Macrophage-enhanced formation of cholesteryl ester–core aldehydes during oxidation of low density lipoprotein

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Abstract Oxidation of low density lipoproteins (LDL) results in changes to the lipoprotein particle that are potentially pro-atherogenic. To investigate mechanisms contributing to the formation of cholesteryl ester (CE)–core aldehydes (9-oxononanoyl- and 5-oxovaleroyl-cholesterol; 9-ONC and 5-OVC, respectively) LDL was incubated in the presence of mouse macrophages (J774 cells) under different culture conditions. Here we demonstrate that the formation of core aldehydes occurs only in transition metal-containing HAM's F10 medium but not in Dulbecco's modified Eagle's medium (DMEM), independent of supplementation with iron and copper at concentrations up to ten times higher than present in HAM's F10. The antioxidative properties of DMEM could be ascribed to the higher amino acid and vitamin content as compared to HAM's F10 medium. Supplementation with these components efficiently inhibited LDL oxidation in HAM's F10. Stimulation of J774 cells with phorbol ester (PMA) resulted in significantly enhanced 9-ONC and 5-OVC formation rates that were accompanied by increased consumption of LDL cholesteryl linoleate (Ch18:2) and cholesteryl arachidonate (Ch20:4) in the cellular supernatant. In PMA (10 ng/ml) activated cells, approximately 5% of Ch18:2 contained in LDL was converted to 9-ONC and 4% of Ch20:4 was converted to 5-OVC. With respect to core aldehyde formation, lipopolysaccharide (LPS, 10 μg/ml) was a less effective stimulant as compared to PMA. Part of the core aldehydes accumulated within the cells. Our study demonstrates that *i*) J774 macrophages are able to promote/accelerate core aldehyde formation in HAM's F10 medium, and *ii*) that core aldehyde formation rates can be increased by stimulation of the cells with PMA, and, although to a lesser extent, with LPS. Finally we could show that *iii*) a small amount of the core aldehydes is internalized by J774 macrophages.—Karten, B., H. Boechzelt, P. M. Abuja, M. Mittelbach, and W. Sattler. **Macrophage-enhanced formation of cholesteryl ester–core aldehydes during oxidation of low density lipoprotein.** ***J. Lipid Res.*** **1999.** 40: **1240–1253.**

Supplementary key words transition metals • J774 cells • lipopolysaccharide • phorbol-12-myristate-13-acetate

Oxidative modification of low density lipoproteins (LDL) results in numerous changes to the lipoprotein particle that are potentially pro-atherogenic (1). Depending on the nature of the oxidant, LDL oxidation might be a sequential process, with oxidation of the lipid moiety preceding the oxidation/modification of the apoprotein domain (2), an effect which can ultimately result in uncontrolled uptake of these lipoproteins by different cells (3). Most of the cell types present in the intima can promote the oxidation of LDL in vitro, however, the presence of transition-metals in the culture medium appears to be an absolute requirement for cell-mediated oxidation of lipoproteins (4). The mechanisms by which cells oxidize LDL in the presence of transition metals are not entirely clear. It appears, however, that the cells can accelerate ongoing metal-catalyzed LDL oxidation. This ability is (at least in part) due to redox cycling of transition metals by the action of trans plasma membrane electron transport (TPMET)-system(s) (5). Recently Garner and colleagues (6) provided evidence that macrophages of human and mouse origin are able to promote direct copper reduction by the action of a TPMET-system, thus accelerating metal-dependent LDL oxidation in vitro.

Abbreviations: AA, amino acids; BCS, bathocuproine disulfonic acid; BHT, butylated hydroxy toluene; CE, cholesteryl ester; Ch18:2, cholesteryl linoleate; Ch20:4, cholesteryl arachidonate; Ch(O)OH, cholesteryl ester hydro(pero)xide; CHD, 1,3-cyclohexanedione; cyt C, cytochrome C; DNPH, dinitrophenyl hydrazine; DTNB, 5,5′-dithiobis(nitrobenzoic acid); EDTA, ethylene diamino tetraacetic acid; FCS, fetal calf serum; GC-FID, gas chromatography–flame ionization detection; HBSS, Hank's balanced salt solution; HDL, high density lipoprotein; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; LPDS, lipoprotein-deficient serum; LPO, lipid peroxidation; LPS, lipopolysaccharide; 9-ONC, 9-oxononanoyl cholesterol; 5-OVC, 5-oxovaleroyl cholesterol; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate-13-acetate; PL, phospholipid; PUFA, polyunsaturated fatty acid; REM, relative electrophoretic mobility; SOD, superoxide dismutase; SPE, solid-phase extraction; TBARS, thiobarbituric acid-reactive substances; TCA, trichloroacetic acid; TEP, tetraethoxy propane; TPMET, trans plasma membrane electron transport; Vit, vitamins.

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Lipid peroxidation (LPO) leads to the formation of a broad array of different products with diverse and powerful biological activities, among them are a variety of different aldehydes (7). The primary products of LPO, lipid hydroperoxides (8), can undergo carbon–carbon bond cleavage via alkoxyl radicals in the presence of transition metals giving rise to the formation of short-chain, unesterified aldehydes (8, 9), or a second class of aldehydes still esterified to the parent lipid. These esterified aldehydes are commonly termed core aldehydes (10). Although these compounds have received less attention, the formation of phospholipid (PL)– and cholesteryl ester (CE)–core aldehydes during oxidation of LDL has been demonstrated (11, 12). Due to their structural similarity to platelet activating factor (PAF) and their rather hydrophilic nature, PL–core aldehydes display a number of PAF-related biological activities. The properties of oxidized phospholipids in relation to atherosclerosis have been reviewed recently (10).

In contrast to PL–core aldehydes, much less is known about the formation, metabolism, and biological properties of CE–core aldehydes. Kamido and colleagues (12) have isolated and identified C5 (5-oxovaleroyl cholesterol, 5-OVC) and C9 (9-oxononanoyl cholesterol, 9-ONC) CE–core aldehydes from copper-oxidized LDL. 9-ONC was shown to be derived from oxidation of cholesteryl linoleate while cholesteryl arachidonate was the 5-OVC precursor (13). Oxidation of HDL resulted in the formation of primarily 5-OVC and 9-ONC and, although to a lesser extent, the corresponding 7-ketocholesterol derivatives (14). In line with a potential pathophysiological role of these compounds, CE- and 7-ketoCE–core aldehydes of varying chain length were identified in human atheromas (15, 16).

Due to this finding and the fact that almost all cell types present in plaques are able to promote LDL oxidation in vitro, we have investigated whether the major CE–core aldehydes (i.e., 9-ONC and 5-OVC) could be generated during cell-mediated oxidation of LDL and serve as a sensitive marker for cell-mediated oxidation. In the present study we have used mouse macrophages (J774 cells) cultured in transition metal-containing (HAM's F10) and transition metal-free (DMEM) medium. Another intention of our study was to clarify whether activation of J774 cells by phorbol ester (PMA) or lipopolysaccharide (LPS) affects their ability for core aldehyde formation.

MATERIALS AND METHODS

Chemicals

Chemicals were obtained from Sigma (Vienna, Austria) or Merck (Vienna, Austria) unless indicated otherwise. LPS from *Escherichia coli*, cytochrome C from horse heart, Cu/Zn-superoxide dismutase (SOD), phorbol-13-myristate-12-acetate (PMA), amino acids, and vitamins (cell culture tested) were obtained from Sigma. Solid-phase extraction cartridges were obtained from Isolute (ICT, Vienna, Austria). 1,3-Cyclohexanedione (CHD; stabilized with 3% w/w NaCl) was purchased from Sigma and used without further purification. EconoPac size exclusion columns were obtained from BioRad (Vienna, Austria). Cell culture material was obtained from Costar (Acton, MA); media were from Boehringer Ingelheim (Vienna, Austria), and phenol red-free DMEM was obtained from Sigma.

Synthesis of cholesteryl ester–core aldehydes

9-Oxononanoyl cholesterol (9-ONC) was prepared by ozonolysis of oleic acid and subsequent condensation of the resulting 9,9-dimethoxy nonanoic acid with cholesterol in the presence of N,N′-dicyclohexyl-carbodiimide (DCC) as described (17). 5-Oxovaleroyl cholesterol (5-OVC) was produced by ozonolysis of cyclopenten, alkaline cleavage of the resulting ozonide and DCC-catalyzed esterification with cholesterol (H. Boechzelt and M. Mittelbach, manuscript in preparation).

LDL isolation

Plasma from overnight fasted normolipemic donors was obtained by venipuncture with EDTA as anticoagulant (1 mg/ml). LDL was prepared using discontinuous gradient ultracentrifugation in a Beckman SW41 rotor (40,000 rpm, 22 h, 10°C) as described (18). LDL was recovered by direct syringe aspiration, filtered through a 0.2 m sterile filter (Corning Glass Works, Corning, NY), transferred to evacuated glass vials (Techne Vial, Mallinckrodt-Diagnostica, Vienna, Austria), and stored under argon at 4°C in the dark. Prior to oxidation experiments, excess KBr and EDTA were removed from LDL by size exclusion chromatography on EconoPac columns. Aliquots of LDL tested were negative for endotoxin in *Limulus* amebocyte lysate assay (BioWhittaker, sensitivity 0.06 EU/ml).

Cell culture

J774 macrophages were grown to confluency in 6-well (oxidation experiments) or 12-well (copper reduction, superoxide production and thiol release) cell culture dishes in RPMI 1640 medium supplemented with fetal calf serum (FCS; 10%, v/v). Prior to the experiment, cells were incubated in RPMI 1640 medium containing lipoprotein-deficient serum (LPDS; 10%, v/v; 15 h), then washed twice with PBS before adding HAM's F10 or Hanks balanced salt solution (HBSS). Prior to some oxidation experiments, cells were preincubated in FCS-containing RPMI 1640 medium (10%, v/v; 15 h). Where indicated, cells were activated with PMA (10 ng/ml) or LPS (10 μg/ml). Control incubations without added stimulant as well as cell-free controls were carried out in parallel. All incubations were performed in triplicate.

Cell-mediated oxidation of LDL

J774 cells were grown to confluency as described above. Confluent cells were then incubated in HAM's F10 or DMEM (1 ml/well) in the presence or absence of PMA (20 ng/ml medium) or LPS (20 μg/ml medium). After 4 h, oxidation experiments were initiated by the addition of LDL in HAM's F10 or phenol red-free DMEM (1 ml; 0.5 mg LDL total mass). At the time points indicated, plates were put on ice and the medium was removed and centrifuged (2 min at 13,000 rpm). The supernatant was mixed with EDTA (20 μl, 100 mg/ml) and butylated hydroxy toluene (BHT; 20 μl, 2 mg/ml in ethanol) and kept under nitrogen at −80°C until analysis. The cellular lipids were extracted with 2 ml hexane–2-propanol 3:2 (v/v) (19) containing BHT (20 μg/ml) on an orbital shaker (25 min at room temperature). Lipid extracts were kept under nitrogen at −80°C until further analysis. After evaporation of the solvent, the dry residue was redissolved in toluene (750 μl) containing heptadecanoic acid (25 μg as internal standard for fatty acid analysis); 250 μl of this solution was used for 9-ONC analysis and 500 μl was used for fatty acid analysis. The cellular protein content was determined according to Lowry et al. (20) in the NaOH lysate (0.3 N, 2 ml) of the adherent cellular residue re-

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maining after hexane–2-propanol extraction. The average protein content on 6-well trays was 0.85 mg/well.

