Uptake of Oxidized LDL by Macrophages Results in Partial Lysosomal Enzyme Inactivation and Relocation

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Abstract—The cytotoxicity of oxidized LDL (oxLDL) to several types of artery wall cells might contribute to atherosclerosis by causing cell death, presumably by both apoptosis and necrosis. After its uptake into macrophage lysosomes by receptor-mediated endocytosis, oxLDL is poorly degraded, resulting in ceroid-containing foam cells. We studied the influence of oxLDL on lysosomal enzyme activity and, in particular, on lysosomal membrane stability and the modulation of these cellular characteristics by HDL and vitamin E (vit-E). Unexposed cells and cells exposed to acetylated LDL (AcLDL) were used as controls. The lysosomal marker enzymes cathepsin L and N-acetyl-β-glucosaminidase (NAβGase) were biochemically assayed in J-774 cells after fractionation. Lysosomal integrity in living cells was assayed by the acridine orange (AO) relocation test. Cathepsin D was immunocytochemically demonstrated in J-774 cells and human monocyte-derived macrophages. We found that the total activities of NAβGase and cathepsin L were significantly decreased, whereas their relative cytosolic activities were enhanced, after oxLDL exposure. Labilization of the lysosomal membranes was further proven by decreased lysosomal AO uptake and relocation to the cytosol of cathepsin D, as estimated by light and electron microscopic immunocytochemistry. HDL and vit-E diminished the cytotoxicity of oxLDL not only partially inactivates lysosomal enzymes but also destabilizes the acidic vacuolar compartment, causing relocation of lysosomal enzymes to the cytosol. Exposure to AcLDL resulted in its uptake with enlargement of the lysosomal apparatus, but the stability of the lysosomal membranes was not changed. (Arterioscler Thromb Vasc Biol. 1998;18:177-184.)

Key Words: atherosclerosis ■ lysosomes ■ macrophages ■ oxidized LDL ■ antioxidants

LDL oxidation is considered to be an initial and important step in atherogenesis. A large body of evidence indicates that oxidized lipoproteins are present both intracellularly and extracellularly within the arterial intima of human and animal atherosclerotic lesions.1-3 OxLDL has a number of atherogenic properties, including cytotoxicity to a variety of artery wall cells, eg, endothelial and smooth muscle cells.4-6 As to the effects of oxLDL on macrophage viability, however, there are a number of somewhat conflicting reports.7-11

LDL is a lysosome-destined particle that is typically transported into cells via receptor-mediated endocytosis. Although most cells have LDL receptors on their plasma membrane, the expression of this receptor on macrophages is low. However, oxLDL is readily endocytosed by macrophages. It has been clearly shown that oxLDL accumulates in macrophage lysosomes, probably after binding to the nonspecific scavenger receptor(s).12,13 It has also been demonstrated that oxLDL is poorly degraded within macrophage lysosomes and that ceroid/lipofuscin accumulates intralysosomally after uptake of oxLDL. This is in contrast to the fate of nLDL or AcLDL, which are easily degraded by lysosomal enzymes without leaving any undegradable material.13-16 Studies in cellular and cell-free model systems have shown that oxLDL seems to inactivate lysosomal proteases, although the mechanisms involved are still not well understood.14-16 A better knowledge of that process would certainly be helpful in the elucidation of the mechanisms behind foam cell formation and atherogenesis.

UV irradiation–induced LDL oxidation has been used earlier in studies on effects of oxLDL on cultured cells.17,18 In a recent study, we used this method for preparation of oxLDL, which avoids the addition of potential cytotoxic substances such as copper, iron, or azo compounds. We found that the cytotoxicity to macrophages of UVoxLDL involved lysosomal membrane destabilization.19

In this study, we aimed to examine further the effects of UVoxLDL on macrophage lysosomes, especially their enzyme activity and membrane permeability and, in addition, the influence of HDL and vit-E on these cellular functions.

