Formation of monohydroxy derivatives of arachidonic acid, linoleic acid, and oleic acid during oxidation of low density lipoprotein by copper ions and endothelial cells

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Abstract An important event in the formation of atherosclerotic lesions is the uptake of modified low density lipoprotein (LDL) by macrophages via scavenger receptors. Modification of LDL, which results in its recognition by these receptors, can be initiated by peroxidation of LDL lipids. The first step in this process is the formation of monohydroperoxy derivatives of fatty acids, which are subsequently degraded to the corresponding monohydroxy compounds, or to a variety of secondary oxidation products. In order to understand this process more completely, we have developed a mass spectrometric procedure to measure the amounts of specific hydroperoxy/hydroxy fatty acids formed by oxidation of the major unsaturated fatty acids in human LDL, oleic acid, linoleic acid, and arachidonic acid. Oxidation of human LDL in the presence of a relatively strong stimulus (20 μM $CuSO_4$) resulted in very large increases in the amounts of the major monohydroxy derivatives of linoleic acid (9- and 13-hydroxy derivatives) and arachidonic acid (5-, 8-, 9-, 11-, 12-, and 15-hydroxy derivatives) in LDL lipids in the early stages of the reaction. After 20 h, the amounts of these products declined due to substrate depletion, but large amounts of monohydroxy derivatives of oleic acid (8-, 10-, and 11-hydroxy derivatives) were detected. Although thiobarbituric acid-reactive substances clearly increased under these conditions, the changes were not nearly so dramatic as those observed for monohydroxy fatty acids. Oxidation of LDL in the presence of endothelial cells, a much milder stimulus, resulted in 2.5- to 3-fold increases in the amounts of monohydroxy derivatives of linoleic and arachidonic acids, as well as thiobarbituric acid-reactive substances, with more modest increases in the amounts of hydroxylated derivatives of oleic acid. There was little positional specificity in the oxidation of the above fatty acids in the presence of either stimulus, suggesting that the formation of these products proceeds primarily by lipid peroxidation, rather than by catalysis by lipoxygenases. However, an important role for lipoxygenases in the initiation of these reactions cannot be excluded. In conclusion, oxidation of LDL in the presence of copper ions or endothelial cells results in the formation of a large number of monohydroxy derivatives of oleic, linoleic, and arachidonic acids. The relative amounts of products formed from each of these fatty acids depends on the strength of the stimulus as well as the incubation time.—**Wang, T., W-g. Yu, and W. S. Powell**. Formation of monohydroxy derivatives of arachidonic acid, linoleic acid, and oleic acid during oxidation of low density lipoprotein by copper ions and endothelial cells. *J. Lipid Res.* 1992. **33:** 525–537.

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One of the pathological characteristics of atherosclerosis is the formation of lipid-laden cells (foam cells) underneath the endothelium of arteries. Many of these foam cells are present in the early stages of the lesion and are postulated to be derived from circulating monocytes which have taken up low density lipoprotein (LDL) (1, 2). Although normal LDL is not well taken up by monocytes (3), LDL that has been

Abbreviations: LDL, low-density lipoprotein; TBARS, thiobarbituric acid-reactive substances; WHHL, Watanabe heritable hyperlipidemic; EDTA, ethylenediamine tetraacetic acid; ETYA, 5,8,11,14-eicosatetraynoic acid; MSTFA, *N*-methyl-*N*-trimethylsilyltrifluoroacetamide; HPLC, high pressure liquid chromatography; ODS, octadecylsilyl GC, gas chromatography; MS, mass spectrometry; HETE, hydroxyeicosatetraenoic acid; PUFA, polyunsaturated fatty acids; h-18:0, hydroxyoctadecanoic acid, h-20:0, hydroxyeicosanoic acid; 8h-18:1, 8-hydroxy-9-octadecenoic acid; 10h-18:1, 10-hydroxy-8-octadecenoic acid; 11h-18:0, 11-hydroxy-9-octadecenoic acid; 9h- 18:2, 9-hydroxy-10,12-octadecadienoic acid; 13h-18:1, 13-hydroxy-9,11-octadecadienoic acid; 5h-20:4, 5-hydroxy-6,8,11,14-eicosatetraenoic acid; 8h-20:4, 8-hydroxy-5,9,11,14-eicosatetraenoic acid; 9h-20:4, 9-hydroxy-5,7,11,14-eicosatetraenoic acid; 11h-20:4, 11-hydroxy-5,8,12,14-eicosatetraenoic acid; 12h-20:4, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; 15h-20:4, 15-hydroxy-5,8,11,13-eicosatetraenoic acid.

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chemically modified by acetylation (3) or biologically modified by treatment with endothelial cells (4, 5), arterial smooth muscle cells (6), activated human monocytes (7, 8), activated platelets (9–11) as well as by extracts from human atherosclerotic lesions (12), is avidly taken up via monocyte scavenger receptors.

The biological modification of LDL is accompanied by increases in the amounts of thiobarbituric acid-reactive substances (TBARS) and other lipid peroxidation products and is inhibited by superoxide dismutase (8, 13), antioxidants (4), and lipoxygenase inhibitors (14), depending on the cell type. This suggests that this process is initiated by lipid peroxidation due either to superoxide, which is released from smooth muscle cells and monocytes, or to radicals formed during the lipoxygenase-catalyzed oxidation of fatty acids by endothelial cells. The importance of lipid peroxidation in the development of atherosclerosis is supported by the finding that probucol, due to its antioxidant properties, prevented the formation of lesions in WHHL rabbits (15–17).

Lipid peroxidation is characterized by the abstraction of a hydrogen atom from an allylic methylene group to form an alkyl radical, followed by migration of one of the double bonds and insertion of molecular oxygen to give a peroxyl radical. Peroxyl radicals are converted to hydroperoxides as a result of abstraction of a hydrogen atom from another molecule of unsaturated fatty acid, initiating a chain reaction. The position at which oxygen is inserted depends on which hydrogen is abstracted. In the case of a monounsaturated fatty acid, oxygenation occurs at either end of the two possible allylic radicals. Therefore, four positional isomers, substituted in the 8, 9, 10, and 11 positions, will be formed from oleic acid (18). In the case of polyunsaturated fatty acids, each 1,4-*cis*,*cis*-pentadiene group will give rise to two positional isomers. For example, 9- and 13-hydroperoxy isomers will be formed from linoleic acid (18), whereas 5-, 8-, 9-, 11-, 12-, and 15-hydroperoxy isomers will be formed from arachidonic acid (19).

The objectives of the present study were to characterize the monohydroxy fatty acids formed by peroxidation of unsaturated fatty acids in LDL, before and after treatment with copper ions or endothelial cells. Two distinct patterns for the formation of monohydroxy fatty acids in the presence of Cu^{2+} were apparent. Products formed from oleic acid increased linearly with time, so that after 20 h, they were the major monohydroxy long-chain fatty acids in LDL. On the other hand, monohydroxy fatty acids derived from arachidonic and linoleic acids reached maximal levels after 5 h, and then declined, presumably due to further oxidative reactions. In the case of the milder oxidative stimulus provided by endothelial cells, all monohydroxy fatty acids increased with time up to 20 h.

