Low Density Lipoprotein Undergoes Oxidation Within Lysosomes in Cells

Yichuan Wen, David S. Leake

Abstract—The oxidized low density lipoprotein (LDL) hypothesis of atherosclerosis proposes that LDL undergoes oxidation in the interstitial fluid of the arterial wall. We have shown that aggregated (vortexed) nonoxidized LDL was taken up by J774 mouse macrophages and human monocyte-derived macrophages and oxidized intracellularly, as assessed by the microscopic detection of ceroid, an advanced lipid oxidation product. Confocal microscopy showed that the ceroid was located in the lysosomes. To confirm these findings, J774 macrophages were incubated with acetylated LDL, which is internalized rapidly to lysosomes, and then incubated (chase incubation) in the absence of any LDL. The intracellular levels of oxysterols, measured by HPLC, increased during the chase incubation period, showing that LDL must have been oxidized inside the cells. Furthermore, we found that this oxidative modification was inhibited by lipid-soluble antioxidants, an iron chelator taken up by fluid-phase pinocytosis and the lysosomotropic drug chloroquine, which increases the pH of lysosomes. The results indicate that LDL oxidation can occur intracellularly, most probably within lysosomes. (Circ Res. 2007;100:1337-1343.)

Key Words: atherosclerosis ■ ceroid ■ lysosome ■ iron ■ oxidized low density lipoprotein

The local oxidation of low density lipoprotein (LDL) within atherosclerotic lesions is widely believed to be of importance in the pathogenesis of atherosclerosis.1 LDL is thought to be oxidized within the extracellular space of atherosclerotic lesions and then to be bound by scavenger receptors and taken up by macrophages, which become cholesterol-laden foam cells, a major feature of atherosclerotic lesions.2 Among many other effects, oxidized LDL increases the expression of cellular adhesion molecules and chemokines,3,4 increases the production of metalloproteinases,5 which probably destabilize the fibrous caps over advanced lesions, and induces apoptosis in cells.6 The mechanisms by which LDL is oxidized in atherosclerotic lesions remain uncertain, despite a great deal of work.7

The oxidation hypothesis of atherosclerosis needs to address the high antioxidant capacity of extracellular fluids. Even a few percent of serum or interstitial fluid can inhibit greatly the oxidation of LDL by cells.8,9 We postulated that LDL oxidation might occur not within the interstitial fluid of atherosclerotic lesions but within lysosomes in macrophages in atherosclerotic lesions.

Materials and Methods

LDL Isolation and Modification

Blood was taken from healthy volunteers with EDTA as the anticoagulant (final concentration 3 mmol/L). LDL (1.019 to 1.063 g/mL) was isolated from the plasma by sequential density ultracentrifugation at 4°C, as described previously.10 LDL was stored in the dark under argon at 4°C and used within 1 month. Aggregation of LDL was achieved by vortexing11 or acetylation.12 Acetylation of LDL was confirmed by agarose gel electrophoresis (Paragon gels; Beckman), as seen by an increase of about 4.5 in electrophoretic mobility relative to native LDL.

Cell Culture

Cell culture media (DMEM, RPMI 1640, and Ham’s F-10) and phosphate buffered saline (PBS) (without calcium or magnesium) were obtained from Gibco Life Technologies. The media used in this study were supplemented with 20% (v/v) fetal calf serum, Glutamax (2 mM/L), penicillin (50 IU/mL), streptomycin (50 µg/mL), and amphotericin B (0.95 µg/mL), unless otherwise stated. Humidified 95% air/5% carbon dioxide at 37°C was used for cell culture. J774 cells were regularly cultured in supplemented DMEM, whereas human monocytes were prepared from the blood of healthy adults using a commercially available kit (Nycodet 1.086, AXIS-SHIELD PoC AS)13 and incubated in DMEM without serum for 24 hours before culturing in supplemented DMEM. Human monocytes were cultured on glass coverslips for 7 days to allow them to differentiate into macrophages before LDL was added. For measuring the intracellular oxysterols, J774 were cultured at a density of 150 000/mL in 6-well plates (35 mm diameter, 1 mL/well) for 24 hours before acetylated LDL was added.

