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Lysosomal Cholesterol Accumulation Inhibits Subsequent Hydrolysis Of Lipoprotein Cholesteryl Ester

W. Gray Jerome^{1,2}, Brian E. Cox¹, Evelyn E. Griffin¹, and Jody C. Ullery¹

¹ Department of Pathology, Vanderbilt University School of Medicine, 1161 21st Ave, South, Nashville, TN 37232-2561

² Department of Cancer Biology, Vanderbilt University School of Medicine, 1161 21st Ave, South, Nashville, TN 37232-2561

Abstract

Human macrophages incubated for prolonged periods with mildly oxidized LDL (oxLDL) or cholesteryl ester-rich lipid dispersions (DISP) accumulate free and esterified cholesterol within large, swollen lysosomes similar to those in foam cells of atherosclerosis. The cholesteryl ester (CE) accumulation is, in part, the result of inhibition of lysosomal hydrolysis due to increased lysosomal pH mediated by excessive lysosomal free cholesterol (FC). To determine if the inhibition of hydrolysis was long lived and further define the extent of the lysosomal defect, we incubated THP-1 macrophages with oxLDL or DISP to produce lysosome sterol engorgement and then chased with acetylated LDL (acLDL). Unlike oxLDL or DISP, CE from acLDL normally is hydrolyzed rapidly. Three days of incubation with oxLDL or DISP produced an excess of CE in lipid-engorged lysosomes, indicative of inhibition. After prolonged oxLDL or DISP pretreatment, subsequent hydrolysis of acLDL CE was inhibited. Coincident with the inhibition, the lipid-engorged lysosomes failed to maintain an acidic pH during both the initial pretreatment and subsequent acLDL incubation. This indicates that the alterations in lysosomes were general, long-lived and affected subsequent lipoprotein metabolism. This same phenomenon, occurring within atherosclerotic foam cells, could significantly affect lesion progression.

Keywords

macrophage; atherosclerosis; foam cell; cholesterol; lysosome; lipoprotein metabolism

INTRODUCTION

Quantitative microscopic techniques provides an efficient means of correlating structural information with biochemical data. In this report, we employ a variety of microscopy approaches, including quantitative microscopy, to compare structural changes with biochemical measures of lipid accumulation and rates of critical reactions. This scheme provided an in depth assessment of the changes in lysosome function produced by sterol accumulation.

In humans and animal models of atherosclerosis, substantial amounts of lipid in atherosclerotic foam cells accumulate within large, lipid-swollen lysosomes (Jerome & Yancey, 2003). However, the defect(s) involved in lysosomal accumulation remain an understudied area. We

Address correspondence to: W. Gray Jerome, PhD, FAHA, Department of Pathology, Vanderbilt University School of Medicine, 1161 21st Ave, South, Nashville, TN 37232-2561, Phone: 615-322-5530, Fax: 615-343-7023, jay.jerome@vanderbilt.edu.

have modeled lysosomal cholesterol accumulation in macrophages by incubating cells in culture with mildly oxLDL, aggregated LDL, or CE-containing lipid dispersions (Griffin, et al., 2005; Jerome, et al., 1998). With these particles, lipid accumulation occurs in two stages. For the initial 1–2 days (FC phase), the accumulation is primarily as FC (Griffin, et al., 2005; Yancey & Jerome, 1998). This suggests that hydrolysis is functioning normally but there is a failure of efficient cholesterol export from lysosomes. After day 2, however, even though FC levels in lysosomes remain high, it is primarily CE that accumulates (CE Phase) because of inhibition of lysosomal CE hydrolysis (Griffin, et al., 2005; Yancey & Jerome, 1998; Yancey & Jerome, 2001). Recently, we showed that the inhibition of lysosomal CE hydrolysis during the CE Phase is, at least in part, due to a failure of sterol-engorged lysosomes to maintain a low pH (Cox, et al., 2007). The increased lysosomal pH is mediated by an inhibition of vacuolar type-ATPases (v-ATPase) in the lysosome membrane. This inhibition occurs after the accumulation of unesterified cholesterol accumulation during the FC phase and our studies indicate that this initial FC build-up is a mediator of the reduced v-ATPase activity (Cox, et al., 2007).

In contrast to the lysosomal accumulation produced by oxLDL, aggregated LDL (aggLDL) or DISP, macrophages incubated with acLDL show very little lysosomal sequestration; rather, the cholesterol in these cells is found within cytoplasmic CE droplets (Griffin, et al., 2005; Yancey & Jerome, 1998). The cytoplasmic CE droplets are the result of reesterification of FC liberated from the lysosomes (Yancey & Jerome, 1998). Thus, for reasons that are not clear, FC derived from acLDL does not accumulate within lysosomes and lysosome function is not inhibited (Brown, et al., 1979; Jerome, et al., 1998; Yancey & Jerome, 1998; Yancey & Jerome, 2001). The enzyme acyl coenzyme A:cholesterol acyltransferase 1 (ACAT-1) is responsible for the cytoplasmic reesterification of cytoplasmic FC to CE (Rudel, et al., 2001).

Recently we showed that if FC derived from lysosomal hydrolysis of acLDL is inhibited from exiting the lysosome by treatment of cells with progesterone, FC does accumulate within lysosomes and lysosomal CE hydrolysis is eventually blocked (Cox, et al., 2007). When lysosomal FC accumulation was allowed to continue for 3 days, the FC content of lysosomes remained high and CE hydrolysis continued to be blocked even after the progesterone inhibition was removed (Cox, et al., 2007).

