


Predominance of esterified hydroperoxy-linoleic acid in human monocyte-oxidized LDL¹

V. A. Folcik and M. K. Cathcart¹

Cleveland Clinic Foundation, Cleveland, OH 44194, and Cleveland State University, Cleveland, OH 44115

Abstract Low density lipoprotein that was oxidized by activated human monocytes was analyzed to determine the identity of oxidized fatty acids present and the conditions required for their formation. The oxidized lipids were also analyzed under conditions allowing preservation of their oxidation state. Using reversed-phase high performance liquid chromatography (HPLC) analysis of native and saponified lipid extracts of oxidized low density lipoprotein (LDL), we found that the major fatty acid oxidation product was esterified hydroperoxyoctadecadienoic acid (HPODE), the oxidized product of the most abundant polyunsaturated fatty acid in human LDL, linoleic acid. Although some esterified hydroxyoctadecadienoic acid (HODE) was also detected, the reduction of HPODE to HODE did not appear to be monocyte-dependent. Essentially all of the HPODE was found to be esterified with the majority being esterified to cholesterol followed by phospholipids and generally following the abundance of esterified linoleic acid within the lipid classes. The percent of cholesteryl linoleate converted to cholesteryl HPODE and cholesteryl HODE at the end of the 24-h incubation was determined to be approximately 13.5%. The formation of oxidized esterified linoleic acid in the LDL was shown to require immunological activation of the human monocytes, a previously observed requirement for general LDL oxidation in this culture system. The oxidized esterified linoleic acid was present in the supernatant with the LDL and was not cell-associated. HPODE formation on LDL was prevented by including superoxide dismutase (SOD) or eicosatetraynoic acid (ETYA) during the 24-h coincubation of activated monocytes with LDL whereas indomethacin was without effect.  The analysis of the lipid oxidation products in oxidized LDL can provide insight into the mechanisms involved in oxidation of LDL by activated human monocytes.—Folcik, V. A., and M. K. Cathcart. Predominance of esterified hydroperoxy-linoleic acid in human monocyte-oxidized LDL. *J. Lipid Res.* 1994. 35: 1570–1582.

Supplementary key words human monocytes • low density lipoprotein • oxidized lipoproteins • hydroperoxyoctadecadienoic acid • hydroxyoctadecadienoic acid • cholesteryl HPODE • cholesteryl HODE • fatty acid oxidation

Many of the cell types present in the arterial wall are capable of oxidatively modifying LDL lipids (reviewed in ref. 1). Our laboratory has focused on the oxidation of LDL lipids by activated human monocytes, with emphasis on investigating the cellular mechanisms involved.

Results from our previous studies have shown that activated human monocyte-mediated LDL oxidation is completely inhibited by superoxide dismutase, and that general inhibitors of monocyte lipoxygenase activity also prevent LDL oxidation (2–4). Our laboratory has also shown requirements for Ca²⁺ influx (5), release of Ca²⁺ from intracellular stores (5), and protein kinase C activity (6) in the process of LDL oxidation by activated human monocytes and U937 cells, a monocyte cell line also capable of oxidizing LDL. The present studies were designed to identify the major oxidation products found in monocyte-oxidized LDL.

LDL modification has traditionally been quantitated by determining the degree of lipid oxidation on LDL using assays for thiobarbituric acid reactive substances (TBARS; 7), conjugated dienes, total lipid peroxides (8), enhanced electrophoretic mobility, or uptake and degradation of the LDL by mouse peritoneal macrophages (9–12). In the present study we have used HPLC to analyze the fatty acid oxidation products present in LDL modified by activated human monocytes. We compared the profile of oxidized linoleic acid products formed by oxidation of LDL using CuSO₄ and soybean 15-lipoxygenase (LO) under conditions that yielded levels of oxidation (measured as TBARS) similar to those found after monocyte-mediated modification (3, 13).

Abbreviations: BHT, butylated hydroxytoluene; DMSO, dimethylsulfoxide; DPBS, Dulbecco's phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; ETOH, ethanol; ETYA, eicosatetraynoic acid; HPODE, hydroperoxyoctadecadienoic acid; HODE, hydroxyoctadecadienoic acid; 15-LO, 15-lipoxygenase; SLO, soybean 15-lipoxygenase; HETE, hydroxyeicosatetraenoic acid; TBARS, thiobarbituric acid reactive substances; ZOP, opsonized zymosan; [³H]PC, 1- α -dipalmitoyl-[2-palmitoyl-9,10-³H(N)]phosphatidylcholine; MTBE, methyl-*t*-butyl ether; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; SOD, superoxide dismutase; MHC, major histocompatibility complex.

¹Presented in part at the 66th Scientific Session of the American Heart Association, November 1993.

²To whom correspondence should be addressed at: Department of Cell Biology, Building NN1, Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195.

LDL functions to transport cholesterol in the blood, and contains considerable quantities of cholesterol esterified to fatty acids, as well as free cholesterol, phospholipids, and triglycerides (14). LDL also contains relatively minor quantities of diglycerides, monoglycerides, and free fatty acids, approximately 0.4%, < 0.7%, and 1.1% by weight, respectively, of total lipids (15). The most abundant available fatty acid substrate for monocyte-mediated lipid oxidation in LDL is esterified linoleic acid. Saturated fatty acids and monounsaturated fatty acids that are also abundant in LDL are not as susceptible to non-enzymatic oxidative modification as are polyunsaturated fatty acids, due to their relative lack of reactive sites. Upon oxidation, linoleic acid (octadecadienoic acid) initially forms several different isomers of hydroperoxyoctadecadienoic acid or HPODE. These isomers include (ZE)-13-HPODE and (EZ)-9-HPODE, and their isomerization products (EE)-13-HPODE and (EE)-9-HPODE. Reduction of the HPODE products results in the formation of hydroxyoctadecadienoic acids or HODEs.

We wished to determine the oxidation state of monocyte-modified LDL, i.e., the quantity of hydroperoxy-linoleic acid versus hydroxy-linoleic acid formed, as this could indicate the biochemical and enzymatic pathways involved in monocyte oxidation of LDL lipids. Previous reports have not directly addressed the ratio of cholesteryl HPODE to cholesteryl HODE products formed in cell-mediated LDL fatty acid oxidation (16-18).

Other aspects of activated human monocyte-mediated LDL lipid oxidation not examined previously are the extent to which various lipid classes contribute to the production of oxidized fatty acids formed, as well as the extent of oxidation of available substrate. We have quantitatively examined these aspects of LDL lipid oxidation using activated monocytes as this cell system may relate to LDL oxidation in atherosclerotic lesions and sites of inflammation.

MATERIALS AND METHODS

LDL isolation

LDL (solvent density 1.019-1.063 g/ml) was isolated from human plasma obtained from apheresis performed on donors at the Cleveland Clinic Blood Bank. The method of isolation was sequential ultracentrifugation according to Hatch and Lees (19) and Havel, Eder, and Bragdon (20) with some modifications. Five mM EDTA was included during all isolation steps to inhibit oxidation during preparation. Prior to use in experiments, LDL was dialyzed in Spectra/Por 2 Molecularporous membrane tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) for 3-5 h against DPBS without calcium or magnesium at 4°C in the dark to remove the EDTA added during preparation. Chelating resin (Sigma Chemical

Co., St. Louis, MO) was added to the dialysis buffer (1.0 mg/ml) to remove any transition metal ions present. The LDL was also filter-sterilized with a 0.45-micron Sterile Acrodisc syringe filter (Gelman Sciences, Ann Arbor, MI) prior to addition to cells in culture. LDL endotoxin levels were determined to be < 0.03 units/mg LDL cholesterol as determined with a QCL-1000 Limulus Amoebocyte Lysate kit (Whittaker Bioproducts, Walkersville, MD).

Monocyte isolation and monocyte-mediated LDL oxidation

Human monocytes were isolated from freshly drawn, heparinized whole blood as previously described (3, 4). After removal from tissue culture flasks using 5 mM ethylenediaminetetraacetic acid (EDTA) in Dulbecco's phosphate-buffered saline (DPBS; Gibco Laboratories, Grand Island, NY) human monocytes were plated in Costar (Cambridge, MA) 12-well tissue culture plates at 1.0×10^6 cells per well in 10% bovine calf serum-Dulbecco's modified Eagle's medium (BCS-DMEM) and allowed to adhere. After adherence the medium was aspirated and replaced with 1 ml of RPMI-1640 medium (Whittaker, Bioproducts). At this time LDL was added to the wells at 0.5 mg cholesterol/ml. Inhibitors including ETYA (Cayman Chemical Co., Ann Arbor, MI), indomethacin, and SOD (Sigma Chemical Co.) or the drug vehicles ethanol (ETOH) or dimethylsulfoxide (DMSO) were added at this time. Opsonized zymosan (ZOP; 2 mg/ml; 21) was then added to activate the cells and the incubation was continued for 20-24 h. The optimal concentrations of ZOP, human monocytes, and LDL and the optimal incubation conditions were determined previously for LDL oxidation by activated human monocytes (22).