Detection of Ceroid

For detection of ceroid, cells were treated with vortexed LDL at 200 µg protein/mL in DMEM with serum for 7 days, with the medium being changed every 2 days. Control cells were cultured without vortexed LDL. Cells were coincubated with fluorescent dextran (50 µg/mL; Alexa Fluor 647, Molecular Probes) and vortexed LDL...
(200 μg protein/mL) for the study of the colocalization of lysosomes and ceroid. Coverslips were fixed with 4% (v/v) formaldehyde in PBS. They were stained with either Oil Red O to demonstrate intracellular lipid droplets or with Oil Red O after treatment with ethanol and xylene, 5 minutes each, to demonstrate ceroid. Ceroid was detected using light microscopy (Axioskop 2, Carl Zeiss Ltd) and the colocalization of ceroid and lysosomes was shown using confocal microscopy (TCS-NT, Leica Microsystems). To detect fluorescent dextran, a krypton/argon laser with an excitation wavelength of 647 nm was used with a 610-nm dichroic mirror combined with a long pass 665-nm filter. For measuring Oil Red O stained lipidic extracellular oxidation that consists of insoluble polymerized lipids and is found within foam cells in atherosclerotic lesions. Ceroid (lipofuscin) is a final product of lipid oxidation and is formed in both J774 and HMDM cells after 7 days of incubation with LDL. Ceroid was present in cells that had been incubated for 7 days with native LDL (Figure 2C). Furthermore, we demonstrated that ceroid was also formed in HMDM and was colocalized with some of the fluorescent dextran-labeled lysosomes in both J774 and HMDM cells (Figure 2E to 2J).

Results
J774 cells (a mouse macrophage-like cell line) were cultured for 24 hours in Dulbecco modified Eagle medium (DMEM) containing nonoxidatively modified aggregated LDL produced by vortexing, which is taken up rapidly by macrophages. Numerous lipid droplets were present in cells incubated with aggregated LDL, but not in control cells, when stained by Oil Red O and examined by light microscopy (Figure 1A and 1B) or in an unstained state when examined by UV microscopy (Figure 1C and 1D). Coincubating cells with fluorescent dextran and aggregated LDL and examining them by confocal microscopy revealed that the lipid droplets were localized mainly in dextran-labeled lysosomes (Figure 1E through 1G). Cells incubated without aggregated LDL showed fluorescent lysosomes, but no lipid droplets (Figure 1H and 1I). HPLC showed that cells incubated with aggregated LDL were rich in both cholesteryl arachidonate and cholesteryl linoleate, but these lipids were absent in control cells (results not shown). Nonesterified cholesterol was increased by about 40% in cells incubated with aggregated LDL.

Ceroid (lipofuscin) is a final product of lipid oxidation that consists of insoluble polymerized lipids and is found within foam cells in atherosclerotic lesions. Ceroid is formed in lysosomes attributable to an iron-catalyzed oxidative process, and its production can be diminished by antioxidants or iron chelators. It can be detected as Oil Red O-stained lipid after other lipids have been removed by organic solvents.

We cultured J774 cells and human monocyte-derived macrophages (HMDM) in DMEM containing aggregated LDL for 7 days to examine the formation of ceroid. Many droplets of lipids, detected by fluorescence (Figure 2A) or Oil Red O staining (Figure 2B), were present in the J774 cells after 7 days, whereas none were visible in cells incubated with native LDL for 7 days (supplemental Figure IA and IB, available online at http://circres.ahajournals.org). Cells on coverslips were treated with ethanol and xylene to remove soluble lipids, followed by Oil Red O to stain ceroid. In contrast to the situation after 24 hours, ceroid was clearly visible in the form of irregularly shaped granules after the cells were treated with ethanol/xylene and stained with Oil Red O (Figure 2D). No ceroid was present in cells that had been incubated for 7 days with native LDL (Figure 2C). Furthermore, we demonstrated that ceroid was also formed in HMDM and was colocalized with some of the fluorescent dextran-labeled lysosomes in both J774 and HMDM cells (Figure 2E to 2J).

