Oxidation products of cholesteryl linoleate are resistant to hydrolysis in macrophages, form complexes with proteins, and are present in human atherosclerotic lesions

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Abstract Accumulation of the insoluble lipid–protein complex, ceroid, is a characteristic of atherosclerotic plaques. To determine whether deficient processing of cholesteryl esters in oxidized (ox) low density lipoprotein (LDL) contributes to ceroid formation, we studied the hydrolysis of internalized [^{3}H]cholesteryl linoleate (CL) in oxLDL by mouse peritoneal macrophages (MPM). The hydrolysis by MPM of [^{3}H]CL incorporated into oxLDL or LDL did not differ, suggesting that products of lipid and/or apoB oxidation had no impact on the lysosomal hydrolysis of [^{3}H]CL. To evaluate the hydrolysis of oxCL by MPM, we subjected extensively ox[^{3}H]CL to fractionation by TLC. The predominant fraction (D) consisted of sterols and oxysterols esterified to scission products of oxidized fatty acids containing terminal carbonyl groups, i.e., lipid core aldehydes. The extent of hydrolysis of [^{3}H]-fraction D by MPM cultures, as well as by MPM extracts at pH 4.0, was significantly reduced when compared to the hydrolysis of intact [^{3}H]CL. Fraction D also formed complexes with serum proteins, and the purified core aldehyde, cholesteryl 9-oxononanoate reacted with ε-amino group of lysines. Finally, several cholesteryl ester aldehydes were detected in lipid extracts of human atheroma. These results suggest that decomposition products of extensively oxidized cholesteryl linoleate that are also present in atherosclerotic lesions, are not adequately degraded by mouse peritoneal macrophage lysosomes and could interact with proteins to form ceroid.—**Hoppe, G., A. Ravandi, D. Herrera, A. Kuksis, and H. F. Hoff.** Oxidation products of cholesteryl linoleate are resistant to hydrolysis in macrophages, form complexes with proteins, and are present in human atherosclerotic lesions. *J. Lipid Res.* 1997. **38:** 1347–1360.

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Lipid accumulation in macrophages and smooth muscle cells of the arterial intima is a characteristic feature of early as well as late phases of atherosclerosis (1). It was demonstrated that at least part of the accumulation of intracellular cholesterol occurs in lysosomes (2, 3). ApoB of oxidized LDL (oxLDL) was shown to be degraded more poorly by mouse peritoneal macrophages (MPM) than apoB in acetylated LDL (acLDL), resulting in the lysosomal accumulation of undegraded oxLDL protein (4–8). Chronic exposure of cultured MPM to oxLDL also led to lipid accumulation within lysosomes, primarily in the form of fluorescent lipid–protein complexes called ceroid, which are similar to the autofluorescent pigment granules found in aging (9). Intracellular lipid peroxidation of polyunsaturated fatty acids (10) was shown to lead to ceroid deposition within cells. Ceroid was consistently found in atherosclerotic lesions (11) and chemically represents a polymer of oxidized lipid and protein that is insoluble in organic solvents.

Deficient processing of oxLDL in MPM was initially attributed to the resistance to proteolysis of apoB in oxLDL due to intra- and intermolecular cross-linking by reactive aldehydes formed during lipid peroxidation such as 4-hydroxynonenal (4, 5). We recently provided an alternative explanation, namely that oxLDL can di-

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ACEH, acid cholesteryl ester hydrolase; acLDL, acetylated LDL, N-BOC-Lys, (Nα-*tert*-butoxycarbonyl)- L-lysine; CapEx, capillary exit; CID, collision induced dissociation; CO, cholesterol oxidase; CE, cholesteryl esters; CL, cholesteryl linoleate; DNPH, 2,4-dinitrophenylhydrazone; ESI, electrospray ionization; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; MPM, mouse peritoneal macrophages; MS, mass spectrometry; NCEH, neutral cholesteryl ester hydrolase; oxCL, oxidized CL; oxLDL, oxidized LDL; PC, phosphatidylcholine; PS, phosphatidylserine; UC, unesterified cholesterol; vxLDL, vortexed LDL.

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rectly inactivate cathepsin B in vitro at acidic pH (8). Furthermore, MPM pretreated with oxLDL showed a reduced ability to degrade lipoproteins. This mechanism could also be responsible for the formation of ceroid, as inhibition of lysosomal thiol proteases alone was found to be sufficient to cause ceroid accumulation in experimental animals (12).

Because both lipids and proteins are components of ceroid, it is possible that the lipid comes from the deficient hydrolysis in lysosomes of cholesteryl esters from internalized oxLDL. Deficient hydrolysis could be the result of one or more of several events, e.g., *i*) direct inactivation of acid cholesteryl ester hydrolase (ACEH) by constituents of oxLDL; *ii*) inhibition of cholesteryl ester (CE) hydrolysis by incomplete proteolysis of apoB in oxLDL; and/or *iii*) oxidized CE being a poor substrate for lysosomal ACEH. In this report we show that oxidized cholesteryl linoleate (CL), consisting of scission products of CL, some containing terminal carbonyl groups, is a poor substrate for ACEH and can form covalent complexes with lysine side chains. We also document the presence of such cholesteryl ester aldehydes in human atherosclerotic lesions.

MATERIALS AND METHODS

Materials

[1,2,6,7-^{3}H(N)]cholesteryl linoleate was obtained from NEN Research Products (Boston, MA). Cholesterol oxidase (EC 1.1.3.6, *Brevibacterium* sp.), fatty acid-free bovine serum albumin, egg phosphatidylcholine (PC), phosphatidylserine (PS), (Nα-*tert*-butoxycarbonyl)-L-lysine (N-BOC-Lys), and filipin were purchased from Sigma Chemical Co. (St. Louis, MO). Neutral cholesteryl ester hydrolase (NCEH) (EC 3.1.1.13, *Candida cylindracea*) was obtained from Boehringer Mannheim (Indianapolis, IN). Compound SAH 58-035/6, an inhibitor of acyl-CoA:cholesterol acyltransferase (ACAT), was a kind gift from Sandoz Pharmaceutical Corp. (East Hanover, NJ). Standard 5-oxovaleroyl cholesterol and 5-oxovaleroyl ketocholesterol were provided by Dr. G. Tirzitis, Institute of Organic Synthesis (Riga, Latvia). 9-Oxononanoyl cholesterol and the 9-oxononanoyl esters of 7α- and 7β-cholesterol were available in the laboratory from previous studies (13). Organic solvents (HPLC grade) were purchased from Baxter (McGaw Park, IL). Rosewell Park Memorial Institute (RPMI-1640) medium was from Whittaker Bioproducts Inc. (Walkersville, MD); fetal calf serum and L-glutamine were from Gibco Laboratories (Grand Island, NY); and tissue culture plates were obtained from Costar (Cambridge, MA).

Lipoproteins

LDL was isolated from fresh plasma obtained from 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). The LDL was dialyzed against 0.15 M NaCl containing 0.5 mM EDTA, pH 8.5, filter-sterilized and stored at 4°C. Radiolabeling of lipoproteins with [^{3}H]CL was performed by modification of the procedure originally described by Sparks et al. (15). [^{3}H]CL (50 μCi in 50 μl of toluene) was added to 200 μl of lipoprotein (500 μg protein/ml), e.g., native LDL, acLDL, or oxLDL, and the mixture was dried under a stream of N_2 in a glass vial. After adding 4 ml of the corresponding lipoprotein at a protein concentration of 500 μg/ml, the vial was flushed with N_2 and gently stirred by gentle inversion for the first 4 h at 20°C, then overnight at 4°C. After incubation the labeled lipoprotein was spun at 10,000 *g* and passed through a 0.2-μm filter. A typical specific activity of labeled lipoproteins ranged between 2000 and 6000 dpm/μg of lipoprotein protein. Oxidation of LDL was performed by dialyzing LDL at a protein concentration of 500 μg/ml against PBS containing 10 μM $CuSO_4$ for 24 h at 37°C. The concentration of EDTA in LDL preparations was reduced prior to oxidation by overnight dialysis against EDTA-free PBS. Oxidation was terminated by extensively dialyzing samples against PBS, 0.3 mM EDTA, pH 7.4. Aggregated LDL was produced by vortexing solutions of LDL (500 μg/ml) in PBS, pH 7.4, containing 0.3 mM EDTA, and 40 μM butylated hydroxytoluene (6, 8). Acetylation of LDL was performed by repeated additions of acetic anhydride as described elsewhere (16).

Generation and fractionation of [^{3}H]CL oxidation products

Oxidative derivatization of CL was achieved through incubating dried CL at 100°C under air (17). Five- to 15-μl aliquots of stock solution containing 1 mg of unlabeled CL and 20 μCi of [^{3}H]CL in 1 ml of chloroform were dried in a borosilicate glass test tube and incubated at 100°C in an oven for different time periods with free access to air. Chloroform extracts of the reaction mixture were resolved by TLC on silica gel G using hexane–ethyl ether–glacial acetic acid 60:70:1.5 (by vol) as the developing solvent. The location of oxidation products was revealed by ^{3}H-autoradiography as described below. Oxidized CL (oxCL) separated by TLC was arbitrarily divided into four fractions, i.e., A, B, C, and D, based on their R_fs (0.73, 0.57, 0.45, and 0.4, respectively). Similar patterns of distribution of ^{3}H-radioactivity were obtained when oxidation was initiated by other well-established oxidation systems, e.g., 10 μm

Cu^{2+} in PBS, or 100 μm Fe^{2+} in 70% *tert*-butyl hydroperoxide (not shown). The above fractions of ox[^{3}H]CL were recovered from the silica gel by extraction with 2 × 4.5 ml of chloroform–methanol 9:1 (by vol) and washed with 2 ml of water. All stock and working solutions of lipid samples or standards were stored as chloroform solutions under N_2 at −20°C.

