Aggregation as well as chemical modification of LDL during oxidation is responsible for poor processing in macrophages

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Abstract Aggregation is a characteristic of extensively oxidized (ox-) LDL. We wished to determine whether this structural change contributed even more to the documented poor degradation in macrophages of ox-LDL than the chemical changes. When protein degradation of the soluble and insoluble portions of extensively ox-LDL was compared to that of acetyl LDL in mouse peritoneal macrophages (MPM), we found that the percent of internalized LDL that was degraded was lowest for the insoluble portion (insol. ox-LDL), intermediate for the soluble portion (sol. ox-LDL), and highest for the acetyl LDL, regardless of whether the binding and uptake mechanisms had been excluded, e.g., by performing appropriate pulse-chase studies. As the same order of degradation was found after long-term degradation under cell-free conditions by a mixture of cathepsin B and D, it is likely that poor degradation of ox-LDL by lysosomal proteases is partially responsible for the deficient processing of ox-LDL in MPM. However, when MPM were incubated in a pulse-chase design with LDL that was induced to aggregate by vortexing without oxidizing (vx-LDL), degradation over an 18-h interval of accumulated vx-LDL was almost as low (25%) as that of insol. ox-LDL (18%), in contrast to sol. ox-LDL (60%). Yet, in a cell-free system cathepsin degradation of vx-LDL was as efficient as that of acetyl LDL and LDL. Also, the differences in degradation between sol. and insol. ox-LDL were smaller than in MPM. Thus, it appears that alternative mechanisms to poor proteolysis of substrate were responsible for poor intracellular processing of such aggregated lipoproteins. These results suggest that, although the poorer processing of insol. ox-LDL than sol. ox-LDL may be due, in part, to more deficient proteolytic degradation, particle aggregation per se may play at least as important a role in such deficiencies. This may occur by such mechanisms as altered intracellular trafficking leading to poorer fusion in macrophages of phagosomes containing aggregated lipoproteins with lysosomes.—**Hoff, H. F., N. Zyromski, D. Armstrong, and J. O'Neil.** Aggregation as well as chemical modification of LDL during oxidation is responsible for poor processing in macrophages. *J. Lipid Res.* 1993. **34:** 1919–1929.

Supplementary key words acetyl LDL • apoB • 4-hydroxynonenal • vortex-aggregated LDL

Oxidation of LDL is characterized by several structural and chemical changes to the lipoprotein particle that lead to numerous modifications in its functional properties (1–3). Lipid hydroperoxides formed during oxidation of LDL rapidly decompose to form a myriad of products including reactive aldehydes such as malondialdehyde, 4-hydroxynonenal, heptadienal, and hexanal (4). Some of these aldehydes interact covalently with amino acid side chains in apoB in LDL such as lysines to form Schiff-base adducts (3–5) and possibly other bonds. The former interactions are responsible for increasing the fluorescence of apoB in LDL at 360 ex/430 em (2, 4), and increasing the particle electrophoretic mobility because of the increased net negative charge (3–5). We recently showed that, in addition to the above-described properties of oxidized (ox-)LDL, particle aggregation occurred at high degrees of oxidation (6). Furthermore, oxidation was more extensive at 37°C than at 20°C, and was highly dependent on the concentration of LDL. We have previously shown that LDL modified directly with the reactive aldehyde, 4-hydroxynonenal (HNE) formed during lipid peroxidation (4), underwent particle aggregation (7, 8). We speculated that this aggregation was, in part, the result of intermolecular cross-linking induced by the bifunctional HNE, as only about 50% of the apoB from aggregated HNE-modified LDL (7) or from insoluble ox-LDL (6) entered into a 3% polyacrylamide gel during SDS-PAGE.

Abbreviations: LDL, low density lipoproteins; ox-LDL, oxidized LDL; sol. ox-LDL, soluble oxidized LDL: insol. ox-LDL, insoluble oxidized LDL: acetyl LDL, acetylated LDL; MPM, mouse peritoneal macrophages; RPMI, Rosewell Park Memorial Institute; TNBS, trinitrobenzene sulfonic acid; MDA, malondialdehyde; HNE, 4-hydroxynonenal; REM, electrophoretic mobility relative to LDL; BSA, bovine serum albumin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; BHT, butylated hydroxytoluene; em, emission; ex, excitation; BCA, bicinchoninic acid; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline containing EDTA; OD, optical density; FCS, fetal calf serum; cyt D, cytochalasin D; DTT, dithiothreitol; vx-LDL, vortex-aggregated LDL.

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LDL extracted from human atherosclerotic lesions (A-LDL), shown to have undergone oxidation, also underwent aggregation following concentrating to levels of over 1 mg/ml (9).

Recently, several studies have reported that ox-LDL is not processed as efficiently as acetyl LDL in cultured macrophages, as indicated by an increase in intracellular accumulation of unprocessed apoB-100 relative to its corresponding degradation (2, 10–13). This accumulation in macrophages after the uptake of unaggregated ox-LDL was suggested to be due to inefficient degradation of apoB by lysosomal enzymes (11–13). Since aggregation is a characteristic of LDL after extensive oxidation at concentrations shown to be present in atherosclerotic lesions (6), we asked whether particle aggregation as well as chemical modification of apoB-100 during oxidation contributed to this processing deficiency.

MATERIALS AND METHODS

Cells and reagents

C57BL/6 mice (16–20 weeks of age) were purchased from the Trudeau Institute (Saranac Lake, NY). Rosewell Park Memorial Institute (RPMI) medium and fetal calf serum were obtained from Gibco Laboratories (Grand Island, NY). Bovine serum albumin (fatty acid-free), trichloroacetic acid, vitamin E, BHT, cathepsin D, cathepsin B, and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Corp. (St. Louis, MO). N-α-carbobenzoyl-L-lysine was obtained from Aldrich Chemicals (Milwaukee, WI). BCA protein assay reagents were purchased from Pierce (Rockford, IL). Tissue culture plates were from Costar (Cambridge, MA). Carrier-free Na ^{125}I was purchased from ICN Pharmaceuticals, Inc. (Irvine, CA). Pre-made 1% agarose gels were purchased from Corning (Palo Alto, CA). Spectra/Por 2 and 3 dialysis tubing was obtained from Spectrum Medical Industries Inc., (Los Angeles, CA). 4-Hydroxynonenal was a kind gift from Dr. Hermann Esterbauer, University of Graz, Austria.

