7-Hydroperoxycholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plaque

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Abstract 7-Hydroperoxycholesterols (7OOHs) are intermediates in cholesterol oxidation and potential cytotoxins. A normal-phase HPLC method with UV (205 nm) detection was developed that could resolve 7αOOH, 7βOOH, 7-ketocholesterol (7K), and the epimeric 7-hydroxycholesterols (7OHs). 7OOH formation was investigated when LDL was exposed to four different oxidizing systems: Cu^{2+}; Ham's F-10; mouse peritoneal macrophages in Ham's F-10; and a metal-independent peroxyl-radical generating system (AAPH). With all four oxidizing systems, 7OOH (both free and esterified, mostly as the β-isomer) was the major oxysterol formed at early times, with 7K dominating at later stages (≥24 h) in Cu-oxLDL. When LDL was oxidized in the presence of cells there was transfer of free oxysterols from LDL to the cells. Negligible 7OOH, but significant amounts of 7OH, accumulated in the cells suggesting efficient cellular reduction of 7OOH. Lipid extracts from eight plaque samples obtained from patients undergoing carotid endarterectomy were analyzed. Only trace amounts of 7OOH (<0.02% of total cholesterol) could be detected using this normal-phase HPLC method with UV detection or with a more sensitive reverse-phase method utilizing chemiluminescence detection. 7K was the major 7-oxygenated sterol detected, at least 20-fold in excess of that calculated for 7OOH, followed by 7βOH and 7αOH. The trace concentrations of 7OOH in plaque indicate its lability in biological/cellular systems and may signify the ability of cells in the artery wall to metabolize it further.—**Brown, A. J., S-l. Leong, R. T. Dean, and W. Jessup.** 7-Hydroperoxycholesterol and its products in oxidized low density lipoprotein and human atherosclerotic plaque. *J. Lipid Res.* 1997. **38:** 1730–1745.

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Low density lipoprotein (LDL), the major carrier of cholesterol in the circulation, is strongly implicated in the development of atherosclerosis, the pathological process underlying much vascular disease. From its earliest detectable stages, atherosclerosis is characterized by the presence of intimal, cholesterol-loaded, macrophage-derived 'foam cells' (1). In vitro studies have shown that LDL can only induce cellular cholesterol accumulation when presented to macrophages in a modified form such that its uptake is mediated either by receptors other than the LDL-receptor or by gross phagocytic uptake. In either case, the cell is unable to down-regulate the route of LDL uptake, resulting in the progressive deposition of cholesterol, principally in esterified form, for example, as cytoplasmic 'foamy' droplets within the cells. In vitro work has identified oxidation as a modification that transforms LDL into such a 'high-uptake' form (2) and provided evidence that in the presence of metals such oxidation can be mediated by intimal cells (3), and that oxidized forms of LDL are present in atheromatous tissue (4).

Copper ion (Cu^{2+})-catalyzed oxidation of LDL provides a useful model system for studying the effects of oxidized LDL on cells. When LDL undergoes oxidation in vitro, a number of changes in lipid composition occur, including a substantial loss of free and esterified cholesterol as these are converted into oxidation products of cholesterol (oxysterols). We (5) and others (6–11) have previously shown that sterols oxygenated in the 7-position predominate in Cu^{2+}-oxidized LDL. In particular, gas chromatographic (GC) analysis indicated that 7-ketocholesterol (7K) is the major oxysterol

Abbreviations: 5αOOH, 5α-hydroperoxycholesterol; 6βOH, 6β-hydroxycholesterol; 7OOH, 7-hydroperoxycholesterol; 7K, 7-ketocholesterol; 7OH, 7-hydroxycholesterol; 19OH, 19-hydroxycholesterol; 27OH, 27-hydroxycholesterol; AAPH, 2,2′-azobis(2-amidinopropane) hydrochloride; BHT, butylated hydroxy toluene; BSA, bovine serum albumin; Ch18:2OOH, cholesteryl hydroperoxy-octadecadienoates; Ch18:2OH, cholesteryl hydroxy-octadecadienoates; Ch18:2=O, cholesteryl keto-octadecadienoates; Cu-oxLDL, Cu^{2+}-oxidized low density lipoprotein; DMEM, Dulbecco's minimum essential medium; EDTA, ethylenediaminetetra acetate; LDL, low density lipoprotein; GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; PBS, phosphate-buffered saline.

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in 24 h Cu^{2+}-oxidized low density lipoprotein (Cu-oxLDL) (5). At earlier times (≤8 h), 7K levels measured by GC were consistently higher than those detected using a reverse-phase high-performance liquid chromatographic (HPLC) method. We speculated (5) that the discrepancy may have been due to the presence of 7-hydroperoxycholesterols (7OOH), intermediates in cholesterol oxidation, which would decompose to 7K and the corresponding 7-hydroxycholesterol (7OH) during the high temperatures encountered in GC analysis but are stable during HPLC analysis. 7OOH has been identified in Cu-oxLDL (12, 13) but quantification of 7OOH in LDL oxidized by Cu^{2+} and cells is lacking. Reliance on GC, traditionally the method of choice for measuring oxysterols (14), will give a distorted profile of oxysterols if 7OOH is a significant oxysterol. In particular, 7βOOH has been reported to occur in human atherosclerotic plaque although quantitative data have not been provided (12). It is important to know whether GC estimates of 7K in human atherosclerotic plaque are accurate or reflect a combination of 7K and 7OOH in these tissues.

In this study we report a normal-phase HPLC method with UV (205 nm) detection that can resolve and detect all products of cholesterol oxygenated at the 7-position: 7K, 7αOOH, 7βOOH, 7αOH, and 7βOH. Using this method, significant 7OOH formation was detected in LDL exposed to several different oxidizing systems at 37°C (up to 48 h): Cu^{2+}; Ham's F-10; mouse peritoneal macrophages in Ham's F-10; and a metal-independent system (AAPH). Evidence is presented to indicate that unesterified 7OOH is transferred to cells during macrophage-mediated oxidation of LDL. The present study substantiates a previous observation that 7OOH occurs in human atherosclerotic plaque (12) but places it into perspective with other oxidized lipids found at much higher concentrations in plaque. Oxysterols including 27OH, 7K, and 7OH have long been known to occur in human atherosclerotic plaque (15–20) but this is the first study to report on their relative proportions as free and esterified oxysterol.

MATERIALS AND METHODS

Reagents

All solvents were HPLC grade unless otherwise specified. Low UV cut-off isopropanol (EM-Science, Cherry Hill, NJ) was used for all HPLC analyses. Dried diethyl ether (Fluka Chemie AG, Buchs, Switzerland) contained negligible peroxides (detection limit 0.5 mg/l) determined by the Merckoquant Peroxide Test (1.10011, E. Merck, Darmstadt, Germany). Cholesteryl ester standards were all from Sigma-Aldrich (Castle Hill, N.S.W., Australia). Cholest-5-en-3β-ol (cholesterol) and cholest-5-en-3β,19-diol (19-hydroxycholesterol) were also from Sigma-Aldrich; cholest-5-en-3β-ol-7-one (7-ketocholesterol), cholest-5-en-3β,7α-diol (7α-hydroxycholesterol), cholest-5-en-3β,7β-diol (7β-hydroxycholesterol) were from Steraloids Inc. (Wilton, NH) and (25R)-cholest-5-en-3β,26-diol (27-hydroxycholesterol) was obtained from Research Plus Inc. (Bayonne, NJ). Cholest-4-en-3β,6β-diol (6β-hydroxycholesterol) was synthesized by the reduction of cholest-4-en-6β-ol-3-one with lithium aluminium borohydride (5). Cholest-6-en-3β-ol-5α-hydroperoxide (5αOOH) was synthesized by photo-oxidation of cholesterol using the method of Schenk, Gollnick and Neumuller (21) as described in (22). 7-Ketocholesteryl esters were synthesized using the appropriate fatty acyl chloride (Nu-Chek Prep, Elysian, MN) according to the method for synthesizing cholesteryl esters described by Deykin and Goodman (23). Cholesteryl hydroxy-octadecadienoate (Ch18:2OH) was from Cayman Chemical Co. (Ann Arbor, MI). Cholesteryl octadecadienoate hydroperoxide (Ch18:2OOH) was prepared by incubating cholesteryl linoleate (6.5 mg) with 2,2′-azo*bis*[2,4-dimethylvaleronitrile] (25 mg AMVN; Polysciences Inc., Warrington, PA) in isopropanol (1.0 ml) at 65°C for 2 h. Ch18:2OOH was purified by reverse-phase HPLC and quantified using our previously published HPLC response factor (24). Standards used for quantitation were ≥99% pure (assessed by HPLC or GC (5)).

Isolation of LDL

Plasma was obtained from individual normolipidemic healthy donors and LDL ($1.05 > d > 1.02$ g/ml, 10°C) was isolated on a discontinuous gradient as described previously (5). The LDL was dialyzed against four exchanges of deaerated Dulbecco's modified phosphate-buffered saline (PBS, pH 7.4, Sigma-Aldrich) containing chloramphenicol (0.1 g/l) and ethylendiaminetetra-acetic acid (EDTA; 1 g/l), then filter-sterilized (0.45 μm), and stored at 4°C in the dark until use (within 7 d).