Nutrient supplementation of HAM's F10

In comparison to DMEM, HAM's F10 has a lower content of certain amino acids and water-soluble vitamins. To evaluate the effects of these compounds with regard to cell-mediated oxidation of LDL, HAM's F10 was supplemented to reach equal or higher (5- or 10-fold) concentrations of amino acids and vitamins as present in DMEM. Subsequently, oxidation experiments were performed in these supplemented media.

HAM's F10 medium was supplemented as follows: *a*) amino acids: 438 mg glutamine, 22.49 mg glycine, 21 mg histidine, 102.4 mg isoleucine, 91.7 mg leucine, 116.7 mg lysine, 25.52 mg methionine, 61.52 mg phenylalanine, 31.5 mg serine, 91.43 mg threonine, 15.4 mg tryptophane, 101.18 mg tyrosine, and 90.5 mg valine were dissolved in 200 ml HAM's F10 to constitute a medium 5-fold concentrated as compared to DMEM; *b*) vitamins: 33.02 mg choline chloride, 26.8 mg folic acid, 66.59 mg myoinositol, 33.58 mg niacinamide, 32.85 mg calcium pantothenate, 37.94 mg pyridoxal hydrochloride, 2.4 mg riboflavin, and 30 mg thiamine hydrochloride were dissolved in 100 ml of HAM's F10 medium to constitute a medium 100-fold concentrated as compared to DMEM. These stock solutions were diluted with HAM's F10 to reach an equal, 5- or 10-fold higher amino acid and vitamin content as DMEM.

Cell-mediated oxidation in HAM's F10 supplemented with amino acids and/or vitamins

J774 cells were grown to confluency as described above, incubated in LPDS-containing RPMI 1640 (10%, v/v; 15 h) and washed with PBS prior to the addition of LDL (0.25 mg/ml) in HAM's F10 (2 ml) supplemented with the indicated concentrations of amino acids and/or vitamins. J774-mediated LDL oxidation was also performed in DMEM containing $CuSO_4$ (10 or 100 nm) and $FeSO_4$ (3 or 30 μm). To investigate possible effects of preconditioning in LPDS-containing medium, J774 macrophages were cultivated in either LPDS- or FCS-containing RPMI 1640 (15 h) prior to the oxidation experiments. LDL was then coincubated with J774 cells in HAM's F10 as described above. All incubations were carried out at 37°C for 10 h, then plates were put on ice and samples were prepared as described above.

Analytical procedures

Fatty acid analysis of the cellular supernatant. Medium samples (1 ml, spiked with 20 μg heptadecanoic acid as internal standard) were lyophilized and transmethylated with 1 ml MeOH containing BF_3 (20%, v/v; BF_3/MeOH) in toluene (500 μl) at 110°C for 60 min. For transmethylation of cellular lipids, 500 μl of cellular lipids in toluene (containing 25 μg heptadecanoic acid as internal standard) was mixed with 1 ml BF_3/MeOH and transmethylated as described above. Separation of fatty acid methyl esters (2 μl samples) was performed as described (21). Briefly, methyl esters were analyzed on a WCOT fused silica 25 m FFAP-CB column (0.32 mm I.D.; Chrompack, Austria) using an HP 5890 gas chromatograph equipped with a flame ionization detector and a split/splitless injector (Hewlett-Packard Co., Austria). Helium was used as carrier gas; the split ratio was about 10:1. The temperature was programmed from 150°C to 215°C at 2.5°/min with a hold for 10 min then to 250°C at 10°/min and a hold for 12.5 min. Concentrations of the individual fatty acids were calculated by peak area comparison with the internal standard.

Core aldehyde analysis. CE–core aldehyde analysis was performed as described (16). Eight hundred μl of medium was extracted twice with 1.75 ml of hexane–2-propanol 3:2 (v/v). The organic phase was dried under vacuum and redissolved in 800 μl CH_3Cl–MeOH 2:1 (v/v). Three hundred μl was dried and mixed with 40 μl CH_3Cl–MeOH 2:1 (v/v) and 20 μl CHD reagent (100 mg/ml 1,3-CHD in 20% acetic acid–ethanol 1:1 (v/v)). After incubation for 70 min at 70°C, the derivatization mixture was diluted with 1 ml acetonitrile and applied onto a solid-phase extraction cartridge (Isolute MF C18) equilibrated with acetonitrile. The cartridge was washed with 2 ml of acetonitrile and 125 μl 2-propanol and eluted with 3 ml chloroform. The eluate was dried under vacuum and redissolved in 30 μl CH_3Cl–MeOH 2:1 (v/v) and 120 μl CH_3CN–MeOH–2-propanol 68:17:15 (v/v/v). Five μl was injected and analyzed on a SupelcoSil LC18 column (250 × 2.1 mm) with a solvent system of CH_3CN–MeOH–2-propanol 68:17:15 (v/v/v) at a flow rate of 0.3 ml/min and fluorescence detection (366 nm excitation, 455 nm emission). Quantitation was performed by peak area comparison with external standards. 9-ONC and 5-OVC in cellular lipid extracts were determined by drying 250 μl cellular lipid extract under vacuum, derivatization with 20 μl CHD reagent in 40 μl CH_3Cl–MeOH 2:1 (v/v) and subsequent HPLC analysis as described above. The limit of quantitation of the method described is 50 fmol; the absolute limit of detection (signal: noise = 6) is about 15 fmol for both 9-ONC and 5-OVC (16).

Cholesteryl ester analysis. CE analysis was performed as described (22). Five hundred μl of the medium lipid extracts was dried under vacuum and redissolved in 100 μl MeOH–2-propanol 1:1 (v/v). Fifteen μl of this solution was analyzed on a SupelcoSil LC18 column (250 × 2.1 mm) in a solvent system of MeOH–2-propanol 1:1 (v/v) at a flow rate of 0.2 ml/min and UV-detection (210 nm). Quantitation was performed by peak area comparison with external standards.

Relative electrophoretic mobility (REM). The relative electrophoretic mobility of LDL in the medium was determined on precasted agarose gels (LipoGel, Beckman, Austria) (21).

TBARS. For determination of the thiobarbituric acid reactive substances (TBARS; ref. 23), 300 μl of the medium was mixed with 20% TCA (300 μl) and kept on ice for 5 min. After centrifugation (5 min; 13,000 rpm; 4°C), 500 μl of the supernatant was mixed with thiobarbituric acid (500 μl) and incubated for 15 min at 95°C. The absorption at 532 nm was measured within 15 min against water. TBARS concentration was calculated by comparison with an external calibration curve obtained from tetraethoxy propane (TEP) solutions.

LDL oxidation in transition metal-supplemented DMEM and PBS

LDL (0.25 mg/ml) was incubated on 6-well cell culture plates in DMEM (phenol red-free) or PBS containing $CuSO_4$ (10 nm) and $FeSO_4$ (3 μm) or HAM's F10. All solutions were adjusted to a pH of 7.4. After 23 h, oxidation was stopped by the addition of 20 μl EDTA (100 mg/ml) and 20 μl BHT (2 mg/ml in ethanol). One ml of the oxidation mixture was mixed with heptadecanoic acid (20 μg), lyophilized, and analyzed for its fatty acid content by transmethylation and subsequent GC analysis of the respective fatty acid methyl esters as described above. Eight hundred μl of the oxidation mixture was extracted twice with 1.75 ml of hexane–2-propanol 3:2 (v/v) and analyzed for core aldehyde content.

Copper reduction, superoxide production, and thiol release by J774 macrophages

Following a commonly used protocol for copper reduction measurement (6), cells were incubated in 1 ml HBSS containing $CuSO_4$ (50 μm), bathocuproine disulfonic acid (BCS, 125 μm), and PMA (10 ng/ml) or LPS (10 μg/ml). At the time points indicated, 1 ml buffer was removed, centrifuged (2 min at 13,000 rpm, 4°C) and the absorbance was measured at 482 nm against a

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solution of BCS (250 μm) in HBSS. Copper concentration was calculated using an absorption coefficient of $\varepsilon_{482\ nm}$ = 12.154 $M^{-1}cm^{-1}$ for the Cu(I)-BCS complex (6).

To evaluate the effect of BCS in the incubation mixture, copper reduction was also determined in the absence of BCS. Cells were incubated in 1 ml HBSS containing $CuSO_4$ (50 μm) and PMA (10 ng/ml) or LPS (10 μg/ml). At the time points indicated, 1 ml buffer was removed, mixed with 25 μl BCS (5 mm) and centrifuged for 2 min at 13,000 rpm at 4°C. The absorption at 482 nm was measured and copper concentrations were calculated as described above.

For superoxide determination, cells were incubated in 1 ml HBSS containing cytochrome C (80 μm) and PMA (10 ng/ml) or LPS (10 μg/ml) as described (24). Parallel incubations were carried out in the presence of horse heart Cu/Zn–superoxide dismutase (SOD; 20 μg/ml). At the time points indicated, 1 ml buffer was removed and centrifuged (2 min at 13,000 rpm). Cytochrome C reduction was measured at 550 nm and corrected for SOD-inhibitable cytochrome C reduction, using an absorption coefficient of $\varepsilon_{550\ nm}$ = 50,000 $M^{-1}cm^{-1}$ for $cytc^{3+}$.

For thiol release measurement, cells were incubated up to 8 h in 1 ml HBSS containing PMA (10 ng/ml) or LPS (10 μg/ml) essentially as described (6). At the time points indicated, 1 ml buffer was removed, centrifuged (2 min at 13,000 rpm) and assessed for thiol content by the DTNB method. Two hundred μl of the supernatant was mixed with 750 μl reaction solution (200 mm Na_2HPO_4, 20 mm EDTA, pH 8) and 50 μl DTNB solution (4 mm DTNB, in 50 mm Na_2HPO_4/NaH_2PO_4 buffer, pH 7) and incubated for 30 min at 37°C. Absorption was measured at 412 nm against HBSS. Calibration was carried out with cysteine solutions in a concentration range of 2–32 μm.

The cellular protein content on 12-well plates was 0.33 ± 0.06 mg/well (mean ± SD) of all experiments.

Miscellaneous

All oxidation experiments were carried out at a final LDL concentration of 0.25 mg/ml LDL total mass, corresponding to a molar concentration of approx. 0.1 μm. Rates are expressed as the difference of concentrations at two time points (Δc/Δt). Statistical significance was calculated by the Student's *t*-test of independent samples. Significance was denoted as $P < 0.05$.