As oxyesters are present in significant amounts in human atherosclerotic lesions and 7-ketocholesterol is a major oxidation product of cholesterol in LDL oxidized in vitro, we measured their intracellular levels in macrophages. J774 cells were incubated with acetylated LDL (50 μg protein/mL) for 24 hours in RPMI 1640 medium containing 20% (v/v) fetal calf serum. RPMI 1640 was chosen because it does not contain any added transitional metal ions and does not support the oxidation of LDL by cells. LDL was washed off and the medium was replaced by DMEM, RPMI 1640, or Ham’s F-10 containing 20% (v/v) lipoprotein-deficient fetal calf serum, but no LDL, for a further 48 hours (chase incubation). Using a medium containing lipoprotein-deficient fetal calf serum for the chase incubation eliminated the possibility that extracellular lipoproteins would be oxidized in the medium and taken up by the cells, so that oxidized lipids appearing in the cells must have been formed by the intracellular oxidation of LDL. Compared with the baseline level at the end of 24 hours incubation with acetylated LDL, 7-ketocholesterol increased significantly in cells subsequently incubated in DMEM and more so in Ham’s F-10 medium, but did not increase in RPMI 1640 medium (Figure 3A). Both DMEM and F-10 media are formulated to contain transition metal ions (0.25 μmol/L iron in DMEM; 3 μmol/L iron in F-10 medium).
iron and 10 nmol/L copper in F-10), whereas RPMI has no added iron or copper.

The total intracellular levels of oxysterols were assessed after 48 hours of chase incubation with Ham’s F10 as described above. Esterified oxysterols were converted into free oxysterols by saponification and then measured by normal phase HPLC. Compared with free oxysterol levels in unsaponified samples, 7-ketocholesterol increased by 4 times (7\( \beta \)-OOH-cholesterol and 7\( \beta \)-OH-cholesterol increased by 5 and 7 times, respectively).

Significant inhibition of 7-ketocholesterol production was produced by the lipid-soluble antioxidants probucol (5 \( \mu \)mol/L), \( \alpha \)-tocopherol (50 \( \mu \)mol/L), and butylated hydroxytoluene (30 \( \mu \)mol/L) (Figure 3B), but no effects were seen with the water-soluble antioxidant Trolox (100 \( \mu \)mol/L). The cellular levels of these antioxidants increased from zero to 7.5±3.3 for probucol, from 0.42±0.16 to 2.16±0.61 for \( \alpha \)-tocopherol, and from zero to 0.65±0.23 nmol/mg cell protein (mean±SEM, n=3) for butylated hydroxytoluene after 48 hours incubation.

The lysosomotropic agent, chloroquine (100 \( \mu \)mol/L), was added during the chase incubation after loading cells with acetylated LDL and resulted in a significantly reduced 7-ketocholesterol level in the cells, suggesting that the lysosomes were responsible for the oxidation of LDL (Figure 3C and 3D).

Adding FeSO\(_4\) or ferritin to the macrophages during the chase incubation period approximately doubled the production of 7-ketocholesterol, whereas the iron-chelator, desferrioxamine, significantly inhibited the generation of 7-ketocholesterol in the presence of FeSO\(_4\) by about 31% (Figure 3D).

A low concentration of FeSO\(_4\) (5 \( \mu \)mol/L) alone oxidized LDL effectively in a cell-free system at pH 4.5, but not at pH 7.4, whereas the opposite was the case for CuSO\(_4\), (Figure 4). The increase in absorbance up to about 0.8 with iron at pH 4.5 was attributable to conjugated diene formation (ie, to lipid oxidation). The further increase in “absorbance” was attributable to the scattering of ultraviolet radiation resulting from the aggregation of LDL (as demonstrated by measuring light scattering at 680 nm; results not shown), and the late decrease in absorbance is attributable to the sedimentation of aggregated LDL.

**Discussion**

The present study was designed to test the possibility that LDL may be oxidized inside the lysosomes of macrophages, the primary source of foam cells in atherosclerosis.