CuSO₄ and SLO-mediated LDL oxidation

CuSO₄ and soybean lipoxygenase (SLO)-mediated LDL oxidations were carried out in 12-well tissue culture plates in a 1-ml volume of RPMI-1640 and incubated for 24 h at 37°C in 10% CO₂ to imitate monocyte assay conditions, but without cells (3, 13). CuSO₄ (Fisher Scientific Co., Fair Lawn, NJ) was used at 2 or 5 μ M and affinity-purified SLO type V (Sigma Chemical Co.) was used at approximately 5000 units/ml to oxidize LDL at 0.5 mg cholesterol/ml.

Sample processing procedures

Prior to extraction, butylated hydroxytoluene (BHT; Sigma Chemical Company) was added to the culture wells to yield a final concentration of 20 μ M or 500 μ M as indicated. [³H]-12-HETE (12(S)-hydroxy [5,6,8,9,11,12,14,15(n)]-[³H]eicosatetraenoic acid, Amersham Corporation, Arlington Heights, IL) was also added to the wells as an internal standard for experiments in which the oxidized fatty acids were to be analyzed. Internal standards of cholesteryl [¹⁴C]HPODE (obtained from HPLC repurifica-

tion of cholesteryl [^{14}C]linoleate; NEN Research Products, DuPont Company, Wilmington, DE) and L- α -dipalmitoyl-[2-palmitoyl-9,10- $^3\text{H}(\text{N})$]phosphatidylcholine (^3H PC; NEN Research Products, DuPont Company) were added to sample wells to be quantitatively analyzed for oxidized linoleic acid products esterified to neutral lipids versus phospholipids. These standards enabled calculation of recovery of fatty acids from both lipid classes as well as detection of contamination of the fatty acids from one lipid class with those from the other.

Lipid extraction methods. At the end of the incubation, the lipid was either extracted from the sample supernatants using chloroform and methanol (23, 24) or, in some experiments, the samples were lyophilized overnight, then extracted with 3–4 ml of ether-ethanol 3:1, vortexed, and centrifuged to remove particulate matter. Unless otherwise indicated, all material present in the wells, including the supernatant and cells, was extracted and analyzed for oxidized fatty acids. The lipid extracts were dried under nitrogen and processed further or stored at -20°C until HPLC analysis.

Lipid class separation. Neutral lipids and phospholipids of oxidized LDL were separated so that the fatty acid oxidation products from these lipid classes could be analyzed and quantitatively compared. The separation of neutral lipids from phospholipids was accomplished as follows (25). The chloroform phase was dried under N_2 , resuspended in 2.0 ml hexane-methyl-*t*-butyl ether (MTBE; 9:1) and applied to Silica Sep-pak cartridges pre-equilibrated with hexane (Waters, Millipore Corp., Cidra, PR). The oxidized and unoxidized neutral lipids were eluted from the silica cartridges with 20 ml of hexane-MTBE 1:1. The phospholipids were eluted from the cartridges with 20 ml methanol. The separated fractions were dried under N_2 and saponified followed by solid-phase extraction of the fatty acids.

Saponification. The lipid was reconstituted in 0.5 ml of absolute ethanol (Aaper Alcohol and Chemical Co., Shelbyville, KY) and saponified with a final concentration of 1 N NaOH (Sigma Chemical Co.) for 20 min at 60°C . At the end of the incubation the samples were acidified with 30 μl of acetic acid (Mallinckrodt Inc., Paris, KY).

Solid-phase fatty acid extraction. Samples were adjusted to a final concentration of 25% ethanol and the 2-ml samples were acidified with 133 μl of 10% formic acid. The samples were passed through Sep-pak C18 columns (Millipore) followed consecutively by 20-ml volumes of water, 25% ethanol, water, and petroleum ether (EM Science, Gibbstown, NJ). The fatty acids were finally eluted with 5 ml of ethyl acetate (EM Science) and dried under N_2 , then stored at -20°C in polypropylene tubes until HPLC analysis (26).

Reversed-phase HPLC

Reversed-phase HPLC was performed with a Rainin

Microsorb C18 column (250 mm \times 4.6 mm, Rainin Instrument Co., Inc., Woburn, MA), a Beckman Model 421A controller, Beckman Model 114M pumps and a Beckman Model 163 Variable Wavelength detector. Radioactivity was detected with an online Radiomatic Instruments Flow-one Beta Radioactive Flow Detector. Data were collected on an IBM Personal Computer XT and processed utilizing Lotus 1-2-3 (Lotus Development Corporation) and GraphPAD InPlot Software (GraphPAD Software, San Diego, Ca.). Packard Radiomatic Flo-Scint II (Radiomatic Instruments and Chemical Co., Meriden, CT) was used as scintillant. The samples were reconstituted in mobile phase immediately before injection and filtered with Rainin Nylon-66, 0.45 micron filters.

Solvent system A. The oxidized fatty acids were eluted isocratically at 1 ml/min. with a solvent mixture consisting of water-acetonitrile-tetrahydrofuran-acetic acid at a ratio of 44:36:20:0.05, as previously described (27). Claeys et al. (27) verified the identity of the monohydroxylinoleic acid metabolites separated with this solvent system by gas chromatography-mass spectrometry (GC-MS). All solvents used were of HPLC grade from Fisher Scientific or EM Science. After the oxidized fatty acids had eluted (approx. 26 min) the column was purged with acetonitrile.

Standards of 13(S)-HODE, 9(S)-HODE, 13(S)-HPODE, and 9(S)-HPODE, and a mixture of 5, 8, 11, 12, and 15-hydroxyeicosatetraenoic acids (HETEs) were purchased from Cayman Chemical Co. Oxidized linolenic acid standards were prepared by oxidation of linolenic acid with soybean lipoxygenase. Standards were run daily to ensure proper identification of metabolites.

Solvent system B. Alternative methods for separating lipids of oxidized LDL were obtained from G. M. Chisolm and P. E. DiCorleto (personal communication). Reversed-phase HPLC was performed using the same column described previously and a gradient solvent system beginning with 100% acetonitrile for 5 min, changing from 100% acetonitrile to 85% acetonitrile and 15% isopropanol over the next 10 min, followed by an increase in isopropanol to 80% over the next 36 min. The solvent was restored to 100% acetonitrile over the next 10 min. Standards of cholesterol and cholesteryl linoleate were purchased from Sigma Chemical Co. Cholesteryl 13-HODE was purchased from Cayman Chemical Co.

Data calculations

Data presented as "normalized" UV absorbance were processed to take into account the recovery of the internal standard so that a quantitative comparison of the data could be made. Recoveries for saponified fatty acids were 30–40%, for cholesteryl esters, 50–60%, and for phospholipids 30–40%. Initial recovery of oxidized cholesteryl esters without lipid class separation ranged from 70 to 90%. The calculation of the inhibition of oxidation in-

cluded subtraction of the spontaneous oxidation found in the absence of cells in each experiment. All chromatograms are representative of two to four experiments performed. Quantitative data were calculated using extinction coefficients calculated from injection of known amounts of oxidized fatty acid standards, or from extinction coefficients calculated by determination of cholesterol content in collected peaks (28).

RESULTS

To examine the total oxidized fatty acids in monocyte-oxidized LDL, saponified samples were analyzed. The oxidized fatty acid products were identified by their retention times in common with standards. Analysis of the oxidized fatty acid composition using solvent system A revealed three major peaks, products of linoleic acid oxidation, followed by smaller peaks, products of arachidonic acid oxidation (Fig. 1). In some samples oxidized linolenic acid products were also detected. Thus, oxidized linoleic acid was the predominant oxidized fatty acid in these preparations.

Figure 1 also demonstrates the marked difference in quantity of oxidized fatty acid products found when saponification was included in the extraction procedure. When the lipids extracted from three wells of monocyte-

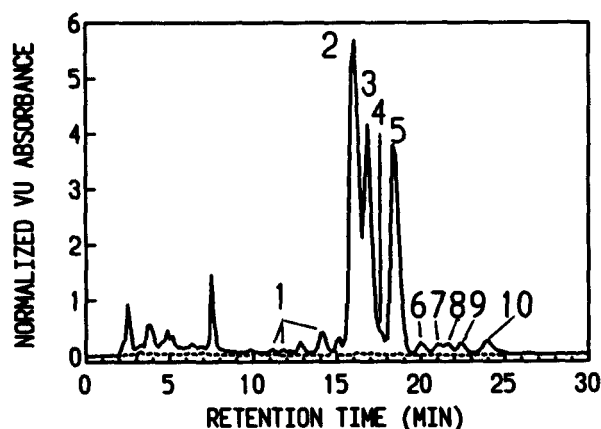


Fig. 1. Oxidized fatty acids detected in monocyte-oxidized LDL are esterified. Monocytes (1×10^6 cells/ml) were incubated at 37°C for 24 h with the activator ZOP (2.0 mg/ml) and LDL (0.5 mg cholesterol/ml). A final concentration of $20 \mu\text{M}$ BHT was added and three culture wells/sample (1 ml/well) were spiked with the internal standard [^3H]12-HETE, pooled and the oxidized fatty acids were extracted for HPLC analysis using solvent system A with UV detection at 236 nm (solid line). The dashed line indicates a sample that was processed similarly but without saponification. The identity of peaks is as follows: 1, oxidized linolenic acid products; 2, (ZE)-13-HODE; 3, (EZ)-9-HODE; 4, 15-HETE; 5, (EE)-13- and (EE)-9-HODE; 6, 11-HETE; 7, 12-HETE; 8, 8-HETE; 9, unknown; 10, 5-HETE.

