polystyrene beads did not show a change in pH over time, indicating that time duration was not the cause of the increase in pH (see supplementary Fig. I). The shift in vesicle pH was also not attributable to the loss of lysosomes, as shown by positive staining for the lysosome/late endosome marker LAMP-1. For instance, in a representative experiment, 73% of vesicles in untreated cells showed positive staining for LAMP-1. This increased to 81% after 2 days of incubation with 75 μg/ml DISP and 84% at day 6. In a separate experiment, 64% of vesicles were LAMP-1-positive at day 0. This increased to 86% and 84%, respectively, with 2 and 6 days of incubation with 100 μg/ml DISP. A similar pattern was seen when macrophages were incubated with 75 μg/ml AggLDL. With AggLDL, 66% of vesicles were LAMP-1-positive at day 0, 72% of vesicles were positive after 2 days of incubation, and 90% were positive after 6 days. This indicates that the majority of the vesicles in macrophages, even untreated macrophages, represent lysosomes/late endosomes, and most of these showed increased pH in conjunction with lysosome FC and CE accumulation.

The DISP data suggest that cholesterol, phospholipids, or hydrolytic products of the two directly or indirectly

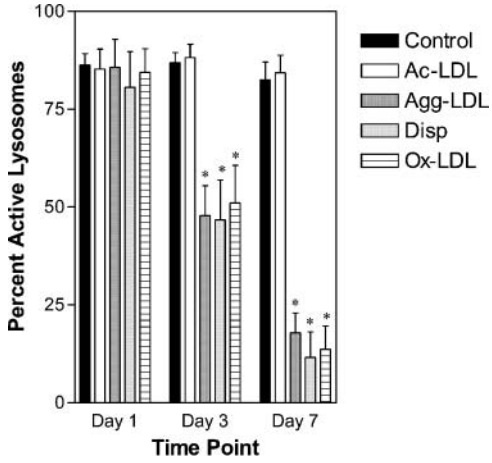


**Fig. 2.** Quantification of the percentage of vesicles showing an active lysosome pH (pH < 4.8) after treatment with various cholesteryl ester (CE) sources. After 1 day of treatment with mildly OxLDL (75 μg/ml), AggLDL (75 μg/ml), or DISP (60 μg/ml), most vesicles exhibited an active pH. However, after 3 days of loading with each type of particle, there was a decrease in the number of active lysosomes. This trend continued such that <25% of the lysosomes exhibited an active pH after 7 days. However, untreated (control) macrophages and macrophages treated with 75 μg/ml AcLDL, which had similar total cholesterol levels to the OxLDL-treated cells, did not undergo changes in the number of active lysosomes over the 7 day period. Values shown are means ± SEM. Asterisks indicate means that were statistically different ($P < 0.001$) from those of untreated control cells.

