Lipid hydroperoxy and hydroxy derivatives in copper-catalyzed oxidation of low density lipoprotein

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Abstract Oxidation of low density lipoprotein (LDL) causes changes in the biological properties of LDL that may be important in atherogenesis. That LDL oxidation is accompanied by lipid peroxidation has been demonstrated, but previous analyses of the products of LDL oxidation have not included measurement of specific lipid hydroperoxy and hydroxy derivatives. In this study, LDL was isolated from plasma of normal volunteers and exposed to oxygenated buffer and 5 μM $CuSO_4$ for 24 h. Oxidized LDL showed decreased linoleate (18:2) and arachidonate (20:4) content with increased concentrations of thiobarbituric acid reactive substances (TBARS) and hydroxy and hydroperoxy 18:2 and 20:4. The electrophoretic mobility of the LDL protein also was increased by oxidation. After reduction, the hydroxy fatty acids were characterized by gas chromatography–mass spectrometric analysis of the trimethylsilyl ether methyl ester derivatives. The hydroperoxy and hydroxy derivatives accounted for approximately 70% of the linoleate consumed during LDL oxidation and represented 45-fold more product than was measured by TBARS analysis. Numerous biological properties, including cytotoxic and chemoattractant activities of hydroperoxy and hydroxy fatty acids, have been reported, but the manner in which they may contribute to atherogenesis requires further study. —**Lenz, M. L., H. Hughes, J. R. Mitchell, D. P. Via, J. R. Guyton, A. A. Taylor, A. M. Gotto, Jr., and C. V. Smith.** Lipid hydroperoxy and hydroxy derivatives in copper-catalyzed oxidation of low density lipoprotein. *J. Lipid Res.* 1990. **31:** 1043–1050.

Supplementary key words lipid peroxidation • lipid hydroperoxides • hydroxyoctadecadienoates

A major risk factor for atherosclerosis is an elevated level of low density lipoprotein (LDL), the major carrier of cholesterol in the blood. However, the mechanisms involved in the pathogenic relationship between elevated LDL and atherogenesis remain unknown. LDL subjected to oxidative conditions in vitro exhibits altered biological activity in cell culture systems. Oxidized LDL shows potent chemotactic activity for monocytes, increased uptake by scavenger receptors on macrophages (leading to cholesterol accumulation and foam cell formation), and cytotoxicity for cultured fibroblasts and endothelial cells (1–4). Similar events involving oxidized LDL may occur during the formation of atherosclerotic plaques in the arterial wall. Atherosclerotic plaque development is characterized by recruitment of monocyte-macrophages into the vessel intima (5, 6), cellular and extracellular lipid accumulation (7–9), smooth muscle cell proliferation and death (10), and potential disruption of the nonthrombogenic endothelial lining (11, 12). The hypothesis that oxidized LDL is involved in this process has been strengthened by the demonstration that probucol, an inhibitor of LDL oxidation, significantly reduces atherosclerosis in the Watanabe heritable hyperlipidemic rabbit (13, 14).

Several investigators have concluded that the alterations in biological characteristics of LDL exposed to oxidative conditions are at least in part due to lipid peroxidation. This hypothesis is based on the observations that oxidation of LDL is accompanied by an increase in thiobarbituric acid reactive substances (TBARS) and that the formation of the lipid peroxidation products as well as changes in LDL biological activity are inhibited by antioxidants (3, 4). Although lipid peroxidation may be involved in pathophysiologically significant LDL modifications, limited data are available concerning specific products formed in this process.