MATERIALS AND METHODS

Materials

Ham's F10 medium was purchased from Flow Laboratories, Inc. 1,1,3,3-Tetramethoxypropane, propyl gallate, and Diazold were obtained from Aldrich. Sodium chloride, potassium bromide, ethylenediamine tetraacetic acid (EDTA, disodium salt), ricinoleic acid (12h-18:1), and thiobarbituric acid were purchased from the Sigma Chemical Company. Anhydrous methanol and other solvents were from Fisher Scientific. Oleic, linoleic, and arachidonic acids were purchased from Nu-Chek Prep, Inc.

Preparation of standards for quantitation of monohydroxy fatty acids by mass spectrometry

Monohydroxy derivatives of oleic acid. Hydroxy derivatives of oleic acid (h-18:1) were prepared by autooxidation of oleic acid by a procedure similar to the one described in the literature for the preparation of hydroxy derivatives of arachidonic acid (20). Oleic acid (10 mg; 99% purity) was dissolved in methanol (40 ml) followed by the addition of 10 ml of 0.2 M Tris buffer containing cupric sulfate (final concentration of 100 μM). The reaction mixture was stirred for 5 h at room temperature in the presence of hydrogen peroxide (0.36 mmol; 400 μl of a 30% solution) which was added at 0, 1, and 3 h of the incubation. The reaction mixture was then diluted with H_2O (100 ml) and extracted on an ODS-silica Sep-Pak (21). The hydroperoxy products were reduced with sodium borohydride and purified using an open column of silicic acid (22). This was followed by normal-phase-high pressure liquid chromatography (NP-HPLC) purification on a 5 μm RoSil silicic acid column (350 × 4.6 mm; Alltech Associates) using a mobile phase consisting of isopropanol–hexane–acetic acid 0.9:99:0.1. 8H-18:1 had a retention time of 48 min (**Fig. 1**). Fractions containing either 10h-18:1 (27.5–29.5 min) or 11h-18:1 (26–27.5 min) were further purified by reversed-phase-high pressure liquid chromatography (RP-HPLC) on a 5-μm Novapak octadecylsilyl silica (ODS-silica) column (150 × 3.9 mm; Waters-Millipore) using a gradient between water–acetonitrile–acetic acid 60:40:0.02 and water–acetonitrile–acetic acid 35:65:0.02 over 30 min followed by isocratic elution with the latter mobile phase. 10H-18:1 and 11h-18:1 had retention times of 33.4 min and 30.1 min, respectively. The identities of these three monohydroxy derivatives of oleic acid were confirmed by gas chromatography–mass spectrometry (GC–MS) using linear scanning. They were quantitated by gas chromatography (GC) using stearic acid (18:0) as an internal standard.

Monohydroxy derivatives of linoleic acid. 9-Hydroxy-10,12-octadecadienoic acid (9h-18:2) and 13-hydroxy-

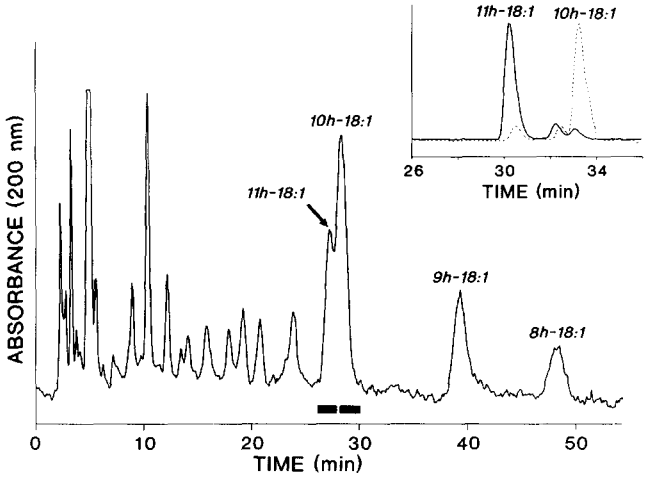

Fig. 1. High-pressure liquid chromatograms of monohydroxy C_{18} fatty acids formed by autoxidation. Oleic acid (10 mg; 99% purity) was autoxidized in the presence of 100 μM copper sulfate in methanol:0.2 M Tris buffer (4:1; 50 ml) for 5 h at room temperature. Hydrogen peroxide (0.35 mmol) was added at 0, 1, and 3 h. The medium was then diluted with H_2O (100 ml), extracted on an ODS-silica Sep-Pak cartridge and the products were separated by open column chromatography. Monohydroxy derivatives of C_{18} fatty acids were purified by NP-HPLC with a mobile phase of isopropanol–hexane–acetic acid 0.9:99:0.1 (2 ml/min). Fractions containing either 10h-18:1 or 11h-18:1 (underlined fractions) were further purified by RP-HPLC (inset) using a linear gradient between water–acetonitrile–acetic acid 60:40:0.02 and water–acetonitrile–acetic acid 35:65:0.02. Fractions containing 8h-18:1 were also collected during NP-HPLC purification.

9,11-octadecadienoic acid (13h-18:2) were prepared by incubating linoleic acid (18:2) with tomato lipoxygenase (23) and soybean lipoxygenase (24), respectively.

Monohydroxy derivatives of arachidonic acid. 5-Hydroxy-6,8,11,14-eicosatetraenoic acid (5h-20:4) and 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12h-20:4) were prepared by incubating arachidonic acid (20:4) and A23187 with porcine leukocytes in the presence or absence, respectively, of 5,8,11,14-eicosatetraynoic acid (ETYA) (25). 8H-20:4, 9h-20:4, and 11h-20:4 were prepared by autoxidation of 20:4 in the presence of cupric sulfate and hydrogen peroxide (20), whereas 15h-20:4 was prepared by incubation of 20:4 with soybean lipoxygenase (24).

Endothelial cells

Endothelial cells from human umbilical veins and endothelial cell growth medium were obtained from Clonetics, San Diego, CA. Endothelial cell growth medium (EGM-UV) was a sterile-filtered liquid culture medium formulation based on MCDB 131 medium supplemented with epidermal growth factor (10 ng/ml), bovine brain extract containing heparin (2 ml aliquot), cortisol (1 μg/ml), fetal bovine serum (2%), gentamycin (50 μg/ml), and amphotericin (0.5 μg/ml). On the second day after receiving the endothelial cells, they were subcultured onto culture dishes (32 cm^2) at a density of 2500 to 5000 cells/cm^2. The cells were grown in EGM-UV until they reached approximately 75% confluence.

Preparation of LDL

Human blood was withdrawn from male volunteers after at least 12 h of fasting. LDL was prepared from plasma according to the method by Havel, Eder, and Bragdon (26). Briefly, four volumes of plasma were mixed with one volume of a salt solution containing NaCl, KBr, and EDTA (density 1.071 g/ml) to yield a density of 1.019 g/ml. After centrifugation at 125,000 *g* using a Beckman type 50 Ti rotor, four volumes of the resulting infranatant were mixed with one volume of NaCl/KBr solution (density 1.239 g/ml) and the mixture was adjusted to a density of 1.063 g/ml for a second ultracentrifugation at 105,000 *g* for 20 h. The antioxidant propyl gallate (final concentration, 50 μM) was added to the plasma and the 125,000 *g* infranatant.