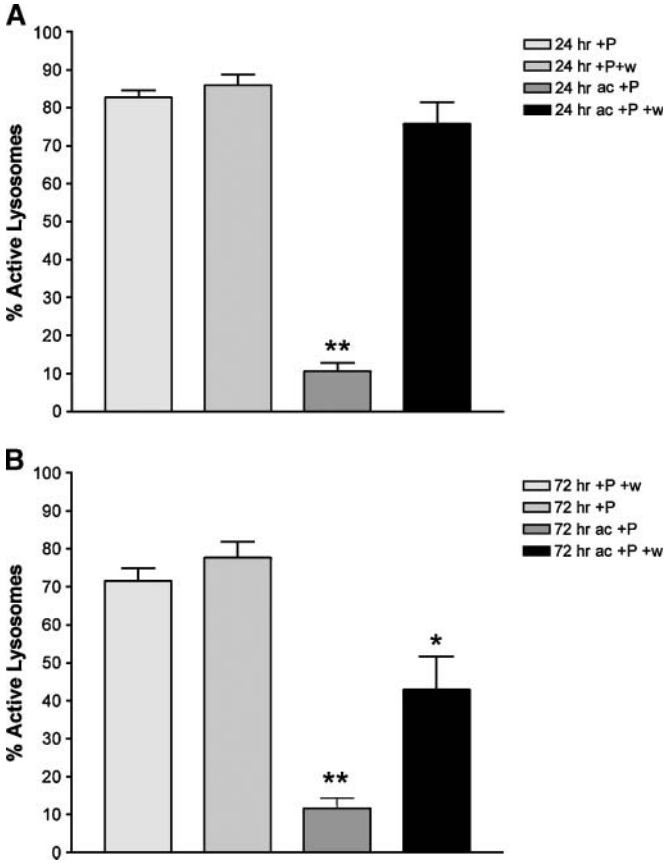


**Fig. 3.** Effects of AcLDL and progesterone treatment on the percentage of vesicles having an active lysosome pH (pH < 4.8). Values shown are means ± SEM. A: THP-1 macrophages were treated for 24 h in medium with 10 μg/ml progesterone (+P) alone or in conjunction with 100 μg/ml AcLDL (ac+P). Most lysosomes in cells treated with progesterone alone had an active pH (pH < 4.8). The progesterone-alone cells were similar to control cells receiving no treatment and to cells treated with AcLDL alone (see Fig. 2). Progesterone treatment followed by 24 h of incubation in medium alone (P+w) had little effect on the number of active lysosomes. However, treatment of cells with AcLDL (as a source of CE) in the presence of progesterone significantly reduced the number of active lysosomes (** $P < 0.001$). This effect was reversed by removing the progesterone block through incubation for 24 h in medium alone (ac+P+w). B: Similar responses were seen when cells were treated for 72 h with progesterone alone (+P), progesterone plus washout (P+w), or AcLDL in the presence of progesterone (ac+P), with a significant reduction in the number of active lysosomes seen only when AcLDL provided a source of cholesterol (** $P < 0.001$). However, unlike the 24 h incubations, macrophages that were incubated in medium alone for an additional 72 h after AcLDL and progesterone treatment (ac+P+w) still had a significant reduction in the number of active lysosomes compared with control cells (* $P < 0.05$).

mediated the inhibition of lysosomal pH, because these are the only components in the DISP. To test whether unesterified cholesterol was a mediating factor, we treated cells with 100 μg/ml AcLDL in the presence of 10 μg/ml progesterone. This treatment has been shown to produce FC accumulation in the lysosomes of AcLDL-treated cells by inhibiting the trafficking of unesterified cholesterol out of the lysosomes (25–27). Incubation with AcLDL in the presence of progesterone for 24 h produced an increase in lysosomal pH > 4.8 in almost all (>90%) lysosomes (**Fig. 3A**, ac+P, and supplementary Fig. IIA). However, the lysosomes of these cells rapidly regained the ability to acidify within 24 h of removing the progesterone block by washing (Fig. 3A, ac+P+w, and supplementary Fig. IIC). It is important to note that neither AcLDL alone (Fig. 2) nor progesterone treatment alone (Fig. 3, P) resulted in a significant inactivation of lysosomes, nor did a 24 h wash affect lysosomes of cells treated with progesterone alone (Fig. 3, P+w). Because the inhibition of acidification with short-term progesterone treatment was reversible, we sought to determine whether longer treatment with progesterone would lead to an irreversible change in lysosomes. Cells that had been incubated with AcLDL in the presence of progesterone for 72 h also contained primarily inactive lysosomes (>90%) (Fig. 3B, ac+P, and supplementary Fig. IIB). After removal of the progesterone block, some of the lysosomes were able to reestablish an active pH, but the majority (>60%) retained an inactive pH (Fig. 3B, ac+P+w, and supplementary Fig. IID).

To confirm that progesterone treatment led to an accumulation of FC in lysosomes, we used filipin staining to identify unesterified cholesterol in cells. AcLDL alone did not produce FC accumulation in vesicles. However, macrophages that were loaded with AcLDL in the presence of progesterone for 24 h had numerous intracellular sites of FC accumulation (**Fig. 4A**). These were of a size, number, and location consistent with our previous studies