RESULTS

Formation of 9-ONC and 5-OVC by unactivated J774 macrophages

To assess whether and under which conditions J774 macrophages are able to promote formation of CE–core aldehydes during 'cell-mediated oxidation' of LDL, we initially examined the formation of 5-OVC and 9-ONC in HAM's F10 (a transition metal-containing and thus 'oxidation permissive' medium) and in DMEM (a copper-free medium). The time-dependent formation of 5-OVC and 9-ONC along with the concentrations of linoleic acid (9-ONC being primarily formed from Ch18:2) and arachidonic acid (5-OVC being primarily formed from Ch20:4) in LDL incubated in HAM's F10 medium in the presence (closed symbols) and absence (open symbols) of J774 macrophages is displayed in **Fig. 1A–D**. In addition, two parameters commonly used to assess LDL oxidation, i. e., TBARS and the increase in LDL's relative electrophoretic mobility (REM, an index of the surface charge of the LDL particle) are shown in Figs. 1E and F. Incubation of LDL in HAM's F10 medium for 48 h in the absence of cells resulted in oxidation of the lipoprotein, as indicated by alterations of all oxidation parameters. However, incubation of LDL in the presence of cells markedly accelerated lipoprotein oxidation during the early stages of LDL oxidation (up to 24 h). After 24 h the LDL oxidation parameters of the cell-free control approached those of LDL incubated in the presence of cells.

Incubation of LDL in the presence of J774 macrophages led to the formation of 5-OVC immediately after initiation of the oxidation experiment and proceeded at a maximal rate of 34 nmol/L per h in the presence of cells (Fig. 1A). 5-OVC was also detected in the cell-free controls; however, the onset was delayed by about 4 h and the maximum rate of formation was lower (25 nmol/L per h). The decrease in arachidonic acid concentrations (Fig. 1B) closely reflected the formation of 5-OVC. In contrast to 5-OVC concentrations, reaching a plateau after 10 h of oxidation, 9-ONC concentrations increased continuously throughout the whole incubation period (Fig. 1C; up to 4.4 μm, corresponding to 44 mol 9-ONC per mol LDL). In the presence of cells, maximum 9-ONC formation was observed between 8 and 12 h; in that time interval formation rates were ≈4 times higher than in the cell-free control (240.8 vs. 61.7 nmol/L per h, J774 vs. cell-free control). In the cell-free controls, maximum formation rates were reached after 23 h.

The higher oxidation rates of LDL-lipids in the presence of J774 macrophages are reflected by the difference in the maximum disappearance rates of linoleic acid (Fig. 1D; 3.4 μmol/L per h vs. 2 μmol/L per h in the cell-free controls). During the experiments described in Figs. 1B and D, arachidonic and linoleic acid were determined after transmethylation of total LDL lipids (i.e., without separation of lipid subclasses), and therefore no direct precursor–product relationship for 9-ONC and 5-OVC formation can be established. However, ≈46% of arachidonic acid and ≈75% of linoleic acid in LDL are confined to the cholesteryl ester fraction (25), and cholesteryl ester oxidation rates are comparable to phospholipid oxidation rates (25), so that the consumption of total PUFAs closely reflects cholesteryl ester oxidation. In support of the findings described above, enhanced LDL oxidation in the presence of J774 cells was also indicated by increased TBARS (Fig. 1E) and increased REM (Fig. 1F).

At the 48 h time point, the cellular lipids were extracted and analyzed for their 5-OVC, 9-ONC, and fatty acid content. While 5-OVC and 9-ONC were undetectable in J774 cells prior to the oxidation experiments, the cells had accumulated 21 and 173 pmol/mg cell protein of 5-OVC and 9-ONC, respectively. These findings indicate that the formation of core aldehydes occurs in the cellular supernatant and that part of the core aldehydes could be internalized by the macrophages. This is further supported by the intracellular accumulation of fatty acids (255 vs. 408 nmol total fatty acids/mg cell protein, cells incubated in the absence or presence of LDL). No CE–core aldehydes were detected in lipid extracts of J774 macrophages incubated in the absence of LDL.

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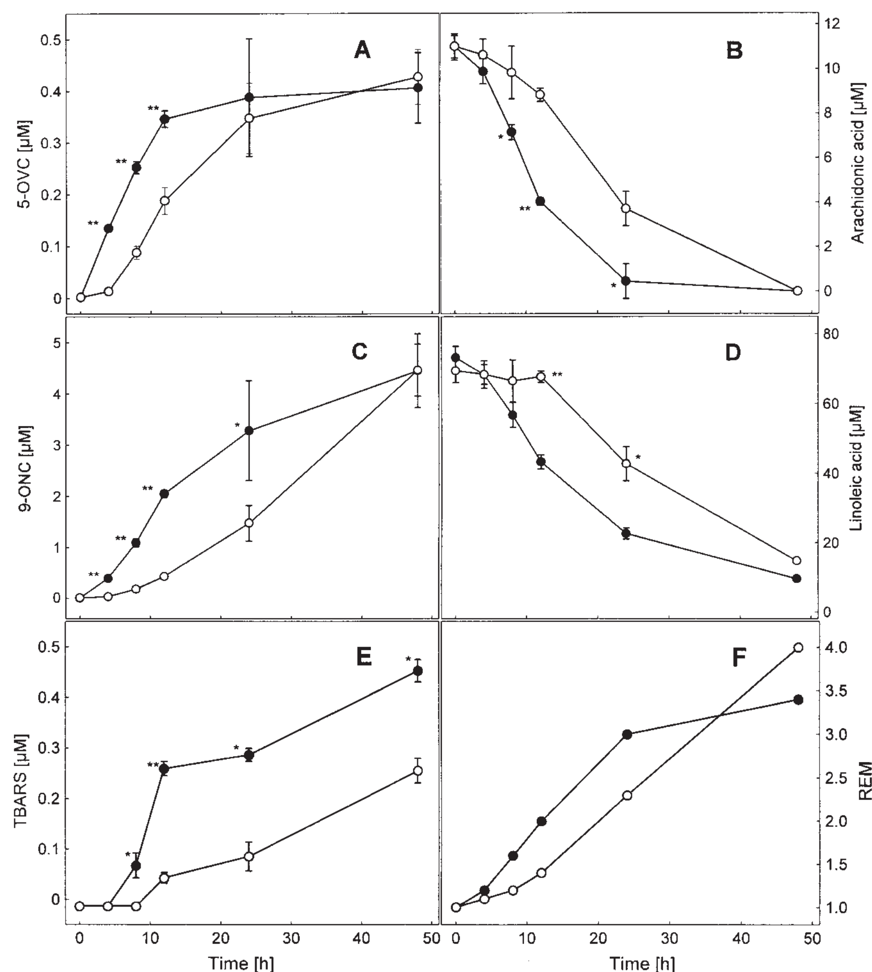

Fig. 1. Cell-mediated oxidation of LDL by J774 macrophages in HAM's F10 medium. Oxidation of LDL (0.25 mg/ml) in HAM's F10 medium was performed in the presence (filled symbols) or absence (open symbols) of J774 macrophages. 5-OVC (A) and 9-ONC (C) concentrations in the medium were determined after extraction with hexane–2-propanol 3:2 (v/v), derivatization with 1,3-cyclohexanedione, and RP-HPLC analysis as described in Materials and Methods. Fatty acids were analyzed by GC-FID after transmethylation of total LDL lipids present in the medium. Only arachidonic- (B) and linoleic acid (D) concentrations are shown. TBARS (E) in the medium were determined photometrically as described; the REM (F) of LDL present in the cellular supernatant was determined on agarose gels. Data represent mean values ± SD from one representative experiment (out of three) performed in triplicate; REM values represent one experiment representative of three. * $P < 0.05$; ** $P < 0.005$; calculated by the Student's t-test of independent samples; J774 cells vs. cell-free control.

In contrast, when the oxidation experiments were performed in DMEM, TBARS and REM remained unchanged (not shown). In the cell-free control, low concentrations of 9-ONC and 5-OVC (approximately 200 nm at 24 h) were detected (**Fig. 2A**). Whether the decrease of 9-ONC and 5-OVC concentrations after 24 h is due to further oxidation or binding to apoB-100 is presently not clear. However, the amounts formed were too small to be reflected in decreased linoleic and arachidonic acid concentrations in the LDL-containing cellular supernatant (Fig. 2B). In the presence of J774 cells, the content of 9-ONC in the medium was near or below the absolute limit of detection (⩾15 fmol) for 9-ONC (Fig. 2B). Whether this was a result of antioxidant properties of J774 macrophages under these conditions or due to cellular uptake of 9-ONC was not further investigated.

Comparison of core aldehyde formation in transition metal-supplemented DMEM and PBS and HAM's F10 medium

To test whether the difference in core aldehyde formation between HAM's F10 and DMEM medium was solely due to the lack of transition metal ions, LDL was incubated in Fe^{2+}/Cu^{2+} supplemented DMEM. The formation of 9-ONC and 5-OVC was compared to incubations in HAM's F10 or transition metal-supplemented PBS. As can be seen from **Table 1**, the supplementation of DMEM with Cu/Fe was almost without effect on the formation of 9-ONC and 5-OVC. On average, the formation of 9-ONC in DMEM or metal-supplemented DMEM was 44- and 16-fold lower as compared to HAM's F10 and metal-supplemented PBS. This effect was even more pronounced for 5-

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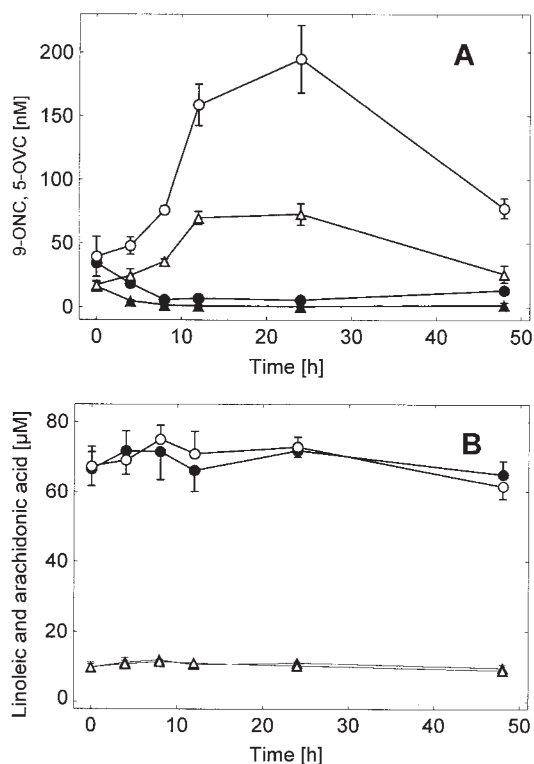

Fig. 2. Cell-mediated oxidation of LDL by J774 macrophages in DMEM. Oxidation of LDL (0.25 mg/ml) in DMEM medium was performed in the presence (filled symbols) or absence (open symbols) of J774 macrophages. A: 9-ONC (circles) and 5-OVC (triangles) concentrations in the medium were determined after extraction with hexane–2-propanol 3:2 (v/v), derivatization with CHD, and RP-HPLC analysis. B: Linoleic acid (circles) and arachidonic acid (triangles) analysis was performed by GC/FID of the corresponding methyl esters as described in Materials and Methods. Data represent mean values ± SD from one representative experiment (out of three) performed in triplicate.

OVC production (60- and 37-fold lower, HAM's F10 and PBS, respectively). The reason for the higher 9-ONC formation during LDL oxidation in cell-free DMEM shown in Fig. 2A as compared to this experiment is not fully clear. We have noticed, however, that the extent of LDL oxidation was dependent on the storage time of the cell culture media used and varied between different LDL preparations.