Our data to date, then, suggests that excess sterol accumulation from atherogenic lipoproteins like oxLDL, aggLDL, or CE-containing lipid particles are capable of producing long-lived, if not permanent, disruption of lysosome function. This could have profound effects on the biology of foam cells as it suggests that any material internalized subsequent to inhibition would also fail to be processed in the lysosomes and thus could not participate in cellular metabolism, intracellular signaling, or be cleared from the cell. In recent studies comparing uptake of mildly oxLDL and acLDL within human macrophages, we found that the rate of uptake and delivery to lysosomes of the two are similar. Moreover, when incubated together or sequentially, greater than 80% of mildly oxLDL and acLDL co-localized to the same lysosomes (Yancey, et al., 2002). These uptake studies confirm that the lipid engorged-lysosome is a long-lived structure continually accepting new lipoprotein. This observation provides an opportunity to determine the extent to which lysosomal processing of subsequently internalized lipoproteins is affected by the lysosomal sterol engorgement. In the studies reported here, we show that even though acLDL-derived CE is normally efficiently hydrolyzed and cleared, a 3 day pre-incubation of macrophages with oxLDL or DISP impaired the subsequent ability of lysosomes to hydrolyze acLDL-derived CE. Moreover, this inhibition occurred coincident with a continued failure of lysosomes to regain an acidic pH.

MATERIALS AND METHODS

Materials

BSA, filipin, and EDTA were purchased from Sigma Chemical Company. Heat-inactivated FBS was purchased from Atlanta Biologicals. RPMI, L-glutamine, Eagle's vitamins, streptomycin, and penicillin were obtained from Mediatech. Nile Red was purchased from ICN, Aurora, IL. ACAT inhibitor, compound CP113,818, was a generous gift from Pfizer Central Research. Lysosomal-associated membrane protein (LAMP-1) mouse IgG antibody was purchased from BD Biosciences (San Jose, CA). Early endosomal antigen (EEA-1) rabbit IgG was purchased from Affinity BioReagents (Golden, CA). TRITC-conjugated F(ab)₂ fragment goat anti-mouse and goat anti-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Phospholipids were purchased from Avanti (Alabaster, AL).

Lipoprotein Isolation, Modification, and Production of Lipid Dispersions

Plasma was obtained from human volunteers following procedures approved by the Human Subjects Institutional Review Board. Human LDL (1.006 # d # 1.063) was isolated from plasma by sequential ultracentrifugation as previously described (Yancey & Jerome, 1998) and was filter sterilized then stored refrigerated under N₂. LDL was acetylated as described by Basu *et. al.* (Basu, et al., 1976). LDL was oxidized as described by Yancey (Yancey & Jerome, 1998). Anisotropic CE-rich DISP were prepared under sterile conditions, as described by Mahlberg *et al.* (Mahlberg, et al., 1990). Briefly, cholesteryl oleate (30 mg), phosphatidylcholine (1 mg), and phosphatidylserine (0.1 mg) were combined in a sterile 50 ml Corex glass tube and dried under nitrogen. RPMI medium (17 ml) supplemented with HEPES (12.5 mM) was then added and subjected to a water bath at 80degree C for 20 min to melt the dried lipids. The solution was sonicated for 20 min using a Branson sonifier (50% duty cycle). Thiobarbituric acid-reactive substance (TBARS) levels showed no oxidation after sonication of DISP. Dispersion size was confirmed by negative staining with 2% phosphotungstic acid. Oxidation or acetylation of LDL was confirmed by agarose gel electrophoresis (Barakat & St Clair, 1985). The level of oxidation was assayed by measuring TBARS (Yancey & Jerome, 1998) and presence of conjugated dienes (Esterbauer, et al., 1989). Only mildly oxLDL (TBARS 5 to 10 nmols MDA equivalents/mg LDL protein with minimal conjugated dienes) was used. Our previous studies showed that this mildly oxidized LDL is not toxic to cells and contains sufficient unoxidized cholesterol to induce levels of CE loading typical of foam cells in atherosclerotic lesions (Yancey & Jerome, 1998). TLC and GC were used to confirm that little if any oxysterol was present in acLDL or DISP.

Cell Isolation and Cell Culture

THP-1 cells (American Type Culture Collection) were plated onto 35-mm wells at a density of 1.5×10^6 cells and differentiated to macrophages by incubated for 3 to 4 d at 37°C in RPMI containing 10% FBS and 50 ng/ml of PMA (Auwerx & Schoonjans, 1991). Culture media for all cell incubations was supplemented with HEPES (20 mmols/liter), bicarbonate (23 mmols/liter), Eagle's vitamins, L-glutamine (200 mmols/liter), glucose (1.5 mg/ml), streptomycin (100 µg/ml), and penicillin (100 IU/ml). Some cells for immunohistochemistry were grown on 22 mm square, sterile coverslips placed in the bottom of the wells. All cells were incubated in a humid atmosphere at 37°C with 95% air and 5% CO₂.

Human monocytes were obtained from whole blood using techniques approved by the Human Subjects Institutional Review Board. Histopaque 1.077 (Sigma) density gradient was used to isolate cells which were then purified using cell depletion with magnetic beads (Miltenyi Biotech). Monocytes were differentiated into macrophages (HMM) during 6 days of culture in RPMI-1640 medium with 10% human serum. We use adherence as our principal stimulus

for human monocyte differentiation since cytokine treatment has been shown to stimulate cell division (Antonov, et al., 1997; Munn & Armstrong, 1993). Both THP-1 and HMM were preincubated with 1% fatty acid-free BSA for 24 hours to minimize excess triglyceride in cells.

Cholesterol Loading, CE Hydrolysis and Efflux

Cholesterol loading was accomplished by incubating either THP-1 or human macrophages at 37°C in culture media containing 1% FBS alone or with 100 µg protein/ml of oxLDL or acLDL for times indicated. Media was changed every three days. Cells were incubated with DISP at a concentration of 60 µg CE/ml. To ensure contact of cells with DISP, an inverted cell culture technique was used (Griffin, et al., 2005). In some experiments, ACAT activity was inhibited by treatment with 1.5 µg/ml compound CP113,818.