Isolation of plasma LDL, iodination, and chemical modification

LDL was isolated from fresh plasma obtained from the American Red Cross Blood Services of Cleveland or the Cleveland Clinic Blood Bank by sequential ultracentrifugation as a $1.019 < d < 1.063$ g/ml fraction using the procedure of Hatch and Lees (14), and stored in 0.15 M NaCl containing 0.5 mM Na_2EDTA, pH 8.5. Acetylation of LDL was performed by repeated additions of acetic anhydride (15) and then extensively dialyzed against 0.15 M NaCl, 0.3 mM Na_2EDTA, pH 7.4. The extent of lysine modification, measured by changes in TNBS reactivity (16) using N-α-carbobenzoxy-L-lysine as a standard, exceeded 60% of the lysine residues on LDL. LDL was modified with 6 mM 4-hydroxynonenal in the presence of 0.1 M phosphate buffer, pH 7.4, at 37°C for 5 h as reported earlier (7). Oxidation of ^{125}I-labeled LDL was performed by dialysis in the presence of 10 μM $CuSO_4$ at a protein concentration of 500 μg/ml for 24 h at 20°C. Oxidation was terminated by dialyzing samples into 0.15 M NaCl, 0.3 mM Na_2EDTA, and 20 mM Na phosphate, pH 7.4 (PBS) for 2 h using Spectra-Por 2 dialysis membranes. LDL and acetyl LDL were labeled for cell experiments with Na^{125}I using the iodine monochloride procedure of McFarlane (17) as modified by Bilheimer, Eisenberg, and Levy (18); the specific activity of labeled preparations was between 100–200 cpm/ng protein. Lipoprotein concentrations are routinely expressed as μg protein/ml. The protein content of lipoproteins was determined by the bicinchoninic acid (BCA) assay as described by Smith et al. (19) except that a 1 h, 60°C heating step was used. Bovine serum albumin (BSA) was used as a standard.

Quantification of LDL oxidation

The degree of Cu^{2+}-induced oxidation was assessed by several procedures. In one, conjugated diene formation was determined by the absorbance at 234 nm at a final concentration of 50 μg protein/ml and adjusting for the background of 1% SDS in PBS buffer as reported previously (6). Increases in electrophoretic mobility of ^{125}I-labeled ox-LDL were determined by one-dimensional agarose electrophoresis. This was performed by running at 90 V for 75 min using pre-made 1% agarose gels and staining with 0.025% Fat Red 7B in 60% methanol following the manufacturer's instructions. Results were expressed as relative electrophoretic mobilities (REM) by comparing the migration distances to those of unmodified LDL. Fluorescence of LDL was measured at 360ex/430em using a Perkin-Elmer Fluorometer LS-3 (Oak Brook, IL) as reported earlier (4, 6), but using a concentration of 50 μg protein/ml and adjusting for the background fluorescence of 1% SDS in PBS buffer.

Preparation of soluble and insoluble forms of oxidized LDL and of vortex-aggregated LDL

Samples of ^{125}I-labeled LDL (500 μg protein/ml) that had been oxidized for 24 h with 10 μM Cu^{2+} at 20°C, were centrifuged at 10,000 *g* for 10 min and the percent precipitated was determined from the difference in label in the original sample and the supernatant fraction after centrifugation. The supernatant fraction was designated sol. ox-LDL while the precipitated fraction was designated insoluble (insol.) ox-LDL and typically represented about 80% of the total LDL. In select experiments ^{125}I-labeled LDL to which butylated hydroxytoluene (BHT) had been added to prevent oxidation was induced to aggregate by vortexing for 30 sec (20). Under these condi-

tions no increases in conjugated dienes or fluorescence were detected. Only the insoluble portion, prepared as described above, was used for uptake studies.

Enzyme degradation

LP degradation by a mixture of cathepsin B and D was determined by a procedure published recently (11). Briefly, cathepsin B and D were activated by incubating 1 unit of each enzyme in 1 ml of a 0.2 M acetate buffer, pH 5.0, containing 10 mM DTT and 1 mM EDTA for 15 min at 37°C. ^{125}I-labeled LP (30 μg protein/ml, sp act = 150 cpm/ng LP) was then incubated at 37°C for the indicated times with the medium containing activated cathepsin B and D at a final concentration of 0.1 unit of each enzyme, 1 mM DTT, and 27 μg of ^{125}I-labeled LP. Aliquots (50 μl) of degraded ^{125}I-labeled LP were removed at the prescribed time intervals and mixed with 50 μl BSA (30 mg/ml) and 900 μl TCA (20%), centrifuged at 10,000 *g* for 10 min, and the amount of TCA soluble and insoluble label was determined.

Lipoprotein interactions with macrophages in culture

Mouse peritoneal macrophages (MPM) were harvested 2–3 days after thioglycollate stimulation of C57BL/6 mice. Primary cultures were prepared at a density of 10^6 cells/16 mm diameter well in RPMI containing 10% FCS and used 48 h after plating. The uptake and subsequent degradation of ^{125}I-labeled lipoproteins by macrophages were measured similarly to the procedure described previously (21). Briefly, the cells were incubated in triplicate with ^{125}I-labeled lipoproteins in RPMI containing 2 mg/ml of fatty acid-free BSA. The extent of degradation was determined as the trichloroacetic acid-soluble (TCA), organic iodide radioactivity present in the media. Non-organic iodide was precipitated with $AgNO_3$ as reported earlier (10). Radioactivity in cell-free control wells was routinely determined for each condition and subtracted from that obtained in the presence of cells. Degradation was expressed as μg protein degraded per mg cell protein per time of incubation period. Cell-associated label was determined by solubilizing PBS-washed cells with 0.25 N NaOH and counting an aliquot. Cell protein was also determined from such NaOH-solubilized extracts. The experiments described are representative of two or more studies.

To identify the presence of immunoreactive apoB in macrophages, an immunoperoxidase technique employing an avidin–biotin amplification was used as previously described (22). Our anti-apoB was shown to cross-react with acetyl LDL after immunoblotting of gels after SDS-PAGE or agarose electrophoresis (not shown). Control incubations in which nonimmune serum was used in place of primary antibody were negative. In select cases cells were also stained with Oil Red O.