LDL oxidation in amino acid- and vitamin-supplemented HAM's F10

To investigate the nature of the antioxidative properties of DMEM, oxidation experiments were performed in HAM's F10 medium which was supplemented with amino acids and/or vitamins to reach equal or higher concentrations as in DMEM (see Materials and Methods for details). In parallel, oxidation experiments were performed in transition metal-supplemented DMEM. Results of these experiments are shown in **Table 2** (fatty acids) and **Table 3** (CE, Ch(O)OH, and CE–core aldehydes). Amino acid or vitamin supplementation of HAM's F10 strikingly affected the degree of J774-mediated LDL oxidation. After a 10 h incubation in unsupplemented HAM's F10, the 9-ONC and 5-OVC concentrations were 4.8 and 0.7 μm, respectively. Amino acid or vitamin supplementation of HAM's F10 significantly reduced the amounts of 9-ONC (2.36 μm) and 5-OVC (0.5 μm) (Table 3). This protective effect was also reflected by reduced consumption of LDL-PUFAs (2- to 3-fold lower in the supplemented media; Table 2) and polyunsaturated LDL–CEs (Table 3). HAM's F10 supplementation with amino acids or vitamins exceeding DMEM concentrations 5- or 10-fold or with a combination of amino acids and vitamins (equal concentrations as in DMEM) completely inhibited cell-mediated LDL oxidation (Tables 2 and 3). In line with these findings, no J774-mediated LDL oxidation was observed in DMEM even when supplemented with 10-fold higher Cu/Fe concentrations than present in HAM's F10 (Tables 2 and 3).

The extent of cell-mediated LDL oxidation was also clearly dependent on the composition of the medium used during preincubation of J774 cells prior to the oxidation experiment: When macrophages were preincubated in FCS-containing RPMI 1640, the extent of LDL modification was approximately 1.8-fold lower as compared to similar experiments where cells were preconditioned in LPDS-containing RPMI 1640. This is reflected by lower CE–core aldehyde concentrations and reduced consumption of PUFA and PUFA esters (Tables 2 and 3).

The results obtained during analysis of the cellular supernatants were confirmed by analyzing 9-ONC and 5-OVC in the corresponding cellular lipid extracts. The cellular accumulation of 9-ONC and 5-OVC was also clearly dependent on the amino acid or vitamin content of HAM's F10 used during the oxidation studies (**Table 4**). LDL oxidation in supplemented HAM's F10 resulted in 2-fold reduced cellular 9-ONC and 5-OVC accumulation as compared to oxidation in non-supplemented medium. A further increase in amino acid or vitamin concentrations (5- and 10-fold higher as compared to DMEM, respectively) resulted in either undetectable (5-OVC) or significantly reduced 9-ONC (10 vs. 290 μm, Table 4) concentrations in the cellular lipid extracts. A reduction of the cellular core aldehyde accumulation was also observed when the cells were preconditioned in FCS- rather than in

TABLE 1. Comparison of LDL–core aldehyde formation in different media in the absence of cells

	Core Aldehydes				
	DMEM	DMEM[a]	DMEM[b]	PBS[a]	HAM's F10
	nM				
9–ONC	17 ± 2.7	14 ± 1.3	17 ± 4.1	260 ± 109.0	704 ± 70.5
5–OVC	4 ± 0.5	4 ± 0.2	6 ± 1.3	166 ± 30.9	268 ± 27.6

LDL (0.25 mg/ml) was incubated for 23 h in DMEM, DMEM supplemented with $CuSO_4$ and $FeSO_4$ (10 nm and 3 μm or 100 nm and 30 μm, respectively), PBS (10 nm Cu and 3 μm Fe), and HAM's F10 at 37°C in 6-well cell culture plates. 9-ONC and 5-OVC were determined by RP-HPLC as described in Materials and Methods. Values represent mean ± SD from one representative experiment (out of three) performed in triplicate.
[a] 10 nm Cu^{2+}, 3 μm Fe^{2+}.
[b] 100 nm Cu^{2+}, 30 μm Fe^{2+}.

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TABLE 2. Fatty acid composition of LDL incubated in the presence of J774 macrophages in media supplemented with amino acids, vitamins, or transition metals

	Fatty Acids								
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	18:3	20:4
					μM				
HAM's	2.8 ± 0.21	49.2 ± 3.79	3.9 ± 0.33	13.8 ± 0.83	37.7 ± 2.30	29.2 ± 2.35	n.d.	n.d.	2.1 ± 0.10
HAM's + AA	3.0 ± 0.47	49.0 ± 1.58	4.1 ± 0.19	13.8 ± 1.36	38.5 ± 0.58	49.9 ± 6.14	0.4 ± 0.62	n.d.	5.9 ± 1.35
HAM's + Vit	3.3 ± 0.35	49.6 ± 0.96	4.1 ± 0.14	13.7 ± 0.44	38.1 ± 0.50	47.3 ± 2.82	0.7 ± 0.60	n.d.	5.4 ± 0.60
HAM's + AA + Vit	3.0 ± 0.78	50.1 ± 2.29	4.4 ± 0.18	14.2 ± 0.86	41.1 ± 2.20	83.0 ± 2.55	1.7 ± 0.16	1.2 ± 0.27	15.9 ± 0.85
HAM's + 5 × AA	3.2 ± 0.43	53.0 ± 0.09	4.7 ± 0.04	14.5 ± 0.27	43.1 ± 0.42	87.9 ± 0.44	1.8 ± 0.18	1.1 ± 0.02	16.8 ± 0.03
HAM's + 10 × Vit	3.1 ± 0.39	50.5 ± 1.92	4.5 ± 0.22	13.9 ± 0.44	40.6 ± 1.21	81.6 ± 3.12	1.7 ± 0.02	0.7 ± 0.63	15.4 ± 0.49
DMEM + 1 × Fe/Cu	3.1 ± 0.50	49.7 ± 0.58	4.3 ± 0.09	14.2 ± 0.20	39.9 ± 0.60	80.4 ± 0.74	1.7 ± 0.02	n.d.	15.4 ± 0.54
DMEM + 10 × Fe/Cu	2.9 ± 0.94	48.6 ± 1.91	4.0 ± 0.49	13.6 ± 0.49	38.9 ± 1.52	78.5 ± 2.11	1.7 ± 0.18	0.3 ± 0.57	15.0 ± 0.54
HAM's/FCS	3.0 ± 0.76	50.3 ± 2.32	4.2 ± 0.25	14.2 ± 0.49	40.3 ± 1.98	51.0 ± 1.75	0.7 ± 0.63	n.d.	6.0 ± 0.17

LDL (0.25 mg/ml) was coincubated with J774 macrophages as described in Materials and Methods. HAM's F10 or DMEM was supplemented with amino acids and/or vitamins or transition metals. Final concentrations in HAM's F10 were equal to (HAM's + AA, HAM's + Vit, HAM's + AA + Vit) or exceeded DMEM concentrations 5-fold (HAM's + 5 × AA) or 10-fold (HAM's + 10 × Vit). DMEM was supplemented with $CuSO_4$ and $FeSO_4$ (10 nm and 3 μm (DMEM + 1 × Fe/Cu) or 100 nm and 30 μm (DMEM + 10 × Fe/Cu), respectively). LDL oxidation was also performed in the presence of cells that were preincubated in FCS-containing RPMI 1640 prior to the oxidation experiments (HAM's/FCS). Fatty acids were analyzed by GC-FID after transmethylation of total LDL-lipids present in the medium. Data represent mean values ± SD from one representative experiment (out of three) performed in triplicate; n.d., not detectable.

LPDS-containing medium prior to the oxidation experiment (143 vs. 290 μm 9-ONC; Table 4).

Formation of 9-ONC and 5-OVC by PMA- and LPS-stimulated J774 macrophages

Previous studies have shown that the ability of human monocytes to oxidize LDL is dependent on their state of activation (26). To test whether activation of J774 macrophages results in enhanced 5-OVC and 9-ONC production, the cells were stimulated with PMA (10 ng/ml) or LPS (10 μg/ml). In a first series of experiments, the effect of these compounds known to stimulate the respiratory burst response in macrophages was tested by incubation of J774 cells in PMA- or LPS-containing HBSS and subsequent measurement of copper reduction, superoxide ($O_2^{\bullet -}$) production and thiol release in the cellular supernatant (**Figs. 3A–D**). All of these parameters have been suggested to promote lipoprotein oxidation in the presence of cells. The experiments described below were carried out in the absence of LDL to avoid copper reduction by LDL-associated α-tocopherol (27, 28) or preformed lipid hydroperoxides (29, 30).

Unstimulated J774 macrophages were able to reduce Cu(II) added to the incubation buffer so that up to 76 nmol Cu(I) per mg cell protein accumulated in the cellular supernatant within 8 h in the presence of bathocuproine disulfonic acid (BCS), as shown by the formation of the stable BCS-Cu(I) complex (BCS-Cu(I)) (Fig. 3A). BCS-Cu(I) formation was already observed 1 h after addition of Cu(II). Stimulation of J774 macrophages with

TABLE 3. Free cholesterol, cholesteryl ester, cholesteryl ester hydroperoxide, and CE–core aldehyde content of LDL incubated in the presence of J774 macrophages in media supplemented with amino acids, vitamins, or transition metals

	Analyte					
	FC	Ch18:2	Ch20:4	Ch(O)OH	9-ONC	5-OVC
				μM		
HAM's	49.4 ± 2.46	19.6 ± 4.26	0.8 ± 0.07	8.3 ± 1.69	4.8 ± 0.47	0.7 ± 0.11
HAM's + AA	47.6 ± 2.97	41.3 ± 4.79	2.0 ± 0.43	8.0 ± 1.44	2.3 ± 0.44	0.5 ± 0.10
HAM's + Vit	46.5 ± 6.28	37.6 ± 2.76	1.8 ± 0.01	8.2 ± 1.12	2.3 ± 0.16	0.5 ± 0.02
HAM's + AA + Vit	45.9 ± 2.12	72.9 ± 3.56	5.5 ± 0.09	n.d.	n.d.	n.d.
HAM's + 5 × AA	38.4 ± 4.23	56.8 ± 2.14	4.4 ± 0.17	n.d.	0.02 ± 0.03	0.01 ± 0.00
HAM's + 10 × Vit	49.4 ± 3.79	75.5 ± 6.43	5.6 ± 0.41	n.d.	n.d.	n.d.
DMEM + 1 × Fe/Cu	47.7 ± 4.10	72.5 ± 7.02	5.5 ± 0.53	n.d.	0.05 ± 0.08	n.d.
DMEM + 10 × Fe/Cu	46.7 ± 4.70	73.5 ± 6.64	5.5 ± 0.43	n.d.	n.d.	n.d.
HAM's/FCS	49.2 ± 3.56	40.3 ± 3.31	2.0 ± 0.14	8.3 ± 0.62	2.7 ± 0.26	0.5 ± 0.04

LDL (0.25 mg/ml) was coincubated with J774 macrophages as described in Materials and Methods. HAM's F10 or DMEM was supplemented with amino acids and/or vitamins or transition metals. Final concentrations in HAM's F10 were equal to (HAM's + AA, HAM's + Vit, HAM's + AA + Vit) or exceeded DMEM concentrations 5-fold (HAM's + 5 × AA) or 10-fold (HAM's + 10 × Vit). DMEM was supplemented with $CuSO_4$ and $FeSO_4$ (10 nm and 3 μm (DMEM + 1 × Fe/Cu) or 100 nm and 30 μm (DMEM + 10 × Fe/Cu), respectively). LDL oxidation was also performed in the presence of cells that were preincubated in FCS-containing RPMI 1640 prior to the oxidation experiments (HAM's/FCS). Free cholesterol, cholesteryl esters, and cholesteryl ester hydroperoxides were analyzed by RP-HPLC (detection at 210 nm) and quantitated by peak area comparison with external standards of known concentrations. 5-OVC and 9-ONC were analyzed as fluorescent CHD derivatives by RP-HPLC analysis as described in Materials and Methods. Data represent mean values ± SD from one representative experiment (out of three) performed in triplicate; n.d., not detectable.