Methods

Chemicals and Culture Media

RPMI 1640, F-10 (culture media), and FCS were from GIBCO. Acetic anhydride was from Fluka Chemie (AG CH-9470 Buchs). Glutamine, penicillin G, and streptomycin were from Flow, and AO was from Gurr. Substrate Z-Phe-Arg-7AMC was from Cambridge Diagnostics. Substrate 4-methylumbelliferyl-2-acetamido-2-deoxy-


Selected Abbreviations and Acronyms

AcLDL = acetylated LDL
AO = acridine orange
FCS = fetal calf serum
HMDM = human monocyte-derived macrophages
NAβGase = N-acetyl-β-glucosaminidase
nLDL = native LDL
oxLDL = oxidized LDL
UVoxLDL = UV irradiation–induced oxLDL
vit-E = vitamin E

β-D-glucopyranoside and D-α-tocopherol acid succinate (vit-E) were from Sigma Chemical Company. Rabbit anti-human cathepsin D and normal rabbit IgG fractions were from Dakopatts, and goat anti-rabbit IgG tagged with 0.8-nm gold particles was from Aurion. All other reagents used were obtained from standard sources and of the highest purity available.

Cell Cultures

J-774 cells (an established murine macrophage cell line) were grown in F-10 with 10% (vol/vol) FCS, glutamine (2 mmol/L), penicillin G (100 U/mL), and streptomycin (100 µg/mL) in 75 mL Costar plastic culture flasks. The cells were kept at 37°C in a humidified atmosphere (5% CO₂ in air). At confluence they were scraped into suspension and replated into 35-mm Costar plastic culture dishes with or without coverslips.

Mixed human mononuclear cells were separated from the buffy coats of donor blood. Isolation, differentiation, and culture of HMDMs were as previously described.20 Briefly, the mononuclear cells were isolated using Ficoll-paque according to the method of Böyum.21 The isolated cells were seeded in 35-mm Costar plastic culture dishes with coverslips at a concentration of 10⁶ per mL. The cells were rinsed in PBS to remove nonadherent cells and cultivated in RPMI 1640 containing 10% FCS or human serum. Medium was renewed every second day. The cells were used for experiments after 6 to 8 days.

Preparation of Lipoprotein

LDL (1.025<d<1.050 g/mL) and HDL (1.0645<d<1.4598 g/mL) were freshly isolated by sequential ultracentrifugation from human plasma according to previous reports.22,23 Lipoproteins were prepared in the presence of EDTA (1.4 mg/mL) to inhibit lipid peroxidation. It was stored at −70°C and used within 1 month after preparation. They were finally dialyzed for 24 hours at 4°C under nitrogen against 0.01 mol/L phosphate buffer with 0.16 mol/L NaCl, pH 7.4, before incubation with the cells.

LDL Oxidation and Acetylation

Aliquots of LDL solutions (1.8 mg protein per milliliter) were photo-oxidized by ultraviolet C irradiation (254 nm) for 3 hours at room temperature (UVoxLDL) as described before.17–19 LDL (4 mg/mL) was also acetylated by using sequential addition of acetic anhydride.24 After acetylation, excess reagent was removed by dialysis.

OxLDL Exposure and Preparation of Cellular Fractions

J-774 cells were grown in complete culture medium for 24 hours before experiments. The cells were then incubated at 37°C in F-10 culture medium containing 5% FCS for another 24 hours and exposed to UVoxLDL (50 to 150 µg/mL), or not, with or without added vit-E (40 µmol/L) or HDL (100 µg/mL). Finally, the cells were washed twice with cold PBS and gently scraped into fresh PBS (2 mL). To break cells, the suspensions were repeatedly forced through a ball homogenizer (Industrial Tectonics) via attached syringes.25 The device consists of a 3.977-mm precision bore in a stainless steel block containing a 3.942-mm stainless steel ball. Cells required 10 strokes to obtain maximum cell disruption (88.7% to 95.1%), as estimated by the trypan blue viability test, in combination with minimum lysosomal damage, as assayed by measurements of sedimentable and unsedimentable enzyme activities. Optimally ruptured cells in suspension were centrifuged at 14 000 g for 15 minutes at 4°C to sediment intact lysosomes. Procedures are summarized in Fig 1.