Hydrolysis of ^{3}H-labeled lipoprotein by macrophages

Thioglycollate-elicited mouse peritoneal macrophages (MPM) from C57BL/6 mice (Trudeau Institute) were harvested, seeded, and cultured as described elsewhere (6). MPM were incubated with [^{3}H]CL-labeled lipoprotein, in the absence and presence of an ACAT inhibitor, SAH 58-035 (10 μg/ml) in DMSO (18). Control cells (minus ACAT inhibitor) received an equivalent amount of DMSO. Neither the ACAT inhibitor nor DMSO affected cell morphology or viability, nor did they inhibit hydrolysis of [^{3}H]CL by extracts of MPM at pH 4.0. After incubation (as described in the text and figure legends), the media and the MPM monolayers were separately assayed for unesterified [^{3}H]cholesterol (UC) and [^{3}H]CE contents as described below. Cell monolayers remaining after lipid extraction were dissolved in 0.1 N NaOH, and aliquots were assayed for protein content as described below. Calculations of the total cholesterol were based on an assumption that LDL contains 1.65 mg of total cholesterol per mg of LDL protein.

Cholesteryl ester hydrolase activity in a cell-free system

MPM grown in 100-mm Petri dishes (2×10^7/dish) were washed three times with ice-cold PBS, and cells were scraped into a small volume of PBS. For each preparation of the cell extract 4–6 dishes were combined and pelleted by centrifugation at 2,000 *g* for 10 min. Cell pellets were quickly resuspended in 1 ml of water, and ruptured by two 15-s bursts of sonication at 20W using a tipped-type sonicator (Ultrasonics), followed by 5 cycles of quick freeze-thaw. The homogenates were spun for 30 min at 10,000 *g* to remove particulate matter, and supernatant fractions, defined in this study as cell extracts, were collected, aliquoted, frozen at −70°C, and subsequently used in experiments over a period of 2 months. Aliquots of MPM extracts (10–50 μg) were placed into a borosilicate test tube containing 100 μl of 100 mM acetate buffer (pH 4.0), and the mixture was pre-incubated for 10–15 min at room temperature. The assay was started by transferring pre-activated MPM extracts into borosilicate test tubes containing dried aliquots of ^{3}H-labeled fractions A and D. After incubation in a closed tube for 2 h at 37°C, the reaction was terminated by adding 3.5 ml of chloroform–methanol 1:1 (by vol), and the mixtures were assayed for [^{3}H]UC and [^{3}H]CE contents as described below. Substrate blanks were run under identical conditions with homogenization medium added in place of the MPM extract. The NCEH assay was conducted similarly to the ACEH activity assay described above, except that the incubation was carried out in 100 μl of PBS (pH 7.4) containing dried aliquots of ^{3}H-labeled fractions A and D in the presence or absence of 5 μl of NCEH (Boehringer Mannheim) for 2 h at 37°C.

Biopsy samples

Atherosclerotic lesions in carotid endarterectomy samples were obtained at the Cleveland Clinic Foundation and immediately placed into PBS, pH 7.4, containing 0.1% EDTA, 0.15% ε-aminocaproic acid, 40 μM butylated hydroxytoluene, and 1 mM phenylmethylsulfonylfluoride. Plaque material (0.4–1.0 g) was separated from media and adventitia, minced into small (0.5–1.0 mm^2) pieces, and total lipid extracts of atheromas were obtained by extraction with chloroform–methanol using the method of Bligh and Dyer (19). Lipid extracts from atherosclerotic tissue were stored in chloroform, containing 40 μM butylated hydroxytoluene, at −20°C under nitrogen.

Preparation of the cholesteryl core aldehyde/lysine adduct

Cholesteryl 9-oxononanoate (1 mg) was dissolved in isopropanol–methanol 1:1 (2 ml) and the solvent was blown down in a test tube. Two ml of water containing 2 mg N-BOC- Lys were then added followed by 1 ml methanol. The mixture was sonicated for 1 min using short bursts at maximum power (Bronson 1200 Sonicator). The sonicated mixture was left standing (4 h) at room temperature. The Schiff base formed was reduced by adding sodium cyanoborohydride in methanol to a final concentration of 70 mM and the mixture was kept at room temperature for 1 h. The reduced Schiff base was extracted with chloroform–methanol 2:1, the solvent was blown down under N_2, and the residue was redissolved in a small volume of chloroform–methanol 2:1 for injection into the normal phase HPLC/ESI/MS system. A similar extract of the unreduced Schiff base was examined by flow injection/ESI/MS.

Lipid analysis

Lipid extraction procedures. Lipid extraction from aqueous samples was performed by a simplified version of the Bligh and Dyer (19) procedure suitable for small volume samples. Briefly, 250 μl of aqueous medium were added to 3.5 ml of chloroform–methanol 1:1, which formed a one-phase solvent system. After a 10–30 min extraction at room temperature with occasional

shaking, 1.25 ml of H_2O was added, samples were vigorously vortexed, and spun in order to break the mixture into two phases. Cellular lipids were extracted twice with 500 µl of hexane–isoprophyl alcohol 3:2 for 30 min on a rocking platform (20).

Derivatization of cholesterol by cholesterol oxidase. To separate fraction D from UC a procedure originally developed by Lange and Ramos (21) was adopted. Lipid extracts were dried in borosilicate test tubes and 250-µl aliquots of 0.5 mM NaH_2PO_4 buffer (pH 7.5) containing 310 mM sucrose and 5 µl of a stock solution of cholesterol oxidase (CO) (2 units/ml final concentration) were added to each sample. After incubating the mixtures for 60 min at 37°C with intermittent shaking, the reaction was stopped by adding 3.5 ml of a chloroform–methanol 1:1 mixture, and lipid extraction was performed as described above.

Thin layer chromatography (TLC) was performed on silica gel G plates with aluminum backing to facilitate cutting. Chromatography was performed using the following solvent systems: system 1, hexane–ethyl ether–glacial acetic acid 80:20:1 (by vol), for separation of UC and CE; system 2, hexane–ethyl ether–glacial acetic acid 60:70:1.5 (by vol), for separation of UC and oxCL. Neutral lipid standards (Sigma) were visualized by iodine vapors. Respective regions of the TLC plate were removed, and ^{3}H-radioactivity in each region was extracted and counted in an EcoLite(+) (ICN) scintillation liquid. The location of ^{3}H-labeled lipids on a TLC plate was revealed by solid-phase scintillation autoradiography. Dried TLC plates were sprayed with EN^{3}HANCE™ (NEN Research Products), a compound for surface autoradiography, and incubated with Kodak X-ray film for 20–48 h. Developed film was subjected to image analysis using a videocamera interfaced with a Macintosh computer.

Preparation of dinitrophenylhydrazone (DNPH) derivatives. An aliquot of fraction D was treated with 2,4-dinitrophenylhydrazine (0.5 mg in 1 ml of 1 N HCl) for 2 h at 20°C and at 4°C overnight (22). The DNPH derivatives were extracted with chloroform–methanol 2:1. The chloroform layer, which contained the DNPH derivatives of the core aldehydes along with other oxidation products of the cholesteryl linoleate, was subjected to high-performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS) directly or after initial separation by TLC (23).

HPLC/ESI/MS of fraction D. The DNPH derivatives of fraction D were analyzed by HPLC/ESI/MS as described elsewhere (24). A Hewlett-Packard (Palo Alto, CA) model 1090 liquid chromatograph was used that was interfaced via electrospray with a Hewlett-Packard model 5985B quadrupole mass spectrometer. A Supelcosil LC-18 HPLC column (250 mm × 4.6 mm ID, Supelco, Bellefonte, PA) was used, and fractions were eluted using a linear gradient of 20–80% isopropanol in methanol over 30 min at a flow rate of 0.8 ml/min. The detection sensitivity in the negative ion mode was facilitated by adding 1% ammonium hydroxide solution (30%) in isopropanol post-column at a flow rate of 0.2 ml/min. Mass spectra were recorded over the mass range of 200–1200.

HPLC/ESI/MS of atheroma extracts. The chloroform extracts were blown down under nitrogen and redissolved in chloroform–methanol containing dinitrophenylhydrazine as previously described (23). The DNPH derivatives along with the unreacted lipids were subjected to reversed phase HPLC/ESI/MS. A Hewlett-Packard reversed phase C-18 column (100 mm × 2.1 mm I.D.) was installed in the liquid chromatograph and connected to a mass spectrometer via the electrospray interface as described above. The column was developed with a linear gradient of 100% solvent system A (methanol–water–30% ammonium hydroxide, 80:12:0.5, by vol) by changing to 100% solvent system B (methanol–hexane–30% ammonium hydroxide, 80:12:0.5, by vol) in 30 min, after initially holding for 1 min at 100% system A and finally holding at 100% system B for 5 min. For fraction collection, a split ratio of 1:50 was used with a flow rate to the mass spectrometer adjusted to 25 µl/min. Temperature of the drying gas was reduced from 300°C to 200°C and the flow of the nebulizer gas was reduced from 60 to 40 bar.

Normal phase HPLC/ESI/MS on the cholesteryl 9-oxononanoate/N-BOC-Lys adduct. Chromatographic analysis of the reduced reaction products of lysine and cholesteryl core aldehyde were performed on Spherisorb column (3 µm, 100 mm × 4.6 mm ID, Altech, Guelph, Ontario) installed in a Hewlett-Packard (Palo Alto, CA) model 1090 liquid chromatograph connected to a Hewlett-Packard 5988B Quadrupole mass spectrometer, and equipped with a nebulizer assisted electrospray interface (A. Ravandi, A. Kuksis, N. Shaikh and G. Jackowski, unpublished results). The column was eluted with a linear gradient of 100% solvent system C (chloroform–methanol–30% ammonium hydroxide, 80:19.5:0.5, by vol) by changing to 100% solvent system D (chloroform–methanol–30% ammonium hydroxide, 60:34:5.5:0.5, by vol.) in 14 min, followed by 100% system D for 10 min. Positive ionization spectra were taken in the mass range 400–1200. Selected ion spectra were retrieved from the total ion spectra by computer analysis.