Copper-mediated oxidation of LDL

To remove the EDTA prior to oxidation, LDL was dialyzed overnight against four exchanges of PBS containing chloramphenicol (0.1 mg/ml) and pre-washed Chelex-100 resin (mesh 100–200, 4 g/l; Bio-Rad) (25). After filter-sterilization, LDL (1.0 mg protein/ml) was added to 35-mm diameter tissue culture wells (7 ml/well; Falcon, Becton-Dickinson, Lincoln Park, NJ) and oxidized by addition of 20 μM cupric chloride (final concentration) in a humidified 37°C incubator (5% carbon dioxide in air). Time-dependent oxidations were performed in triplicate for 0, 2, 4, 6, 8, 10, 24, 32, and

48 h. Oxidation was arrested by adding the LDL (1.0 mg/ml, 100 μl for saponified and 200 μl for unsaponified) to a screw-cap culture tube (Kimble, Owens-Illinois, Alton, IL) containing EDTA (20 mM, 10 μl) and butylated hydroxy toluene (BHT) in ethanol (2 mM, 10 μl) and cooling on ice. Samples were extracted for lipids as described below.

Metal-independent oxidation of LDL

Metal-independent oxidation of LDL (1.0 mg/ml) was performed using the aqueous peroxyl radical generator, 2,2′-azo*bis*(2-amidinopropane) hydrochloride (AAPH; Polysciences Inc. 50 mM) in the presence of EDTA (1.0 g/l) for 20 h at 37°C.

In two reduction experiments, the AAPH-oxLDL was passed through two Sephadex G-25 columns (PD-10; Pharmacia Biotech, Uppsala, Sweden) to remove AAPH and then incubated with Ebselen (2-phenyl-1,2-benzoisoselenazol; Cayman Chemical Co., Ann Arbor, MI) and reduced glutathione (GSH) (0.15 and 1.5 μmol/mg LDL protein, respectively) for 1 h at 37°C. After further desalting over Sephadex G-25, samples were saponified, extracted and analyzed as described below.

Cells

Resident macrophages were isolated by peritoneal lavage from 6-week-old Quackenbush Swiss (QS) strain mice and cultured as previously described (5) in 35-mm diameter wells. For cell-mediated oxidation experiments, cells were plated at a density of 4×10^6/well and incubated in 2 ml fresh serum-free Ham's F-10 medium (Gibco, Life Technologies Inc., Grand Island, NY) containing LDL (25–100 μg protein/ml) for periods up to 48 h. Cell-free control incubations were set up in parallel. At the designated times, the medium was removed, centrifuged (16,000 *g*, 10 min, 4°C) to remove any detached cells, and 1.2 ml of the supernatant was extracted as below.

For measurement of cellular uptake of 7OOH, LDL that had undergone Cu^{2+}-mediated oxidation for 10 h was treated with EDTA (added in 25-fold molar excess over Cu^{2+} to stop further oxidation) and then dialyzed overnight against 4×1 l of chelex-treated deaerated PBS to remove Cu^{2+} and EDTA. After filter-sterilization, the 10 h Cu-oxLDL (25 μg/ml) was incubated for 24 h with cells plated at a density of 6×10^6/35-mm diameter tissue culture well and cultured in Dulbecco's minimal essential medium (DMEM; Trace BioSciences, Castle Hill, NSW, Australia) containing penicillin G (50 units/ml), streptomycin (50 μg/ml), and 10% (v/v) human lipoprotein-deficient serum ($d > 1.25$; 25 mg protein/ml). After lipid loading, the cells were washed three times with pre-warmed PBS and incubated overnight in DMEM medium (2 ml/well) containing 1 mg/ml BSA (essentially fatty acid-free bovine serum albumin). This equilibration period has previously been shown to allow sufficient time for complete lysosomal degradation of acetylated LDL (26). Cells were washed again with pre-warmed PBS before extraction. Heptane–isopropanol 3:2 (v/v; 2.0 ml) containing 19-hydroxycholesterol (19OH, 5 μg/ml) as internal standard was added and the plates were placed on a platform rocker for 30 min at 4°C. The extraction was repeated twice and the extracts were combined and evaporated using a Savant Speed Vac Plus concentrator system (SC110A/UVS400A). The lipid extract was redissolved in diethyl ether (4.0 ml) which was analyzed for total sterols (saponified: 1.5 ml) and free sterols (unsaponified: 2.5 ml) as described below. Sodium hydroxide (0.2 M, 0.6 ml) was added to solubilize cellular protein for 30 min at 4°C with rocking. Cell-free control incubations in DMEM containing 1 mg/ml BSA were set up in parallel and extracted as below.

Lipid extraction and saponification

Because alkaline saponification may cause decomposition of peroxides, some initial experiments were carried out using bovine pancreatic cholesterol esterase (EC 3.1.1.13, Sigma-Aldrich, 1 mg/100 μg LDL) to hydrolyze steryl esters in native and oxidized LDL for 3 h at 37°C. As this resulted in less than 90% cholesteryl ester hydrolysis, a second bolus of enzyme and a further incubation for 2 h at 37°C was included, which yielded >97% hydrolysis. However, pilot studies indicated that this method caused more extensive decomposition of 7OOH than alkaline saponification at room temperature (data not shown) and so enzymic hydrolysis was not used further.

Cells and LDL were extracted unsaponified or subjected to alkaline saponification. The saponification method was based on that described in (27) but conducted on ice to minimize thermal decomposition of the 7OOHs. LDL samples were added to a screw-cap culture tube containing 19OH (50 μg/ml heptane–isopropanol 95:5 (v/v), 100 μl) as an internal standard, BHT (2.0 mM in ethanol, 10 μl) and EDTA (200 mM, 10 μl). For saponification, a methanolic solution of potassium hydroxide (20% w/v, 2.0 ml) was added while only methanol (2.0 ml) was added to the unsaponified sample. Diethyl ether (2.5 ml) was added, the tubes were flushed with argon and vigorously shaken for 3 h in a polystyrene box packed with ice in an incubator shaker (Series 25, New Brunswick Scientific, New Brunswick, NJ). Cell extracts in diethyl ether (already containing internal standard) were added to tubes containing only BHT and EDTA. Methanol or methanolic KOH were added (as above) and additional ether was

TABLE 1. Human arterial and plaque samples: donor and processing details

Samples	n	Donor Sex	Donor Age (y) Mean ± SD (Range)	Processed Immediately (I) or after Storage (S)
Normal iliac A	4	2M, 2F	46 ± 9 (35–58)	4S
Carotid plaques A	4	3M, 1F	66 ± 15 (43–76)	1I, 3S
Carotid plaques B	4	3M, 1F	68 ± 11 (52–78)	4I

Normal arteries or plaque samples were either extracted with diethyl ether–hexane (A) or acetone after lyophilization (B). M, male; F, female. Samples were either extracted immediately (I) or after storage at −80°C for <4 months (S).

introduced to make up a final diethyl ether volume of 2.5 ml. Saponification was stopped by the addition of acetic acid (20% v/v. 2.0 ml) whereas potassium acetate (20% w/v, 2.0 ml) was used in the unsaponified sample. Hexane (2.5 ml) was added to all tubes which were vortex-mixed (30 s). The ether–hexane phase was evaporated under vacuum and redissolved in isopropanol–acetonitrile 70:30 (v/v) for reverse-phase HPLC analyses, heptane–isopropanol 95:5 (v/v) for normal-phase HPLC analysis, or 100% isopropanol for reverse-phase HPLC with chemiluminescence detection.

In order to quantify any losses of 7OOH due to ice-cold saponification, unilamellar liposomes consisting of egg phosphatidylcholine and unesterified cholesterol (33 mol%, 2 mg/ml) were prepared (28) and oxidized with AAPH (50 mM) at 37°C for 20 h. Samples (representing ≈3 mg of initial cholesterol in total) were extracted twice with hexane to remove the majority of phospholipids and redissolved in heptane–isopropanol 95:5 (v/v). Aliquots (in triplicate) containing 5, 10, and 15 nmol of 7-oxygenated sterols were analyzed by normal-phase HPLC after extraction with or without prior saponification, as described above.

Normal and atherosclerotic artery samples

Normal iliac arteries were obtained from liver transplant donors and human plaques were obtained from patients undergoing carotid endarterectomy. Characteristics of subjects and arterial samples are summarized in **Table 1.** This study was approved by the Central Sydney Area Health Service Ethics Review Committee and full and informed written consent was obtained from the patients. Within an hour of excision, samples were immediately placed into chelex-treated and argon-flushed PBS (20) or saline (0.15 M NaCl; pH 9.0) containing EDTA (1 mM), BHT (0.1 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml) (12). Samples were rinsed three times before storage (n = 3: −80°C for <4 months) or immediate extraction (n = 5). Fine forceps were used to strip off as much of the adventitia and media as possible under a dissecting microscope. The tissue was blotted dry, weighed, and a sample (approx. 0.7 g) was extracted using one of two methods. In the first instance, four samples (3 stored, 1 fresh) were chopped finely in 1.0 ml PBS/EDTA/BHT and shaken vigorously with diethyl ether (2.5 ml) overnight under argon at 0°C. These samples were then subjected to ice-cold saponification as detailed above. The organic phase was removed and the aqueous phase was extracted twice again with ether (2.5 ml)–hexane (2.5 ml). The three extracts were evaporated separately, weighed, dissolved in heptane–isopropanol 3:2 (v/v), and then re-combined.