Lytic Enzyme Assays

Control and pretreated cells were ruptured and the suspensions centrifuged as described above. Supernatants were withdrawn and 1

Scheme on the release of lytic enzymes from macrophage lysosomes due to uptake of UVoxLDL

![Schema on the release of lytic enzymes from macrophage lysosomes due to uptake of UVoxLDL](image)

Figure 1. Schematic of the cellular fractionation procedure. J-774 cells were broken by shear force homogenization. Sedimentable (bound or intralysosomal) and unsedimentable (cytosolic or released) activities of two lysosomal enzymes were estimated after centrifugation at 14 000g for 15 minutes.
mL distilled water with Triton X-100 (final concentration 0.1%) was added to the pellets to induce lysosomal lysis. The sedimentable and unsedimentable activities of two lysosomal marker enzymes were calculated and expressed as arbitrary units per milligram cell protein. Cathepsin L, one of the most powerful lysosomal proteinases, was assayed at pH 5.5 using Z-Phe-Arg-7AMC as a substrate.\textsuperscript{26} NA\textsubscript{b}Gase was assayed using 4-methylumbelliferyl-2-acetamido-2-deoxy-\textbeta-D-glucopyranoside as a substrate.\textsuperscript{27} The fluorescence of the reaction products was measured at ex 370/em 440 nm and ex 356/em 444 nm, respectively.

Light and Electron Microscopic Cathepsin D Immunocytochemistry
J-774 cells and human macrophages were grown on coverslips and exposed, or not, to UVoxLDL as described above. For light microscopic immunocytochemistry, the cells were fixed in 4% paraformaldehyde and then labeled with primary (polyclonal rabbit anti-human cathepsin D) and secondary (goat anti-rabbit IgG Texas Red conjugate) antibodies, as described before.\textsuperscript{28,29} The cells were mounted in Gelvatol (Monsanto) and examined and photographed in a Microphoto-SA fluorescence microscope (Nikon). Immunocytochemical demonstration of cathepsin D at the ultrastructural level was performed as previously described.\textsuperscript{28} Briefly, the cultures were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde in 0.15 mol/L Na cacodylate buffer at pH 7.6 for 20 minutes at 4°C and rinsed with PBS and then exposed to 0.05% sodium borohydride and 0.1% glycine in PBS. The cells were incubated with 1 mL polyclonal anti-human cathepsin D (1:100 in PBS containing 0.1% saponin and 5% FCS) at 4°C overnight. After rinsing, the cells were incubated with 1:100 diluted goat anti-rabbit IgG tagged with 0.8-nm gold particles overnight at 4°C, and the silver enhancement technique was then used to visualize the particles. Control cells, on which the specific cathepsin D polyclonal antibodies were replaced with PBS containing 0.8% BSA, 20 mmol/L NaN\textsubscript{3}, and 0.1% gelatin, remained unstained.

Estimation of lysosomal Integrity Using AO Vital Staining
J-774 cells, growing on coverslips, were exposed for 24 to 48 hours, or not, to UVoxLDL (80 to 100 \textmu g/mL) or Vit-E (40 \textmu mol/L); or cells were exposed to AcLDL (100 \textmu g/mL) on coverslips. After exposure, the cells were vitally stained with AO solution (5 \textmu g/mL in complete medium) for 15 minutes at 37°C and then kept for another 10 minutes in complete medium at 22°C. AO is a lysosomotropic weak base and a metachromatic fluorochrome showing red fluorescence at high and green fluorescence at low concentrations. The intensities of red and green AO-induced fluorescence from 100 individual cells per coverslip were then measured using a static cytofluorometer system based on a computer-assisted MPV III (Leitz) photometer-microscope, as described previously.\textsuperscript{19,29} The cells were also examined with an LSM 410 confocal laser scanning microscope (Carl Zeiss).

