Production of oxidized lipids during modification of low-density lipoprotein by macrophages or copper

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The oxidation of low-density lipoprotein (LDL) is implicated in atherosclerosis. Lipids and oxidized lipids were analysed by gas chromatography and gas chromatography–mass spectrometry in human LDL incubated with mouse peritoneal macrophages (MPM) or copper(II) sulphate in Ham's F-10 medium or medium alone (control). MPM-modification and copper-catalysed oxidation of LDL resulted in the formation of oxysterols, mainly cholest-5-en-3β,7β-diol (7β-OH-CHOL); 7%–19% of the initial cholesterol was converted to 7β-OH-CHOL in 24 h. 7β-OH-CHOL levels in control LDL were very low. The increase in 7β-OH-CHOL in MPM and copper-oxidized LDL was accompanied by decreases in linoleate and arachidonate and increases in the electrophoretic mobility and degradation of LDL protein by 'target' macrophages. The concerted occurrence of these processes and their similarity in both MPM-modification and copper-catalysed oxidation of LDL were suggested by the highly significant cross-correlations. The fall in polyunsaturated fatty acid (PUFA) was accompanied by a directly proportional increase in electrophoretic mobility of the LDL. Production of 7β-OH-CHOL and protein degradation by macrophages showed modest elevations during the initial steep fall in PUFA, and showed their greatest increases as the levels of PUFA slowly approached zero. The levels of 7β-OH-CHOL and the degradation of LDL by macrophages were directly proportional. The degradation of LDL by macrophages increased rapidly as the electrophoretic mobility of LDL was slowly approaching its maximum level.

INTRODUCTION

There is now considerable evidence for oxidation of low-density lipoprotein (LDL) during the pathogenesis of atherosclerosis [1–4]. Modified, probably oxidized, LDL has been detected in human and animal lesions using immunohistochemistry [5–9]; auto-antibodies to oxidized LDL occur in patients with advanced atherosclerosis [10]; and lipophilic antioxidants inhibit atherogenesis in hyperlipidaemic rabbits [11–13] and primates [14].

The exact sites and mechanisms of LDL oxidation *in vivo* are still uncertain, although the consensus is that it occurs within the arterial lesions. Since the recognition of foam cells as macrophages [15–17], the view that macrophages are the source of most of the oxidation [18,19] has been supported by reports that monocytes and macrophages are capable of oxidizing LDL *in vitro* [20–25], including macrophage-derived foam cells isolated from rabbit atherosclerotic lesions [9].

Evidence of monocyte- or macrophage-mediated LDL oxidation has taken various forms, including increased electrophoretic mobility [21], the detection of thiobarbituric acid-reactive substances (TBARS) [21,22,26–28], increased uptake and degradation by target macrophages [21–27], appearance of lipid hydroperoxides [23,27] and toxicity for other cell-types [20] or for the macrophages themselves [28]. However, there are virtually no data on the chemical analysis of lipid oxidation products during LDL oxidation by macrophages.

The aim of this study is therefore to characterize the oxidized lipids produced during LDL modification by macrophages and to measure their production with time. The study also assesses the relationship of lipid oxidation to some of the other changes in modified LDL, namely the increase in negative charge of apolipoprotein B-100 (apo B-100), the protein moiety of LDL, and its degradation by macrophages. We also set out to compare copper oxidation of LDL with macrophage modification.

EXPERIMENTAL

LDL isolation and iodination

LDL (density 1.019–1.063 g/ml) was isolated from the pooled plasma of healthy volunteers (three different donors were used for each LDL preparation) by sequential density ultracentrifugation, and a portion of it labelled (within a week of isolation) with $Na^{125}I$ using an iodine monochloride method as described previously [27]. The iodinated LDL was mixed (immediately after labelling) with non-iodinated LDL to obtain a preparation of specific activity 30–60 c.p.m./ng protein and 3–6 mg protein/ml, which was used for the experiments. ^{125}I-LDL prepared by this method had the same electrophoretic mobility as the corresponding unlabelled native LDL from which it was derived.

A separate LDL preparation was used for each experiment. The LDL used in the preliminary experiment was 9 days old (completion of isolation to start of experiment), that in ex-

Abbreviations used: 7-KETO-CHOL, cholest-5-en-3β-ol-7-one; 7α-OH-CHOL, cholest-5-en-3β,7α-diol; 7β-OH-CHOL, cholest-5-en-3β,7β-diol; 26-OH-CHOL, cholest-5-en-3β,26-diol; Cu-Ox-LDL, copper-oxidized low-density lipoprotein; 3β,5α-DIOL, cholestan-3β,5α-diol; HETEs, hydroxyeicosatetraenoic acids; HODEs, hydroxyoctadecadienoic acids; LDL, low-density lipoprotein; MPM, mouse peritoneal macrophages; MPM-LDL, mouse peritoneal macrophage-modified low-density lipoprotein; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid-reactive substances; apo B-100, apolipoprotein B-100; DMEM, Dulbecco's modified Eagle's medium; TMSi, trimethylsilyl ether; BSTFA, *N,O*-bis(trimethylsilyl)-trifluoroacetamide.

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periment 1 was 4 days old, that in experiment 2 was 17 days old and that in experiment 3 was 4 days old.

Isolation of mouse peritoneal macrophages

Resident peritoneal macrophages were isolated and cultured from female Swiss T.O. mice (A. Tuck and Son, Battlesbridge, Essex, U.K.) [23]. The cells were plated in 22 mm diameter wells in 12-well cluster plates (Costar, from Northumbria Biologicals Ltd., Cramlington, Northumbria, U.K.) at 2×10^6 peritoneal cells per well (corresponding to approximately 1×10^6 macrophages per well) for the macrophages used to modify the LDL ('modifying' macrophages) and at 1×10^6 peritoneal cells per well for the macrophages that were to receive the modified LDL ('target' macrophages). The plated cells were incubated at 37 °C under 5% CO_2 for 2–4 h to allow macrophage adherence, then washed with Dulbecco's modified Eagle's medium (DMEM; Gibco) to remove lymphocytes before use in experiments.

