# Accumulation and metabolism of low density lipoprotein-derived cholesteryl linoleate hydroperoxide and hydroxide by macrophages[1]

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**Abstract** **Cholesteryl linoleate hydroperoxide (CLOOH) and hydroxide (CLOH) are present in human atheroma. The intracellular metabolism of low density lipoprotein (LDL)-derived CLOOH and CLOH remain undefined because extensive free radical-mediated LDL oxidation, which modifies LDL apolipoprotein B sufficiently to allow endocytosis by the scavenger receptor (ScR), also degrades CLOOH and CLOH. This problem was approached by first acetylating LDL lysine residues (AcLDL) to achieve protein modification, then exposing AcLDL to the aqueous radical donor 2,2′-azobis(2-amidinopropane) HCl (AAPH), to generate mildly oxidized AcLDL (OxAcLDL). Murine peritoneal macrophages incubated with OxAcLDL accumulated large quantities of CE and small, non-toxic quantities of CLOOH and CLOH in a time- and concentration-dependent manner, and accumulation was inhibited by fucoidin. Inhibition of acyl CoA: cholesterol acyltransferase during loading did not inhibit the accumulation of either CLOOH or CLOH, whereas $NH_4Cl$ decreased intracellular clearance of accumulated CLOOH from 68.3 ± 1.7% to 35.3 ± 1.0% over 12 h, suggesting lysosomal or pre-lysosomal accumulation. Intracellular clearance of unoxidized lipoprotein-derived CE decreased from 84.0 ± 5.9% to 43.1 ± 2.3% over 12 h when cells were loaded with AcLDL or OxAcLDL, respectively. Aggregation of mildly oxidized LDL, even without acetylation, also promoted cellular accumulation of CLOOH and CLOH. We conclude that intracellular accumulation of cholesteryl linoleate hydroperoxide and cholesteryl linoleate hydroxide can follow charge modification or aggregation of mildly oxidized LDL, and that LDL-derived oxidation products may inhibit hydrolysis of LDL-derived CE in foam cell macrophages.—** Kritharides, L., J. Upston, W. Jessup, and R. T. Dean. **Accumulation and metabolism of low density lipoprotein-derived cholesteryl linoleate hydroperoxide and hydroxide by macrophages.** ***J. Lipid Res.*** **1998.** 39: **2394–2405.**

**Supplementary key words** cholesteryl ester hydroperoxide • foam cell macrophage • scavenger receptor • oxidation • low density lipoprotein • lysosome • atherosclerosis, cholesterol

Lipid peroxidation is a biologically important process relevant to a number of human disease states, including cancer and atherosclerosis. In the latter, the oxidation of low density lipoprotein (LDL) in particular is probably important, and LDL oxidation products have been identified in immunohistological and chemical analyses of human atherosclerotic plaque (1–8).

Lipid hydroperoxides are generated during the early stages of lipoprotein oxidation, and these compounds are relatively easily decomposed to reactive radical species, such as peroxyl and alkoxyl hydroperoxyl radicals which can propagate the oxidative process (9). In addition, lipid hydroperoxides are among a range of LDL lipid oxidation products that have biological activity in addition to cytotoxicity (10, 11). For example, linoleic acid hydroperoxides alter cellular matrix metalloproteinase secretion (12), indicating that as well as causing oxidative damage, lipid hydroperoxides can more subtly and secondarily affect cellular function.

No information is presently available to indicate the consequences or fate of LDL-derived lipid hydroperoxides accumulated intracellularly after receptor-mediated endocytosis. Some previous studies have indicated the selective uptake and clearance by hepatic cells of cholesteryl ester hydroperoxides from oxidized lipoprotein (principally HDL) (13, 14) by a route independent of whole particle uptake. A similar route has been described for selective uptake of unoxidized cholesteryl esters from HDL and LDL by a number of cell types, including macrophages (15–17); however, at least for LDL, this is quite distinct from, and generally quantitatively less significant

Abbreviations: LDL, low density lipoprotein; FC, unesterified cholesterol; CE, cholesteryl ester (unoxidized); CLOOH, cholesteryl linoleate hydroperoxide; CLOH, cholesteryl linoleate hydroxide; 7KC, 7-ketocholesterol; REM, relative electrophoretic mobility; MPM, mouse peritoneal macrophage; AAPH, 2,2′-azobis(2-amidinopropane) HCl; AcLDL, acetylated LDL; OxAcLDL, oxidized AcLDL; ScR, scavenger receptor type A; apoB, apolipoprotein B; GPX, glutathione peroxidase; PhGPX, phospholipid glutathione peroxidase.

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than, whole particle receptor-mediated endocytosis. Efficient cellular reduction of LDL-derived 7-hydroperoxycholesterol has recently been described (18). Neither for LDLs or HDL have any studies demonstrated that lipid hydroperoxides can be detected within cells after exposure to lipoproteins.

The derivatization during oxidation that converts apolipoprotein B into a satisfactory ligand for the type A scavenger receptor ScR (and thereby causes lipid accumulation in cells) depends upon modification of apoB lysine residues by products of the decomposition of lipid hydroperoxides (19–21). After transition metal-mediated oxidation sufficient to modify the relative electrophoretic mobility of LDL to approximately 3.0, which is a requirement for ScR binding and cell lipid accumulation (4), only minor amounts of cholesteryl ester hydroperoxides remain in the LDL (22), and cells loaded with such copper-oxidized LDL do not contain detectable amounts of cholesteryl ester hydroperoxides (23–25). As CLOOH and CLOH are present in the lipids of atherosclerotic plaque (5), and LDL oxidation in plaque may be mild (26), the cell metabolism of lipoprotein-derived lipid hydroperoxides is potentially biologically very important. The uptake processes mediating accumulation of lipoprotein lipids by macrophages in vivo are unknown, but may include ScR-mediated uptake (27) or phagocytosis of LDL aggregates (26).

Recently, mutations preventing ScR expression in apoE knockout mice reduced atherosclerosis (28), emphasizing the potential importance of ScR-mediated uptake of lipoproteins in atherogenesis. Because ScR-mediated endocytosis of acetylated LDL (AcLDL) is not down-regulated by accumulation of cell lipid (27, 29) we hypothesized that unregulated ScR-mediated uptake of LDL containing lipid hydroperoxides could provide a mechanism by which lipid hydroperoxides could accumulate inside cells. Such a process would elucidate the intracellular metabolism of potentially very reactive hydroperoxide species, and may further clarify the atherogenic role of the macrophage ScR. However, as indicated above, LDL which has been sufficiently oxidized by transition metals to be a ligand for the ScR is not a suitable substrate for such a process because it does not contain significant quantities of cholesteryl ester hydroperoxides.

In the present studies we have achieved intracellular accumulation of LDL lipid hydroperoxides by dissociating apoB modification and lipid peroxidation. LDL was first acetylated to reproducibly generate a high-uptake ScR-binding lipoprotein particle (acetylated LDL or AcLDL), and then mildly oxidized using the aqueous radical donor AAPH (2,2′-azobis(2-amidinopropane) HCl under carefully defined conditions to generate OxAcLDL which contained substantial quantities of cholesteryl linoleate hydroperoxide (CLOOH), lesser amounts of cholesteryl linoleate hydroxide (CLOH), but preserved large quantities of unoxidized cholesteryl ester (CE). OxAcLDL was internalized by macrophages via the ScR and, remarkably, caused substantial intracellular accumulation of CLOOH, as well as CLOH, cholesterol, and unoxidized CE, whereas non-acetylated LDL oxidized to the same mild extent did not cause such accumulation. The accumulation of CLOOH and CLOH occurred in a lysosomal or prelysosomal compartment and was compatible with normal cell viability within a defined range of lipoprotein concentrations. Aggregation of oxidized LDL also permitted accumulation of CLOOH and CLOH in macrophages, and we further report that mild oxidation of LDL interfered with the cellular hydrolysis of lipoprotein-derived unoxidized CE. Intracellular accumulation of LDL-derived cholesteryl ester hydroperoxides may thus contribute to foam cell formation in vivo.