Agarose Gel Electrophoresis
The mobility of different LDLs was assayed by electrophoresis on 1.2% agarose gels in barbital buffer (pH 8.6). The gels were fixed with 50% ethanol and the bands visualized by staining with 0.2% Sudan blue in 60% ethanol.\textsuperscript{30}

Statistics
Results are given as mean±SEM. Statistical comparisons were made using the two-tailed Student’s paired t test. Results were considered significant at \(P<.05\).

Results
OxLDL Causes Partial Inactivation and Redistribution of Lysosomal Enzymes
J-774 cells were exposed to UVoxLDL for 24 hours. As shown in Figs 2 and 3, the total activity of NA\textbeta Gase was significantly reduced after exposure to both 50 and 150 \textmu g protein per milliliter of oxLDL, while cathepsin L activity was significantly reduced after 150 \textmu g protein per milliliter of oxLDL exposure. Moreover, relatively enhanced levels of cytosolic (released) enzyme activities were observed (presented as percentage of total), in a dose-dependent manner, being significant at the 150


**OxLDL-Induced Lysosomal Changes**

µg/mL level for both enzymes (Figs 2 and 3, top). These findings indicate that exposure to UVoxLDL results in partial redistribution, as well as inactivation, of lysosomal enzymes.

Using cathepsin D immunocytochemistry at light and electron microscopical levels, we confirmed the UVoxLDL-induced leakage of lysosomal enzymes in both J-774 cells and HMDMs. Light microscopically, most control cells were of normal size and showed a granular type of cathepsin D localization. UVoxLDL-treated cells, however, were generally slightly enlarged and showed an enhanced cytoplasmic distribution of cathepsin D, which also filled many of the cytoplasmic blebs (indicating cytotoxicity) along the cell borders. The localization of cathepsin D within J-774 cells and HMDMs was also examined at the ultrastructural level. The cells were initially exposed, or not, to 100 µg/mL UVoxLDL for 24 hours. The findings were generally the same for both cell types. The UVoxLDL-treated cells showed an increased number of silver-enhanced gold particles, indicating the presence of gold-labeled antibodies against cathepsin D. Many of these particles were in the cytosol, indicating lysosomal membrane damage by oxLDL, with subsequent leakage of cathepsin D. In contrast, control cells showed a few silver particles, and most of them were located within lysosomal vesicles (Fig 4). The size of the lysosomes was about the same in control and UVoxLDL-treated cells. However, the general density of the vesicles (reflecting binding of osmium) was somewhat greater in UVoxLDL-treated cells, suggesting lysosomal lipid accumulation after UVoxLDL treatment.

**Agarose Gel Electrophoresis**

Varying electrophoretic mobility was shown by AcLDL, nLDL, and oxLDL (Fig 5). The mobility of oxLDL and AcLDL was increased compared with nLDL, and the mobility of AcLDL was higher than that of oxLDL.

**Lysosomal Integrity as Reflected by Uptake of AO**

The lysosomal membrane stability (ie, preserved proton gradient) was determined in control, UVoxLDL-, and AcLDL-exposed J-774 cells by the AO test. The differences between control and UVoxLDL-treated cells with respect to AO fluorescence (Fig 6, UVoxLDL, 80 µg/mL) were in accordance with previous findings. In summary, after exposure to UVoxLDL, the red fluorescence decreased, indicating lysosomal membrane damage, with disturbed proton gradients, while green cytosolic fluorescence generally increased, reflecting a lowered cytosolic pH due to the proton redistribution and thus the trapping of protonized HAO- in the cytosol of damaged cells. Confocal scanning microscopy of cells exposed to AcLDL (100 µg/mL) or UVoxLDL (100 µg/mL) for 24 hours is shown in Fig 7. AcLDL-exposed cells showed an enhanced red (due to lysosomal expansion) and unchanged green fluorescence compared with control cells. The increased AO-induced red fluorescence reflects pronounced uptake of AcLDL with expansion of the lysosomal vesicle.