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TABLE 4. CE–core aldehydes in cellular lipid extracts of J774 macrophages coincubated with 0.25 mg/ml LDL in HAM's F10 supplemented with amino acids and/or vitamins

	Core Aldehydes	
	9-ONC	5-OVC
	pmol/mg cell protein	
HAM's	289.3 ± 35.20	72.0 ± 18.82
HAM's + AA	148.4 ± 13.80	48.1 ± 9.62
HAM's + Vit	155.9 ± 51.72	46.7 ± 21.20
HAM's + AA + Vit	10.8 ± 0.52	n.d.
HAM's + 5 × AA	8.1 ± 2.16	n.d.
HAM's + 10 × Vit	10.4 ± 1.9	n.d.
HAM's/FCS	143.3 ± 15.55	42.9 ± 3.59

LDL (0.25 mg/ml) was coincubated with J774 macrophages as described in Materials and Methods. HAM's F10 was supplemented with amino acids and/or vitamins or transition metals. Final concentrations in HAM's F10 were equal to (HAM's + AA, HAM's + Vit, HAM's + AA + Vit) or exceeded DMEM concentrations 5-fold (HAM's + 5 × AA) or 10-fold (HAM's + 10 × Vit). LDL oxidation was also performed in the presence of cells that had been preincubated in FCS-containing RPMI 1640 prior to the oxidation experiments (HAM's/FCS). Cellular lipids were extracted at 25°C for 25 min with hexane–2-propanol 3:2 (v/v). 5-OVC and 9-ONC were determined as fluorescent CHD derivatives by RP-HPLC analysis as described in Materials and Methods. The protein content was determined in NaOH (0.3 N) lysates according to the method of Lowry et al. (20). Data represent mean values ± SD from one representative experiment (out of three) performed in triplicate; n.d., not detectable.

PMA resulted in approximately 3-fold higher initial copper reduction rates as compared to control and LPS-stimulated cells (0.28, 0.29, and 0.69 nmol/min per mg cell protein in control, LPS- and PMA- stimulated cells, respectively). After 8 h, stimulatory effects were no longer apparent. In contrast, the addition of LPS was without effect on the ability for copper reduction. No formation of the BCS-Cu(I) complex was observed in cell-free controls.

To circumvent removal of Cu(I) and a concomitant shift in the Cu(I)/Cu(II) redox-equilibrium by formation of the stable BCS-Cu(I) complex, experiments were performed in a manner identical to that described above except for the omission of BCS during the incubation. Cu(I) concentrations were measured by addition of BCS to the cellular supernatant at the time points indicated. After an apparent lag phase of 15 min, Cu(I) concentrations increased rapidly, reaching a plateau after approx. 2 h. Prolonged incubation did not lead to a further increase in Cu(I) concentrations (Fig. 3B). The maximum Cu(I) concentration measured in these experiments was 15 nmol/mg cell protein and thus clearly lower (≈5-fold) than observed with BCS present during the incubation. However, in line with data shown in Fig. 3A, PMA stimulation led to significantly accelerated Cu(II) reduction between 30 and 60 min (0.21 vs. 0.13 nmol/min; PMA stimulation vs. control) in the absence of BCS but was without effect on the final Cu(I)/Cu(II) ratio. As before, LPS stimulation was without effect on copper reduction. No Cu(I) formation was detectable in the cell-free controls.

The NADPH-dependent respiratory burst oxidase of macrophages is normally present as an inactive form which can be activated by stimuli such as PMA or zymosan to release superoxide radicals at the extracellular face of the plasma membrane (31). The results for $O_2^{\bullet -}$ release (measured as SOD-inhibitable reduction of ferricytochrome C) in unstimulated and stimulated macrophages are shown in Fig. 3C. Both PMA and LPS stimulated superoxide release (13- and 5-fold 30 min after stimulation relative to unstimulated cells, respectively).

It was reported recently that J774 macrophages can release low molecular weight thiols (6), compounds implicated in the formation of $O_2^{\bullet -}$ and cell-mediated copper reduction. Thiol release of control and stimulated J774 macrophages is displayed in Fig. 3D. The concentration of free SH groups in the cellular supernatant increased continuously over the whole time period of 12 h in all incubations. Thiol release induced by LPS was, however, markedly higher than that induced by PMA and in the unstimulated control (58, 50, and 82 nmol SH/12 h per mg cell protein for controls, PMA- and LPS-stimulated cells).

The time-dependent formation of 5-OVC, 9-ONC and the concomitant consumption of 20:4, 18:2, Ch20:4, and Ch18:2 in the supernatant of control and stimulated J774 macrophages are shown in **Figs. 4A–F**. As oxidation parameters in the absence of cells were not affected by the presence of PMA or LPS, data shown for cell-free controls represent average values of all incubation conditions. The alterations in core aldehyde formation and PUFA consumption in the absence of cells were similar to those shown in Fig. 1.

From data shown in Figs. 4A and B it is evident that PMA- and LPS-stimulated J774 cells produced higher concentrations of core aldehydes as compared to control cells. The formation of 9-ONC in PMA-stimulated cells was 3.6- (62 vs. 17 nm; 5 h) and 2.6-fold (564 vs. 220 nm; 12 h) higher than in unstimulated cells (Fig. 4A). PMA stimulation resulted in comparable augmentation of 5-OVC production: At the 5 and 12 h time points, the amount of 5-OVC produced in the presence of PMA-stimulated macrophages was 4- (26.9 vs. 6.65 nm; 5 h) and 1.5-fold (173.6 vs. 116.8 nm; 12 h) higher as compared to unstimulated macrophages (Fig. 4B). The effect of PMA stimulation on LDL oxidation was even more pronounced for PUFA decomposition (Fig. 4C and D), resulting in significantly enhanced consumption of 18:2 (3.2-fold) and 20:4 (2.5-fold) at the 12 h time point compared to control and LPS-treated cells. LPS treatment of cells was less effective than PMA; although LDL oxidation appeared to be consistently higher as compared to controls; this effect was, however, not statistically significant (except for the 5 h time point).

To be able to present an improved precursor–product relationship for 9-ONC and 5-OVC formation, we have also analyzed the Ch18:2 and Ch20:4 content in the cellular supernatant. A comparison of Figs. 4A and E shows that the decrease in Ch18:2 is matched by 9-ONC formation. At the end of the incubation period, about 5% of cholesteryl linoleate consumed during the oxidation experiment had been converted to 9-ONC (calculated on a molar basis). The decrease in Ch20:4 (Fig. 4F) is accompanied by the formation of 5-OVC (Fig. 4B; on a molar basis about 4% of Ch20:4 was converted to 5-OVC).

After 23 h the cellular lipids were extracted and ana-

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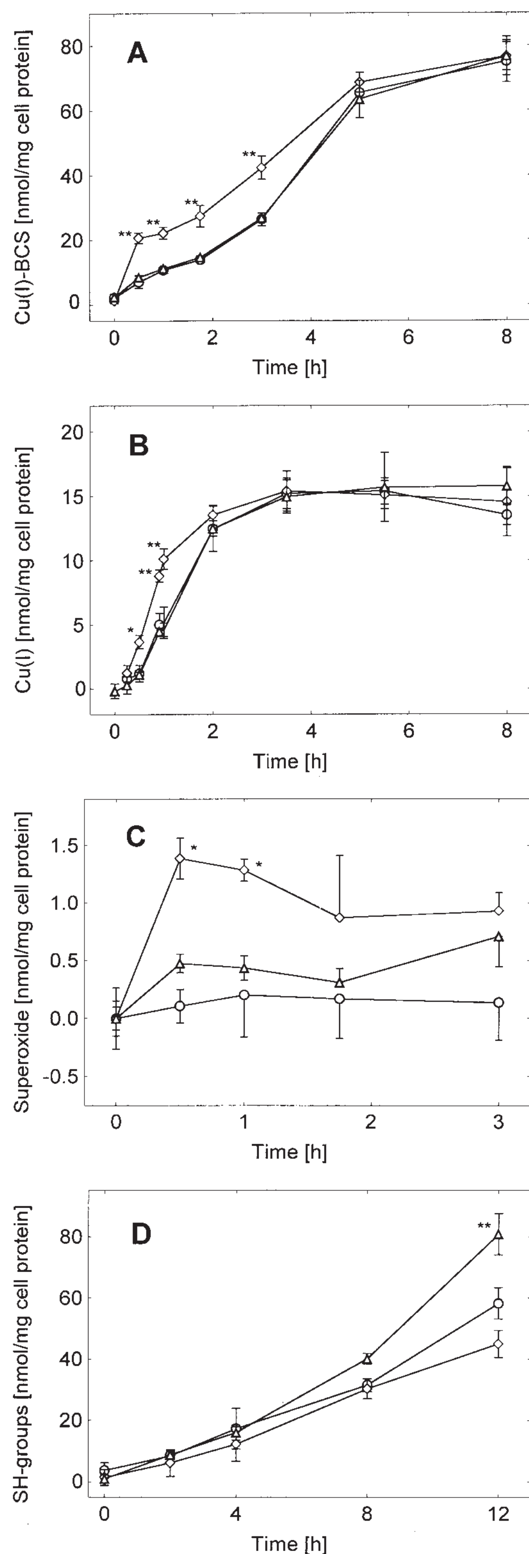

Fig. 3. Copper reduction, $O_2^{\bullet -}$ production and thiol-release by control and activated J774 macrophages. J774 macrophages (confluent on 12-well trays) were incubated in HBSS buffer containing no stimulant (circles), PMA (10 ng/ml; diamonds), or LPS (10 μg/ml; triangles). Formation of Cu(I)-BCS (A), Cu(I) (B), superoxide (C), and free thiol groups (D) in the cellular supernatant was determined as described in Materials and Methods. A: 50 μm $CuSO_4$, 125 μm BCS in HBSS incubation buffer, B: 50 μm $CuSO_4$, no BCS in HBSS incubation buffer. Average cellular protein content was 0.33 ± 0.06 mg/well as determined in NaOH (0.3 N) lysates according to the method of Lowry et al. (20). Data represent mean values ± SD from one representative experiment (out of three) performed in triplicate; values of the cell-free control incubations were subtracted. * $P < 0.05$; ** $P < 0.005$; calculated by the Student's *t*-test of independent samples; stimulated vs. unstimulated cells.

lyzed for their core aldehyde and fatty acid content. As observed in the experiments with unstimulated cells, coincubation of LDL and J774 cells led to intracellular fatty acid, 9-ONC and 5-OVC accumulation. The detailed results of these analyses are displayed in **Table 5**. Macrophages stimulated with LPS contained about twice as much free 9-ONC (62.4 pmol/mg cell protein) as cells treated with PMA (34 pmol/mg cell protein) or unstimulated cells (29 pmol/mg protein). 5-OVC concentrations in lipid extracts obtained from LPS-stimulated cells were approximately 1.5-fold higher as compared to control and PMA-stimulated cells. As can be seen from Table 5, the intracellular fatty acid composition of J774 macrophages cultured in the absence of LDL was considerably different from cells cultured in the presence of LDL with or without PMA or LPS. The most pronounced increase in cellular fatty acid concentrations was observed for stearic (18:0), oleic (18:1), and linoleic (18:2) acids (Table 5).