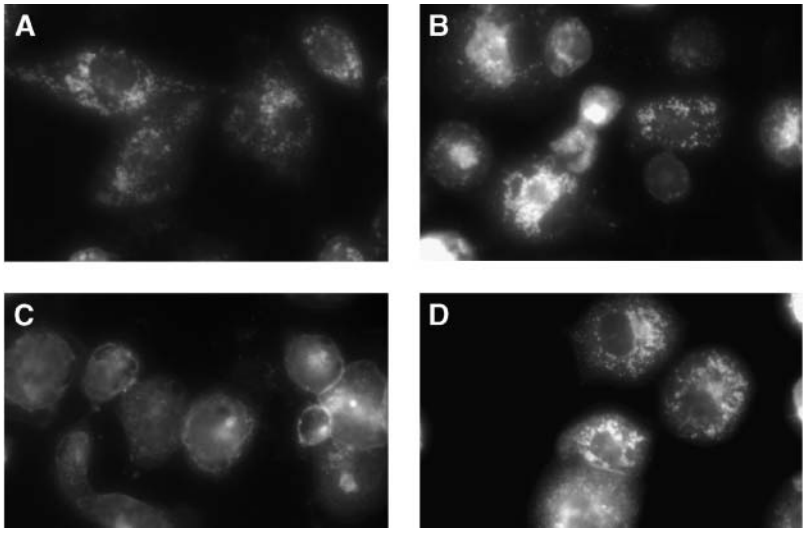


**Fig. 4.** Filipin staining for free cholesterol (FC) in macrophages treated with progesterone and AcLDL. A, B: Cells treated with 100 μg/ml AcLDL and 10 μg/ml progesterone for 24 h (A) and 72 h (B) had many FC-enriched vesicles with a size and distribution pattern consistent with macrophage lysosomes. C: However, removal of the progesterone block after 24 h of AcLDL/progesterone treatment resulted in an almost complete reduction in intracellular FC stores after 24 h. D: In contrast, intracellular accumulations of unesterified cholesterol were not completely removed when cells treated with AcLDL and progesterone for 72 h were allowed even a 72 h washout period in medium alone. Magnification = 300×.

demonstrating FC accumulation in lysosomes (5, 6). The filipin studies also demonstrated a nearly complete loss of intracellular FC stores after a 24 h chase in medium alone (Fig. 4C). Cells that were treated for 72 h with AcLDL and progesterone accumulated even greater amounts of intracellular cholesterol (Fig. 4B). In contrast to cells incubated for 24 h, cells incubated with AcLDL and progesterone for 72 h retained much of the FC within intracellular stores even after the block was removed (Fig. 4D). Untreated cells showed little intracellular filipin staining; rather, the staining was localized primarily to the plasma membrane (data not shown). Together, our data suggest that lysosomal unesterified cholesterol accumulation plays a role in the inhibition of lysosomal acidification.

Lysosomes are acidified by v-ATPases, which pump protons into the lysosome lumen. One possible reason for the loss of lysosomal activity could be that there is a decrease in the levels of the v-ATPase. The v-ATPase is a multiprotein complex with a transmembrane domain, $v_0$, and a cytosolic domain, $v_1$. It has been demonstrated that subunit H of the $v_1$ domain, a protein in the stalk region, is required for activation of the complex (23). We used Western blotting of cell lysates to examine cellular levels of subunit H. We observed no difference in levels of subunit H between controls (untreated or bead-treated) and all of our lipid treatments (**Fig. 5**). This finding suggests that inactivation of lysosomes is not a function of the reduction in v-ATPase levels.

The inactivation of lysosomes correlated temporally with the inhibition of CE hydrolysis (6, 7) and the accumulation of lysosomal CE, suggesting a cause-and-effect relationship. Failure to maintain an active pH should also inhibit the lysosomal hydrolysis of other molecules as well. To test this notion, we determined whether degradation of the apoB component of mildly OxLDL and AggLDL particles was also inhibited (**Fig. 6**). Cells incubated with AcLDL for up to 6 days did not accumulate immunostainable apoB. The lack of apoB staining was not attributable to a lack of uptake of AcLDL, because cells treated with both AcLDL and OxLDL had equivalent cholesterol levels. This indicates that the AcLDL was taken up by the macrophages and delivered to lysosomes, where the lipoprotein was actively degraded within the cells. This is consistent with previous studies from our laboratory and others (7, 9, 12). The lack of staining was also not attributable to a lack of immunoreactivity of apoB after acetylation, because apoB from AcLDL was readily detectable by our antibody before incubation of the AcLDL with cells (see supplementary Fig. III). On the other hand, macrophages incubated with mildly OxLDL or AggLDL showed a time-dependent accumulation of immunostainable apoB and apoB fragments, with AggLDL producing a greater intracellular accumulation than OxLDL (Fig. 6).