Modification of LDL by macrophages or copper

^{125}I-labelled LDL or homologous unlabelled LDL (100 μg protein/ml) was incubated for up to 24 h (or 26 h in the case of the experiment in Figure 1) with modifying macrophages (macrophage-modified LDL), or with 5 μM or 25 μM $CuSO_4$ (copper-modified LDL), or in wells devoid of cells or $CuSO_4$ supplements (control LDL). The culture medium (1 ml per well) consisted of Ham's F-10 medium (Flow Laboratories Ltd., High Wycombe, Bucks., U.K.), supplemented with additional 3 μM $FeSO_4$, thus bringing the total $FeSO_4$ concentration to 6 μM, and gentamicin (50 μg/ml) (Gibco Ltd., Uxbridge, Middlesex, U.K.). The Ham's F-10 medium also had the Phenol Red level adjusted to 12 mg/l by addition of a Phenol Red solution (0.5% w/v) (Sigma Chemical Co., Poole, Dorset, U.K.). The basal $CuSO_4$ concentration in the medium as supplied was stated by the manufacturers to be 0.01 μM. After incubation for the specified time, the medium was collected, oxidation stopped by addition of 1 mM EDTA and 20 μM butylated hydroxytoluene, centrifuged (1500 *g* for 10 min at 4 °C) to remove any detached cells, and then stored at 4 °C until the end of the experiment and used for measurement of macrophage degradation (^{125}I-labelled LDL) and electrophoretic mobility (^{125}I-labelled LDL), or further processed for analysis of lipids and oxidized lipids (unlabelled LDL).

Measurement of modified LDL degradation by target macrophages

Modified or control ^{125}I-labelled LDL was diluted to 10 μg protein/ml with DMEM containing 10% (v/v) fetal calf serum (Gibco) and gentamicin (50 μg/ml). The LDL was then incubated for 20 h–24 h with 'target' macrophages or cell-free wells (1 ml of medium per well). Its rate of uptake was estimated by the measurement of the release of radioactive non-iodide trichloroacetic acid-soluble degradation products into the medium, using modification of the method of Drevon et al. [29], as previously described [27]. This method underestimates the rate of uptake of LDL [30], but is still a valid measure of a late stage of its oxidation.

Measurement of electrophoretic mobility

Agarose gel electrophoresis was carried out using pre-cast MULTITRAC agarose electrophoresis film [1 mm thick film; 1% (w/v) agarose/5% (w/v) sucrose/0.035% (w/v) EDTA in 65 mM barbitone buffer, pH 8.6, from Ciba-Corning, Sussex, U.K.] and 50 mM barbitone, 1 mM EDTA buffer, pH 8.6. The samples of LDL in a volume of 0.6 μl (i.e. 60 ng of protein) were applied to each well. The gel was then run at 90 V for 3 h. The agarose film was fixed by immersion in 1.2 M acetic acid for 5 min and dried thoroughly at 55 °C. The electrophoresis film was autoradiographed, using X-ray film, for 7 days. The film was developed, and the mobility was measured as the distance moved by the centre of the bands from the front edge of the well.

Processing of samples for gas chromatography and gas chromatography–mass spectrometry

The non-labelled LDL samples were dialysed against dialysis buffer (154 mM NaCl/16.7 mM NaH_2PO_4/21.1 mM Na_2HPO_4/100 μM Na_2EDTA, pH 7.4) (3 × 5 litres) over 24 h, and stored at −20 °C under nitrogen until analysis. The analytical work-up, described previously [31], essentially consisted of addition of internal standards (n-heptadecanoic acid, 5α-cholestane and coprostane), Bligh and Dyer extraction, sodium borohydride reduction (to convert hydroperoxides to the more stable alcohols; sodium borohydride also converts aldehydes and ketones to alcohols), saponification using potassium hydroxide (to convert esters to alcohols and acids), and derivatization using diazomethane (to convert carboxylic acids to methyl esters) followed by *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (to convert alcohols to trimethylsilyl ethers (TMSi)). After processing, samples were stored at −20 °C under nitrogen or argon until analysis by g.c. At all times, care was taken to minimize exposure of samples to air, and sodium borohydride reduction, saponification, derivatization and storage of samples were all carried out under nitrogen or argon.

For some samples, sodium borodeuteride (98 atom% ^{2}H) was used instead of sodium borohydride [to enable estimation of the proportion of the measured cholest-5-en-3β,7β-diol (7β-OH-CHOL) which arose from cholest-5-en-3β-ol-7-one (7-KETO-CHOL); see below].

Chemicals and cleaning of analytical glassware were as described previously [31,32]. Reagent blanks were run to check for contamination. In addition, medium blanks were run (i.e. culture medium which had been incubated for 24 h both with and without macrophages, in the absence of LDL) to check that no interfering substances were present.

Gas chromatography and gas chromatography–mass spectrometry

After the above work-up, g.c. analysis was performed for all the samples, using a 25 m OV-1 Crossbond fused-silica capillary column. G.c. conditions and instrumentation were as described previously [32]. Quantification of components was by peak areas measured electronically using an integrator, relative to internal standards. Repeat injections on g.c. of the same processed sample gave results which differed by less than 2% from the mean values of the amounts of the various components. Duplicate extraction and work-up of aliquots of the same sample gave results which differed by 10% or less from their mean value for components lower than 20 μg, and by 5% or less for components greater than 20 μg. Selected samples were analysed by g.c.–m.s. using a similar capillary column. G.c.–m.s. conditions and instrumentation were as described previously [31]. Standards for g.c. and g.c.–m.s. were as described previously [31,32]. In addition to these, authentic campesterol and β-sitosterol were obtained from Professor D. Kirk (Steroid Reference Collection, Queen Mary College, University of London, U.K.).

Abundance of m/z 457 relative to m/z 456 was measured on g.c.–m.s. using peak heights of the mass fragmentogram peaks for 7β-OH-CHOL for the samples which had been reduced with sodium borodeuteride. The same measurements were carried out on duplicate samples which had been reduced with sodium

borohydride. The ion m/z 456 and its deuterated analogue m/z 457 are the key ions for the TMSi ether derivatives of cholest-5-en-3β,7-diols, arising from elimination of TMSiOH from the molecular ion. During processing prior to g.c. and g.c.–m.s., any cholest-5-en-3β-ol-7-one present in the lipid extract will pick up a deuterium upon reduction with sodium borodeuteride, and will thus give m/z 457 on g.c.–m.s. Measurement of the enhancement in abundance of m/z 457 above its natural abundance, relative to m/z 456, enables the proportion of the measured cholest-5-en-3β,7β-diol which originates from cholest-5-en-3β-ol-7-one to be estimated.

