Free and esterified oxysterol: formation during copper-oxidation of low density lipoprotein and uptake by macrophages

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Abstract We have defined the lipid composition of copper-oxidized LDL (Cu-oxLDL) and a macrophage-foam cell model generated by the uptake of this modified lipoprotein. An HPLC method previously developed by our group for the measurement of lipid oxidation products of LDL was extended to permit the analysis of an array of 7-ketocholesteryl esters. Gas chromatography was used for the quantitation of oxysterols (free and esterified) in Cu-oxLDL and their subsequent uptake by macrophages. LDL (1.0 mg protein/ml) was oxidized using Cu(II) (20 μM) for up to 48 h at 37°C. Resident mouse peritoneal macrophages were incubated with 24 h Cu-oxLDL (50 μg/ml) for 24 h. In 24 h Cu-oxLDL, cholesterol comprised approximately 50% of total sterols, 7-ketocholesterol comprised approximately 30%, with five other oxysterols comprising the remainder (7α- and 7β-hydroxycholesterol, cholesterol α- and β-epoxides, and 6β-hydroxycholesterol). Macrophages that were incubated with 24 h Cu-oxLDL displayed a profile of oxysterols remarkably similar to that of 24 h Cu-oxLDL itself. The majority of cholesteryl esters and 7-ketocholesteryl esters in Cu-oxLDL and in Cu-oxLDL-loaded macrophages contained fatty acyl chains which are presumed oxidized. This work represents a comprehensive survey of free and esterified oxysterols in Cu-oxLDL and Cu-oxLDL-loaded macrophages and provides a basis for exploring how oxysterols are metabolized by macrophages and authentic human foam cells, and how, in turn, these oxysterols influence cellular metabolism.—**Brown, A. J., R. T. Dean, and W. Jessup.** Free and esterified oxysterol: formation during copper-oxidation of low density lipoprotein and uptake by macrophages. *J. Lipid Res.* 1996. **37:** 320–335.

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The uptake of modified low density lipoprotein (LDL) by macrophages to form foam cells has become a central focus of research into the development of atherosclerosis. One way that LDL can be modified which may be of physiological relevance is through oxidation (1). When LDL undergoes oxidation in vitro, a number of changes in lipid composition occur, including a substantial loss of free and esterified cholesterol and the concomitant generation of oxidation products of cholesterol (oxysterols) (2–9).

Oxysterols are present in human atherosclerotic plaques (10, 11) and in human foam cells (12) and can exert potent biological effects, some of which may implicate them in the initiation and/or progression of atherosclerosis. A number are chemotactic for macrophages (13) and can be toxic in vitro to major cell types present in the arterial wall (endothelial cells, smooth muscle cells, and fibroblasts), whereas purified cholesterol has no such effect (10, 14, 15). Epoxy-, keto-, and hydroxy-derivatives of cholesterol affect de novo sterol biosynthesis, DNA synthesis, plasma membrane structure, cellular functions, and cellular growth and proliferation (10). We have recently studied reverse sterol transport from oxidized LDL-loaded macrophages (16), and have shown that at least one cell-associated oxysterol (7-ketocholesterol) is hardly accessible for export and is associated with impaired export of cholesterol to an extracellular acceptor. Thus, oxysterols such as 7-ketocholesterol may promote foam cell formation by impairing reverse cholesterol transport.

Copper-catalyzed oxidation of LDL provides a useful model system for studying the effects of oxidized LDL metabolism by macrophages. We aim to precisely define

Abbreviations: ACAT, acyl coenzyme A:cholesterol acyltransferase; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; Cu-oxLDL, copper-oxidized low density lipoprotein; DMEM, Dulbecco's minimum essential medium; EDTA, ethylenediaminetetraacetate; LDL, low density lipoprotein; GC, gas chromatography; HPLC, high performance liquid chromatography; MS, mass spectrometry; PBS, phosphate-buffered saline; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; TMS, trimethylsilyl.

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the lipid composition of copper-oxidized LDL (Cu-oxLDL) and of model foam cells that are formed as a consequence of Cu-oxLDL uptake by macrophages (17). Several papers have presented data on the oxysterol content of Cu-oxLDL but the results are still incomplete and equivocal. Even the identity of the major oxysterol formed during copper-catalyzed oxidation of LDL is unclear with 7-ketocholesterol (3, 4), cholest-3,5-dien-7-one (5), and 7-hydroxycholesterol (7–9) all being possible contenders. Furthermore, there are very few data indicating the proportion of oxysterols that are free or esterified. It is important to characterize the chemical composition of Cu-oxLDL when studying its metabolism by and its effects on cells.

In this study we report a high-performance liquid chromatographic (HPLC) method developed by our group for the measurement of lipid oxidation products of LDL (17), which has been extended to permit the analysis of an array of oxysteryl esters. HPLC was used in conjunction with gas chromatography (GC) to investigate the quantitation of oxysterols (free and esterified) in Cu-oxLDL and their subsequent uptake by macrophages. Our novel approach enables us to measure both free and esterified oxysterols and, in the case of the most abundant oxysterol, 7-ketocholesterol, we can distinguish between esters with oxidized or unoxidized fatty acyl chains. Presented in this report is a more complete characterization of oxysterols in Cu-oxLDL and Cu-oxLDL-loaded macrophages than previously published. We present a novel method for the detection of 7-ketocholesteryl esters, evidence of steryl esters with extensively oxidized fatty acyl chains in Cu-oxLDL, and we demonstrate the occurrence of these sterols and their esters in macrophages that have been incubated with Cu-oxLDL.

MATERIALS AND METHODS

Reagents

All solvents were HPLC grade unless otherwise specified (Mallinckrodt, Biolab Scientific, Clayton, South Australia). Cholesteryl ester standards were all obtained from Sigma-Aldrich (Castle Hill, N.S.W., Australia) except for cholesteryl docosahexaenoate (Nu Chek Prep, Elysian, MN). Cholest-5-en-3β-ol (cholesterol), cholest-5-en-3β,19-diol, cholest-5-en-3β,20α-diol, and cholestan-3β,5α,6β-triol were also purchased from Sigma-Aldrich. The following oxysterols were purchased from Steraloids Inc. (Wilton, New Hampshire): 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), cholest-5-en-3β,22(R)-diol, cholest-5-en-3β,25-diol, cholestan-5α,6α-epoxy-3β-ol (cholesterol α-epoxide), cholest-4-en-3-one, 5α-cholestan-3β-ol-6-one, cholest-3,5-dien-7-one, cholest-4-en-6β-ol-3-one, and cholestan-3β,5α-diol. Cholest-5-en-3β,26S-diol, cholest-5-en-3β,26R-diol, cholestan-5β,6β-epoxy-3β-ol (cholesterol β-epoxide) were obtained from Research Plus Inc. (Bayonne, NJ). Cholest-4-en-3β,6β-diol (6β-hydroxycholester o l) is not readily available from commercial sources and thus was synthesized by the reduction of cholest-4-en-6β-ol-3-one with lithium aluminum borohydride (18, 19). 7-Ketocholesteryl esters were synthesized using the appropriate fatty acyl chloride (NuChek Prep) according to the method for synthesizing cholesteryl esters described by Deykin and Goodman (20). Cholesteryl linoleate hydroperoxide was made by incubating cholesteryl linoleate (6.5 mg) with 2,2′-azobis[2,4-dimethylvaleronitrile] (25 mg AMVN; Polysciences Inc., Warrington, PA) in isopropanol (1.0 ml) at 65°C for 2 h, as previously described (21). Cholesteryl linoleate hydroperoxide was purified by HPLC (detailed below) and quantified using our previously published HPLC response factor (17).

Isolation of LDL

Whole blood, obtained from individual normolipidemic healthy donors, was collected into 50-ml tubes containing disodium ethylenediaminetetraacetate (EDTA: 200 mM, 0.5 ml), aprotinin (5–10 trypsin inhibitory units, 50 μl; Sigma-Aldrich), PPACK (1 μM final concentration, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; Calbiochem) and soybean trypsin inhibitor (0.2 mg/ml final concentration; Sigma-Aldrich). Plasma was separated by centrifugation (3000 rpm (1600 *g*), 20 min, 10°C). LDL (1.05 > d > 1.02 g/ml, 10°C) was isolated on a discontinuous gradient (22) using a Beckman L8-M ultracentrifuge (Palo Alto, CA) and a VTi50 rotor (206,000 *g* (average), 2.5 h, 10°C). A second centrifugation at a density of 1.063 g/ml using a Ti70 rotor (184,000 *g* (average), 22 h, 10°C) ensured a washed preparation of LDL. 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 EDTA (1 g/l), then filter-sterilized (0.45 μm), and stored at 4°C under nitrogen in the dark until use (within 7 days).

Oxidation of LDL

In order to remove the EDTA prior to oxidation, LDL was dialyzed overnight against four exchanges of PBS containing chloramphenicol (0.1 mg/ml) and prewashed Chelex-100 resin (mesh 100–200, 4 g/l) (23). LDL (1.0 mg protein/ml) was added to a 6-well cell culture plate (Costar, Cambridge, MA) and oxidized by addition of 20 μM cupric chloride (final concentration) in a humidified 37°C incubator (5% carbon dioxide in

air). We have found that this copper and LDL protein concentration produced a very similar profile of oxidation products (A. J. Brown, R. T. Dean, W. Jessup, unpublished observations) as previously reported by our group when lower concentrations of copper (10 μM) and LDL protein (400 μg/ml) were used (17). Time-dependent oxidations were performed in triplicate and results are presented as the mean and standard deviation at the following time-points: 0, 1, 2, 4, 6, 8, 24, and 48 h.