TABLE 1. Oxidation state of oxidized cholesteryl linoleate in LDL

LDL Treatment	% Cholesteryl HPODE ^a	Ratio ^b
Cells + ZOP	84.2 ± 1.1	5.3:1
ZOP	73.6 ± 1.5	2.8:1
CuSO_4	86.2 ± 1.1	6.3:1
SLO*	88.1 ± 2.8	7.4:1

LDL (0.5 mg cholesterol/ml) was incubated for 24 h in RPMI-1640 medium in the presence of activated monocytes ($1 \times 10^6/\text{ml}$), the activator, ZOP (2 mg/ml), CuSO_4 ($5 \mu\text{M}$), or SLO (approx. 5000 units/ml) as indicated. At the end of the incubation $500 \mu\text{M}$ BHT ($20 \mu\text{M}$ in some samples) was added, followed by lipid extraction and analysis with solvent system B.

^aThe percentage indicates the fraction of total cholesteryl HPODE and cholesteryl HODE detected. The mean \pm the standard error of the means of five experiments (* $n = 2$ experiments) is shown. The percent cholesteryl HPODE determined for oxidation mediated by activated monocytes and CuSO_4 was significantly different than that obtained in the cell-free control ($P = 0.0079$, $P = 0.0159$, respectively; Mann-Whitney Two Sample Test).

^bRatio of cholesteryl HPODE to cholesteryl HODE detected.

oxidized LDL were saponified, HODEs were detected in considerable quantity compared to almost none found when saponification was omitted. A quantitative comparison could be made because the recovery of an internal standard, [^3H]12-HETE, was used to normalize the UV absorbance data. The samples shown in Fig. 1 contained 342.8 nmol HODEs and 23.9 nmol/mg LDL cholesterol of HETEs in the saponified sample and 2.8 nmol/mg LDL cholesterol of HODEs (0.8%) in the sample that was not saponified. Thus, nearly all of the oxidized fatty acids detected in monocyte-oxidized LDL were present in esterified form.

The HETEs appear to be formed by autooxidation, as the same quantity of these metabolites was formed in the presence or absence of activated monocytes (see Fig. 2, B and C). In addition, positional isomers of HETE were formed that are not known to be formed enzymatically.

It should be noted that although HODEs appeared to be the products of oxidation of esterified linoleic acid in these samples, their oxidation state was found to be influenced by the fact that the sample processing procedure reduced hydroperoxy-polyunsaturated fatty acids in the samples to hydroxy-polyunsaturated fatty acids. We therefore determined the actual oxidation state of the cholesterol-esterified oxidized linoleic acid products by other means as discussed below (Table 1; see Fig. 7).

In this study we sought to examine the oxidized fatty acid products formed under the same conditions that had previously been used to study cell-mediated LDL oxidation. Previously, LDL oxidation had been quantified using general methods to detect lipid peroxides, conjugated dienes, or malondialdehyde-like products (7, 8). Monocyte-mediated LDL oxidation has been consistently dependent

upon monocyte activation in our hands; freshly isolated human blood monocytes that are not activated do not oxidize LDL in RPMI-1640 medium (2-6, 22). Monocyte oxidation of esterified linoleic acid in LDL, in a fashion similar to the formation of TBARS or lipid peroxides, was also found to be dependent on monocyte activation. Only 2.2 nmol HODEs/mg LDL cholesterol was detected in the supernatants of monocytes incubated with LDL in the absence of activator (**Fig. 2A**). LDL oxidation in the presence of activator without cells was detectable (**Fig. 2B**; 95.7 nmol HODEs and 32.9 nmol HETEs/mg LDL cholesterol), consistent with the low but detectable TBARS formation noted in our previous findings (2-5, 22). Results displayed in **Fig. 2C** (and **Fig. 1**, solid line) show that the formation of oxidized linoleic acid was maximal when the monocytes were activated during incubation with LDL. In samples of monocytes activated in the absence of exogenously added substrate such as LDL, cell-derived fatty acid oxidation products, if formed, were in quantities below the limits of detection of the HPLC-UV absorbance detection system used to analyze the samples (data not shown; UV absorbance detection limit for HODEs was approximately 100 pmol).

We also determined whether the esterified HODEs were in the supernatant, as are the oxidation products detected by the assay for TBARS, or whether some remained associated with the cell layer. For these studies, the supernatants were extracted separately from the cell layer (**Fig. 3**). HODEs (273.9 nmol) and HETEs (59.1 nmol) were detected in the supernatant while only 7.7 nmol HODEs (and no HETEs) were detected in the fraction associated with the cells (per mg LDL cholesterol). Thus, the HODEs and HETEs were detected almost entirely in the supernatant.

We have previously shown that inhibitors of the lipoxygenase pathway, but not the cyclooxygenase pathway inhibit LDL oxidation by activated monocytes at concentrations that are not toxic and under conditions where the inhibitors do not appear to act as general antioxidants (3). ETYA is an arachidonic acid analog and an inhibitor of both lipoxygenase and cyclooxygenase enzymes. **Figure 4** shows a representative experiment where 30 μ M ETYA inhibited monocyte oxidation of LDL lipids by 62.4% (quantitated as esterified HODE formation). In **Fig. 4A**, 443.0 nmol HODEs and 51.5 nmol HETEs were detected; in **Fig. 4B**, 260.0 nmol HODEs and 55.37 nmol HETEs were detected per mg LDL cholesterol. HODEs (149.7 nmol) and HETEs (44.6 nmol) were detected in the cell-free control (not shown) and subtracted from both values to calculate inhibition of cell-mediated oxidation. This degree of inhibition is in agreement with our previous results for 30 μ M ETYA inhibition of monocyte-mediated LDL oxidation quantitated as TBARS (A. K. McNally, M. K. Cathcart and V. A. Folcik, unpublished observations). We have found that 30 μ M ETYA is not toxic to

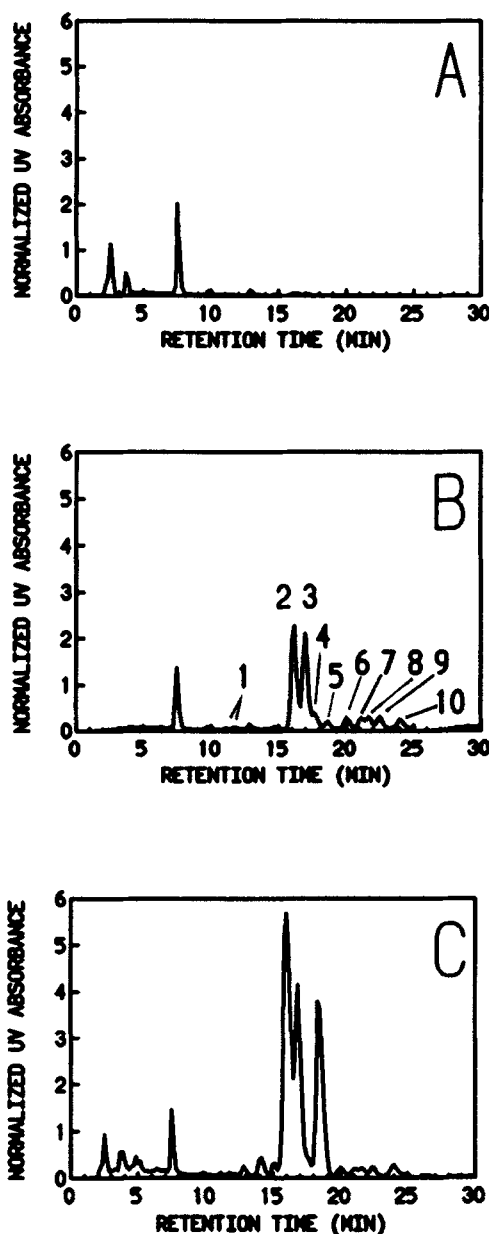


Fig. 2. Monocyte activation is required for monocyte-mediated LDL oxidation. LDL (0.5 mg cholesterol/ml) was incubated in the presence of A, monocytes (1×10^6 cells/ml) that were not activated; B, the activator ZOP (2.0 mg/ml); or C, monocytes and ZOP at 37°C for 24 h. A final concentration of 20 μ M BHT and the internal standard [3 H]12-HETE were added to each sample well; the sample wells were pooled, then the oxidized fatty acids were extracted for HPLC analysis using solvent system A with UV detection at 236 nm (3 wells/sample, 1 ml/well). See **Fig. 1** legend for identity of labeled peaks. Chromatogram shown in panel C is labeled in **Fig. 1**.