RESULTS

Lipids and oxidized lipids

Representative g.c. traces for native LDL, control LDL and macrophage-modified LDL (MPM-LDL) are shown in Figure 1. The traces were plotted at a sensitive setting in order to display the oxidized lipids. As stated in the Experimental section, peak areas were measured electronically, so that although some of the peaks went off the top of the chart, their areas were accurately measured. Linoleic acid (18:2; peak 5 in Figure 1) in MPM-LDL (26 h) has fallen to 17.5% of its original level in native LDL. Arachidonic acid (20:4; peak 12 in Figure 1) in MPM-LDL (26 h) has fallen to 6% of its original level in native LDL. Copper-oxidized LDL (Cu-Ox-LDL) gave g.c. traces with similar features to those of MPM-LDL (results not shown). It should be noted that these lipids and oxidized lipids are the sum of the free and esterified forms; no information on esterification-state was sought in the present study. Precise determination of lag phases of the various parameters, which would necessitate many time-points over the first few hours, was not attempted, owing to the constraint of numbers of cells. Peak identifications were confirmed by g.c.–m.s. analysis of the samples in Figure 1, and also of 24 h samples of control LDL, MPM-LDL and Cu-Ox-LDL (both 5 μM Cu and 25 μM Cu) from Experiments 2 and 3 (see below). The main features of the g.c. and g.c.–m.s. results were as follows.

Oxysterols

The major oxysterol in both MPM-LDL and Cu-OX-LDL was identified as 7β-OH-CHOL (peak 22 in Figure 1) on the basis of coelution on g.c. with an authentic standard, and also its mass spectrum on g.c.–m.s. 7β-OH-CHOL rose over the 24 h time-courses, above the very low levels found in control LDL, after an initial delay of a few hours (Figure 2). This represents a conversion of 7%–19% of the cholesterol originally present (Figure 3) into 7β-OH-CHOL. Both macrophages and 25 μM copper produced 7β-OH-CHOL faster than 5 μM copper when run in parallel time-courses. 7β-OH-CHOL levels in all the batches of native LDL used in this study were either zero or trace (< 0.015 μg/100 μg of LDL protein). Similarly, in the zero-time samples (for MPM-LDL, Cu-Ox-LDL and control LDL) the 7β-OH-CHOL levels were either zero or trace (< 0.018 μg/ml; LDL protein concentration 100 μg/ml).

For MPM-LDL at 24 h, approximately 22% of the measured 7β-OH-CHOL was derived from 7-KETO-CHOL present in the original incubation products (i.e. prior to analytical work-up) as determined by substituting sodium borodeuteride for sodium borohydride in the reduction step of the processing of samples above, whilst for LDL oxidized by 5 μM $CuSO_4$ at 24 h approximately 16%, and for LDL oxidized by 25 μM $CuSO_4$ at 24 h approximately 28% of the measured 7β-OH-CHOL was derived from 7-KETO-CHOL.

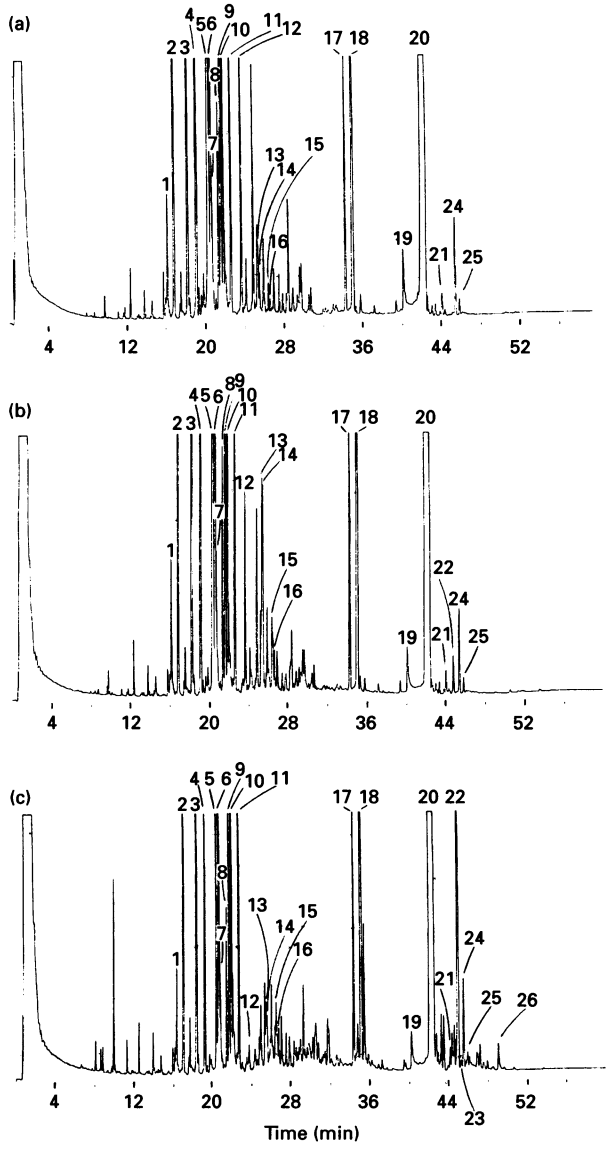

Figure 1 G.c. traces of lipids and oxidized lipids in (a) native LDL, (b) control LDL (no cells) (26 h) and (c) macrophage-modified LDL (MPM-LDL) (26 h)

The same amount was injected for all three traces. The attenuation setting was 16. OV-1 capillary column temperature programmed 65–120 °C at 8 °C/min, 120–200 °C at 4 °C/min, 200–280 °C at 3 °C/min, and held at 280 °C for 10 min. The injector temperature was 280 °C. The carrier gas was hydrogen. Peak identifications are as follows: 1 = 16:1 fatty acid methyl ester (FAME), 2 = 16:0 FAME, 3 = 16:0 fatty alcohol trimethylsilyl ether (FALTMSi), 4 = 17:0 FAME internal standard, 5 = 18:2 FAME, 6 = 18:1 FAME, 7 = 18:1 FAME isomer, 8 = 18:0 FAME, 9 = 18:2 FALTMSi, 10 = 18:1 FALTMSi, 11 = 18:0 FALTMSi, 12 = 20:4 FAME, 13 = HODE 1 methyl ester, trimethylsilyl ether (ME,TMSi), 14 = HODE 2 ME,TMSi, 15 = HODE 3 ME,TMSi, 16 = HODE 4 ME,TMSi, 17 = coprostane internal standard, 18 = 5α-cholestane internal standard, 19 = cholesterol unsilylated, 20 = cholesterol TMSi, 21 = campesterol TMSi, 22 = 7β-OH-CHOL TMSi, 23 = 3β,5α-DIOL TMSi*, 24 = unknown cholestandiol TMSi, 25 = β-sitosterol TMSi, 26 = 26-OH-CHOL TMSi*. Components designated * are tentative identifications based on g.c. only. For further details see text.