LDL obtained from the second ultracentrifugation was dialyzed in the dark for 20 h against 3×30 volumes of phosphate-buffered saline (pH 7.4) containing 0.01% EDTA with two buffer changes after 3 and 10 h. The buffer had previously been degassed and bubbled with argon for 30 min to minimize lipid peroxidation during dialysis.

Incubation of LDL with copper sulfate

LDL (200 μg/ml) was incubated in Ham's F10 medium (2 ml) at 37°C in the presence or absence of copper sulfate (20 μM). After 1, 5, or 20 h, the incubation medium was withdrawn from the tubes, which were then washed with 1 ml of phosphate-buffered saline. The two portions were combined. An aliquot (1 ml) of the resulting mixture was diluted with phosphate-buffered saline (0.5 ml) for measurement of conjugated dienes and TBARS. The remainder of the incubation medium (2 ml) was mixed with 3.3 ml of NaCl/KBr solution (density 1.239 g/ml) containing propyl gallate and EDTA (final concentrations of 50 μM and 0.1% (w/v), respectively) to yield a final density of 1.15 g/ml. LDL was reisolated by centrifugation at 125,000 *g* for 16 h in a Beckman type 50 Ti rotor. The top portion (ca. 1.5 ml) containing reisolated LDL was used for measurement of lipid peroxidation products in LDL, whereas the infranatant was used for measurement of lipid peroxidation products released from LDL into the medium. The recovery of LDL protein after reisolation was 92%.

Incubation of LDL with endothelial cells

LDL (200 μg/ml) was incubated in Ham's F10 medium (2 ml) at 37°C in the presence or absence of endothelial cells. After various times the medium was

removed and the LDL was reisolated as described above for measurement of monohydroxy fatty acids.

Extraction and purification of monohydroxy fatty acids

The procedure for the analysis of hydroxylated fatty acids from LDL is summarized in **Fig. 2**. In detail, LDL was extracted as described by Folch, Lees, and Sloane Stanley (27) after addition of the methyl ester of 14h-19:2 (200 ng) as an internal standard. After reduction of hydroperoxy lipids to hydroxy lipids with triphenylphosphine (1 mg) at room temperature for 1 h in diethyl ether, LDL lipids were transmethylated by reaction with 0.3 N sodium methoxide in anhydrous methanol (0.3 ml) at 60°C under argon for 30 min. Tris-HCl (50 mM) was added to the reaction mixture to give a final concentration of methanol of 15%, and fatty acid and monohydroxy fatty acid methyl esters were extracted with 3 × 5 ml of methylene chloride. Monohydroxy fatty acids were separated from their fatty acid precursors by open column chromatography (22). All of the above steps were completed on the same day in order to minimize autooxidation of lipids.

Monohydroxy fatty acids were further purified by RP-HPLC on a Novapak ODS-silica column (150 × 3.9 mm; Waters-Millipore) which was eluted with a gradient between water–acetonitrile 70:30 and water–acetonitrile 25:75 over 30 min followed by a gradient to water–acetonitrile 15:85 over 8 min. A single fraction containing all the monohydroxy fatty acids of interest was collected between 26 and 33 min.

Unesterified monohydroxy fatty acids released into the incubation medium were extracted using a cartridge containing ODS-silica (Waters C_{18} Sep-Pak) as described previously (21), followed by methylation with diazomethane (Fig. 2) and open column chromatography.

Following purification, monohydroxy fatty acid methyl esters were hydrogenated for 5 min at room temperature in the presence of rhodium on alumina (1 mg) in methanol (300 µl). They were then converted to their trimethylsilyl ether derivatives by reaction with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) for 30 min at room temperature.

Rigorous attempts were made to minimize lipid peroxidation during preparation of the samples. All procedures prior to the separation of monohydroxy fatty acids from their fatty acid precursors were performed under argon or nitrogen. Addition of propyl gallate prior to the extraction of lipids further prevented lipid peroxidation during workup. The effectiveness of propyl gallate was demonstrated by its ability to nearly completely block peroxidation of LDL lipids in the presence of copper sulfate (20 µM) (data not shown).

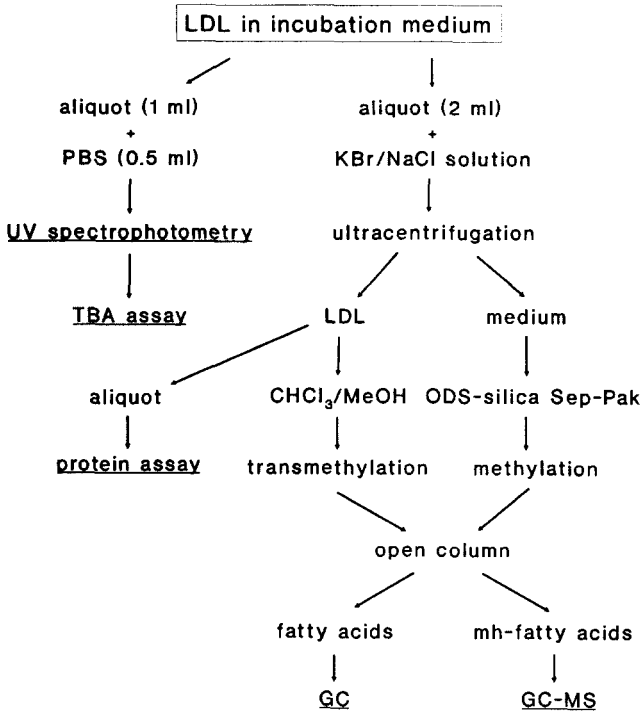

Fig. 2 Experimental protocol for the measurement of monohydroxy metabolites of unsaturated fatty acids in human LDL.

Gas chromatographic–mass spectrometric measurement of monohydroxy fatty acids

Methyl ester-trimethylsilyl ether derivatives of monohydroxy fatty acids were quantitated on a Hewlett-Packard model 5890 gas chromatograph with a 15 m DB-1 fused capillary column coupled to a model 5988 mass spectrometer in the electron impact mode. The temperature program used for the analysis was as follows: 0 min (50°C; 7.6 min (240°C), 12.6 min (260°C); 13.6 min (280°C). Monohydroxy fatty acids were divided into three groups according to their carbon numbers (C_{18}, C_{19}, and C_{20}). The *m/z* values monitored for each group were as follows: C_{18} (245 (8h), 259 (9h), 273 (10h), 287 (11h), 301 (12h), and 315 (13h)); C_{19} (329 (14h)); C_{20} (313 (5h), 245 (8h), 259 (9h), 287 (11h), 301 (12h), and 343 (15h)) (**Fig. 3**). The dwell time was 30 msec in each case with the exception of *m/z* 329, for the internal standard, in which case a dwell time 60 msec was used. The peak area for each monohydroxy fatty acid was compared to that for the internal standard and the amount was calculated from the appropriate standard curve.