activated monocytes in the presence of LDL in RPMI-1640 medium (4) using an assay of [14 C]adenine metabolite release (29). ETYA had no effect on the formation of HETEs (**Fig. 4A** and **B**). Indomethacin, an inhibitor of the cyclooxygenase pathway, was without effect on

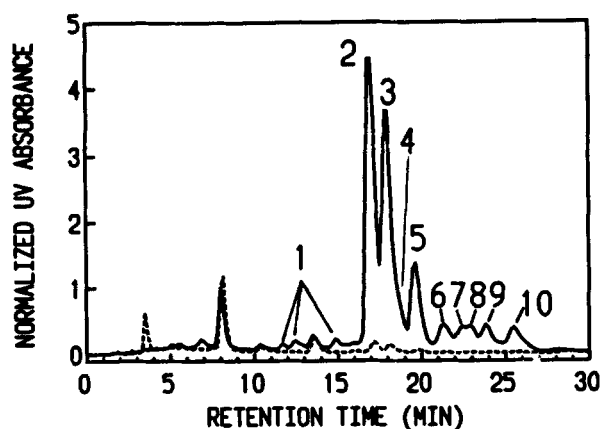


Fig. 3. Oxidized fatty acids in monocyte-oxidized LDL are in the supernatant. Monocytes (1×10^6 cells/ml) were incubated at 37°C for 24 h with the activator ZOP (2.0 mg/ml) and LDL (0.5 mg cholesterol/ml). A final concentration of $20 \mu\text{M}$ BHT was added; the cell culture plates were centrifuged; supernatants from three culture wells (1 ml/well) were harvested and pooled, then the internal standard $[^3\text{H}]12\text{-HETE}$ was added, and the oxidized fatty acids were extracted (solid line). The cells remaining in the wells were scraped with a rubber policeman and reconstituted in 1 ml of RPMI-1640 medium; BHT was added to a final concentration of $20 \mu\text{M}$; the internal standard $[^3\text{H}]12\text{-HETE}$ was added; and the oxidized fatty acids were extracted (dashed line). The samples were analyzed by HPLC using solvent system A with UV detection at 236 nm. Identities of labeled peaks are given in Fig. 1 legend.

HODE formation (data not shown) when added to yield $30 \mu\text{M}$, a concentration shown to inhibit the cyclooxygenase pathway in macrophages (30).

We have previously shown that SOD added during the exposure of LDL to activated monocytes completely inhibits monocyte-mediated LDL oxidation as measured with the assay for TBARS (2). In the presence of SOD, only 8.9 nmol HODEs and 5.7 nmol HETEs were detected, compared to 365.7 nmol HODEs and 97.2 nmol HETEs/mg LDL cholesterol oxidized by activated monocytes in the absence of SOD (Fig. 5).

The profile of products formed in monocyte-oxidized LDL, LDL oxidized by soybean 15-lipoxygenase and by CuSO_4 were compared. We had previously shown that soybean 15-lipoxygenase oxidizes LDL and causes it to become cytotoxic much like monocyte-oxidized LDL (13). We previously established conditions for soybean 15-lipoxygenase and CuSO_4 -mediated LDL oxidation that caused levels of LDL oxidation similar to those obtained by oxidation with activated monocytes as measured by TBARS (3, 13). Our TBARS levels for LDL oxidation by activated monocytes normally range between approximately 2–4 nmol/0.5 mg LDL cholesterol. For LDL oxidized by soybean 15-LO (Fig. 6A, solid line) the TBARS level was 2.35 nmol/0.5 mg LDL cholesterol, and the TBARS level for LDL oxidized by CuSO_4 (Fig. 6B) was 1.42 nmol/0.5 mg LDL cholesterol. The sample shown in Fig. 6A (solid line) contained 187.7 nmol

HODEs and 56.2 nmol HETEs; LDL incubated without a catalyst for oxidation (Fig. 6A, dashed line) contained 9.5 nmol HODEs and 3.9 nmol HETEs; and the sample shown in Fig. 6B contained 152.9 nmol HODEs and 44.9 nmol HETEs/mg LDL cholesterol. Just as with oxidation

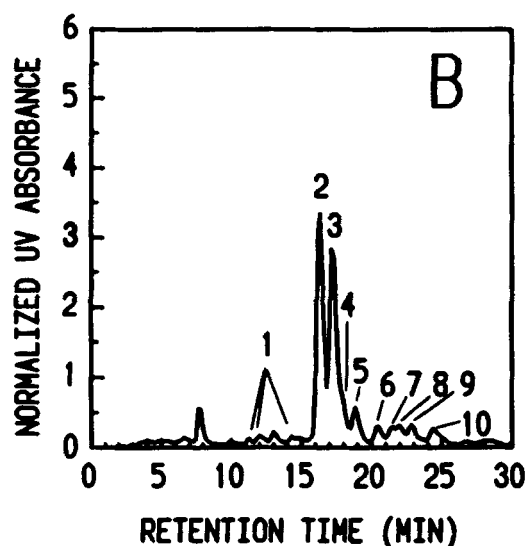
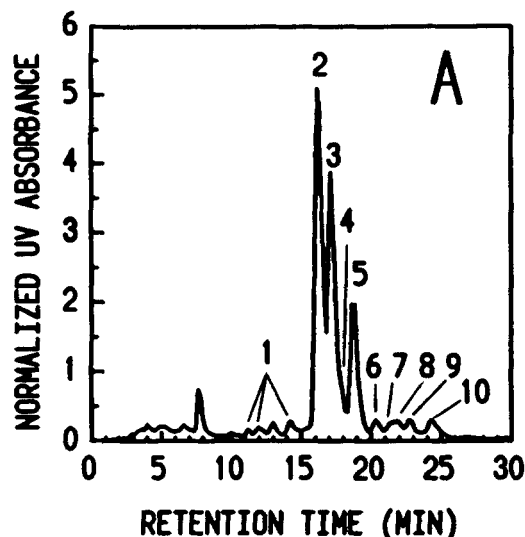


Fig. 4. ETYA inhibits LDL fatty acid oxidation by activated monocytes. Monocytes (1×10^6 cells/ml) were incubated at 37°C for 24 h with the activator, ZOP (2.0 mg/ml), and LDL (0.5 mg cholesterol/ml) in the presence of A, the drug vehicle, ETOH; or B, $30 \mu\text{M}$ ETYA prepared fresh for the experiment. At the end of the incubation a final concentration of $20 \mu\text{M}$ BHT was added; the sample wells (two 1-ml culture wells/sample) were spiked with an internal standard, $[^3\text{H}]12\text{-HETE}$; the sample wells were pooled; and the oxidized fatty acids were extracted for HPLC analysis using solvent system A with UV detection at 236 nm. See Fig. 1 legend for identities of labeled peaks.

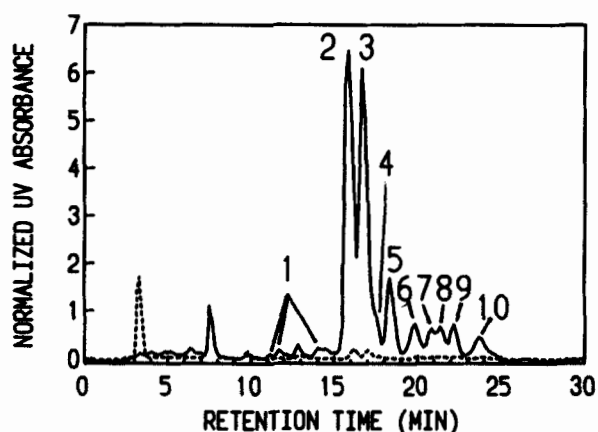


Fig. 5. SOD inhibits LDL fatty acid oxidation by activated monocytes. Monocytes (1×10^6 cells/ml) were incubated at 37°C for 24 h with the activator ZOP (2.0 mg/ml) and LDL (0.5 mg cholesterol/ml) in the presence (dashed line) and absence (solid line) of SOD (150 U/ml). A final concentration of $20 \mu\text{M}$ BHT was added, then the samples were spiked with an internal standard, $[^3\text{H}]12\text{-HETE}$, pooled (three 1-ml culture wells/sample), and the oxidized fatty acids were extracted for HPLC analysis using solvent system A with UV detection at 236 nm. Labeled peaks are identified in Fig. 1 legend.

mediated by activated monocytes, the most abundant oxidation products detected were (ZE)-13- and (EZ)-9-HODE.