Determination of electrophetic mobility

At the various time-points indicated, oxidation was arrested by adding the LDL (1.0 mg/ml, 20 μl) to an Eppendorf tube containing EDTA (20 mM, 20 μl) and butylated hydroxy toluene (BHT) in ethanol (20 mM, 1 μl) and refrigerating to 4°C. Samples preserved in this way were found to be stable for at least 48 h in terms of relative electrophoretic mobility (data not shown). Samples (6 μl = 3 μg LDL protein) were subjected to electrophoresis on an 1.0% Universal Agarose gel (Ciba-Corning, Palo Alto, CA) in Tris-barbitone buffer (pH 8.6) at 90 V for 45 min (Propak-1000, Australian Chromatography Co.). LDL that had not been incubated with copper was used as reference and bands were visualized by staining with Fat Red 7B (Sigma-Aldrich). The relative electrophoretic mobility of oxidized samples was calculated by dividing the distance travelled during electrophoresis by the median distance travelled by the reference native LDL.

Extraction of cholesterol, cholesteryl esters, and their oxidation products from LDL for HPLC analysis

A rapid lipid extraction method described by Rosseneu et al. (24) was used. LDL (1.0 mg/ml, 50 μl) was added to a 2.0-ml glass vial (Chromocol, Activon, Thornleigh, NSW, Australia) containing EDTA (20 mM, 50 μl). As an internal standard, cholesteryl heptadecanoate dissolved in chloroform (5 mg/10 ml, 50 μl) and including BHT (0.2 mM final concentration) was added and the vial was vortex-mixed (5 sec). Isopropanol was added (600 μl) and the mixture was vortex-mixed (30 sec) again before centrifugation (2000 rpm (700 *g*), 10 min, 10°C). One hundred μl samples were used directly for HPLC analysis.

HPLC analyses

The HPLC system comprised a SIL-10A autosampler (Shimadzu, South Rydalmere, NSW, Australia), a pump (2150, LKB-Pharmacia, North Ryde, NSW, Australia) running at a flow rate of 1.0 ml/min, a Linear UV 200 detector (Activon), and a Chromatopac integrator (C-R4A, Shimadzu). A Supelco reverse-phase C18 column was used (Sigma-Aldrich: 0.46 × 25 cm, 2 cm Pelliguard column, 5 μm particle size). Analysis of cholesterol and cholesteryl esters was performed by detecting 210-nm absorbance after elution with acetonitrile–isopropanol 30:70 (v/v) (17, 25). Analysis of several oxidized derivatives of cholesterol and cholesteryl esters was performed as detailed by Kritharides et al. (17) at 234-nm absorbance using a mobile phase of acetonitrile–isopropanol–water 44:54:2 (v/v/v).

Quantitation of 7-ketocholesteryl esters

Six 7-ketocholesteryl esters were synthesized: palmitate, stearate, oleate, linoleate, arachidonate, and docosahexaenoate. As these products were not 100% pure (containing amounts of residual unesterified 7-ketocholesterol and unreacted fatty acyl chloride), an indirect method was used to calculate the HPLC response factors for each. The individual 7-ketocholesteryl esters were saponified as described below or extracted in similar fashion but in the absence of alkali and then analyzed by the 234 nm HPLC system. Saponification resulted in the formation of free 7-ketocholesterol, the response factor of which has been determined previously (17). The response factors of the 7-ketocholesteryl ester were then calculated from the area units of 7-ketocholesteryl ester that gave rise to the amount of free 7-ketocholesterol derived after saponification (**Table 1**). Cholesterol was included as an internal standard in both the saponified and unsaponified samples and detected simultaneously at 210-nm to account for any possible differential losses. This indirect approach was checked for one of the synthesized 7-ketocholesteryl esters: 7-ketocholesteryl oleate was purified, weighed and analyzed by HPLC, yielding a very similar response factor (mean ± SD: 464 ± 27 units/pmol, n = 9) as displayed in Table 1.

Extraction of oxysterols from LDL for GC analysis

LDL was subjected to room temperature saponification (26) or extracted unsaponified. LDL (1.0 mg/ml, 200 μl) was added to a Kimax tube (Kimble, Owens-Illinois) containing 19-hydroxycholesterol (1.0 mg/ml tetrahydrofuran, 50 μl) as an internal standard, BHT (0.2 mM in ethanol, 50 μl), and EDTA (20 mM, 100 μl). For saponification, a methanolic solution of potassium hydroxide (20% w/v, 2.0 ml) was added whereas only methanol (2.0 ml) was added to the unsaponified sample. Diethyl ether (4.0 ml) was added and the tubes were vigorously shaken at room temperature for 3 h in an incubator shaker (Series 25, New Brunswick Scientific, New Brunswick, NJ). 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 to produce two phases in the unsaponified sample. Hexane (4.0 ml) was added to all tubes that were vortex-mixed (30 sec). The

TABLE 1. HPLC response factors of 7-ketocholesterol and synthesized 7-ketocholesteryl esters

7-Ketocholesteryl Ester[a]	Peak Area 234 nm	Peak Area Ratio 234/210 nm
	units/pmol	
Unesterified 7-ketocholesterol	450 ± 19	2.69 ± 0.03
7-Ketocholesteryl palmitate (16:0)	483 ± 13	2.27 ± 0.11
7-Ketocholesteryl stearate (18:0)	513 ± 19	2.52 ± 0.08
7-Ketocholesteryl oleate (18:1)	485 ± 12	2.15 ± 0.10
7-Ketocholesteryl linoleate (18:2)	410 ± 21	1.57 ± 0.02
7-Ketocholesteryl arachidonate (20:4)	469 ± 45	0.73 ± 0.05
7-Ketocholesteryl docosahexaenoate (22:6)	541 ± 40	0.51 ± 0.01

The HPLC response factors were calculated as described in the Materials and Methods. Values are the mean ± standard deviation of three determinations.

[a]The numbers in parentheses refer to the carbon chain length and number of double bonds of the fatty acyl chain.

ether–hexane phase (7 ml) was evaporated under vacuum by rotary evaporation. Trimethylsilyl ether (TMS) derivatives of the oxysterols were prepared by adding 100 μl of Fluka II Silylating Mixture [Fluka: N,O-bis(trimethylsilyl) acetamide–1-(trimethylsilyl)imidazole–trimethylchlorosilane 3:3:2 (v/v/v)] and leaving the sample at room temperature for at least 30 min before analysis. Five-μl samples were used for GC analysis.

Gas chromatography

Cholesterol and oxysterols were analyzed using a method adapted from Pie, Spahis, and Seillan (27). The TMS ether derivatives of oxysterols were analyzed by flame ionization gas chromatography (Hewlett-Packard 5890A capillary Gas Chromatograph, North Ryde, Australia), using a fused carbon-silica column (30 m × 0.25 mm i.d.) coated with (5%-phenyl) methylpolysiloxane (DB-5MS; film thickness 1 μm) (J&W Scientific, Folsom, CA) with ultra-high purity hydrogen as carrier gas (split ratio = 12; flow rate = 1.2 ml/min). The injector temperature was 300°C and the detector temperature was 310°C. An isothermal oven temperature of 300°C was used to separate the TMS sterols of interest (run time = 50 min).

Authentic standard mixtures were run and the retention times relative to 19-hydroxycholesterol were used to identify the peaks (**Table 2**). Response factors were calculated relative to 19-hydroxycholesterol (Table 2) from the slope of the line of the peak area ratios versus the corresponding molar ratios. Responses were linear for all the sterols up to molar ratios in excess of those found in samples ($r^2 \geq 0.97$), and the line of best fit passed very close to the origin. The limit of detection of oxysterols by this GC method was approximately 4 ng. All relevant standards were subjected to the extraction procedure (with or without saponification) to check for generation of any artifactual oxysterols. The amount of steryl esters in samples was calculated by subtracting the measured free sterol content (unsaponified) from the total sterol content measured in saponified samples. Efficiency of saponification was tested on native LDL and established by HPLC as measured by the complete disappearance of cholesteryl esters and concomitant increase in free cholesterol.

