Uptake of Oxidized LDL by Macrophages Differs From That of Acetyl LDL and Leads to Expansion of an Acidic Endolysosomal Compartment

Marilee Lougheed, Edwin D.W. Moore, David R.L. Scriven, Urs P. Steinbrecher

Abstract—Accumulation of cholesterol by macrophage foam cells in atherosclerotic lesions is thought to involve the uptake of modified low density lipoproteins (LDLs). Previous studies have shown that there is impaired degradation of oxidized LDL in macrophages. The present study was done to determine whether the differences in intracellular metabolism of oxidized LDL and acetyl LDL were associated with delivery to different intracellular compartments. Mouse peritoneal macrophages were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate–labeled oxidized LDL or 3,3′-dioctadecyloxacarbocyanine perchlorate–labeled acetyl LDL and examined by fluorescence microscopy. Deconvolution image analysis showed <10% colocalization of the 2 lipoproteins at incubation times ranging from 30 minutes to 6 hours. Subcellular fractionation of macrophages after incubation with 99mTc-labeled oxidized LDL revealed accumulation of the tracer in a compartment with a d = 1.042 g/mL, consistent with endosomes. Surprisingly, there was a concurrent dramatic shift of the density of lysosomal marker enzymes from d = 1.1 g/mL to the same fractions that contained 99mTc, indicating that this compartment was formed after fusion with primary lysosomes. Parallel experiments in J774 cells, a murine macrophage–like cell line, did not show a similar density shift, perhaps because of the slower rate of accumulation of oxidized LDL by these cells. Fluorescence microscopy of macrophages labeled with a lysosomotropic dye revealed a marked expansion of the acidic compartment after exposure of cells to oxidized LDL. We conclude that oxidized LDL and acetyl LDL are internalized by morphologically distinct pathways. Furthermore, because of its impaired lysosomal degradation, oxidized LDL causes expansion of and a decrease in the density of the lysosomal compartment in macrophages. (Arterioscler Thromb Vasc Biol. 1999;19:1881-1890.)

Key Words: oxidized LDL ■ macrophages ■ lysosomes ■ endocytosis ■ scavenger receptors

Low density lipoprotein (LDL) is believed to be the source of excess cholesterol deposits in macrophage foam cells, which are the hallmarks of developing atherosclerotic lesions. Because uptake of normal LDL via the LDL receptor is subject to feedback regulation, accumulation of excessive amounts of LDL-derived cholesterol by macrophages is thought to require modification of LDL in a way that permits rapid unregulated internalization.1 Several types of LDL modification have been shown to permit cholesterol accumulation in vitro, including acetylation, oxidation, and aggregation.2–4 Acetylated LDL and oxidized LDL are both ligands for the scavenger receptor class A type I/II (SR-AI/II). Recent studies with macrophages from SR-AI/II–knockout mice have shown that nearly all of the high-affinity uptake of acetyl LDL is due to SR-AI/II, but 70% of the uptake of oxidized LDL is attributable to different, as-yet-incompletely characterized receptors.5 Although the rates of uptake of oxidized LDL and acetyl LDL are similar, the degradation of oxidized LDL is much less efficient than that of acetyl LDL, and as a result, significant amounts of oxidized LDL accumulate intracellularly in an undegraded form.6–8

The inefficient degradation of oxidized LDL by macrophages has been attributed to resistance of oxidized apoB to cathepsins9 and to inactivation of cathepsins by reactive aldehydes contained in oxidized LDL.10,11 Surprisingly, despite the evidence supporting lysosomal dysfunction induced by oxidized LDL, exposure of macrophages to oxidized LDL does not interfere with the capacity of these cells to degrade acetyl LDL.9,12,13 The simplest hypothesis that reconciles these observations is that oxidized LDL and acetyl LDL are internalized by separate uptake pathways that deliver the 2 ligands to different endocytic compartments.