Other analytical methods

Fatty acids in LDL were extracted and subjected to transmethylation as described above for esterified

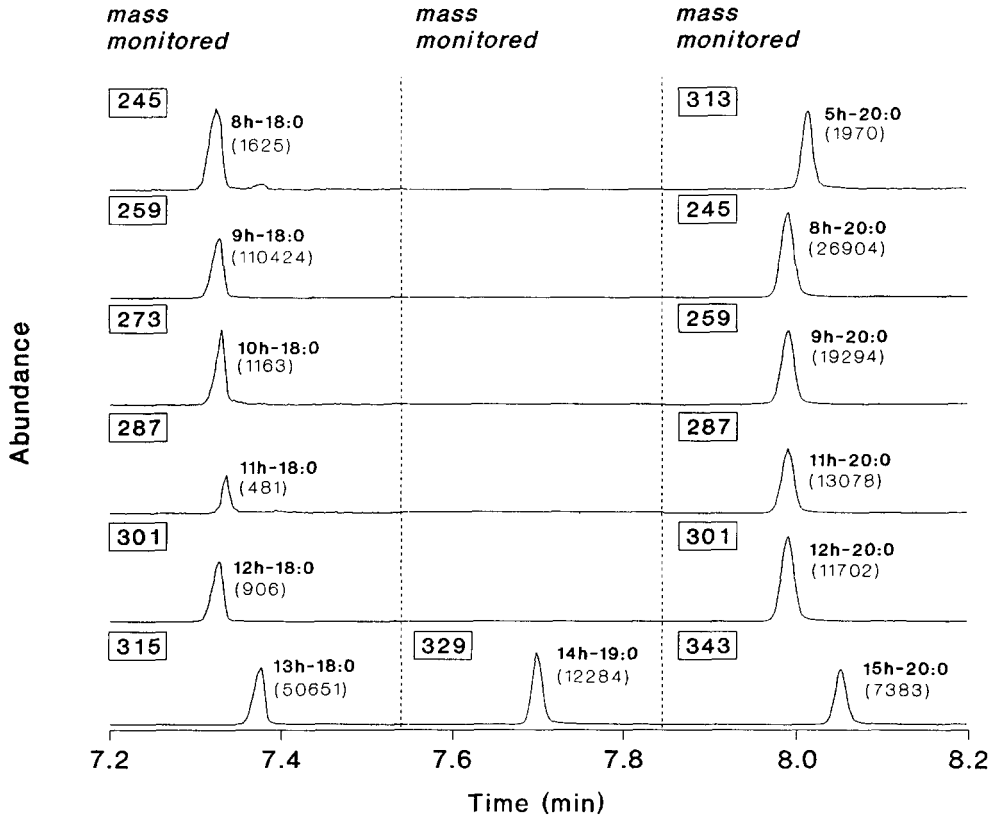

Fig. 3. Selected ion chromatograms of monohydroxy derivatives of unsaturated fatty acids in human LDL incubated with endothelial cells for 20 h. Monohydroxy fatty acids were hydrogenated and converted to their trimethylsilyl ether derivatives before being measured by GC-MS using electron impact ionization and selected ion monitoring. The *m/z* values monitored were changed at 7.55 min and 7.85 min to measure the specific compounds eluted from the GC column. The area of each peak (in brackets) was compared to the area of the peak for the internal standard (14h-19:0) and the ratio was compared to the standard curve for that compound constructed in each experiment. Note that the peak heights are normalized by the computer and only the areas in brackets were used for quantitation.

monohydroxy fatty acids. They were separated from their monohydroxy derivatives by open column chromatography (22) and measured using a Varian Model 3300 gas chromatograph equipped with a flame ionization detector using a DB-23 capillary column (0.25 mm × 30 m). Fatty acids were quantified by comparing their peak areas to that of the internal standard (20:0), which was added to LDL prior to extraction.

Conjugated dienes were measured using a Hitachi model 2000 UV/visible spectrophotometer. Conjugated dienes were quantified at 234 nm using an extinction coefficient of 30,500 (28).

Thiobarbituric acid-reactive substances (TBARS) were measured by spectrofluorometry using an excitation wavelength of 515 nm and an emission wavelength of 565 nm (29). Freshly prepared solutions of 1,1,3,3-tetramethoxypropane were used to construct a standard curve.

LDL protein content was measured using a modified Lowry assay (30).

RESULTS

Analysis of monohydroxy fatty acids by GC–MS

Monohydroxy fatty acids were measured using an approach similar to that which we previously used to measure the 9- and 13-hydroxy metabolites of linoleic acid and the 11-, 12-, and 15-hydroxy metabolites of arachidonic acid (22, 31). However, in this case we extended the assay to include the 5-, 8-, and 9-hydroxy derivatives of arachidonic acid. Since oleic acid is a major unsaturated fatty acid in LDL, we also measured monohydroxy fatty acids derived from this substrate. As discussed above, oxidation of oleic acid would be expected to give rise to 8-, 9-, 10-, and 11-monohydroxy derivatives. These products were synthesized by autoxidation of oleic acid in the presence of cupric sulfate and were purified by NP-phase HPLC (Fig. 1); 10h-18:1 and 11h-18:1 were only partially separated under these conditions, but could be resolved by RP-

HPLC (Fig. 1, inset). The identities of each of these products was confirmed by GC-MS analysis. They were used to construct standard curves which enable the corresponding products formed from endogenous oleic acid in LDL to be quantitated by selected ion monitoring as shown in Fig. 3. In addition to the above compounds, we also measured 12-hydroxy C_{18} fatty acids, since we previously detected these products in aorta (31).

Formation of monohydroxy metabolites of C_{18} fatty acids during incubation of LDL with copper ions

The time courses for the formation of various 18-carbon monohydroxy fatty acids, presumably derived principally from oleic acid and linoleic acid, during incubation of human LDL in the presence or absence of 20 μM $CuSO_4$ are shown in **Fig. 4**. Three distinct patterns for the formation of these products are apparent. The amounts of 8-hydroxy, 10-hydroxy, and 11-hydroxy C_{18} fatty acids increased nearly linearly with time up to 20 h in the presence of Cu^{2+}, by which time the levels were about 50 to 60 times greater than in control incubations in the absence of Cu^{2+} (Fig. 4A, 4B, and 4C). The pattern observed for the formation of 9-hydroxy and 13-hydroxy C_{18} fatty acids was strikingly different, in that the amounts increased rapidly to reach maximal levels after 5 h, but then decreased to control levels by 20 h (Fig. 4D and 4E). Only relatively small amounts of 12-hydroxy C_{18} fatty acids were detected after incubation of LDL, and, unlike the other monohydroxy C_{18} isomers discussed above, the amounts of this substance were not affected to a great extent by the presence of cupric ions (Fig. 4F). These data would suggest that there are two major groups of monohydroxy C_{18} fatty acids that are formed in LDL in response to incubation with copper ions. The first group (8h-, 10h-, and 11h-18:0) appear to be more stable, since their formation is linear with time, suggesting that they may have only one double bond and may be derived from oleic acid. The second group (9h- and 13h-18:0) appear to be less stable, since their amounts decline rapidly after 5 h, suggesting that they may be more highly unsaturated and may be derived principally from linoleic acid.