Esterbauer and colleagues (15) have identified a number of lipid aldehydes resulting from LDL oxidation. Aldehydes, through Schiff base formation with amino acid

Abbreviations: HODE, hydroxyoctadecadienoate; HETE, hydroxyeicosatetraenoic acid; TBARS, thiobarbituric reactive substances; GC–MS, gas chromatography–mass spectrometry; apoB, apolipoprotein B; MDA, malondialdehyde; LDL, low density lipoprotein; BHT, butylated hydroxytoluene.

residues of apoB, may lead to increased electronegativity of LDL and enhanced affinity for scavenger receptors on macrophages (16). Two aldehydes identified in oxidized LDL by Esterbauer et al. (15), 4-hydroxyoctenal and 4-hydroxynonenal, have previously been demonstrated to have chemotactic properties (17). However, the aldehydes measured in these studies accounted for only 2% of the polyunsaturated fatty acids consumed by the oxidation in vitro.

The course of nonezymatic lipid peroxidation is directed largely by the preferential abstraction of *bis*-allylic hydrogen atoms from fatty acids containing two or more homoconjugated carbon–carbon double bonds and by the rapid reaction of the ensuing pentadienyl radical with molecular oxygen. However, a number of sequential and sometimes competing reaction pathways are available to the peroxyl radicals thus produced and complex product mixtures are not unexpected (18, 19). The low molar yields of lipid-derived aldehydes are not surprising, but a further description of LDL oxidation requires a marked improvement in accountability of the fates of the fatty acids lost during oxidation. Therefore, the present study was undertaken to identify and quantitate other products of lipid peroxidation in oxidized LDL in order that we might better understand its altered and potentially pathological biological properties.

MATERIALS AND METHODS

Hydroxyeicosatetraenoate standards were prepared from arachidonic acid using cupric oxide and hydrogen peroxide as described by Boeynaems et al. (20). Chemicals and solvents were purchased from Fisher or Sigma.

Whole blood was obtained by venipuncture from four healthy volunteers who had fasted for 12 h. Butylated hydroxytoluene (BHT) (20 μM) and ethylenediamine tetraacetic acid (EDTA) (0.5 mg/ml) were added to the citrated plasma. Plasma LDL was prepared by ultracentrifugation (21) within a density cut-off of 1.020 to 1.055 g/ml. LDL was dialyzed for 48 h at 4°C in the dark against vacuum-degassed 0.01 M phosphate buffer, pH 7.4, containing 0.16 M NaCl and 0.1 mg/ml chloramphenicol (15). The dialysis bag was Spectropor 20.4 diameter tubing that was boiled in distilled deionized water for 30 min prior to use.

For oxidation of LDL, the dialyzed LDL solution was diluted with the dialysis buffer to a final concentration of 1.5 mg LDL protein/ml, transferred into a dialysis bag, and immersed in a 3-fold volume of dialysis buffer with 5 μM $CuSO_4$. The system was kept in the dark at room temperature for 24 h and oxygen was bubbled continuously through the external buffer. Samples were withdrawn at 0, 2, 4, 6, 14, and 24 h for electrophoretic mobility determinations and for chemical analysis of peroxidation products.

Oxidized LDL was analyzed for lipid peroxidation products using high performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) as previosly described by Hughes et al. (22, 23). Briefly, total lipids were extracted from LDL samples (250 μl of 1.5 mg LDL protein/ml) according to Folch et al. (24) with 20 vol of chloroform–methanol 2:1 (v/v). After transesterification of the total lipid extract with sodium methoxide, the resulting methyl esters were analyzed by normal phase HPLC. Hexane–isopropanol–acetic acid 993:6:1 at 2.3 ml/min was used as mobile phase and UV absorbance was monitored at 235 nm. Analysis was carried out on a 5 μm 250 mm × 4.6 mm HPLC Econosphere silica column, Alltech Associates. Samples were subjected to HPLC analysis with and without reduction of hydroperoxides with triphenylphosphine (0.7 mg in 700 μl methanol–ether 1:1). The identity of the oxidation products in HPLC fractions was confirmed by GC–MS analysis. Samples were analyzed, both with and without hydrogenation (platinum oxide catalyst), as their trimethylsilyl (TMS)-ether derivatives as previously described (22). GC–MS analyses were performed using an HP5890A gas chromatograph (Hewlett-Packard, Avondale, PA) coupled to a VG TS250 mass spectrometer (VG Tritech, Manchester, U.K.). A fused silica capillary column (20 m × 0.32 mm, i.d.) of the DB-1 bonded phase-type (J&W Scientific, Rancho Cordova, CA) was used for the analyses with temperature programming from 200°C to 300°C at 5°/min. Spectra were recorded under EI (70 eV) ionization conditions. Using HPLC, methyl hydroxyoctadecadienoates (HODEs) and methyl hydroxyeicosatetraenoates were quantitated relative to standard methyl hydroxyeicosatetraenoates based on peak area calculations. Methyl 11-hydroxyeicosatetraenoate coeluted with the *cis,trans* isomer of 13-HODE but represented only a minor fraction (<10%) of this peak as determined by GC–MS analysis. Since similar amounts of all other hydroxyeicosatetraenoate isomers (i.e., 5, 8, 9, 12, and 15-hydroxyeicosatetraenoate) were formed during LDL oxidation, the average peak area of the measured hydroxyeicosatetraenoate isomers was subtracted from the peak area containing both 11-hydroxyeicosatetraenoate and *cis,trans* 13-HODE for calculation of *cis,trans* 13-HODE peak area.