To measure CE hydrolysis, LDL was radiolabeled with [³H]CE essentially as described by Brown, Dana, and Goldstein (Brown, et al., 1975). Briefly, 598 mCi [³H]CE was solubilized in 10 µl of toluene, and 1 ml of rewarmed dimethyl sulfoxide was added to the [³H]CE solution. This mixture was incubated for 15 min at 40 C. The [³H]CE solution was then diluted by addition of 1 ml of Tris-HCL buffer prewarmed to 40 C, and 16 mg of LDL in 6 ml of Tris-HCL buffer was added to the [³H]CE solution. The mixture was incubated overnight at 40 C. Immediately before addition to cells, thin-layer chromatography (TLC) analysis was done on the [³H]cholesteryl oleate acLDL using hexane-ethyl ether-acetic acid 80:20:1 (v/v/v) as the solvent system to confirm that >85 ± 3% of the total tritium label remained as unoxidized CE. Sterol-loaded cells were washed three times with MEM containing HEPES then further incubated for 24 hours with 100 µg/ml acLDL that had been radiolabeled prior to acetylation. Incubation was done in culture medium containing 1.5mg/ml of compound CP113,818. At times during the final incubation, aliquots of cells and media were taken to measure the changes in the distribution of [3H] between FC and CE as an indicator of CE hydrolysis. To remove any floating cells, the media aliquots were centrifuged for 15 min at 2,5000 rpm. Media lipids were extracted by the method of Bligh and Dyer (Bligh & Dyer, 1959).

Mass loss of cholesterol from cells to cyclodextrins was determined by methods we have previously described (Yancey & Jerome, 2001). Essentially, at the end of loading, the cell monolayers were washed three times with MEM containing HEPES (20 mmol/l). Cells were then incubated for 2 hours at 37degree C in culture medium containing 1.5 mg/ml of compound CP113,818 then incubated for 8 h in media containing 1.5 µg/ml of compound CP113,818 alone or with 15mM 2-hydroxypropylbeta-cyclodextrins (CD) as a cholesterol efflux acceptor.

Cellular Lipid Analysis

Cell lipids were extracted in isopropanol (Yancey & St Clair, 1992) and cellulr free and esterified cholesterol content was quantified by gas chromatography using the procedure reported by Klansek (Klansek, et al., 1995). Cell proteins were solubilized by addition of 1N NaOH to the wells and protein content measured using the Lowry method (Lowry, et al., 1951). Triglyceride mass was determined by the quantitative chromatography technique described by Brasaemle (Brasaemle, et al., 1997). For all analyses, triplicate samples were measured to determine mean and assess experimental variability.

Colloidal Gold Formation, Lipoprotein Labeling

Colloids of gold were made by reduction of gold chloride using the method described by Frens (Frens, 1973). A 0.01% aqueous solution of gold chloride was brought to a boil in a flask attached to a reflux column. A small amount of sodium citrate solution was then rapidly injected into the boiling mixture. The mixture was refluxed for 60 min. After reflux, the solution was cooled and the diameter of the resulting colloidal gold measured by electron microscopy. Only solutions with a reasonably uniform size of colloidal gold were used in these studies.

Preparations having more than a 62% spread from the mean size were discarded. The size of the gold colloidal produced can be varied by varying the concentration of the sodium citrate. We used gold of 15 nm and 30 nm diameter. Modified LDL was conjugated to colloidal gold particles using standard methodology (Handley, et al., 1981). Essentially, the pH was adjusted to 5.5 and then the lipoprotein was rapidly injected into a vortexing mixture of colloidal gold. The gold and lipoprotein concentrations were adjusted to minimize the presence of unconjugated gold or lipoprotein. The quality of the conjugate was assessed by negative stain transmission electron microscopy. A satisfactory conjugate preparation was characterized by complete adsorption of lipoprotein particles to the gold with no excess unlabeled lipoprotein. If necessary, unlabeled lipoprotein was separated from labeled conjugates by centrifugation over a sucrose cushion and reisolated. Ac- and oxLDL were labeled with gold of contrasting sizes. In repeated experiments sometimes AcLDL received the 15 nm gold and in others the 30 nm gold. The size of the colloid in the range we used ~15–30 nm had no effect on uptake and processing. Cells were incubated with 75 µg protein/ml oxLDL for 3 days followed by 75 µg protein/ml acLDL for 3 days or vice versa. Each experiment was repeated at least 3 times.

Microscopy

Filipin staining identified cell FC as described previously (Yancey & Jerome, 2001). Anti-LAMP 1 (10 µg/ml) and anti-EEA-1 (5 µg/ml) were used to localize lysosomes/late endosomes and early endosomes, respectively. The secondary antibody was a TRITC-conjugated F(ab)2 fragment goat anti-mouse or goat anti-rabbit IgG secondary antibody. Controls included eliminating the primary antibody from the staining and including pre-immune serum in place of the primary antibody. Previously we have shown that the LAMP 1-positive lysosomes/late endosomes are equally affected by cholesterol accumulation and, many vesicles stain positive for both late endosome and lysosome markers (Cox, et al, 2007; Jerome et al, 1998). Thus, for simplicity, throughout the rest of the text we use the generic term lysosome to refer to LAMP-1 positive hydrolytic compartments (lysosomes/late endosomes). Transmission electron microscopy (TEM) was used to confirm lysosomal accumulation and quantify the distribution of lipid between lysosomes and cytoplasmic lipid droplets after staining to demonstrate acid phosphatase as a lysosomal marker (Jerome, et al., 1998).

Nile red staining was used to identify neutral lipid deposits (Greenspan, et al., 1985). Stain was made from a 0.5mg Nile red/ml acetone stock. Cells, grown on coverslips, were fixed in 1.5% glutaraldehyde buffered with 0.1M cacodylate (pH 7.4) then rinsed in PBS. Two microliters of stain stock was added to 1 ml of 75% glycerol solution as a mounting media. A drop of the glycerol solution was then placed on the cells and the cells coverslipped. Nile red was excited at 488 nm and wavelengths less than 580 nm were imaged. Under these conditions Nile red indicates only neutral lipids (Greenspan & Fowler, 1985).