## MATERIALS AND METHODS

### LDL preparation

Human LDL was isolated from healthy fasting volunteers in the presence of ethylenediaminetetraacetic acid (EDTA, 3 mm, Sigma), aprotinin (90 kallikrein inhibitory units/ml; Sigma), and soybean trypsin inhibitor (20 mg/ml: Sigma) by discontinuous density-gradient ultracentrifugation ($1.02 < d < 1.05$), as previously described (22). The isolated LDL was filtered (0.45 μm), and stored in the dark in phosphate-buffered saline (PBS) containing 1.0 mg/ml EDTA and 0.1 mg/ml chloramphenicol (Sigma) at 4°C under $N_2$. LDL preparations were always used within 24–48 h of isolation. All dialyses were performed using deoxygenated Chelex-100® (Bio-Rad)-treated buffers in filled, stoppered bottles, in the dark, at 4°C, to prevent oxidation. Lipoprotein-deficient serum ($d > 1.25$ g/ml) was prepared as described (23). We have previously demonstrated that Chelex-100 treament of PBS to remove adventitious metals effectively prevents unregulated lipoprotein oxidation during dialysis (30).

### LDL modifications

*Acetylation*. LDL (6–8 mg protein/ml) was acetylated as described (23). Excess reagents were removed by dialysis against 4 × 1 litre exchanges of Chelex-100-treated PBS containing chloramphenicol (0.1 mg/ml).

*Oxidation*. Oxidation with the thermolabile aqueous radical donor AAPH (Polysciences Inc., Warrington, PA) was performed using a modification of a previous method (31). LDL or AcLDL (both at 1.0 mg protein/ml) were incubated with 50 mm AAPH in Chelex-100-treated PBS for 2 h at 37°C. Samples were then placed on ice and AAPH was removed by immediately passing oxidized lipoprotein solutions through two successive PD10 columns (Sephadex G-25M, Pharmacia Biotech Sweden) that had been previously equilibrated with ice-cold, Chelex-100-treated PBS.

### Non-denaturing agarose gel electrophoresis

Acetylation was assessed using non-denaturing agarose gel electrophoresis on 1% Universal agarose gels (Ciba-Corning) in Tris-barbitone buffer (pH 8.6) at 90 V for 45 min. The LDL band was visualized with Fat Red 7B stain. A relative electrophoretic mobility of ≥3, using native LDL as a reference, was routinely obtained.

### Isolation and culture of macrophages

Resident mouse peritoneal macrophages (MPM) were isolated by lavage from unstimulated QS mice as described (32) and plated in 35-mm-diameter tissue culture wells (Costar) at 5–6 × $10^6$ cells per well. Cultures were incubated at 37°C for 1–2 h to establish adherence, then washed three times with pre-warmed PBS

before incubation with Dulbecco's minimal essential medium (DMEM) containing LPDS (10%, v/v, equivalent to final protein concentration of 2.5 mg/ml), penicillin G, and streptomycin (50 units/ml and 50 μg/ml respectively, Sigma), plus modified lipoproteins as indicated.

### Incubation of macrophages with lipoproteins

The concentrations and incubation times of macrophages with lipoproteins were systematically varied in different experiments and are described in Results, but generally represented only minor modifications of experimental protocols we have previously described (23, 33, 34). Unless otherwise indicated, LDL and OxAcLDL were supplied at 50 μg protein/ml of DMEM containing 10% (v/v) LPDS for up to 24 h. LPDS was omitted in some specified experiments in order to investigate the effects of LPDS on cell viability and where extraction of lipoprotein lipids from cell culture media was required. We have previously determined that extraction of LDL lipids from DMEM without LPDS was approximately 100%, but that in the presence of LPDS, the extraction yield of unoxidized cholesteryl esters was reduced by 60.5 ± 7.6% (22, 24).

### Protocol for pulse-chase studies

In experiments studying the kinetics of cellular metabolism of cell-incorporated cholesteryl ester hydroperoxides and hydroxides, cells were loaded with 50 μg OxAcLDL protein/ml in DMEM–LPDS for 24 h (pulse phase), washed three times in PBS, and incubated in fresh DMEM containing 1.0 mg/ml bovine serum albumin (DMEM–albumin) without LPDS or lipoproteins for up to 12 h (chase phase). LPDS was omitted during the chase incubation to exclude significant efflux of cell lipids to LPDS (35). To confirm that unoxidized cholesteryl esters (CE), cholesteryl linoleate hydroperoxide (CLOOH), and cholesteryl linoleate hydroxide (CLOH) analyzed during the chase period were derived from OxAcLDL and did not represent cellular re-esterification of oxidized fatty acids, pulse and chase incubations were repeated in the presence of the acyl-CoA:cholesterol acyltransferase (ACAT) inhibitor Sandoz-58035 (Sandoz Pharmaceuticals, East Hanover, NJ). Sandoz-58035 was used at a final concentration of 5 μg/ml (final ethanol concentration in medium of <0.1 % v/v) as previously described (23, 36).

### Conditions for supply of ammonium chloride

The weak base and lysosomotropic agent $NH_4Cl$ was added to lipid-loaded MPM to determine the effect of lysosomal alkalinization on cellular clearance of CLOOH and CLOH. Because $NH_4Cl$ can interfere with endocytosis, receptor cycling, and a number of other cellular processes upstream of its inhibitory effects on lysosomal hydrolases (37), it was added after the pulse phase (i.e., at the beginning of the chase phase) to allow cellular accumulation of lipoprotein-derived material without interference. $NH_4Cl$ stock solution (1.0 m in water) was diluted in DMEM to a final concentration of 10 mm as previously described (38). This low concentration of $NH_4Cl$ was used to effectively inhibit lysosomal enzymatic hydrolysis (38) without causing the significant secretion of lysosomal enzymes evident at higher concentrations (39).

### Analysis of oxidized and unoxidized lipids

Prior to extraction, cells were gently washed three times with ice-cold, Chelex-treated PBS and scraped from the culture dish into 1 ml of ice-cold PBS. Two 50-μl aliquots of cell suspension were lysed with 50 μl of 0.4 m NaOH for protein assay by the bicinchoninic (BCA) acid method using BSA as standard (23). Aliquots of the cell suspension (0.8 ml) were placed into glass Kimax tubes (Kimble, MA), containing 0.2 ml ice-cold PBS containing BHT and EDTA (Sigma; final concentrations 20 μm and 2 mm, respectively), and the total was extracted into HPLC grade, Chelex-treated methanol (2.0 ml) then hexane (5 ml) (Mallinckrodt) (22). A sample of the hexane layer was evaporated to dryness, redissolved in the appropriate mobile phase, and analyzed by HPLC as described below (22) . LDL (20 μg protein) was diluted to 1.0 ml with ice-cold PBS (containing BHT and EDTA as above) before extraction into methanol/hexane.

Separation of lipids at room temperature on a C-18 column (25 × 0.46 cm length, 5 μm pore size, Supelco) was performed by reverse phase high performance liquid chromatography (HPLC). Cholesterol and cholesteryl esters (specifically cholesteryl docosahexanoate, cholesteryl arachidonate, cholesteryl linoleate, cholesteryl oleate, and cholesteryl palmitate) were separated in acetonitrile–isopropanol 30:70 (v/v) and detected at 210 nm (22). Oxidation products including CLOOH, CLOH, and 7-ketocholesterol were separated using a solvent system of acetonitrile–isopropanol–water 44:54:2 (v/v/v) and detected at 234 nm (22).