There is considerable evidence for the existence of morphologically and functionally distinct endocytic compartments in macrophages. It has been shown that there is an abundance of tubular (vacant) lysosomes in unstimulated, thioglycollate-elicited macrophages, and that thioglycollate-elicted macrophages and in J774 cells and that after active endocytosis of particulate ligands, the morphol-
ogy changes to predominantly spherical forms. As well, it has been shown that when mouse bone marrow–derived macrophages are presented with poorly degradable compounds such as horseradish peroxidase or dextran, these initially appear in a perinuclear lysosomal compartment but then move to a distinct lysosome-derived compartment of small vesicles dispersed randomly throughout the cells. In mouse peritoneal macrophages, β-VLDL was found to be routed to peripherally distributed vesicles, whereas LDL was delivered to more centrally located vesicles. This difference in distribution was dependent on the presence of apoE3 in VLDL. It was found that β-VLDL produced a much greater stimulation of acyl CoA:cholesterol acyltransferase (ACAT) activity than did LDL, even though the degradation of β-VLDL protein and cholesterol ester was slower than that of LDL. A recent ultrastructural study of pigeon monocyte–sphingomyelin, and LysoSensor yellow/blue DND-160 were observed in all breeding animals, as well as in randomly selected experimental AI/II genotypes were verified by Southern blot analysis of tail DNA from Dr T. Kodama, University of Tokyo, Tokyo, Japan. The construct and the phenotypic expression in homozygous knockout mice have been reported elsewhere. SR-AI/II– knockout mice by peritoneal lavage with ice-cold Ca2+-free PBS. Cells were suspended in α-MEM with 10% FBS and seeded onto sterile coverslips at a density of 1.x 106 cells/mL. Adherent macrophages were cultured overnight, washed with α-MEM, and then incubated for 0.5 to 6 hours with 10 μg/mL DiI-labeled oxidized LDL together with 10 μg/mL DiO-labeled acetyl LDL or DiO-labeled aggregated LDL. LDL-derived membrane compartments were then visualized by fluorescence microscopy. Cells were examined on the stage of a Nikon Diaphot 200 inverted microscope, in epifluorescent mode, by using a Planapo 100×/1.3 nominal-aperture glycerin-immersion objective. A rhodamine filter set was used for imaging DiI and a fluorescein filter set for DiO. A series of 2D images were acquired at 0.25-μm steps, through focus, onto a thermoelectrically cooled CCD camera (Tektronix TK512CB chip). Image stacks were transferred to a Silicon Graphics Indigo 2 XZ workstation where they were corrected for the dark current of the camera, background fluorescence, and nonuniformity across the field of illumination and detection. The optical transfer function of the microscope was measured under the same optical conditions as the experimental samples. Image stacks were then deconvolved using the empirically determined optical transfer function of the microscope and a constrained, iterative deconvolution algorithm based on regularization theory. Small fluorescent beads (Molecular Probes) with broad excitation and emission spectra were added to the mounting medium and acted as fiduciary markers in aligning data sets. The deconvolved and aligned data sets were then thresholded with the threshold value being selected from a small region of the deconvolved image containing no specific lipid staining. The thresholded and aligned data sets were then compared voxel by voxel for coincidence of the 2 fluorescent markers.

Conventional Epiﬂuorescence Microscopy

Macrophages were harvested from wild-type mice or SR-AI/II mice and cultured on sterile coverslips as described above. Cells were incubated for 6 hours with 10 μg/mL DiI-labeled oxidized LDL or DiI-labeled acetyl LDL in α-MEM containing 2.5% lipoprotein-deficient serum, washed with α-MEM, and then incubated with 4 μg/mL LysoSensor yellow/blue in serum-free medium for 1 hour at 37°C. Unfixed live macrophages were washed with PBS containing Ca2+ and immediately photographed using a Zeiss Ax-
Subcellular Fractionation