The profiles of the saponified and reduced (by the extraction procedure) LDL-derived linoleic acid oxidation products formed by all three means differed in the formation of the (EE)-13- and (EE)-9-HODE isomers in the samples shown (Fig. 2C, Fig. 6A and B). The quantity of *trans,trans* isomers present in other samples of CuSO_4 and soybean 15-LO-oxidized LDL varied (not shown). The ratio of HODE products detected as *trans,trans* isomers also varied among experiments in which LDL was oxidized by activated monocytes (Fig. 2C, Fig. 3, Fig. 4A, and Fig. 5). Thus, the factors promoting the formation of *trans,trans* isomers of 13- and 9-HODE were not determined. Such isomerizations can occur spontaneously to varying degrees in different solvents and under different conditions (31, 32).

As the available linoleic acid substrate for oxidation within LDL is esterified to both neutral lipids and phospholipids, we analyzed the percentage of the oxidized linoleic acid in monocyte-oxidized LDL present in each of these lipid classes. After extraction of the total lipids from oxidized LDL, the neutral lipids and phospholipids were separated and the oxidized fatty acids were analyzed and quantitated. The percentages of oxidized linoleic acid products found esterified within neutral lipids versus phospholipids are summarized in Table 2. The molar percentages of linoleic acid molecules esterified to cholesterol, triglycerides, and phospholipids in LDL were estimated from published reports to be 79% esterified to cholesterol, 6% esterified in triglycerides, and 15% es-

terified to phospholipids (14). The quantity of oxidized free linoleic acid that contributes to the total amount detected in the saponified samples was less than 1.0% (Fig. 1). The oxidation of linoleic acid appears to follow the abundance of linoleic acid molecules estimated to be present in the three lipid classes.

These calculations and the verification of the separation of neutral lipids and phospholipids were accomplished using the recovery and separation of cholesteryl $[^{14}\text{C}]\text{HPODE}$ and $[^3\text{H}]\text{PC}$ internal standards. The fate of oxidized triglycerides in the separation is not certain, although native triglycerides were found in the neutral lipid fraction (data not shown).

For the purpose of determining the oxidation state of the oxidized linoleic acid in LDL, total lipid extracts from

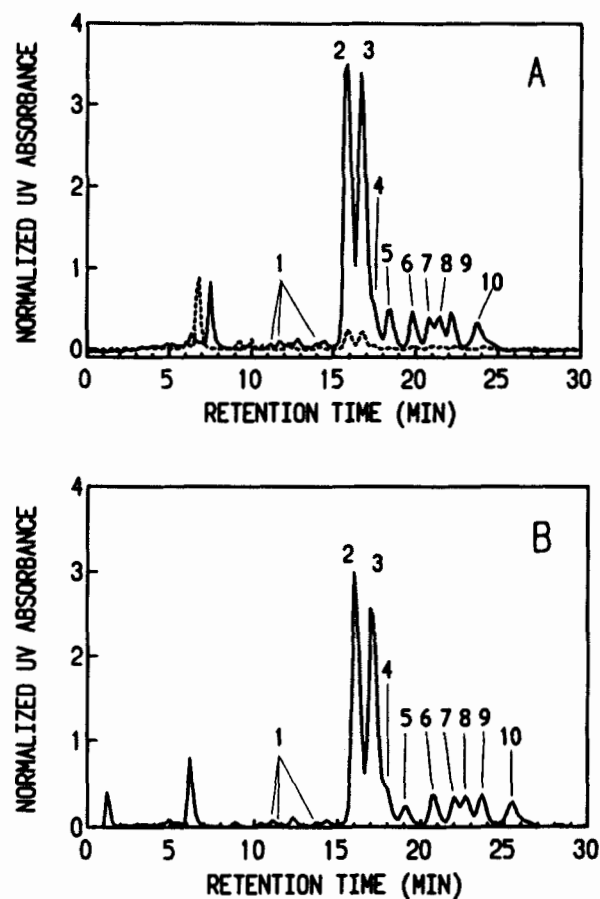


Fig. 6. SLO- and CuSO_4 -mediated LDL fatty acid oxidation. LDL (0.5 mg cholesterol/ml) was incubated at 37°C for 24 h in the presence (solid line) or absence (dashed line) of A, soybean 15-LO (approximately 5000 units/ml) or B, $5 \mu\text{M}$ CuSO_4 in RPMI-1640 medium. A final concentration of $20 \mu\text{M}$ BHT was added then the samples were spiked with an internal standard, $[^3\text{H}]12\text{-HETE}$, pooled (three 1-ml culture wells/sample), and the oxidized fatty acids were then extracted and analyzed by reversed-phase HPLC using solvent system A with UV detection at 236 nm. Labeled peaks are identified in Fig. 1 legend.

TABLE 2. Determination of lipid class origin of esterified, oxidized linoleic acid

LDL Treatment	% from NL Fraction ^a
Cells + ZOP	80.4 ± 2.5
ZOP	75.0 ± 6.1
SLO	79.4 ± 0.8
CuSO ₄ ^b	78.6 ± 0.7

LDL (0.5 mg cholesterol/ml) was incubated for 24 h in RPMI-1640 medium in the presence of activated monocytes (1×10^6 /ml), the activator, ZOP (2 mg/ml), CuSO₄ (5 μ M), or SLO (approx. 5000 units/ml) as indicated. Samples of oxidized LDL or cell-free controls were labeled with cholesteryl [¹⁴C]HPODE and [³H]PC; BHT (500 μ M) was added; the samples were extracted; the neutral lipids (NL) were separated from the phospholipids and saponified; then the oxidized fatty acids were extracted and analyzed by reversed phase HPLC with solvent system A. The percentage of the total oxidized linoleic acid products originating from the neutral lipids or the phospholipids was calculated based on the recovery of the internal standards added to the samples. Data are means \pm data spread from two experiments performed in duplicate or triplicate.

^aThe percent of the linoleic acid oxidation products detected in the neutral lipid fraction, the remainder were detected in the phospholipid fraction.

^bMean \pm data spread within an experiment.

monocyte-oxidized LDL were analyzed by a reversed-phase HPLC method that allows resolution and detection of free fatty acids, cholesterol, cholesteryl-HPODEs, cholesteryl-HODEs, triglycerides, and cholesterol esterified to native fatty acids (Fig. 7). Samples were monitored for absorbance at 206 nm to detect carbon-carbon double bonds (Fig. 7A), or at 236 nm to detect conjugated dienes of oxidized lipids (Fig. 7B). The peaks of absorbance with known identity are labeled. Peak 1 shares the retention time of free cholesterol. Peak 2 shares the retention time of a peak previously reanalyzed with cyano-HPLC and identified using fast atom bombardment mass spectrometry and infrared spectroscopy. The re-analyzed peak was identified as a mixture of cholesteryl HPODE isomers (M. VanHeek, D. Schmidt, P. Toren, and P. E. DiCorleto, unpublished results). Peak 3 shares the retention time of cholesteryl HODE. Additionally, when peak 2 was collected, chemically reduced and re-analyzed by reversed-phase HPLC it subsequently eluted with the retention time of peak 3 (P. E. DiCorleto and D. Schmidt, unpublished results). For the purpose of verifying the identity of peaks 2 and 3 in our samples as oxidized cholesteryl linoleate products, these peaks were collected upon elution from the column and subjected to saponification, followed by re-analysis by reversed-phase HPLC with solvent systems A and B. Re-analysis of the products by solvent system A resulted in detection of oxidized linoleic acid, as expected. Re-analysis with solvent B resulted in disappearance of the original peak and detection of cholesterol and free fatty acids (data not shown; solvent system B does not resolve individual fatty acids).

Peaks 2 and 3 also absorbed at 236 nm, verifying the presence of conjugated dienes (Fig. 7B). Peak 4 shares the retention time of triolein (G. M. Chisolm, personal communication) and peak 5 shares the retention time of cholesteryl linoleate (Fig. 7A). Other cholesteryl esters may also co-elute with cholesteryl linoleate. Cholesteryl 13- and 9-HPODE (or HODE) and the *cis,trans* and *trans,trans* isomers of each positional isomer are not separated by solvent system B.