We have demonstrated that nonoxidized aggregated (vortexed) LDL causes large scale lipid accumulation in macrophages and that this lipid accumulates in lysosomes (Figure 1). When large amounts of LDL are delivered to lysosomes after endocytosis, it overwhelms the capability of the lysoso-
mal acidic lipases to degrade it and release the lipids from these organelles. It is known that large amounts of cholesterol and cholesteryl esters accumulate in the lysosomes of foam cells in atherosclerotic lesions.\(^{25}\) The accumulated lipid within the lysosomes of the J774 macrophages and the human monocyte-derived macrophages became oxidized because ceroid, an advanced lipid oxidation product present in human atherosclerotic lesions,\(^{20}\) accumulated in these organelles (Figure 2). The possibility that the aggregated LDL was oxidized in the extracellular medium was eliminated by using a medium, DMEM, that does not support the extracellular oxidation of LDL by cells,\(^{26}\) especially in the presence of 20% (v/v) serum, a strong antioxidant.\(^{8,9}\) The ceroid was present in lysosomes because confocal microscopy showed that it colocalized with a lysosomal marker (Figure 2).

To confirm this finding, J774 macrophages were incubated with nonoxidized acetylated LDL, which is taken up rapidly and causes large scale lipid accumulation in macrophages.\(^{27}\) The acetylated LDL was then washed away and the cells were chased with a medium supplemented with lipoprotein-deficient fetal calf serum in the absence of LDL, so that no lipoprotein would be present extracellularly for the cells to oxidize. Therefore any oxidized lipids generated in the cells must have been formed by the intracellular oxidation of LDL already taken up. Oxysterols (7-ketocholesterol, 7β-OH-cholesterol and 7β-OH-cholesterol) were generated in the cells during the chase incubation period (Figure 3).

The increase in oxysterols was inhibited by the lipid-soluble antioxidants probucol, α-tocopherol, and butylated hydroxytoluene, which were shown to be taken up by the cells, but not by the water-soluble antioxidant Trolox. The water-soluble antioxidant Trolox may not be able to enter the lysosomes efficiently and therefore failed to provide protection against the intralysosomal oxidation of LDL. If the oxidation of LDL had been extracellular, it should have been inhibited by Trolox.\(^{28}\)

Increasing the pH of lysosomes may inhibit the oxidation of LDL within these organelles, as LDL oxidation can be promoted by acidic pH.\(^{20}\) Weak bases, such as chloroquine, may accumulate to about 1000 times their extracellular

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**Figure 2.** Macrophages incubated with aggregated LDL generate intralysosomal ceroid. J774 cells were cultured in DMEM containing serum and aggregated LDL (200 μg protein/mL) throughout the experiment. The medium was changed every 2 days and after 6 days the cells were allowed to adhere to coverslips for 24 hours. Cells were examined by confocal microscopy or stained with either Oil Red O to demonstrate intracellular lipid droplets or with Oil Red O after treatment with ethanol and xylene to demonstrate ceroid. A shows autofluorescent material, and B shows Oil Red O-stained lipid in cells incubated with aggregated LDL for 7 days. C shows cells that had been incubated with native LDL, and D shows cells that had been incubated with aggregated LDL for 7 days and then treated with ethanol/xylene and stained with Oil Red O to show ceroid. Scale bar, 40 μm. J774 and HMDM cells were cultured in DMEM containing aggregated LDL (200 μg protein/mL) and Alexa Fluor 647 dextran (50 μg/mL). The medium was changed every 2 days and after 7 days the cells were treated with ethanol and xylene, followed by Oil Red O staining, and examined by confocal microscopy to demonstrate ceroid. Ceroid colocalized with dextran-labeled lysosomes. Dextran and ceroid were detected with excitation wavelengths at 647 nm and 568 nm, respectively. Dextran-labeled lysosomes appear blue and are shown in a group of J774 cells (E) and HMDM (H); Oil Red O–stained ceroid appears red and is shown in the same J774 cells (F) and HMDM (I). G and J are overlay images to show the colocalization of lysosomes and ceroid as a pink color in J774 and HMDM cells, respectively. Scale bars, 40 and 10 μm, for J774 and HMDM, respectively.
concentration within lysosomes and increase the pH of lysosomes in macrophages from about 4.8 to about 6.4.30 Adding chloroquine during the chase incubation after incubation with acetylated LDL inhibited the generation of oxysterols in the cells, suggesting that the lysosomes were the sites of generation of these oxysterols and that the low pH of these organelles was important in the oxidation of LDL.