**HDL and Vit-E Protect Lysosomes Against OxLDL-Induced Damage**

To examine the protective effects of HDL and vit-E on UVoxLDL-induced lysosomal membrane damage, J-774 cells were exposed to either UVoxLDL alone or to UVoxLDL together with HDL or vit-E. The AO test for lysosomal stability (see above) was performed. The protective effects of vit-E and HDL are illustrated in Fig 6. The UVoxLDL-induced decrease in red fluorescence (lysosomal rupture) was partially inhibited by both HDL and vit-E. A significant cytoprotective effect was obtained with HDL.

Protective effects of HDL and vit-E on UVoxLDL-treated cells were also detected by lysosomal enzyme assays on fractionated cells, as shown in Figs 8 and 9. HDL caused a significant decrease in enzyme relocation of NA½Gase and cathepsin L, whereas enzyme inactivation was significantly restored by both HDL and vit-E.

**Discussion**

Understanding the interaction between oxLDL and the acidic vacuolar compartment (late endosomes, prelysosomes, and lysosomes) of macrophages is of critical importance for grasping the mechanisms responsible for foam cell formation and the development of the atherosclerotic process. In this study, we used the AO vital staining technique and biochemical and immunocytochemical assays of lysosomal enzymes on intact cells and, after subcellular fractionation, evaluated lysosomal membrane stability and activity and relocation of some lysosomal enzymes. We have demonstrated that UVoxLDL induces partial inhibition of enzyme activity and destabilizes lysosomal membranes, with redistribution of lysosomal enzymes to the cytosol. Moreover, we found that HDL and vit-E, if mixed with UVoxLDL before its endocytic uptake, have the capacity to partially protect cells from UVoxLDL-induced lysosomal damage.

A number of techniques have been used for subcellular fractionation of cells after their interaction with lipoproteins. In this study, a ball homogenizer was used, which, in combination with the estimation of lysosomal marker enzyme activity, permitted us to identify alterations in lysosomal membrane stability and enzyme inactivation in a simple and efficient way on a limited number of cells.

The lysosomotropic weak base AO is a useful marker for lysosomal integrity in living cells, and we described its use on lysosomal UVoxLDL-induced damage in an earlier study. The results of the biochemical assays of the present study are in good agreement with the cytochemical findings of the present and our previous study. The AO technique was also found to be of critical importance to show the absence of lysosomal change by endocytosed AcLDL.

It has been shown that activities of several lysosomal hydrolases, including NA½Gase, are elevated twofold to four fold with increased atherosclerosis in lipid-rich atherosclerotic lesions compared with fibrous or complicated lesions or with normal arterial walls. There is, however, significant decrease of intracellular acid hydrolases, which is particularly striking for the NA½Gase. It has even been suggested that cholesterol might be a stabilizer of lysosomal membranes in cells of atherosclerotic lesions, thus preventing the relocation of lysosomal enzymes. However, based on our data and those of others, we conclude that the effect of lipoprotein cholesterol on lysosomal stability is largely dependent on the physicochemical status of the former. If the lipids are oxidized, the
resulting oxysterols, hydroperoxides, and their toxic carbonylic fragments may affect lysosomal enzymes and membranes, whereas nLDL or AcLDL would not.

Since the early 1990s, several authors have shown that oxLDL is more resistant than nLDL to cathepsins and, moreover, causes partial inactivation of macrophage lysosomal proteases.\textsuperscript{14,16,36} Our data are consistent with those findings.

Further, we have now shown that in addition to partial inactivation of lysosomal enzymes, there are signs that these enzymes relocate to the cytosol. This observation would indicate that oxLDL, when present in lysosomes, not only affects their content but also damages their membranes, resulting in leakage of these enzymes to the cytosol of hydrolytic enzymes. At later stages of the formation of degenerated foam cells, oxLDL itself