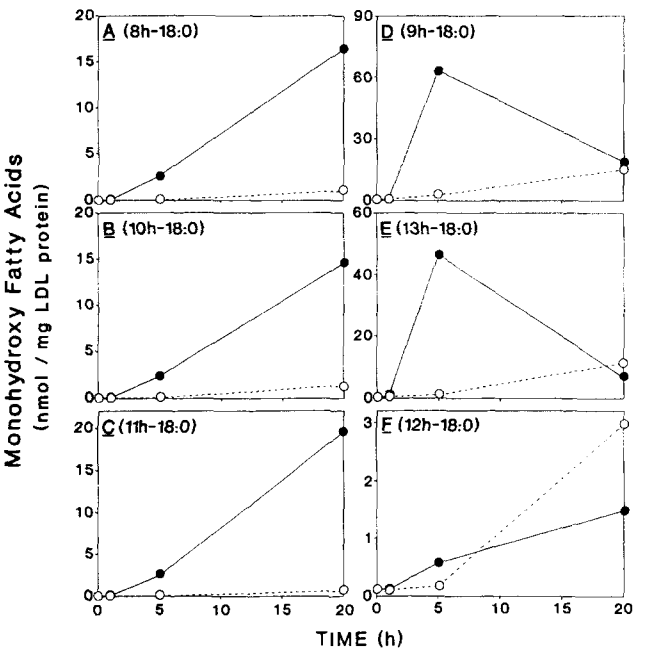

Fig. 4. Formation of monohydroxy C_{18} fatty acids in LDL during copper-catalyzed oxidation. Monohydroxy C_{18} fatty acids in control LDL (o----------o) or LDL incubated with 20 μM $CuSO_4$ (●——●) were extracted from reisolated LDL by the Folch method with methyl 14h-19:2 as an internal standard. After transmethylation, these monohydroxy metabolites were first separated from their fatty acid precursors by open column chromatography and further purified by RP-HPLC. After hydrogenation and conversion to their trimethylsilyl ether derivatives, they were measured by GC-MS using electron impact ionization and selected ion monitoring. The values are means of triplicate determinations.

Formation of monohydroxy metabolites of C_{20} fatty acids during incubation of LDL with copper ions

The time courses for the formation of individual monohydroxy C_{20} fatty acids (i.e., HETEs), presumably all derived from arachidonic acid, during incubation of LDL with copper ions were all similar to those shown for 9h-18:0 and 13h-18:0 in Figs. 4D and 4E, except that after 20 h, negligible amounts of these substances could be detected (data not shown). The maximal amounts of HETEs were observed after 5 h and were all approximately 3 to 5 times the amounts in control incubations performed in the absence of copper ions (**Fig. 5A**).

Effects of endothelial cells on the formation of monohydroxy derivatives of unsaturated fatty acids

Incubation of LDL with 20 μM copper sulfate would be expected to strongly stimulate lipid peroxidation in LDL. To investigate the effects of a physiologically more relevant stimulus, human LDL was incubated with human endothelial cells for various times, and the amounts of esterified monohydroxy fatty acids were measured as described above. For simplicity, monohydroxy fatty acids in each of the three major groups discussed above were combined (**Fig. 6**). The time courses for the formation of monohydroxy C_{18} and C_{20} fatty acids by endothelial cells (Fig. 6D, 6E, and 6F) were quite different from those observed after incubation with copper ions (Fig. 6A, 6B, and 6C). Only small increases in the formation of these products were observed after 5 h, but after 20 h, the amounts of monohydroxy derivatives of linoleic acid (Fig. 6E) and arachidonic acid (Fig. 6F) were about 2.5–3 times those in control incubations. A smaller in-

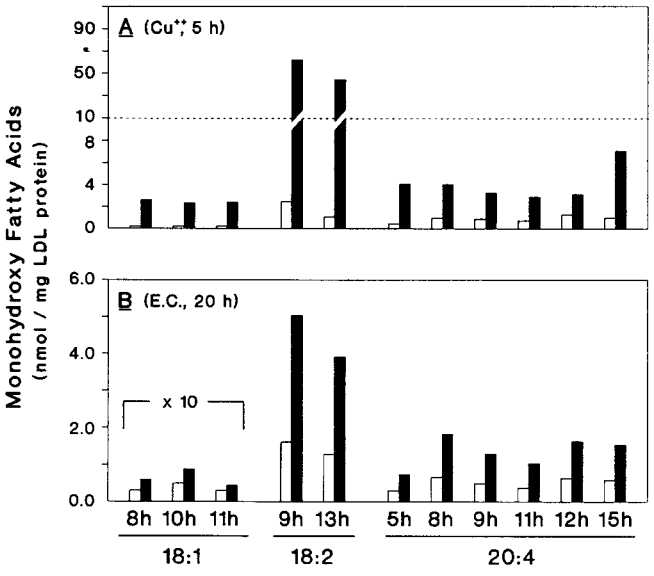

Fig. 5. Formation of individual monohydroxy unsaturated fatty acids in human LDL. Monohydroxy fatty acids in LDL incubated with 20 μM of $CuSO_4$ (solid bars, A) for 5 h or with endothelial cells (solid bars, B) for 20 h or in control LDL (open bars) at the corresponding time points were quantitated as described in Materials and Methods. The values are means of triplicates ($CuSO_4$) or duplicates (endothelial cells). Note the change of scale above 10 nmol/mg LDL protein in Panel A.

crease (ca. 1.8 times control) was observed for monohydroxy compounds derived from oleic acid. However, in contrast to the results obtained with copper-induced oxidation of LDL, the total amounts of the latter products were at least 30 times lower than those of monohydroxy derivatives of linoleic acid and arachidonic acid. The amounts of individual monohydroxy C_{18} and C_{20} fatty acids formed after incubation of LDL with endothelial cells for 20 h are shown in Fig. 5B. The increases in the amounts of monohydroxy C_{20} derivatives were nearly as great as the maximal increases observed in incubations with copper ions (i.e., after 5 h). However, the increases observed for monohydroxy derivatives of oleic and linoleic acids were much less with endothelial cells (Fig. 5A and 5B). There was little selectivity with regard to the formation of positional isomers of monohydroxy fatty acids, suggesting that they were formed principally by nonenzymatic mechanisms.