Determination of intracellular accumulation of LP

As preliminary immunohistochemical data demonstrated that some insol. ox-LDL was attached to the cell surface of MPM after the incubation period, we attempted to quantify the percent of cell-associated aggregated ox-LDL that was actually inside the cell. As we had previously shown that uptake and subsequent degradation of aggregated forms of LDL was inhibitable by cytochalasin D (cyt D) (7, 8), we assumed that any cell association in the presence of cyt D should be extracellular. Likewise, we assumed that the cell-associated label measured after incubation of MPM with insol. ox-LDL at 4°C represented extracellular LP. When insol. ^{125}I-labeled ox-LDL was incubated with MPM in the presence of cyt D, which induced a 90% inhibition of degradation, cell-associated label was found to be similar to that obtained by incubating cells with this ligand for 1 h at 4°C. The extracellular label in different samples ranged between 30 and 50% of the total cell-associated label. In this study we have used either cell-associated label obtained after incubation for 4 h at 4°C or cell-associated label obtained after incubation at 37°C for the specified time in the presence of cyt D as estimates of extracellular accumulation. These values were subtracted from total cell-associated label after incubation at 37°C or after incubation in the presence of cyt. D, respectively, to give the intracellular accumulation values reported in this study.

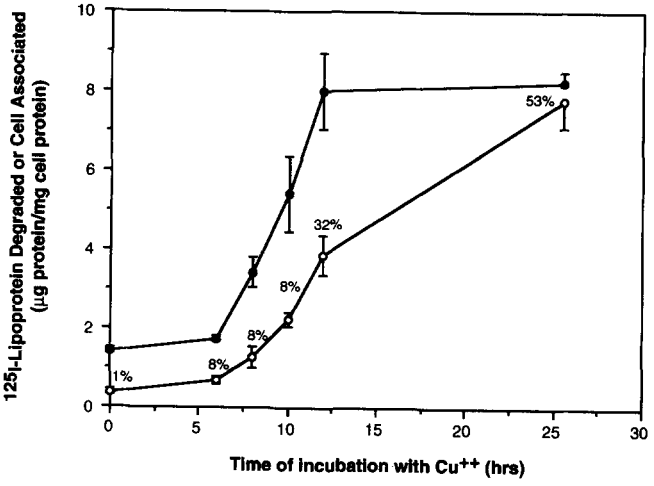


Fig. 1. Degradation and cell-associated label in MPM of ^{125}I-labeled LDL oxidized by incubating with Cu^{2+} for increasing time intervals. LDL (500 μg protein/ml) was incubated at 20°C with 10 μM Cu^{2+} for up to 25 h. The level of aggregation of LDL oxidized for the stated time periods was assessed by measuring the percent of label precipitated by centrifugation at 10,000 *g* (shown in parentheses). Each sample of ^{125}I-labeled ox-LDL was added to media and incubated with MPM at a final concentration of 20 μg protein/ml for 4.5 h at 37°C. Degradation (●) and cell-associated levels of label (○) were determined as described in Materials and Methods. Data points represent the mean ± SD of triplicate determinations.

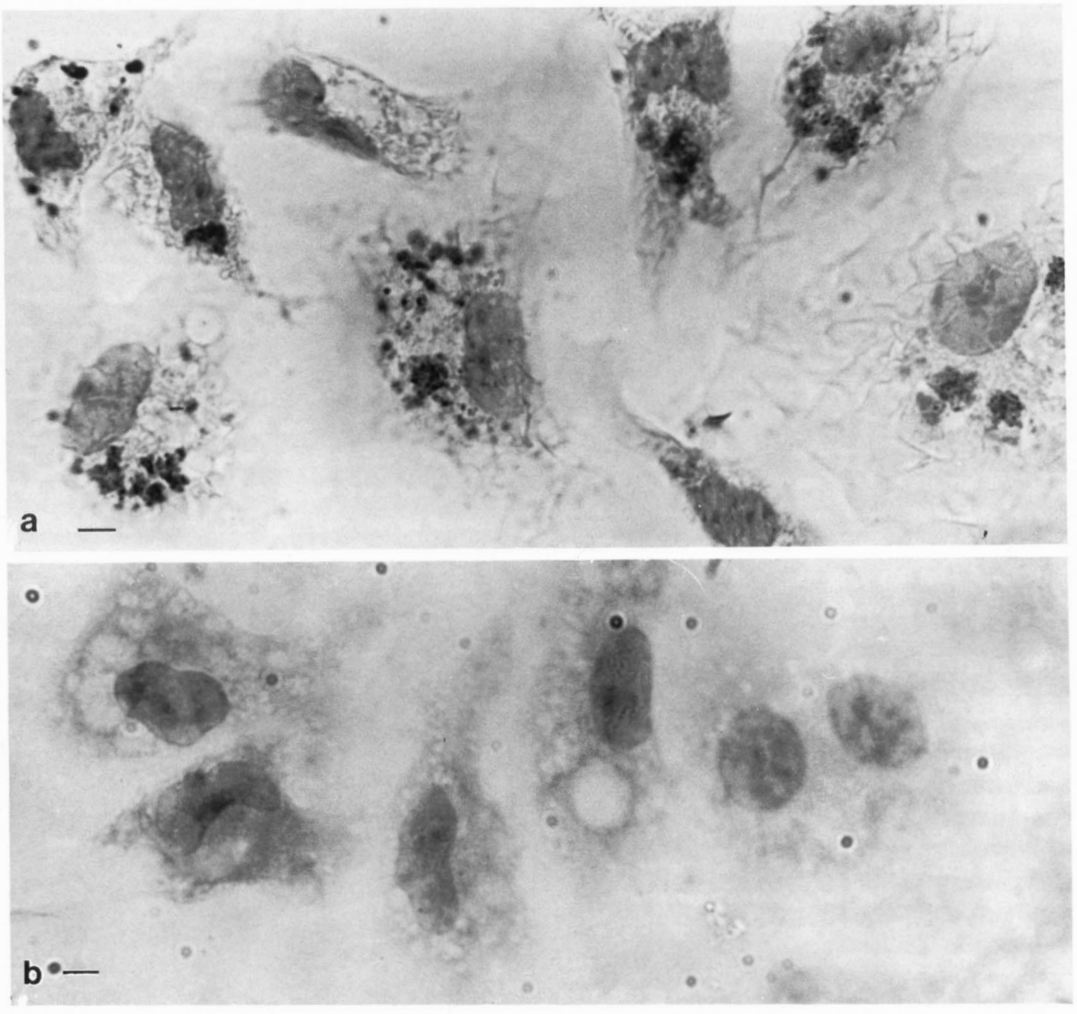


Fig. 2. Micrographs of MPM after immunohistochemical staining for apoB or Oil Red O staining. a: Immunoperoxidase staining of MPM for apoB after incubation for 4.5 h with insol. ox-LDL (20 μg protein/ml). b: Immunoperoxidase staining of MPM for apoB after incubation for 4.5 h with acetyl LDL (20 μg protein/ml). c: Oil Red O-stained MPM after incubation for 4.5 h with insol. ox-LDL (20 μg protein/ml). d: Oil Red O-stained MPM after incubation for 4.5 h with acetyl LDL (20 μg protein/ml). e: Micrograph of MPM stained with immunoperoxidase for apoB after incubation with 20 μg protein/ml of insol. ox-LDL for 4.5 h. Bar indicates 10 microns.