Loading mouse peritoneal macrophages with oxidized LDL

Six-week-old Quackenbush Swiss (QS) strain mice were asphyxiated using carbon dioxide gas. Resident macrophages were isolated by peritoneal lavage with ice-cold Dulbecco's minimum essential medium (DMEM; Trace BioSciences, Castle Hill, NSW, Australia) containing 0.38% (w/v) sodium citrate, penicillin G (50 Units/ml) and streptomycin (50 μg/ml). The isolated cells were immediately plated in 75-cm^2 flasks at a density of 0.6×10^6 cells/cm^2, incubated at 37°C for 2 h, then washed three times with pre-warmed PBS to remove non-adherent cells. The macrophages were incubated with LDL that had been oxidized for 24 h with Cu^{2+} (Cu-oxLDL, 50 μg/ml) in DMEM containing 10% (v/v) human lipoprotein-deficient serum for 24 h followed by an overnight equilibration period (1 mg essentially fatty acid-free bovine serum albumin (BSA)/ml DMEM). Control flasks of non-loaded cells were prepared in the same way except that Cu-oxLDL was omitted. After the equilibration period, cells were washed three times with ice-cold PBS. Ice-cold sodium hydroxide (0.2 M, 3.0 ml) was added and the flasks were placed on a platform rocker (Edwards Instrument Co., NSW, Australia) for 15 min at 4°C. Aliquots (1.0 ml) were extracted as described above for total oxysterols (saponified) and free oxysterols (unsaponified). The unsaponified samples were neutralized by adding an equimolar amount of hydrochloric acid (10 M, 20 μl). A further aliquot (0.5 ml) was extracted for HPLC analysis as described previously (17). In brief, the aliquot was added to tubes containing BHT (0.2 mM in ethanol, 100 μl) and EDTA (20 mM, 100 μl). PBS (0.4 ml) was added to bring the aqueous volume to 1.0 ml. Methanol (2.5 ml) was

TABLE 2. Retention times and response factors of oxysterols present in copper-oxidized LDL analyzed by gas chromatography relative to an internal standard, 19-hydroxycholesterol

Sterol	Relative Retention Time[a]	Molar Response Factor[b]
7α-Hydroxycholesterol	0.84	1.12
Cholesterol	0.94	1.27
6β-Hydroxycholesterol	0.96	nd
19-Hydroxycholesterol (internal standard)	1.00	1.00
7β-Hydroxycholesterol	1.07	1.16
Cholest-3,5-dien-7-one	1.09	1.72
Cholestan-3β,5α-diol	1.15	nd
Cholesterol β-epoxide	1.17	nd
Cholest-4-en-3-one	1.17	nd
Cholest-4-en-6β-ol-3-one	1.18	nd
Cholest-5-en-3β,22(R)-diol	1.19	nd
Cholesterol α-epoxide	1.20	1.35
Cholest-5-en-3β,20α-diol	1.25	nd
Cholestan-3β,5α,6β-triol	1.37	nd
Cholest-5-en-3β,25-diol	1.49	nd
5α-Cholestan-3β-ol-6-one	1.54	nd
7-Ketocholesterol	1.58	1.27
Cholest-5-en-3β,26(S)-diol	1.71	nd
Cholest-5-en-3β,26(R)-diol	1.71	0.90

Trimethylsilyl ether derivatives of standard sterols were injected into the gas chromatograph.

[a]The retention time of 19-hydroxycholesterol was typically 21 min under the conditions used. Each relative retention time varied less than 2%.

[b]Molar response factors were calculated from the slope of the line of the ratios of the peak area to 19-hydroxycholesterol area versus the corresponding molar ratios; nd, not determined.

added and the tubes were vortex-mixed (10 sec). Hexane (10 ml) was then added and again the tubes were vortex-mixed (30 sec) before centrifugation (2000 rpm (700 *g*), 10 min, 10°C). After rotary evaporation of 8 ml of the hexane upper phase, samples were resuspended in isopropanol (300 μl). Half of this was reserved for cholesterol and cholesteryl ester analysis (100 μl being injected). Acetonitrile–water (95.7:4.3 (v/v) 128 μl) was added to the remainder of the solution to make up the mobile phase that was used for the HPLC analysis of oxidized derivatives of cholesterol and cholesteryl esters (150 μl being injected).

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-ketocholesteryl esters

We have previously identified free 7-ketocholesterol as a major product of prolonged copper oxidation (17). In the present study, in order to determine whether 7-ketocholesteryl esters also form during copper oxidation, a range of 7-ketocholesteryl esters were synthesized and were found to be resolved by our HPLC system (**Fig. 1A, B**). This system offers a sensitive method for separating free and esterified 7-ketocholesterol. The order of elution of the 7-ketocholesteryl esters follows that of the corresponding cholesteryl esters present in LDL (Fig. 1A, B; cf. ref. 17).

The HPLC response factors of the 7-ketocholesteryl esters were similar to 7-ketocholesterol itself, and apparently not related to chain-length or degree of unsaturation of the fatty acyl chain (Table 1).

Chromatograms of lightly (4 h) and extensively (24 h) Cu-oxLDL are shown in Figs. 1C and 1D, respectively. In 24 h Cu-oxLDL (Fig. 1D), the previously unidentified peaks "C" and "D" discussed in Kritharides et al. (17) were identified as 7-ketocholesteryl oleate and 7-ketocholesteryl palmitate (retention times 14.1 and 15.0 min, respectively). This was based on the re-chromatography of the material collected from these peaks. Using three different solvent systems (acetonitrile–isopropanol–water 50:50:0; 54:44:2; 52:44:4 (v/v/v)), "C" and "D" co-chromatographed with synthesized 7-ketocholesteryl oleate and 7-ketocholesteryl palmitate, respectively. 7-Ketocholesteryl oleate and 7-ketocholesteryl palmitate formed in similar proportions in Cu-oxLDL after 6 h and a small amount of 7-ketocholesteryl stearate was detected at much later times of oxidation (24 and 48 h). No 7-ketocholesteryl esters possessing polyunsaturated fatty acids were detected at any stage of oxidation.

Gas chromatographic determination of oxysterols

Gas chromatography resolved cholesterol and seven major oxysterols previously identified in Cu-oxLDL (6β-

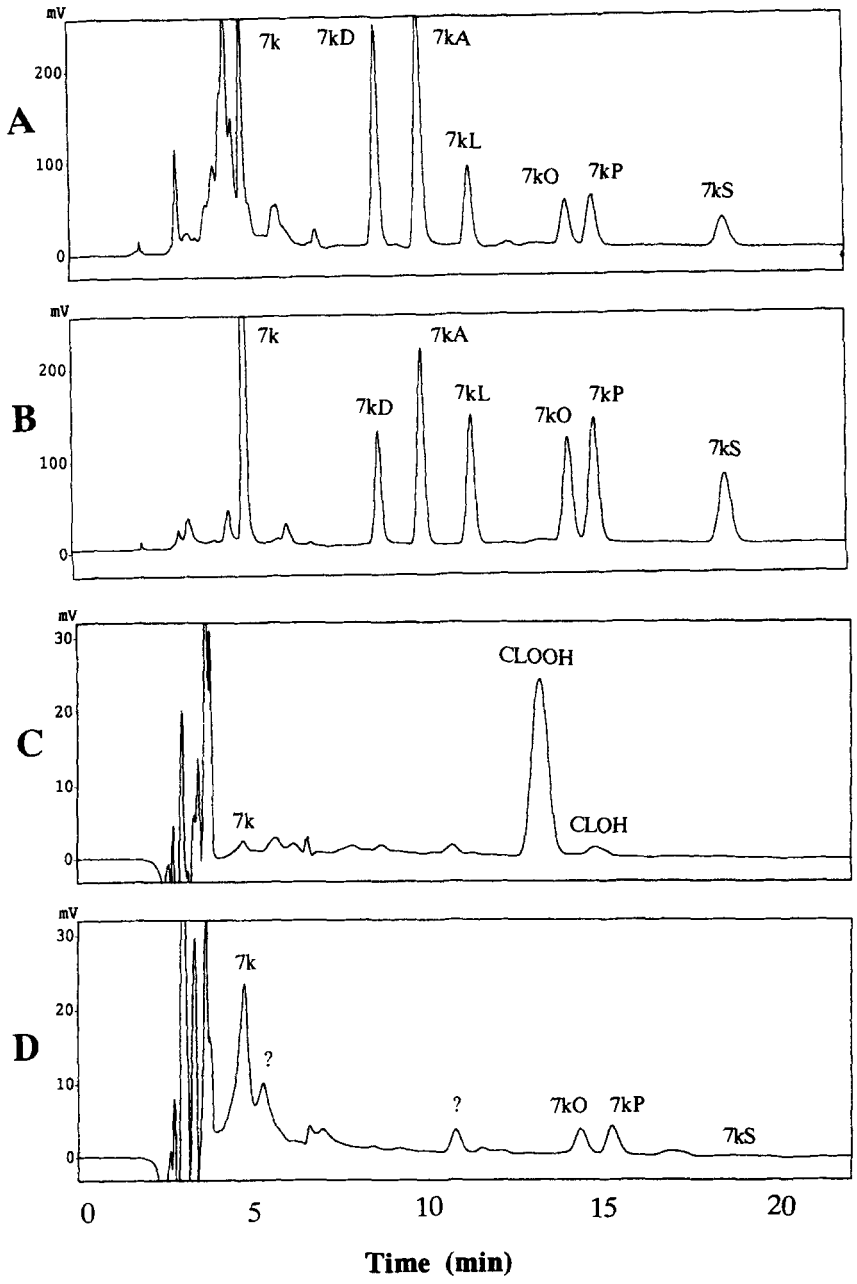

Fig. 1. HPLC chromatograms of A) a mixture of synthesized 7-ketocholesteryl ester standards monitored at 210-nm; and B) 234-nm; C) 4 h copper-oxidized LDL monitored at 234-nm; and D) 24 h copper-oxidized LDL monitored at 234-nm. Separation was achieved using a reverse-phase column and a mobile phase of acetonitrile–isopropanol–water 44:54:2 (v/v/v) at a flow rate of 1.0 ml/min. 7k, unesterified 7-ketocholesterol; 7kD, 7-ketocholesteryl docosahexaenoate; 7kA, 7-ketocholesteryl arachidonate; 7kL, 7-ketocholesteryl linoleate; 7kO, 7-ketocholesteryl oleate; 7kP, 7-ketocholesteryl palmitate; 7kS, 7-ketocholesteryl stearate; ?, unidentified; CLOOH, cholesteryl linoleate hydroperoxide; CLOH, cholesteryl linoleate hydroxide.

hydroxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, cholest-3,5-dien-7-one, cholesterol α- and β-epoxides, and 7-ketocholesterol) (2–9). The retention times of these relative to the internal standard (19-hydroxycholesterol) are given in Table 2.