Effects of incubation of LDL with copper and endothelial cells on other parameters of lipid peroxidation

Fatty acids. Incubation of LDL with copper ions resulted in relatively modest changes in esterified

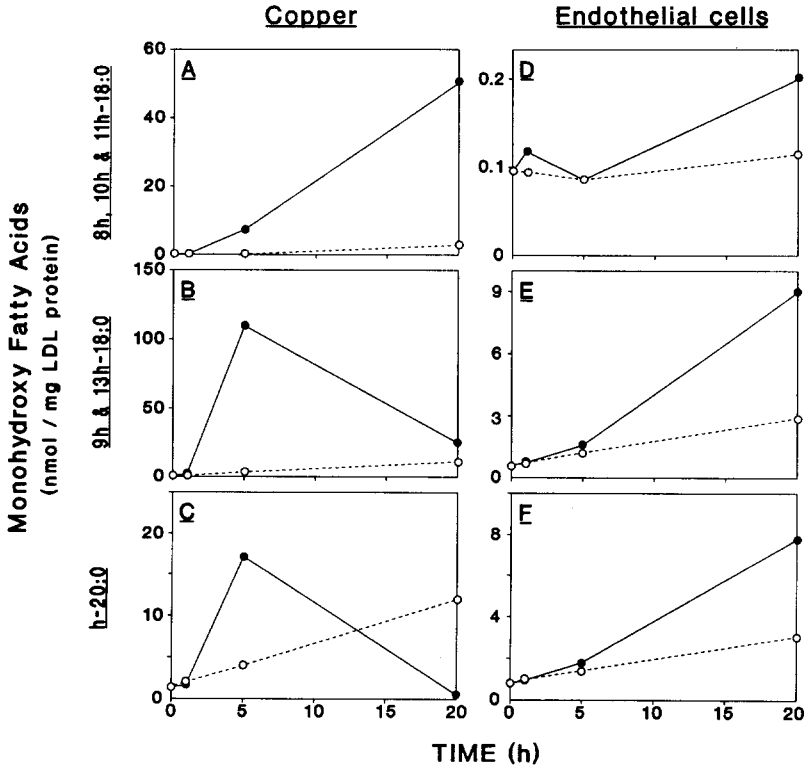

Fig. 6. Time course for the formation of monohydroxy metabolites of unsaturated fatty acids in human LDL. Monohydroxy fatty acids in control LDL (○----------○) or LDL incubated with 20 μM $CuSO_4$ (●———●) (A, B, C) or endothelial cells (●———●) (D, E, F) were quantitated as described in Materials and Methods. The values are means of triplicates ($CuSO_4$) or duplicates (endothelial cells).

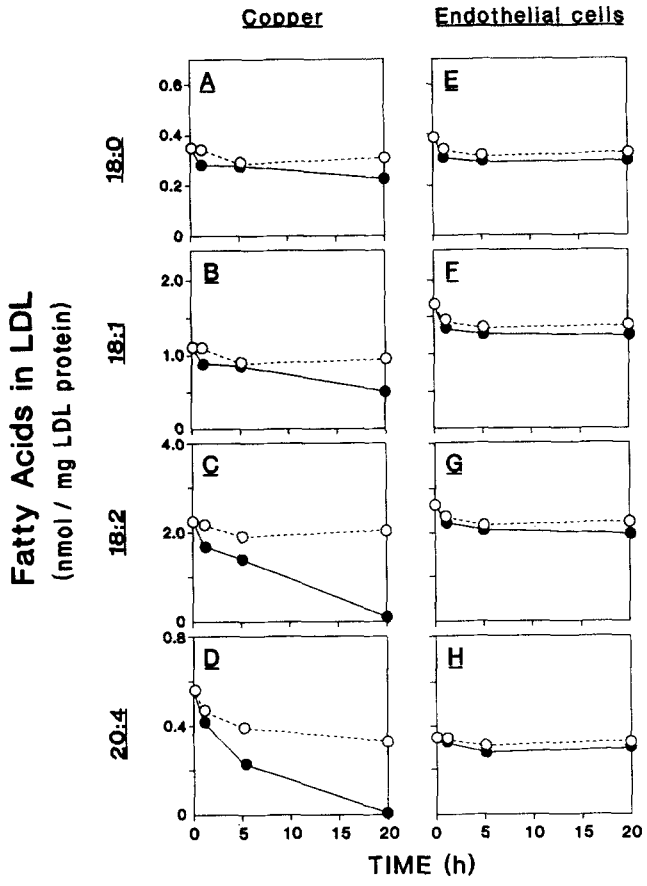

Fig. 7. Changes in the amounts of fatty acids in LDL during oxidation. Control LDL (○----------○) or LDL incubated with 20 μM $CuSO_4$ (●——●) (A, B, C, D) or endothelial cells (●——●) (E, F, G, H) was reisolated by ultracentrifugation and extracted by the Folch method with methyl arachidic acid (20:0) as an internal standard. After transmethylation, fatty acids were separated from their oxygenation products by open column chromatography and measured by gas chromatography. Quantitation of fatty acids was performed by comparing their peak areas to that for the internal standards. The results are means of triplicates ($CuSO_4$) or duplicates (endothelial cells).

stearic acid in LDL (**Fig. 7A**), and a reduction in oleic acid content by nearly 50% after 20 h (Fig. 7B). In contrast, almost all the linoleic acid (Fig. 7C) and arachidonic acid (Fig. 7D) was lost from LDL after incubation for 20 h. On the other hand, incubation of LDL with endothelial cells led to negligible changes in esterified fatty acids (Fig. 7, E to H). In all cases there was an initial decrease in fatty acid content of LDL, possibly due to the loss of loosely bound lipids into the medium.

Conjugated dienes. Incubation of LDL with $CuSO_4$ resulted in marked increases in UV absorption at about 234 nm (conjugated dienes) and about 270 nm (conjugated trienes) after 5 and 20 h (**Fig. 8**). However, no increases in UV absorption at these wavelengths were observed as a result of incubation of LDL with endothelial cells for up to 20 h (data not shown).

Thiobarbituric acid-reactive substances (TBARS). Lipid peroxidation results in the generation of malondialdehyde as well as other aldehydes which react with thiobarbituric acid to produce adducts which have absorbance maxima at ca. 565 nm with an excitation wavelength of 515 nm (29). Incubation of LDL with both copper ions and endothelial cells resulted in increased production of TBARS (**Fig. 9**). The amounts of TBARS formed were much greater after incubation with copper ions, the levels being much higher than controls after incubation for only one 1 h.

Distribution of monohydroxy fatty acids and TBARS in LDL and incubation medium after incubation with copper ions

We measured the amounts of monohydroxy fatty acids and TBARS in LDL and the incubation medium after incubation of LDL with 20 μM $CuSO_4$ at 37°C. The times chosen corresponded to the times for which the level of the substances in question were maximal (5 h in the cases of monohydroxy derivatives of 18:2 and 20:4, and 20 h in the cases of monohydroxy-18:1 and TBARS) (**Table 1**). After the incubations, LDL was reisolated by ultracentrifugation. Esterified monohydroxy fatty acids were extracted from LDL, whereas free monohydroxy fatty acids were extracted from the incubation medium (infranatant after centrifugation) as shown in Fig. 2. TBARS were also measured in these two fractions. The results indicate that nearly all of the monohydroxy fatty acids remained bound to LDL lipids, whereas about 80% of the TBARS were released into the medium (Table 1).