A role for lysosome FC accumulation in mediating lysosome pH increases is suggested by the fact that lysosomal FC accumulation precedes lysosome inactivation and that

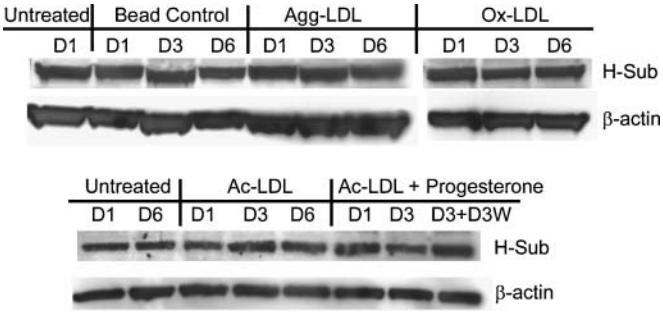


**Fig. 5.** Western blot of the H-subunit (H-Sub) of the vacuolar $H^+$-ATPase (v-ATPase) in cells loaded with AggLDL or OxLDL showed no difference over time in the H-subunit concentration compared with untreated cells. Likewise, AcLDL loading or AcLDL loading in the presence of progesterone did not alter H-subunit levels. Ten micrograms of protein was loaded in each lane. Incubation conditions were as described in Experimental Procedures. Cells loaded with inert beads acted as a control for lysosome stimulation. Some macrophages incubated with AcLDL and progesterone for 3 days were allowed a further 3 day washout before analysis (D3+D3W). D1 = day 1, D3 = day 3, and D6 = day 6. β-Actin was used as a loading control.

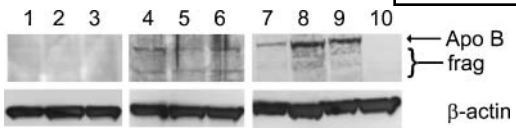


**Fig. 6.** Apolipoprotein B (apoB) accumulation in THP-1 macrophages. THP-1 macrophages incubated with mildly OxLDL (75 μg/ml) for 1 day (lane 4), 3 days (lane 5), or 6 days (lane 6) or AggLDL (75 μg/ml) for 1 day (lane 7), 3 days (lane 8), or 6 days (lane 9) and then blotted for the presence of apoB showed progressive accumulation of immunoreactive apoB [apoB and fragments (frag)]. In contrast, THP-1 macrophages incubated with AcLDL (75 μg/ml) for 1 day (lane 1), 3 days (lane 2), or 6 days (lane 3) showed no increase in apoB staining over control (lane 10) levels, indicating that apoB present in AcLDL was efficiently degraded. β-Actin was used as a loading control.