Cholest-5-en-3β,7α-diol (7α-OH-CHOL) was detected in MPM-LDL and Cu-Ox-LDL at about one-third of the level of 7β-OH-CHOL, but detailed quantification was not attempted because of its very close proximity to the large cholesterol peak.

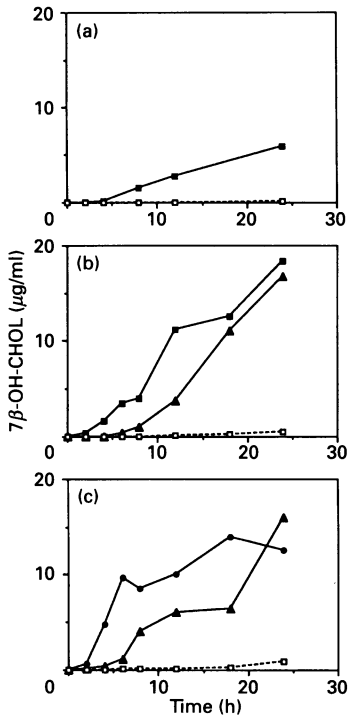

Figure 2 Levels of 7β-OH-CHOL (μg/ml of medium) plotted versus time (h) from incubations of LDL with MPM (■) or copper sulphate [5 μM (▲) or 25 μM (●)] or control (□) (no cells, no added copper sulphate) in Ham's F-10 medium (see Materials and methods section)

The LDL protein concentration in the medium was 100 μg/ml. A different batch of LDL was used for each of the experiments 1(**a**), 2(**b**) and 3(**c**). For each set of conditions at each time-point, the contents of at least three culture wells were pooled; each culture well contained 1 ml of medium and (where appropriate) approximately 1×10^6 MPM (2×10^6 peritoneal cells initially plated). For experiment 1(**a**), an aliquot of 4 ml of culture medium was used for each analysis (each data point); for experiments 2 and 3 (**b** and **c**), an aliquot of 2 ml of medium was used for each analysis (each data point). The results of a preliminary experiment (quantitative data not shown) in which LDL (from a separate batch) was incubated for a single time-point (26 h) with MPM and in cell-free wells (see Figure 1; all other conditions the same as in the above experiments), support the 24 h results shown here.

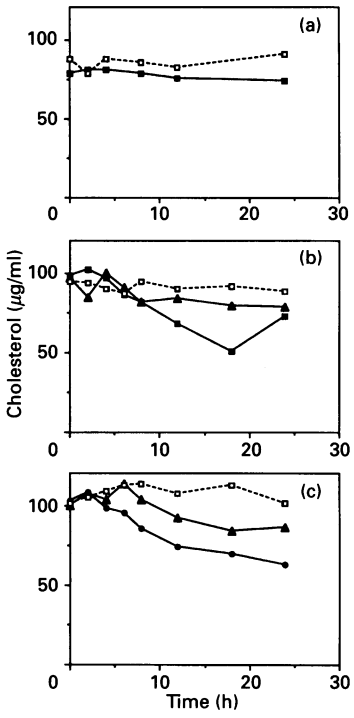

Figure 3 Levels of cholesterol (μg/ml of medium) versus time (h) for incubations of LDL with MPM (■) or copper sulphate [5 μM (▲) or 25 μM (●)] or control (□) (no cells, no added copper sulphate) in Ham's F-10 medium.

Further details are as in the legend to Figure 2.

7α-OH-CHOL is not visible in the example shown in Figure 1 because the cholesterol peak is so large.

A small peak tentatively assigned as cholest-5-en-3β,26-diol (26-OH-CHOL) (peak 26 in Figure 1) was detected, rising from negligible levels in native LDL and zero-time samples to no more than a few micrograms per ml by 24 h in the MPM-LDL and Cu-Ox-LDL samples. This assignment was based solely on g.c. evidence, as the peak coeluted with authentic 26-OH-CHOL, but was too weak to be confirmed by g.c.–m.s.

Other oxysterols, detected only in very small amounts, were an unknown compound (peak 24 in Figure 1), assigned as a cholestandiol on the basis of its mass spectrum (*m/z* 548, 458, 369, 329, 147, 129) [32], and a compound that may have been cholestan-3β,5α-diol (3β,5α-DIOL; peak 23 in Figure 1). The unknown cholestandiol appeared to be an artefact since it showed no significant trends with time and was also detected in native LDL and control LDL at comparable levels to those seen in MPM-LDL and Cu-Ox-LDL (Figure 1). The tentative 3β,5α-DIOL showed a slight increase over 24 h (results not shown) and might be a very minor oxidation product of LDL. However, 3β,5α-DIOL appears to be an artefact in atheroma and normal intima [32], where it is more abundant than in the present study, and is also accompanied by the unknown cholestandiol.

Cholesterol

Cholesterol levels (peak 20 in Figure 1) gradually fell over 24 h for MPM-LDL and Cu-Ox-LDL, but not significantly for control LDL (Figure 3). The fall was greater for MPM-LDL and Cu-Ox-LDL using 25 μM $CuSO_4$ than for Cu-Ox-LDL using 5 μM $CuSO_4$ in parallel time courses, thus mirroring the trends for 7β-OH-CHOL in these experiments (see above). The fall in cholesterol was roughly equal to the rise in 7-oxysterol, apart from Cu-Ox-CHOL using 25 μM $CuSO_4$, where the fall was greater than the 7-oxysterol production.