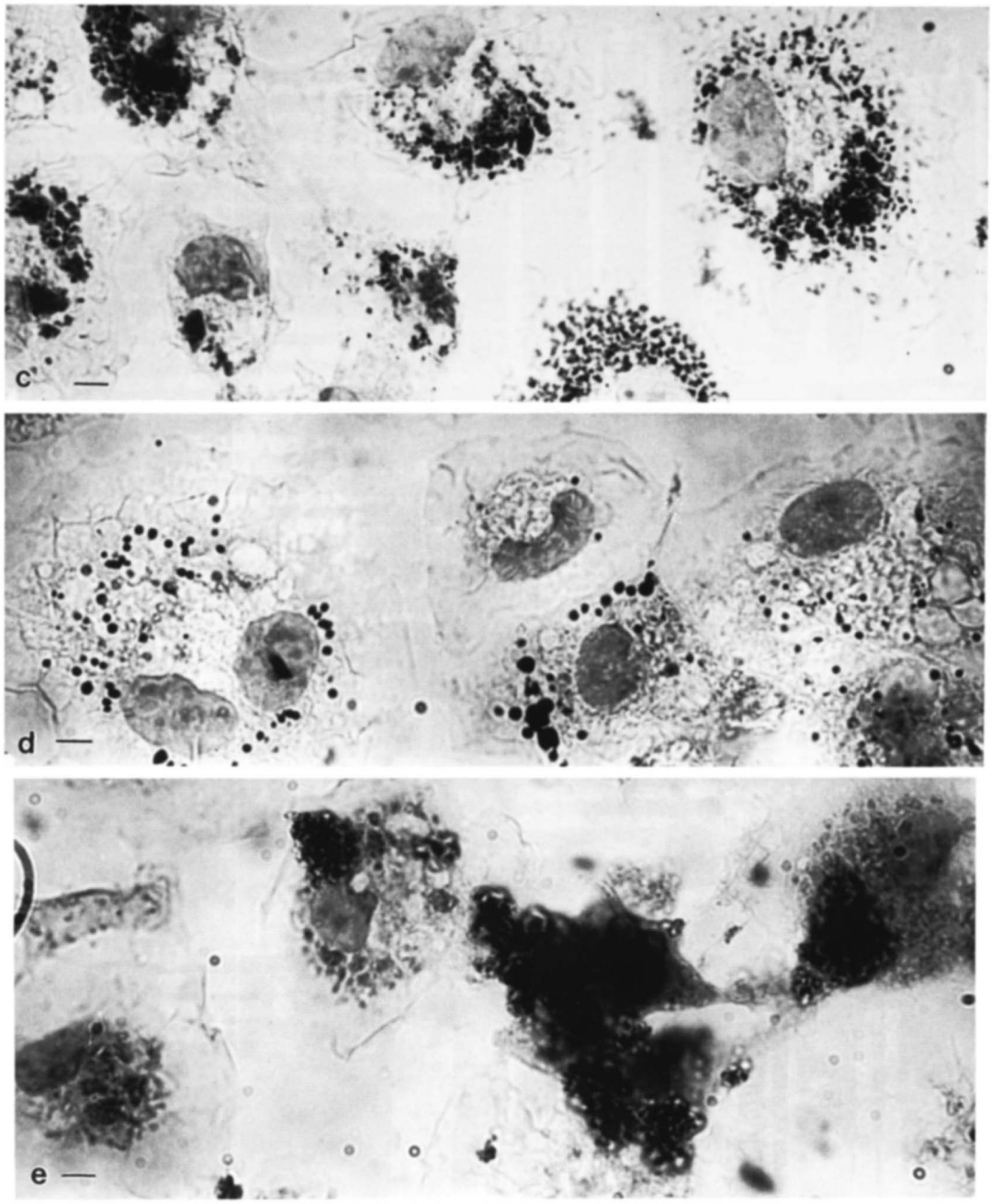
RESULTS

Association of aggregation and poor processing in MPM of oxidized forms of LDL

Deficiency of intracellular processing of lipoproteins, defined as the percent of internalized lipoprotein (LP) that remains undegraded, has been observed previously for ox-LDL in MPM (3, 10–13). To determine whether this processing deficiency of ox-LDL was a function of the degree of oxidation, we compared degradation and cell-associated label after incubating with MPM for 4.5 h of ^{125}I-labeled LDL oxidized for periods of up to 25 h. Degradation and cell-associated label both increased for oxidation up to 12 h, but only the latter continued to increase between oxidation periods of 12 and 25 h (**Fig. 1**). Particle aggregation also showed increases between 10 and 25 h. When intracellular accumulation of undegraded LP, e.g., difference in cell-associated label at 37°C and at 4°C, was measured for ^{125}I-labeled insol. ox-LDL, e.g., the precipitated portion of LDL oxidized with Cu^{2+} for 24 h, ^{125}I-labeled acetyl LDL, and ^{125}I-labeled LDL, only insol. ^{125}I-labeled ox-LDL showed a major increase during a 4.5-h incubation period and was linear with time (not shown).

Morphology of undegraded insol. ox-LDL accumulating in macrophages

To demonstrate morphologically that insol. ox-LDL accumulated in macrophages in an undegraded form, we documented the localization of immunoreactive apoB



using an immunoperoxidase technique. Most cells incubated with insol. ox-LDL for 4 h showed the presence of immunoreactive apoB intracellularly (**Fig. 2a**). By contrast, no immunoreactive apoB was detected in cells incubated with acetyl LDL (Fig. 2b). It was of note that Oil Red O staining of these cells after incubation with insol. ox-LDL was focal and granular and present close to the periphery of the cell (Fig. 2c). This contrasted with the localization of lipid in droplet form throughout the cells after incubation with acetyl LDL (Fig. 2d). These droplets presumably represented the formation of cholesteryl oleate (15). Many macrophages incubated with insol. ox-LDL demonstrated attachment of apoB-positive material to their surfaces (Fig. 2e). This result was consistent with the presence of appreciable insol. ox-LDL associated with the surface of cells contributing to total cell-associated lipoprotein as described in Materials and Methods.