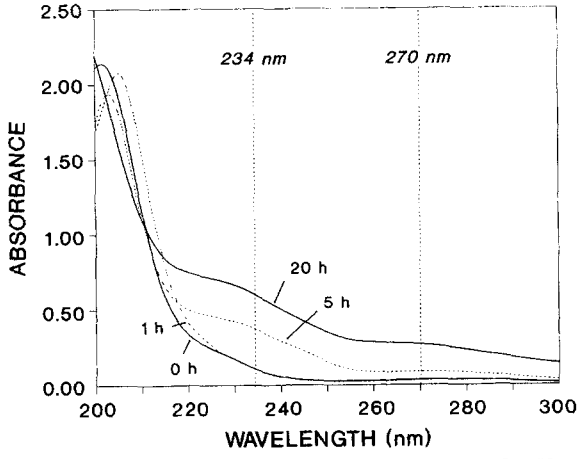

Fig. 8. UV spectra of human LDL incubated with $CuSO_4$. Human LDL was incubated in the presence of 20 μM $CuSO_4$ for 1, 5, and 20 h, respectively. LDL was diluted with phosphate-buffered saline and the ultraviolet spectra were recorded. The baseline was established by recording the spectrum of the incubation medium without LDL.

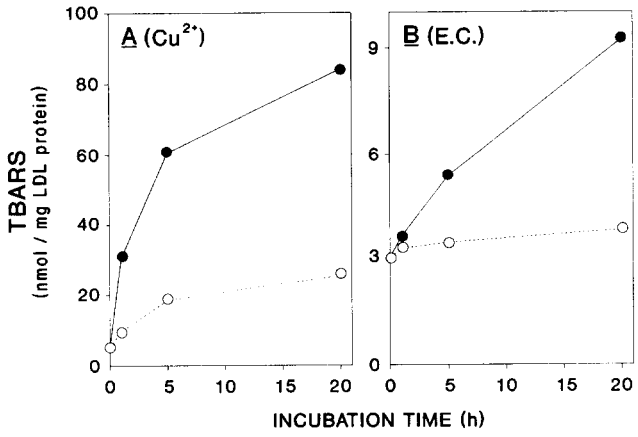

Fig. 9. Time course for the formation of thiobarbituric acid-reactive substances (TBARS) during the oxidation of human LDL. Control LDL (○----------○) or LDL incubated with either 20 μM $CuSO_4$ (●——●) (A) or endothelial cells (●——●) (B) was diluted with phosphate-buffered saline and TBARS were measured using spectrofluorometry with an excitation wavelength of 515 nm and an emission wavelength of 565 nm. The concentration of TBARS was calculated from using a calibration curve obtained using a freshly prepared solution of 1,1,3,3,-tetramethoxypropane. The values are means of triplicates ($CuSO_4$) or duplicates (endothelial cells).

DISCUSSION

Lipid peroxidation is a complicated series of reactions that results in the conversion of polyunsaturated fatty acids to a large number of products, many of which can serve as indicators for this process. However, the relative amounts of different products formed are dependent on the reaction conditions, and may vary considerably. The most commonly used method to evaluate the extent of lipid peroxidation is to measure thiobarbituric acid-reactive substances (TBARS), which consist primarily of malondialdehyde, along with other aldehydes (32). This procedure is relatively simple, but lacks specificity and is complicated by the fact that malondialydehyde is derived principally from polyunsaturated fatty acids (PUFA) containing at least three double bonds (33, 34). In this study we have measured the formation of various monohydroxy fatty acids, which are formed during the initial stages of lipid peroxidation. Although the procedure may be rather complicated for routine use, it provides important information concerning the identity of the fatty acids being oxidized as well as the natures of the products initially formed.

Various approaches could be used to measure monohydroxy fatty acids by mass spectrometry. The most sensitive method would be to measure the amounts of these products by negative ion-chemical ionization mass spectrometry, after derivatization to their pentafluorobenzyl esters (35). However, this method lacks specificity, since all of the many positional isomers of monohydroxy fatty acids derived from the same fatty acid precursor will have similar fragmentation patterns due to loss of the pentafluorobenzyl group. The advantage of electron impact mass spectrometry, which was used in the present study, is that different positional isomers of these compounds have characteristic mass spectra, making it possible to quantitate many different isomers at the same time. However, there are several problems with this approach. Monohydroxy fatty acid derivatives of PUFA formed by lipid peroxidation are mixtures of *trans-cis* and *trans-trans* isomers, making the GC profile rather complicated. Second, the fragmentation patterns for some positional isomers are similar to one another, differing only in the relative intensities of certain ions (e.g., 9h-18:2 and 13h-18:2). Third, some monohydroxy derivatives of 20:4 are thermally unstable, resulting in rather poor recoveries after gas chromatography. To circumvent these problems, monohydroxy fatty acids can be converted to hydrogenated derivatives, which are much more stable and, after methylation and conversion to their trimethylsilyl ethers, have distinctive mass spectra with single major

TABLE 1. Distribution of monohydroxy fatty acids in LDL and in medium

	h-18:1[a]		h-18:2[b]		h-20:4[c]		TBARS	
	LDL	Medium	LDL	Medium	LDL	Medium	LDL	Medium
	nmol product/mg LDL protein							
"Native"	0.2	0.2	0.7	0.2	1.5	0.1	0.3	1.8
Oxidized	50.3	0.4	109.6	0.2	17.1	0.3	11.9	48.4

Human LDL was incubated for either 5 h (h-18:2 and h-20:4) or 20 h (h-18:1 and TBARS) with 20μM $CuSO_4$ and then reisolated by ultracentrifugation. The times chosen corresponded to those when the amounts of the products in question were maximal. Monohydroxy derivatives of fatty acids in LDL (top fraction after centrifugation) and medium (infranatant after centrifugation) were extracted by the Folch method and using an ODS-silica Sep-Pak, respectively. They were purified and measured by GC–MS as described in Materials and Methods. TBARS were also measured in these two fractions. All values are means of triplicates. Similar results were obtained for h-18:1 after 5 h (7.4 nmol/mg protein in LDL vs. 0.1 ng/mg in the medium).

[a]Sum of 8h-18:0, 10h-18:0, and 11h-18:0.
[b]Sum of 9h-18:0 and 13h-18:0.
[c]Sum of 5h-20:0, 8h-20:0, 9h-20:0, 11h-20:0, 12h-20:0, and 15h-20:0.

ions due to fragmentation of one of the carbon–carbon bonds adjacent to the hydroxyl group.

The major problem with the approach described above using hydrogenated derivatives is that it is not possible to determine the number of double bonds originally present in the underivatized monohydroxy fatty acid. Since arachidonic acid is by far the major C_{20} fatty acid in mammalian lipids (except in cases where the diet contains large amounts of fish oils), one can safely assume that the hydrogenated C_{20} monohydroxy fatty acids that we measured are nearly all derived from the corresponding tetraenes (i.e., HETEs). The situation is more complicated with C_{18} fatty acids, since mammalian lipids contain large amounts of linoleic acid and oleic acid, both of which can be oxidized as a result of lipid peroxidation. However, it is well known that linoleic acid is oxidized primarily in the 9- and 13-positions, whereas oleic acid is oxidized principally in the 8-, 9-, 10-, and 11-positions (36). The data in Fig. 4 clearly show that the time courses for the formation of 9- and 13-hydroxy C_{18} derivatives are different from those for the other C_{18} monohydroxy fatty acids. The fact that the amounts of both the 9- and 13-hydroxy products decline after 20 h suggests that they can be readily oxidized to other products due to the presence of multiple double bonds. It is therefore likely that both of these products are derived from linoleic acid. Fig. 4 also indicates that the time courses for the formation of the 8-, 10-, and 11-hydroxy C_{18} fatty acids are similar to one another and that these compounds are much more stable than the 9- and 13-hydroxy compounds, suggesting that they have fewer double bonds. We have therefore assumed, although it is not proven, that these compounds are derived from oleic acid. We also measured the amounts of 12-hydroxy C_{18} fatty acids in LDL, since we previously detected this product in aortic lipids (31). Only small amounts of this substance were present in human LDL, and the amounts did not change substantially in the presence of Cu^{2+} compared to the other monohydroxy compounds measured. The origin of the 12-hydroxy C_{18} fatty acid is still not clear.