Resident peritoneal macrophages obtained from CD-1 mice were suspended in α-MEM with 10% FBS and plated into 100-mm dishes at a density of 1.5 × 10^6 cells/mL. Nonadherent cells were removed by medium exchange after 1 hour, and macrophages were used the next day for experiments. J774 macrophages were plated into 100-mm dishes in Dulbecco’s modified Eagle’s medium containing 10% FBS and grown to 80% confluence before use in experiments. Macrophages were incubated for 2 or 15 hours with 20 μg/mL radiolabeled, oxidized or acetyl LDL in medium containing 2.5% lipoprotein-deficient serum to minimize toxicity. Cells were washed twice with ice-cold PBS without Ca^2+ and incubated with PBS containing 0.02% EDTA for 10 minutes at 4°C. Macrophages were disrupted and fractionated essentially as described by Dean and Martin.22 Cells were gently removed from the plates by using cell lifters and sedimented at 1100 g for 10 minutes at 4°C. Between 10^6 and 2 × 10^6 cells were resuspended in 2 mL of cold 10 mmol/L Tris, 0.25 mol/L sucrose, and 1 mmol/L EDTA (pH 7.5) and disrupted by N_2 cavitation at 445 kPa (mouse peritoneal macrophages) or 310 kPa (J774 cells) for 15 minutes in a precooled Kontes mini cell-disruption chamber (Mandel Scientific). Nuclei, mitochondria, and intact cells were removed by centrifugation at 1000 g for 5 minutes at 4°C. An aliquot of the resultant postnuclear supernatant was reserved for latency determination, and the remainder was mixed with 10 μg of bovine albumin and layered over 10 mL of 40% Percoll in a cushion of 0.5 mL of 2 mol/L sucrose. Self-forming gradients were generated by centrifugation at 20 000 rpm for 3 hours at 4°C in a Beckman SW-40 Ti rotor. Fractions of 0.5 mL were collected from the bottom of the gradient and assayed for radioactivity by using an LKB 1282 gamma counter. Recoveries of radiolabel consistently exceeded 90%. Individual fractions were frozen at −20°C and assayed the following day for neutral α-glucosidase and acetylglucosaminidase. Density profiles of blank gradients spun in parallel were measured using a digital densitometer.

Enzyme Assays

Endoplasmic reticulum and lysosomes were identified in density-gradient fractions by assay of neutral α-glucosidase and β-N-acetylglucosaminidase activities by using the corresponding 4-methylumbelliferyl substrates.23,24 Latency of β-N-acetylglucosaminidase was determined as the ratio of activity in the postnuclear supernatant without Triton X-100 to that with 0.1% Triton X-100.

Analytic Procedures

Protein was assayed by the Lowry method in the presence of 0.05% sodium deoxycholate with BSA as the standard. Lipoprotein electrophoresis was performed in 50 mmol/L barbital buffer (pH 8.6) with a Corning apparatus and Universal agarose film. Lipoprotein bands were visualized by staining with fat red.

Results

Different Intracellular Localization of Oxidized LDL and Acetyl LDL in Normal Macrophages

To determine whether oxidized LDL and acetyl LDL were trapped within the organelle in which the labeled lipoprotein is ultimately degraded or deposited,22 Results in Figure 1 show that there was minimal colocalization of oxidized LDL and acetyl LDL in macrophages from wild-type mice. Calculated values for coincidence derived from deconvolution analysis confirmed that the 2 fluorophores are present in different compartments (the Table).

Partial Colocalization of Acetyl LDL With Oxidized LDL in SR-AI/II–Knockout Macrophages

We recently reported that ≈80% of the uptake of acetyl LDL is mediated by SR-AI/II and that ≈70% of the uptake of

Colocalization of Oxidized LDL and Acetyl LDL in Macrophages

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Incubation Time, h</th>
<th>Colocalization, %</th>
<th>n</th>
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<tbody>
<tr>
<td>Wild-type mice</td>
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<tr>
<td>Oxidized LDL with acetyl LDL</td>
<td>0.5</td>
<td>6.0±3.4</td>
<td>4</td>
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<tr>
<td></td>
<td>1.5</td>
<td>13.5±5.2</td>
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<td></td>
<td>3</td>
<td>24.5±11.5</td>
<td>4</td>
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<td></td>
<td>6</td>
<td>10.3±5.1</td>
<td>4</td>
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<tr>
<td>Oxidized LDL with aggregated LDL</td>
<td>6</td>
<td>4.9±2.6</td>
<td>5</td>
</tr>
<tr>
<td>SR-AI/II–knockout mice</td>
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<tr>
<td>Oxidized LDL with acetyl LDL</td>
<td>0.5</td>
<td>22.6±15.6</td>
<td>4</td>
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<tr>
<td>Acetyl LDL with oxidized LDL</td>
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<td>87.6±3.3</td>
<td>4</td>
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<tr>
<td>Oxidized LDL with acetyl LDL</td>
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<td>51.5±21.8</td>
<td>4</td>
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<td>62.1±10.1</td>
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<td>Oxidized LDL with acetyl LDL</td>
<td>3</td>
<td>55.2±15.0</td>
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<td>Acetyl LDL with oxidized LDL</td>
<td>50.3±13.6</td>
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<td>Oxidized LDL with acetyl LDL</td>
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<td>36.8±8.8</td>
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<tr>
<td>Acetyl LDL with oxidized LDL</td>
<td>56.5±9.1</td>
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</table>