Hydroxyoctadecadienoic acids

Four g.c. peaks were identified as hydroxyoctadecadienoic acids (HODEs) (Figure 1, peaks 13–16). On g.c.–m.s., HODEs 1 and 2 eluted together as a single, slightly broadened peak. The HODE peaks showed the ions *m/z* 382, 311 and 225, which are characteristic of 9- and 13-HODEs [31]. Each HODE peak may represent more than one isomer. HODE 1 coeluted on g.c. with 13-hydroxyoctadeca-9,11-dienoic acid produced by soybean lipoxygenase-mediated oxidation of linoleic acid [31]. The HODEs were rather difficult to quantify because they are partially resolved peaks in a complex region of the chromatograms, but the following statements can be made. Individual

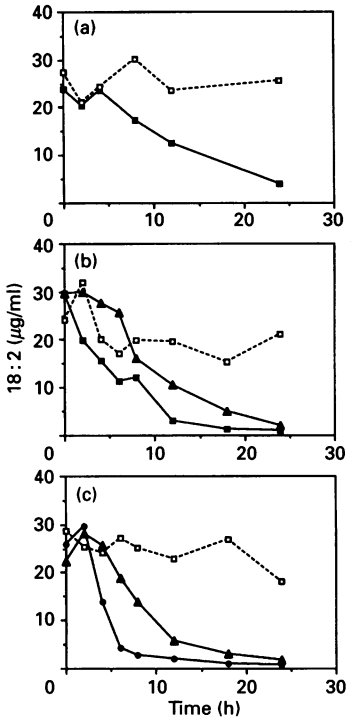

Figure 4 Levels of linoleic acid (18:2) (μg/ml of medium) versus time (h) for incubations of LDL with MPM (■) or copper sulphate [5 μM (▲) or 25 μM (●)] or control (□) (no cells, no added copper sulphate) in Ham's F-10 medium.

Further details are as in the legend to Figure 2.

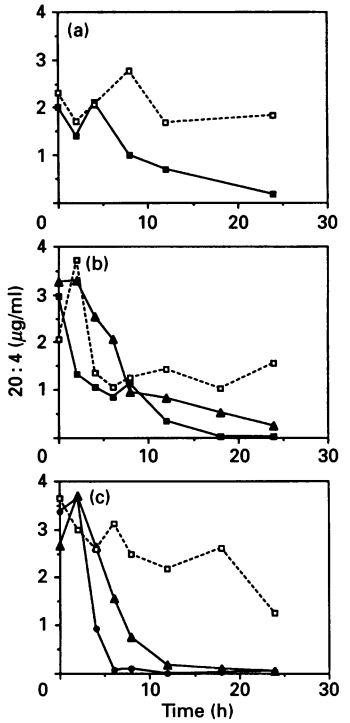

Figure 5 Levels of arachidonic acid (20:4) (μg/ml of medium) versus time (h) for incubations of LDL with MPM (■) or copper sulphate [5 μM (▲) or 25 μM (●)] or control (□) (no cells, no added copper sulphate) in Ham's F-10 medium

Further details are as in the legend to Figure 2.

HODE peaks varied in their behaviour; HODEs 1 and 2 were the most abundant and increased in MPM-LDL over the first few hours and then fell. HODE 3 showed somewhat erratic variation, and HODE 4, which was usually the least abundant, rose in MPM-LDL over 24 h, after an initial delay (data not shown). In control LDL, HODEs 1 and 2 rose after an initial delay, so that by 24 h they were more abundant than in MPM-LDL or Cu-Ox-LDL. HODE 4 rose in control LDL after a lag phase, although by 24 h it had risen less in control LDL than in MPM-LDL. In Cu-Ox-LDL, the behaviour of HODEs 1 and 2 in the early stages was somewhat variable, but they fell in the later stages. HODE 4 rose in Cu-Ox-LDL, after an initial delay; its behaviour thereafter was variable.

Hydroxyeicosatetraenoic acids (HETEs) were not found. Some minor peaks were seen in the region of the g.c. trace between the HODEs and coprostane, where HETEs, if present, would be expected to elute (Figure 1); however, by mass fragmentography on g.c.–m.s., the diagnostic ions for HETEs [33] were not present in significant amounts above background.

Linoleic acid and arachidonic acid

The concentrations of linoleic acid (18:2; peak 5 in Figure 1) and arachidonic acid (20:4; peak 12 in Figure 1) fell progressively over the 24 h (Figures 4 and 5) with a similar delay for the fall in 18:2 and 20:4 as for the rise in 7β-OH-CHOL. The relative order of the rate of their decline was the same as for the increase in 7β-OH-CHOL, so that 18:2 and 20:4 fell faster for MPM-LDL and LDL oxidized by 25 μM $CuSO_4$ than for LDL oxidized by 5 μM $CuSO_4$ in parallel time-courses. In control LDL, 18:2 and 20:4 generally fell much less.

Oleic and stearic acids

Oleic acid (18:1; peak 6 Figure 1), the major monounsaturate in LDL, and stearic acid (18:0; peak 8 in Figure 1) showed little significant variation in any of the time-courses, except for LDL oxidized by 25 μM $CuSO_4$, in which 18:1 fell somewhat over the 24 h time-course (data not shown).

Degradation by target macrophages

Increase in degradation by target macrophages of MPM-LDL and Cu-Ox-LDL followed the same trend over the 24 h time-courses as 7β-OH-CHOL formation, though sometimes the delay periods for the increased degradation by macrophages appears to be a few hours longer than those for 7β-OH-CHOL formation (Figure 6), although a precise comparison of the lag phases of the respective parameters would require a larger number of early time-points. Over the 24 h time-courses, control LDL showed no increase in degradation (Figure 6).