Impurities in the cholesterol β-epoxide standard (a major component occurring with relative retention time of 1.28) hampered quantitation of this oxysterol so we have assumed that the response factor of cholesterol β-epoxide is the same as cholesterol α-epoxide, an assumption supported by other studies using GC with flame ionization detection (27, 28). The 6β-hydroxycholesterol synthesized in the present study by the reduction of cholest-4-en-6β-ol-3-one was probably a mix-

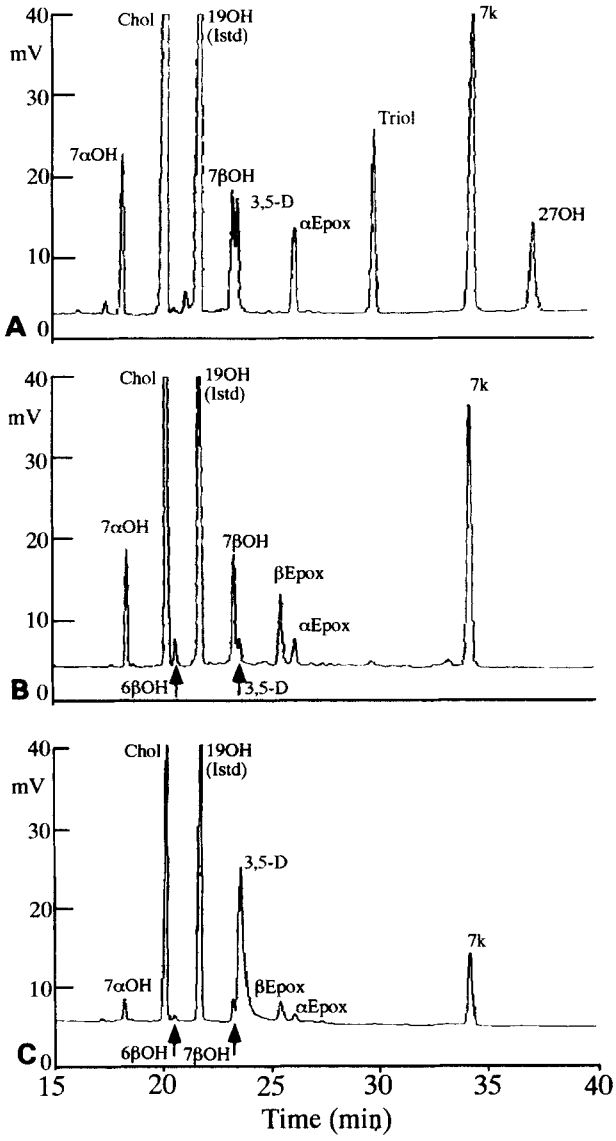

Fig. 2. Gas chromatograms of A) a standard mixture of trimethyl silyl ether derivatives of oxysterols; B) 24 h copper-oxidized LDL after saponification; and C) 24 h copper-oxidized LDL without prior saponification. 7αOH, 7α-hydroxycholesterol; Chol, cholesterol; 19OH, 19-hydroxycholesterol (internal standard); 7βOH, 7β-hydroxycholesterol; 3,5-D, cholest-3,5-dien-7-one; αEpox, cholesterol α-epoxide; Triol, 5α-cholestan-3β,5,6β-triol; 7k, 7-ketocholesterol; 27OH, 27-hydroxycholesterol (cholest-5-en-3β,26(R)-diol); 6βOH, 6β-hydroxycholesterol; βEpox, cholesterol β-epoxide.

ture of the 3α- and 3β-hydroxy derivatives that apparently were not separable on the GC system used, only one major peak representing 82% of the total area unit being present. Because of the impurities present in this preparation of 6β-hydroxycholesterol, an average of the response factors of the 7-hydroxycholesterols was assumed (1.14).

Figure 2 shows chromatograms of a standard mixture of sterols and of 24 h Cu-oxLDL with and without prior saponification. Cholest-4-en-3-one co-chromatographed with cholesterol β-epoxide using the isothermal oven temperature (300°C) but could be separated using another oven temperature program (290°C for 33 min, rise of 10°C/min up to 300°C; relative retention time = 1.15 vs. 1.17). Cholest-4-en-3-one was not detected in the Cu-oxLDL samples. The relative retention times of other oxysterols not detected in Cu-oxLDL are listed in Table 2. Cholestan-3β,5α,6β-triol was evident at more advanced stages of LDL oxidation (24 and 48 h) but only at trace concentrations (< 1% total sterols) which may arise artifactually from hydrolysis of the epimeric epoxides (29).

Standard 7-ketocholesterol produced a single peak on GC. However, when standard 7-ketocholesterol was injected onto GC after being subjected to the saponification procedure, 11.8 mol% (± range: 1.9 mol%, n = 2) was dehydrated to form choles-3,5-dien-7-one. This is similar to the molar proportion of choles-3,5-dien-7-one: (7-ketocholesterol + choles-3,5-dien-7-one) which appeared in Cu-oxLDL. A consistent proportion of choles-3,5-dien-7-one was detected in samples after 2 h copper-oxidation (relative to 7-ketocholesterol, mean ± SD: 14.4 ± 0.9 mol%, n = 6). On this basis it is assumed that all choles-3,5-dien-7-one was an artifact of the saponification process. Therefore choles-3,5-dien-7-one has been included in the quantification of 7-ketocholesterol. None of the oxysterol standards tested nor cholesterol gave rise to artifactual oxysterols during the saponification procedure.

A peak that occurred in the nonsaponified sample of Cu-oxLDL (relative retention time = 1.09, Fig. 2C) co-chromatographed with the single peak generated from purified authentic 7-ketocholesteryl esters (palmitate and oleate) and was absent in the saponified sample (Fig. 2B). Similarly, when the synthesized 7-ketocholesteryl esters were saponified, this peak also disappeared. This peak has been identified as cholest-3,5-dien-7-one by GC–MS (data not shown) and apparently arises from the decomposition of 7-ketocholesteryl esters during GC analysis.

The only oxysterol detectable in unoxidized LDL was 7-ketocholesterol, present in trace amounts in saponified samples (mean ± SD: 4.6 ± 1.5 nmol/mg LDL protein), within the range found by others (30). That these amounts are artifactual cannot be excluded (31).

Time-course of copper-catalyzed LDL oxidation

Under the oxidation conditions used in this study, relative electrophoretic mobility increased rapidly during the first 8 h of LDL oxidation accompanying the generation and decomposition of cholesteryl linoleate hydroperoxide. After the complete decomposition of cholesteryl linoleate hydroperoxide (> 8 h), the relative electrophoretic mobility remained relatively stable (**Fig.**

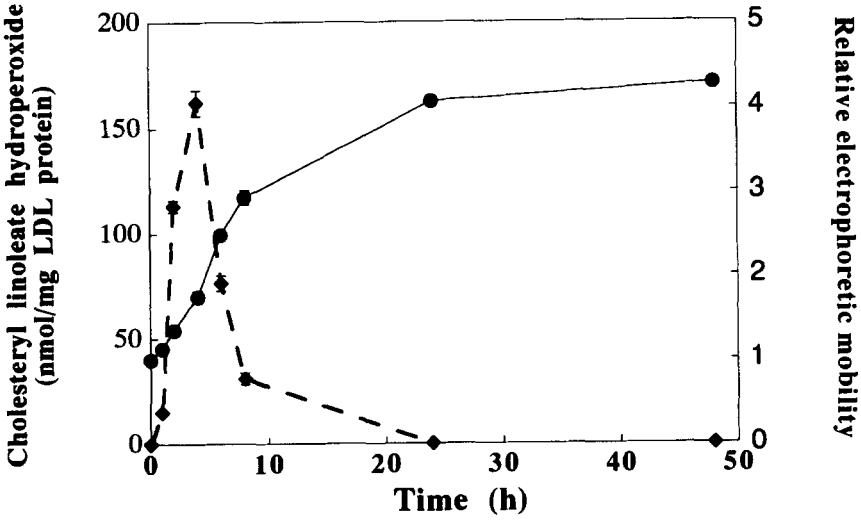

Fig. 3. Changes in relative electrophoretic mobility (solid line, filled circles) and formation of cholesteryl linoleate hydroperoxide (dashed line, filled diamonds) during copper-oxidation of LDL. LDL (1.0 mg protein/ml) was oxidized using copper ions (20 μM) for up to 48 h at 37°C. Cholesteryl linoleate hydroperoxide was determined by HPLC analysis with 234-nm detection as described in Materials and Methods. Values shown are the mean ± standard deviation of triplicate oxidations of the same LDL sample.

3) but oxidation continued as discussed below. These data are consistent with those presented in Kritharides et al. (17).

Figure 4 shows the loss of unoxidized free cholesterol and individual cholesteryl esters during copper-catalyzed oxidation of LDL. The rate of oxidation was proportional to the degree of unsaturation of the fatty acyl ester. Thus free cholesterol was most resistant, followed by cholesteryl palmitate (no double bonds), whereas cholesteryl docosahexaenoate (6 double bonds) was the most susceptible to oxidation. The only cholesteryl esters to survive 24 h copper-oxidation were the oleate, palmitate, and stearate esters (data for latter not shown). These esters were also the only 7-keto-cholesteryl esters possessing unoxidized fatty acids to be detected.