Resident peritoneal macrophages were incubated with DiI-labeled oxidized LDL and DiO-labeled acetyl LDL for varying times. Cells were then fixed and examined by deconvolution fluorescence microscopy as described in Methods. Colocalization was calculated as the percentage of voxels containing the fluorophore tracing the first listed molecule, which also contains the fluorophore tracing the second listed molecule. The total number of cells analyzed from 2 independent experiments is indicated by n.
oxidized LDL is mediated by a different receptor. The clearance of radioiodinated acetyl LDL from the circulation of SR-AI/II–deficient mice was as rapid as that in wild-type mice, and we hypothesized that acetyl LDL could act as a ligand for the oxidized LDL receptor, although its apparent affinity was an order of magnitude lower than that of oxidized LDL. If this were the case, then one would expect a greater degree of colocalization of acetyl LDL with oxidized LDL in SR-AI/II–knockout macrophages. To test this hypothesis, macrophages from SR-AI/II–deficient macrophages were examined after incubation with DiI-labeled oxidized LDL and DiO-labeled acetyl LDL. As shown in Figure 2 and the Table, >50% of internalized acetyl LDL colocalized with oxidized LDL in macrophages lacking SR-AI/II. This finding supports the notion that the different intracellular localization of oxidized LDL and acetyl LDL in macrophages may in part be related to specific uptake pathways.

**Localization of Oxidized LDL Cannot Be Explained by Aggregation**

Extensively oxidized LDL has a tendency to aggregate, and it is possible that the intracellular localization as well as the catabolism of oxidized LDL could differ from that of acetyl LDL because of accumulation of aggregates of oxidized LDL in phagosomes. As well, it has recently been shown that aggregated LDL accumulates within a “surface-connected compartment” in human macrophages, and this could be another explanation for the different localization of oxidized LDL and acetyl LDL. To test these possibilities, we compared the intracellular location of oxidized LDL to that of LDL that had been induced to aggregate by vortexing. Macrophages from wild-type mice were incubated with DiI-labeled oxidized LDL together with DiO-labeled, vortex-aggregated LDL and analyzed by digital deconvolution microscopy. Results in the Table indicate only 5% coincidence in the distribution of oxidized LDL and aggregated LDL. In contrast to ligands internalized by endocytosis or phagocytosis, ligands internalized in the “surface-connected compartment” are trypsin releasable. To rule out the possibility that oxidized LDL is sequestered in a surface-connected compartment (and is therefore trypsin releasable), macrophages from CD-1 mice were preincubated with radioiodinated or Di labeled, oxidized LDL or the corresponding vortex-aggregated LDL for 4 hours at 37°C; washed; and incubated for an additional 30 minutes in α-MEM or in α-MEM containing 50 μg/mL trypsin. This concentration of trypsin was previously shown to release 90% of aggregated 125I-LDL from macrophages, and this was interpreted as evidence that the aggregated LDL was in a surface-connected compartment. Neither the cell-associated radioactivity nor the DiI fluorescence with oxidized LDL was altered by trypsin treatment (not shown). As expected with vortexed LDL, large extracellular aggregates were seen by microscopy, and these were partly degraded by trypsin, but intracellular fluorescence was not altered by trypsin. Thus, aggregation cannot account for the different intracellular localization of oxidized LDL compared with acetyl LDL.

**Subcellular Fractionation of Macrophages Incubated With Oxidized LDL**

As endosomal vesicles traverse the endocytic pathway, they are thought to become acidified, acquire proteolytic enzymes, and increase in density. Uptake of oxidized LDL by a receptor(s) other than the SR-AI/II could result in inefficient delivery of ligand to lysosomes, with accumulation of oxidized LDL in an endosomal compartment. To determine whether oxidized LDL is localized in a prelysosomal compartment, macrophages from CD-1 mice were incubated for 2 or 16 hours with oxidized LDL that had been labeled with 99mTc and then disrupted by N2 cavitation, and the postnuclear supernatant was fractionated on Percoll gradients. 99mTc crosses organelle membranes very slowly and serves as a “trapped label” that marks the site of accumulation or degradation of the protein to which it was coupled. As shown in Figure 3, after a 2-hour incubation of macrophages with 99mTc–oxidized LDL, the activity of the lysosomal enzyme marker β-N-acetylglucosaminidase was concentrated primarily at a density of 1.13 g/mL, indicative of dense lysosomes. A small peak of activity was localized at a density of 1.045 g/mL, consistent with endosomes. At 2 hours’ incubation, 99mTc was equally distributed between the endosomal and lysosomal density fractions, but after 16 hours nearly all of the radioactivity was in the lower-density fraction. To confirm that the 99mTc was still within intact organelles and did not represent free 99mTc or undegraded, 99mTc–oxidized LDL that had been released from lysosomes, macrophages were disrupted and then 99mTc-LDL was added to the postnuclear supernatant before fractionation. More than 98% of the radioactivity was recovered at the top of the gradient at d<1.030 g/mL (data not shown).