Protein assay

The protein content of lipoproteins, cells, and of cell extracts was measured by the bicinchoninic acid (BCA, Pierce) assay using bovine serum albumin as a standard (25). Samples were diluted in 1% SDS to minimize turbidity.

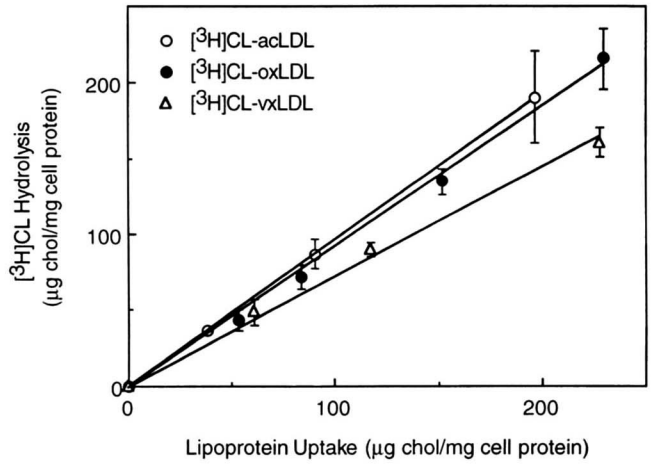

Fig. 1. Hydrolysis by MPM of [^{3}H]CL incorporated into various forms of modified LDL. MPM were incubated in RPMI medium containing 0.2% bovine serum albumin and the indicated concentrations of [^{3}H]CL-oxLDL, [^{3}H]CL-acLDL, or [^{3}H]CL-vxLDL for 20 h in the presence of 10 µg/ml of the ACAT inhibitor, SAH 58-035. Culture media were collected at the end of the incubation, and cell monolayers were washed three times with PBS. Media and cellular lipid extracts were resolved by TLC and analyzed for [^{3}H]UC and [^{3}H]CE content. [^{3}H]CE hydrolysis ([^{3}H]UC content of media and cells) is presented as a function of [^{3}H]-lipoprotein uptake (cell-associated ^{3}H-label plus medium [^{3}H]UC).

RESULTS

Cellular hydrolysis of intact [^{3}H]CL incorporated into oxLDL

To explore whether components within an oxLDL particle affect the intracellular hydrolysis of CL, we compared the hydrolysis by MPM of [^{3}H]CL incorporated into oxLDL with that of [^{3}H]CL incorporated into either native LDL or acLDL. To increase the delivery of native LDL into MPM, we aggregated [^{3}H]CL-LDL by vortexing (vxLDL) immediately prior to addition to the cells. The amount of [^{3}H]UC formed by MPM over 20 h in the presence of the ACAT inhibitor, SAH 58-035, increased linearly with the lipoprotein uptake (cell-associated ^{3}H-label plus [^{3}H]UC in the media) (**Fig. 1**). However, the rate of hydrolysis was not reduced for [^{3}H]CL incorporated into oxLDL when compared to that of vxLDL or acLDL. Media [^{3}H]UC was due entirely to intracellular hydrolysis, as we found no CE-hydrolyzing activity in the MPM-conditioned media at either pH 7.4 or pH 4.0. In addition, cell extracts prepared from MPM pretreated with oxLDL for 24 h demonstrated no decrease in hydrolytic activity towards [^{3}H]CL when compared to extracts of control MPM pre-incubated with either acLDL or native LDL (not shown). These results suggests that it is unlikely that ACEH in MPM was inactivated by some components of oxLDL.

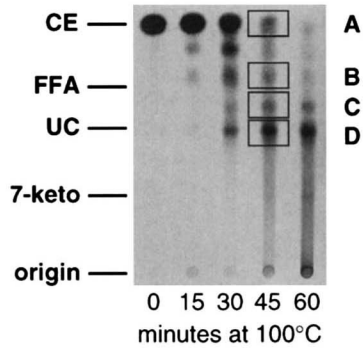

Fig. 2. Oxidation of [^{3}H]CL. Aliquots of [^{3}H]CL (5 µg, 0.1 µCi) were incubated at 100°C in an oven with free access to air for the indicated time periods. At the end of each incubation, lipid-soluble products of oxidation were extracted according to Bligh and Dyer (19) and resolved by TLC using system 2 as a developing solvent (see Methods). Solid-phase scintillation autoradiography was performed as described in Methods. Standards are: CE, cholesteryl oleate; FFA, free fatty acid (oleic acid); UC, cholesterol; 7-keto, 7-ketocholesterol.

Isolation of products of [^{3}H]CL peroxidation by TLC

Shown in **Fig. 2** are representative TLC profiles of [^{3}H]CL oxidized to different degrees by incubating at 100°C (17). Four characteristic bands were detected on TLC of oxCL, designated fractions A, B, C, and D. With increasing degrees of oxidation, fractions A, B, and C were found to be transient and finally disappeared (Fig. 2), i.e., were intermediate products of CL oxidation, whereas fraction D steadily increased, and appears to be a final stage in the oxidative modification of CL. For these reasons we chose to study the hydrolysis of fractions A and D only. When fractions of oxCL were first purified by extraction from respective regions of the TLC plate and then saponified, the hydrolyzed fractions A, B, C, and D were shown to contain oxysterols and UC (**Fig. 3**). The ratio of oxysterol/UC increased in

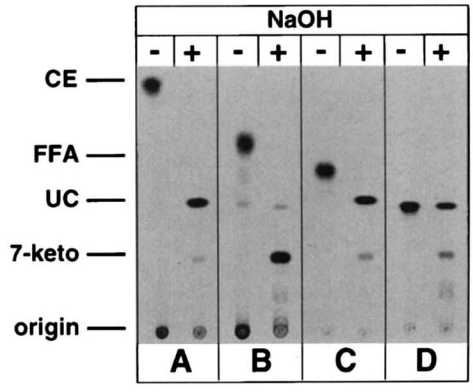

Fig. 3. Saponification of fractions of oxCL. Aliquots of ^{3}H-labeled fractions A, B, C and D were dried in borosilicate glass test tubes, and 200 µl of ethanol with or without 1 N NaOH (±NaOH) was added for 30 min at 60°C. After evaporation of ethanol under N_2, samples were further processed for lipid extraction, TLC, and autoradiography as described in Methods. Standards are the same as in Fig. 3.

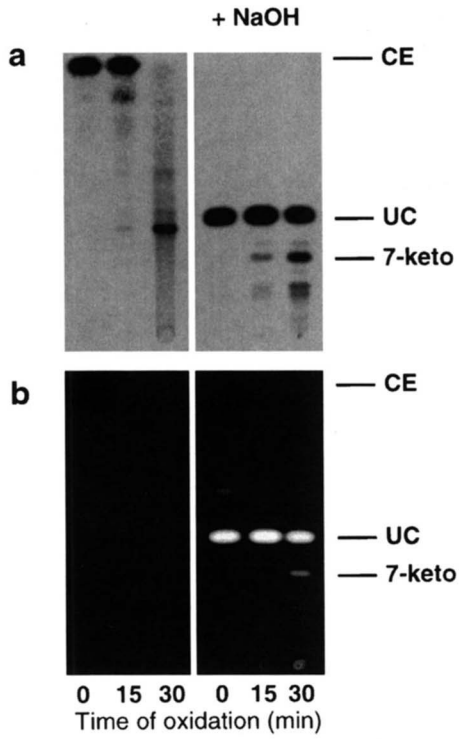

Fig. 4. Filipin staining of TLC. Aliquots of 5 μg of [^{3}H]CL (0.1 μCi) were incubated in an oven with free access to air at 100°C for the indicated time periods. One half of each sample was then saponified (+NaOH); the remainder of each sample received similar treatment at 60°C except that no NaOH was added. Lipid extracts of intact (left panel) and saponified (right panel) samples were developed on two separate TLC plates. (a) Autoradiogram of the TLC plate. (b) Filipin staining was performed as described elsewhere (26). Briefly, TLC plates were incubated for 30 min in the filipin suspension (120 μg in PBS) at 37°C. After staining and washing 2 × 10 min in H_2O, cholesterol spots were visualized by simple UV-transillumination. Standards are the same as in Fig. 3.

fraction D relative to that in fraction A. It is likely that fraction D is still at least partially acylated, as saponification of fraction D resulted in the appearance of additional bands, presumably oxysterols (Fig. 3).

To determine whether fraction D contained any UC, we utilized the fluorescent dye filipin, previously shown to interact with UC or oxysterols, but not esterified forms of cholesterol (26, 27). As seen in **Fig. 4,** neither intact nor oxidized CL interacted with filipin, whereas saponification of the above samples resulted in the appearance of filipin-positive spots with Rfs corresponded to UC and oxysterol. This result suggests that the cholesteryl ester bond is still preserved in extensively oxidized CL.

Hydrolysis of fractions of oxCL by MPM

As fraction D co-migrated with UC on TLC, it was not possible to use this separation technique to assess the formation of UC from fraction D (Fig. 3). However, we found that treatment with cholesterol oxidase (CO) specifically changed the TLC mobility of UC yielding 4-cholesten-3-one (21), but not that of fraction D (**Fig. 5**). Thus, treating the sample with CO before TLC enabled us to separate UC from fraction D and thereby measure hydrolysis of the latter.