Hydroperoxides of cholesteryl linoleate (CLOOH) were also analyzed by HPLC using chemiluminescence detection as previously described (40, 41). Briefly, hexane extracts were injected onto a LC-18 column (25 × 0.46 cm length, 5 μm pore size, Supelco) and eluted with ethanol–methanol–2-propanol 736: 225:39 (v/v/v) at 1 ml/min. Compounds eluting from the column were detected by post column chemiluminescence using a Tohoku CLD-100 chemiluminescence detector (Tohoku Electronic Industry Co. Ltd., Japan). The reaction solution for chemiluminescence comprised methanol–100 mm sodium borate 1:1 (v/v) pH 10, 1 mm isoluminol (Sigma), and 1 mg/l microperoxidase (Type MP-11; Sigma) run at 1.5 ml/min.

Normal phase HPLC was utilized for the analysis of the four geometrical cholesteryl linoleate hydroperoxides (CLOOH) and corresponding hydroxides (CLOH)(5). Saponification of the oxidized CE was not required, with CLOOH and CLOH being analyzed directly on a silica column (LC-Si, 25 × 0.46 cm, 5 μm, Supelco) eluted with heptane–diethyl ether–isopropanol 100:0.5: 0.175 (v/v/v) at 2 ml/min and monitored at 234 nm. Authentic standards of 13-(S)(*Z*,*E*) and 9-(S)(*E*,*Z*) CLOH (in heptane) were subjected to chromatography to allow assignment of the different isomers.

Commercial standards (hydroxy 13-(S)(*Z*,*E*) and 9-(S)(*E*,*Z*) CLOH (Cayman Chemicals, Ann Arbor, MI), 7-ketocholesterol (Steraloids, Wilmington, MA), cholesterol and unoxidized cholesteryl ester (CE) (Sigma)) of each of the oxidation products and unoxidized lipids were used to determine the elution time of unknowns and response factors as described (22). Standards of cholesteryl linoleate hydroperoxides, and hydroxides and racemic cholesteryl linoleate hydroxides (CLOH) were also prepared as previously described (31, 40, 42). Lipoprotein lipids were calculated as nmol/mg apolipoprotein B or nmol/nmol LDL cholesterol, and cell lipids as nmol/cell culture or nmol/mg cell protein as indicated in Results.

### Protein estimation

The protein content of LDL samples and cell lysates was measured using the bicinchoninic acid (BCA) method (Sigma) using BSA in water (LDL) or in 0.2 m NaOH (cell lysates) as standard (23). Samples were incubated for 60 min at 60°C and the absorbance at 562 nm was determined. All samples were assayed in duplicate or triplicate for each culture dish or each lipoprotein sample.

### Cell viability

Cell viability was routinely assessed by light microscopic morphology, by estimation of cell protein, and by absence of cell

staining with 0.5% (w/v) Trypan blue (Gibco) after 2 min incubation. Counting was performed from at least two high-powered fields in representative dishes, and viability under various loading conditions was independently confirmed in several replicate experiments.

### Presentation, statistical analysis

A minimum of three separate incubations was performed for each condition in each experiment. Individual calculations were made for each cell culture dish from which cells were removed, results for each experiment are expressed as the mean ± SD of triplicate cultures, and all experiments described are representative of several. Where data have been pooled from multiple experiments, these are expressed as the mean ± SEM of "n" experiments. Statistical comparisons were made using Student's unpaired *t*-test using Mystat software, and significance was concluded if $P < 0.05$.

## RESULTS

### Characterization of the lipid composition of oxidized and acetylated LDL (OxAcLDL)

In order to generate a high-uptake form of LDL that contained large quantities of CLOOH, LDL was first acetylated and then AcLDL (1 mg/ml) was incubated with 50 mm AAPH for 120 min (**Table 1**). This duration of exposure reproducibly generated CLOOH while preserving over 70% of LDL cholesteryl ester (CE) and only mildly modifying lipoprotein apoB as assessed by relative electrophoretic mobility (REM). REM of LDL oxidized by AAPH was 1.3 ± 0.2 relative to control, unoxidized LDL. Acetylation without oxidation always increased the REM to between 3.5 and 4.0, and oxidation of AcLDL (to generate OxAcLDL) did not significantly change the REM beyond that achieved by acetylation alone (Table 1).

Although the oxidation procedure utilized to generate OxAcLDL was intentionally limited by the duration of incubation, OxAcLDL was clearly oxidized, with consistently lower cholesterol and CE values than that of AcLDL not subjected to oxidation. OxAcLDL contained only small amounts of 7KC, consistent with its identification as a product of more extensive LDL oxidation (22, 25, 43, 44). As indicated in Table 1, CLOOH was always more abundant (177 ± 20.9 nmol/mg) than CLOH (8.45 ± 0.97 nmol/mg). In AcLDL that had not been exposed to AAPH, total CLOOH+CLOH was always less than 1.0 nmol/mg AcLDL protein.

OxAcLDL analyzed as previously described (40, 41) did not contain α-tocopherol (data not shown). Thus, in the experiments described below, cells incubated with OxAcLDL were exposed to a modified LDL of enhanced electrophoretic mobility (negative charge), containing large quantities of unoxidized CE and cholesterol, small but defined quantities of CLOOH and CLOH, and lesser quantities of 7KC.

### Macrophages incubated with OxAcLDL contain cholesteryl linoleate hydroperoxide and hydroxide

Mouse peritoneal macrophages (MPM) incubated for 24 h with OxAcLDL accumulated the lipid oxidation products CLOOH, CLOH, and 7KC (**Fig. 1**). In contrast, control MPM not exposed to OxAcLDL did not contain any of these oxidation products. The identity of 7KC, CLOOH, and CLOH was confirmed by the co-elution of the unknown peaks with authentic standards of these compounds, both when standards and unknowns were injected separately, and by simultaneous injection after mixing standards and unknowns ("spiked" samples, Fig. 1A and 1D). Co-elution of standards and cell lipids during simultaneous injection was also confirmed with a second more polar mobile phase (acetonitrile–isopropanol 60:40 (v/v), chromatograms not shown). MPM incubated with freshly prepared, unoxidized, acetylated LDL (AcLDL) did not contain any detectable 7KC, CLOH, or CLOOH, but did accumulate large quantities of unoxidized CE (described in detail below) as previously observed (23, 29).

Hexane extracts of MPM loaded with OxAcLDL were also analyzed for CLOOH by a reverse phase HPLC system with chemiluminescence detection, which is both sensitive and specific for the detection of hydroperoxides (40). This analysis independently confirmed the presence of CLOOH in the cell extract on the basis of a clear chemiluminescence signal eluting at the same time as authentic CLOOH standard, and by the complete quenching of the signal after reduction of the cell lipid extract by sodium borohydride (40) (data not shown). In addition, separation of CLOOH and CLOH isomers by normal phase HPLC indicated that the profile of isomers in OxAcLDL and in MPM loaded with OxAcLDL were identical (data

TABLE 1. Lipid composition of OxAcLDL

| | FC | CE | CLOOH | CLOH | 7KC | REM |
|---|---|---|---|---|---|---|
| | *nmol/mg LDL protein* | | | | | |
| A | | | | | | |
| AcLDL | 781 ± 60.0 | 1504 ± 90.2 | 0 | 0 | 0 | 4.0 |
| OxAcLDL | 531 ± 1.6 | 940 ± 17.9 | 209 ± 11.8 | 8.5 ± 0.43 | 1.49 ± 0.11 | 3.9 |
| B | | | | | | |
| OxAcLDL | 613 ± 52.6 | 1242 ± 130 | 177 ± 20.9 | 8.45 ± 0.97 | 1.23 ± 0.38 | 3.6 ± 0.11 |

Fresh LDL was acetylated (AcLDL) or acetylated and then exposed to AAPH to generate OxAcLDL as indicated in Methods. FC, unesterified cholesterol; CE, cholesteryl ester; CLOOH, cholesteryl linoleate hydroperoxide; CLOH, cholesteryl linoleate hydroxide; 7KC, 7-ketocholesterol; REM, relative electrophoretic mobility (relative to unmodified native LDL used to prepare AcLDL and OxAcLDL). A: Comparison of the composition of OxAcLDL and AcLDL from the same preparation (mean ± SD of triplicate extractions). B: Variations in the composition of OxAcLDL from four different preparations of LDL used in different experiments (mean ± SEM).