Unexpectedly, almost all of the β-N-acetylglucosaminidase activity had also shifted to low-density fractions. The disappearance of the dense lysosomal peak in subcellular fractions after overnight incubation of macrophages with oxidized LDL suggests that oxidized LDL is not accumulating in a prelysosomal compartment but rather that LDL-containing endosomes fuse with lysosomes and cause them to undergo a density shift. The density shift is not an artifact of the 99mTc trapped label, because a similar shift was observed using 125I-labeled oxidized LDL (not shown).

Oxidized apoB has recently been shown to accumulate in the lysosomes of J774 macrophages, but no lysosomal density shift was reported after a 24-hour exposure of J774 macrophages to oxidized LDL. To determine whether this discrepancy with our findings reflected a difference between...
J774 cells and primary macrophages, we repeated the above experiment with J774 cells, and there was indeed no lysosomal density shift in J774 macrophages incubated with oxidized LDL (Figure 4). The total uptake of oxidized LDL by J774 cells in this experiment was 13.3 µg/mg of cell protein. This is <20% of that seen with mouse peritoneal macrophages, consistent with previous observations that SR activity is lower in J774 macrophages than in primary macrophages.32 Hence, it is possible that J774 cells simply do not accumulate oxidized LDL rapidly enough to cause a lysosomal density shift.

Expansion of the Acidic Compartment in Macrophages Incubated With Oxidized LDL

Oxidized LDL contains reactive aldehydes that are capable of forming covalent adducts with amino or thiol groups of proteins.33,34 This reaction is favored at lysosomal pH and can induce lysosomal dysfunction.10,11 To determine whether the lysosome-derived compartment in which oxidized LDL accumulates retains the capacity for acidification, macrophages from wild-type mice or SR-AI/II–knockout mice were incubated with DiI-labeled, oxidized LDL or DiI-labeled, acetyl LDL; washed; and then incubated with the pH-sensitive fluorescent probe LysoSensor yellow/blue DND-160. This probe is a weak base that accumulates in acidic organelles as a result of protonation and has pH-dependent excitation and emission spectra such that at pH <5, it has predominantly yellow fluorescence and at pH 6 to 7 it has blue fluorescence. Results in Figures 5C and 5F show that there is intense yellow fluorescence of the LysoSensor probe that colocalized with DiI fluorescence from acetyl LDL (Figure 5B) and oxidized LDL (Figure 5E), indicating that both acetyl LDL and oxidized LDL are delivered to acidic compartments consistent with functional lysosomes. Many more organelles with fluorescence from the LysoSensor probe were seen in macrophages incubated with oxidized LDL (Figure 5F) compared with macrophages that had been incubated without LDL or with acetyl LDL (Figure 5C), demonstrating that the lysosomal compartment appears to increase substantially in volume with exposure of macrophages to oxidized LDL.

Accumulation of Oxidized LDL Is Associated With Impaired Lysosomal Hydrolysis of LDL Sphingomyelin

Although resistance of oxidized LDL to cathepsins has been reported by several groups to account for intracellular accumulation of undegraded oxidized LDL in macrophages,5,12,13 inactivation of lysosomal hydrolases may also contribute.10

Figure 3. Subcellular fractionation of primary macrophages exposed to oxidized LDL. Resident peritoneal macrophages were incubated for 2 hours (A) or 16 hours (B) with 20 µg/ml 99mTc–oxidized LDL (electrophoretic mobility 2.7 relative to native LDL). Cells were washed and disrupted by N2 cavitation, and postnuclear supernatants were fractionated on 40% Percoll and 250 mmol/L sucrose at 45 000 g for 3 hours. Fractions were assayed for radioactivity (○), the lysosomal marker enzyme β-N-acetylglucosaminidase (■), and the endoplasmic reticulum marker enzyme neutral α-glucosidase (▲). The upper 2 curves have been shifted along the y axis to minimize overlap.