Detection of sterols by HPLC and GC was compared in samples of LDL oxidized for various times. HPLC and GC gave comparable estimates of free cholesterol content of LDL at all stages of oxidation (compare **Fig. 5A** vs. **5B**). Similar values for unesterified 7-ketocholesterol were determined by both the GC and HPLC methods for the later stages of oxidation (24 and 48 h). However, at earlier stages of LDL oxidation (≤ 8 h), GC consistently gave a concentration of free 7-ketocholesterol at least 2-fold greater than that obtained by HPLC determination (Fig. 5C vs. 5D). At 8 h, the difference between the two methods was 43 nmol/mg LDL protein or 6% free initial cholesterol. This discrepancy is addressed in the Discussion.

Direct HPLC detection of cholesteryl and 7-keto-cholesteryl esters in Cu-oxLDL produced a significantly lower estimate than the amounts calculated from GC (with and without saponification) (compare Fig. 5A vs. 5B, 5C vs. 5D). The HPLC system used here measures

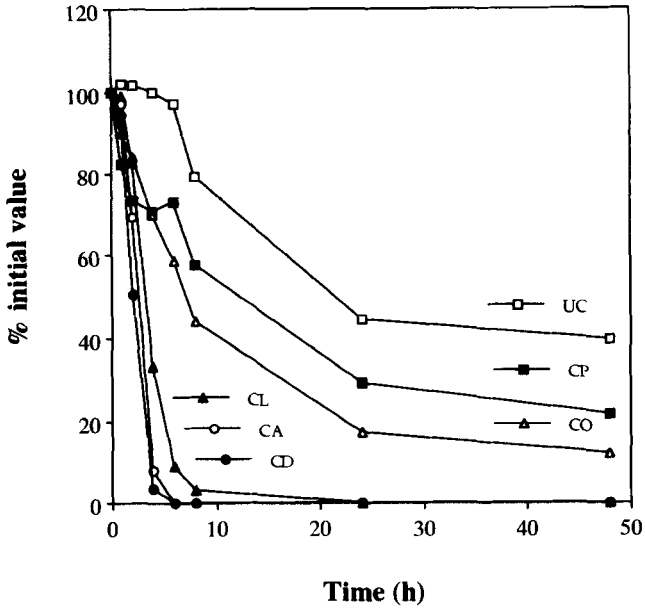

Fig. 4. Loss of cholesterol and individual cholesteryl esters during copper-oxidation of LDL. LDL (1.0 mg protein/ml) was oxidized using copper ions (20 μM) for up to 48 h at 37°C. Cholesterol and cholesteryl esters were determined by HPLC analysis with 210-nm detection as described in Materials and Methods. UC, unesterified cholesterol (100% = 711 nmol/mg LDL protein); CP, cholesteryl palmitate (100% = 169 nmol/mg); CO, cholesteryl oleate (100% = 329 nmol/mg); CL, cholesteryl linoleate (100% = 1075 nmol/mg); CA, cholesteryl arachidonate (100% = 110 nmol/mg); CD, cholesteryl docosahexaenoate (100% = 15 nmol/mg). Cholesteryl stearate was at or below the limits of detection (≤ 12 nmol/mg) and therefore not included. Values shown are the mean. Error bars are not presented.

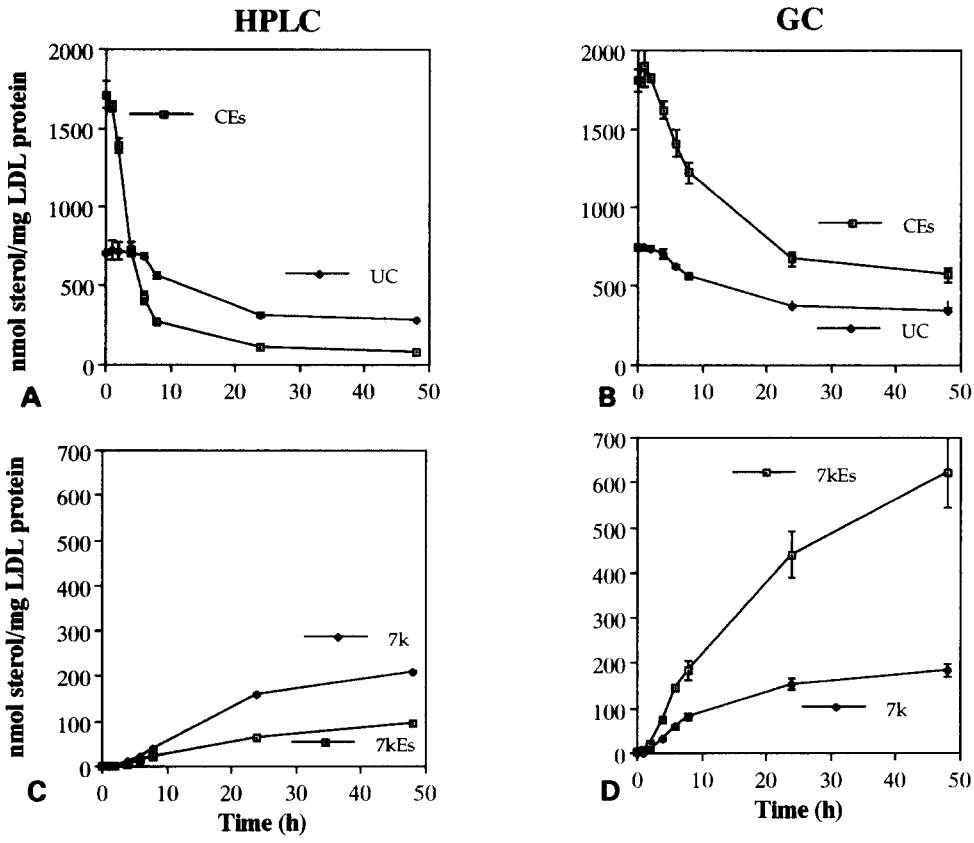

Fig. 5. Loss of free and esterified cholesterol and the formation of free and esterified 7-ketocholesterol during copper-oxidation of LDL: comparison of HPLC and GC determinations. A: HPLC analysis of unesterified cholesterol (UC) and cholesteryl esters (CEs) determined by 210-nm detection. B: Gas chromatographic analysis of UC (without saponification) and CEs (total cholesterol after saponification minus free cholesterol). C: HPLC analysis of unesterified 7-ketocholesterol (7k) and 7-ketocholesteryl esters (7kEs) determined by 234-nm detection. D: Gas chromatographic analysis of 7-ketocholesterol (without saponification) and 7kEs (total 7-ketocholesterol after saponification minus free 7-ketocholesterol). Details are given in Materials and Methods. Concentrations shown are the mean ± standard deviation of triplicate oxidations of the same LDL sample.

only steryl esters that have non-oxidized fatty acyl chains whereas the GC approach measures all esters by difference between the saponified and unsaponified sample, irrespective of whether the fatty acyl chains are oxidized or not. It was concluded that the difference between the values for the two methods represents steryl esters containing oxidized fatty acids.

Oxysterol formation was evident as early as 1 h after initiating oxidation (**Fig. 6**). The total oxysterol content of LDL increased steadily during the course of oxidation, a higher proportion originating from esterified than free cholesterol by 6 h (Fig. 6A). This was reflected in the higher proportion of free cholesterol that survived copper-oxidation compared with the cholesterol moiety of cholesteryl esters (50% vs. 37% at 24 h).

The proportion of the total initial sterol pool (free and esterified cholesterol at 0 h) recovered by GC analysis decreased progressively with prolonged oxidation. But even by 48 h, 86% of the initial sterol content could be accounted for, six oxysterols comprising half of the total sterol. Of these, 7-ketocholesterol was by far the predominant oxysterol formed during copper-oxidation of LDL (Fig. 6). 7-Ketocholesterol arose at similar rates from both the free and esterified pools until 24 h, after which a higher proportion was derived from cholesteryl esters (Fig. 6B).

Like 7-ketocholesterol, the cholesterol epoxides increased steadily during the 48 h of copper-oxidation with similar proportions of free and esterified cholesterol epoxide formed (Fig. 6C, D). The proportion of 7α- and 7β-hydroxycholesterol arising from esters was consistently greater than the proportion arising from free sterol for all time-points examined. Both began to plateau after 8 h (Fig. 6E, F). Thus, the 7-hydroxycholesterols seemed to arise mostly from cholesteryl esters whereas less selectivity was seen in the case of 7-ketocholesterol and the cholesterol epoxides.