Marked differences in the chromatograms representing LDL lipids that have been oxidized by activated monocytes and the control incubation without cells are evident at both wavelengths shown (Fig. 7). The quantitative differences, however, cannot be estimated by simple in-

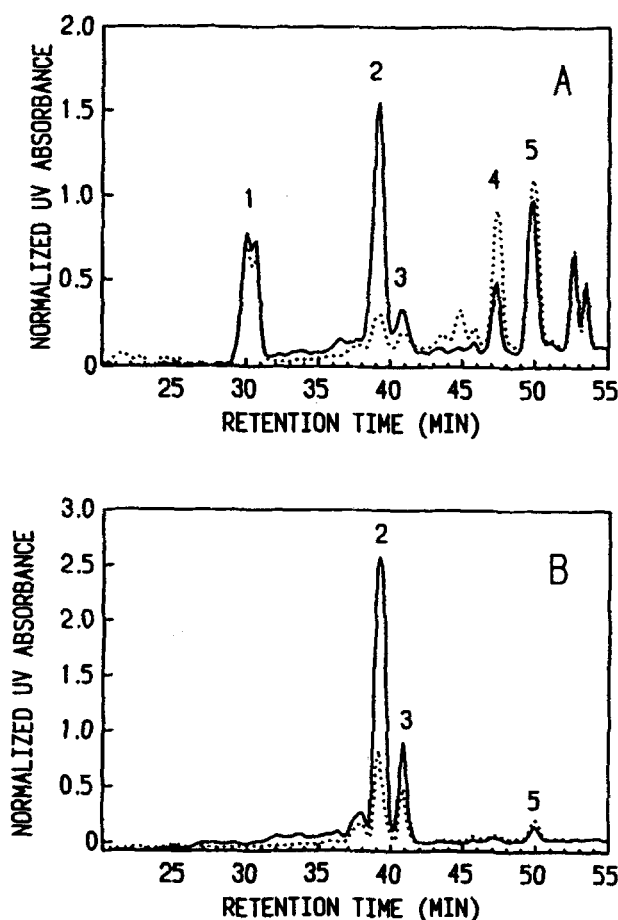


Fig. 7. Reversed-phase HPLC analysis of neutral lipids from LDL oxidized by activated monocytes. LDL (0.5 mg cholesterol/ml) was incubated with the activator ZOP (2.0 mg/ml) in the presence (solid line) or absence (dashed line) of monocytes (1×10^6 cells/ml). A final concentration of 500 μ M BHT and an internal standard, cholesteryl [¹⁴C]HPODE, were added to each sample. The pooled sample wells (3 culture wells/sample, 1 ml/well) were then extracted with chloroform and methanol and analyzed by reversed-phase HPLC using solvent system B with UV detection at 206 nm (A) and 236 nm (B). Peak 1, cholesterol; peak 2, cholesteryl HPODE; peak 3, cholesteryl HODE; peak 4, triolein; peak 5, cholesteryl linoleate.

spection of the UV absorbance as shown in Fig. 7A because each of the metabolites has a unique extinction coefficient at 206 nm.

The recoveries of the metabolites for the chromatograms shown in Fig. 7A and B were based upon the recovery of cholesteryl [^{14}C]HPODE. Using cholesteryl [^{14}C]HPODE as the internal standard also allowed for detection of reduction of the oxidized products during the processing of the samples. When cholesteryl [^{14}C]HPODE was included in samples processed for analysis of oxidized fatty acids, only [^{14}C]HODE was detected. The reduction of the hydroperoxy products was shown to be due to the saponification step following the chloroform-methanol extraction; a step that involved both extremes of pH and heating of the samples, and the subsequent solid phase fatty acid extraction that involved acidification of the samples. The sample processing method used for the results shown in Figs. 7A and B was simply the chloroform-methanol extraction and caused no conversion of the cholesteryl [^{14}C]HPODE to cholesteryl [^{14}C]HODE. Thus, the oxidation state of the products at the end of the 24-h incubation could be determined from samples processed in this manner. The majority of the oxidation products detected were in the hydroperoxy form (Table 1). The ratio of cholesteryl HPODE to cholesteryl HODE found in monocyte-oxidized LDL lipids was compared to the LDL from control incubations without monocytes and to CuSO_4 - and SLO-oxidized LDL. There was a significant difference ($P = 0.0286$, Mann-Whitney Two Sample Test) in the percentage of cholesteryl HPODE product in the presence and absence of cells, but not between cell-mediated LDL oxidation and CuSO_4 - or SLO-mediated oxidation.

A time course experiment was also performed to follow the formation of cholesteryl HPODE and cholesteryl HODE products over a 24-h period (Fig. 8). The time course of LDL oxidation (measured as TBARS) by activated human monocytes has previously been published (2). It appears that the most rapid increase in cholesteryl HPODE formation occurs in the first 12 h of incubation in the presence of activated monocytes, while, in the absence of monocytes, the formation of cholesteryl HPODE is slow but continuous over the entire 24 h. The formation of cholesteryl HODE follows a similar pattern but in lesser quantities.

The percent of available cholesteryl linoleate substrate that was oxidized was calculated based on the quantity of substrate accounted for in the HPLC analysis; $13.5 \pm 1.1\%$ (SEM, $n = 5$) of the available cholesteryl linoleate substrate in LDL was converted to cholesteryl HPODE or HODE when oxidized by activated monocytes in a 24-h incubation, compared to about $4.2 \pm 0.7\%$ (SEM, $n = 5$) in cell-free controls. The percent conversion of available substrate is likely an underes-

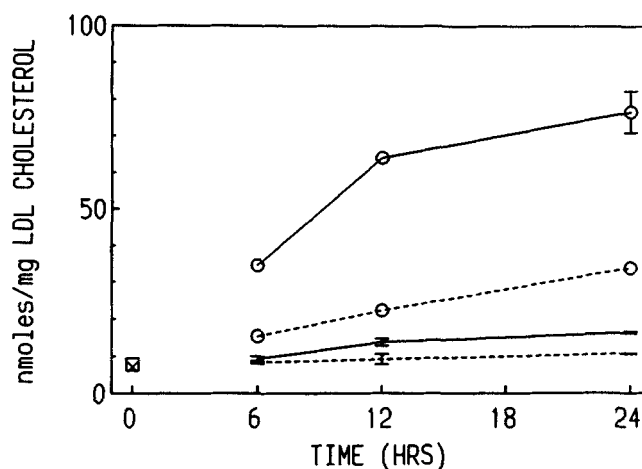


Fig. 8. Time course of cholesteryl HPODE and cholesteryl HODE formation in LDL oxidized by activated human monocytes. LDL (0.5 mg cholesterol/ml) was incubated with the activator ZOP (2.0 mg/ml) in the presence (solid line) or absence (dashed line) of monocytes (1×10^6 cells/ml). A final concentration of 500 μM BHT and an internal standard, cholesteryl [^{14}C]HPODE, were added to each sample at each of the time points indicated. The pooled samples (3 culture wells/sample, 1 ml/well) were then extracted with chloroform and methanol and analyzed by reversed-phase HPLC using solvent system B with UV detection at 206 nm. Extinction coefficients were used to calculate the quantities of cholesteryl HPODE and HODE present. Error bars indicate the mean \pm data range of duplicates; where error bars are not visible error is within the size of the symbols. Circles represent cholesteryl HPODEs formed, dots represent cholesteryl HODEs. LDL was also analyzed at zero time to determine oxidation levels before incubation (open squares, cholesteryl HPODEs; X, cholesteryl HODEs).

timate because the primary oxidation products or fatty acid hydroperoxides may become further oxidized to form breakdown products such as aldehydes during the incubation (33). BHT was added to all samples at the end of the incubation, before extraction of the lipids, to minimize the breakdown of primary oxidation products during the sample processing and analysis. Further, the appearance of cholesteryl HPODE and HODE accounted for approximately $43.4 \pm 1.7\%$ (SEM, $n = 3$) of the disappearance of available substrate. This percentage reflects both the loss of primary oxidation products to further oxidation and the possibility that other cholesteryl esters may co-elute with cholesteryl linoleate when neutral lipids are separated using solvent system B.

DISCUSSION

We have used HPLC analysis to characterize the oxidized fatty acids on LDL that had been oxidized by activated human monocytes. The major oxidation products were esters of 13- and 9-HPODE (Figs. 1, 7A and B). Thomas and Jackson (17) and Ku et al. (34) reported the formation of HODE but not HPODE on LDL oxidized by human monocytes in RPMI-1640 medium, a result

quite different from our observations. They suggest that the HPODE products formed have been degraded by trace amounts of copper or iron present in RPMI-1640 medium or converted to HODEs by cellular peroxidase activity. They and others have also reported studies involving the HPLC and GC-MS analyses of lipid oxidation products in LDL oxidized by CuSO_4 or peroxy radical (16–18). Oxidized linoleic acid was found to be the major oxidation product in chemically oxidized LDL, a result similar to that obtained with cells, CuSO_4 and SLO presented herein. Pre-existence of trace quantities of lipid hydroperoxides in LDL was shown to be necessary for CuSO_4 -catalyzed fatty acid oxidation to occur (17). Our analysis differed in that it included internal standards to account for the recovery and preservation of esterified oxidation products, as well as determination of the oxidation state of the linoleic acid oxidation products using a method capable of detecting both cholesteryl HPODEs and HODEs.

In time course studies of the formation of primary oxidation products in activated human monocyte-oxidized LDL, the rate of formation of cholesteryl HPODE was greatest during the first 12 h of incubation in the presence of activated monocytes, while formation was slow but continuous in samples without monocytes (Fig. 8). The pattern of cholesteryl HODE formation follows that of cholesteryl HPODE formation, but in lesser quantities. This is to be expected as HODEs are the reduction products of HPODEs and the reduction appears to be occurring spontaneously in RPMI medium (Table 1). A similar pattern of oxidized product formation was obtained when the time course of LDL oxidation by activated human monocytes was measured as TBARS, an assay for general lipid oxidation (2).