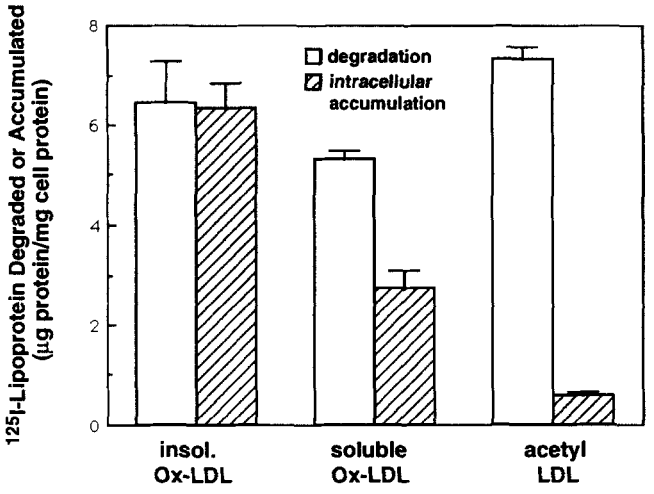


Fig. 3. Degradation and intracellular accumulation in MPM of the soluble (sol.) and insoluble (insol.) portions of extensively oxidized ^{125}I-labeled LDL (24 h at 25°C with 10 μM Cu^{2+}). Samples of sol. and insol. ^{125}I-labeled ox-LDL were prepared as described in Materials and Methods. Each sample was incubated with MPM for 3 h at 37°C at a final concentration of 20 μg/ml, and cell-associated label and degradation was measured as described in the text. Intracellular accumulation was estimated as the difference in cell-associated label at 37°C and at 4°C. Data points represent the mean ± SD of triplicate determinations.

Role of chemical modification and/or aggregation of LDL on intracellular processing in MPM

Although several groups have reported poor processing of ox-LDL in macrophages (2, 10-12), it was not readily clear from the description of these reports whether any particle aggregation had occurred, although from the conditions used, it appeared to be minor. To assess whether the soluble and insoluble portions of extensively oxidized LDL (separated by centrifugation at 10,000 *g*) differed in terms of intracellular processing efficiency, we incubated MPM with equal amounts of soluble (sol.) ^{125}I-labeled ox-LDL and of insol. ^{125}I-labeled ox-LDL for 3 h. As seen in **Fig. 3,** the accumulation of undegraded LP was about twice as great for insol. ^{125}I-labeled ox-LDL as for sol. ^{125}I-labeled ox-LDL. As degradation rates did not differ appreciably, the percent of internalized lipoprotein remaining undegraded was much higher for insol. ox-LDL (50%) than for sol. ox-LDL (25%). This result suggests that particle aggregation also plays an important role in the documented poor intracellular processing.

Interparticle crosslinking by reactive aldehydes such as HNE formed during lipid peroxidation (4) was suggested to be responsible for particle aggregation during extensive oxidation of LDL (7). We therefore asked whether modification of LDL with HNE also led to an intracellular processing deficiency in MPM. As seen in **Fig. 4a,** aggregated HNE-modified LDL showed a large accumulation of undegraded lipoprotein relative to that for acetyl LDL, the percentage of internalized lipoprotein remaining undegraded being 44% for HNE-modified LDL and 6% for acetyl LDL. Significant variability in this percentage was found in separate samples for both insol. ox-LDL and HNE-LDL that ranged from 25 to 70%. To ascertain whether particle aggregation per se could induce this processing deficiency, we also compared process-

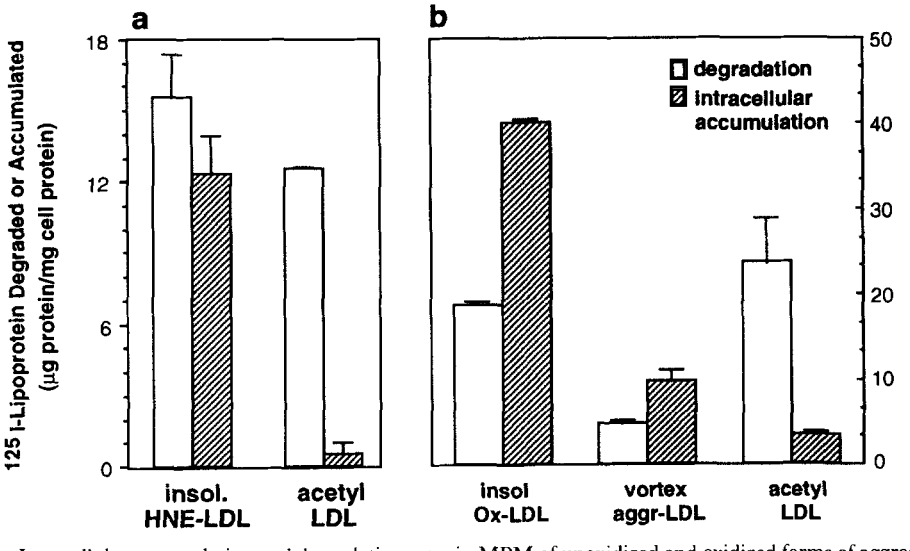


Fig. 4. Intracellular accumulation and degradation rates in MPM of unoxidized and oxidized forms of aggregated LDL. Samples of insoluble HNE-modified ^{125}I-labeled LDL (insol. HNE-LDL), insol. ^{125}I-labeled ox-LDL, and vortex-aggregated ^{125}I-labeled LDL were prepared as described in Materials and Methods. MPM were incubated in one experiment with HNE-LDL and acetyl LDL (a), and in another experiment with insol. ^{125}I-labeled ox-LDL, vortex-aggregated (aggr.) ^{125}I-labeled LDL, and ^{125}I-labeled acetyl LDL (b), each at a final concentration of 20 μg protein/ml for 4.5 h at 37°C; cell-associated label and degradation was then measured as described in the text. Intracellular accumulation was determined as described in Fig. 3. Data points represent the mean ± SD of triplicate determinations.

ing in MPM for insol. ox-LDL with that of LDL that had been induced to aggregate by vortexing without oxidation. As seen in Fig. 4b, the accumulation of undegraded lipoprotein, and, therefore, the percent of internalized lipoprotein remaining undegraded, also increased for vortex-aggregated LDL, although the absolute level of degradation and of accumulation was not as high as that for insol. ox-LDL. These data suggest that processing deficiency of aggregated LDL internalized by MPM occurred even without interparticle crosslinking.