Quantitative Microscopy

The lipid volume of lysosomes and cytoplasmic lipid droplets was estimated using point counting stereology (Weibel, et al., 1966), as routinely done in our laboratory (Jerome & Lewis, 1985). Five thin sections from multiple parts of the embedded block were cut for each sample. From each thin section, ten to fifteen areas were chosen using unbiased techniques. From these images, the average volume density (volume per volume of cell cytoplasm) was computed for lipid in both lysosomes and cytoplasmic lipid droplets.

To determine the percent of cells with intracellular FC, a grid of random points was used to unbiasedly select cells. Cells underneath points were scored as positive or negative for intracellular filipin-positive vesicles. Five hundred cells were scored. In addition, for positive cells the number of filipin-positive vesicles were counted.

Lysosome pH was evaluated in living cells using ratio imaging of cells treated with 5 μ M LysoSensor Yellow/Blue DND-160 (Molecular Probes). This dye exhibits a pH dependant fluorescent pattern of yellow fluorescence in acidic environments with a significant blue shift as pH increases (Diwu, et al., 1999; Lin, et al., 2001). Vesicles were scored as active if the pH was less than 4.8 and inactive if the pH was greater than 4.8 (Cox, et al., 2007). One hundred arbitrarily selected cells were counted for each condition. All imaging was done within 10 minutes following a 30 second equilibration.

The size distribution of Nile red-positive lipid accumulations were determined using the same selection scheme employed for Filipin stained samples. For each selected lipid accumulation, the diameter of the droplet in the longest direction was taken. More than five hundred droplet diameters were measured for each condition. On average, our sampling process selected no more than 3 droplets per cell.

To quantify uptake of gold-labeled lipoprotein, following uptake cells were fixed in 2.5% glutaraldehyde, washed, postfixed in 1% osmium tetroxide, dehydrated through a graded series of ethanols, and embedded in spur resin. To quantify co-localization, 100 cell thin cross sections for each condition were chosen arbitrarily and vesicles containing gold were scored as containing a single gold label or both sizes of gold.

Statistics

The mean, standard deviation, and standard error of the mean were determined from at least 3 separate experiments. Two group comparisons used Student's T test. For multiple comparisons, following an analysis of variance, group comparisons were performed with the multiple comparison HSD method of Tukey (Kirk, 1968). Percentage data was corrected to a normal distribution using the arcsin transform before analysis. Two means were considered significantly different if the possibility of a Type I error was less than 0.05.

RESULTS

Cells were sequentially incubated for 3 days with oxLDL followed by 3 days with acLDL (or vice versa) to determine if pre-incubation with oxLDL would perturb metabolism of acLDL. Cells incubated with oxLDL or acLDL alone acted as controls. Electron microscopy of colloidal gold-labeled lipoprotein uptake showed co-localization of both acLDL and oxLDL after sequential incubation (Figure 1). Since the gold is not digestible, it acts as a residualizing label indicating the amount of lipoprotein internalized and delivered to lysosomes. When oxLDL was labeled with 15 nm colloidal gold and acLDL was labeled with 30 nm colloidal gold, 78% (+/- 8%) of all labeled vesicles contained both sizes of gold. When oxLDL was labeled with 30 nm colloidal gold and acLDL with 15 nm colloidal gold, 82% (+/- 14%) of all labeled vesicles contained both sizes of gold. This confirms earlier studies showing that both types of lipoprotein are delivered to the same lysosomes (Yancey, et al., 2002).

When cells were incubated for 6 days with acLDL or mildly oxLDL alone they accumulated between 190 and 260 μ g total cholesterol/mg cell protein (Figure 2A). This was roughly twice the amount accumulated after 3 days of incubation with oxLDL or acLDL. No significant differences were detected in total cholesterol accumulation between the different treatment groups. However, differences were seen in the distribution between free and esterified forms (Figure 2A). Incubation of cells with acLDL for either 3 or 6 days primarily led to an accumulation of CE. At the 6 day time point, the CE levels in acLDL-treated cells were statistically different ($p < 0.05$) compared to the levels seen in cells incubated with oxLDL or sequentially with both forms of modified lipoproteins. In these latter groups, large amounts of both FC and CE were present (differences between these groups were not statistically significant). At day 3 of accumulation, the CE levels between acLDL and oxLDL groups were

not different at $p < 0.05$ but the FC levels were different. Consistent with similarities in total cellular cholesterol levels, the volume of cytoplasm occupied by lipid was similar in all groups (Figure 2B). However, at day 6 only two percent of the cell volume of acLDL-treated macrophages was lysosomal lipid. The majority of the stored lipid in these cells occurred as cytoplasmic lipid droplets. In contrast, the bulk of the lipid in oxLDL-treated cells was within lysosomes. Importantly, cells incubated first with oxLDL and then with acLDL showed similar amounts and lysosomal distribution of lipid to that seen when cells were incubated with oxLDL alone. Given that oxLDL pretreatment reduced the volume of cytoplasmic lipid droplets produced by acLDL and produced increases in both lysosomal FC and CE accumulation, the simplest explanation of the results is that preincubation with oxLDL inhibited lysosomal processing of subsequently internalized acLDL. In contrast, cells incubated first with acLDL followed by oxLDL showed a distribution pattern in-between acLDL alone and oxLDL alone. This suggests little or no impairment of the initial acLDL metabolism but subsequent inhibition of oxLDL metabolism.

We pre-incubated cells for 3 days with oxLDL before incubating them with acLDL because previous experiments suggested that inhibition of CE hydrolysis was not immediate but required 2–3 days (Yancey & Jerome, 1998). To determine if inhibition of acLDL processing by oxLDL also required extended incubation, we incubated THP-1 cells for 3 days with acLDL with or without a 6 hour preincubation with oxLDL (Figure 2). This short preincubation with oxLDL did not effect acLDL-induced cholesterol accumulation (Figure 3A) or lipid distribution (Figure 3B). Thus, the effect of oxLDL treatment on lysosomes was not immediate.