Formation of cholesterol β-epoxide was favored over

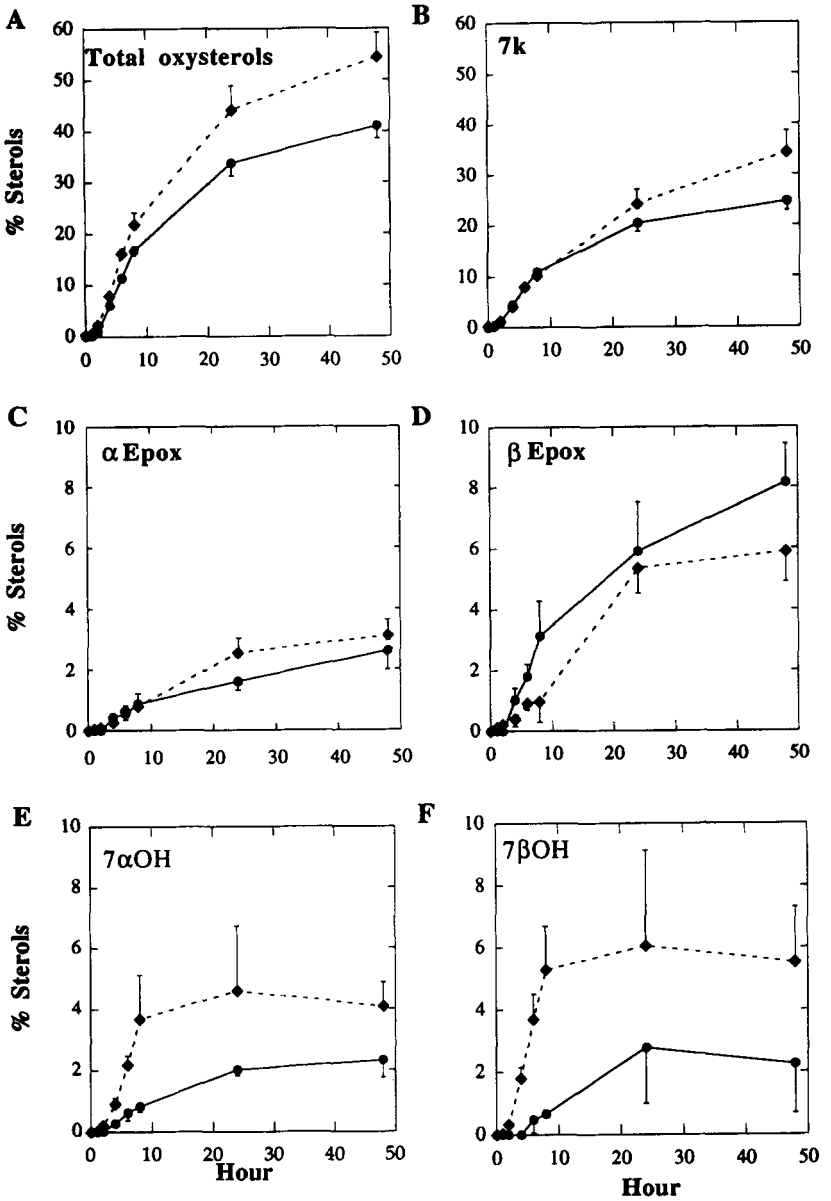

Fig. 6. Percent proportion of oxysterols derived from free and esterified cholesterol during copper-oxidation of LDL. A: Total oxysterols, sum of all oxysterols measured. Abbreviations given in Fig. 1. The unesterified proportions are represented by solid lines and filled circles. The esterified proportions are represented by dashed lines and filled diamonds. Details are given in Materials and Methods. Percent proportions shown are the mean ± standard deviation of triplicate oxidations of the same LDL sample. The initial amount of free and esterified cholesterol in native LDL was 749 ± 22 and 1806 ± 72 nmol/mg LDL protein, respectively.

the α-isomer. During the 48 h incubation, twice as much β-epoxide formed compared with cholesterol α-epoxide (β-epoxide/α-epoxide ratio, mean ± SD: 2.13 ± 0.24). The production of 7-hydroxycholesterol also showed β-stereospecificity. The average ratio of 7β-hydroxycholesterol to 7α-hydroxycholesterol over the 48 h was 1.55 (± SD: 0.24).

The individual oxysterol compositions (free and esterified) of different preparations of 24 h Cu-oxLDL were very similar (data not shown). The concentrations of cholesterol β-epoxide and 7α- and 7β-hydroxycholesterol were roughly one-fifth that of 7-ketocholesterol, whereas cholesterol α-epoxide and 6β-hydroxycholesterol were approximately a tenth and a twentieth of 7-ketocholesterol, respectively (**Fig. 7**). The contribution of total 7-ketocholesterol to total measured sterols ranged from 28% to 33% in 24 h Cu-oxLDL prepared from three different donors on different occasions. The proportion of total 7-ketocholesterol that was unesterified was also similar between the three

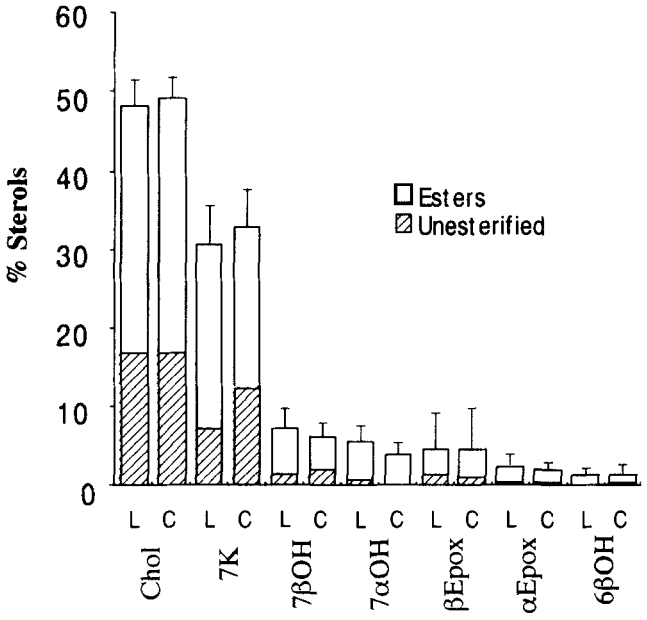

Fig. 7. Percent composition of sterols (free and esterified) in copper-oxidized LDL (L) and macrophage cells (C) that have been loaded with copper-oxidized LDL. Details are given in Materials and Methods. The cell composition represents the increment of sterols accumulated, that is, after adjusting for the cellular free cholesterol content of non-loaded cells. Abbreviations given in Fig. 1. Percentages shown are the mean ± range of two experiments. For one typical experiment, the total amount of sterol (oxidized and unoxidized) was 427 ± 36 nmol/mg cell protein.

donors in the range 22% to 26%.

Cellular accumulation of oxysterols

Free cholesterol but no cholesteryl esters (< 10 nmol/mg cell protein, that is, below detection limit) could be detected in the non-loaded control cells. Upon incubation with 24 h Cu-oxLDL, resident mouse peritoneal macrophages accumulated both oxidized and unoxidized cholesterol. The cholesterol content was 5-fold greater in the Cu-oxLDL-loaded cells compared with the non-loaded cells. For one typical experiment, the cholesterol content of non-loaded cells was 56 ± 4 nmol/mg cell protein (mean ± SD, n = 3) and after loading with Cu-oxLDL (50 μg protein/ml), the total amount of cholesterol (free and esterified) was 244 ± 15 nmol/mg cell protein, the total amount of 7-ketocholesterol was 110 ± 14 nmol/mg cell protein, while the total amount of sterol (oxidized and unoxidized) was 427 ± 36 nmol/mg cell protein. The increment in the sterol content of macrophages caused by uptake of Cu-oxLDL produced a sterol composition remarkably similar to Cu-oxLDL itself (Fig. 7). However, there were some differences. The proportion of free versus total 7-ketocholesterol in Cu-oxLDL-loaded cells was greater than that in the Cu-oxLDL supplied to the cells (mean ± range: 37.5 ± 2.7% vs. 24.2 ± 3.4%). This was also evident for 7β-hydroxycholesterol (31.9 ± 9.3% vs. 17.4 ± 2.8%) whereas less 7α-hydroxycholesterol was in the free form in Cu-oxLDL-loaded cells (5.8 ± 2.5%) compared with Cu-oxLDL (15.7 ± 0.7%).

Table 3 shows the distribution of the cholesterol and 7-ketocholesterol pools in Cu-oxLDL and macrophages loaded with Cu-oxLDL between free and esterified forms. Also shown is the proportions of esterified cholesterol and 7-ketocholesterol that possess unoxidized fatty acyl chains (visible to our HPLC system) or oxidized fatty acids (calculated by difference). Modified esters, which represent between one- and two-thirds of the total cholesterol and 7-ketocholesterol pools, contain a presumably oxidized fatty acyl group. Compared with Cu-oxLDL, there was a higher proportion of unesterified cholesterol and a lower proportion of esterified cholesterol in the loaded cells. But when adjustment was made for the pre-existing amount of free cholesterol present in non-loaded cells, there was excellent agreement between Cu-oxLDL and loaded cells in the distribution of cholesterol, apart from a slightly lower proportion of unoxidized esters in the cells. The proportion of unoxidized 7-ketocholesteryl esters was higher in the cells when compared with Cu-oxLDL. As already noted, a higher proportion of 7-ketocholesterol was in the free form in cells with a concomitant lower proportion of oxidized esters.