Iron may be the key factor in promoting oxidation reactions in lysosomes.31 LDL oxidation in vitro can be catalyzed by iron, and it is much faster at acidic pH.29 Adding FeSO₄ or ferritin, the main storage site for iron in the body,32 into the culture medium during the chase incubation increased the generation of oxysterols, presumably by supplying extra iron to the lysosomes, by endocytosis or by other means. Desferrioxamine, which selectively chelates iron and is taken up by cells by pinocytosis,33 inhibited the generation of oxysterols. It may be of interest that Ren et al.34 have shown that atherosclerosis in cholesterol-fed rabbits is inhibited by desferrioxamine.

It has been reported by Yuan et al.15 that lysosomes contain iron and that this iron is catalytically active. Iron staining has been reported to be common in human advanced atherosclerotic lesions and to colocalize with ceroid.36 This iron may either be derived by autophagy (ie, the normal turnover of organelles together with their iron-containing proteins) or from the endocytosis of iron-containing proteins.37 We have found that FeSO₄ is highly effective in oxidizing LDL at pH 4.5, the approximate pH of lysosomes, but very poor at doing so at pH 7.4, whereas the opposite was true for CuSO₄ (Figure 4). This finding, together with the inhibition of intracellular LDL oxidation by macrophages by desferrioxamine (Figure 3), strongly suggests that iron was catalyzing LDL oxidation in lysosomes. The lysosomal oxidation process required the combined effect of acidic pH and catalytically active iron, and was inhibited by lipid-soluble antioxidants.

We propose that LDL undergoes nonoxidative aggregation in the extracellular space of the arterial wall, by the action of factors such as sphingomyelinase,38 phospholipase A₂,39 phospholipase C,39 proteases,40,41 or proteoglycans,42 and is endocytosed rapidly by macrophages and maybe by other cells and is then oxidized in lysosomes. The initial oxidation in the lysosomes may be of aggregated LDL, but the oxidation may continue as the LDL particles are degraded by the lysosomal lipases and proteases. The release of oxidized lipids from the lysosomes into the rest of the cell, the engorgement of the lysosomes partly attributable to ceroid, or the inactivation of lysosomal enzymes by oxidized LDL,43 may possibly lead to the alteration of cell function in an
Figure 4. Native LDL (50 μg protein/mL) was incubated at 37°C with 5 μmol/L FeSO₄ or 5 μmol/L CuSO₄ at pH 4.5 or 7.4 and the Aₐ₃₈₅ monitored.

atherogenic manner. If the cells were to die and lyse, the contents of the lysosomes may be released into the interstitial fluid of atherosclerotic lesions and may include some oxidized LDL that has not yet been completely degraded (but at least some of this may be aggregated LDL).

Our findings may help to explain why the recent large clinical trials of antioxidants and cardiovascular disease have been disappointing. The antioxidants may not have entered the lysosomes efficiently or may not have remained active for long enough compared with the residence time of LDL or its lipids in these organelles.

Acknowledgments
We are grateful to Ms Jessica del Rio for skillfully isolating LDL and Dr Peter D. Weinberg for valuable discussions.

Source of Funding
This work was supported by the Wellcome Trust.

Disclosures
The University of Reading applied for a patent entitled “UK patent application no. 0413634.7, Inhibition of LDL oxidation” based on this work.

References
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Supplemental Figure 1. Macrophages incubated with native LDL do not accumulate lipid droplets. J774 cells were cultured in DMEM containing serum and native LDL (200 µg protein/ml) throughout the experiment. The medium was changed every two days and after six days the cells were allowed to adhere to coverslips for 24 hours. Cells were examined by UV microscopy (A) or stained with Oil Red O to detect intracellular lipid droplets (B). This figure should be compared to Figure 2A and B in the paper.