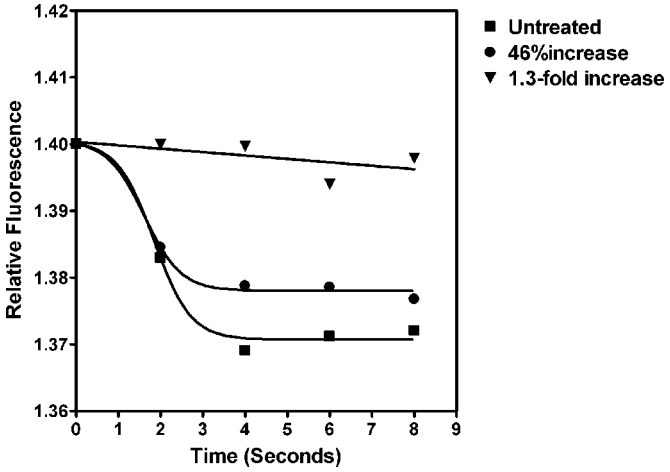


**Fig. 7.** Quenching of acridine orange fluorescence in isolated lysosomes before or after cholesterol enrichment. Once stimulated by the addition of ATP and valinomycin (time 0), untreated lysosomes (squares) exhibited a rapid quenching of acridine orange fluorescence, as indicated by the decrease in the relative fluorescence intensity. Acridine orange quenching is the result of the activation of v-ATPase and the pumping of hydrogen ions into the lysosomal lumen. Lysosomes with a 46% increase in membrane unesterified cholesterol (circles) exhibited a reduction in overall quenching of acridine orange. Larger increases in cholesterol levels to >1.3-fold (inverted triangles) almost complete inhibited v-ATPase activity.

the inhibition of AcLDL-derived FC egression from lysosomes is accompanied by lysosome inactivation. These experiments, however, do not differentiate between FC accumulation in the lysosome lumen versus possible augmentation of the lysosome membrane FC. To assess what role increased lysosome membrane FC may play in lysosome inactivation, lysosomes were isolated from non-cholesterol-enriched cells and the activity of the v-ATPase was assessed by monitoring the quenching of acridine orange when the lysosomal v-ATPases were stimulated with ATP (23, 28). In a representative experiment, control lysosomes (1.50 μg cholesterol/mg lysosome protein) exhibited rapid quenching of the acridine orange, showing that the v-ATPase in non-cholesterol-enriched lysosomes was pumping protons into the lysosomal lumen (**Fig. 7**). When the lysosomal membrane cholesterol was increased by 46% (2.19 μg cholesterol/mg lysosome protein) by incubating the isolated lysosomes with FC-containing CD, there was a reduction in the ability of lysosome membranes to quench acridine orange in the lysosome lumen. Further increases in cholesterol to 1.3 times (3.46 μg cholesterol/mg lysosome protein) that seen in controls almost completely inhibited acridine orange quenching. Each cholesterol augmentation experiment was repeated at least three times with similar results each time.

Just as FC-enriched CD can transfer cholesterol to membranes, CD lacking cholesterol can remove cholesterol from membranes (20). When we increased lysosomal membrane FC in isolated lysosomes to inhibitory levels (3.5-fold increase) using FC-containing CD and then reduced it by incubation for 15 min with 1 mg/ml FC-poor CD to a 1.5-fold increase (3.21 μg cholesterol/mg lysosome protein), we regained some v-ATPase activity. The level of activity seen with this final 1.5-fold cholesterol increase over control levels was similar to that seen in lysosomes in which we obtained a modest 1.3-fold increase in lysosome membrane cholesterol (Fig. 7) through brief incubation with FC-enriched CD.

As a control for cyclodextrin treatment, isolated lysosomes were treated with α-cyclodextrin, which does not transfer cholesterol. This did not inhibit the quenching of acridine orange fluorescence (see supplementary Fig. IV).

Further controls included incubating isolated lysosomes with ATP in the absence of valinomycin and testing whether magnetic beads alone (without v-ATPase-containing membranes) in the presence of activation buffer could affect acridine orange fluorescence (see supplementary Fig. IV). None of these controls produced a quenching of acridine orange fluorescence, confirming that inhibition of quenching was related to the inhibition of v-ATPase activity.

## DISCUSSION

Lysosomal lipid accumulation is a ubiquitous but underexplored feature of macrophage foam cells in atherosclerotic lesions (29). It is probably most prevalent at later stages of lesion development rather than in the early, fatty streak development (4). Previously, we showed that cells treated in culture with mildly OxLDL, AggLDL, or DISPs exhibit significant lysosomal free and esterified cholesterol accumulation similar to that seen in lesions (6, 7). There is ample evidence that OxLDL, AggLDL, and CE-containing lipid particles are present in atherosclerotic lesions and are internalized by macrophages and thus deliver sterol to lysosomes (30–34). However, despite the variety of loading vehicles present in the artery wall, foam cells in advanced lesions have remarkably similar phenotypes, including substantial free and esterified cholesterol accumulation in lysosomes (29). One explanation for this would be that there is a key component common to all of these atherogenic particles that produce a similar defect in the lysosomes of most foam cells. Our previous studies

indicate that, in tissue culture, CE accumulation was, in part, the result of the failure of lysosomal lipoprotein CE hydrolysis (6, 8). However, both FC and CE accumulated in lysosomes, so differentiating the effects of lysosomal CE and FC on hydrolysis was not possible in those studies. In the current study, using progesterone to specifically inhibit FC egression from lysosomes, we show that lysosomal FC accumulation alone has the potential to inhibit lysosome function by inhibiting the ability of lysosomes to maintain the correct pH. Initial accumulation of FC is an event common to all three loading vehicles and thus a strong candidate for the common mediator of the subsequent lysosomal CE accumulation.