DISCUSSION

Considerable changes occur in the composition of LDL during its oxidation catalyzed by transition metals, including increased negative surface charge, loss of cholesterol and cholesteryl esters, and simultaneous formation of free and esterified oxysterols. Here we have used a combination of GC and HPLC methods to comprehensively characterize oxysterol formation during copper-oxidation of LDL and their cellular accumulation after uptake of this Cu-oxLDL by cultured macrophages. GC, with and without prior cold alkali saponification, enabled us to measure esterified and free forms of both cholesterol and oxysterols. This approach complemented our previously published HPLC methods (17), by which we could measure cholesterol, individual cholesteryl esters, cholesteryl linoleate hydroperoxide, and a major unesterified oxysterol, 7-ketocholesterol. In the present study, one of these HPLC methods was extended to measure a range of 7-ketocholesteryl esters with unoxidized fatty acyl chains. The authors are aware of one other HPLC method that was used to separate only saturated 7-ketocholesteryl esters (myristate, palmitate, and stearate)

TABLE 3. Percent distribution of cholesterol and 7-ketocholesterol pools in copper-oxidized LDL and macrophages that have been loaded with copper-oxidized LDL

	Cholesterol (%)			7-Ketocholesterol (%)	
Fraction	oxLDL	Cells	Cells[a]	oxLDL	Cells
Unesterified	34.5 ± 2.1	55.1 ± 8.1	34.1 ± 4.1	24.2 ± 3.4	37.5 ± 2.7
Unoxidized esters[b]	9.5 ± 0.6	6.6 ± 0.3	6.6 ± 0.3	9.2 ± 1.3	14.3 ± 1.0
Oxidized esters[c]	56.0 ± 2.7	38.3 ± 8.4	59.3 ± 4.4	66.6 ± 4.6	48.2 ± 3.8

LDL (1.0 mg protein/ml) was oxidized using copper ions (20 μM) for 24 h at 37°C. Resident mouse peritoneal macrophages were incubated with this copper-oxidized LDL (50 μg/ml) for 24 h, followed by an overnight equilibration period. Percentages shown are the mean ± range of two experiments. For one typical experiment, the cholesterol content of non-loaded cells was 56 ± 4 nmol/mg cell protein (mean ± SD, n = 3) and after loading with Cu-oxLDL (50 μg protein/ml), the total amount of cholesterol (free and esterified) was 244 ± 15 nmol/mg cell protein, the total amount of 7-ketocholesterol was 110 ± 14 nmol/mg cell protein, while the total amount of sterol (oxidized and unoxidized) was 427 ± 36 nmol/mg cell protein.

[a]Gives the incremental distribution of cholesterol in oxidized LDL-loaded macrophages, that is, after correcting for the cellular free cholesterol content of non-loaded cells.

[b]Unoxidized esters refer to those steryl esters that possess unoxidized fatty acyl chains and were determined by HPLC (210 or 234-nm detection) as described in Materials and Methods.

[c]Oxidized esters (i.e., those containing presumed oxidized fatty acyl groups) were calculated by difference: total sterols (from GC determination after saponification) minus free sterol (from GC determination without saponification) minus esters with unoxidized fatty acids (from HPLC determination).

(32). Our method has applications beyond the present investigation of in vitro lipid oxidation studies. 7-Ketocholesteryl esters may be physiologically significant as they have been found in human tissue: in patients with Wolman's disease by thin-layer chromatography (33), in liver and plasma by HPLC (34), and our method has been successful in detecting 7-ketocholesteryl esters in human atherosclerotic plaque (C. Suarna, R. T. Dean, J. May, and R. Stocker, unpublished observations).

Several studies have reported on oxysterols formed in LDL during copper-oxidation using a variety of conditions (**Table 4**). The value of many of the studies is limited by the small number of oxysterols that could be adequately resolved or detected by the GC systems used. One is a preliminary report (2) and not all of the other studies presented quantitative data on oxysterol formation during copper-oxidation (3, 5, 6). Only two studies have presented any data on the relative amounts of free and esterified oxysterols (4, 7), though neither make any direct determinations of oxysteryl esters.

Jialal, Freeman, and Grundy (4) do not present absolute data on their deduced oxysteryl ester levels, and hence it is difficult to compare their data with ours. Tanaka and Kanamaru (7) found that the amount of esterified 7-hydroxycholesterol (isomer not specified) equalled that of unesterified 7-hydroxycholesterol in oxidized LDL, while esterified 7-ketocholesterol was less abundant than the free form. By contrast, we found that both 7-hydroxycholesterol and 7-ketocholesterol were present mostly in the esterified form, 7-hydroxycholesterol arising mostly from cholesteryl esters whereas less preference was evident in the case of 7-ketocholesterol. Tanaka and Kanamaru (7) probably underestimated the amount of steryl esters in their study, as oxysteryl esters with oxidized fatty acyl chains are much more polar than their unoxidized counterparts and so are not readily extracted into hexane (A. J. Brown, unpublished observations). Hence it is also likely that some of the sterol was lost during the hexane–isopropanol 3:1 (v/v) extraction performed before alkaline saponification in this earlier study (7).

The presence of 7-ketocholesterol in Cu-oxLDL has been consistently reported in all previous studies (Table 4). Like many of the studies listed, we found 7-ketocholesterol to be the major oxysterol. We consider that technical and procedural issues explain the differences between our data and other published data. Three critical issues determine the quantitative significance of a particular oxysterol. First, the capacity of the analytical system to clearly resolve all the relevant oxysterols. The second issue is that the precise conditions of oxidation may well determine the relative proportions of oxysterols. And third, the analytical procedures themselves may cause artefactual oxidation or conversion of (oxy)sterols. For example, choles-3,5-dien-7-one observed by Bhadra et al. (5) to be the predominant oxysterol in Cu-oxLDL is probably an artifact arising from the dehydration of 7-ketocholesterol. We have shown that even the room temperature (routinely described in the literature as 'cold') alkali saponification used in the present study resulted in conversion of at least 10% 7-ketocholesterol to choles-3,5-dien-7-one. We found that standards of 7α-hydroxycholesterol and 7β-hydroxycholesterol did not give rise to 7-ketocholesterol during the sample processing procedure. Because others have failed to address artefactual oxidation or conversion of (oxy)sterols, some of the claims (Table 4) that 7-hydroxycholesterols are the major sterol product of oxidation may be insecure. Carpenter et al. (8) relied on an indirect method for measuring 7-ketocholesterol, because they reduced their samples prior to analysis, converting 7-ketocholesterol to 7-hydroxycholesterol.

TABLE 4. Summary of studies that have examined oxysterols in copper-oxidized LDL

Reference	Cu Conc	LDL Protein	Cu/LDL Molar Ratio	Time Points	Oxysterols Found[b]	Comments[c]
	μM	μg/ml		h		
Warner et al. (2)	5.0	na[a]	na	48	7αOH, 7βOH, 7K, α-Epox, β-Epox	Abstract; qualitative; GC; no triol or 25OH.
Zhang et al. (3)	5.0	200	12.8	20	**7K,** 7OH, Epox	Qualitative; Unsap; GC/FID/MS; 2 unidentified peaks. No 25OH.
Jialal et al. (4)	2.5	200	6.4	5,8,24	**7K,** ?22OH	Sap and Unsap; GC/FID; 1 major unidentified peak. No 7αOH, 7βOH, 25OH, cholest-4-en-3-one.
Bhadra et al. (5)	10	na	na	72	**3,5-D,** β-Epox, 7K, 25OH, 7αOH	Qualitative; Sap; GC/FID/MS; 1 unidentified peak.
Malavasi et al. (6)	20	200	51	0,3,5,8,12,18,24	**7K,** 7βOH, 7αOH, ?Epox, 3,5-D. 7OOH	Qualitative; Unsap; GC/MS; TLC.
Tanaka and Kanumara (7)	5.0	200	12.8	20	**7OH,** 7K	Sap and Unsap; GC/MS; no mention of whether 7αOH or 7βOH.
Carpenter et al. (8)	5.0/25	100	26/128	2,4,6,8,12,18,24	**7βOH,** 7K, 7αOH, ?26OH	Sap; Ham's F10 incl. Fe(11) (6 μM); sample reduced, 7K measured indirectly; GC/FID/MS.
Mori et al. (9)[d]	8.0	62	64	1,2,4,20	**7βOH,** 7K, α-Epox, 6βOH, 4βOH	Sap; GC/MS–SIM
Present study	20	1000	10	1,2,4,6,8,24,48	**7K,** 7βOH, β-Epox, 7αOH, α-Epox, 6βOH.	Sap and Unsap; GC/FID. No cholest-4-en-3-one, 22OH, 25OH, 26OH, 27OH

[a]na, not available.

[b]Oxysterols found in approximate descending order of quantity. Major oxysterol found in boldface (where stated). ? refers to a tentatively identified oxysterol. 7αOH, 7α-hydroxycholesterol; 7βOH, 7β-hydroxycholesterol; 7K, 7-ketocholesterol; α-Epox, cholesterol α-epoxide; β-Epox, cholesterol β-epoxide; 3,5-D, cholest-3,5-dien-7-one; 25OH, 25-hydroxycholesterol; 22OH, 22(R)-hydroxycholesterol; 7OOH, cholesterol 7-hydroperoxide; 26OH, 26-hydroxycholesterol; 6βOH, 6β-hydroxycholesterol; 4βOH, 4β-hydroxycholesterol.

[c]Sap, looked only at total oxysterols after saponification; Unsap, looked only at free oxysterols (without saponification); Sap and Unsap, presented at least some data on free and esterified oxysterols; GC, gas chromatography with detection by FID (flame ionization detection) and/or MS (mass spectrometry); TLC, thin-layer chromatography; SIM, selective ion monitoring. 26OH, 26-hydroxycholesterol (cholest-5-en-3β,26(S)-diol); 27OH, 27-hydroxycholesterol (cholest-5-en-3β,26(R)-diol).

[d]Details supplied by T. A. Mori (personal communication).

Whether this conversion was quantitative was not established. In addition, their GC method did not permit accurate determination of 7α-hydroxycholesterol. For both these reasons, 7-ketocholesterol may have been underestimated. We conclude that all the studies published in detail are consistent with our view that 7-ketocholesterol is a major product of copper-mediated LDL oxidation. There may be some circumstances, yet to be defined, in which 7-hydroxycholesterol predominates.