Figure 4. Subcellular fractionation of J774 macrophages exposed to oxidized LDL. Macrophages were incubated for 16 hours without (A) or with (B) 20 µg/ml 125I–oxidized LDL (electrophoretic mobility 3.2 relative to native LDL). Cells were washed and disrupted by N2 cavitation, and post-nuclear supernatants were fractionated as described in Methods. Symbol assignments are as indicated in the legend to Figure 3.
We previously reported that the degradation of acetyl LDL was normal in macrophages simultaneously incubated with oxidized LDL and interpreted this phenomenon as evidence against global lysosomal dysfunction. However, if oxidized LDL and acetyl LDL are in fact in different intracellular compartments, then this conclusion may be incorrect. To address this question, C6 NBD-sphingomyelin was incorporated into oxidized LDL or acetylated LDL, and the labeled LDLs were incubated with mouse peritoneal macrophages. As shown in Figure 6C, in macrophages incubated with oxidized LDL, there was extensive accumulation of C6 NBD-sphingomyelin within dispersed vesicles. In contrast, in cells incubated with acetyl LDL, there was no retention of C6 NBD (Figure 6D), indicating hydrolysis and release of the fluorophore from the lysosomes. This supports the suggestion that intralysosomal accumulation of oxidized LDL results in inactivation of hydrolyses in the lysosomal compartment to which oxidized LDL is delivered. However, it remains possible that resistance of oxidized apoB to degradation somehow protects sphingolipids in the same LDL particle from degradation.

Discussion

It is generally assumed that after receptor-mediated endocytosis of lipoproteins by macrophages, lipoprotein-derived cholesterol is released from lysosomes, enters a cellular compartment accessible to ACAT, becomes esterified, and is stored as cytoplasmic lipid droplets. However, ultrastructural studies of macrophage-derived foam cells within atherosclerotic lesions in animals suggest that a significant part of the lipid deposits in these cells is in lysosomes. Similarly, in macrophage foam cells in human atherosclerotic lesions, a substantial proportion of lipid is found within membrane-bound vacuoles, at least some of which appear to be of lysosomal origin.

We previously showed that cholesterol delivered to macrophages by oxidized LDL does not readily enter the ACAT substrate pool and that this was due to impaired lysosomal degradation of oxidized LDL. Thus, in contrast to acetyl LDL, which is efficiently degraded and is a potent stimulator of ACAT that leads to formation of cytoplasmic cholesterol ester droplets, oxidized LDL causes intralysosomal lipid deposition, analogous to findings in foam cells in atherosclerotic lesions. Such intralysosomal lipid deposits are of potential pathophysiological importance because they are inaccessible to regulated cholesterol-efflux pathways. For example, Mahlberg and coworkers showed that cholesterol ester droplets were internalized by J774 cells, resulting in foam cell morphology. Most of the internalized lipid remained within lysosome-like structures, and only ~10% of the free cholesterol that was generated by lysosomal cholesterol esterases was subsequently reesterified by ACAT. Subcellular fractionation confirmed that the accumulation of free cholesterol was within lysosomes and that this pool of cholesterol could not be mobilized by addition of cholesterol acceptors, such as HDL, to the medium.