The ability of progesterone to inhibit unesterified cholesterol traffic out of lysosomes is a major and well-characterized effect of progesterone treatment (25). Although the largest effect of progesterone on macrophages is inhibition of cholesterol movement (25), progesterone has other effects on macrophages, including stimulating the production of growth factors and increases in the synthesis of cholesterol and sterol precursors (35, 36). However, these additional effects would be present in cells treated with progesterone alone; thus, they cannot be the explanation for the inhibition of lysosomal acidification. Moreover, treatment of cells with AcLDL in the presence of 2 μg/ml U18666A (3β-[2-(diethylamino)ethoxy]androst-5-en-17-one), which also modulates the trafficking of cholesterol out of lysosomes, equally inhibits lysosomal CE hydrolysis (unpublished observation).

A decline in lysosome or cell viability is also not the explanation for the progesterone effect, because AcLDL plus progesterone treatment did not change cell viability and the lysosomes of cells treated for 24 h could regain activity after the progesterone block was removed. Thus, we conclude that the lysosomal unesterified cholesterol accumulation produced by our modified particles, or by progesterone treatment in the presence of AcLDL, is the most likely candidate for the decreased ability of lysosomes to maintain a pH level conducive to lysosomal lipase activity.

Not all vesicles in macrophages are lysosomes, although in active macrophages a significant proportion of intracellular vesicles are lysosomes (37). Thus, not all of the vesicles with pH $>$ 4.8 are inactive lysosomes. There is no direct way to determine the pH only of lysosomes, because it is not possible to stain cells for both lysosomal marker proteins, such as LAMP-1, and pH-sensitive dyes. However, in these studies, we have shown that a majority of macrophage vesicles were lysosomes/late endosomes and that treatment with AggLDL or DISP increased the percentage of total vesicles in the cell that represent lysosomes. In contrast, with cholesterol loading the percentage of vesicles with pH $<$ 4.8 declined dramatically. In addition, we have shown previously that the majority of the FC accumulation in macrophages incubated with AggLDL or DISP occurs in LAMP-1-positive vesicles (6). Together, these data indicate that the majority of vesicles with pH $>$ 4.8 in treated cells are inactive lysosomes/late endosomes and that these inactive lysosomes have significant FC accumulation.

Our results are in contrast to a report indicating that fibroblasts from patients with Neimann-Pick type C (NPC) disease do not have increased lysosomal pH (38). Mutations in the NPC-1 protein cause cholesterol to accumulate within lysosomes. It is not clear why the pH of these lysosomes remains acidic, but one possible explanation is that the unesterified cholesterol levels in these cells never reach sufficient concentration to inhibit proton pumping. In our studies, we found no decrease in active lysosomes after 24 h, despite clear evidence that unesterified cholesterol accumulates in lysosomes. It appears that the lysosomes begin to fail at maintaining an active pH only after sufficient FC accumulates. It would be interesting to determine whether challenging NPC-1 mutant cells with lipoprotein to further increase lysosomal cholesterol levels results in an increase in lysosomal pH. Similarly, it is not clear why the AcLDL plus progesterone treatment produced a more rapid increase in lysosome pH (1 day compared with ⩾2 days for particles), except that progesterone treatment, by severely inhibiting lysosomal loss of FC, would be expected to increase lysosomal FC very rapidly.