Mobility on agarose gel electrophoresis

The general trends for the increase with time of the mobility of LDL on agarose resembled those for the increase in 7β-OH-

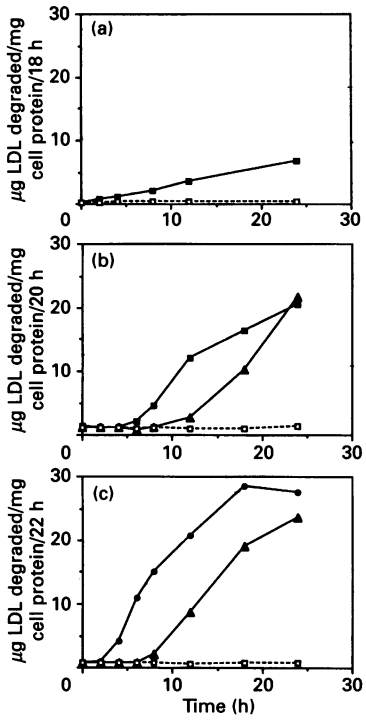

Figure 6 Amounts (μg) of ^{125}I-LDL protein subsequently degraded by 'target' macrophages, per mg cell protein, per 18 h (or 20 h or 22 h) versus time (h) for incubations of ^{125}I-LDL with MPM (■) or copper sulphate [5 μM (▲) or 25 μM (●)] or control (□) (no cells, no added copper sulphate) in Ham's F-10 medium (see Materials and methods section)

Each data point represents the mean of 3 wells. Standard deviations were less than 8% of the mean values. (**a**), (**b**) and (**c**) as in Figure 2.

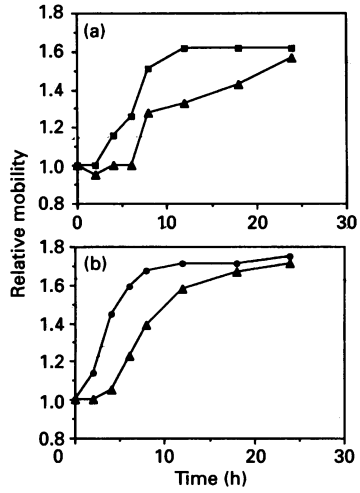

Figure 7 Relative mobilities (i.e. distance migrated by sample divided by distance migrated by control) on agarose gel electrophoresis of ^{125}I-LDL versus time (h) for incubations in Ham's F-10 medium of ^{125}I-LDL with MPM (■) or copper sulphate [5 μM (▲) or 25 μM (●)]; controls consisted of ^{125}I-LDL incubated in the same medium for the same time, but with no cells and no added copper sulphate

Mobility on agarose was measured for experiments 2(**a**) and 3(**b**) but not for experiment 1. The absolute distances migrated (mm) by controls showed little change for any of the timepoints. Each data point in (**a**) represents the distance migrated by one sample, divided by that migrated by the appropriate control. This is also true for (**b**), except that at 0 h and at 8 h, samples and controls were measured in duplicate, and for these the individual relative mobilities differed from their mean by 2% or less.

CHOL, but the increase in mobility appeared to plateau or began to slacken before that of the oxysterol (Figure 7), as judged within the limitations of the time-points sampled.

DISCUSSION

The most striking results of the experiments on both macrophage modification of LDL and copper-catalysed oxidation of LDL are the production of 7β-OH-CHOL, the concomitant fall in cholesterol, the diminution of the polyunsaturated fatty acids (PUFAs), the increase in uptake of the LDL by target macrophages and the increase in electrophoretic mobility on agarose. The significant cross-correlations of these parameters on regression analysis (Figure 8) strongly suggest that the phenomena may be related, though do not prove causality.

A possible explanation of this is as follows. The oxidation of the lipoprotein is probably initiated on the PUFAs; the pentadienyl and peroxyl radicals so formed would then be able to abstract a hydrogen atom from other lipids, including from the 7-position of cholesterol, creating a resonance-stabilized allyl radical, which could react with oxygen to result in the formation of 7-oxysterols. The polyunsaturated fatty acid peroxyl radicals would form hydroperoxides, which would be able to fragment to shorter-chain aldehydes, which would then react with the lysyl residues of apo B-100, thus diminishing their positive charge and so giving the protein an increased net negative charge. The modification of lysyl residues by these aldehydes would also be responsible for the uptake of the oxidized LDL by the scavenger receptors of macrophages. The cross-correlations in Figure 8 show little difference between macrophage modification of LDL and copper-catalysed oxidation of LDL as regards the parameters measured, although measurement of other features of LDL oxidation might reveal differences.

The cross-correlation graphs of 18:2 versus 7β-OH-CHOL and 18:2 versus LDL degradation (Figure 8) are negatively sloped exponentials ($r^2 = 0.910$ and $r^2 = 0.917$ respectively), in which the initial steep fall in 18:2 corresponds to modest increases in 7β-OH-CHOL and LDL degradation; as 18:2 falls to its lowest values, 7β-OH-CHOL and LDL degradation increase dramatically. This implies that 7β-OH-CHOL production and increased LDL degradation are secondary to the fall in 18:2, and indeed the graph of 7β-OH-CHOL versus LDL degradation is a positively sloped straight line ($r^2 = 0.866$). Arachidonic acid (20:4) behaves similarly to 18:2 in terms of its relationships with 7β-OH-CHOL, with LDL degradation and with relative mobility (data not shown). The fatty acid 20:4 falls slightly faster than 18:2, presumably because it is more unsaturated.

This study does not distinguish the states of esterification of the fatty acids but Noguchi et al. [34] have shown that the hydroperoxides of cholesterol esters are formed more rapidly than those of phosphatidylcholine in LDL oxidized by copper because the kinetic chain lengths of free radical-mediated oxidation are greater for cholesterol esters.

The graph of 18:2 versus relative mobility (Figure 8) is a negatively sloped straight line ($r^2 = 0.907$), indicating that the decrease in 18:2 is closely mirrored by the increase in LDL mobility, presumably due to modification of lysyl or other amino-acyl residues. The graph of relative mobility versus LDL degradation is a positive log relationship ($r^2 = 0.896$), i.e. an initial steep increase in relative mobility accompanies modest