To determine the hydrolysis of oxCL in the cell-free system we used either MPM extracts at pH 4.0 as a source of ACEH activity or commercial NCEH (Boehringer Mannheim). Incubation with NCEH for 1 h led to complete hydrolysis of ^{3}H-labeled fraction A (100% of the maximal extent of hydrolysis determined by saponification), but only about 62% of ^{3}H-labeled fraction D (**Fig. 6a**) as assessed by densitometry of autoradiograms. Lysosomal enzymes in MPM extracts were able to hydrolyze 75% of ^{3}H-labeled fraction A, whereas the hydrolysis of ^{3}H-labeled fraction D did not exceed 15% (Fig. 6a). From time-course studies of hydrolysis by MPM extracts (Fig. 6b), hydrolysis of both fractions A and D reached a plateau after an incubation for 3 h, indicating that not only the rate but also the extent of fraction D hydrolysis by lysosomal ACEH was reduced.

We also studied the hydrolysis of fraction D by intact MPM. Fraction D was added to MPM by two different ways, as a lipid emulsion or as anionic liposomes. Incorporation of fraction A into lipoproteins proved to be far less efficient than comparable incorporation of fraction D (not shown) and, therefore, this approach was not used for subsequent hydrolysis studies. Injecting fraction D solubilized in ethanol into the culture media produced a lipid emulsion which would be expected to partition freely into the plasma membrane and/or be internalized by phagocytosis, resulting in a distribution within lysosomal as well as non- lysosomal compartments. However, such a delivery makes it impossible to distinguish between lipid hydrolysis by NCEH and

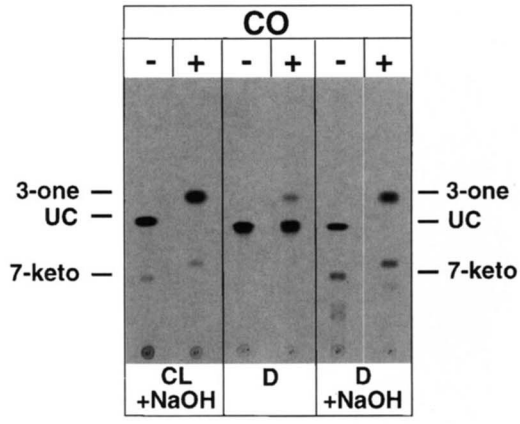

Fig. 5. Sterol derivatization by cholesterol oxidase. Aliquots of saponified [^{3}H]CL, ^{3}H-labeled fraction D, and saponified ^{3}H-labeled fraction D were treated with or without CO (±CO) as described in Methods. Lipid extraction, chromatography, and autoradiography were performed as described in Methods. Standards are: 3-one, 4-cholesten-3-one; UC, cholesterol; 7-keto, 7-ketocholesterol.

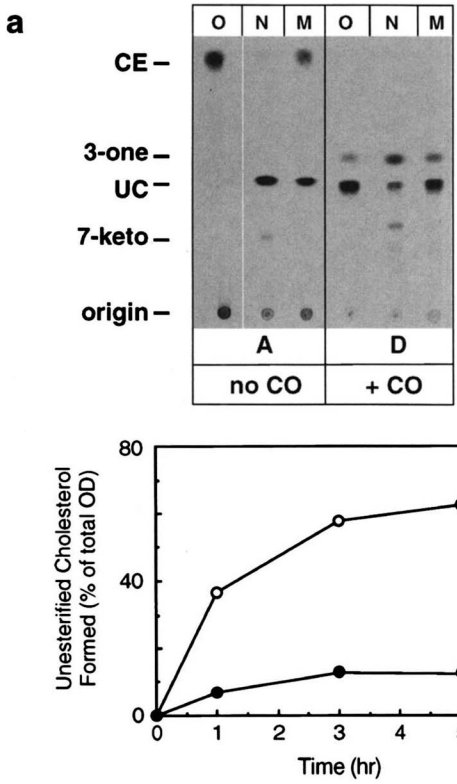

Fig. 6. Hydrolysis of fractions of oxCL by NCEH and ACEH. (a) Aliquots of ^{3}H-labeled fractions A and D were dried in borosilicate glass test tubes (35,000 dpm/tube). Three sets of tubes from each fraction were prepared. One set received 200 µl of PBS containing 2 µl of stock solution of NCEH (N). Another set received 150 µl of acetate buffer (pH 4.0) containing 50 µg of MPM extract (M). A control set received 200 µl of acetate buffer (O). After incubation at 37°C for 2 h, lipid extracts of the samples were obtained and then treated for 60 min in the absence (fraction A, no CO) or presence (fraction D, + CO) of cholesterol oxidase. Lipid extraction, chromatography, and autoradiography were performed as described in Methods. Standards are the same as in Fig. 3. (b) Time-course: ^{3}H-labeled fractions A and D were incubated with MPM extracts in acetate buffer (pH 4.0) for the indicated time periods. Results were expressed as the percent of optical density under the UC spot relative to the optical density measured for the whole lane.

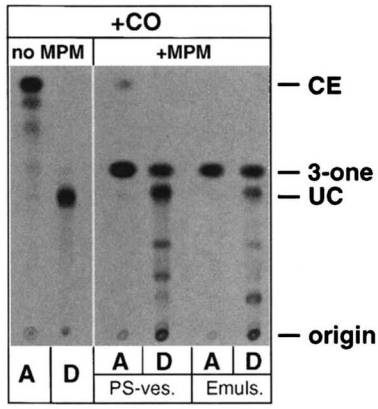

Fig. 7. Hydrolysis of the fraction D by MPM. A PS-containing substrate (PS-ves.) was prepared and was comprised of PC/PS/dicetyl phosphate/UC (1:1:0.2:1.5 mol/mol) and a trace amount of ^{3}H-labeled fraction A or D. Emulsions (Emuls.) of CE were prepared by injection of an ethanol solution of ^{3}H-labeled fraction A or D (25 µl) into 5 ml of culture media. MPM cultures were incubated with the above substrates (200,000 dpm/well) for 20 h in the presence of 10 µg/ml of SAH 58-035 (+MPM). Control samples were incubated on the same plate in the wells containing no cells (no MPM). The cells were then washed three times with PBS, and lipids from cell and control samples were extracted and treated with CO. The [^{3}H]UC formation was analyzed by autoradiography after TLC of the lipid extracts. Standards are the same as in Fig. 3.

ACEH. To direct substrates specifically into lysosomes, we incorporated cholesteryl esters into phosphatidylserine (PS)-containing liposomes. Vesicles containing anionic phospholipids were previously shown to be internalized efficiently by MPM via receptor-mediated endocytosis and, unlike PC-containing liposomes, were rapidly delivered to lysosomes (28). Almost 100% of the cell-associated ^{3}H-label was found in [^{3}H]UC when MPM were incubated for 20 h with ^{3}H-labeled fraction A, as contrasted to ^{3}H- labeled fraction D, in which only 60% of the cell-associated ^{3}H-radioactivity was in the form of [^{3}H]UC (**Fig. 7**). Delivery by PS- containing liposomes consistently caused slightly less hydrolysis of fraction D than that obtained with a lipid emulsion, which would be presumably accessible for both lysosomal ACEH and cytoplasmic NCEH. This is consistent with the lower ability of ACEH to hydrolyze substrates when compared to that of NCEH in cell-free studies (Fig. 6).

Identification of cholesteryl ester core aldehydes in fraction D

The DNPH derivatives of fraction D were subjected to reversed phase HPLC/ESI/MS, and the intensities of both negative and positive ions were recorded for each peak. In the positive ion mode we detected only the characteristic sterol ring moieties associated with each HPLC peak, e.g., *m/z* 367 (cholestenol); *m/z* 369 (cholesterol); *m/z* 385 (5,6-epoxycholesterol); *m/z* 385 (7-hydroxycholesterol); and *m/z* 383 (7- ketocholesterol). These ions were associated with the HPLC peaks also containing the [M–1] ions detected in the negative ion mode (**Fig. 8a,b**). The major peak in the negative ion profile of fraction D (peak 12) showed the same retention time of 15.3 min as that of the DNPH derivative of cholesteryl 19-oxononanoate (Fig. 8c). Synthetic cholesteryl 9- oxononanoate was prepared from cholesteryl linoleate by oxidation with osmium tetroxide and reduction with sodium sulfite (13), for which only a single [M–1] ion corresponding to the molecular mass at *m/z* 719 was detected in the appropriate HPLC elution range (15–16 min) and the mass range (300–900) (not shown). No peak was detected at 12.447 min (Fig. 8a), the retention time for synthetic 5- oxovaleroyl ester (13), indicating the absence of any oxovaleroyl ester in fraction D. Increasing the exit voltage of the ESI block from 170 V to 300 V yielded cholesterol and oxy-