Quantitative EM confirmed lysosomal accumulation but did not differentiate whether the lipid was FC or CE (triglyceride, the other neutral lipid that might accumulate accounted for less than 30 $\mu\text{g}/\text{mg}$ cell protein in oxLDL-treated cells). Comparison of the biochemical data showing However, the significant FC accumulation combined with increases in lysosomal volume suggested significant lysosomal FC sequestration in oxLDL-treated cells. We confirmed this using filipin as a stain for FC. Cells treated for 6 days with acLDL showed FC within plasma membrane but very little within cytoplasmic droplets (Figure 4). In contrast, cells incubated with oxLDL for 6 days generally showed numerous filipin-positive vesicles. These had a size, shape, and cellular location consistent with lysosomes/late endosomes (Figure 4) and co-localized with LAMP-1 staining (Supplement Figure 1). This is not surprising, since most non-lysosomal free cholesterol is effluxed or converted to CE for storage in extralysosomal CE droplets (McGookey & Anderson, 1983; Yancey, et al., 1995). Macrophages incubated with oxLDL before acLDL also showed numerous filipin-positive accumulations (Figure 4). Finally, cells incubated with acLDL first and then oxLDL had fewer filipin-positive cytoplasmic deposits than those incubated with oxLDL alone. Thus, like with other parameters, cells that received acLDL before oxLDL had a phenotype in-between acLDL alone and oxLDL alone.

Not all cells showed filipin positive accumulations. Cultures incubated with oxLDL alone or oxLDL followed by acLDL had FC accumulation in 81% and 77% of cells, respectively. In both cases, more than 93% of positive cells contained more than 16 such deposits. Thus, cells pretreated for 3 days with oxLDL before receiving acLDL mimicked those receiving 6 days of oxLDL treatment. In contrast, only 5% of cells treated with acLDL and 42% of cells incubated with acLDL followed by oxLDL showed filipin-positive accumulations. Filipin-positive cells treated for 6 days with acLDL had an average of only 3 filipin positive accumulations per cell. Filipin-positive cells receiving acLDL followed by oxLDL averaged 8 per cell.

Comparison of the biochemical and morphologic data suggested both lysosomal CE and FC accumulation. To confirm the CE accumulation, we sequentially incubated cells with

lipoprotein in the presence or absence of an ACAT inhibitor (CP113,818). Our reasoning being that if the CE was unhydrolyzed lipoprotein CE in lysosomes, then inhibition of ACAT should not alter the ratio of FC to CE in the cell. On the other hand, if the accumulated CE was in ACAT-derived cytoplasmic droplets, then ACAT inhibition should reduce CE which should be accompanied by a concomitant increase in FC. Figure 5 shows that ACAT inhibition during acLDL-loading led to a major decrease in cellular CE and increases in FC, indicating that most of the stored CE in acLDL-treated cells was ACAT derived. In contrast, ACAT inhibition had little effect on either oxLDL-treated macrophages or on cells sequentially incubated with oxLDL followed by acLDL, indicating that the lysosomal CE accumulation seen with these conditions was primarily unhydrolyzed lipoprotein CE. Cells incubated with acLDL followed by oxLDL exhibited a phenotype in-between the oxLDL and acLDL extremes, suggesting that these cells had significant amounts of CE in both lysosomes and cytoplasmic lipid droplets.

Taken together our results suggest that in acLDL-treated cells most of the CE was contained in cytoplasmic droplets and most of the FC was within membranes. In contrast, much of the FC and most of the CE in oxLDL treated cells was trapped in lysosomes. The data also suggested that much of the FC and most of the CE in cells pre-treated for 3 days with oxLDL before incubation with acLDL had a similar lysosomal distribution. In earlier work, we showed that the FC and CE within lysosomes of oxLDL treated cells is sequestered and unavailable for efflux (Yancey & Jerome, 2001). To determine if the lysosomal CE and FC in sequentially oxLDL and acLDL treated cells was also trapped, we measured the loss of FC and CE mass from loaded cells by incubation for 8 hours in the presence of CD, a potent efflux promoter (Yancey, et al., 1996). Cells loaded for 6 days with acLDL effluxed 69 (+/- 5)% of their total sterol while cells loaded with oxLDL or oxLDL followed by acLDL effluxed only 31 (+/- 7)% and 36 (+/- 13)%, respectively. This was a statistically significant ($p < 0.05$) difference. Cells treated with acLDL followed by oxLDL effluxed 51 (+/- 7)% of their sterol. The differences were even more dramatic comparing CE loss from cells. AcLDL-treated cells lost 66 (+/- 5)% of their CE compared to only 5 (+/- 5) or 16 (+/- 9) for oxLDL-treatment or oxLDL- followed by acLDL-treatment. Cells treated first with acLDL and then oxLDL lost 32 (+/- 14)% of their CE. This value was intermediate between acLDL only and oxLDL only treated cells. The free cholesterol loss values were 79%, 72%, 62%, and 65% for acLDL, acLDL/oxLDL, oxLDL, and oxLDL/acLDL-treated cells, respectively. Although the differences in FC loss were not statistically different, the trend for oxLDL-treated and oxLDL/acLDL-treated cells to be similar and acLDL/oxLDL-treated cells to be intermediate between oxLDL-treated and acLDL-treated cells was also seen for this parameter.