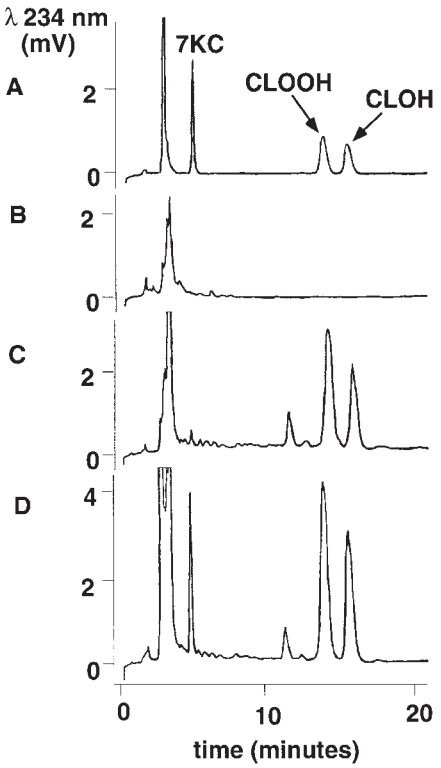


**Fig. 1.** Detection of lipid oxidation products in macrophages incubated with OxAcLDL by reverse phase HPLC. After 24 h incubation with OxAcLDL, macrophages (panel C) had accumulated CLOOH, CLOH, and small quantities of 7KC. Macrophages incubated without OxAcLDL (panel B) did not contain these lipids, and the identity of cellular lipids was confirmed by authentic standards (panel A) and co-injecting or "spiking" these standards with cell extract (panel D). Reverse phase HPLC analysis with a mobile phase of acetonitrile–isopropanol–water 44:54:2 (v/v/v) and detection by 234 nm absorbance as described in Methods.

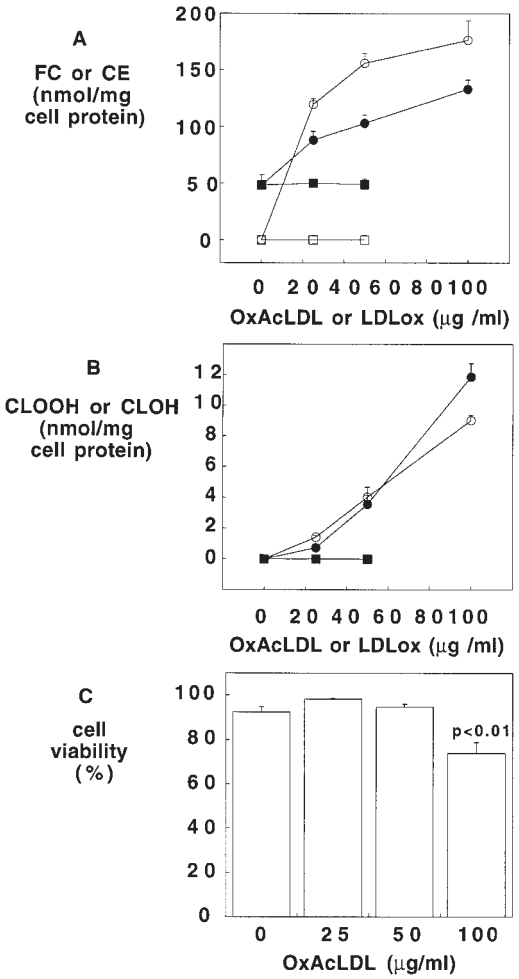


**Fig. 2.** Concentration-dependent accumulation of CLOOH and CLOH in macrophages. MPM were incubated for 24 h with 0–100 μg/ml OxAcLDL (circles) or with LDL oxidized to the same degree but without acetylation (LDLox, squares). (A) Macrophages incubated with OxAcLDL accumulated unesterified (FC, closed circle) and esterified cholesterol (CE, open circle), but macrophages incubated with LDLox did not accumulate FC (closed square) or CE (open square). (B) Simultaneously, macrophages incubated with OxAcLDL accumulated CLOOH (closed circle) and CLOH (open circle), whereas cells incubated with LDLox accumulated neither (CLOOH and CLOH closed and open square, respectively, CLOH values obscured by symbol for CLOOH). (C) Cell viability was quantified by the absence of staining with trypan blue as described in Methods. In most experiments, concentrations of 100 μg/ml were toxic; $P < 0.01$ for comparison of viability after incubation of cells with 100 μg/ml compared with each of the other indicated concentrations of OxAcLDL.

not shown), consistent with the cellular CLOOH and CLOH being lipoprotein-derived (discussed further below). Thus three separate chromatographic analytical systems confirmed that macrophages incubated with OxAcLDL accumulated CLOOH.

### Concentration-dependent accumulation of unoxidized and oxidized lipids

The capacity of oxidized LDL and OxAcLDL to cause cellular accumulation of LDL oxidation products was compared. LDL that was oxidized but not acetylated and OxAcLDL (both from the same preparation of LDL) contained similar concentrations of CLOOH and CLOH (oxidized LDL contained 100.9 ± 4.7 nmol CLOOH and 8.93 ± 0.89 nmol CLOH per mg apoB, and OxAcLDL contained 115.5 ± 13.0 nmol CLOOH and 8.2 ± 1.1 nmol CLOH per mg apoB). Unlike OxAcLDL, however, AAPH-oxidized LDL did not promote cellular accumulation of unoxidized or oxidized lipids (**Fig. 2**).

Consistent with previous literature describing the effects of unoxidized AcLDL (45), cells loaded with OxAcLDL accumulated increasing quantities of unoxidized CE as the concentration of OxAcLDL in the incubation medium increased, with apparent maximal CE accumulation at between 50 and 100 μg/ml OxAcLDL (Fig. 2). In contrast, there was simultaneous concentration-dependent accumulation of CLOOH and CLOH, without evident saturation at higher concentrations of OxAcLDL. The lack of saturation may indicate that the endocytosis of CLOOH and CLOH also occurred by routes additional to whole-particle uptake of OxAcLDL, such as "selective uptake," or that the rate of their intracellular accumulation greatly exceeded the capacity of macrophages to metabolize these lipids.