The second extraction procedure (four fresh plaque samples) was based on the method reported by Chisolm et al. (12). Samples were freeze-dried overnight and weighed. The tissue was ground using a small ceramic mortar and pestle on dry-ice and transferred to a screw-cap culture tube containing EDTA (10 μl, 200 mM). The mortar and pestle were rinsed with acetone (5.0 ml) containing BHT (0.1 mM) and 19OH (5.0 μg/ml) followed by two washes with cold acetone/BHT (2 × 5.0 ml). The acetone washes were added to the powdered tissue in the screw-cap culture tubes. The tubes were vortex-mixed (5 min) and centrifuged (1600 *g*, 4°C, 15 min). This extraction was repeated twice, first with 10 ml of cold acetone/BHT and then with 5.0 ml. The combined extracts were evaporated under vacuum and stored at −80°C in heptane–isopropanol 3:2 (v/v) before analysis by HPLC (within 3 wk). A portion of the extract was subjected to ice-cold saponification as described above.

To determine the recovery of 7OOH, a carotid plaque from a single donor (male; 73 y; tissue stored at −80°C for 3 months) was cut longitudinally and 5 nmol of 7βOOH was injected into each half, before lyophilization, and acetone extraction as above. 7βOOH alone (without tissue) was lyophilized and extracted in parallel.

HPLC analyses

The HPLC system comprised a SIL-10A autosampler with cooler (Shimadzu, South Rydalmere, NSW, Australia), a pump (2150, LKB-Pharmacia, North Ryde, NSW, Australia), a SPD-10A UV-vis detector (Shimadzu), and a personal computer running Class-LC10 software (Shimadzu). For reverse-phase analyses, a Supelco C18 column was used (Sigma-Aldrich: 0.46 × 25 cm, 2 cm Pelliguard column, 5 μm particle size) at a flow-rate of 1.0 ml/min. Analysis of cholesterol and cholesteryl esters was performed by detecting 210-nm absorbance after elution with isopropanol–acetonitrile 70:30 (v/v) (24, 29). Analysis of several oxidized derivatives of choles-

TABLE 2. Retention times and response factors of sterols analyzed by normal-phase HPLC

Sterol	Relative Retention Time[a]	HPLC Response Factors[b] (area units/pmol)
Cholesterol	0.147	117 ± 5
27-Hydroxycholesterol	0.347	115 ± 3
7β-Hydroperoxycholesterol	0.563	138 ± 17
7-Ketocholesterol	0.595	180 ± 5
7-Ketocholesterol (234 nm)	0.595	497 ± 14
7α-Hydroperoxycholesterol	0.609	131 ± 12
19-Hydroxycholesterol (internal standard)	1.000	121 ± 4
7β-Hydroxycholesterol	1.127	189 ± 5
7α-Hydroxycholesterol	1.162	156 ± 8

Separation was achieved using a silica column and a mobile-phase of hexane–isopropanol–acetonitrile 95.8:3.9:0.3 (v/v/v) at a flow-rate of 1.5 ml/min. Detection was at 205 nm except where indicated.

[a]The retention times are relative to the internal standard, 19-hydroxycholesterol (typically 24.8 min). Each relative retention time had a coefficient of variation (%SD/mean) <2%.

[b]The HPLC response factors were calculated as described in the Material and Methods. Values are the mean ± SD of at least three determinations.

terol and cholesteryl esters was performed as detailed previously (5, 24) at 234-nm absorbance using a mobile phase of isopropanol–acetonitrile–water 54:44:2 (v/v/v). In some cases, 7OOHs were analyzed using a method modified from (30) using the same C18 column, but with a mobile phase of methanol–water 90:10 (v/v) run at 1.2 ml/min and chemiluminescence detection (CLD-110, Tohuku Electronic Industrial Co., Japan) using isoluminol/microperoxidase reagent pumped at 1.5 ml/min (31).

The major method of oxysterol analysis was modified from Sevanian and McLeod (32). Separation was achieved by two silica columns in series (0.46 × 10 cm) with a 3-cm guard column (Ultremex; 3 μm particle size from Phenomenex, Australian Chromatography Co., Thornleigh, N.S.W., Australia). The mobile phase consisted of hexane–ispropanol–acetonitrile 95.8:3.90:0.30 (v/v/v) run at a flow-rate of 1.5 ml/min. The mobile phase was degassed thoroughly by sonication under vacuum immediately prior to use and was continually sparged with argon during HPLC analysis.

Response factors for sterols analyzed by the normal-phase HPLC system were derived using commercial standards (**Table 2**). As no commercial standards were available for 7αOOH and 7βOOH, response factors for these compounds were determined as follows. 7αOOH and 7βOOH standards were purified from saponified samples of 20 h AAPH-oxLDL using normal-phase HPLC and fraction collection. These standards were analyzed using normal-phase HPLC with or without prior reduction using lithium aluminium hydride (2 mg in 1.0 ml dried diethyl ether stirred at room temperature for 2 h). After reduction, the corresponding alcohols were quantified using commercial standards to give the number of moles of 7αOOH and 7βOOH present in the standards and hence the response factors (Table 2).

Fatty acid analysis

Fatty acids were analyzed as their methyl ester derivatives by capillary GC with flame ionization detection as previously described (33) in five saponified plaque extracts after acetone extraction.

Protein determination

The protein content of the cell extracts and LDL preparations was measured using the bicinchoninic acid method (Sigma-Aldrich) using BSA (Fraction V, Sigma-Aldrich) as standard. Standards were prepared in 0.2 M sodium hydroxide and PBS for the cell extracts and LDL preparations, respectively. After incubation (60°C, 60 min), absorbance was measured at 562 nm.

RESULTS

HPLC analysis of 7-hydroperoxycholesterol

The normal-phase HPLC method was modified from a published method (32) in which oxysterols were detected by differential refractometry. Because the focus of the present study was on sterols oxygenated at the 7-position, all of which absorb in the lower UV region, detection was at 205 nm. In the present system a small proportion of acetonitrile was required to separate 7K from 7αOOH while the β-isomer eluted just before 7K (**Fig. 1**). Identification of the 7OOHs was based on the following criteria: *a*) occurrence in cholesterol/phospholipid liposomes oxidized by AAPH detected by normal-phase HPLC (Fig. 1B); *b*) the purified 7OOHs giving a chemiluminescent signal in the isoluminol/microperoxidase HPLC system; *c*) reduction of the purified 7OOHs to the corresponding alcohols by lithium

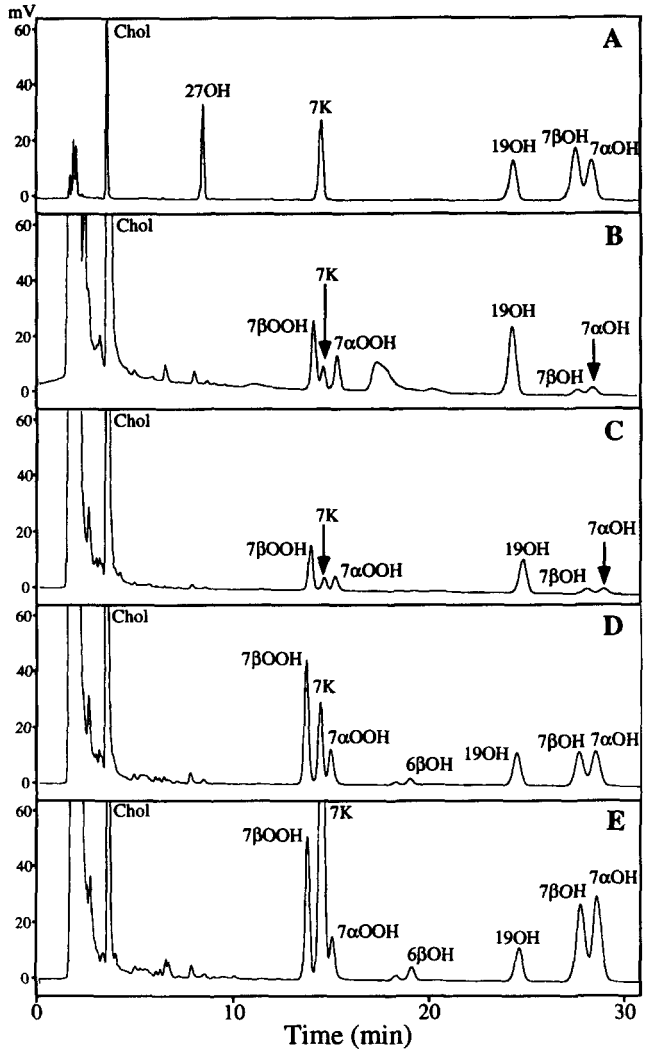

Fig. 1. Normal-phase HPLC analysis of oxysterols. Detection was at 205 nm while separation was achieved using normal-phase silica columns and a mobile phase of n-hexane–isopropanol–acetonitrile 95.8:3.9:0.3 (v/v/v) at a flow-rate of 1.5 ml/min. A: a mixture of sterol standards (2 μg each injected); B: unilamellar liposomes consisting of egg phosphatidylcholine and unesterified cholesterol were oxidized with AAPH (50 mM) for 20 h at 37°C. LDL (1.0 mg protein/ml) was oxidized with Cu^{2+} (20 μM) for: C: 4 h; D: 8 h; and E: 24 h and saponified extracts were analyzed. Chol, cholesterol; 27OH, 27-hydroxycholesterol; 7K, 7-ketocholesterol; 19OH, 19-hydroxycholesterol (internal standard); 7βOH, 7β-hydroxycholesterol; 7αOH, 7α-hydroxycholesterol; 7βOOH, 7β-hydroperoxycholesterol; 7αOOH, 7α-hydroperoxycholesterol, 6βOH, 6β-hydroxycholesterol.

aluminium hydride; *d*) reduction of the 7OOHs (in LDL oxidized for 20 h with AAPH) to the corresponding alcohols by Ebselen; and *e*) decomposition of the purified 7OOHs to the corresponding alcohols (and 7K) during analysis by GC/MS (data not shown). Retention times and response factors for the normal-phase HPLC method are given in Table 2. Limit of detection for the sterols was ≈5000 AU, equivalent to ≈50 pmol or ≈20 ng.