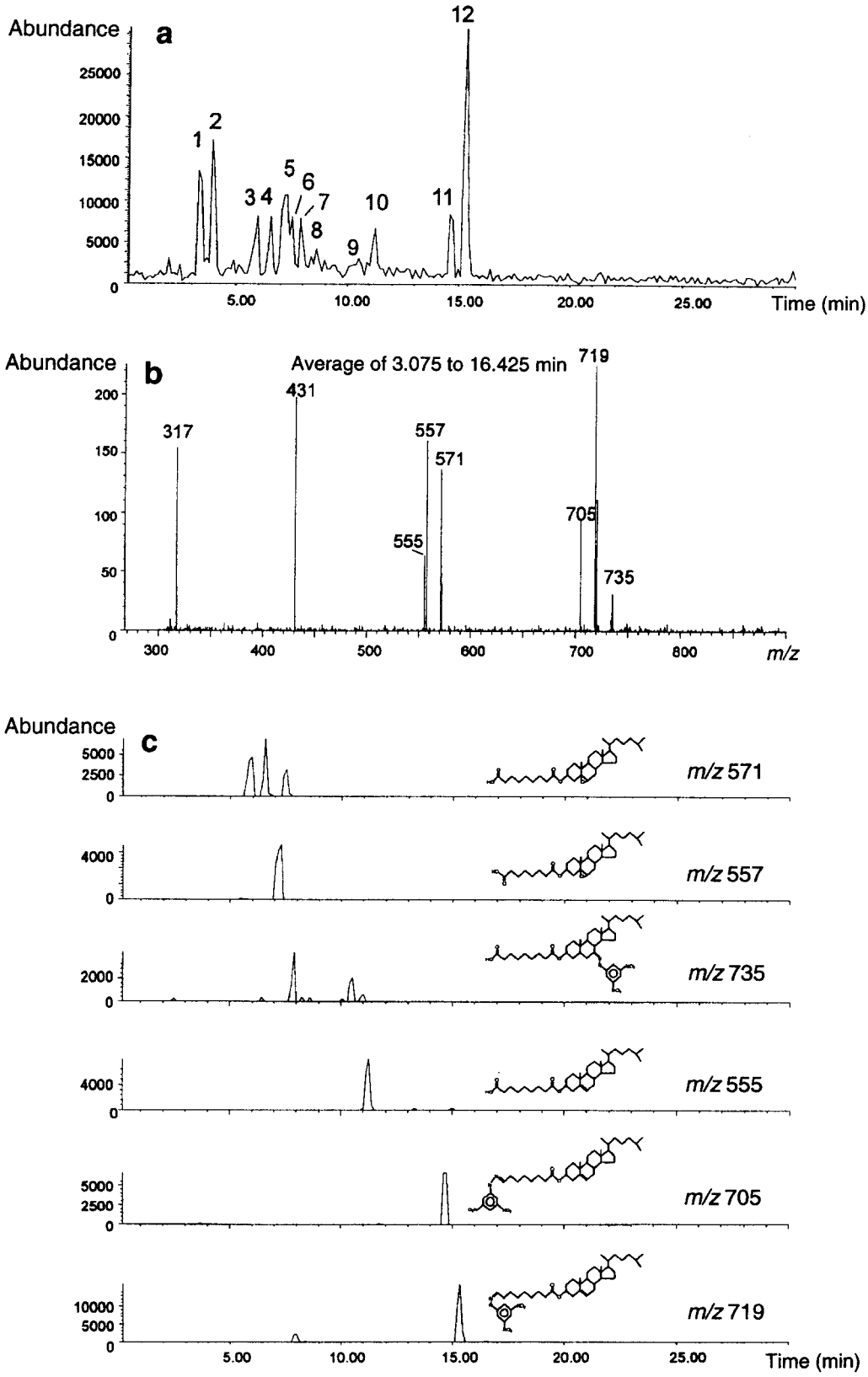

Fig. 8.

genated cholesterol ring moieties. Apparently, there was no cleavage of the hydrazone bond with the release of the cholesteryl ester core aldehydes from the DNPH derivative under the present working conditions.

Interaction of fraction D with proteins

The presence of lipid aldehydes in fraction D suggests the possibility of covalent interactions between oxCL and proteins (29, 30). When MPM were incubated with RPMI containing 10% lipoprotein-deficient serum and ^{3}H-labeled fraction D or [^{3}H]CL incorporated into PS-liposomes, we obtained incomplete recovery of the internalized label of ^{3}H-labeled fraction D from cell monolayers by conventional lipid extraction with hexane–isopropyl alcohol 3:2. To explore whether the label was still associated with MPM, we solubilized cell monolayers in NaOH after extensive lipid extraction, and measured ^{3}H-radioactivity present in the NaOH extract. As seen in **Fig. 9a,** four times more ^{3}H-label was found in the cell layer after incubation with ^{3}H-labeled fraction D than with [^{3}H]CL. Likewise, when the incubation medium was subjected to lipid extraction and the protein interface was extensively washed with chloroform, a significantly higher portion of the ^{3}H-labeled fraction D-label was found still associated with the protein interface (Fig. 9a). Binding of fraction D to serum proteins was cell-independent, as virtually identical increases in protein-bound ^{3}H-label were observed when lipoprotein-deficient serum was incubated with ^{3}H-labeled fraction D but not with [^{3}H]CL in the absence of cells (not shown). These data suggest that components in fraction D had interacted with cell proteins resulting in their insolubility in organic solvents. The formation of detergent-resistant complexes between lipoprotein-deficient serum and fraction D was further confirmed by SDS/PAGE (Fig. 9b), suggesting a covalent interaction between fraction D and serum proteins.

Schiff base adducts of core aldehydes

In order to demonstrate the reactivity of the C_9 aldehyde ester of cholesterol with amino groups of amino acids and polypeptides, we examined the Schiff base formation between cholesteryl 9-oxononanoate and the amino acid lysine with blocked α-amino group, N-BOC-

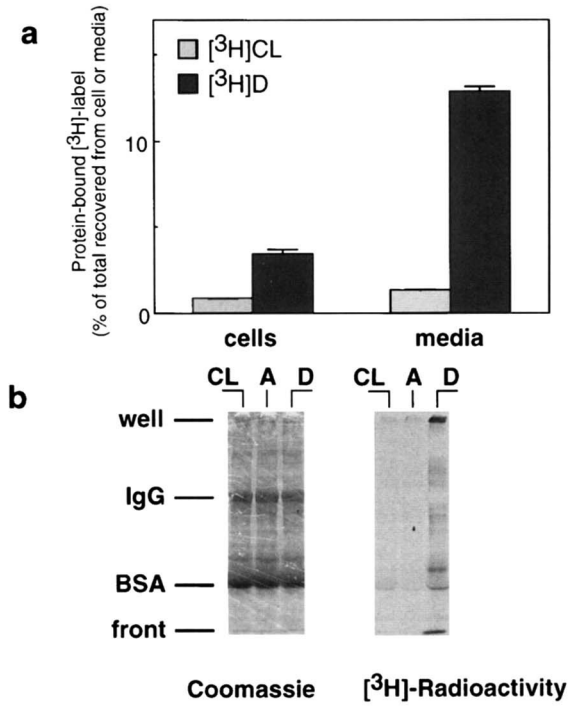

Fig. 9. Complexing of ^{3}H-radioactivity with cellular and serum proteins. (a) Aliquots of 25 µl of the PS-containing substrates prepared as described in Fig. 8 (200,000 dpm/well) were incubated with MPM cultures for 20 h. Conditioned media and cell monolayers were collected separately and subjected to lipid extraction. The protein interface resulting from Bligh-Dyer lipid extraction of the media was extensively washed 3 times with 3 ml of chloroform. Likewise, cellular proteins were washed repeatedly with hexane–isopropanol. The protein residues were then dissolved in 0.1 N NaOH for measurement of tritium label. Prior to mixing with scintillation fluid, samples were neutralized with an equinormal amount of HCl. (b) Aliquots of [^{3}H]CL, ^{3}H-labeled fractions A, or D (10^6 dpm) in chloroform were dried on a 2 × 2 mm glass filter and placed on the bottom of borosilicate glass test tubes, and 50 µl of 10% LPDS in PBS containing 2 mM EDTA, and 40 µM of butylated hydroxytoluene were added. After incubation at 37°C for 24 h the unbound lipid was extracted 3 times with 1 ml of ethyl ether. For SDS/PAGE, aqueous phases were mixed with the sample buffer containing 63 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, and 0.0025% bromophenol blue. The samples were electrophoretically resolved on precast 4–12% Tris-glycine gels (NOVEX, San Diego, CA). Gels were then fixed, stained, soaked in EN^{3}HANCE™ according to the manufacturer's specifications before drying and autoradiography.

Fig. 8. Reverse phase HPLC/ESI/MS of fraction D after reaction with 2,4-dinitrophenylhydrazine. (a) Total negative ion [M−1] current profile of a chloroform–methanol extract of the reaction mixture. Peak identification (based on both [M−1] and sterol ring ions (m/z 369–385) detected in positive ion mode): Peak 1, unknown (m/z 317); Peak 2, unknown (m/z 431); Peak 3, 7α-hydroxycholesteryl 9-carboxynonanoate (m/z 571, 385); Peak 4, 7β-hydroxycholesteryl 9-carboxynonanoate (m/z 571, 385); Peak 5, 5,6-epoxycholesteryl 8-carboxyoctanoate (m/z 557, 385); Peak 6, 5,6-epoxycholesteryl 9-carboxynonanoate (m/z 751, 385); Peak 7, 7α-hydroxycholesteryl 9-oxononanoate DNPH (m/z 735, 385); Peak 8, 7β-hydroxycholesteryl 9-oxononanoate DNPH (m/z 735, 385); Peak 9, 7-ketocholesteryl 9-carboxynonanoate DNPH (m/z 735, 383); Peak 10, cholesteryl 9-carboxynonanoate (m/z 555, 369); Peak 11, cholesteryl 8-oxooctanoate DNPH (m/z 705, 369); Peak 12, cholesteryl 9-oxononanoate DNPH (m/z 719, 369). Instrumentation and operating conditions are given in Methods. CapEx = 170 V; (b) full mass spectrum averaged over the entire elution profile in (a); (c) Single ion chromatograms of major ions detected in the full spectrum corresponding to [M−1] of known cholesteryl linoleate peroxidation products.