Comparison of degradation in MPM of insol. ox-LDL and acetyl LDL after exclusion of binding and uptake mechanisms

Intracellular processing is a function of a number of events beginning with ligand binding to a specific receptor, internalization into early endosomal or phagosomal (for aggregates) compartments, fusion of early endosomes or phagosomes with late endosomes/lysosomes, and degradation in late endosomes/lysosomes (23). Thus, one potential caveat with assessing intracellular processing by measuring degradation after incubating MPM for a given time interval is that one cannot exclude the role of each of the above-described events in the ultimate degradation in lysosomes. One way of, at least, minimizing the possible role of binding and uptake on resultant degradation rates is to first pre-load MPM with labeled lipoproteins during a pulse period in the presence of a lysosomal inhibitor such as chloroquine (24). Following removal of all labeled lipoprotein from the incubation medium, a chase period is introduced in which the degradation rate of the remaining undegraded lipoprotein that had been internalized is measured. When MPM were preloaded for 3 h at 37°C with ^{125}I-labeled insol. ox-LDL (5 μg/ml) and ^{125}I-labeled acetyl LDL (40 μg/ml) in the presence of chloroquine, similar amounts of undegraded lipoprotein accumulated, e.g., 10 and 7 μg/mg cell protein, respectively. Degradation rates during the pulse period in the presence of chloroquine were reduced by 83 and 87%, respectively (**Table 1**). To ascertain whether degradation, presumably in lysosomes, of the two ligands differed, we "chased" cells in the absence of chloroquine and lipoprotein for an additional 3 h and found that intracellular degradation was three times higher for acetyl LDL than for insol. ox-LDL. Based on the amounts of lipoprotein accumulated during the "pulse" period, only 12% of the accumulated insol. ox-LDL but 52% of the acetyl LDL were degraded in the 3-h chase. This result suggested that degradation was clearly lower for insol. ox-LDL than for acetyl LDL, even at roughly equal levels of internalized lipoprotein.

Comparison of degradation in MPM of sol. and insol. ox-LDL and of vortex-aggregated LDL after exclusion of binding and uptake mechanisms

We also needed to reappraise the processing deficiency just described in MPM of insol. and sol. ox-LDL as well as vortex-aggregated LDL, as binding and uptake mechanisms of these LP might have differed from one another. We chose to perform these pulse-chase studies in the absence of chloroquine because appreciable accumulation of undegraded insol. ox-LDL or vortex-aggregated LDL occurred in MPM during the pulse phase even in the absence of a lysosomal inhibitor. To determine whether short-term (3 h) degradation of internalized lipoprotein depended on the amount of lipoprotein that had accumulated in an undegraded form, we first pulsed MPM with increasing amounts of insol. ^{125}I-labeled ox-LDL. After removing the labeled LP from the medium, we measured the degradation of this internalized LP over a 3-h chase period. As seen in **Fig. 5** this initial degradation rate during the chase phase increased with the amount of insol. ox-LDL accumulated during the pulse phase, eventually leveling off at very high concentrations of accumulated lipoprotein. This result suggested that if unequal accumulations of undegraded lipoprotein are obtained in the pulse phase of pulse-chase studies, subsequent initial

TABLE 1. Lysosomal degradation in MPM of insoluble oxidized LDL and acetyl LDL

	3 h Pulse				
	Degradation[a]		Intracellular Accumulation[a]		3 h Chase
Radiolabeled Ligand	Alone	+ Chloroquine	Alone	+ Chloroquine	Degradation[a]
Insol. ox-LDL	4.0 ± 0.1	0.5 ± 0.1	7.1 ± .1	10.1 ± .3	1.2 ± 0.1
Acetyl LDL	11.4 ± 0.5	1.9 ± 0.2	1.6 ± .2	7.1 ± .2	3.6 ± 0.4

MPM were incubated with insol. ^{125}I-labeled ox-LDL or with ^{125}I-labeled acetyl LDL in medium at a final concentration of 5 and 40 μg protein/ml respectively for 3 h at 37°C in the presence or absence of 300 μM chloroquine. Other cells were incubated with these lipoproteins for 3 h at 4°C without chloroquine. Degradation and intracellular accumulation levels were then determined as described in Materials and Methods. After the "pulse" incubation, cells were washed three times with saline and incubated for an additional 3 h in medium not containing lipoproteins or chloroquine. After the 3-h "chase" period, degradation was again determined.

[a] μg degraded or accumulated/mg cell protein.

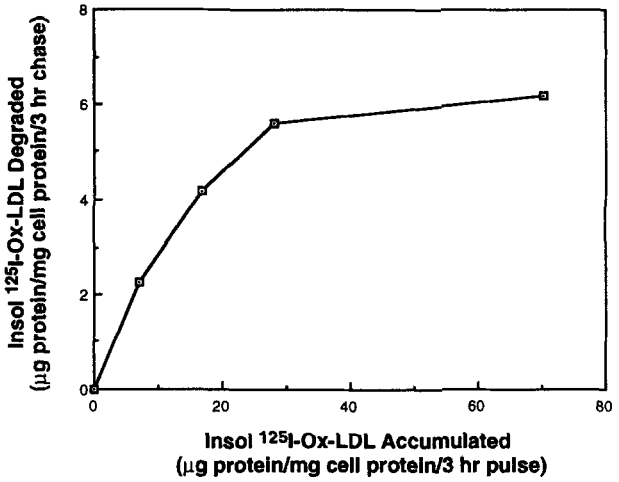


Fig. 5. Degradation of insoluble (insol. ^{125}I-labeled ox-LDL) that had accumulated in MPM. During the pulse phase MPM were incubated at 37°C for 3 h with increasing amounts of insol. ^{125}I-labeled ox-LDL, prepared as described in the text. This resulted in the accumulation of the indicated amounts of undegraded insol. ox-LDL. After removing the medium containing the labeled insol. ox-LDL, MPM were incubated during the chase phase with fresh medium containing no lipoprotein for 3 h and the resultant degradation was determined as described in Materials and Methods. Data points represent the mean ± SD of triplicate determinations.