The retention times of other hydroperoxycholesterols on the normal-phase HPLC system were determined using cholest-6-en-3β-ol-5α-hydroperoxide (5αOOH) and cholest-4-en-3β-ol-6β-hydroperoxide (6βOOH) prepared by photo-oxidation of cholesterol (21, 22, 34). Photo-oxidized cholesterol contained in addition to unreacted cholesterol two major peaks with retention times relative to the internal standard (19OH) of 0.41 and 0.28 (data not shown). On reduction with lithium aluminium hydride, the corresponding relative retention times were 0.75 and 0.78. The latter co-elutes with synthesized 6βOH. From this it was deduced that the relative retention times for the other compounds were: 5αOOH, 0.41; 6βOOH, 0.28; and 5αOH, 0.75.

Estimating total 7-hydroperoxycholesterol

Measurement of both free and esterified 7OOH in biological samples using this normal-phase HPLC system requires hydrolysis of esters before HPLC analysis. Because alkaline saponification has been purported to cause decomposition of peroxides in general (35) and 7OOH in particular (13, 36), we tested the effect of temperature of alkaline saponification on 7OOH decomposition. LDL oxidized for 20 h with AAPH was used as this contains predominantly 7OOH with only minor amounts of 7K (<10% of total oxysterols). Saponification was complete at all temperatures (established by absence of cholesteryl esters and concomitant increase in free cholesterol measured by reverse-phase HPLC of the same samples). However, the recovery of 7OOH decreased progressively with increasing saponification temperature and was accompanied by increases in 7K and 7OH (**Fig. 2**). Saponification on ice was therefore adopted as the standard method for sample hydrolysis.

The absolute recovery of 7OOH during ice-cold saponification was determined in extracts from 20 h AAPH-oxidized cholesterol/phospholipid liposomes that were extracted using methanolic KOH as with the saponified samples or extracted using methanol as with the unsaponified samples (**Fig. 3**). There was a very slight but consistent difference in the levels of 7αOOH and 7βOOH between the saponified and unsaponified samples ($P < 0.05$, by two-way ANOVA). In total, ≈0.2 nmol of 7OOH was lost per saponification across the concentration range. For the amount of oxidized LDL extracted (100 μg), this represents ≈4% of 20 h AAPH-oxLDL and ≈1% each of 24 h cell-mediated oxLDL and 24 h Cu-oxLDL. Therefore the recovery of 7OOH after saponification on ice is ≥95% under these conditions.

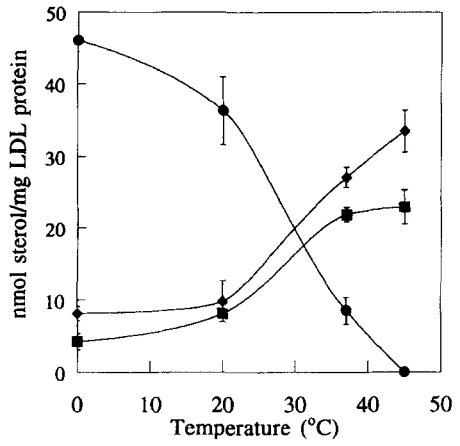

Fig. 2. Effect of temperature on the decomposition of 7-hydroperoxycholesterol (7αOOH + 7βOOH, ●) to 7-ketocholesterol (■) and 7-hydroxycholesterol (7αOH + 7βOH, ◆) during alkaline saponification. LDL (1.0 mg protein/ml) was oxidized using AAPH (50 mM) for 20 h at 37°C. Samples (100 μg LDL protein) were saponified with methanolic potassium hydroxide for 3 h in a shaking temperature-controlled incubator. Oxysterols were analyzed by normal-phase HPLC with 205-nm detection as described in Materials and Methods. Values shown are from a single experiment (which are consistent with a pilot study) and represent the mean ± SEM of triplicate saponifications.

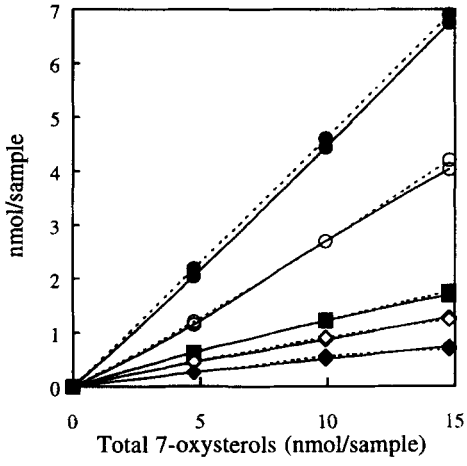

Fig. 3. Effect of ice-cold alkaline saponification on oxysterol recovery. Cholesterol (2.0 mg/ml) in phospholipid liposomes was oxidized using AAPH (50 mM) for 20 h at 37°C. Samples (in triplicate) containing 5, 10, and 15 nmol of 7-oxygenated sterols were subjected to ice-cold saponification and extraction (solid lines) or extracted unsaponified (dotted lines). 7β-Hydroperoxycholesterol, ●; 7α-hydroperoxycholesterol, ○; 7-ketocholesterol, ■; 7α-hydroxycholesterol, ◇; 7β-hydroxycholesterol, ◆. Oxysterols were analyzed by normal-phase HPLC with 205-nm detection as described in Materials and Methods. Values shown are the mean from a single experiment and are consistent with a pilot study. Standard deviations were ≤ 0.2 nmol/sample for each oxysterol.

7-Hydroperoxycholesterol formation in LDL oxidized in vitro

Kinetics of 7OOH formation were measured in LDL exposed to three different oxidizing systems at 37°C for durations up to 48 h: *1*) Cu^{2+} (20 μM) at an LDL protein concentration of 1.0 mg/ml; *2*) mouse peritoneal macrophages in Ham's F-10; and *3*) Ham's F-10 medium alone.

With all three oxidizing systems (**Fig. 4**), 7OOH was the first detectable and major oxysterol formed at earlier times of oxidation (most apparent in Fig. 4A), with 7K and 7OH dominating at later times (≥24 h), although some 7OOH persisted even at 48 h. In LDL oxidized with Cu^{2+} or cells, the maximum level of 7OOH occurred later than the fatty acid hydroperoxide arising from cholesteryl linoleate oxidation (Ch18:2OOH) but the quantities accumulated of the steryl and fatty acid hydroperoxides were comparable.

As expected, cell-mediated oxidation of LDL was faster than the cell-free control (Ham's F-10 alone) as evidenced by the faster consumption of cholesteryl arachidonate and linoleate (not shown) and the kinetic of Ch:2OOH generation. Indeed, oxysterol content in the cell-free condition at 48 h (Fig. 4D) was similar to that observed in LDL oxidized in the presence of cells between 8 and 24 h (Fig. 4C). As might be expected in a system containing abundant peroxides, there was some loss of cell viability during prolonged exposure to LDL as assessed by trypan blue exclusion (viabilities as assessed by two observers were: 4 h, 94 ± 3%; 8 h, 85 ± 5%; 24 h, 66 ± 11%; 48 h, 65 ± 6% (n = 3)).

In all oxidizing conditions examined, 7βOOH was the major isomer formed (shown for Cu^{2+}-mediated oxidation in **Fig. 5A**) whereas little or no isomeric preference was seen in the case of 7OH (Fig. 5B). The ratio of 7βOOH to 7αOOH for samples taken over 48 h of Cu^{2+}-oxidation was 2.37 (±SD: 0.42; n = 8).

Analysis of samples with or without prior alkaline saponification allowed measurement of free and total oxysterols, respectively. The proportion of total 7OOH that was unesterified reflected the proportion of unesterified cholesterol in the starting material (27.4 ± 3.9%) and was similar to that of other oxysterols. For example, in 24 h Cu-oxLDL, free 7OOH and 7K comprised 20.9 ± 2.0% and 21.3 ± 2.0%, respectively; in cell-free Ham's F-10 medium they were 19.6 ± 1.5% and 16.2 ± 3.7%, respectively. By contrast, in LDL oxidized by macrophages, unesterified 7K comprised 20.6 ± 0.5% of the total 7K but free 7OOH comprised only 10.7 ± 2.3%. One possible explanation for this difference is that free 7OOH generated in the presence of macrophages is selectively metabolized by and/or transferred to the cells.