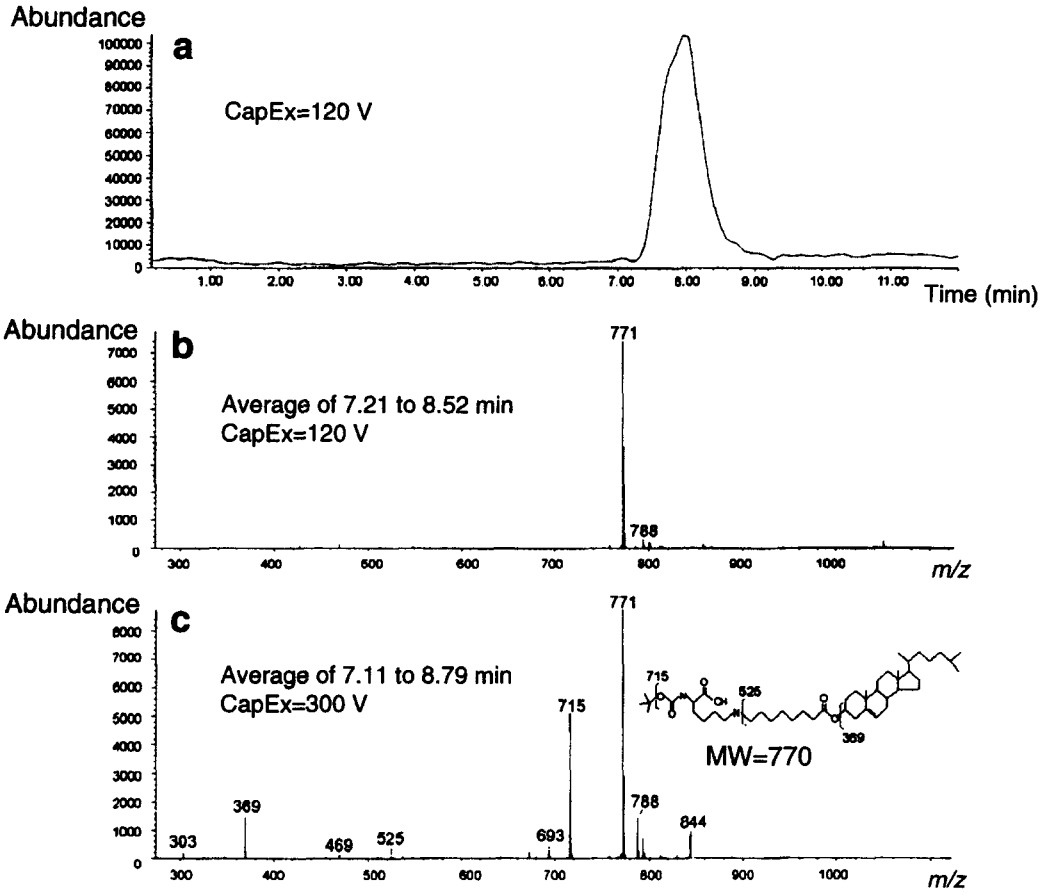

Fig. 10. Demonstration of Schiff base formation between cholesteryl 9-oxononanoate and the ε-amino group of N-BOC-Lys. (a) Total positive ion current of $NaCNBH_3$ reduced base as obtained by HPLC/ESI/MS at CapEx voltage of 120. (b) Full mass spectrum at CapEx = 120 V averaged over the entire peak eluted in (a). (c) Full mass spectrum at CapEx = 300 V averaged over the entire peak eluted in (a). Ion identification: *m/z* 771, $[M + 1]^+$; *m/z* 788 $[M + NH_4]^+$; *m/z* 715, [M–*tert*-butyl + 1]$^+$; *m/z* 845, $[M + 57 + 18]^+$; *m/z* 525, [M–N-BOC-Lys]$^+$; *m/z* 369, cholesterol ring; *m/z* 303, reduced Schiff base of N-BOC-Lys and 9-oxononanoate, HPLC and mass spectrometry conditions are as given under Methods.

Lys. The free ε-amino group readily reacted with the core aldehyde to yield a Schiff base. The base gave a correct molecular mass of *m/z* 769 $[M + 1]^+$ on flow injection/ESI/MS at a capillary exit (CapEx) of 120V (not shown). The base was stabilized by sodium cyanoborohydride reduction which increased its mass to *m/z* 771 (**Fig. 10b**) and permitted its chromatographic purification. Fig. 10a shows the total negative ion current profile as obtained by normal phase HPLC/ESI/MS along with the full mass spectrum averaged over the entire HPLC peak (Fig. 10b) and the averaged full mass spectrum averaged over the entire HPLC peak after increasing the exit voltage of the ESI block from 120 to 300 (Fig. 10c). The fragment ions formed upon ESI/CID/MS clearly support the Schiff base structure of this compound by yielding the fragment ions anticipated from a loss of the *tert*-butyl group $[M–57]^+$ at *m/z* 715, [M–N-BOC- Lys]$^+$ at *m/z* 525, and [M–N-BOC-Lys + 9- oxononanoate]$^+$ at *m/z* 369 (cholesterol ring) (Fig. 10c). Although HPLC/ESI/MS at two different CapEx voltages is not a conventional MS/MS, it is considered adequate for the characterization of chromatographic peaks selected from efficient HPLC separations (31).

Detection of cholesteryl ester core aldehydes in human atherosclerotic lesions

Finally, we have examined total lipid extracts from six human atheroma biopsies and have been able to show that all of them contain readily detectable amounts of cholesteryl ester core aldehydes. HPLC/ESI/MS analysis of the parts of the total negative ion profiles corresponding to the UV absorbing peaks of the DNPH derivatives of standard cholesteryl ester core aldehydes yielded a homologous series ranging in chain length from 4 to 9 or 10 carbons (**Fig. 11**). In the representative sample analysis shown, the major species were the C_9 and C_8 aldehydes, but the C_5 aldehyde was also found in significant amounts. There was some variation from

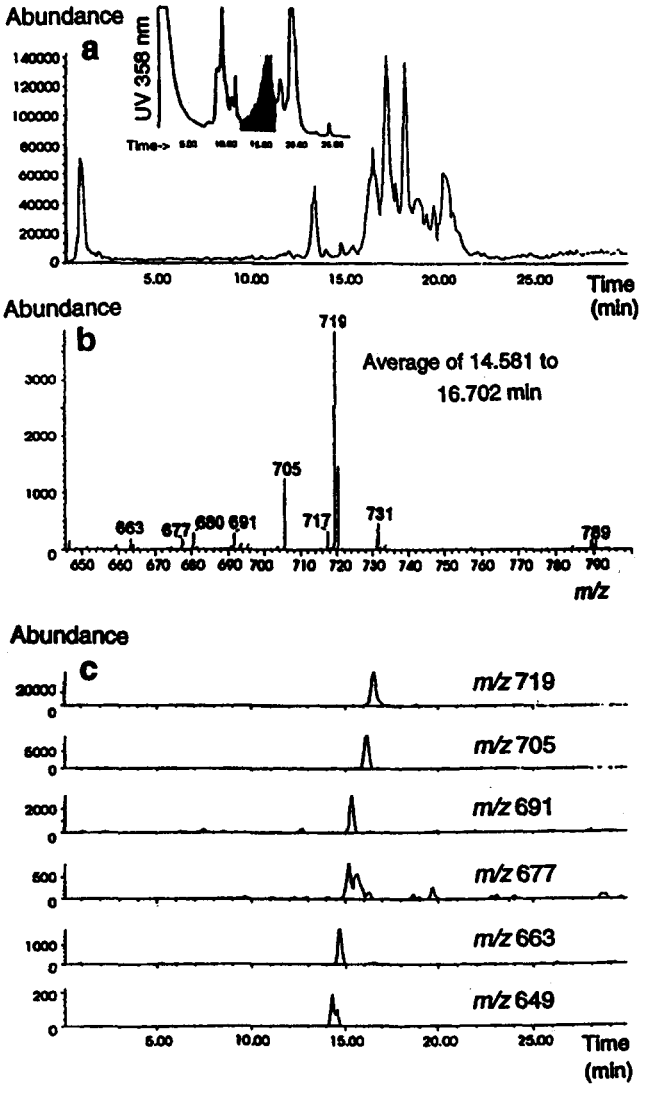

Fig. 11. Demonstration of the presence of cholesteryl ester core aldehydes in human atheroma. (a) Total negative ion current profile of 2,4-dinitrophenylhydrazine-treated chloroform–methanol extract of human atheroma as obtained by reverse phase HPLC/ESI/MS. (a) Inset: Simultaneously recorded UV absorption profile with aldehyde region shaded in. (b) Full spectrum averaged over the total ion current peak corresponding to the shaded area in the inset in (a) (14.581–16.702 min). Ion identification: *m/z* 731, 10-oxodecenoyl cholesterol; other ions are identified as show in (c). (c) [M–1] Ion chromatograms for cholesteryl ester core aldehydes: C_9, *m/z* 719, C_8, *m/z* 705; C_7, *m/z* 691; C_6, *m/z* 677; C_5, *m/z* 663; C_4, *m/z* 649. HPLC and mass spectrometry conditions are as given under Methods. CapEx = 170 V.

sample to sample in the relative proportion of the different chain lengths of the aldehydes as well as in the total amounts of the aldehydes, but none of the lesions appeared to be aldehyde free. Kamido et al. (22) had reported that inclusion of dinitrophenylhydrazine in the extraction solvent increased the recovery of the aldehydes because the hydrazine caused the displacement of the aldehydes from their protein-bound forms. In the present study, readily detectable amounts of core aldehyde were recovered by a simple chloroform–methanol extraction of the atheroma samples. Atheroma-free areas of one human aorta sample obtained post mortem failed to show the presence of core aldehydes (not shown). These studies are consistent with an earlier report in abstract form (32) demonstrating the presence of cholesteryl ester core aldehydes in human atheromas.

DISCUSSION

Extensive oxidation of [^{3}H]CL resulted in almost complete consumption of authentic CL, consistent with other studies (22, 33–36). However, products of such oxidative breakdown appear to preserve the ester bond, as all fractions were filipin negative, and UC and oxysterols were readily released by saponification. Based on HPLC/ESI/MS analysis, the most abundant fraction of oxCL, fraction D, contained lipid core aldehydes, i.e., cholesterol or oxysterol esters of 9-oxononanoate. Esterbauer and Zollner (37) predicted the formation of lipid esters containing C_5, C_9, and C_{13} fatty acid chains, as a result of terminal peroxidation of polyunsaturated acids. This idea was confirmed by the identification of "core aldehydes" in copper-oxidized LDL (38), as well as in CL treated with *tert*-butyl hydroperoxide/Fe^{2+} (23). C_8, C_9, C_{11}, C_{13} core aldehydes, derived from the oxidation of CL, were previously identified (23) and later also detected in oxLDL and oxidized high density lipoproteins (22). In a recent report by Itabe et al. (39) the presence of chemically related phospholipid-based core aldehydes, i.e., C_8 and C_9 aldehydes, was found in the fractions of oxidized PC.