In addition to esterified, oxidized linoleic acid products; esterified, oxidized arachidonic acid products were also detected in the samples of oxidized LDL. As there was little difference in the quantities of HETEs detected in the presence and absence of activated monocytes, and because the positional isomers of HETE were detected in approximately equal quantities, including those not enzymatically formed, we conclude that the oxidation of the esterified arachidonic acid was autooxidative. The formation of HETEs was not inhibited by ETYA (Fig. 4A and B), further evidence that formation was not cell-mediated. The low quantities of oxidized arachidonic acid products prevented determination of the lipid class to which these products were esterified.

The fact that detection of > 99% of the oxidized fatty acids was dependent on saponification indicates that nearly all of the fatty acid oxidation products were esterified (Fig. 1). Whether intact, esterified linoleate is oxidized by the cells or whether de-esterification, oxidation and re-esterification of esterified linoleic acid from LDL

take place cannot be determined from the experiments performed.

We have previously reported that our system for LDL oxidation is unique in that activation of human monocytes is required (2–6, 22). We found no esterified linoleic acid oxidation products from LDL incubated with unactivated monocytes in RPMI-1640 medium without added metal ions, in the cells or supernatant (Fig. 2A). This is in contrast to the results of Ku et al. (34) and Thomas and Jackson (17) who reported LDL oxidation by human monocytes in RPMI-1640 medium in the absence of any additional activator. The differences in our results may be explained either by differences in cell isolation procedures or differences in LDL isolation procedures or both. Our monocyte isolation procedure involves adherence as a method of separating monocytes from other nonadherent mononuclear cells. We perform the adherence step in serum-coated tissue culture flasks, allowing the monocytes to adhere physiologically to the serum proteins that have attached to the polystyrene, instead of allowing the cells to attach directly to the polystyrene. We also include serum in the medium when allowing the monocytes to adhere to the 12-well tissue culture plates for experiments. Additionally, we use EDTA to detach the adherent monocytes, rather than scraping them from the tissue culture flask surface. These differences may account for the requirement for activation of freshly isolated human monocytes in our hands.

The necessity for immunological activation of monocytes in order for oxidation of LDL lipids to occur in vitro has relevance for the atherosclerotic process in vivo. Immunocytochemical studies of atherosclerotic plaques have demonstrated that foam cells express the major histocompatibility complex (MHC) class II marker, a marker known to be expressed by macrophages that are activated and presenting antigen to T-lymphocytes (35–37). There are also T-lymphocytes in the atherosclerotic lesions, and these also display markers associated with activation such as the interleukin 2 receptor (37, 38). Fluorescently labeled macrophages and T-lymphocytes have been photomicrographed in close association (touching) as if in the process of antigen presentation within atherosclerotic lesions (38).

Further evidence exists that our studies of LDL oxidation are relevant to in vivo disease processes. The same products that we have found in monocyte-modified LDL in vitro have also been shown to be present in atherosclerotic plaques (39–42). Belkner et al. (43) extracted lipids from human atherosclerotic lesions and found oxidized cholesteryl linoleate in those lesions, verifying the earlier findings of Brooks et al. (40, 41) and Harland et al. (42). Belkner et al. (43) also observed that increasing severity of the lesion was correlated with an increasing proportion of oxidized versus non-oxidized cholesteryl linoleate in

those lesions, in agreement with the earlier report of Glavind et al. (39). These results provide another link between monocyte oxidation of LDL lipids *in vitro* and the processes that may be taking place *in vivo* in atherosclerosis.

We have previously shown that our system for studying LDL oxidation is unique in that it is completely inhibited by superoxide dismutase (2; Fig. 5). Others studying the oxidation of LDL by mouse peritoneal macrophages in metal ion-containing Ham's F-10 medium find that SOD (336 units/ml) only inhibits LDL oxidation by about 25% (11). The iron in Ham's F-10 medium has been shown to be necessary for mouse peritoneal macrophage-mediated LDL oxidation (10) and may serve to promote the peroxidation of LDL lipids, decreasing the need for oxygen radicals. The mechanism of SOD-inhibition in metal ion-dependent LDL oxidation systems has recently been questioned (44). The implications of these results for the SOD inhibition of LDL oxidation in our system that does not require addition of metal ions are not known. The role of oxygen radicals in monocyte oxidation of LDL lipids may be that of initiation, involving exhaustion of endogenous antioxidants within LDL as well as initiation of peroxidation on LDL lipids (18, 33).

Our laboratory has previously reported the inhibition of human monocyte-mediated LDL oxidation by ETYA, an arachidonic acid analog and inhibitor of both the cyclooxygenase and lipoxygenase pathways (3). Consistent with our previous results, ETYA but not indomethacin (a cyclooxygenase inhibitor) inhibited formation of esterified linoleic acid oxidation products (Fig. 4; data not shown). We have ruled out the possibility that 5-LO may be involved as part of the activation pathway necessary for human monocytes to oxidize LDL (4). The involvement of another lipoxygenase as indicated by ETYA inhibition remains to be verified.

Analysis of the oxidation state of the esterified, oxidized linoleic acid indicated that about 84.2% of the oxidized linoleic acid products were in the HPODE form in activated monocyte-oxidized LDL, while only 73.6% were in the unreduced form in the cell-free control (Table 1). Possible explanations for these results are that the reduction of the products to cholesteryl HODEs are due to reducing agents in the cell culture medium (RPMI-1640 contains reduced glutathione, 1 mg/l) or reducing activity inherent within the LDL itself. The ratio of hydroperoxy products to hydroxy products in LDL oxidized by activated monocytes or CuSO₄ was greater in samples with higher levels of oxidation, and lesser at the levels of oxidation in control incubations without any added catalyst for oxidation. A limited reducing capacity of the culture medium is likely responsible for this observation, i.e., the reducing capacity of the medium may be overcome at the higher levels of LDL lipid oxidation.

As the oxidized linoleic acid in monocyte-oxidized LDL was esterified, we sought to determine the per-

centage coming from each of the lipid classes present in LDL (Table 2). We have concluded that the percentages of the oxidized, esterified linoleic acid found esterified to cholesterol and phospholipids approximated the abundance of the substrate linoleic acid present in these LDL lipid groups. This suggests that at the LDL oxidation level we are studying, the linoleic acid within LDL is equally susceptible to oxidation independent of the lipid class to which it is esterified. This is interesting to note because phospholipids are located at the surface of the LDL particle where they would be expected to be more susceptible to oxidation than cholesteryl esters and triglycerides which are found in the inner hydrophobic core. Even in one study examining the initiation of LDL oxidation, oxidation was detected in cholesteryl esters, triglycerides, and phospholipids (18).

Although oxidized triglycerides were not isolated, the apparent disappearance of lipids that elute with retention times similar to triolein (peak 4 and several peaks between peak 3 and peak 4, Fig. 7A) in the chromatogram of monocyte-oxidized LDL lipids suggests that the triglycerides are becoming oxidized. In any case, only approximately 6% of the linoleic acid in LDL is found esterified within triglycerides (14), so one might expect that this lipid group would contribute an equally small percentage of the oxidizable substrate.

After 24 h of exposure to activated monocytes, 13.5% of the total cholesteryl linoleate in the samples was converted to cholesteryl HPODE/HODE. This determination of the percentage of available substrate oxidized does not take into account the formation of other oxidation products such as aldehydes, the breakdown products of lipid peroxides (33). In this study our aim was to analyze the primary products of LDL oxidation, especially those whose formation was cell-mediated. In our previous studies the assay for TBARS was used to quantitate LDL oxidation. The TBARS assay detects aldehyde-like oxidation products and indicates general lipid oxidation.

The quantity of monocyte-mediated formation of cholesteryl HPODE and HODE was also compared to the quantity of monocyte-mediated disappearance of cholesteryl ester substrate in a 24-h incubation. The percentage of substrate lost was greater than that detected as oxidized products. This was likely due to the limitations of our analysis methods in terms of resolving and detecting all possible oxidation products formed and all substrates available for oxidation.

We have found that in LDL that has been oxidized by activated monocytes, CuSO₄, or soybean 15-LO, the most abundant primary oxidation product is cholesteryl HPODE. We have also determined that the percentage of oxidized linoleic acid esterified in each of the lipid classes present in oxidized LDL follows the abundance of linoleic acid among the lipid classes in LDL. Finally, the extent of the oxidation of available substrate within LDL in our

system for studying LDL oxidation by activated human monocytes was estimated to be 13.5%. These studies further the understanding of LDL oxidation by activated human monocytes in vitro. ■

We wish to thank Guy M. Chisolm and Paul E. DiCorleto for expert technical advice and help in reviewing the manuscript; Dave Schmidt for technical advice; the Cleveland Clinic Blood Bank for supplying the plasma for LDL isolation; and the Department of Cardiovascular Biology Research for use of the HPLC and online beta-counter. This investigation was supported by a grant from the National American Heart Association, #91012010.