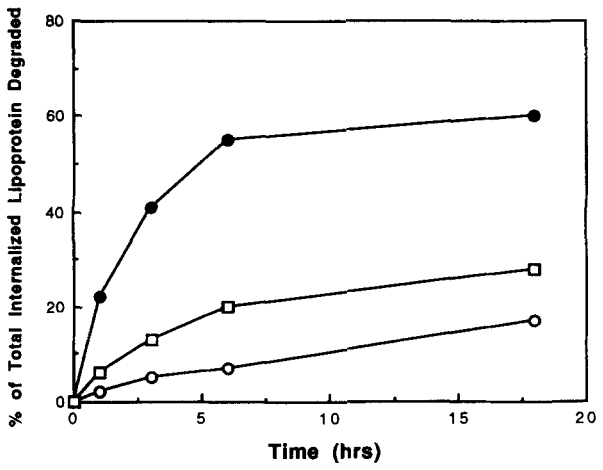


Fig. 6. Percent of internalized lipoprotein degraded of insoluble and soluble forms of oxidized and unoxidized LDL after minimizing the contribution of binding and uptake. Samples of ^{125}I-labeled insol. ox-LDL (○), sol. ox-LDL (●), and ^{125}I-labeled vortex-aggregated LDL (□) were prepared as described in Materials and Methods. MPM were incubated for 3 h at 37°C and at 4°C with the amounts of ^{125}I-labeled sol. and insol. ox-LDL as described in Table 2, or with labeled vortex-aggregated LDL in the presence and absence of cyt D. Levels of intracellular accumulation were determined from the differences in cell-associated label obtained at 37°C and at 4°C or with and without cyt D, respectively. Other cells were washed several times with PBS and then incubated at 37°C for the indicated periods with medium containing no labeled lipoproteins, and degradation rates were determined. Data points represent the mean ± SD of triplicate determinations.

degradation rates during the chase period would also reflect these differences.

To assess the relative roles of chemical changes and particle aggregation that both occur during extensive oxidation on intracellular degradation, we compared the degradation rate in MPM during a 3-h chase period after a 3-h pulse period for sol. ^{125}I-labeled ox-LDL, insol. ^{125}I-labeled ox-LDL, and vortex-aggregated (vx-) ^{125}I-labeled LDL. Although MPM were incubated with four to five times more sol. ox-LDL than insol. ox-LDL or vx-LDL, the resultant accumulation of undegraded lipoprotein was about three- to fourfold higher for both insoluble forms than for the soluble LP. Nevertheless, we found a severalfold higher initial degradation rate for sol. ox-LDL than for insol. ox-LDL and vortex-aggregated LDL (**Table 2, Fig. 6**). This occurred even though the accumulation during the pulse phase of the insoluble LP was threefold higher than that of sol. ox-LDL (Table 2) which, if anything, would have increased, not decreased this initial

TABLE 2. Intracellular processing in MPM of soluble and insoluble forms of oxidized LDL and vortex-aggregated LDL

	Sol. Ox-LDL (40 μg/ml sol.)	Insol. Ox-LDL (10 μg/ml insol.)	Vortex-Aggreg. LDL (10 μg/ml)
Accumulation of unprocessed lipoprotein after 3-h pulse	4.2 ± 0.8	14.7 ± 2.0	8.3 ± 0.8
Degradation after 3-h chase	2.0 ± 0.3	0.7 ± 0.1	1.1 ± 0.1
Degradation after 18-h chase	2.8 ± 0.6	2.5 ± 0.2	2.4 ± 0.1
% of internalized lipoprotein degraded after 18 h	66	17	29

MPM were incubated for 3 h with the indicated amounts of ^{125}I-labeled soluble (sol.) and insoluble (insol.) fractions of oxidized LDL at 37°C or at 4°C or with vortex-aggregated LDL at 37°C in the presence or absence of cytochalasin D, and were prepared as described in Materials and Methods. The amounts of accumulated lipoprotein were determined from the differences in cell-associated label at 37°C and at 4°C or at 37°C in the presence or absence of cytochalasin D, respectively. Other cells were washed several times with PBS and were then further incubated at 37°C for 18 h with medium not containing labeled lipoproteins. Degradation and cell-associated label were then determined, and intracellular accumulation was calculated as described above. Data points represent mean ± SD of triplicate determinations.

degradation, based on the influence of initial accumulation on subsequent degradation (Fig. 5).

When degradation, expressed as percent of total accumulated LP that became degraded, was measured over an 18-h time interval, we found that it eventually began to level off for both sol. and insol. LP (**Fig. 7**). The percentage of accumulated LP that had been degraded at this time interval was much lower for insol. ox-LDL than for the corresponding sol. ox-LDL (Fig. 7, Table 2). This suggested that particle aggregation occurring during extensive oxidation might have contributed more to the poor processing of ox-LDL than the chemical modifications. When we compared chemically modified and unmodified forms of aggregated LDL, e.g., insol. ox-LDL and vx-LDL, respectively, we found that the percent of internalized LP that was degraded after 18 h was only slightly higher for the latter. Thus, even after minimizing the possible effects of binding and uptake on subsequent degradation, we confirmed our earlier conclusion that a processing deficiency in macrophages could occur for aggregated forms of LDL, but independent of oxidation.

These above-described studies indicate that unoxidized, aggregated LDL showed the same processing deficiency in MPM as insol. ox-LDL. To assess whether the poor processing was due directly to poor degradation by lysosomal enzymes, we compared the hydrolysis of ^{125}I-labeled forms of LDL, acetyl LDL, insol. ox-LDL, sol. ox-LDL, and vx-LDL by a mixture of cathepsin B and D as had been previously reported to mimic protein degradation by lysosomal enzymes (11). As seen in Fig. 7 when the degradation of each labeled LP was assessed, acetyl LDL, LDL and vx-LDL all showed similar values of percent of total LP applied that was degraded to TCA soluble fragments after 33 h. By contrast, degradation of both sol. and insol. ox-LDL showed a lower percent after 33 h, insol. ox-LDL even lower than sol. ox-LDL. As vx-LDL was degraded as well as LDL, it does not appear that lysosomal degradation, as modeled by digestion of LP by a mixture of cathepsin B and D, was responsible for its poor processing in macrophages. It is of note that initial degradation rates (over the first few hours) were appreciably higher for acetyl LDL than for LDL, consistent with similar studies recently reported using mixtures of cathepsin B and D (11) or extracts of macrophages to degrade ox-LDL (12, 13).

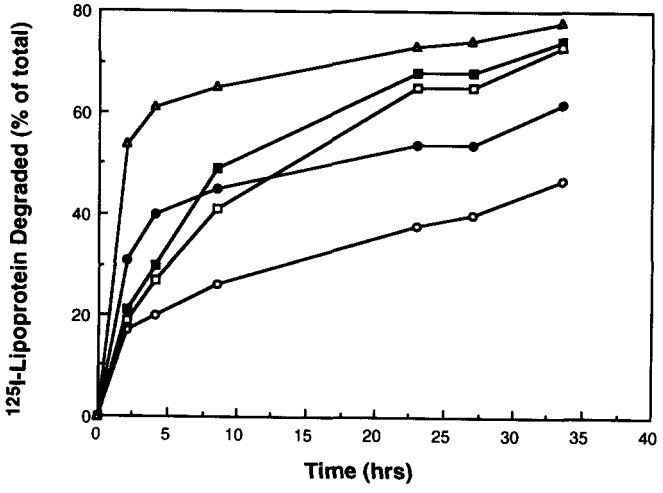


Fig. 7. Degradation of ^{125}I-labeled lipoproteins in vitro by a mixture of cathepsin B and D. Samples of ^{125}I-labeled forms of LDL (■), acetyl LDL (△), vortex-aggregated (aggr.) LDL (□), insoluble oxidized LDL (insol. ox-LDL) (○), and soluble oxidized LDL (sol. ox-LDL) (●) were incubated for the indicated time with a mixture of activated cathepsin B and cathepsin D, and the percent of added lipoprotein that became TCA-soluble was determined as described in Materials and Methods.