### Concentration-dependent effects of OxAcLDL on cell viability

Lipid hydroperoxide cytotoxicity is usually dose-dependent, and has hitherto been principally characterized after adding isolated lipid hydroperoxides to culture media in high concentrations, rather than by assessing the effect of intracellular incorporation of lipid hydroperoxides within a lipoprotein particle. CLOOH-containing OxAcLDL could be cytotoxic in a concentration-dependent manner, but was only so when added to cells at concentrations of at least 100 μg/ml (Fig. 2C). In some experiments, even 100 μg/ml was not detectably cytotoxic, on the basis of morphology, cell protein, and trypan blue staining. The uninterrupted concentration-dependent accumulation of CLOOH (Fig. 2B) despite mild toxicity at 100 μg/ml may indicate that cellular uptake of CLOOH preceded the development of cytotoxicity. Omission of serum (10% lipoprotein-deficient human serum, LPDS) from OxAcLDL-containing medium reduced cell viability even at the lower concentrations used in most experiments. In one typical experiment, the viability of MPM incubated with 50 μg/ml OxAcLDL decreased from 95.6 ± 1.0% in the presence of LPDS to 88.3 ± 1.1% without LPDS. However, as the intracellular accumulation of CLOOH was very similar in cells incubated in media with or without LPDS (data not shown) the cytoprotective effects of serum are not simply related to accumulated intracellular CLOOH, and the cytotoxicity of OxAcLDL may be contributed to by other oxidation products internalized by the cell. LPDS was routinely included in all incubations unless otherwise specified.

### Time-dependent accumulation of lipids in macrophages incubated with OxAcLDL

Over 24 h, macrophages incubated with 50 μg/ml OxAcLDL accumulated large amounts of CE (**Fig. 3A**), and smaller but time-dependent quantities of CLOOH, and CLOH (Fig. 3B and 3C). The cellular accumulation of CLOOH as assessed by 234 nm absorbance was paralleled by the accumulation of CLOOH as assessed by chemiluminescence (Fig. 3B).

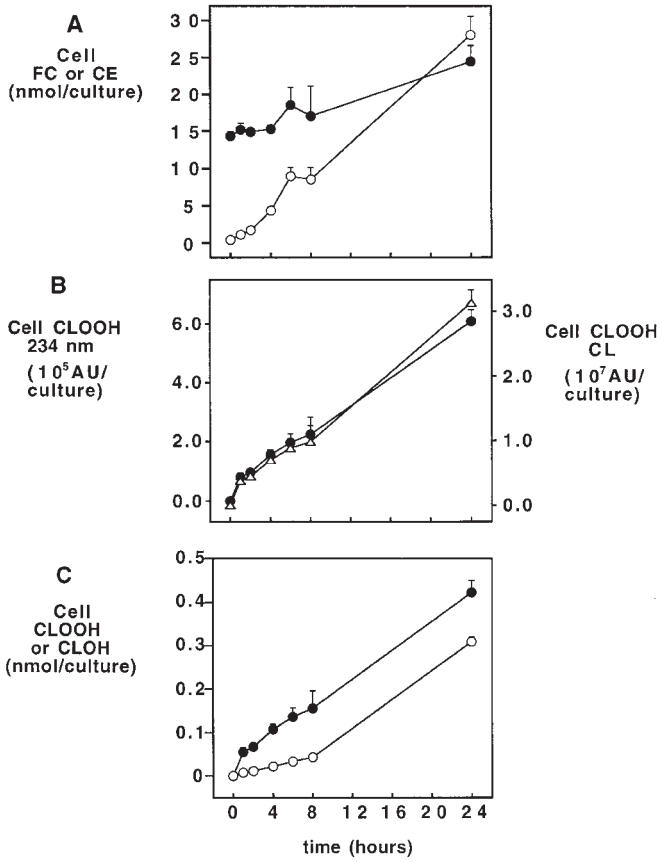


**Fig. 3.** Time-dependent cellular accumulation of CLOOH and CLOH during incubation of macrophages with OxAcLDL. Macrophages were incubated with 50 μg/ml OxAcLDL for up to 24 h, then lipids were extracted and analyzed by HPLC. Cells simultaneously accumulated unesterified (FC, closed circles) and esterified (CE, open circles) cholesterol (panel A) and CLOOH and CLOH (panel C, closed and open circles, respectively). Accumulation of CLOOH was confirmed by chemiluminescence detection (CL, panel B). Mass data in panels A and C are expressed as nmol/culture. In panel B, 234 nm (closed circle) and CL detection (open triangle) are expressed as total AU (area units of 234 nm absorbance or CL) per cell culture. The data for CLOOH in panel C are the same as those used in panel B after correction for the molar absorbance of CLOOH at 234 nm.

### Cell-mediated clearance of CLOOH from OxAcLDL during incubations with cells

During incubation of OxAcLDL with macrophages, there was extracellular clearance of CLOOH and CLOH from OxAcLDL as well as intracellular accumulation of these lipids (**Table 2**). To confirm apparent cell-mediated clearance of CE, CLOOH, and CLOH from OxAcLDL, lipid concentrations for each sample were calculated per nmol of extracted lipoprotein cholesterol. The molar ratio of CLOOH to CLOH (CLOOH/CLOH) in OxAcLDL was also calculated to quantify the depletion of CLOOH relative to CLOH. Cell-dependent extracellular clearance of CLOOH was greater than concurrent clearance of CLOH or unoxidized CE (Table 2A). To account for reduction of CLOOH to CLOH, the sum of CLOOH and CLOH (CLOOH + CLOH) was calculated, and the residual CLOOH + CLOH after incubation with or without cells was used to estimate cell-mediated clearance (Table 2B). Cell-mediated clearance of CLOOH+CLOH from OxAcLDL was much greater than could be accounted for by the net accumulation of CLOOH and CLOH within cells.

### Scavenger receptor-mediated uptake of OxAcLDL

The requirement for acetylation of LDL (prior to its oxidation with AAPH) for cellular accumulation of CLOOH and CLOH indicated that uptake via the macrophage ScR-mediated uptake was important in this process. The observed cell-dependent depletion of extracellular CE, CLOOH, or CLOH relative to cholesterol (Table 2A) could be due to selective uptake of some LDL esterified lipids by macrophages (e.g., (17)), particularly CLOOH (13). However, fucoidin, a competitive ligand for the ScR, very effectively inhibited the accumulation of cholesterol, CE,

TABLE 2. Cell-mediated clearance of CLOOH from OxAcLDL

A: Residual lipids in OxAcLDL after 24-h incubation with macrophages

| | CE | CLOOH | CLOH | CLOOH/CLOH |
|---|---|---|---|---|
| | *mol/mol lipoprotein cholesterol* | | | *molar ratio* |
| Cell-free | 2.10 ± 0.09 | 0.29 ± 0.015 | 0.033 ± 0.002 | 8.9 ± 0.77 |
| With cells | 1.65 ± 0.02 | 0.17 ± 0.024 | 0.029 ± 0.004 | 6.04 ± 0.29 |
| (% of cell-free incubation) | (78.6) | (58.6) | (87.9) | (67.9) |

B: Clearance from OxAcLDL and accumulation in macrophages of CLOOH and CLOH

| | (i) OxAcLDL 24 h, no cells | (ii) OxAcLDL 24 h, +cells | (iii) OxAcLDL net difference (cell clearance) | (iv) Cellular CLOOH + CLOH | (v) % Clearance accounted for by cellular CLOOH + CLOH |
|---|---|---|---|---|---|
| | *nmol/culture* | | | | *%* |
| CLOOH + CLOH | 11.36 ± 1.48 | 6.40 ± 1.10 | 4.96 | 1.34 ± 0.027 | 27.0 |

OxAcLDL was added to DMEM (without LPDS, see Methods) and added to empty culture dishes (cell-free) or with macrophages (with cells, both at 50 μg apoB protein/ml) for 24 h, then extracted and analyzed for cholesterol, unoxidized CE, CLOOH, and CLOH as described in Methods. Values are expressed as mol per mol of lipoprotein cholesterol (panel A) or nmol per cell culture (panel B). Panel A: Values after cell-free incubation were defined as 100%, and mol/mol values after incubation with cells were calculated as a % of cell-free values to generate figures in parentheses. Panel B: To account for reduction of CLOOH to CLOH during exposure of OxAcLDL to cells, the sum of CLOOH and CLOH (CLOOH + CLOH) present after 24 h incubation without cells (column (i)) was calculated per culture. The cell-mediated clearance of CLOOH + CLOH from OxAcLDL for each cell culture (column (iii)) was calculated by subtracting column (ii) from column (i). Total CLOOH+CLOH accumulated in cells (column (iv)) was divided by (iii) to calculate the proportion of net clearance accounted for by intracellular accumulation (v).