DISCUSSION

The formation of lipid-esters containing C5, C9, and C13 fatty acid chains during lipid-peroxidation of PUFAs was predicted by Esterbauer, Zollner, and Schaur (32). Although these core aldehydes represent only a small percentage of lipid peroxidation products, the presence of cholesteryl ester–core aldehydes of different chain length was demonstrated in tert-butyl hydroperoxide/Fe^{2+} oxidized cholesteryl linoleate (13), copper-oxidized LDL and HDL (14), and lipid extracts of atherosclerotic lesions (15, 16). However, reports on the mechanism(s) responsible for the formation of these compounds are lacking. During the present study we have shown that J774 macrophages markedly accelerate the formation of cholesteryl ester–core aldehydes (9-ONC and 5-OVC) and promote oxidation of Ch18:2 and Ch20:4 in LDL containing HAM's F10 medium. The presence of cells enhances LDL oxidation particularly during the earlier stages of oxidation, resulting in a shorter lag phase and increased oxidation rates. At time points ⩾24 h, LDL incubated in the absence or presence of cells is modified to a similar extent. Cell-mediated oxidation of LDL in the presence of transition metals is probably a valid model for LDL oxidation in the arterial wall as copper and iron ions were found in atherosclerotic lesions (33). Kritharides, Jessup, and Dean (34) have shown that macrophages require a combination of copper and iron ions to enhance LDL oxidation in vitro. Caeruloplasmin-released copper ions are available to the arterial wall under certain pathological conditions and transition metals contained in human atherosclerotic lesions can catalyze the oxidation of LDL by macrophages (35).

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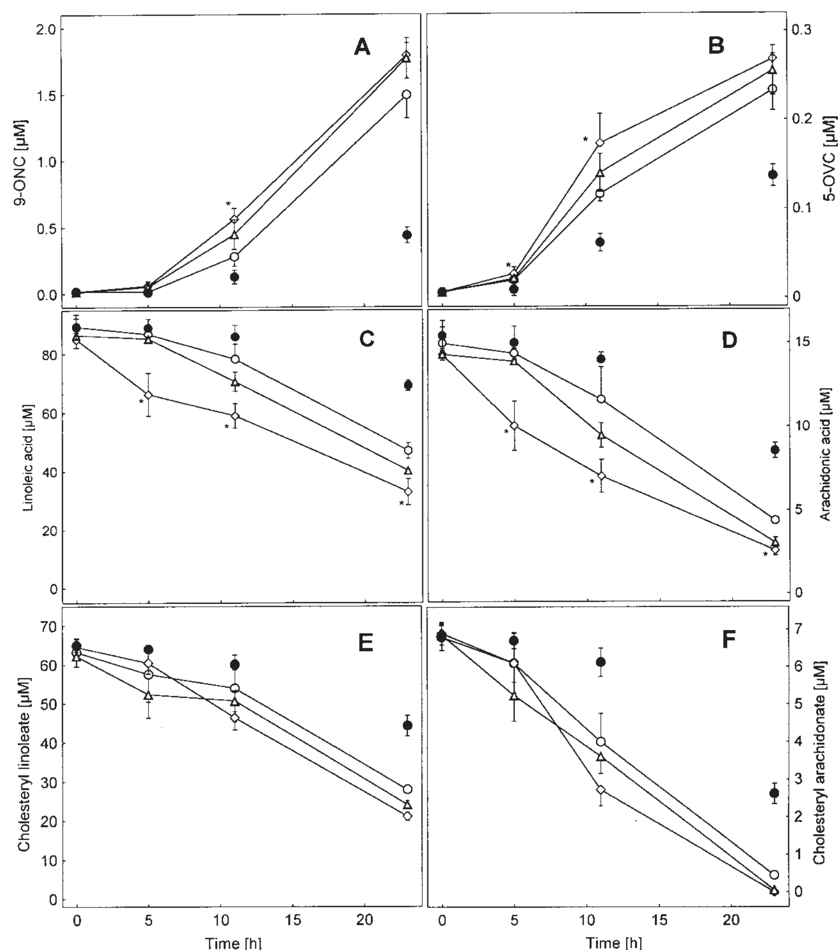

Fig. 4. Effect of PMA and LPS stimulation on J774-mediated LDL oxidation. LDL (0.25 mg/ml) was incubated in the presence of unstimulated (circles), PMA- (10 ng/ml; diamonds) or LPS-stimulated (10 µg/ml; triangles) J774 cells in HAM's F10 medium. 9-ONC (A) and 5-OVC (B) concentrations in the medium were determined after extraction with hexane–2-propanol 3:2 (v/v), derivatization with 1,3-cyclohexanedione and RP-HPLC analysis. Linoleic acid (C) and arachidonic acid (D) concentrations in the medium were determined after lyophilization of 1 ml medium, transmethylation of total LDL lipids with BF_3/MeOH and GC/FID analysis. Cholesteryl linoleate (E) and cholesteryl arachidonate (F) concentrations in the supernatant were determined by RP-HPLC analysis with UV detection at 210 nm. Filled symbols represent averaged data for all cell-free controls (the presence of PMA or LPS was without effect on analyte concentrations). Data represent mean values ± SD from one of two experiments performed in triplicate. * $P < 0.05$; ** $P < 0.005$; calculated by the Student's *t*-test of independent samples; stimulated vs. unstimulated cells.

We have detected only marginal LDL oxidation (as indicated by REM, TBARS, PUFA consumption, and formation of 9-ONC and 5-OVC) in DMEM supplemented with concentrations of Cu^{2+}/Fe^{2+} 10-fold higher than present in HAM's F10 medium. Compared to HAM's F10 medium, DMEM contains about 2- to 4-fold higher concentrations of amino acids and water-soluble vitamins. To further investigate the antioxidative properties of these components, HAM's F10 was supplemented with amino acids and/or vitamins. Addition of either amino acids or vitamins significantly reduced the extent of LDL modification and intracellular accumulation of CE–core aldehydes. When HAM's F10 was supplemented with a combination of both amino acids and vitamins, LDL oxidation was almost completely inhibited. Antioxidative properties of amino acids (e.g., histidine, cysteine, glutamic acid, and serine, which are particularly effective in inhibiting Cu-mediated oxidation of LDL) have been reported (36). Moreover, it was shown that glutamate, a competitor for the cystine transporter, effectively suppressed the oxidation of LDL by endothelial cells in the presence of cystine (24). In the present study we cannot ascribe the effects of vitamin supplementation to single components. However, there are several lines of evidence relating B vitamins and their derivatives to lipid peroxidation and/or atherogenesis. Impaired LDL oxidation as observed during the present study could, at least in part, be a result of iron complexation as suggested for pyridoxal, pantothenate, and myo-inositol (37). Other B vitamins might also be able to chelate transition metal ions (38). Radical-scavenging abilities were shown for nearly all vitamins with pyridoxal being the most effective $OH^{\bullet}$ and superoxide scavenger, fol-

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TABLE 5. Cellular lipid composition of unstimulated and stimulated J774 macrophages after a 23 h coincubation with LDL in HAM's F10 medium

Addition to Cells	Core Aldehydes		Fatty Acids					
	5-OVC	9-ONC	16:0	16:1	18:0	18:1	18:2	20:4
	pmol/mg cell protein		*nmol/mg cell protein*					
No LDL	n.d.	0.35 ± 0.46	83.5 ± 5.6	25.4 ± 1.3	24.4 ± 1.3	99.0 ± 2.2	7.2 ± 0.6	7.2 ± 0.7
LDL	7.4 ± 1.4	28.5 ± 7.7	90.0 ± 9.1	31.9 ± 5.1	42.0 ± 5.0	120.4 ± 13.7	17.0 ± 1.8	10.9 ± 1.3
LDL, PMA (10 ng/ml)	6.4 ± 1.8	33.8 ± 11.3	86.5 ± 4.4	28.1 ± 1.7	41.9 ± 3.3	115.9 ± 5.6	14.0 ± 0.5	10.5 ± 0.6
LDL, LPS (10 μg/ml)	10.3 ± 4.5	62.4 ± 9.8	98.1 ± 4.6	27.1 ± 3.8	43.4 ± 1.6	113.2 ± 1.6	16.0 ± 1.0	10.3 ± 0.3

After a 23 h coincubation of J774 cells in the absence or presence of LDL (0.25 mg/ml) in HAM's F10 or HAM's F10 containing PMA (10 ng/ml) or LPS (10 μg/ml), cells were washed and the cellular lipids were extracted with hexane–2-propanol (3:2, v/v; 25 min on an orbital shaker at room temperature). The fatty acid and core aldehyde content was analyzed by GC-FID of the corresponding fatty acid methyl esters and RP-HPLC of the fluorescent core aldehyde derivatives as described in Materials and Methods. The protein content was determined in NaOH (0.3 N) lysates according to the method of Lowry et al. (20). Data shown represent mean values ± SD from one of two experiments performed in triplicate; n.d., not detectable.

lowed by pantothenate (OH$^{\bullet}$) and thiamine (OH$^{\bullet}$ and superoxide) (37). The reaction constants of choline chloride, myo-inositol, and niacinamide for OH$^{\bullet}$-scavenging were markedly lower (37). The same authors found a significant inhibition of Fe/ascorbate-induced LPO in liver microsomes in the presence of pyridoxal or pantothenate (37). Riboflavin as a known photosensitizer also reacts with free radicals; this might, however, mediate oxidative damage rather than prevent it (39).

In addition, endotoxin-mediated activation of macrophages is counteracted by choline chloride (40) and niacinamide in vivo and in vitro (41, 42). Niacinamide treatment protected cardiac myoblasts against oxidant injury (43). Epidemiological studies have shown a correlation between low plasma folate concentrations and increased plasma levels of homocysteine, a independent risk factor for atherogenesis (44). Thiamine deficiency is implicated in various neurodegenerative diseases (45), possibly in conjunction with increased cerebral formation of reactive oxygen species (46). Evidence presented above indicates that vitamins present in cell culture media can act directly as redox-active compounds or indirectly via modulation of intracellular processes, thus affecting cell-mediated LDL oxidation. Our findings demonstrate that in addition to the transition metal content, the amino acid and vitamin concentrations are important factors determining whether a given cell culture medium is 'oxidation-permissive' or not.