To confirm that the accumulation of CE in lysosomes of cells treated with oxLDL followed by acLDL was due to an inhibition of hydrolysis, we measured the rate of acLDL-derived CE hydrolysis in cells incubated for 6 days with acLDL (Figure 6A) or cells incubated for 3 days with oxLDL followed by 3 days with acLDL (Figure 6B). After loading, cells were washed and then incubated with 100 µg protein/ml [³H]CE-labeled acLDL for 24 hours. Reesterification of any [³H]FC generated was inhibited by treating the cells with 1.5mg/ml of the ACAT-inhibitor, CP113,818. The distribution of cell [³H]CE, cell [³H]FC and [³H]FC in the media indicates that acLDL-treated cells generated primarily [³H]FC a portion of which was effluxed to the media. In contrast, much less [³H]FC was generated in cells pretreated for 3 days with oxLDL even after 3 days of unlabeled acLDL incubation indicating a continued inhibition of lysosomal CE hydrolysis. To confirm that the CE hydrolysis in acLDL-only treated cells was lysosomal, in some incubations we included 50 µM chloroquine, a weak base that raises intracellular pH and thus inactivates lysosomal acid lipase (De Duve, et al., 1974). Chloroquine produced an 87% reduction in the generation of [³H]FC in acLDL treated cells.

In previous experiments we've shown that treating human macrophages with lipid dispersions also produces lysosomal sterol accumulation and inhibition of CE hydrolysis (Griffin, et al.,

2005). To determine whether pretreatment of cells with DISP would also produce a long term lysosomal inhibition, we treated THP-1 with 60 μ g CE/ml DISP for 3 days followed by a 3 day incubation with 100 μ g protein/ml acLDL (Figure 7). Like oxLDL, DISP produced significant CE accumulation (Figure 7A) and the lipid accumulation in these cells was primarily within lysosomes (Figure 7B). Moreover, 3 days of DISP pretreatment followed by acLDL produced a phenotype very similar to that of DISP-treatment alone. Treatment of cells with acLDL followed by DISP produced an intermediate phenotype.

To confirm that human macrophages other than THP-1 showed this long-term inhibition of hydrolysis, we investigated sequential incubation of human monocyte-derived macrophages with ox- and acLDL (Figure 8). The results were similar to those with THP-1. After 6 days with acLDL, HMM accumulated an average of 184 μ g cholesterol/mg cell protein (Figure 8A). The majority of this (84%) was within cytoplasmic lipid droplets (Figure 8B). In contrast, HMM incubated with oxLDL alone or oxLDL followed by acLDL accumulated significantly more total cholesterol ($p<0.05$) and this occurred as a mixture of FC and CE. Most of the cholesterol (>82%) was within lysosomes. The total cholesterol levels in cells receiving acLDL first followed by oxLDL was significantly different ($p<0.05$) than in cells receiving only acLDL but not different from cells receiving oxLDL alone or oxLDL followed by acLDL. However, the cells had a cholesterol distribution intermediate between that of oxLDL alone and acLDL alone. Both the volume of cytoplasmic droplets and the volume of lysosomes was significantly different ($p<0.05$) across all three groups.

The prolonged inhibition of CE hydrolysis seen with oxLDL or DISP suggested a general alteration in lysosome function rather than a direct inhibition of enzyme by substrate. A principal attribute of lysosomes is their reduced pH. We have previously shown that incubation of macrophages with oxLDL or DISP leads to a failure of lysosomes to maintain an acidic pH (Cox, et al., 2007). To test whether the oxLDL-induced increase in lysosome pH was maintained during the 3 subsequent days of acLDL, we determined the number of LysoSensor Yellow/Blue-containing vesicles that had an active pH (pH<4.8). Incubation with oxLDL for 3 days produced a significant ($p<0.05$) reduction (compared to untreated cells) in the percent of lysosomes with an active pH and this number was not reversed with an additional 3 days incubation in media without lipoprotein (Figure 9). Treatment with acLDL alone did not produce this decrease in active lysosomes. However, cells receiving 3 days of oxLDL followed by 3 days of acLDL contained reduced numbers of active lysosomes. Thus, it is probable that at least part of lysosomal CE accumulation was due to reduced hydrolysis because of increased lysosome pH.

DISCUSSION

Previously we demonstrated that macrophages incubated with mildly oxLDL, aggLDL or CE-rich dispersions accumulate significant amounts of both FC and CE within cholesterol-swollen lysosomes (Griffin, et al., 2005; Yancey & Jerome, 2001). This mimics the phenotype of macrophage foam cells found in atherosclerotic lesions (Peters & De Duve, 1974). Several lines of evidence in animal studies of atherosclerosis, including the resistance of lysosomal cholesterol to efflux, suggest that the lysosomal cholesterol accumulation influences atherogenesis (see review (Jerome, 2006)). The accumulation of significant CE within lysosomes indicates a failure of CE hydrolysis. This has been confirmed for mildly oxLDL treatment where a reduction in CE hydrolysis was directly measured (Yancey & Jerome, 1998). In the present study, we used a combined biochemical and structural approach to extend our understanding of lysosomal cholesterol accumulation by demonstrating that the lipoprotein-induced inhibition of CE hydrolysis is generalized to other CE sources and is long-lived.