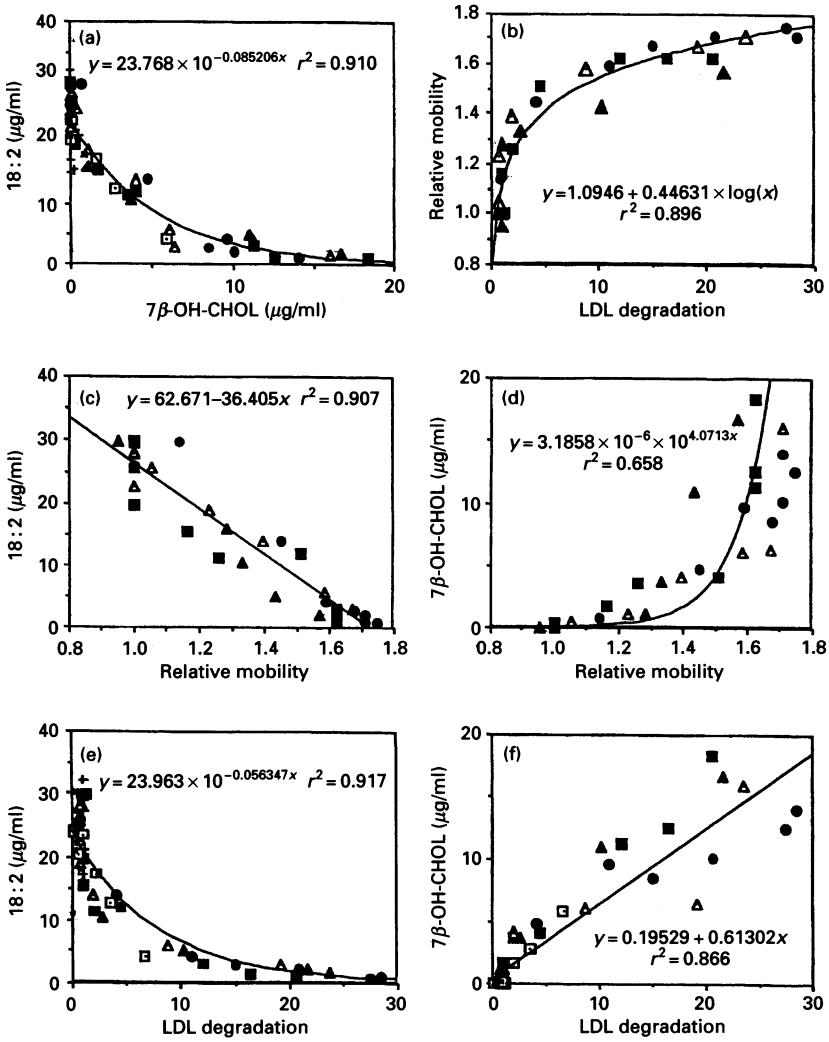

Figure 8 Correlation plots using data from Figures 2, 4, 6 and 7. □ = MPM-LDL, experiment 1; × = control LDL, experiment 1; ■ = MPM-LDL, experiment 2; ▲ = Cu-Ox-LDL (5 μM Cu^{2+}), experiment 2; + = control LDL, experiment 2; △ = Cu-Ox-LDL (5 μM Cu^{2+}), experiment 3; ● = Cu-Ox-LDL (25 μM Cu^{2+}), experiment 3; ✱ = control LDL, experiment 3.

As in previous figures, linoleic acid (18:2) and 7β-OH-CHOL are expressed as μg/ml of medium, LDL degradation is expressed as μg ^{125}I-LDL protein degraded by 'target' macrophages per mg of cell protein per 18 h (or 20 h or 22 h) and relative mobility is the distance migrated on agarose gel electrophoresis by ^{125}I-LDL incubated with MPM or copper sulphate (5 μM or 25 μM) divided by distance migrated by control ^{125}I-LDL incubated with no cells and no added copper sulphate. Curve fitting was performed using Cricket Graph software. Equations of fitted curves and correlation coefficients for the regressions are given on each graph; (**a**), (**e**) and (**f**) combined data from experiments 1, 2 and 3; (**b**), (**c**) and (**d**) combined data from experiments 2 and 3.

increases in LDL degradation, whilst further, more modest increases in mobility correspond to dramatic increases in LDL degradation. The graph of 7β-OH-CHOL versus relative mobility is a positively sloped exponential ($r^2 = 0.658$), indicating that small increases in 7β-OH-CHOL are associated with the initial large increases in relative mobility, whilst large increases in 7β-OH-CHOL are accompanied by further modest increases in mobility.

The slackening in rate of increase of LDL mobility with progressive oxidation (Figures 7 and 8) could be due to the increase in charge being offset by an increase in aggregation of the protein, thus limiting the rise in electrophoretic mobility, or simply to the consumption of PUFAs, so that the supply of aldehyde for modifying the protein was becoming limited. In contrast to LDL mobility, LDL degradation by macrophages (Figures 6 and 8) showed little slackening, perhaps suggesting that enhanced LDL degradation is the result of a more complex process than simple lysine modification.

There is little previous information about the lipid oxidation products of macrophage-mediated LDL oxidation, but experiments on Cu^{2+}-catalysed oxidation of LDL have shown falls in the PUFAs 18:2 and 20:4 relative to 18:0, accompanied by increased electrophoretic mobility and TBARS, in proportion to the concentrations of copper [35]. A less pronounced decline in PUFAs was found in LDL oxidized in oxygen-saturated buffer, without copper [36]. Lenz et al. [37], using Cu^{2+}-catalysed oxidation of LDL, demonstrated that, after a lag phase, decreases in 18:2 and 20:4 fatty acids were accompanied by production of their hydroxy and hydroperoxy derivatives; the derivatives of 18:2 being more abundant than those of 20:4. Wang et al. [38]

reported hydroxy derivatives of 18:2, 20:4 and 18:1 fatty acids in Cu^{2+}-catalysed oxidation of LDL and in endothelial cell-modified LDL. In copper-oxidized LDL, the 18:1, 18:2 and 20:4 fatty acids fell markedly, but for the endothelial cell-modified LDL little fall was apparent, suggesting that LDL modification by endothelial cells is a milder process [38].