Figure 4. Immunocytochemical demonstration of cathepsin D of HMDMs at an ultrastructural level. The precipitates are silver-enhanced gold particles and indicate the binding of gold-tagged anti–cathepsin D antibodies (for further information on the techniques used, see “Methods”). A and B, Control cells with only a few silver particles, which are found only within lysosomes (arrows). C and D, Cells incubated with UVoxLDL (100 μg/mL) for 24 hours before immunocytochemistry. These cells show many more silver particles than control cells, and most of them are found in the cytosol (arrowheads), indicating enhanced amounts of cathepsin D, as well as leakage of this enzyme from the acidic vacuolar apparatus into the cytosol.
also may leak to the cytosol. The resistance of oxLDL to lysosomal enzymes may explain the formation of ceroid in atherosclerosis.\textsuperscript{37} It is well known that peroxidized lipid and protein residues polymerize to form lipofuscin/ceroid.\textsuperscript{38} The strong immunostaining for cathepsin D that was induced by UVoxLDL suggests that, unlike cathepsins B or L, macrophage synthesis of cathepsin D can be induced by oxLDL. Our finding is consistent with previous reports about high cathepsin D expression in human macrophage-derived foam cells.\textsuperscript{39} It has also been shown in cell-free systems that oxLDL inactivates cathepsin B but does not affect cathepsin D.\textsuperscript{16,36}

Epidemiological data strongly indicate an inverse correlation between plasma HDL and atherosclerosis. The proposed antiatherogenic activity of HDL would be explained by stimulated reverse cholesterol transport from foam cells and cholesterol efflux from peripheral tissues to the liver, as well as inhibited...

Figure 5. Electrophoretic mobility of nLDL (A), AcLDL (B), and oxLDL (C).

Figure 6. Alterations of lysosomal membrane stability as estimated by the AO relocation test. J-774 cells were grown for 48 hours on coverslips while exposed, or not, to UVoxLDL (80 \( \mu \)g/mL) with or without HDL (80 \( \mu \)g/mL) or vit-E (40 \( \mu \)mol/L). Red fluorescence intensity, indicating the presence of AO within intact lysosomes with preserved proton gradients, was measured by static cytofluorometry. Data are mean \( \pm \) SEM (n=3). *\( P < .05 \); **\( P < .01 \).

Figure 7. Confocal scanning micrographs of J-774 cells stained with AO. Control cells (unexposed cells) and cells exposed to UVoxLDL (100 \( \mu \)g/mL) or AcLDL (100 \( \mu \)g/mL) for 24 hours are shown. Note the decreased number of intact lysosomes in many of the UVoxLDL-exposed cells and enlarged but intact lysosomes in AcLDL-exposed cells. The increased green fluorescence of nuclei and cytosols in oxLDL-exposed cells indicates decreased cytosolic pH.
LDL oxidation. However, other HDL antiatherogenic mechanisms cannot be ruled out. In this study, a protective role of HDL on UVoxLDL cytotoxicity was demonstrated, which is consistent with earlier reports about HDL inhibition of oxLDL-induced cytotoxicity to smooth muscle, endothelial, and lymphoblastoid cells. 

Antioxidants have been supposed to play a protective role against atherosclerosis, because LDL oxidation seems to be a critical step in its formation. Several cell types are reportedly protected against oxLDL cytotoxicity by α-tocopherol. Data from the present study suggest that the antiatherogenic properties of α-tocopherol are due not only to inhibition of LDL oxidation but also to preservation of the lysosomal membrane stability against internalized oxLDL.

The absence of vital macrophages in the central part of an atheroma with its partially calcified gruel, in combination with the finding that oxLDL, but not AcLDL, is toxic to cells in culture, is evidence for oxLDL cytotoxicity. Whether oxLDL-induced cell death is apoptotic or necrotic is not well understood. Recently, however, it has been demonstrated that abundant apoptotic cells exist in human atherosclerotic lesions. Moreover, oxLDL has been found to induce apoptosis of cultured macrophages and other cells. We noticed previously that UVoxLDL did indeed induce both an apoptotic morphology in macrophages and TUNEL (terminal deoxyribonucleotidyl transferase-mediated dUTP-biotin nick end-labeling) positivity of their nuclei. This finding is consistent with findings by us and others that relocation of lysosomal enzymes to the cytosol may occur during apoptosis and perhaps even initiate this process. We hypothesize that oxLDL may induce apoptosis by rupturing lysosomal membranes, resulting in the leakage of lysosomal endonucleases and cathepsins into the cytosol.

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