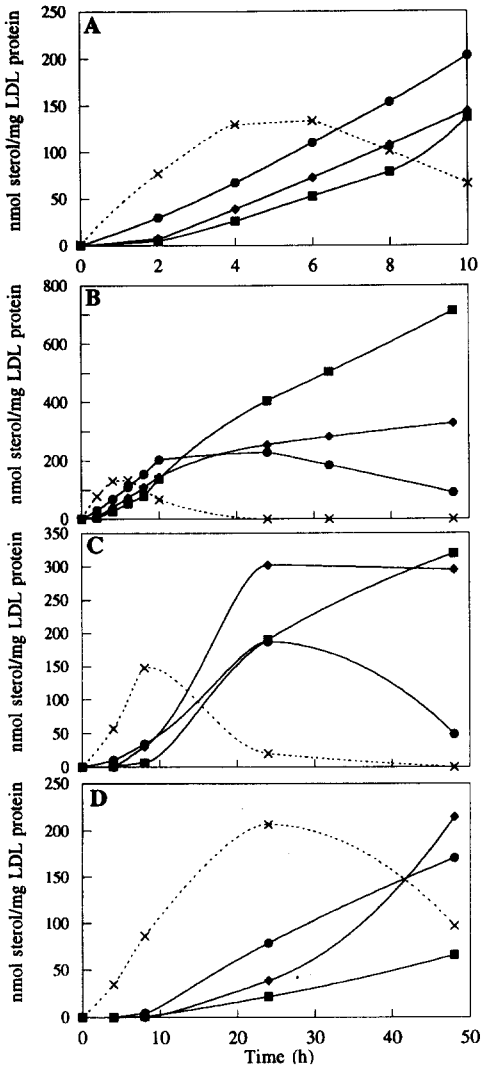

Fig. 4. Kinetics of appearance of oxysterols and cholesteryl hydroperoxy-octadecadienoate in LDL oxidized in vitro under three conditions: LDL (1.0 mg/ml) with Cu^{2+} (20 μM) at 37°C for up to A: 10 h; B: 48 h; C: LDL (25 μg/ml) in the presence of mouse peritoneal macrophages in Ham's F-10 medium for up to 48 h; and D: LDL (25 μg/ml) in Ham's F-10 medium without cells for up to 48 h. Cholesteryl hydroperoxy-octadecadienoate, dotted lines; 7-hydroperoxycholesterol (α- + β-isomers), ●; 7-ketocholesterol, ■; 7-hydroxycholesterol (α- + β-isomers), ◆. Oxysterols were analyzed by normal-phase HPLC with 205-nm detection as described in Materials and Methods. Values shown are from single experiments (consistent with a pilot study for Cu^{2+}-mediated oxidation) and are the mean of triplicate oxidations. Standard deviations were <18% of the mean or <14 nmol/mg for values <100 nmol/mg.

Interactions of 7-hydroperoxycholesterol with mouse peritoneal macrophages

To investigate the interaction of macrophages with 7OOH, oxysterol accumulation was measured in both cells and medium after macrophage-mediated oxidation of LDL. LDL was added at 25, 50, or 100 μg/ml. As LDL concentration increased, the oxidation products per particle decreased (±cells) as shown by the reduction in the proportion of total sterols that were oxygenated at the 7-position (**Fig. 6A**) and consumption of polyunsaturated cholesteryl esters (data not shown). This is presumably because the ratio of oxidants to LDL particle is lower with increasing LDL concentration. The composition of oxysterols also indicated a lesser degree of oxidation at higher LDL concentrations by higher proportions of 7OOH relative to 7K (Fig. 6B). In the cells some oxysterols were detected but only as 7K and 7OH. No 7OOH was detectable in the cells; however the 7OH content of cells correlated with 7OOH in the medium (Fig. 6B), consistent with cellular uptake of 7OOH and reduction to 7OH.

To further study cellular accumulation of 7-oxysterols in the absence of ongoing cell–mediated oxidation, LDL was first Cu^{2+}-oxidized for 10 h, then transferred to DMEM. No further LDL oxidation occurs in this 'non-permissive' medium (37). The oxysterol content of cells exposed to this 10 h Cu-oxLDL was analyzed after incubation at 37°C for 24 h and a further overnight incubation to permit time for lysosomal degradation. While 7OOH was stable in cell-free DMEM under identical conditions (not shown), little 7OOH could be detected in cells, although 7K and 7OH did accumulate (**Fig. 7**). Again, these data are compatible with the notion that cells can metabolize 7OOH.

7-Hydroperoxycholesterol in human atherosclerotic plaque

The 7OOH content of eight human atherosclerotic plaque samples excised from human carotid arteries was measured using two different extraction methods. One was based on the extraction procedure developed in the present study to measure 7OOH in LDL oxidized in vitro while the other was based on a previous study that reported 7OOH in human carotid atherosclerotic plaque (12). The first method involved extraction of four plaque samples with ether–hexane after alkaline saponification. This extraction was exhaustive based on the mass of lipid recovered after each of the three extractions (mean ± SD (n = 4): 84% ± 2.6%; 15 ± 2.6%; and 1 ± 0.3% of total lipid recovered after each successive extraction). The second method involved exhaustive extraction of four plaque samples with acetone after lyophilization. All extracts were then analyzed directly by normal-phase HPLC (unesterified sterols), or after ice-cold saponification (total sterols).

Only trace amounts of 7OOH were detected by normal-phase HPLC with 205 nm detection and then only in four freshly processed samples where ≥0.28 mg of total cholesterol was injected onto the column. Of these four plaques, one had been extracted by the ether–hexane method (after saponification) while three had been

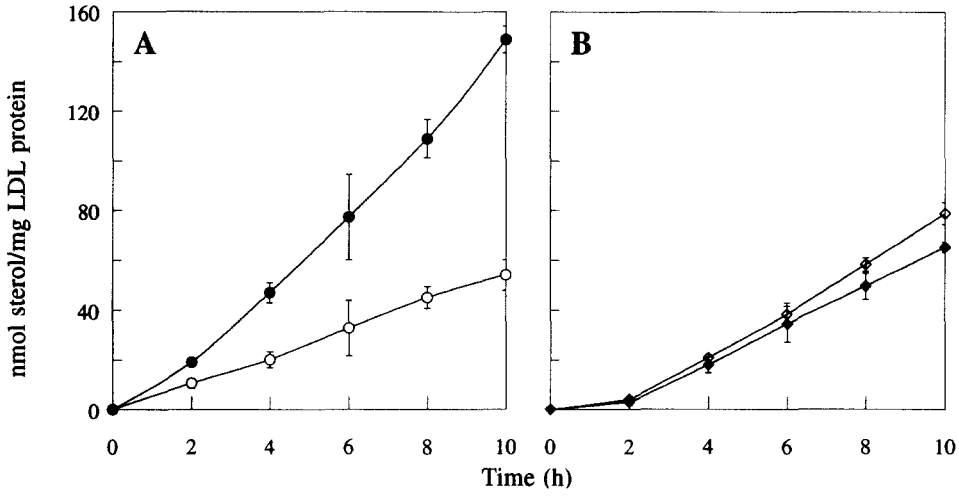

Fig. 5. The β-isomer (solid symbol) dominates over the α-isomer (empty symbol) for 7-hydroperoxycholesterol (A., circles) but not for 7-hydroxcholesterol (B., diamonds) in Cu^{2+}-oxidized LDL. LDL (1.0 mg/ml) was oxidized with Cu^{2+} (20 μM) at 37°C. Oxysterols were analyzed by normal-phase HPLC with 205-nm detection as described in Materials and Methods. Concentrations shown are from a single experiment and are the mean ± SD of triplicate oxidations of the same LDL sample.

lyophilized and extracted with acetone (both with and without saponification). Normal-phase HPLC analysis showed a small peak corresponding in retention time to 7βOOH (compare **Fig. 8A and 8C**) preceded by an extremely large 7K peak that may have obscured any 7αOOH. A reverse-phase (as opposed to normal-phase) system utilizing chemiluminescence detection was 10-fold more sensitive than 205 nm detection and revealed a complex peak eluting in the region of 7βOOH (compare Fig. 8B and 8D), with the bulk eluting more closely with the α-isomer (standard not shown). This complex peak was assumed to represent total 7OOH as there was a positive association (r = 0.786; P = 0.002; n = 12) between its area and that for 7βOOH detected by the normal-phase system. The amount of 7βOOH in all four samples was estimated to be 1.05 ± 0.46 nmol/g wet weight of tissue (mean ± SD) by normal phase HPLC and 2.35 ± 0.99 nmol/g by the chemiluminescence system. Even then, this higher estimate represents only 0.0051 ± 0.0016% of total cholesterol and is less than 5% of total 7K (2.63 ± 0.92%). Comparing saponified and unsaponified values from the chemiluminescent system (available for three samples), the majority of this peroxide appeared to be esterified (78 ± 8%). This value changed little when the marginal loss of 7OOH during saponification (0.2 nmol/sample) was taken into account (82 ± 6%).

The recovery of 7βOOH alone (without tissue) when lyophilized and taken through the acetone extraction as described above was 90% (10% was converted to 7K while a trace amount was reduced to 7βOH). However, when 7βOOH was injected into plaque tissue its recovery was ≈50% (after saponification) and quite different between the two longitudinal sections of the same plaque sample analyzed (62% vs. 42%). Thus, it seems that it is the interaction with the plaque tissue rather than the processing itself that caused the greatest loss of added 7OOH. But even assuming that only ~40% of endogenously produced 7OOH is recovered from the tissue, the levels of 7OOH must be regarded as trace (<0.02% of total cholesterol; the sample that displayed the highest level of 7OOH was 0.0064%).

The possibility that freeze-drying might cause artifactual production of oxysterols was investigated. Samples of a cholesterol/egg phosphatidylcholine emulsion were extracted and analyzed by normal-phase HPLC with and without prior freeze-drying overnight. No production of oxysterols was detected after freeze-drying. Sufficient cholesterol was analyzed so that a level of 7βOOH > 0.002% of total cholesterol would have been detectable.