In addition to monohydroxy fatty acids, we have also measured other indicators of lipid preoxidation, including TBARS, the formation of conjugated dienes and trienes, and losses of fatty acids from LDL lipids. Measurement of TBARS was a good indicator of lipid peroxidation induced by both mild (endothelial cells) and strong (copper ions) stimuli. The increases in the amounts TBARS (Fig. 9) were comparable to the increases in the amounts of monohydroxy derivatives of 18:2 and 20:4 (Fig.7) after incubation of LDL with endothelial cells. However, measurement of conjugated dienes or fatty acids was not very useful for measurement of lipid peroxidation under these conditions.

All the methods used to estimate lipid peroxidation gave positive results when a strong stimulus (20 μM $CuSO_4$) was used to induce lipid peroxidation. The best indicators of lipid peroxidation at shorter time points (5 h) were monohydroxy derivatives of 18:2 (about 30 times control values) and 18:1 (about 60 times control values) (Fig. 6). At longer time points (20 h), the best indicators were monohydroxy derivatives of oleic acid (about 20 times control values) (Fig. 6) and polyunsaturated fatty acids (almost complete loss of 18:2 and 20:4) (Fig. 7). The increases in the amounts of monohydroxy derivatives of 20:4 after incubation of LDL with $CuSO_4$ for 5 h were much less than those observed for monohydroxy C_{18} fatty acids, due to the instability of the former substances.

Our results indicate that both linoleic acid and arachidonic acid are extensively oxidized during the copper-catalyzed oxidation of LDL, since both of these fatty acids were almost completely depleted after 20 h. Since linoleic acid is present in larger amounts than arachidonic acid, it would appear to be the major source of lipid peroxidation products. However, it is also evident that the initial oxidation products derived from arachidonic acid are considerably less stable than those derived from linoleic acid, since monohydroxy derivatives derived from the former fatty acid were almost completely consumed by 20 h. Similar conclusions were reached from a recent study by Lenz et al. (37), who used normal phase-HPLC to measure the amounts of monohydroxy derivatives of linoleic acid and arachidonic acid in LDL subjected to copper-induced oxidation. These authors found that monohydroxy derivatives of 18:2 accounted for 67% of the amount of this fatty acid consumed after incubation of LDL with 5 μM $CuSO_4$ for 24 h, whereas monohydroxy derivatives of 20:4 accounted for only 25% of the amount of the 20:4 which was consumed during this period (37). Thus, arachidonic acid should be a good source of secondary oxidation products such as the various aldehydes which appear to be important for the modification of apolipoprotein B (38, 39) and the subsequent uptake of modified LDL by the macrophage scavenger receptor (38–41). Although our studies have shown that oleic acid can be oxidized extensively in the presence of a strong stimulus for lipid peroxidation, its rate of oxidation is much less than those of polyunsaturated fatty acids. Replacement of arachidonic acid and linoleic acid in LDL by other fatty acids, such as oleic acid, which are less readily oxidized may thus protect LDL from modification. This would be consistent with the recent finding of Parthasarathy et al. (42) that LDL from rabbits fed an oleic acid-enriched diet was resistant to oxidative modification induced by copper ions and endothelial cells.

Modification of LDL induced by copper ions is presumably due to nonenzymatic lipid peroxidation. However, various types of cells, including endothelial cells (4, 5), vascular smooth muscle cells (6), and monocytes/macrophages (7, 8), can modify LDL by release of superoxide (6, 8, 13, 43) or by lipoxygenase-mediated reactions (14, 44). It would seem unlikely that lipoxygenases in endothelial cells, which have been shown to contain 15-lipoxygenase (14, 45), modify LDL lipids principally by direct oxidation of esterified polyunsaturated fatty acids (44), since we did not observe any positional specificity with respect to the oxidation of arachidonic acid when LDL was incubated in the presence of these cells. It has been observed that modification of LDL by endothelial cells is dependent upon the presence of low concentrations of transition metal ions such as Cu^{2+} and Fe^{2+}, which are present in low concentrations (10 nM and 3 μM, respectively) in Ham's F-10 medium (4). In the presence of transition metal ions, hydroperoxy-PUFA, initially formed by the action of a lipoxygenase, could be converted to alkoxyl or peroxyl radicals, which could abstract hydrogen atoms from LDL lipids and thereby initiate a chain of lipid peroxidation. This would explain the lack of positional specificity in the formation of hydroxy-PUFA in LDL incubated with endothelial cells. Furthermore, the alkoxy and hydroperoxy fatty acids formed in this way could decompose to give various aldehydes, including malondialdehyde, which react with lysine amino groups of apolipoprotein B (39, 41, 46, 47). Since we and others (4, 48) have shown that TBARS (principally malondialdehyde) are found principally in the incubation medium, more hydrophobic aldehydes such as 4-hydroxynonenal (41, 47) could also play important roles in the derivatization of these amino groups.

The extent of oxidation of LDL by human umbilical vein endothelial cells in our study was somewhat less than that observed by Steinbrecher et al. (49) who used rabbit aortic endothelial cells. This could possibly be due to differences in the experimental conditions used in these studies. Marked differences have been observed in the abilities of various types of endothelial cells to oxidize LDL. Whereas both human umbilical vein and rabbit aortic endothelial cells can oxidize LDL, bovine aortic endothelial cells are relatively inactive (50). Other factors, such as lipoprotein concentration, media volume, and numbers of cells, are also important factors in determining the extent of cell-mediated oxidation of LDL (7).

In conclusion, we have shown that incubation of human LDL in the presence of a strong stimulus for lipid peroxidation (20 μM $CuSO_4$) results in extensive oxidation of linoleic acid and arachidonic acid to give rise to monohydroperoxy/monohydroxy products, which are themselves further degraded to secondary products. Although monohydroxy derivatives of these fatty acids are good indicators of the earlier stages of the peroxidation of LDL lipids, monohydroxy derivatives of oleic acid are excellent indicators of the later stages of this reaction because of their increased stability. Relatively smaller increases in thiobarbituric acid-reactive substances were observed with copper-induced oxidation of LDL. A different pattern of lipid peroxidation products was observed when oxidation of LDL was induced by incubation with endothelial cells, a much milder and more physiological stimulus. In this case, increases in the formation of monohydroxy derivatives of polyunsaturated fatty acids were sustained for a longer period of time, and were similar to the increases observed for TBARS. In comparison, the increases observed in the formation of monohydroxy derivatives of oleic acid were more modest.

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