To define the pathophysiological importance of the identification of core aldehydes, it is necessary to demonstrate their existence in vivo. The presence of "polar lipids" with characteristic TLC mobility, and identified as oxysterol esters of linoleic acid, was first demonstrated in atherosclerotic aortas as early as in the beginning of the 1970s by Brooks et al. (40). Recently Itabe et al. (39) found that a monoclonal antibody directed against an epitope in foam cells of human atherosclerotic lesions (41) can also recognize a protein-bound C_9 aldehyde of oxidized PC. However, up to now, chemical evidence for the presence of core aldehydes in vivo was missing. In this study we were able to show by mass spectrometric analysis the existence of a variety of core aldehydes in human atheroma. However, given the chemical complexity of atherosclerotic plaques, further work will be needed to fully identify and characterize the

cholesterol-containing aldehydic species found in lipid extracts of human atherosclerotic lesions.

In a previous publication we showed that oxLDL is capable of inactivating the lysosomal thiol protease, cathepsin B (8). It was, therefore, conceivable that ACEH, which is sensitive to sulfhydryl modifying reagents (42), might also be inactivated by compounds in oxLDL. Some lipid constituents of oxLDL, such as lipid hydroperoxides, were reported to cause inactivation of both acid and neutral cholesteryl ester hydrolase (43), whereas other constituents, such as oxysterols, appear to have no effect (44). Based on data obtained from the literature, one would anticipate that the extensively oxidized LDL used in our study is depleted of hydroperoxides (33) but is rich in oxysterols (34, 35). Indeed, in this present study components of extensively oxidized LDL did not inhibit the hydrolysis of [^{3}H]CL by MPM. This assumption is supported by our observation that incorporation of [^{3}H]CL into oxLDL did not lead to reduced hydrolysis of this CL relative to CL in LDL. Likewise, Maor and Aviram (45) recently reported that authentic [^{3}H]CL present in oxLDL particles was efficiently hydrolyzed by J774 macrophages.

One can postulate several mechanisms by which core aldehydes present in oxCL could cause the deficient hydrolysis of CL. First, the length of the fatty acid chain in CE is critical for the rate of its enzymatic hydrolysis. CE with an acyl chain shorter than 10 carbons were shown to be hydrolyzed by ACEH much slower than C_{16} or C_{18} cholesteryl esters (42). Thus, it is conceivable that some of the resistance of fraction D to hydrolysis by MPM extracts under acidic conditions could be due to its suboptimal recognition by lysosomal ACEH. A potential alternative explanation for the resistance of the secondary peroxidation products of cholesteryl linoleate to ACEH and NCEH could be provided by aldol condensation of the core aldehydes. We observed that synthetic cholesteryl 5-oxovalerate tends to dimerize upon storage giving a m/z value of 969 (A. Ravandi and A. Kuksis, unpublished observation). However, the corresponding dimer of the C_9 aldehyde (m/z 1181) was not detected in fraction D. Finally, poor hydrolysis of oxCL could be also explained by the ability of some products of CL peroxidation to bind covalently to proteins and thereby become unavailable for enzymatic degradation. If poor hydrolysis of oxCL by MPM were to occur, it would lead to the retention of fraction D in lysosomes of MPM. Given the UC-like mobility of the fraction D on TLC, the above mechanism could provide an alternative explanation for the apparent accumulation of UC in lysosomes after the internalization of oxLDL by macrophages (45).

The ability of oxidized lipids to cross-link proteins is not limited only to low molecular weight aldehydes such as malondialdehyde and 4-hydroxynonenal. It was recently demonstrated that, after oxidation, radiolabeled phospholipids and cholesteryl esters that were incorporated into LDL became associated with apoB and were suggested to be bound covalently (46). In addition, another recent study provides evidence that oxidized PC forms complexes with lysyl residues on proteins, due to the presence of 9-oxononanoyl PC (39). It is possible that the formation of lipid–protein complexes could occur via interactions between the carbonyl group of lipid core aldehydes (22) and lysine groups on proteins (29). In this study we demonstrated such Schiff base adduction of synthetic C_9 cholesteryl ester core aldehyde with ε-amino groups of lysines. In view of their high reactivity with proteins, it is likely that the core aldehydes detected in lipid extracts of human atheroma represent only a fraction of the total core aldehydes present in atherosclerotic lesions. In addition, unsaturated and α-hydroxy-core aldehydes can be formed by the decomposition of linoleic acid via dioxygenated fatty acids (47), which, in turn, appear to be more robust in their interaction with protein side-chains than carbonyl groups alone. In a recent study, we also found that oxLDL can irreversibly bind other proteins, but only when exposed to acidic pH (48).

The precise mechanism of the interaction of oxLDL with other biological molecules is still not resolved. However, it is clear that the possibility of interactions occurring between products of lipid peroxidation in oxLDL and cellular proteins opens up a variety of potential biological implications. For instance, some properties of oxLDL, such as cytotoxicity attributed to 7-hydroperoxycholesterol (49) or 25-hydroxycholesterol (50), could also be caused by core aldehydes. Furthermore, it is conceivable that aldehydic products of cholesteryl ester oxidation could be sequestered into the hydrophobic core of oxLDL (22), while hydrophilic short-chain aldehydes, such as malondialdehyde (51), could easily diffuse away from the LDL particle. OxLDL might remain unreactive in the circulation, but become highly reactive when core aldehydes in oxLDL are exposed. This could occur, for instance, in lysosomes as a result of possible pH-dependent conformational changes in the oxLDL particle or partial proteolytic cleavage of apoB. Such site-specific release of aldehydes could lead to irreversible intra-lysosomal deposition of protein–lipid polymers and eventually to lysosomal dysfunction. Thus, the localization predominantly to lysosomes of epitopes specific for oxidized proteins (52, 53), as well as lysosomal accumulation of ceroid (11) and partially degraded apoB from oxidized LDL (7), could be explained by the liberation in lysosomes of such hydrophobic reactive components as lipid core aldehydes. Further studies are in progress to test these hypotheses.

In summary, we have shown that after extensive oxi-

dation of cholesteryl linoleate, which is the principal cholesteryl ester in LDL, the resulting shorter-chain esters are poorly hydrolyzed in MPM. We have demonstrated that: *1*) oxLDL did not affect the activity of ACEH towards intact CL, nor did the previously documented retarded proteolysis of apoB in oxLDL result in poor lipolysis of native CL; *2*) the ester bond of all cholesterol-containing products of lipid peroxidation of CL was preserved; *3*) extensively oxidized forms of CL were resistant to enzymatic hydrolysis, especially by lysosomal ACEH; *4*) some oxidized products of CL contain carbonyl groups that can covalently interact with proteins; and *5*) cholesteryl ester core aldehydes exist in human atherosclerotic lesions.

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REFERENCES

1. Stary, H. C., A. B. Chandler, S. Glagov, J. R. Guyton, W. Insulls, Jr., M. E. Rosenfeld, S. A. Schaffer, C. J. Schwartz, W. D. Wagner, and R. W. Wissler. 1994. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation.* **89:** 2462–2478.
2. Shio, H., N. Haley, and S. Fowler. 1978. Characterization of lipid-laden aortic cells from cholesterol-fed rabbits. II. Morphometric analysis of lipid-filled lysosomes and lipid droplets in aortic cell populations. *Lab. Invest.* **39:** 390–397.
3. Jerome, W. G., and J. C. Lewis. 1985. Early atherogenesis in White Carneau pigeons. Ultrastructural and cytochemical observations. *Am. J. Pathol.* **119:** 210–222.
4. Jessup, W., E. L. Mander, and R. T. Dean. 1992. The intracellular storage and turnover of apolipoprotein B of oxidized LDL in macrophages. *Biochim. Biophys. Acta.* **1126:** 167–177.
5. Lougheed, M., H. Zhang, and U. P. Steinbrecher. 1991. Oxidized low density lipoprotein is resistant to cathepsins and accumulates within macrophages. *J. Biol. Chem.* **266:** 14519–14525.
6. Hoff, H. F., N. Zyromski, D. Armstrong, and J. O'Neil. 1993. Aggregation as well as chemical modification of LDL during oxidation is responsible for poor processing in macrophages. *J. Lipid Res.* **34:** 1919–1929.
7. Mander, E. L., R. T. Dean, K. K. Stanley, and W. Jessup. 1994. Apolipoprotein B of oxidized LDL accumulates in the lysosomes of macrophages. *Biochim. Biophys. Acta.* **212:** 80–92.
8. Hoppe, G., J. O'Neil, and H. F. Hoff. 1994. Inactivation of lysosomal proteases by oxidized LDL is partially responsible for its poor degradation by macrophages. *J. Clin. Invest.* **94:** 1506–1512.
9. Ball, R. Y., J. P. Bindman, K. L. H. Carpenter, and M. J. Mitchinson. 1986. Oxidized low density lipoprotein induces ceroid accumulation by murine peritoneal macrophages in vitro. *Atherosclerosis.* **60:** 173–181.
10. Hunt, J. V., M. A. Bottoms, S. E. Taylor, V. Lyell, and M. J. Mitchinson. 1994. Differing effects of probucol and vitamin E on the oxidation of lipoproteins, ceroid accumulation and protein uptake by macrophages. *Free Radical Res.* **20:** 189–201.
11. Ball, R. Y., K. L. H. Carpenter, and M. J. Mitchinson. 1987. What is the significance of ceroid in human atherosclerosis? *Arch. Pathol. Lab. Med.* **111:** 1134–1140.
12. Ivy, G. O., S. Kanai, M. Ohta, G. Smith, Y. Sato, M. Kobayashi, and K. Kitani. 1990. Lipofuscin-like substances accumulate rapidly in brain, retina and internal organs with cysteine protease inhibition. *In* Lipofuscin and Ceroid Pigments. E. A. Porta, editor. Plenum Press, New York, N.Y. 31–47.
13. Kamido, H., A. Kuksis, L. Marai, J. J. Myher, and H. Peng. 1992. Preparation, chromatography, and mass spectrometry of cholesteryl ester and glycerolipid-bound aldehydes. *Lipids.* **27:** 645–650.
14. Hatch, F. T., and R. S. Lees. 1968. Practical method for plasma lipoprotein analysis. *Adv. Lipids Res.* **6:** 2–63.
15. Sparks, D. L., J. Frohlich, P. Cullis, and P. H. Pritchard. 1987. Cholesteryl ester transfer activity in plasma measured by using solid-phase-bound high-density lipoprotein. *Clin. Chem.* **33:** 390–393.
16. Goldstein, J. L., Y. K. Ho, S. K. Basu, and M. S. Brown. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA.* **76:** 333–337.
17. Steinbrecher, U. P., M. Lougheed, W-C. Kwan, and M. Dirks. 1989. Recognition of oxidized low density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein B byproducts of fatty acid peroxidation. *J. Biol. Chem.* **264:** 15216–15223.
18. Ross, A. C., K. L. Go, J. G. Heider, and G. H. Rothblat. 1984. Selective inhibition of acyl-coenzyme A: cholesterol acyltransferase by compound 58-035. *J. Biol. Chem.* **259:** 815–819.
19. Bligh, E. J., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
20. Hara, A., and N. S. Radin. 1978. Lipid extraction of tissues with low-toxicity solvent. *Anal. Biochem.* **90:** 420–426.
21. Lange, Y., and B. V. Ramos. 1983. Analysis of the distribution of cholesterol in the intact cell. *J. Biol. Chem.* **258:** 15130–15134.
22. Kamido, H., A. Kuksis, L. Marai, and J. J. Myher. 1995. Lipid ester-bound aldehydes among copper-catalyzed peroxidation products of human plasma lipoproteins. *J. Lipid Res.* **36:** 1876–1886.
23. Kamido, H., A. Kuksis, L. Marai, and J. J. Myher. 1993. Identification of core aldehydes among in vitro peroxi-