Lipid analysis of human atherosclerotic plaque

The lipid content of atherosclerotic arteries measured after ether–hexane extraction was 30-fold greater than in the normal samples (6.3 ± 1.7 vs. 0.20 ± 0.05 g/ 100 g). This difference is reflected in the total cholesterol contents of the normal and diseased arteries (**Table 3**). For the second batch of four plaque samples lyophilized and extracted with acetone, dry weight represented 29.1% (±4.7%) of the wet weight. 27OH was the major oxysterol in all nine plaque samples measured (includes that plaque sample used for the 7βOOH recovery experiment). 7K was the major 7-

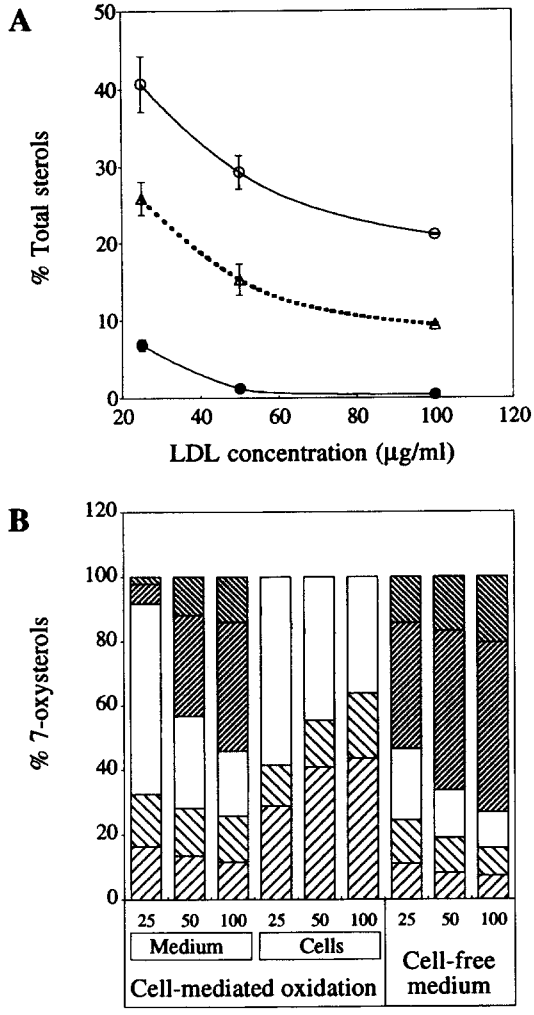

Fig. 6. Oxysterol content (A.) and composition (B.) in media and cells after cell-mediated oxidation of LDL. LDL at three concentrations (25, 50, and 100 μg/ml) were oxidized in the presence or absence of mouse peritoneal macrophages in Ham's F-10 medium for 24 h. Values in panel A are the percent proportion of all sterols oxygenated at the 7-position (mean ± SD of triplicate oxidations of the same LDL sample from a single experiment). Solid lines show the values from the cell-mediated oxidation (medium, ○; cells, ●). A dotted line (△) shows the values from the cell-free medium. Panel B shows the constituent oxysterols as percent composition. From top to bottom: 7α-hydroperoxycholesterol, densely hatched section slanting up towards the left; 7β-hydroperoxy-cholesterol, densely hatched section slanting up towards the right; 7-ketocholesterol, empty; 7α-hydroxycholesterol, sparsely hatched section slanting up towards the left; 7β-hydroxycholesterol, sparsely hatched section slanting up towards the right. Average 100% values expressed as nmoles of 7-oxysterols per culture (±SD) are for the three increasing LDL concentrations (25, 50, and 100 μg/ml, respectively): 22.0 ± 1.1, 42.2 ± 2.0, 64.1 ± 2.3 for medium in the cell-mediated oxidation condition; 3.7 ± 0.2, 1.8 ± 0.2, 1.3 ± 0.4 for cells in the cell-mediated oxidation condition; and 13.8 ± 0.6, 23.6 ± 3.0, 29.3 ± 0.2 for medium in the cell-free condition. Oxysterols were analyzed by normal-phase HPLC with 205-nm detection as described in Materials and Methods.

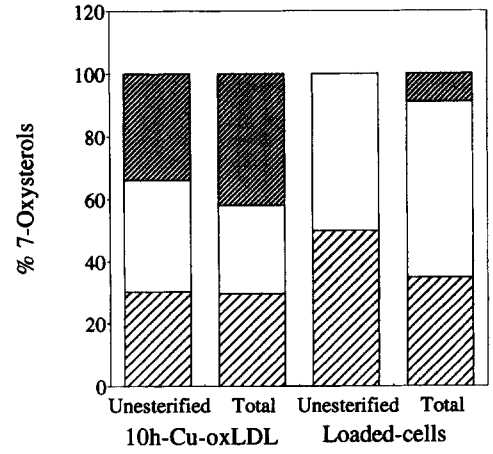

Fig. 7. Percent composition of oxysterols (unesterified and total) in 10 h Cu^{2+}-oxidized LDL and mouse peritoneal macrophages that have been loaded with 10 h Cu^{2+}-oxidized LDL. LDL (1.0 mg/ml) was oxidized with Cu^{2+} (20 μM) at 37°C for 10 h. 10 h-Cu-oxLDL (25 μg/ml) was incubated with mouse peritoneal macrophages followed by an overnight equilibration period. From top to bottom: 7-hydroperoxycholesterol (α- + β-isomers), densely hatched section slanting up towards the right; 7-ketocholesterol, empty; 7-hydroxycholesterol (α- + β-isomers), sparsely hatched section slanting up towards the right. Oxysterols were analyzed by normal-phase HPLC with 205-nm detection as described in Materials and Methods. The contents of unesterified and total oxysterols in the 10 h Cu-oxLDL were 85 ± 2 and 485 ± 18 nmol/mg LDL protein, respectively, and 10.2 ± 0.4 and 22.6 ± 1.1 nmol/mg cell protein, respectively, in macrophages loaded with 10 h Cu-oxLDL (mean ± SD; n = 3).

oxygenated sterol, followed by 7βOH and 7αOH (Table 3). 7βOH was greater than the α-isomer in eight out of nine cases. This difference was more pronounced in the unesterified 7OHs; the 7βOH/7αOH ratio was consistently higher for the unsaponified samples relative to the saponified samples (2.43 ± 0.75 vs. 1.40 ± 0.13; n = 5). As shown for 7OOH above, the sterols were predominantly esterified (Table 3) and of these the proportion of oxysterols present as esters (≥ 83%) was larger than than for cholesterol (58% esterified).

Analysis of cholesteryl esters by reverse-phase HPLC (24) with 205 nm detection gave a relatively consistent profile for all five plaque samples analyzed without prior saponification. Cholesteryl linoleate was the major ester (49.8 ± 1.1%), followed by cholesteryl oleate (27.0 ± 3.1%), cholesteryl arachidonate (10.3 ± 2.1%), and cholesteryl palmitate (9.6 ± 3.6%). The total fatty acid composition measured by GC with flame ionization detection yielded a comparable profile. Twenty three different fatty acids were detected. Of these, linoleate and oleate dominated (18:2n–6, 31.8 ± 5.6%; 18:1n–9, 27.7 ± 2.2%), followed by palmitate (16:0, 11.9 ± 1.5%) and arachidonate (20:4n–6, 7.4 ± 1.1%). Other fatty acids that comprised more than 2% of the total fatty acids were palmoleate (16:1n–7, 4.4 ± 1.4%), *cis-*

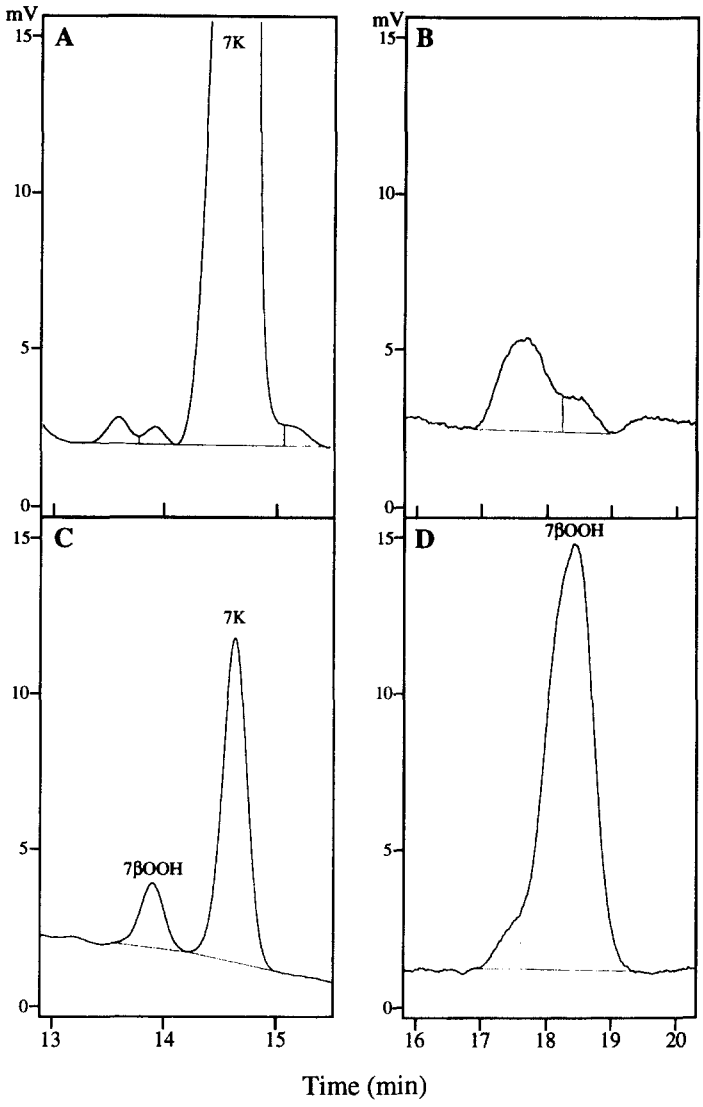

Fig. 8. Trace amounts of 7-hydroperoxycholesterol are detected in human carotid atherosclerotic plaque. Two plaque samples were extracted with acetone after lyophilization and analyzed by normal-phase HPLC with 205-nm detection (A, C) or by reverse-phase (as opposed to normal-phase) HPLC with post-column chemiluminescence detection (B, D). One of the plaque samples (C, D) had been injected with 7β-hydroperoxycholesterol prior to lyophilization and extraction. 7βOOH, 7β-hydroperoxycholesterol; 7K, 7-ketocholesterol.