DISCUSSION

Poor processing in macrophages of the protein portion of ox-LDL could be due to substrate modification, which decreases the ability of lysosomal proteases to degrade such modified proteins, or to a change in intracellular trafficking that might diminish the fusion of endosomes/phagosomes with lysosomes. In this study we have confirmed and extended studies demonstrating the role of apoB modification on poor proteolytic degradation (2, 10–13), as well as providing novel data indicating that particle aggregation may result in poor processing in macrophages by a mechanism alternative to poor proteolysis in lysosomes.

Since particle aggregation is a characteristic of extensively oxidized LDL (6), we asked whether the documented deficiency in degradation of ox-LDL would be even greater for the insoluble portion of extensively ox-LDL. We found that macrophage degradation was far poorer for the insoluble fraction than for the corresponding soluble fraction, regardless of whether determined by measuring accumulation of undegraded LP after a 4-h incubation or after performing a pulse-chase study to minimize the contribution to degradation of the binding and uptake mechanisms. The poor degradation of both forms of ox-LDL relative to acetyl LDL could be due to deficient degradation by lysosomal cathepsins. This was shown by the observation in our study that ox-LDL degradation by a mixture of cathepsin B and D over a protracted period (33 h) under cell-free conditions was appreciably less than that of acetyl LDL or LDL. However, the differences between sol. and insol. ox-LDL degradation under cell-free conditions were not as great as found intracellularly. These results confirm results obtained by Lougheed, Zhang, and Steinbrecher (11) on the degradation by cathepsins of ox-LDL with undetermined degrees of aggregation. The poorer degradation of the insoluble than the soluble portions of ox-LDL shown by us in this report is consistent with data by Jessup, Mander, and Dean (13) showing that more extensively oxidized LDL is

degraded more poorly by proteases derived from macrophage extracts than less extensively oxidized LDL. In addition, they showed that the ability to be degraded by such extracts decreased with the degree of crosslinking of apoB in LDL by HNE (13), which is believed to be partly responsible for the aggregation of extensively oxidized LDL (6).

What this study has shown that was not addressed in previous reports (2, 11–13) is that the state of aggregation appears to be responsible for poor processing of insol. ox-LDL by a mechanism independent of deficient protease degradation. This assumption is based on the observation that when LDL that had been induced to aggregate by vortexing without oxidizing (vx-LDL) was incubated with macrophages in a pulse-chase experiment, the percent of internalized LP that was degraded after 18 h resembled that of insol. ox-LDL. By contrast, when vx-LDL was incubated under cell-free conditions for 33 h with a mixture of cathepsin B and D, it was degraded as well as acetyl LDL or LDL. Thus, the poor intracellular degradation cannot be explained by deficient proteolysis. Likewise, the larger differences in long-term degradation by cathepsin B and D for sol. and insol. ox-LDL under intracellular conditions than under cell-free conditions cannot be explained by deficient lysosomal proteolysis alone. A possible explanation for the poor processing in MPM of aggregated forms of LDL, whether oxidized or not, would be that such LP undergo a different intracellular trafficking route than monomeric LP. This would likely be the case for monomeric sol. ox-LDL entering endocytic pathways (2, 8), and insol. ox-LDL entering phagocytotic pathways. Why aggregated, unoxidized LDL would be channeled differently from insol. ox-LDL after phagocytosis is currently unclear. Complex sorting mechanisms by which ligands are directed to a final destination based on their unique structural and/or chemical features could explain differences in processing. Shuttling of internalized LP to different endosomal compartments with resultant differences in subsequent degradation was recently reported for LDL and β-VLDL following internalization in macrophages (25). If the fusion of phagosomes with lysosomes were incomplete, a reduced degradation of internalized aggregated LP could occur. Since insol. ox-LDL possesses both the characteristics that could lead to deficient degradation by cathepsins as well as the putative reduction in fusion with lysosomes, such LP would show an even lower degradation rate than their soluble counterpart. Based on the similarity in poor intracellular degradation between insol. ox-LDL and vx-LDL, it would appear that particle aggregation may play an even greater role than apoB crosslinking in decreasing the processing of extensively oxidized LDL in macrophages.

Intracellularly, the degradation of both sol. and insol. ox-LDL appears to have reached a maximum by about 18 h. This result would suggest that the accumulation of undegraded LP was not the result of a transient overloading of the lysosomal degradation system, especially after the phagocytosis of aggregated LP. When macrophages in culture were incubated with ox-LDL for at least 48 h, they accumulated ceroid, a residual lipid that cannot be extracted with organic solvents, which is believed to be a remnant of unprocessed ox-LDL (26). Such ceroid as well as epitopes unique for oxidized (27) or aldehyde-modified LDL (28) could be demonstrated in macrophage-derived foam cells derived from rabbit lesions. Ceroid was also shown to be present in secondary lysosomes of foam cells and in the extracellular space of atheromas of advanced atherosclerotic lesions in humans (29), especially within plaques of coronary bypass grafts (30). It is possible that when such foam cells lyse, this ceroid is extracted into the extracellular space and may play a role in the further development of the atherosclerotic lesion.

In summary, we have found that the insoluble portion of extensively oxidized LDL was processed even more poorly than its soluble portion. Whereas the deficiency of the soluble portion could be attributed to modifications of apoB by reactive aldehydes formed during oxidation, the deficiency of insoluble ox-LDL could be due to at least two mechanisms. One is by prevention of degradation of apoB after intermolecular crosslinking by such reactive aldehydes. The other is by aggregation per se, which could lead to poor processing, by being routed to different intracellular pathways.

The authors wish to thank Drs. Richard Morton and Guy Chisolm for their helpful suggestions during the preparation of the manuscript, Gary Smejkal for excellent technical assistance, and Susan Cothren for editorial assistance. This work was supported by NIH Program Project Grant HL 29582.

Manuscript received 10 March 1993 and in revised form 7 June 1993.

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