dation products of cholesteryl esters. *Lipids.* **28:** 331–336.
24. Ravandi, A., A. Kuksis, J. J. Myher, L. Marai. 1995. Determination of lipid ester ozonides and core aldehydes by high-performance liquid chromatography with on-line mass spectrometry. *J. Biochem. Biophys. Methods.* **30:** 271–285.
25. Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150:** 76–85.
26. Smejkal, G., G. Hoppe, and H. F. Hoff. 1996. Filipin as a fluorescent probe of lipoprotein-derived oxysterols on thin-layer chromatograms. *Anal. Biochem.* **239:** 115–117.
27. Kruth, H. S. 1983. Filipin-positive, oil red O-negative particles in atherosclerotic lesions induced by cholesterol feeding. *Lab. Invest.* **50:** 87–93.
28. Nishikawa, K., H. Arai, and K. Inoue. 1990. Scavenger receptor-mediated uptake and metabolism of lipid vesicles containing acidic phospholipids by mouse peritoneal macrophages. *J. Biol. Chem.* **265:** 5226–5231.
29. Esterbauer, H., R. J. Schaur, and H. Zollner. 1991. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radical Biol. Med.* **11:** 81–128.
30. Uchida, K., and E. R. Stadtman. 1993. Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase. A possible involvement of intra- and intermolecular cross-linking reaction. *J. Biol. Chem.* **268:** 6388–6693.
31. Voyksner, R. D., and T. Pack. 1991. Investigation of collisional-activation decomposition process and spectra in the transport region of an electrospray single quadruple mass spectrometer. *Mass Spectrom.* **5:** 263–268.
32. Kamido, H., K. Nonaka, A. Kuksis, L. Marai, and A. Ravandi. 1996. Identification of lipid ester core aldehydes and hydroperoxides in human atherosclerotic lesions. *Circulation.* **94(Suppl):** I–707.
33. Brown, A. J., R. T. Dean, and W. Jessup. 1996. Free and esterified oxysterol: formation during copper-oxidation of low density lipoprotein and uptake by macrophages. *J. Lipid Res.* **37:** 320–335.
34. Carpenter, K. L. H., G. M. Wilkins, B. Fussel, J. A. Ballantine, S. E. Taylor, M. J. Mitchinson, and D. S. Leake. 1994. Production of oxidized lipids during modification of low-density lipoprotein by macrophage or copper. *Biochem. J.* **304:** 625–633.
35. Kritharides, L., W. Jessup, J. Gifford, and R. T. Dean. 1993. A method for defining the stages of low-density lipoprotein oxidation by the separation of cholesterol- and cholesteryl ester-oxidation products using HPLC. *Anal. Biochem.* **213:** 79–89.
36. Nikkari, T., U. Malo-Ranta, T. Hiltunen, O. Jaakkola, S. Ylä-Herttuala. 1995. Monitoring of lipoprotein oxidation by gas chromatographic analysis of hydroxy fatty acids. *J. Lipid Res.* **36:** 200–207.
37. Esterbauer, H., and H. Zollner. 1989. Methods for determination of aldehydic lipid peroxidation products. *Free Rad. Biol. Med.* **7:** 197–203.
38. Kamido, H., A. Kuksis, L. Marai, and J. J. Myher. 1992. Identification of cholesterol-bound aldehydes in copper-oxidized low density lipoprotein. *FEBS Lett.* **304:** 269–272.
39. Itabe, H., H. Yamamoto, M. Suzuki, Y. Kawai, Y. Nakagawa, A. Suzuki, T. Imanaka, and T. Takano. 1996. Oxidized phosphatidylcholines that modify proteins. Analysis by monoclonal antibody against oxidized low density lipoprotein. *J. Biol. Chem.* **271:** 33208–33217.
40. Brooks, C. J. W., G. Steel, J. D. Gilbert, and W. A. Harland. 1971. Lipid of human atheroma. Part 4. Characterization of a new group of polar sterol esters from human atherosclerotic plaques. *Atherosclerosis.* **13:** 223–237.
41. Itabe, H., E. Takeshima, H. Iwasaki, J. Kimura, Y. Yoshida, T. Imanaka, and T. Takano. 1994. A monoclonal antibody against oxidized lipoprotein recognizes foam cells in atherosclerotic lesions. Complex formation of oxidized phosphatidylcholines and polypeptides. *J. Biol. Chem.* **269:** 15274–15279.
42. Négre, A., R. Salvayre, P. Rogalle, Q. Q. Dang, and L. Douste-Blazy. 1987. Acyl-chain specificity and properties of cholesterol esterases from normal and Wolman lymphoid cell lines. *Biochim. Biophys. Acta.* **918:** 76–82.
43. Maehira, F. 1994. Inhibitory effect of lipid hydroperoxide on cholesteryl esterases. *Biochem. Mol. Biol. Int.* **32:** 221–231.
44. Morin, R. J., and S-K. Peng. 1989. Effects of cholesterol oxidation derivatives on cholesterol esterifying and cholesteryl ester hydrolytic enzyme activity of cultured rabbit aortic smooth muscle cells. *Lipids.* **24:** 217–220.
45. Maor, I., and M. Aviram. 1994. Oxidized low density lipoprotein leads to macrophage accumulation of unesterified cholesterol as a result of lysosomal trapping of the lipoprotein hydrolyzed cholesteryl ester. *J. Lipid Res.* **35:** 803–819.
46. Tertov, V. V., V. V. Kaplun, S. N. Dvoryantsev, and A. N. Orekhov. 1995. Apolipoprotein B-bound lipids as a marker for evaluation of low density lipoprotein oxidation in vivo. *Biochem. Biophys. Res. Commun.* **214:** 608–613.
47. Loidl-Stahlhofen, A., K. Hannemann, and G. Spiteller. 1994. Generation of α-hydroxyaldehydic compounds in the course of lipid peroxidation. *Biochim. Biophys. Acta.* **1213:** 140–148.
48. O'Neil, J., G. Hoppe, L. M. Sayre, and H. F. Hoff. 1997. Inactivation of cathepsin B by oxidized LDL involves complex formation induced by binding of putative reactive sites exposed at low pH to thiols on the enzyme. *Free Radical Biol. Med.* **23:** In press.
49. Chisolm, G. M., G. Ma, K. C. Irwin, L. L. Martin, K. G. Gunderson, L. F. Linberg, D. W. Morel, and P. E. DiCorleto. 1994. 7β-Hydroperoxycholest-5-en-3β-ol, a component of human atherosclerotic lesions, is the primary cytotoxin of oxidized human low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **91:** 11452–11456.
50. Guyton, J. R., B. L. Black, and C. L. Seidel. 1990. Focal toxicity of oxysterols in vascular smooth muscle cell culture. A model of the atherosclerotic core region. *Am. J. Pathol.* **137:** 425–434.
51. Esterbauer, H., G. Jürgens, O. Quehenberger, and E. Koller. 1987. Autooxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J. Lipid Res.* **28:** 495–509.
52. Rosenfeld, M. E., W. Palinski, S. Ylä-Herttuala, S. Butler, and J. L. Witztum. 1990. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arteriosclerosis.* **10:** 336–349.
53. Palinski, W., V. A. Ord, A. S. Plump, J. L. Breslow, D. Steinberg, and J. L. Witztum. 1994. ApoE-deficient mice are a model of lipoprotein oxidation in atherogenesis: demonstration of oxidation-specific epitopes in lesions and high titers of autoantibodies to malondialdehyde-lysine in serum. *Arterioscler. Thromb.* **14:** 605–616.