Although numerous studies have shown that cultured macrophages can promote lipoprotein oxidation by a transition metal-dependent process, the exact mechanisms responsible for cell-mediated LDL oxidation are not entirely clear and may vary considerably among different cell types. During the present study the extent of LDL modification by J774 macrophages was strongly dependent on the preincubation conditions (LPDS- vs. FCS-containing media, Tables 2, 3, and 4). Whether this is due to altered receptor expression or due to stress-induced activation was not further investigated. Activated phagocytes are able to produce $O_2^{\bullet -}$, to release low molecular weight thiols, and to reduce Cu(II) and Fe(III). All of these pathways have been implicated to be involved in cell-mediated oxidation of LDL. Accordingly it was demonstrated that the rate of LDL oxidation is proportional to the production of $O_2^{\bullet -}$ in arterial smooth muscle cells (47) and endothelial cells (48). Moreover, overexpression of human Cu/Zn–SOD in bovine arterial endothelial cells led to significantly reduced LDL oxidation (49). On the other hand, the participation of $O_2^{\bullet -}$ during macrophage-mediated LDL oxidation has been questioned (24) and overexpression of Cu/Zn–SOD in mice did not support an antiatherogenic effect of Cu/Zn–SOD (50). The uptake of extracellular cystine, intracellular reduction, and release of the reduced thiol was proposed as another factor accelerating LDL oxidation in the medium (51–54) either by formation of oxygen-derived free radicals or by reduction of transition metals in the medium, maintaining a high reactivity towards lipid hydroperoxides (36). Finally, Garner and colleagues (6) have demonstrated that macrophages are able to promote direct copper reduction in the absence of thiols, a process mediated via TPMET systems (5, 55). Copper reduction by macrophages was assessed in the absence of LDL (6) as α-tocopherol and/or preformed lipid hydroperoxides can directly promote copper reduction (27–30).

Our own experiments have shown that PMA activation of J774 macrophages significantly stimulated $O_2^{\bullet -}$ release and accelerated copper reduction. Although the final Cu(I)/Cu(II) ratio was independent of the activation state, the rate of copper reduction was significantly increased by PMA. In line with other findings (56), LPS was less effective in activating $O_2^{\bullet -}$ release when applied as single stimulus. However, in contrast to PMA, LPS stimulated the release of thiols in the cellular supernatant. A similar effect was observed for mouse peritoneal macrophages where the presence of LPS significantly enhanced thiol production (57). LDL oxidation was enhanced by PMA activation of J774 cells as indicated by increased PUFA, Ch18:2 and Ch20:4 consumption, and enhanced 5-OVC and 9-ONC production (Fig. 4). This indicates that either increased $O_2^{\bullet -}$ production or accelerated Cu(II) reduction is responsible for enhanced LDL modification. Redox-cycling of copper is assumed to be particularly important for LPO initiation and, in further consequence, for core aldehyde formation. Although the

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mechanisms of core aldehyde formation are not yet elucidated, cholesteryl ester hydroperoxides appear to be precursors of CE–core aldehydes (13, 15, 32). A Fenton-type reaction, involving Cu(I) and/or Fe(II), and e.g., 9-hydroperoxy cholesteryl linoleate, would lead to the formation of an alkoxy radical and, via subsequent β-scission, the formation of 9-ONC and the corresponding alkyl radical. This reaction would require Cu(I) which is formed at higher rates in PMA-stimulated cells as compared to the unstimulated controls. In line with enhanced redox-cycling of Cu, we have observed markedly increased 9-ONC and 5-OVC formation in PMA-stimulated cells as compared to LPS-stimulated and unstimulated cells (Fig. 4). The finding that this effect was most pronounced at the shorter incubation times is in line with the time course of direct copper reduction in PMA-stimulated cells. The observation that coincubation of LDL with isolated J774 plasma membranes resulted in higher 9-ONC formation, as compared to other cell organelles, further substantiates Cu(I) generation via a TPMET system (B. Karten and W. Sattler, unpublished results).

After a 23-h coincubation of J774 macrophages in the presence of LDL, we could detect small amounts of 5-OVC and 9-ONC in the cellular lipid extracts. This indicates that part of the core aldehydes is internalized by J774 cells. The concentration of CE–core aldehydes in the cellular lipid extract is dependent on the degree of LDL oxidation in the cellular supernatant, indicating that cellular core aldehydes originate from LDL. This was, however, not experimentally verified, due to the lack of a radioactively labeled core aldehyde standard. Intracellular accumulation was more pronounced in the presence of LPS as compared to PMA-stimulated and control cells; these observations are in line with LPS-mediated CE accumulation by RAW-macrophages cultured in the presence of βVLDL or LDL (58). It is noteworthy that the amounts of 5-OVC and 9-ONC detected in the cell lipid extracts could be an underestimation due to the formation of Schiffs-base adducts (15), as protein-bound core aldehydes would escape the analytical method used during the present study (16). On the other hand, Hoppe et al. (15) have provided evidence that core aldehydes produced from oxidized [^{3}H]Ch18:2 are hydrolyzed by an acidic macrophage cholesteryl ester hydrolase less efficiently than native [^{3}H]Ch18:2. Moreover, these authors (15) have suggested that the site-specific release of core aldehydes in lysosomes could lead to irreversible intralysosomal deposition of lipid–protein complexes. This is compatible with lipid engorgement in lysosomes (59) and partial lysosomal enzyme inactivation after uptake of ox-LDL (60). Also, predominant lysosomal localization of epitopes specific for oxidized proteins (61), lysosomal accumulation of ceroid (62) and partially degraded apoB from oxidized LDL (63) indicate that the lysosome is the site where potential pathophysiological consequences of core aldehydes could be manifested. In line with the suggestion of a relatively slow turnover, CE–core aldehydes of different chain length were found in lipid extracts of atherosclerotic plaques (15, 16). We have attempted to clarify whether 9-ONC and 5-OVC were subjected to lysosomal or extralysosomal hydrolysis by the addition of chloroquine during the oxidation experiments. However, the presence of chloroquine completely suppressed cell-mediated oxidation (not shown).

Fatty acid analysis of cellular lipid extracts revealed that individual fatty acids accumulated in a ratio incompatible with the fatty acid composition of native or modified LDL (25). Consequently, fatty acid accumulation could not be attributed solely to LDL particle uptake. It most probably reflects a superimposition of endogenous de novo fatty acid synthesis (64), selective uptake of LDL–CEs (65, 66) and probably increased PL synthesis as an adaptive response to FC loading of macrophages (67). Which one of these mechanisms is predominant under the experimental conditions used during the present study is not clear.

In summary, we have shown that J774 macrophages accelerate the formation of CE–core aldehydes in transition metal-containing HAM's F10 medium, an effect even more pronounced when the cells were stimulated with PMA. CE–core aldehydes were internalized by the cells, however, which pathway is responsible for cellular uptake remains to be elucidated.

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REFERENCES

1. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
2. Esterbauer, H., and P. Ramos. 1995. Chemistry and pathophysiology of oxidation of LDL. *Rev. Physiol. Biochem. Pharmacol.* **127:** 31–64.
3. Goldstein, J. L., and M. S. Brown. 1977. Low-density lipoprotein pathway and its relation to atherosclerosis. *Annu. Rev. Biochem.* **46:** 897–930.
4. Chait, A., and J. W. Heinecke. 1994. Lipoprotein modification: cellular mechanisms. *Curr. Opin. Lipidol.* **5:** 365–370.
5. Crane, F. L., I. L. Sun, M. G. Clark, C. Grebing, and H. Löw. 1985. Transplasma-membrane redox systems in growth and development. *Biochim. Biophys. Acta.* **811:** 233–264.
6. Garner, B., D. van Reyk, R. T. Dean, and W. Jessup. 1997. Direct copper reduction by macrophages. Its role in low density lipoprotein oxidation. *J. Biol. Chem.* **272:** 6927–6935.
7. Witz, G. 1989. Biological interaction of α,β-unsaturated aldehydes. *Free Radical Biol. Med.* **7:** 333–349.
8. Girotti, A. W. 1998. Lipid hydroperoxide generation, turnover, and effector action in biological systems. *J. Lipid Res.* **39:** 1529–1542.
9. Terao, J. 1990. Reactions of lipid hydroperoxides. *In* Membrane Lipid Oxidation. C. Pelfrey, editor. CRC Press, Boca Raton. 219–238.
10. Itabe, H. 1998. Oxidized phospholipids as a new landmark in atherosclerosis. *Prog. Lipid Res.* **37:** 181–207.
11. Watson, A. D., N. Leitinger, M. Navab, K. F. Faull, S. Hörkkö, J. L. Witztum, W. Palinski, D. Schwenke, R. G. Salomon, W. Sha, G. Subbanagounder, A. M. Fogelman, and J. A. Berliner. 1997. Structural identification by mass spectrometry of oxidized phospholipids in minimally oxidized low density lipoprotein that induce monocyte/