Jerome and colleagues recently reported that prolonged incubation of mildly oxidized LDL with pigeon monocye-
derived macrophages or human THP-1 cells resulted in accumulation of cholesterol and cholesterol ester in lysosomes and in acid phosphatase–positive tubular organelles that were thought to represent the Golgi and trans-Golgi network. Similar incubations with acetyl LDL caused lipid accumulation in cytoplasmic droplets, but no expansion of Golgi-like organelles.18 The oxidized LDL preparations used in these studies had the same electrophoretic mobility as native LDL and had thiobarbituric acid–reactive substances values of 5 to 10 nmol/mg protein, thereby fulfilling the physical criteria of “minimally modified LDL.”47–50 By definition, such minimally oxidized LDL is not recognized by SRs but remains a ligand for LDL receptors and, therefore, does not cause cholesterol accumulation in macrophages.5,8,20,47–50 Jerome et al18 also found no evidence of other modifications, such as LDL aggregation, to account for the increased uptake, and so the most obvious explanation for their apparently paradoxical finding that cholesterol loading with mildly oxidized LDL was the same as that with acetyl LDL is that during the prolonged incubation of mildly oxidized LDL with macrophages, there was further oxidation of LDL, sufficient to permit uptake via SRs. If one accepts this explanation, then our findings and those of Jerome et al, in that extensively oxidized LDL was used, intracellular localization of modified LDLs was done simultaneously and at early as well as late time points by using fluorescent rather than ultrastructural techniques, expansion of the lysosomal compartment was demonstrated morphologically with a lysosomotropic fluorophore, and cell fractionation studies were done to show that oxidized LDL caused a density shift of the lysosomal enzyme pool. We were unable to resolve Golgi from endosomes and other organelles by Percoll density centrifugation and so are unable to support or refute the suggestion that oxidized LDL accumulates in a Golgi-derived compartment. However, the microscopic studies with a pH-sensitive fluorophore (LysoSensor yellow/blue DND 160) suggest that expansion involves a highly acidic compartment, which would be more consistent with a lysosome-derived compartment rather than the Golgi. On the other hand, lysosomes and the Golgi are functionally related, and it is conceivable that oxidized LDL is indeed targeted to the Golgi, but that the sorting of membrane domains is impaired in lysosomes containing oxidized LDL, resulting in persistent acidification after fusion with Golgi elements.

The lysosomal compartment in macrophages exists as an extensive, tubuloreticular network built on a “scaffolding” of microtubules that can undergo extensive rearrangements.14,15 Typically, tubular elements are transformed to spherical vesicles on fusion with endosomes or phagosomes or when an osmotic load is applied to lysosomes.14,15 Lang and col-

Figure 6. Accumulation of sphingomyelin in macrophages incubated with oxidized LDL. Resident peritoneal macrophages from wild-type mice were incubated for 6 hours at 37°C with 10 μg/mL C6NBD-sphingomyelin–labeled oxidized LDL (A, C) or C6NBD-sphingomyelin–labeled acetyl LDL (B, D). Cells were then washed and examined with a 100× objective and phase contrast (A, B) or a fluorescein filter set (C, D).
leagues\textsuperscript{51} reported the interesting observation that when murine bone marrow--derived macrophages were incubated with \textit{Bacillus subtilis}, the intracellular location of levan (a slowly degraded polysaccharide antigen of \textit{B subtilis}) was different from that of rapidly degraded bacterial antigens. Levan and other slowly degraded substances such as horseradish peroxidase and dextran initially appeared in a perinuclear lysosome-like compartment but were then routed to a “dispersed compartment” of small vesicles distributed throughout the cell.\textsuperscript{16} This dispersed compartment accumulated a weakly basic fluorophore, contained immunoreactivity for cathepsins D and L as well as for the 100-kDa lysosomal glycoprotein, and was therefore thought to be derived from lysosomes.\textsuperscript{16} The appearance and location of this dispersed compartment are strikingly similar to the pattern shown in Figure 5E with oxidized LDL. Our data are insufficient to confirm or refute the suggestion by Lang et al that this compartment is derived from secondary lysosomes. However, several indirect lines of evidence from the present studies are more consistent with the hypothesis that oxidized LDL and acetyl LDL proceed along independent endocytic pathways throughout their intracellular course. First, even at 0.5-hour incubation with macrophages, there was very little colocalization of oxidized LDL with acetyl LDL. Second, colocalization of oxidized LDL with acetyl LDL was much higher in SR-AI/II--knockout macrophages than in wild-type cells (>50\% versus 10\%). Acetyl LDL is a weak ligand for oxidized LDL receptors, and in the absence of SR-AI/II, part of its internalization is expected to occur through oxidized LDL receptors.\textsuperscript{5,27} Hence, increased colocalization in this setting suggests that the specific cell-surface receptors responsible for internalizing oxidized LDL play a role in directing its intracellular processing.