A number of studies indicate that oxLDL can inhibit lysosomal degradation. However, the nature of the inhibition remains controversial. Clearly, acLDL delivery of cholesterol does not inhibit hydrolysis but oxLDL does (Yancey & Jerome, 1998). This has led investigators to focus on oxidized lipids as the culprit. A number of studies, including our own, have shown inhibition of lipoprotein degradation in the presence of oxLDL (Hoppe, et al., 1994; Kritharides, et al., 1998; Lougheed, et al., 1991; Maor & Aviram, 1994; Yancey & Jerome, 2001). In some of these studies, an immediate, direct inhibition of lipoprotein degradation was demonstrated. For instance, Lougheed and colleagues showed that the apolipoprotein B on heavily oxLDL particles is resistant to lysosomal proteolysis (Lougheed, et al., 1991). Oxidized sterol esters are also resistant to lysosomal hydrolysis (Brown, et al., 2000). Some of the impaired lysosomal degradation has been attributed to direct inhibition of enzyme activity by 7-keto-cholesterol and oxidized phospholipids (Dhaliwal & Steinbrecher, 2000; Hoppe, et al., 1994; Kritharides, et al., 1998; Lougheed, et al., 1991; O'Neil, et al., 2003; Roma, et al., 1992). Our previous studies, however, indicate that less heavily oxLDL, such as used in the present studies, lacks detectable 7-keto-cholesterol and contains only minimal oxidized phospholipids (Yancey & Jerome, 2001). Moreover, the effect with mildly oxLDL is not immediate but requires up to two days to become established (Yancey & Jerome, 1998). A lack of direct inhibition by mildly oxLDL is also suggested by the fact that short-term (6 hour) incubation with mildly oxLDL did not inhibit subsequent processing of acLDL. These observations indicate that the inhibition of hydrolysis is not an inherent property of the mildly oxLDL particle but, rather, is due to oxLDL-induced alterations in the lysosome, which are maintained even after oxLDL influx has ceased. This suggests that mildly oxLDL produces a general effect on lysosomes. This effect probably does not require oxidized lipids since aggLDL and CE-rich lipid dispersions can also produce an inhibition of hydrolysis (Griffin, et al., 2005).

It is clear from the pH analysis that oxLDL produced a long-term increase in lysosome pH to levels inconsistent with efficient lysosomal acid lipase activity. Moreover, Cox showed that oxLDL and aggLDL also inhibits apolipoprotein B degradation after prolonged incubation (Cox, et al., 2007). The failure of lysosomes to maintain an active pH coincident with the inhibition of CE hydrolysis suggests that the pH change is, at least in part, the mechanism by which CE hydrolysis is inhibited. The failure of lysosomes to regain an active pH after 3 days and the inhibition of acLDL-derived CE hydrolysis following oxLDL treatment indicates that the inhibition is long-lived. Thus, once a lysosome becomes altered, it may not be able to regain function. This intriguing possibility and the expected influence on subsequent foam cell lipid metabolism is an important concept that deserves further study.

Our previous studies showing an initial accumulation of unesterified cholesterol in lysosomes as well as those of Maor and Aviram (Maor & Aviram, 1994) implicates the generation of lysosomal free cholesterol as a possible modulator of lysosome pH. In this regard, it is known that cholesterol levels in membranes can modulate the activity of membrane pumps (Crider & Xie, 2003). The lysosomal pumps are vacuolar type-ATPases responsible for pumping hydrogen ion into the lysosome to maintain a low pH (Crider & Xie, 2003; Grabe & Oster, 2001). Recently, using isolated lysosomes, we showed that increasing FC levels in isolated lysosomes can inhibit lysosome acidification, presumably through inhibition of v-ATPase activity (Cox, et al., 2007). Thus, it is plausible that excess FC generated within lysosomes can produce long-term alterations in v-ATPase activity and contribute to the failure of sterol-engorged lysosomes to maintain an active pH.

Although v-ATPases are the primary pumps involved in internalization of hydrogen ions into lysosomes, other lysosomal membrane proteins also play roles in mediating the lysosome pH. Recently Soyombo has shown that the lysosomal membrane protein TRP-ML1 can act as a safety valve helping to remove hydrogen ions from over acidified lysosomes (Soyombo, et al.,

2006). It is possible that, in addition to effects on v-ATPases, perturbations such as increased lysosomal membrane cholesterol concentration could affect ancillary proteins such as TRP-ML1 as well. Heightened activity of TRP-ML1 could produce an increase in lysosome pH. The study by Soyombo also highlights the importance of correct lysosome pH to lysosome function (Soyombo, et al., 2006).

After 3 days of oxLDL or DISP, not all lysosomes had a pH above 4.8, suggesting some lysosomes remained active. Despite this, though, there was a dramatic decrease in overall CE hydrolysis and a significant increase in lysosomal CE. One possible explanation for this apparent paradox would be if oxLDL and DISP were preferentially delivered to a subset of lysosomes during the first incubation period but that acLDL in the subsequent incubation went to all lysosomes. Arguing against this is the fact that prolonged incubation with oxLDL, aggLDL or DISP for 6 days produces an increase in pH in greater than 80% of all cellular vesicles (Cox, et al., 2007). A more plausible explanation would be that the active lysosomes represent newer lysosomes formed at later time points. Consistent with this possibility, the active lysosomes at 3 days tended to be smaller than the inactive ones, suggesting less lipid engorgement perhaps because of a shorter period of exposure to lipoprotein uptake. Much less CE, if any, would be contained in these smaller lysosomes and so, despite their activity, their contribution to the overall CE hydrolysis rate would be minimal.

With respect to implications of our study for understanding atherogenesis, it is known that macrophage foam cells contain significant amounts of exogenously derived cholesterol in both free and esterified forms. However, the nature of the delivery vehicle(s), exact mechanism(s) of entry and reasons for excess accumulation are still not known (Jerome, 2006). Our studies suggest that at least some of the CE accumulation is related to inhibition of CE hydrolysis involving a general, long-lived alteration in the lysosome. Thus, once a lysosome has been inhibited, we would expect that CE arriving on any type of particle and taken up by any mechanism would not be hydrolyzed. In addition to oxLDL, possible sources in lesions for this exogenous cholesterol include aggLDL, VLDL and its remnants, and cholesteryl-rich extracellular lipid droplets which have been identified within the extracellular regions of the plaque (Guyton & Klemp, 1994; Hoff & Morton, 1985; Kruth, 1984; Smith, et al., 1968). Since macrophage foam cells can remain in lesions for extended periods of time (Jerome & Lewis, 1997) the long-lived (and perhaps permanent) inhibition of CE hydrolysis of any subsequent CE delivery would be expected to produce significant lysosomal CE accumulation, consistent with observations in animal models (Jerome & Yancey, 2003). Moreover, by being trapped in lysosomes, this inert pool of cholesterol would not be easily removed nor would it be expected to participate as part of the regulatory pool of cholesterol in cells. This could exacerbate atherogenesis. Moreover, the studies by Cox indicate that proteolysis is also inhibited by the increase in lysosomal pH (Cox, et al., 2007). This suggests an all around inhibition of lysosome function; a factor which could make the macrophages even more atherogenic.