TABLE 3. Cholesterol and oxysterol content of extracts prepared from normal human iliac arteries and human carotid atherosclerotic plaque

Sample	n	Extraction	Cholesterol	27-Hydroxy-cholesterol	7-Keto-cholesterol	7β-Hydroxy-cholesterol	7α-Hydroxy-cholesterol	7-Keto-cholesterol/ Cholesterol	Oxysterols/ Cholesterol
			μmol/g		*nmol/g*			%	%
Normal	4	Ether–hexane; saponified	1.3 ± 1.3	ND	ND	ND	ND	ND	ND
Plaques A	4	Ether–hexane; saponified	33.2 ± 8.6	292 ± 274	117 ± 42	37 ± 31	27 ± 15	0.35 ± 0.08	1.32 ± 0.70
Plaques B	4	Acetone; saponified	39.9 ± 15.5	331 ± 162	86 ± 59	38 ± 28	27 ± 22	0.22 ± 0.13	1.35 ± 0.29
Plaques B	4	Acetone; unsaponified	16.7 ± 9.4	53 ± 57	17 ± 21	2.6 ± 2.3	1.5 ± 1.6	0.09 ± 0.07	0.38 ± 0.19
% Esters			(58.9 ± 11.0)	(85.9 ± 8.5)	(83.6 ± 8.8)	(93.3 ± 1.8)	(95.3 ± 1.9)		

Normal arteries or plaque samples were either extracted with diethyl ether–hexane (A) or acetone after lyophilization (B). All samples were analyzed after saponification by normal-phase HPLC as described in Materials and Methods to give total sterol per gram of tissue (wet weight). The second batch of plaques (B) was also analyzed unsaponified to give free sterol. The difference between total sterol (after saponification) and free sterol (without saponification) allowed the percentage of esters to be calculated. Values are mean ± SD; ND, not detected. The molar ratio of 7-ketocholesterol to cholesterol and sum of oxysterols to cholesterol are expressed as a percentage.

TABLE 4. Content of unoxidized and oxidized forms of cholesteryl linoleate in extracts prepared from human carotid atherosclerotic plaque

	Ch18:2	Ch18:2OH	Ch18:2=O	7K18:2
Mean	28.5	0.74	0.47	0.06
SD	5.5	0.32	0.25	0.03
Range	21.3–36.2	0.27–1.15	0.15–0.82	0.02–0.09

Five plaque samples were extracted with acetone after lyophilization and analyzed by three reverse-phase HPLC systems. Cholesteryl linoleate (Ch18:2) was detected at 205 nm with a mobile phase of isopropanol–acetonitrile 70:30 (v/v). Cholesteryl hydroxy-octadecadienoate (Ch18:2OH) was detected at 234-nm and cholesteryl ketooctadecadienoate (Ch18:2=O) was detected at 279-nm using a mobile phase of isopropanol–acetonitrile–water 54:44:2 (v/v/v). Ch18:2=O was estimated using published extinction coefficients for keto-octadecadienoate (at 279-nm) relative to Ch18:2OH (at 234-nm). 7-Ketocholesteryl (7K18:2) was detected at 234-nm with a mobile phase of isopropanol–acetonitrile–water 54:42:4 (v/v/v). The molar ratios of the components to total cholesterol are expressed as a percentage (i.e., 100 × component/total cholesterol).

vaccenate (18:1n–7, 3.1 ± 0.6%), stearate (18:0, 2.7 ± 0.4%), and dihomo-γ-linolenate (20:3n–6, 2.1 ± 0.9%).

As linoleate was the major cholesteryl ester, five of the plaque samples were analyzed for oxidized forms of this ester. Reverse-phase HPLC with 234 nm detection (5) revealed a component that corresponded in retention time to 7K linoleate using three different solvent systems (isopropanol–acetonitrile–water 40:60:0, 54:44:2, 54:42:4 (v/v/v)) and represented 24.7 ± 4.9% of the 7K esters (**Table 4**). Two other major 7K esters anticipated, oleate and palmitate, may have been masked by a prominent peak eluting in the region containing cholesteryl linoleate oxidation products: cholesteryl hydroxy-octadecadienoate (Ch18:2OH) at 234 nm (24) and cholesteryl ketooctadecadienoate (Ch18:2=O) at 279 nm (20). Ch18:2OH may be slightly overestimated using this approach since Ch18:2=O also contributes some absorbance at 234 nm. However, Suarna et al. (20) comparing this reverse-phase approach with a normal-phase system which separates Ch18:2OH and Ch18:2=O found that Ch18:2OH was only overestimated by 7% by the reverse-phase method (range: 0–16%; n = 8). This slight overestimation by the reverse-phase approach may at least partially explain why Ch18:2OH appeared to predominate over Ch18:2=O in molar terms, comprising 62.1% (±4.8%). Together Ch18:2OH and Ch18:2=O represented 1.2 ± 0.6% of total cholesterol (Table 4) or 4.5 ± 2.0% of cholesteryl linoleate. The content of these linoleate oxidation products in plaque was similar to that of total oxysterols measured and six-fold greater than the level of total 7K (Tables 3 and 4). There was a positive association between the proportion of cholesteryl linoleate in the form of the hydroxide and keto and %7K/cholesterol (r = 0.866, P = 0.026, n = 6).

The relative proportions of individual oxysterols among plaque samples were variable. Even within a single sample, variation was extreme; two halves of a single plaque sample contained similar total cholesterol (52 vs. 47 μmol/g tissue) and %oxysterols/cholesterol (1.9 vs. 1.5%), yet the profile of the oxysterols was very different (27OH, 30 vs. 69%; 7K, 40 vs. 17%; 7βOH, 16 vs. 8%; 7αOH, 14 vs. 6%).

DISCUSSION

In the present study, a normal-phase HPLC method with UV (205 nm) detection was developed to avoid thermal decomposition of 7OOH which occurs during GC analysis and resolve all products of cholesterol oxygenated at the 7-position: 7K, 7αOOH, 7βOOH, 7αOH, and 7βOH. This method does not detect the cholesterol epoxides which, lacking a double bond, do not absorb at 205 nm (38) but these epoxides do not appear to be major oxysterols in oxidized LDL (5, 9) and in human atherosclerotic plaque (18). Analysis by normal-phase HPLC with and without prior ice-cold alkaline saponification enabled us to measure formation of free and total 7OOH in LDL oxidized under various conditions as well as in human atherosclerotic plaque samples. Contrary to expectations (13, 36), 7OOH survived alkaline saponification. The amount of 7OOH that survived was highly dependent on the temperature at which the saponification was carried out. When conducted at 0°C both 7OOH and 7K were preserved (≥ 95% recovery).

7-Hydroperoxycholesterol is a major oxysterol in LDL oxidized in vitro

Several studies have detected 7OOH in Cu-oxLDL (9, 12, 13, 22, 39) mostly in the free form (in unsaponified samples). However, very little quantitation has been attempted. Chisolm and colleagues (12, 39) incorporated [^{14}C]cholesterol into LDL and measured formation of [^{14}C]oxysterols during Cu^{2+}-mediated oxidation using thin-layer chromatography. While their oxidation conditions were different from those used in the present study (3 μM Cu^{2+}, 3.3 mg LDL protein/ml for 48 h at 25°C (G.M. Chisolm, personal communication) vs. 20 μM Cu^{2+}, 1.0 mg LDL protein/ml at 37°C in the present study) the proportions of LDL free cholesterol converted to 7OOH (~5%) were similar. In LDL oxidized with Cu^{2+} for 6 to 48 h, free 7OOH ranged from 2.6 to 7.2% of initial free cholesterol whereas the proportion of total cholesterol (free and esterified) converted to total 7OOH was slightly higher (4.2–10.7%). Like Colles, Irwin, and Chisolm (39) we found that 7OOH levels were comparable with the sum of the other 7-

oxygenated sterols. At early (≤ 10 h) stages of Cu^{2+}-mediated oxidation, 7OOH comprised more than 40% of the total 7-oxygenated sterols and even at 24 h comprised 25% of all oxysterols measured. 7βOOH predominated over the α-isomer in agreement with others (9, 39). 7αOOH is known to undergo rearrangement to 7βOOH via a fragmentation–readdition process (40), which may explain why the latter form predominates in the case of the hydroperoxide but not in the case of the alcohol. Furthermore, it appears from our data presented here and those of others (9, 34, 39) that reduction of 7OOH (both chemical and enzymatic) leads to stereospecific conversion to the respective 7OH. Thus, we would speculate that rearrangement of 7αOOH to 7βOOH occurs after decomposition/reduction to the corresponding alcohol.