Manuscript received 30 August 1993 and in revised form 26 January 1994.

REFERENCES

- Witzum, J. L., and D. Steinberg. 1991. Role of oxidized low density lipoprotein in atherogenesis. *J. Clin. Invest.* **88**: 1785-1792.
- Cathcart, M. K., A. K. McNally, D. W. Morel, and G. M. Chisolm. 1989. Superoxide anion participation in human monocyte-mediated oxidation of low-density lipoprotein and conversion of low-density lipoprotein to a cytotoxin. *J. Immunol.* **142**: 1963-1969.
- 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.
- Folcik, V. A., and M. K. Cathcart. 1993. Assessment of 5-lipoxygenase involvement in human monocyte-mediated LDL oxidation. *J. Lipid Res.* **34**: 69-79.
- Li, Q., A. Tallant, and M. K. Cathcart. 1993. Dual Ca^{2+} requirement for optimal lipid peroxidation of low density lipoprotein by activated human monocytes. *J. Clin. Invest.* **91**: 1499-1506.
- Li, Q., and M. K. Cathcart. 1994. Protein kinase C activity is required for lipid oxidation of low density lipoprotein by activated human monocytes. *J. Biol. Chem.* **269**: 17508-17515.
- Schuh, J., G. F. Fairclough, and R. H. Haschemeyer. 1978. Oxygen-mediated heterogeneity of apo-low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **75**: 3173-3177.
- El-Saadani, M., H. Esterbauer, M. El-Sayed, M. Goher, A. Y. Nassar, and G. Jürgens. 1989. A spectrophotometric assay for lipid peroxides in serum lipoproteins using a commercially available reagent. *J. Lipid Res.* **30**: 627-630.
- Parthasarathy, S., D. J. Printz, D. Boyd, L. Joy, and D. Steinberg. 1986. Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis.* **6**: 505-510.
- Leake, D. S., and S. M. Rankin. 1990. The oxidative modification of low-density lipoproteins by macrophages. *Biochem. J.* **270**: 741-748.
- Rankin, S. M., S. Parthasarathy, and D. Steinberg. 1991. Evidence for a dominant role of lipoxygenase(s) in the oxidation of LDL by mouse peritoneal-macrophages. *J. Lipid Res.* **32**: 449-456.
- Jessup, W., V. Darley-Usmar, V. O'Leary, and S. Bedwell. 1991. 5-Lipoxygenase is not essential in macrophage-mediated oxidation of low-density lipoprotein. *Biochem. J.* **278**: 163-169.
- Cathcart, M. K., A. K. McNally, and G. M. Chisolm. 1991. Lipoxygenase-mediated transformation of human low density lipoprotein to an oxidized and cytotoxic complex. *J. Lipid Res.* **32**: 63-70.
- Scanu, A. M. 1979. Plasma lipoproteins: an introduction. In *The Biochemistry of Atherosclerosis*. A. M. Scanu, editor. Marcel Dekker, Inc., New York. 3-8.
- Skipski, V. P. 1967. Lipid composition of human serum lipoproteins. *Biochem. J.* **104**: 340-352.
- Lenz, M. L., H. Hughes, J. R. Mitchell, D. P. Via, J. R. Guyton, A. A. Taylor, A. M. Gotto, and C. V. Smith. 1990. Lipid hydroperoxy and hydroxy derivatives in copper-catalyzed oxidation of low density lipoprotein. *J. Lipid Res.* **31**: 1043-1050.
- 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.
- Stocker, R., V. W. Bowry, and B. Frei. 1991. Ubiquinol-10 protects human low density lipoprotein more efficiently against lipid peroxidation than does α -tocopherol. *Proc. Natl. Acad. Sci. USA.* **88**: 1646-1650.
- Hatch, F. T., and R. S. Lees. 1968. Practical methods for plasma lipoprotein analysis. *Adv. Lipid Res.* **6**: 1-68.
- Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34**: 1345-1353.
- Johnson, R. B. 1981. Secretion of superoxide anion. In *Methods of Studying Mononuclear Phagocytes*. D. O. Adams, P. J. Edelson, and H. Koren, editors. Academic Press, New York. 489-497.
- Cathcart, M. K., G. M. Chisolm, A. K. McNally, and D. W. Morel. 1988. Oxidative modification of low density lipoprotein (LDL) by activated human monocytes and the cell lines U937 and HL60. *In Vitro Cell. & Dev. Biol.* **24**: 1001-1008.
- Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**: 497-509.
- Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**: 911-917.
- Hamilton, J. G., and K. Comai. 1988. Rapid separation of neutral lipids, free fatty acids and polar lipids using prepacked silica Sep-pak columns. *Lipids.* **23**: 1146-1149.
- Powell, W. S. 1982. Rapid extraction of arachidonic acid metabolites from biological samples using octadecylsilyl silica. *Methods Enzymol.* **86**: 467-477.
- Claeys, M., G. A. A. Kivits, E. Christ-Hazelhof, and D. H. Nugteren. 1985. Metabolic profile of linoleic acid in porcine leukocytes through the lipoxygenase pathway. *Biochim. Biophys. Acta.* **837**: 35-51.
- Zak, B., N. Moss, A. J. Boyle, and A. Zlatkis. 1954. Reactions of certain unsaturated steroids with acid iron reagent. *Anal. Chem.* **26**: 776-777.
- Shirhatti, V., and G. Krishna. 1985. A simple and sensitive method for monitoring drug-induced cell injury in cultured cells. *Anal. Biochem.* **147**: 410-418.
- Bonney, R. J., and J. L. Humes. 1984. Physiological and pharmacological regulation of prostaglandin and leukotriene production by macrophages. *J. Leukocyte Biol.* **35**: 1-10.
- Kaneko, T., and M. Matsuo. 1984. Isomerization of linoleic acid hydroperoxides under argon and under degassed conditions. *Chem. Pharm. Bull.* **32**: 332-335.

32. Teng, J. I., and L. L. Smith. 1985. High-performance liquid chromatography of linoleic acid hydroperoxides and their corresponding alcohol derivatives. *J. Chromatogr.* **350**: 445-451.
33. Esterbauer, H., G. Jürgens, O. Quehenberger, and E. Koller. 1987. Autooxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J. Lipid Res.* **28**: 495-509.
34. Ku, G., G. E. Thomas, A. L. Akeson, and R. L. Jackson. 1992. Induction of interleukin 1 beta expression from human peripheral blood-derived macrophages by 9-hydroxy-octadecadienoic acid. *J. Biol. Chem.* **267**: 14183-14188.
35. Munro, J. M., J. D. Van der Walt, C. S. Munro, J. A. C. Chalmers, and E. L. Cox. 1987. An immunohistochemical analysis of human aortic fatty streaks. *Hum. Pathol.* **18**: 375-380.
36. Hansson, G. K., L. Jonasson, B. Lojstved, S. Stemme, O. Kocher, and G. Gabbiani. 1988. Localization of T lymphocytes and macrophages in fibrous and complicated human atherosclerotic plaques. *Atherosclerosis*. **72**: 135-141.
37. Van der Wal, A. C., P. K. Das, D. B. Van de Berg, C. M. Van der Loos, and A. E. Becker. 1989. Atherosclerotic lesions in humans: in situ immunophenotypic analysis suggesting an immune mediated response. *Lab. Invest.* **61**: 166-170.
38. Emeson, E. E., and A. L. Robertson. 1988. T lymphocytes in aortic and coronary intimas: their potential role in atherogenesis. *Am. J. Pathol.* **130**: 369-376.
39. Glavind, J., S. Hartmann, J. Clemmesen, K. E. Jessen, and H. Dam. 1952. Studies on the role of lipoperoxides in human pathology. II. The presence of peroxidized lipids in the atherosclerotic aorta. *Acta. Pathol. Microbiol. Scand.* **30**: 1-6.
40. Brooks, C. J. W., W. A. Harland, G. Steel, and J. D. Gilbert. 1970. Lipids of human atheroma: isolation of hydroxy-octadecadienoic acids from advanced aortal lesions. *Biochim. Biophys. Acta.* **202**: 563-566.
41. Brooks, C. J. W., G. Steel, J. D. Gilbert, and W. A. Harland. 1971. Lipids of the human atheroma. Part 4. Characterization of a new group of polar sterol esters from human atherosclerotic plaques. *Atherosclerosis*. **13**: 223-237.
42. Harland, J. D., J. D. Gilbert, G. Steel, and C. J. W. Brooks. 1971. Lipids of the human atheroma. Part 5. The occurrence of a new group of polar sterol esters in various stages of human atherosclerosis. *Atherosclerosis*. **13**: 239-246.
43. Belkner, J., R. Weisner, H. Kuhn, and V. Z. Lankin. 1991. The oxygenation of cholesterol esters by the reticulocyte lipoxygenase. *FEBS Lett.* **279**: 110-114.
44. 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.