SUMMARY AND CONCLUSION

By correlating biochemical and structural data we show that sterol accumulation within lysosomes produces a significant inhibition of CE hydrolysis that is not specific for one type of lipoprotein but generalized to several modified forms of LDL, including acLDL which is normally hydrolyzed efficiently within lysosomes. This inhibition is maintained for at least 3 days and leads to significant accumulation of both FC and CE within lysosomes. The inhibition of hydrolysis occurs concomitant with a prolonged inhibition of lysosomes to maintain an acidic pH. This suggests that at least a partial explanation for the hydrolysis failure is the inability of lysosomes to maintain the proper pH for lysosomal acid lipase to function. Based on the current and previous data, we hypothesize that the initial FC accumulation within lysosomes leads to an inhibition of lysosome proton pumps which leads to the increase in

lysosome pH and failure of CE hydrolysis. Once established, the inhibition is long-lived and affects the ability of lysosomes to hydrolyze other substrates, even those that are normally efficiently degraded.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Electron micrograph of THP-1 incubated for 3 days with oxLDL conjugated to 15 nm colloidal gold followed by 3 day incubation with acLDL conjugated to 30 nm colloidal gold. Lipid engorged lysosomes contain both 15 nm and 30 nm gold particles. Bar= 200 nm.

Figure 2.

THP-1 macrophages incubated for 3 days with acLDL (Ac) or oxLDL (Ox), for 6 days (media refreshed after 3 days) with acLDL (Ac/Ac) or oxLDL (Ox/Ox), for 3 days with acLDL followed by a 3 day incubation with oxLDL (Ac/Ox), or for 3 days with oxLDL followed by a 3 day incubation with acLDL (Ox/Ac). Data represents the mean and standard error for 3 experiments. A) Total cholesterol and its distribution between free (FC) and esterified (CE) form in cells after incubation. B) Volume of cell occupied by lipid stores and the distribution between lysosomes (Lys) and cytoplasm droplets (Drop). Bars with the same letter indicate means which are not statistically different ($p < 0.05$).

Figure 3.

Effect of pretreatment of cells with oxLDL for 6 hours prior to incubation for 3 days with acLDL (Ox/Ac) compared to cells incubated with acLDL alone (Ac). Data represents the mean and standard error for 3 experiments. A) Total cholesterol and its distribution between free (FC) and esterified (CE) cholesterol. B) Volume of cell occupied by lipid stores and the distribution between lysosomes (Lys) and cytoplasm droplets (Drop).

Figure 4. Filipin staining of THP-1 macrophages incubated for 6 days (media refreshed after 3 days) with acLDL (A) or oxLDL (B), for 3 days with acLDL followed by 3 day incubation with oxLDL (C), or for 3 days with oxLDL followed by a 3 day incubation with acLDL (D). Bar= 5 μ m.

Figure 5.

Distribution of free (FC) and esterified (CE) cholesterol determined for THP-1 macrophages incubated without lipoprotein (No) or for 6 days (media refreshed after 3 days) with acLDL (Ac/Ac) or oxLDL (Ox/Ox), for 3 days with acLDL followed by a 3 day incubation with oxLDL (Ac/Ox), or for 3 days with oxLDL followed by a 3 day incubation with acLDL (Ox/Ac). Incubations with lipoprotein were done either in the absence (-) or presence (+) of the ACAT inhibitor, CP113,818.

Figure 6.

Time course of hydrolysis of acLDL-derived [³H]CE. THP-1 macrophages were incubated for 6 days with acLDL (A) or 3 days with oxLDL followed by 3 days with acLDL. Following sterol loading, cells were incubated with [³H]CE-labeled acLDL for up to 24 hours in the presence of the ACAT inhibitor CP113,818. At the indicated times, cells and media were harvested and the [³H]CE and [³H]FC were measured. Results are expressed as mean $\pm$ SD for triplicate dishes.

Figure 7.

THP-1 macrophages incubated for 6 days (media refreshed after 3 days) with acLDL (Ac/Ac) or CE-rich dispersions (DISP/DISP), for 3 days with acLDL followed by a 3 day incubation with dispersions (Ac/DISP), or for 3 days with dispersions followed by a 3 day incubation with acLDL (DISP/Ac). Data represents the mean and standard error for 3 experiments. A) Total cholesterol and its distribution between free (FC) and esterified (CE) form in cells after incubation. B) Volume of cell occupied by lipid stores and the distribution between lysosomes (Lys) and cytoplasm droplets (Drop).

Figure 8.

HMM incubated for 6 days (media refreshed after 3 days) with acLDL (Ac/Ac) or oxLDL (Ox/Ox), for 3 days with AcLDL followed by 3 day incubation with oxLDL (Ac/Ox), or for 3 days with oxLDL followed by a 3 day incubation with acLDL (Ox/AC). Data represents the mean and standard error for 3 experiments. A) Total cholesterol and its distribution between free (FC) and esterified (CE) form in cells after incubation. B) Volume of cell occupied by lipid stores and the distribution between lysosomes (Lys) and cytoplasm droplets (Drop). Bars with the same letter indicate means which are not statistically different ($p < 0.05$).

Figure 9.

Percentage of LysoSensor Yellow/Blue staining vesicles which show an active pH ($\text{pH} < 4.8$) in THP-1 macrophages in media alone (No LP), treated for 3 days with oxLDL (Ox), 3 days with oxLDL followed by 3 days in media alone (Ox/Med), 3 days with acLDL (Ac), 6 days with AcLDL (Ac/Ac), or 3 days with oxLDL followed by 3 days with acLDL. (Ox/Ac). Bars with the same letter indicate means which are not statistically different ($p < 0.05$).