As 7OOH is a major oxysterol in Cu-oxLDL and as previous quantification of oxysterols in Cu-oxLDL has mostly been performed by GC (5–11), these studies will have overestimated 7K and 7OH. For example, we previously reported using GC (5) that 24 h Cu-oxLDL comprised ~30% of total sterols as 7K whereas we now detect ~22% using normal-phase HPLC. The difference can be largely accounted for by the presence of 7OOH (up to 12%) with a lower proportion of 7OH (mostly the β-isomer) accounting for the remainder.

7OOH is a significant oxysterol not only in LDL oxidized with Cu^{2+} but in LDL oxidized using a variety of conditions: cell-mediated oxidation (using resident mouse peritoneal macrophages), a cell-free condition (in Ham's F-10 media), and a metal-independent system using the aqueous peroxyl radical generator, AAPH (consistent with a previous report (41)). 7OOH has also been reported to occur in LDL oxidized with soybean lipoxygenase (9).

Metabolism of 7-hydroperoxycholesterol by cells

Our results support the idea that 7OOH can be efficiently metabolized by macrophages as has been previously shown with protein hydroperoxides (42). That cells have mechanisms for reducing cholesterol hydroperoxides is given credence by our observation that 7OOH can be acted on by the synthetic selenoperoxidase, ebselen, in support of a previous report (43). Moreover, Korytowski, Geiger, and Girotti (34) have shown that purified phospholipid hydroperoxide glutathione peroxidase (EC 1.11.1.12) can reduce 7OOH as well as other photochemically generated cholesterol hydroperoxide isomers to the corresponding alcohols.

Free and esterified 7OOH associated with oxLDL may be metabolized differently by cells. We have previously shown that free 7K incorporated into LDL can be readily transferred to mouse peritoneal macrophages without the need for modification of the lipoprotein and subsequent uptake by the scavenger receptor (44). We assume that at least some free 7OOH is similarly transferred to cells from the surface of the oxidized lipoprotein whereas esterified 7OOH, located more in the core of the lipoprotein particle, will require receptor-mediated endocytosis to be taken up (probably via a scavenger receptor). If reduction occurs on the plasma membrane, esterified 7OOH will be more resistant to reduction to 7OH than the free form but may be converted to other oxysterols including 7K during intracellular processing. This scenario, although speculative, is consistent with the data presented herein.

7-Hydroperoxycholesterol in human atherosclerotic plaque

A previous report that 7OOH is a component of human atherosclerotic plaque provided no quantitative data to indicate whether it is a major or minor component (12). On the other hand, there are several reports of 7K in plaque using GC analysis (15, 16, 18). Therefore it was important to establish whether such estimates of plaque 7K content are correct or reflect 7OOH content. The present study indicates that 7OOH occurs only in trace amounts (<0.02 % of total cholesterol) in human carotid atherosclerotic plaque suggesting that GC is a valid method to measure 7K in plaque samples. The low levels of 7OOH may reflect its lability in biological systems based on: *1*) efficient metabolism by cells; *2*) absence of any detectable 7OOH in stored plaque samples; and *3*) relative lability of 7OOH injected into plaque samples versus tissue-free controls. Moreover, the recovery of 7βOOH injected into two longitudinal sections of the same plaque sample was quite different despite similar total cholesterol content and oxysterol: cholesterol ratios. Interestingly, the relative amounts of individual oxysterols were markedly different between the two segments with the half that had the poorest recovery of 7βOOH also containing the greatest proportion of 7K and noted at the time of preparation to contain more obvious 'gruel'. It is possible that the same component(s) in the plaque responsible for decomposition of injected 7βOOH can also cause decomposition of endogenously produced 7OOH but more work is necessary to investigate this further.

Other oxysterols in human atherosclerotic plaque

Several studies have detected oxysterols in human atherosclerotic plaque (15–20) although not all have presented quantitative data. This is the first study, as far as we are aware, which separately measures both free and esterified forms of the major oxysterols in advanced human atherosclerotic plaques. The material was processed within 30 min to 1 h of excision and extreme

care was taken to minimize the likelihood of artifactual production of oxysterols during sample processing through presence of a metal chelator and antioxidant at all stages of the procedure. The success of these precautions was demonstrated by the absence of detectable oxysterols in normal arteries. Sufficient sample from the normal arterial extracts were extracted so that a level of 7K > 0.03% of total cholesterol would have been detectable.

Björkhem et al. (18) measured nine different oxysterols in human femoral plaques and found that the four measured herein (27OH, 7K, 7αOH, 7βOH) comprised ≈80% of these oxysterols. We found that the enzymically produced 27OH (previously referred to as 26OH (15–17, 19)) was the major oxysterol. This may reflect the activity of the mitochondrial 27-hydroxylase pathway as a mechanism for macrophages to eliminate cholesterol (18). Smith et al. (45) reported similar amounts (as the 27OH diester) in grossly atherosclerotic aorta.

7K was the next most abundant oxysterol in the present study, followed by 7βOH and 7αOH. The 7K:total cholesterol ratio (range: 0.10–0.44%) was higher than that (~0.03%) found by Björkhem et al. (18) in five femoral plaque samples. But in close agreement with our own data for unsaponified (as opposed to saponified) samples, Suarna et al. (20) found an average unesterified 7K: free cholesterol ratio of ~0.06% in femoral and carotid plaque samples. Carpenter et al. (17, 19) analyzed 7βOH in plaque samples (mostly from thoracic and abdominal aorta but also some from carotid arteries) after reduction with sodium borohydride and saponification. Sodium borohydride reduction of 7K results in the production of 7βOH and 7αOH in a ratio of 3.6:1 (S-l. Leong and A. J. Brown, unpublished data). The 7βOH:total cholesterol ratio reported by Carpenter et al. (17, 19) was ~0.14% which is comparable to our results for 7K.

Possible mechanism(s) of oxysterol formation in human atherosclerotic plaque

Oxysterols found at sites of atherosclerosis may be derived from the diet (46), enzymically (18, 47) and/or via non-enzymic free-radical oxidation (48–51). An early target for free radical attack is probably the bisallylic group of polyunsaturated fatty acids consistent with previous in vitro results (5) and present results that fatty acid oxidation products of cholesteryl linoleate in atherosclerotic plaque are several fold greater than sterols oxygenated at the 7-position. Fatty acid radicals may then abstract a hydrogen atom from the susceptible 7-allylic position of cholesterol. Reaction with molecular oxygen and subsequent hydrogen atom abstraction then leads to the formation of 7α- and 7βOOH. The presence of trace amounts of 7OOH in plaque reported herein support the contention that this peroxidation occurs in vivo rather than being derived from dietary sources as Sevanian et al. (52) could not detect 7OOH in the circulation despite a variety of sensitive methods being used including HPLC with mass spectrometric detection.

7OOH can decompose to 7K, 7αOH, and 7βOH by a variety of different mechanisms such as radical combination and/or disproportionation (53). The decomposition of 7OOH to 7K and 7OH is evident in the kinetics observed in our in vitro systems and is a likely route by which these 7-oxygenated sterols are formed in atherosclerotic plaque. Indeed, the ratio of 7K: 7αOH: 7βOH in the eight plaque samples analyzed is 3.20 (±SD: 1.52): 0.77 (±0.17): 1.0 vs. 3.64 (±0.36): 1.29 (±0.02): 1.0 in 24 h Cu-oxLDL and 1.90 (±0.25): 0.74 (±0.10): 1.0 in 48 h cell-mediated oxLDL. The predominance of the β-isomer of 7OH observed in plaques and cell-mediated LDL oxidation, but not in Cu-oxLDL, suggests that some 7OOH (presuming 7βOOH predominates in plaque) may be reduced stereospecifically by cells in the intima.

Esterified rather than free cholesterol is the more likely target of free radical attack because of the proximity of polyunsaturated fatty acids as discussed above and our finding that 7OOH is mostly in the esterified form as are the 7-oxygenated products. However, because linoleate is more readily oxidized than cholesterol in vitro (5), the presence of 7K linoleate indicates that some remodelling (hydrolysis/re-esterification) may occur. It is known that 7K is actively esterified in both mouse peritoneal macrophages (44) and human monocyte-derived macrophages (E. Oates, L. Kritharides, and W. Jessup, unpublished observations).

Possible atherogenicity of oxysterols

An often reported atherogenic effect of oxysterols is their cytotoxicity (34, 36, 39, 54, 55). Even though 7OOH is present in trace amounts in human atherosclerotic plaque compared with the other oxysterols, it is by no means insignificant. 7βOOH has been calculated to be 15- and 11-fold more toxic to cultured fibroblasts than 7βOH and 7K, respectively (39). Furthermore, as discussed above, 7OOH is a probable intermediate in the formation of 7K and 7OH in plaque. It is unclear whether 7OOH, at low concentrations but at high flux, is toxic in vivo. Further work is also needed to clarify the importance of oxysterols in atherogenesis beyond the issue of cytotoxicity. For example, we (26, 44) and others (56) have shown that oxysterols may suppress sterol export from cells. Perturbation of sterol homeostasis in cells such as the macrophage may promote foam cell formation.

In conclusion, we found that 7OOH is a major oxy-

sterol in LDL oxidized in vitro but a trace component among other oxysterols and their esters in human atherosclerotic plaque, perhaps reflecting the ability of cells in the intima to metabolize 7OOH further.

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