CLOOH, and CLOH. In one experiment in which macrophages not exposed to OxAcLDL (control) contained 14.4 ± 0.6 nmol FC/dish, 0.41 ± 0.05 nmol CE/dish and no detectable CLOOH or CLOH, macrophages incubated with 50 μg/ml OxAcLDL contained 24.5 ± 2.2 nmol cholesterol/dish, 28.1 ± 2.5 nmol CE/dish , 0.42 ± 0.027 nmol CLOOH/dish, and 0.31 ± 0.011 nmol CLOH/dish. In contrast, macrophages incubated with OxAcLDL together with 50 μg/ml fucoidin contained 15.5 ± 0.8 nmol cholesterol/dish, 1.1 ± 0.15 nmol CE/dish, and no detectable CLOOH and CLOH; nearly identical to control macrophages not exposed to OxAcLDL. These data indicated that binding to the ScR was essential for accumulation of CE, CLOOH, and CLOH by macrophages. Consequently, under these experimental conditions, ScR-mediated uptake was either quantitatively the most important route of uptake of unoxidized and oxidized esterified lipids, or other selective or non-selective routes of uptake required initial binding of lipoprotein to the ScR.

### Intracellular CLOOH is cleared more rapidly than CLOH or unoxidized cholesteryl esters

To study the intracellular stability of oxidized cholesteryl linoleate, a series of pulse-chase experiments were undertaken. MPM were incubated with 50 μg/ml OxAcLDL for 24 h (pulse), then washed and incubated in control medium (DMEM-albumin) for up to 12 h (chase). This dissociated accumulated intracellular CLOOH and CLOH from a further supply of OxAcLDL, allowing measurement of the intracellular metabolism of CLOOH independently of clearance of extracellular lipoprotein lipids.

Over the chase period, progressive clearance of intracellular CLOOH was observed (**Fig. 4**). Concurrently, intracellular CLOH, cholesterol, and CE values were maintained at their initial concentrations. In the case of CLOH, this most likely suggested either that the rate of reduction of CLOOH to CLOH was sufficient to replenish CLOH depletion caused by hydrolysis of CLOH or that CLOH, but not CLOOH, was being re-generated by intracellular esterification of cholesterol and free linoleic acid hydroxide. In the case of cholesterol and CE, it seemed that an overall steady state was achieved between ongoing cellular re-esterification of cholesterol via ACAT, and lysosomal and non-lysosomal hydrolysis of CE. Each of these possible explanations was investigated below.

### ACAT inhibition during loading affects accumulation of CE but not of CLOOH and CLOH

Macrophages were incubated with OxAcLDL or AcLDL for 24 h, with or without ACAT inhibitor Sandoz-58035 (**Fig. 5**). ACAT inhibition significantly decreased the cellular accumulation of CE in macrophages incubated with either AcLDL or OxAcLDL. In contrast, cellular CLOOH and CLOH concentrations were unaffected by ACAT inhibition. Thus, unlike CE, accumulation of CLOOH and CLOH did not depend upon intracellular re-esterification by ACAT. This is consistent with their being entirely or almost entirely lipoprotein-derived.

As cellular synthesis of CE is catalyzed by ACAT (36, 46), residual CE after ACAT inhibition implies the presence of undegraded, lipoprotein-derived CE in these cells. ACAT inhibition achieved a consistently greater absolute and proportional reduction in cellular CE concentrations in AcLDL-incubated cells (75.4 ± 5.7% reduction in cellular CE) than in macrophages incubated wth OxAcLDL (34.1 ± 4.1% reduction in cellular CE). As the only difference be-

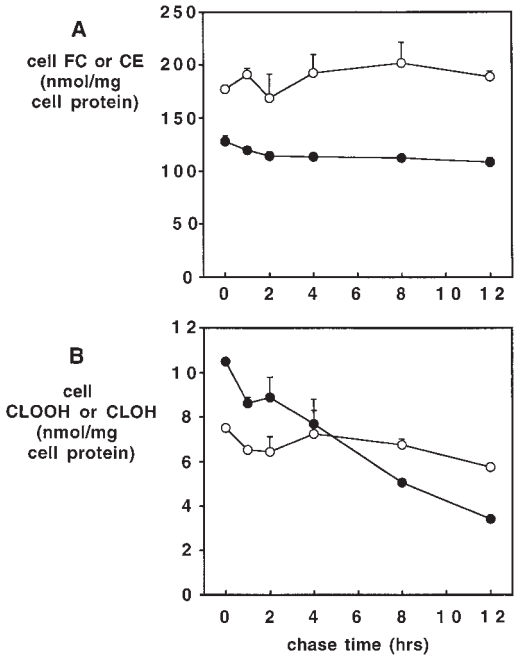


**Fig. 4.** Intracellular clearance of accumulated CLOOH. Macrophages were incubated with OxAcLDL for 24 h (pulse), then washed and incubated in DMEM (chase) for 12 h. Cells and media were extracted at varying intervals during the chase and analyzed for FC and CE (closed and open circles respectively; panel A), and CLOOH and CLOH (closed and open circles respectively, panel B). No CLOOH or CLOH appeared in the medium during chase incubations.

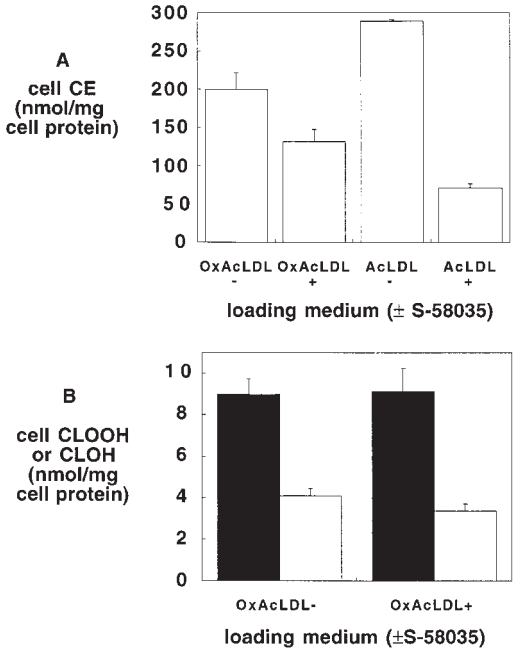


**Fig. 5.** ACAT inhibition does not significantly affect intracellular accumulation of CLOOH or CLOH. Macrophages were incubated with OxAcLDL or AcLDL for 24 h, with (+) or without (−) ACAT inhibitor S-58035, and analyzed for CE (panel A), and CLOOH and CLOH (solid and open bars respectively, panel B). Almost all cellular CLOOH and CLOH is undegraded, lipoprotein-derived material and is not generated by intracellular re-esterification of cholesterol.

tween AcLDL and OxAcLDL used in these experiments was mild oxidation of the latter, processes mediating the intracellular clearance of lipoprotein-derived unoxidized CE (such as lysosomal hydrolysis) may be inhibited by mild lipoprotein oxidation.