Oxidation of LDL catalyzed by copper ions is a complex multi-phase system and it is likely that multiple oxidations take place by a variety of pathways. Some discussion of the mechanisms of sterol oxidation is warranted. The oxysterols formed are the same as seen with cholesterol autoxidation (29) and hence similar mechanisms may apply. Initial free radical attack on LDL lipid probably involves abstraction of a bisallylic hydrogen from a polyunsaturated fatty acid, and then subsequent reaction with oxygen to yield the corresponding peroxyl radical. This is supported by our data showing early production of cholesteryl linoleate hydroperoxide (Fig. 3), and increased rate of consumption of cholesteryl esters with increasing degree of unsaturation (Fig. 4). The resulting fatty acid radical or peroxyl radical would then be able to abstract another hydrogen from other lipids, including the C7 allylic position of the B-ring of cholesterol to yield the epimeric 7-hydroperoxides, and a propagating radical. The peroxide bond may then undergo cleavage yielding the corresponding cholesterol 7-alkoxyl radicals which are subject to hydrogen abstraction to form 7-ketocholesterol. The alcohols, 7-hydroxycholesterol, can also arise via cholesterol 7-alkoxyl radical intermediates (29). Our data support the idea that esterified cholesterol 7-alkoxyl radicals are most likely to abstract a bisallyic hydrogen from a cholesteryl ester possessing a polyunsaturated fatty acyl chain as 7-hydroxycholesterol forms mostly from the cholesteryl ester pool (Fig. 6E, F), and production of

7-hydroxycholesteryl esters levels off by 8 h when most of the polyunsaturated esters have been consumed (Fig. 4). Unesterified cholesterol 7-alkoxyl radicals perhaps abstract a C7 allylic hydrogen from a neighboring free cholesterol molecule as the proportion of free 7-hydroxycholesterol produced increases up to 24 h (when half the initial free cholesterol still remains, yet much of the esterified fatty acids have already been oxidized). Other major products found in Cu-oxLDL are the isomeric cholesterol epoxides, which have been proposed to arise from alkoxyl and peroxyl-radical attack on cholesterol in a process showing marked β-stereospecificity (35, 36), which is again consistent with our own data. 6β-Hydroxycholesterol is a thermal decomposition product of cholesterol 6β-hydroperoxide which has been shown to form by attack of singlet oxygen on cholesterol (37), and was found in small quantities in our analysis.

Our GC method yielded consistently higher concentrations of free 7-ketocholesterol at earlier time-points (≤ 8 h) than our HPLC method. At 8 h, the difference between the two methods was 6% initial free cholesterol. The initial formation of cholesterol 7-hydroperoxide (which is well documented (37)), may explain this discrepancy. Cholesterol 7-hydroperoxide is known to be thermally labile (6), prone to degradation in the alkaline conditions used for alkali saponification (38), and even unstable in aqueous solution (28). Indeed, when cholesterol 7α-hydroperoxide was analyzed by GC–MS, two peaks resulted with the retention times and mass spectra of 7-ketocholesterol and 7α-hydroxycholesterol (6). Hence, it is likely that any cholesterol 7-hydroperoxide present was thermally decomposed in our GC analysis. In contrast, the HPLC approach may have spared cholesterol 7-hydroperoxide as high temperature and alkali saponification were avoided, and samples were immediately extracted into organic solvent where cholesterol 7-hydroperoxide is more stable (28). In the HPLC system, it is likely that cholesterol 7-hydroperoxide elutes in the vicinity of 7-ketocholesterol but because it lacks the enone chromophore it would not absorb at 234-nm. As we have observed for cholesteryl linoleate hydroperoxide, cholesterol 7-hydroperoxide may have all decomposed by 24 h copper-oxidation, and this would account for the agreement of the values given by our GC and HPLC methods at this time-point. At earlier stages of oxidation (< 8 h), the difference between the GC and HPLC determinations of unesterified 7-ketocholesterol actually exceeds 7-ketocholesterol (determined by HPLC). Thus, cholesterol 7-hydroperoxide rather than 7-ketocholesterol may dominate at earlier time-points. Cholesterol 7-hydroperoxide has been recently identified in Cu-oxLDL (6, 39) and also been claimed to be in human atherosclerotic plaque (39). Chisolm et al. (39) reported that approximately 5% of LDL free cholesterol was oxidized to cholesterol 7β-hydroperoxide using copper as the catalyst; this quantity would be sufficient to explain the discrepancy mentioned above.

Twenty-four h Cu-oxLDL can serve as a ligand for scavenger receptors on the macrophage. Scavenger receptors, unlike LDL receptors, are not regulated by intracellular cholesterol and allow cultured macrophages to rapidly accumulate lipid. We have previously shown (16) that under these conditions, 7-ketocholesterol accumulates in cells. We have now used our comprehensive GC- and HPLC-based approaches to show that when resident peritoneal macrophages are incubated with 24 h Cu-oxLDL, they accumulate both free and esterified oxysterols in very similar proportions to those supplied in the Cu-oxLDL itself. It is thus now well established that oxysterols accumulate under these conditions (2, 4, 7), but we can find only one previous publication offering measurements on cellular oxysteryl esters: Jialal et al. (4) presented limited data to the effect that significant quantities of 7-ketocholesterol (some esterified but mostly free) were present in human monocyte-derived macrophages incubated with 24 h Cu-oxLDL. These data indicate more limited loading than we describe, and concern a different cell type.

We have found that unoxidized fatty acyl esters of cholesterol and 7-ketocholesterol are indeed hydrolyzed and so the lysosomal acid lipase is certainly active. Furthermore, we have several lines of evidence to show that 7-ketocholesterol is a substrate for ACAT (acyl coenzyme A:cholesterol acyltransferase) to form esters with unoxidized fatty acids and that ACAT is active in Cu-oxLDL-loaded cells. For example, compounds "C" and "D" (17), identified here as 7-ketocholesteryl esters, are synthesized in loaded macrophages through the action of ACAT as their formation is inhibitable by Sandoz 58-035 (ref. 16; E. Mander, A. J. Brown, R. T. Dean, and W. Jessup, unpublished observations).

In Cu-oxLDL-loaded macrophages, there was also a large pool of esters with modified fatty acyl chains. These were deduced for cholesterol and 7-ketocholesterol from the difference between the GC analysis before and after saponification and HPLC determination of esters with unoxidized fatty acids. The oxidized esters were not detected by the HPLC system, probably eluting in the void volume or even too polar to be extracted into hexane (A. J. Brown, unpublished observations). Some will almost certainly be the core aldehydes associated with sterols (including 7-ketocholesterol) characterized by Kamido et al. (40) as occurring in Cu-oxLDL. What happens to the esters with modified fatty acyl chains after delivery to the macrophage is not clear. One possibility is that similar to esters with unmodified fatty acyl chains, these oxidized esters

are hydrolyzed in lysosomes, but then the resultant sterols are re-esterified to modified fatty acyl chains by ACAT in the cytoplasm. An alternative is that the majority of esters with modified fatty acids are inefficiently hydrolyzed in lysosomes and thus are trapped in the lysosomes, as we have shown to occur for the protein moiety of Cu-oxLDL (41).

Pertinent to the formation of esters of both unoxidized and oxidized fatty acids and sterols is the availability of the co-substrates for esterification and the efficiency with which ACAT can use these substrates. Little unoxidized cholesteryl esters survive 24 h copper-oxidation and consequently little unoxidized fatty acid is available when Cu-oxLDL is incubated with cells. These data suggest that supply of fatty acids may limit ACAT-mediated formation of esters containing unoxidized fatty acids in this Cu-oxLDL-loaded macrophage system. Consistent with this idea is our observation that the unesterified proportion of two of the major oxysterols, 7-ketocholesterol and 7β-hydroxycholesterol, was greater in the loaded cells than in Cu-oxLDL. Unoxidized fatty acids may be diverted to other pools we have not measured, such as phospholipids, which may be expected to increase as the cells increase in size during loading. In contrast, substantially less 7α-hydroxycholesterol was evident in the unesterified form in cells compared with Cu-oxLDL. One possibility is that free 7α-hydroxycholesterol is actively metabolized by the cells. Certainly, 7α-hydroxycholesterol is known to undergo a range of transformations in humans and animals (34), and metabolism of 7α-hydroxycholesterol to primary bile acids has been suggested to occur in human monocytes (42). It is not known whether mouse macrophages possess this ability.

Presented in this report is a more comprehensive characterization of free and esterified oxysterols of Cu-oxLDL than published before. In contradiction to some previous reports, 7-ketocholesterol was by far the predominant oxysterol encountered, being derived from the oxidation of free and esterified cholesterol in similar proportions. Other oxysterols detected in relatively minor amounts also arose from the oxidation of the B-ring of cholesterol: epimeric 7-hydroxycholesterols, cholesterol epoxides, and 6β-hydroxycholesterols. Macrophages that have been incubated with Cu-oxLDL displayed a remarkably similar profile of oxysterols. Our HPLC method in conjunction with GC analysis of samples (with and without saponification), has proved extremely useful for differentiating between 7-ketocholesteryl esters with intact fatty acids (visible by HPLC) or with oxidized fatty acids (by difference from GC value). The majority of cholesteryl esters and 7-ketocholesteryl esters in Cu-oxLDL and in Cu-oxLDL-loaded macrophages had modified fatty acyl chains. This work has helped to define the chemical composition of Cu-oxLDL which we use in our macrophage–foam cell models and has laid the foundation for exploring how oxysterols are metabolized by macrophages and authentic human foam cells, and how in turn these oxysterols influence cellular metabolism.

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