It is noteworthy that oxidized LDL caused a dramatic decrease in the density fraction in which lysosomal marker enzymes were recovered and a marked increase in the number of acidified vesicles in macrophages. At 2 hours of incubation of macrophages with oxidized LDL, there was a bimodal density distribution of lysosomal marker enzyme activity. This result suggests that there is a quantum shift in the density of individual lysosomal elements on fusion with oxidized LDL--containing endosomes rather than a gradual shift in density of the entire lysosomal enzyme pool. After 16 hours, essentially all of the lysosomal marker enzyme activity was in the low-density fractions. One model of the interaction of late endosomes and lysosomes proposes that late endosomes fuse with dense lysosomes to form hybrids where hydrolytic degradation of internalized material occurs.\textsuperscript{52} As the hydrolysis products are released into the cytosol, a wortmannin-sensitive process causes recycling of excess membrane components, leading to the regeneration of small dense lysosomes. Osmotically active trapped components, or, as in the case of oxidized LDL, poorly degradable ligands prevent this recycling process and lead to a marked expansion of the acidic compartment associated with a shift to lower density of the pool of acid hydrolytic enzymes. As well, oxidized LDL contains reactive aldehydes that are capable of directly binding to and inactivating lysosomal cysteine proteases,\textsuperscript{10,11} and it is possible that they could also cause dysfunction of other lysosomal proteins required for processing and recycling of the hybrid lysosomes. The changes in the distribution and density of the lysosomal compartment in macrophages incubated with oxidized LDL are consistent with this model. However, it does not explain the lack of colocalization of oxidized LDL and acetyl LDL, because if both ligands were delivered to the same late endosomal compartment, then one would expect the respective tracers to both be present in the same hybrid compartment (assuming that the fluorescent probes used in these experiments cross lysosomal membranes slowly in relation to the time course of these experiments and that there is no selective sorting of fluorophore from degraded acetyl LDL versus that of undegraded oxidized LDL within the same hybrid organelle). We have not validated these assumptions in the present work, but there is reasonable evidence that DiI and DiO are effectively trapped in lysosomes. It is conceivable that sorting of undegraded oxidized LDL might occur in hybrids, but if that were the case, then the remaining organelle would be able to continue the maturation and regenerate dense lysosomes, which was not observed in our density fractionation experiment.

The present studies extend previous observations by showing that not only is oxidized LDL retained within lysosomes but also that it is found in a compartment that is morphologically distinct from that associated with acetyl LDL or aggregated LDL. This difference was seen with short as well as longer incubation times, and so it appears that oxidized LDL and acetyl LDL follow independent endocytic pathways. There are several mechanisms that could contribute to the divergence of oxidized LDL and acetyl LDL processing. The earliest step at which segregation could occur is at the level of the plasma membrane. About 80\% of the uptake of acetyl LDL is mediated by SR-AI/II, and this receptor leads to internalization via clathrin-coated vesicles. In contrast, 70\% of oxidized LDL uptake is mediated by a different receptor or receptors.\textsuperscript{5} The receptor for oxidized LDL has not been identified, but an increasing number of cell-surface molecules that bind to oxidized LDL are being identified. The list of such candidate oxidized-LDL receptors includes CD36,\textsuperscript{53--55} macrosialin/CD68,\textsuperscript{56,57} F\textsubscript{c}yRIIb\textsubscript{2},\textsuperscript{58} MARCO,\textsuperscript{59,60} LOX-1,\textsuperscript{61} and SREC.\textsuperscript{62} If the receptor(s) that accounts for most of the endocytosis of oxidized LDL is found to be clathrin independent, then it is possible that segregation of endocytic pathways could begin at the receptor level. However, the endocytic pathway is a complex dynamic process that, in addition to receptors and specialized plasma membrane domains, also involves cytoskeletal elements such as actin filaments and microtubules, small GTP-binding proteins of the Rho and Rab families, vesicle formation and maturation controlled by specific targeting and fusion proteins, and signaling pathways including phosphatidylinositol 3-kinase.\textsuperscript{63--67} In view of this complexity, it is certainly possible that the divergence in the processing of oxidized LDL and acetyl LDL occurs at 1 or more of these later steps.

Acknowledgments
This study was supported by grant MT8630 from the Medical Research Council (MRC) of Canada to U.P.S. and by grant MT12875 from the MRC and a grant-in-aid from the Heart and Stroke Foundation of BC and Yukon to E.D.W.M.

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Uptake of Oxidized LDL by Macrophages Differs From That of Acetyl LDL and Leads to Expansion of an Acidic Endolysosomal Compartment
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doi: 10.1161/01.ATV.19.8.1881
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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