To further measure intracellular hydrolysis of lipoprotein-derived CE, MPM were incubated with either OxAcLDL or AcLDL in the presence of S-58035 (pulse), washed, then incubated in DMEM with S-58035 for up to 12 h (chase). MPM cleared 84.0 ± 5.9% of accumulated AcLDL-derived CE compared with only 44.1 ± 9.3% of accumulated OxAcLDL-derived CE in 12 h. In the same OxAcLDL-loaded cells, intracellular clearance of CLOOH (78.7 ± 6.4% in 12 h) was therefore faster than the hydrolysis of unoxidized CE. Comparison of the rates of clearance of CE and CLOOH must be interpreted cautiously because of the 15- to 20-fold different pool sizes of the two lipids at the end of the pulse at $t_0$. For example, in one typical experiment, at $t_0$ cells loaded with OxAcLDL contained 225.1 ± 9.9 nmol CE/mg cell protein and 13.5 ± 1.2 nmol CLOOH/ mg cell protein, and at the end of the 12-h chase period contained 128.2 ± 6.8 nmol CE/mg cell protein and 2.92 ± 0.4 nmol CLOOH/mg cell protein.

### Cellular clearance of accumulated CLOOH and CLOH requires maintenance of acidic lysosomal or endosomal pH

To evaluate whether CLOOH and CLOH were located and metabolized in a lysosomal or prelysosomal compartment, the effect of the lysosomotropic agent $NH_4Cl$ was tested. To avoid interference with endocytosis and to take advantage of its relatively rapid onset of action, macrophages were first loaded with OxAcLDL without $NH_4Cl$, washed, and then incubated with DMEM–albumin and $NH_4Cl$ (**Table 3**).

$NH_4Cl$ substantially preserved CLOOH levels inside macrophages. There were significant differences between cells exposed to $NH_4Cl$ and those not exposed to $NH_4Cl$ within 4 h (data not shown), and by 12 h $NH_4Cl$ had inhibited the intracellular clearance of CLOOH by more than 50%. The pH-dependent clearance of CLOOH suggests that a major metabolic fate of lipoprotein-derived CLOOH is lysosomal hydrolysis, although pH-sensitive lysosomal peroxidase activity cannot be excluded. The persisting 30–40% $NH_4Cl$-independent decline in cell CLOOH may represent reduction of CLOOH to CLOH, or pH-independent clearance of CLOOH in a non-acidic compartment.

CLOH concentrations were also clearly increased by $NH_4Cl$, in some cases to values greater than those present at the start of the chase incubation. This is most likely attributable to additive effects of inhibition of CLOH hydrolysis by $NH_4Cl$ and continued generation of CLOH from pH-independent reduction of CLOOH. As indicated in Table 3, the decline in cellular CLOOH mass was more than sufficient to account for increases in cellular CLOH concentrations. As approximately 85% of CLOOH + CLOH was accounted for after 12 h chase in the presence of $NH_4Cl$ (see note to Table 3), it is likely that pH-indepen-

TABLE 3. $NH_4Cl$ inhibits intracellular clearance of CLOOH and CLOH accumulated in macrophages

| | CLOOH | | CLOH | |
|---|---|---|---|---|
| | *nmol/culture* | | | |
| $t_0$ (end pulse) | 1.62 ± 0.062 | | 1.16 ± 0.041 | |
| | $-NH_4Cl$ | $+NH_4Cl$ | $-NH_4Cl$ | $+NH_4Cl$ |
| $t_{12}$ h (end chase) | 0.55 ± 0.005 | 1.064 ± 0.059 | 0.925 ± 0.029 | 1.32 ± 0.05 |
| (% change/12 h) | (−66.1 ± 0.6) | (−34.3 ± 1.9) | (−20.3 ± 0.6) | (+13.8 ± 0.5) |

Macrophages incubated with OxAcLDL for 24 h pulse were washed and chased in DMEM–albumin for 12 h to monitor clearance of intracellular lipids. Matched cultures were chased in DMEM containing 10 mM $NH_4Cl$. Triplicate cell cultures were extracted at time 0 ($t_0$) (at the end of the pulse) and after 12 h chase. CLOOH and CLOH were analyzed by HPLC, and data are expressed as nmol per culture to indicate absolute changes in CLOOH and CLOH content of cell cultures. The mass of CLOOH and CLOH present after 12 h chase is also expressed as a % of that present in cultures at $t_0$. $NH_4Cl$ inhibited the clearance of both CLOOH and CLOH, even causing a net increment in cell CLOH. The sum of CLOOH and CLOH after 12 h was 1.48 ($-NH_4Cl$) and 2.38 ($+NH_4Cl$) nmol/culture, representing, respectively, 53.2% and 85.6% of that present in cell cultures at $t_0$.

dent hydrolysis is a relatively minor contributor to the intracellular clearance of CLOOH and CLOH.

### Aggregated oxidized LDL also results in cell-associated CLOOH and CLOH

Although macrophage expression of the ScR is demonstrable in human atherosclerosis, a physiological lipoprotein ligand for this receptor has not been defined. Furthermore, the physiological relevance of acetylation as a means of internalizing products of mild lipoprotein oxidation is unclear. Enhanced cellular uptake of mildly oxidized LDL by non ScR-mediated routes might also permit cellular accumulation of CLOOH. LDL within atherosclerotic plaque forms aggregates and is a substrate for macrophage phagocytosis (26). Oxidation of LDL increases its tendency to aggregate (47), and phagocytosis of lipoprotein aggregates may stimulate cell accumulation of LDL–lipid hydroperoxides.

LDL (without acetylation) was oxidized with AAPH and incubated with cells after usual careful handling or after aggregation by vortexing (48) (**Fig. 6**). Although mild oxidation of LDL alone did not cause cellular accumulation of CE or CLOOH or CLOH, aggregation of oxidized LDL did. Less FC and CE, but more CLOOH + CLOH, were accumulated with aggregated, oxidized LDL than after incubation with OxAcLDL. Relative to CLOH, more CLOOH accumulated in cells after exposure to aggregated, oxidized LDL than after exposure to OxAcLDL. This is likely due to less effective reduction of CLOOH to CLOH when delivered within LDL aggregates, as the large concentrations of CLOOH relative to CLOH are very similar to those routinely detected in both oxidized LDL and OxAcLDL before exposure to cells (data not shown).

## DISCUSSION

These studies have described for the first time the cellular accumulation of lipoprotein-derived cholesteryl ester hydroperoxides. That CLOOH can accumulate within cells raises the possibility of intracellular oxidative damage mediated by lipoprotein-derived lipid hydroperoxides, and as we have indicated in this report, the subsequent impairment of intracellular processes such as the hydrolysis of unoxidized lipoprotein-derived cholesteryl esters. Intracellular accumulation of CLOOH supports the concept of biologically relevant accumulation of such lipid peroxidation products without immediate cell death (49), and implies that their intracellular biological activities

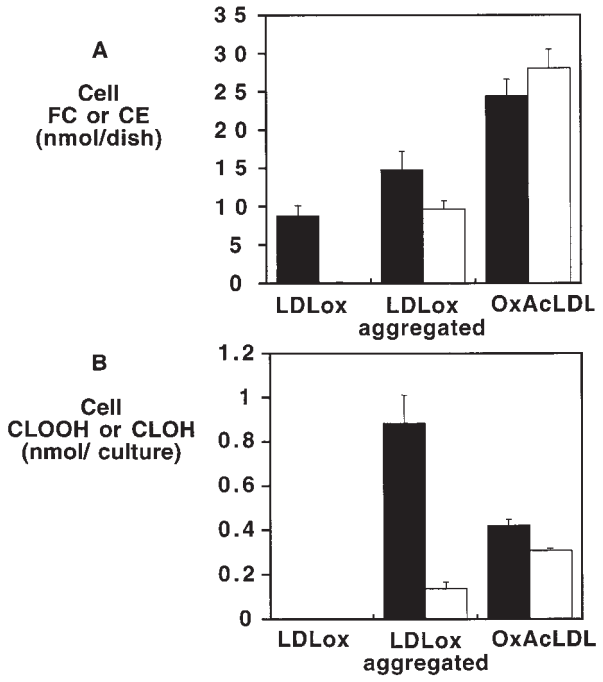


**Fig. 6.** Aggregation of mildly oxidized LDL also permits cell accumulation of CLOOH. LDL was acetylated then oxidized by AAPH (OxAcLDL), or oxidized with AAPH without prior acetylation (LDLox) achieving identical degrees of oxidation. LDLox and OxAcLDL (both at 50 μg protein/ml) were incubated with macrophages for 24 h after usual careful handling or after vortexing LDLox for 60 sec to achieve aggregation (LDLox aggregated). Cell extracts were analyzed for FC and CE (solid and open bars respectively, panel A), and CLOOH and CLOH (solid and open bars respectively, panel B).