Previous information on oxysterol production in oxidized LDL is rather sparse. Bhadra et al. [39] found cholesterol-α-epoxide after endothelial cell modification of LDL, and cholest-3,5-dien-7-one from Cu^{2+}-oxidized LDL, but no time-courses were reported. Jialal et al. reported 7-KETO-CHOL as the main oxysterol product of Cu^{2+} oxidation of LDL; 7-KETO-CHOL rose over 20 h, and showed a lag phase in only one of ten batches of LDL [40]. They also reported that 7-KETO-CHOL was the main oxysterol produced by monocyte-macrophage modification of LDL in Ham's F-10 medium; the only quantitative results given were that in two experiments, 20% and 32% of the total LDL cholesterol were converted in 24 h into 7-KETO-CHOL. 7-KETO-CHOL (plus smaller amounts of 7-hydroxy and other oxysterols) was also reported by Zhang et al. [41] as a product of Cu^{2+} oxidation of LDL. Malavasi et al. reported that copper-oxidized LDL contained 7-KETO-CHOL, 7α- and 7β-hydroperoxycholesterols, 7α-OH-CHOL and 7β-OH-CHOL [42]. The concentrations of the hydroxycholesterols increased as that of the hydroperoxycholesterols decreased. The main oxysterols in LDL oxidized by t-butyl hydroperoxide/Fe^{2+} have been reported as 7-KETO-CHOL, 7α-OH-CHOL and 7β-OH-CHOL and 5α,6α- and 5β,6β-epoxycholestanols [43]. The latter study also shows the conversion of cholesterol esters to aldehydes, cholesteryl linoleate to 9-oxononanoates of cholesterol and cholesteryl arachidonate mainly to 5-oxovalerates of cholesterol. Recently Kritharides et al. [44] have used h.p.l.c. and m.s. to characterize copper-oxidized LDL and have found there was a transient rise of cholesteryl linoleate hydroperoxides followed by a sustained rise in 7-KETO-CHOL and cholesteryl linoleate hydroxides, but the position of the hydroxide group on the cholesteryl linoleate was unknown. In contrast to our results, apparently more 7-KETO-CHOL than cholesterol ester hydroxide was formed. Our own results suggest that the ratio of 7-KETO-CHOL to 7β-OH-CHOL increases with the severity of the oxidation.

The idea that oxidation of PUFAs leads to oxidation of cholesterol at the 7-position is supported by previous results from in vitro incubations of artificial lipoproteins (each consisting of a single lipid species emulsified with BSA) with human monocyte-macrophages, as a result of which 7β-OH-CHOL, 7α-OH-CHOL, and 7-KETO-CHOL were produced from cholesteryl linoleate but not from cholesteryl oleate or from cholesterol [31]. Also supporting this idea are the findings of Teng and Smith, who found 7-oxysterols (7α- and 7β-hydroperoxycholesterols which slowly decomposed to give 7α-OH-CHOL, 7β-OH-CHOL and 7-KETO-CHOL) produced by co-oxidation of a mixture of ethyl linoleate and cholesterol, using soybean lipoxygenase [45]. When ethyl linoleate was omitted, no 7-oxysterols were produced. Further work on a similar system revealed evidence that the pentadienyl radical of the fatty acid was at least partially responsible for abstracting the hydrogen at the 7-position of cholesterol, although hydrogen abstraction by the fatty acid peroxyl radical was also believed likely [46].

The present findings on lipid oxidation in LDL oxidation are thus in general agreement with previous reports showing falls in polyunsaturated fatty acids and production of 7-oxysterols. In addition our experiments show that these transformations occur in a concerted fashion, that they correlate closely with transformation of the protein moiety as demonstrated by increased mobility on agarose and increased degradation by macrophages, and moreover they suggest that, *in vitro*, the chemistry of macrophage modification and copper oxidation of LDL have much in common.

The results of the present study are further supported by those of time-course experiments involving oxidation of LDL using Cu^{2+} in PBS (K. L. H. Carpenter, S. E. Taylor, C. van der Veen and M. J. Mitchinson, unpublished work). Similar trends to those of the present study were seen for the behaviour of 18:2, 20:4 fatty acids, 7β-OH-CHOL and electrophoretic mobility. Modified ^{125}I-LDL degradation by macrophages was not measured. However, anion-exchange f.p.l.c. and fluorescence spectroscopy supported the other measurements. A difference apparent between the results of these experiments in the PBS and those of the present study in Ham's F-10 was that the onset of oxidation was quicker in the former than the latter, being clearly apparent at 2 h as judged by all the techniques used. The results of the present study are also supported by time-courses of macrophage modification of LDL and of Cu^{2+} oxidation of LDL, in which LDL modification was assessed by degradation of modified ^{125}I-LDL by macrophages [24].

The oxysterols 7β-OH-CHOL and 26-OH-CHOL have been detected in the necrotic gruel from human advanced plaques, but were present in negligible amounts in normal, lesion-free intima [32]. 7β-OH-CHOL is the main oxysterol product of LDL oxidation in the present study, but there is very little 26-OH-CHOL production. In the liver, 26-OH-CHOL is produced by the cytochrome P450 sterol 26-hydroxylase, an enzyme which has also been found in various other tissues [47,48]; 26-OH-CHOL is not an auto-oxidation product of cholesterol [49,50]. 26-OH-CHOL in lesions is therefore probably an enzymic product [32]. Cytochrome P450 enzymes decline rapidly in cells *in vitro* [51], possibly explaining why 26-OH-CHOL levels are so low in macrophage-modified LDL in the present study. In contrast to 26-OH-CHOL, 7-oxysterols can be produced both biologically and abiologically, since, as explained above, hydrogen abstraction, by whatever means, from the 7-position of the cholesterol molecule produces a resonance-stabilized allyl radical. 7β-OH-CHOL (accompanied by 7α-OH-CHOL and 7-KETO-CHOL) has previously been detected as a product of oxidation of cholesteryl linoleate in the form of an artificial lipoprotein by human monocyte-macrophages *in vitro* [31].

Oxidized LDL is cytotoxic [20,28,52] and the cytotoxicity is associated with the lipid fraction [52]. Many oxysterols, including the 7-oxysterols and 26-OH-CHOL, are toxic [53] and it is possible that these compounds are at least partially responsible for the necrosis seen in advanced atherosclerotic plaques [28]. Oxidized LDL is now known to have other diverse effects on cells [1–4] and it is conceivable that some of these also may be mediated by various species of oxysterols.

The results of oxidation *in vitro* of LDL by macrophages and by copper thus provide some insight into the processes which might occur within atherosclerotic lesions. The remarkable similarity between copper- and macrophage-mediated LDL oxidation *in vitro* suggests that macrophages can catalyse the oxidation by redox cycling of transition metals, perhaps by a process involving cysteine [54]. However, the virtual absence *in vitro* of the production of 26-OH-CHOL, an oxysterol found in atheroma [32], hints that additional pathways may operate *in vivo*. Complete understanding of the mechanisms of LDL oxidation *in vivo* will require further investigation. The model described for *in vitro* studies here may be appropriate for exploring free radical processes which appear to cause profound changes to LDL, which may well be important in the contribution of macrophages to the progression of atherosclerotic lesions.

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