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endothelial interactions and evidence for their presence *in vivo. J. Biol. Chem.* **272:** 13597–13607.
12. Kamido, H., A. Kuksis, L. Marai, and J. J. Myher. 1992. Identification of cholesterol-bound core aldehydes in copper-oxidized low density lipoprotein. *FEBS Lett.* **304:** 269–272.
13. Kamido, H., A. Kuksis, L. Marai, and J. J. Myher. 1993. Identification of core aldehydes among *in vitro* peroxidation products of cholesteryl esters. *Lipids.* **28:** 331–336.
14. Kamido, H., A. Kuksis, L. Marai, and J. J. Myher. 1995. Lipid ester-bound aldehydes among copper-catalyzed peroxidation products of human plasma lipoproteins. *J. Lipid Res.* **36:** 1876–1886.
15. Hoppe, G., A. Ravandi, D. Herrera, A. Kuksis, and H. F. Hoff. 1997. Oxidation products of cholesteryl linoleate are resistant to hydrolysis in macrophages, form complexes with proteins, and are present in human atherosclerotic lesions. *J. Lipid Res.* **38:** 1347–1360.
16. Karten, B., H. Boechzelt, P. M. Abuja, M. Mittelbach, K. Oettl, and W. Sattler. 1998. Femtomole analysis of 9-oxononanoyl cholesterol by high performance liquid chromatography. *J. Lipid Res.* **39:** 1508–1519.
17. Boechzelt, H., B. Karten, P. M. Abuja, W. Sattler, and M. Mittelbach. 1998. Synthesis of 9-oxononanoyl cholesterol by ozonization. *J. Lipid Res.* **39:** 1503–1507.
18. Sattler, W., G. M. Kostner, G. Waeg, and H. Esterbauer. 1991. Oxidation of lipoprotein Lp(a). A comparison with low-density lipoproteins. *Biochim. Biophys. Acta.* **1081:** 65–74.
19. Goldstein, J. L., S. K. Basu, and M. S. Brown. 1983. Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol.* **98:** 241–260.
20. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
21. Panzenboeck, U., S. Raitmayer, H. Reicher, H. Lindner, O. Glatter, E. Malle, and W. Sattler. 1997. Effects of reagent and enzymatically generated hypochlorite on physicochemical and metabolic properties of high density lipoproteins. *J. Biol. Chem.* **272:** 29711–29720.
22. Sattler, W., D. Mohr, and R. Stocker. 1994. Rapid isolation of lipoproteins and assessment of their peroxidation by high-performance liquid chromatography postcolumn chemiluminescence. *Methods Enzymol.* **233:** 469–489.
23. Esterbauer, H., and K. H. Cheeseman. 1990. Determination of aldehydic lipid peroxidation product: malondialdehyde and 4-hydroxynonenal. *Methods Enzymol.* **186:** 407–421.
24. Jessup, W., J. A. Simpson, and R. T. Dean. 1993. Does superoxide radical have a role in macrophage-mediated oxidative modification of LDL? *Atherosclerosis.* **99:** 107–120.
25. Sattler, W., H. Reicher, P. Ramos, U. Panzenboeck, M. Hayn, H. Esterbauer, E. Malle, and G. M. Kostner. 1996. Preparation of fatty acid methyl esters from lipoprotein and macrophage lipid subclasses in thin-layer plates. *Lipids.* **31:** 1303–1310.
26. McNally, A. K., G. M. Chisolm, D. W. Morel, and M. K. Cathcart. 1990. Activated human monocytes oxidize low-density lipoprotein by a lipoxygenase-dependent pathway. *J. Immunol.* **145:** 254–259.
27. Kontush, A., S. Meyer, B. Finckh, A. Kohlschütter, and U. Beisiegel. 1996. α-Tocopherol as a reductant for Cu(II) in human lipoproteins. Triggering role in the initiation of lipid peroxidation. *J. Biol. Chem.* **271:** 11106–11112.
28. Proudfoot, J. M., K. D. Croft, I. B. Puddey, and L. J. Beilin. 1997. The role of copper reduction by α-tocopherol in low-density lipoprotein oxidation. *Free Radical Biol. Med.* **23:** 720–728.
29. Patel, R. P., D. Svistunenko, M. T. Wilson, and V. M. Darley-Usmar. 1997. Reduction of Cu(II) by lipid hydroperoxides: implications for the copper-dependent oxidation of low density lipoprotein. *Biochem. J.* **322:** 425–433.
30. Thomas, C. E., and R. L. Jackson. 1991. Lipid hydroperoxide involvement in copper-dependent and independent oxidation of low density lipoproteins. *J. Pharmacol. Exp. Ther.* **256:** 1182–1188.
31. Hamilton, T. A., and D. O. Adams. 1987. Molecular mechanisms of signal transduction in macrophages. *Immunol. Today.* **8:** 151–158.
32. Esterbauer, H., H. Zollner, and R. J. Schaur. 1990. Aldehydes formed by lipid peroxidation: mechanisms of formation, occurrence and determination. *In* Membrane Lipid Oxidation. C. Pelfrey, editor. CRC Press, Boca Raton. 239–268.
33. Smith, C., M. J. Mitchinson, O. I. Aruoma, and B. Halliwell. 1992. Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochem. J.* **286:** 901–905.
34. Kritharides, L., W. Jessup, and R. T. Dean. 1995. Macrophages require both iron and copper to oxidize low-density lipoprotein in Hanks' balanced salt solution. *Arch. Biochem. Biophys.* **323:** 127–136.
35. Lamb, D. J., M. J. Mitchinson, and D. S. Leake. 1995. Transition metal ions within human atherosclerotic lesions can catalyse the oxidation of low density lipoprotein by macrophages. *FEBS Lett.* **374:** 12–16.
36. Santanam, N., and S. Parthasarathy. 1995. Cellular cysteine generation does not contribute to the initiation of LDL oxidation. *J. Lipid Res.* **36:** 2203–2211.
37. Hu, M-L., Y-K. Chen, and Y-F. Lin. 1995. The antioxidant and prooxidant activity of some B vitamins and vitamin-like compounds. *Chem. Biol. Interact.* **97:** 63–73.
38. Tandon, S. K., S. J. Flora, and S. Singh. 1987. Chelation in metal intoxication. XXIV: Influence of various components of vitamin B complex on the therapeutic efficacy of disodium calcium versenate in lead intoxication. *Pharmacol. Toxicol.* **60:** 62–65.
39. Lucius, R., R. Mentlein, and J. Sievers. 1998. Riboflavin-mediated axonal degeneration of postnatal retinal ganglion cells in vitro is related to the formation of free radicals. *Free Radical Biol. Med.* **24:** 798–808.
40. Rivera, C. A., M. D. Wheeler, N. Enomoto, and R.G. Thurman. 1998. A choline-rich diet improves survival in a rat model of endotoxin shock. *Am. J. Physiol.* **275:** G862–G867.
41. Zingarelli, B., M. O'Connor, H. Wong, A. L. Salzman, and C. Szabo. 1996. Peroxynitrite-mediated DNA strand breakage activates polyadenosine diphosphate ribosyl synthetase and causes cellular energy depletion in macrophages stimulated with bacterial lipopolysaccharide. *J. Immunol.* **156:** 350–358.
42. Pellat-Deceunynck, C., J. Wietzerbin, and J. C. Drapier. 1994. Nicotinamide inhibits nitric oxide synthase mRNA induction in activated macrophages. *Biochem. J.* **297:** 53–58.
43. Gilad, E., B. Zingarelli, A. L. Salzman, and C. Szabo. 1997. Protection by inhibition of poly (ADP-ribose) synthetase against oxidant injury in cardiac myoblasts in vitro. *J. Mol. Cell. Cardiol.* **29:** 2585–2597.
44. Verhoef, P., M. J. Stampfer, E. B. Rimm. 1998. Folate and coronary heart disease. *Curr. Opin. Lipidol.* **9:** 17–22.
45. Calingasan, N. Y., L. C. H. Park, L. L. Calo, R. R. Trifiletti, S. E. Gandy, and G. E. Gibson. 1998. Induction of nitric oxide synthase and microglial responses precede selective cell death induced by chronic impairment of oxidative metabolism. *Am. J. Pathol.* **153:** 599–610.
46. Langlais, P. J., G. Anderson, S. X. Guo, S. C. Bondy. 1997. Increased cerebral free radical production during thiamine deficiency. *Metab. Brain Dis.* **12:** 137–143.
47. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1986. Superoxide-mediated modification of low density lipoproteins by arterial smooth muscle cells. *J. Clin. Invest.* **262:** 10098–10103.
48. Steinbrecher, U. P. 1988. Role of superoxide in endothelial-cell modification of low-density lipoproteins. *Biochim. Biophys. Acta.* **959:** 20–30.
49. Fang, X., N. L. Weintraub, C. D. Rios, D. A. Chappell, R. M. Zwacka, J. F. Engelhardt, L. W. Oberley, T. Yan, D. D. Heistad, and A. A. Spector. 1998. Overexpression of human superoxide dismutase inhibits oxidation of low-density lipoprotein by endothelial cells. *Circ. Res.* **82:** 1289–1297.
50. Tribble, D. L., E. L. Gong, C. Leeuwenburgh, J. W. Heinecke, E. L. Carlson, J. G. Verstuyft, and C. J. Epstein. 1997. Fatty streak formation in fat-fed mice expressing human copper-zinc superoxide dismutase. *Arterioscler. Thromb. Vasc. Biol.* **17:** 1734–1740.
51. Heinecke, J. W., H. Rosen, and L. A. Suzuki. 1987. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *J. Biol. Chem.* **262:** 10098–10103.
52. Heinecke, J. W., M. Kawamura, L. Suzuki, and A. Chait. 1993. Oxidation of low density lipoprotein by thiols: superoxide-dependent and -independent mechanisms. *J. Lipid Res.* **34:** 2051–2061.
53. Sparrow, C. P., and J. Olszewski. 1993. Cellular oxidation of low density lipoprotein is caused by thiol production in media containing transition metal ions. *J. Lipid Res.* **34:** 1219–1228.
54. Heinecke, J. W. 1994. Cellular mechanisms for the oxidative modification of lipoproteins: implications for atherogenesis. *Coronary Artery Dis.* **5:** 205–210.
55. Rodriguez-Aguilera, J. C., K. Nakayama, A. Arroyo, J. M. Viallalba, and P. Navas. 1993. Transplasma membrane redox system of HL-60 is controlled by cAMP. *J. Biol. Chem.* **268:** 26346–26349.

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56. Jian, Z. J., Z. Yang, G. L. Mason, D. O. Slauson, and P. N. Bochsler. 1995. Regulation of superoxide anion generation in bovine alveolar macrophages by bacterial lipopolysaccharide, serum proteins, and modulators of signal transduction. *Inflammation.* **19:** 637–650.
57. Fujiwara, K., H. Sato, and S. Bannai. 1998. Involvement of endotoxins or tumor necrosis factor-α in macrophage-mediated oxidation of low density lipoprotein. *FEBS Lett.* **431:** 116–120.
58. Funk, J. L., K. R. Feingold, A. H. Moser, and C. Grunfeld. 1993. Lipopolysaccharide stimulation of RAW 264.7 macrophages induces lipid accumulation and foam cell formation. *Atherosclerosis.* **98:** 67–82.
59. Yancey, P. G., and W. G. Jerome. 1998. Lysosomal sequestration of free and esterified cholesterol from oxidized low density lipoprotein in macrophages of different species. *J. Lipid Res.* **39:** 1349–1361.
60. Li, W., X. Yuan, A. G. Olsson, and U. T. Brunk. 1998. Uptake of oxidized LDL by macrophages results in partial lysosomal enzyme inactivation and relocation. *Arterioscler. Thromb. Vasc. Biol.* **18:** 177–184.
61. Rosenfeld, M. E., W. Palinski, S. Yla-Herttuala, S. Butler, and J. L. Witztum. 1990. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arteriosclerosis.* **10:** 336–349.
62. Ball, R. Y., K. L. Carpenter, J. H. Enright, S. L. Hartley, and M. J. Mitchinson. 1987. Ceroid accumulation by murine peritoneal macrophages exposed to artificial lipoproteins. *Br. J. Exp. Pathol.* **68:** 427–438.
63. Mander, E. L., R. T. Dean, K. K. Stanley, and W. Jessup. 1994. Apolipoprotein B of oxidized LDL accumulates in the lysosomes of macrophages. *Biochim. Biophys. Acta.* **1212:** 80–92.
64. Shichiri, G., M. Kinoshita, and Y. Saeki. 1993. Polyunsaturated fatty acid metabolism and acetylated low density lipoprotein uptake in J774A.1 cells. *Arch. Biochem. Biophys.* **303:** 231–237.
65. Rinninger, F., M. Brundert, S. Jäckle, T. Kaiser, and H. Greten. 1995. Selective uptake of low-density lipoprotein-associated cholesteryl esters by human fibroblasts, human HepG2 hepatoma cells and J774 macrophages in culture. *Biochim. Biophys. Acta.* **1255:** 141–153.
66. Green, S. R., and R. C. Pittman. 1991. Selective uptake of cholesteryl esters from low density lipoproteins in vitro and in vivo. *J. Lipid Res.* **32:** 667–678.
67. Tabas, I. 1997. Phospholipid metabolism in cholesterol-loaded macrophages. *Curr. Opin. Lipidol.* **8:** 263–267.

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