may be important in chronic diseases such as atherosclerosis or cancer. The methodological strategy used, of dissociating protein modification (acetylation which resulted in a high uptake ScR-ligand form), or aggregation, and lipoprotein peroxidation (by AAPH), may have broad application to the study of the intracellular fate of a range of biological substrates and intracellular redox processes.

Several methodological developments were essential to the demonstration of intracellular accumulation of CLOOH in these studies: ScR-mediated holoparticle uptake of mildly oxidized LDL; delivery of sufficiently large concentrations of CLOOH-containing OxAcLDL in culture medium that would exceed cell-mediated clearance of CLOOH; and, finally, pulse-chase experiments during concurrent ACAT inhibition that permitted monitoring the metabolism of total lipoprotein-derived CE, CLOOH, and CLOH. We anticipate that previous studies using non-ScR receptor ligands such as LDL, and using very small mass quantities of oxidized lipoproteins, would not have demonstrated intracellular accumulation of CLOOH as the quantity CLOOH delivered to cells would be insufficient to overcome cellular clearance mechanisms. As indicated in Table 2, the sum of CLOOH and CLOH accumulated in cells represents a small proportion of the total extracellular clearance of lipoprotein CLOOH achieved by cells. Further, in initial experiments, low concentrations of OxAcLDL (e.g., 5 μg apoB/ml) did not cause detectable intracellular CLOOH and CLOH accumulation (data not shown).

Hydroperoxides are reactive intermediates in the chain reaction of lipid peroxidation, for example giving rise to free radicals on exposure to transition metals, and are thus potentially more cytotoxic and have a wider range of biological activities than their reduced hydroxides. For example, exposure of erythrocytes to *t*-butyl hydroperoxide modifies oleic acid incorporation into phospholipid species and decreases cellular GSH whereas exposure to *t*-butyl hydroxide does not (50).

Similarly, cholesterol hydroperoxide is more cytotoxic than its reduced 7-hydroxycholesterol form (51). Intracellular accumulation of small quantities of peroxides without cell death is biologically possible, as intracellular lysosomal accumulation of hydrogen peroxide has been demonstrated to cause sublethal cell damage with subsequent repair (49). We have demonstrated that clearly detectable quantities of LDL-derived CLOOH can accumulate in macrophages without toxicity, if LDL are incubated with cells both in the presence of serum and within a defined range of lipoprotein particle concentrations. The cytoprotective effect of serum against exogenous lipid hydroperoxides previously described (51, 52) thus also applies to our model system. It is important to emphasize, however, that components of OxAcLDL other than CLOOH may also contribute to its overall cytotoxicity.

The importance of preventing the accumulation of lipid hydroperoxides in biological systems is supported by the presence of a number of enzymatic and non-enzymatic processes that are capable of reducing hydroperoxides to the less reactive hydroxide species. Of the intracellular systems, the best characterized are the selenium-dependent enzymes, glutathione peroxidase (GPX) and phospholipid hydroperoxide glutathione peroxidase (PHGPX). GPX has been located in mitochondria and in the cytosol (53) but only reduces free fatty acid hydroperoxides (54). In contrast, PHGPX, which has been isolated from mitochondria, nuclei, and microsomes (55), has a broad substrate specificity and does not require hydrolysis of phospholipid or cholesteryl ester hydroperoxides prior to reduction of respective fatty acid hydroperoxide groups (56). PHGPX therefore appears to be a likely potential participant in reduction of intact LDL-derived cholesteryl ester hydroperoxides (57). In our studies, the intracellular reduction of intact CLOOH to CLOH by PHGPX would require that activity is present in either a prelysosomal or lysosomal compartment. Intracellular reduction may be limited if lipid hydroperoxides accumulate in compartments inaccessible to cellular reducing systems, or if lipoprotein aggregation renders CLOOH physically less accessible for reduction to CLOH. The latter may explain the greater preservation of CLOOH after incubation of cells with aggregated oxidized LDL than after incubation with OxAcLDL (Fig. 6). In addition, although PHGPX can reduce intact lipid hydroperoxides, it appears to have a large degree of specificity towards hydroperoxides in membranes (55). This may mean that once CLOOH are intracellular, as described in these studies, clearance may be slow compared to that of extracellular lipoprotein hydroperoxides directly exposed to the plasma membrane.

If LDL lipid hydroperoxides were to accumulate in intracellular compartments after endocytosis, they could impair intracellular metabolism. Cellular catabolism of apoB of extensively copper-oxidized LDL is known to be inefficient (58–60), and although this is probably secondary to modification of apoB occurring during oxidation, under some circumstances there may be inactivation of lysosomal proteases (61). The observation that lipid hydroperoxides appear to directly inhibit neutral and acidic cholesteryl ester hydrolase activity (62) is concordant with our observations on the impaired clearance of lipoprotein-derived, unoxidized CE in cells loaded by incubation with OxAcLDL. During concurrent ACAT inhibition, cells incubated with OxAcLDL contained both a larger residual CE pool immediately after loading and impaired hydrolysis of CE during pulse-chase experiments compared with AcLDL-incubated cells. It is likely that a number of oxidation products generated during conversion of AcLDL to OxAcLDL impaired the hydrolysis of lipoprotein CE, and it is possible that intracellular CLOOH may contribute to this effect (62). At this time, however, we cannot conclude by which mechanism oxidized lipid components may inhibit CE hydrolysis. Recent work using extensively copper-oxidized LDL (500 μg/ml LDL incubated with 10 μm $CuSO_4$ for 24 h) (8) has suggested that hydrolysis of native cholesteryl linoleate was unaffected by lipoprotein oxidation, whereas hydrolysis of certain advanced oxidation products of cholesteryl linoleate was impaired. These data are readily reconciled with our own observations because

of the more extensive LDL oxidation used by these investigators, which would be expected to deplete CLOOH, and other probably many other products of mild LDL oxidation, from their lipoprotein preparations (22, 25).

The precise physiological relevance of our findings has not been established, as the acetylation of LDL is not known to occur in vivo. However, the demonstration herein of the principle of intracellular accumulation of lipid hydroperoxides has the potential to be of biological importance. The likelihood that cellular accumulation of LDL lipid hydroperoxides occurs in vivo is increased by the fact that it may be facilitated by multiple uptake processes, including the phagocytosis of lipoprotein aggregates (26, 47, 63). Finally, in older, complex atherosclerotic lesions, foam cell lipid is predominantly lysosomal whereas in earlier fatty streak lesions, most lipid is cytosolic (64, 65). If intralysosomal lipid hydroperoxides or other products of mild LDL oxidation were to accumulate progressively with foam cell age and lesion complexity, such compounds could inhibit hydrolysis of unoxidized intracellular CE and mediate the lysosomal accumulation of undegraded CE in advanced human atherosclerosis.

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