It is also not clear whether the FC effect on lysosomes is a direct or indirect one. However, using isolated lysosomes, we show that increased FC in the lysosome membrane inhibits v-ATPase. Thus, it is plausible that the FC accumulating in lysosomes partitions into the lysosome membrane and inhibits the v-ATPases. The acidic pH of lysosomes is maintained by these v-ATPases. They are multimeric proteins that pump $H^+$ ions into the lysosome using hydrolysis of ATP as the energy source. It is known that a number of lysosomal disorders are associated with the inhibition of v-ATPase (38, 39) and that the efficiency of the v-ATPase can be affected in some cell types by membrane cholesterol concentration (23, 28, 40). Here, we show that, in human macrophages, augmentation of lysosomal membrane FC concentration can inhibit lysosomal v-ATPases and produce an inactivation of lysosomes, and we demonstrate that lysosome inactivation is not attributable to a decrease in levels of the v-ATPase. Together, our observations suggest a direct effect of FC on lysosome acidification. We are currently pursuing studies to identify more details of how this inhibition occurs.

An alternative explanation for some of our data would be that increased lysosomal unesterified cholesterol could increase the leakiness of the lysosomal membrane. If protons were not retained within the lysosomes, pH would increase. However, several lines of evidence argue against this explanation. First, cholesterol increases order in membranes and makes them less permeable and has been shown to decrease lysosome leakiness (41). Moreover, increased lysosome leakiness produced by a number of different factors, including some oxidized sterols, is generally associated with apoptosis (42, 43). We did not see increased apoptosis in our cells. Finally, the time course of our studies could not account for the decrease in activity, because the acridine orange quenching is rapid and is the result of the protonation of acridine orange. If the v-ATPases were actively transporting the protons into the

lysosomal lumen, it is unlikely that they could leak out of the lysosomes before protonating the acridine orange, which retains the protons within the lysosome. Thus, leakiness would not appear to explain our results. However, we cannot rule out increased leakiness as an exacerbating factor.

The simplest explanation for our observations is that alteration in lysosomal pH accounts for the lack of CE hydrolysis we have seen in other studies and the lack of apoB degradation reported here. Although different lysosomal enzymes have different pH maxima, most require an acidic environment. The lysosomal acid lipase responsible for CE hydrolysis has a very narrow pH range, with almost no activity greater than pH 4.5 (44). Cholesterol accumulation from mildly OxLDL, AggLDL, and DISP increased the lysosomal pH to a point well above that at which lysosomal acid lipase would be active. Some lysosomal proteases have a wider pH range, with some activity greater than pH 4.8, which might explain why some apoB is only degraded to small fragments (45).

Of particular interest is our observation that short-term cholesterol accumulation was completely reversible but that longer term incubation, leading to higher lysosomal FC levels, was less reversible. One explanation is that those lysosomes able to reacidify represent a younger population of lysosomes with less unesterified cholesterol. However, once a high FC level is reached over time, the lysosome cannot recover. Further experimentation will be required to confirm this hypothesis. If lysosomes of macrophages in the atherosclerotic lesions were equally resistant to decreased foam cell cholesterol intake, they would be expected to maintain their lysosome-engorged phenotype and be resistant to regression diets. In a study of pigeon atherosclerosis, it was found that the cholesterol in foam cell lysosomes was resistant to regression (46).

Overall, our data are consistent with the hypothesis that cholesterol accumulation in lysosomes, particularly FC, contributes to the failure of lysosomes to maintain an active pH. Our experiments highlight the fact that cholesterol can modulate lysosome function and that accumulation of cholesterol within lysosomes can produce an acquired lysosomal insufficiency. Moreover, we show that several different types of particles, similar to those implicated in atherosclerosis, can bring about this insufficiency. Thus, our data also suggest a partial explanation for why the foam cells in advanced lesions have a similar lysosomal phenotype despite the presence of multiple sources of cholesterol. Finally, we show here for the first time that the cholesterol delivered to lysosomes as a component of modified lipoproteins has the potential to inhibit v-ATPase activity in human macrophages.

The long-term nature of the inhibition would suggest that once a foam cell's lysosomes are inhibited, they do not regain function. Because a macrophage can exist within a lesion for extended periods, the failure of acidification, and the subsequent trapping of cholesterol within this key organelle for macrophage function, suggest that this defect can influence the nature and progression of the atherosclerotic lesion.

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