The less polar fractions of the transesterified lipid isolated by HPLC contained fatty acid methyl esters and were analyzed by gas chromatography with a flame ionization detector. A Hewlett-Packard 5710A gas chromatograph equipped with a glass column (6 ft × 2 mm, i.d.) packed with 10% SP 2330 on Chromosorb WAW (100/120 mesh) was used for the analyses. Nitrogen (30 ml/min) was used as carrier gas with temperature programming from 160° to 250°C at 4°/min. The fatty acid methyl esters were quantitated relative to the standard methyl heneicosanoate based on peak area calculations.

Electrophoretic mobility of LDL was determined by standard agarose gel electrophoresis. Protein concentrations were determined by the method of Lowry et al. (25). Malondialdehyde content of lipoprotein samples was measured spectrophotometrically using the thiobarbituric assay as described by Steinbrecher et al. (26).

RESULTS

Oxidation of LDL for 24 h in the presence of 5 μM Cu^{2+} resulted in decreases in linoleate and arachidonate of 21 ± 8% and 44 ± 13% (mean ± SEM), respectively (**Fig. 1A and 1B**). Two of the LDL samples showed substantially more polyunsaturated fatty acid loss during oxidation than did the other samples. Measurable losses of linoleate and arachidonate generally were not observed in the first 4–6 h but there were continuous decreases with time thereafter. This lag phase preceding significant polyunsaturated fatty acid loss appears to be related to the time required to overcome the antioxidant effects of vitamin E contained in LDL (15).

HPLC analysis of LDL that had been oxidized 24 h indicated that a mixture of hydroperoxy and hydroxy derivatives of linoleate and arachidonate had been formed. Samples were studied before and after reduction of hydroperoxides to their hydroxy forms with triphenylphosphine (**Fig. 2**). As peaks corresponding to hydroxy derivatives elute later on HPLC than their corresponding hydroperoxides (27), estimations of hydroperoxide content prior to reduction with triphenylphosphine could be made. HPLC profiles of the nonreduced and reduced products revealed that more than 70% of the linoleate products identified in LDL oxidized for 24 h were present in the hydroperoxide form. To confirm the identity of the hydroperoxides, peaks were collected from HPLC, reduced with triphenylphosphine, and reanalyzed by HPLC. The example shown in Fig. 2b indicates the reduction products of four hydroperoxides derived from methyl linoleate. The compounds were identified by GC–MS analysis of the TMS ethers. Peaks 3b and 4 gave the same mass spectrum with diagnostic ions at *m/z* 382 (M^+), 311 ($M-C_5H_{11}$), 292 (M-TMSOH), and 225 ($M-C_9H_{17}O_2$). The two peaks correspond to the *cis,trans* and *trans,trans* iso-

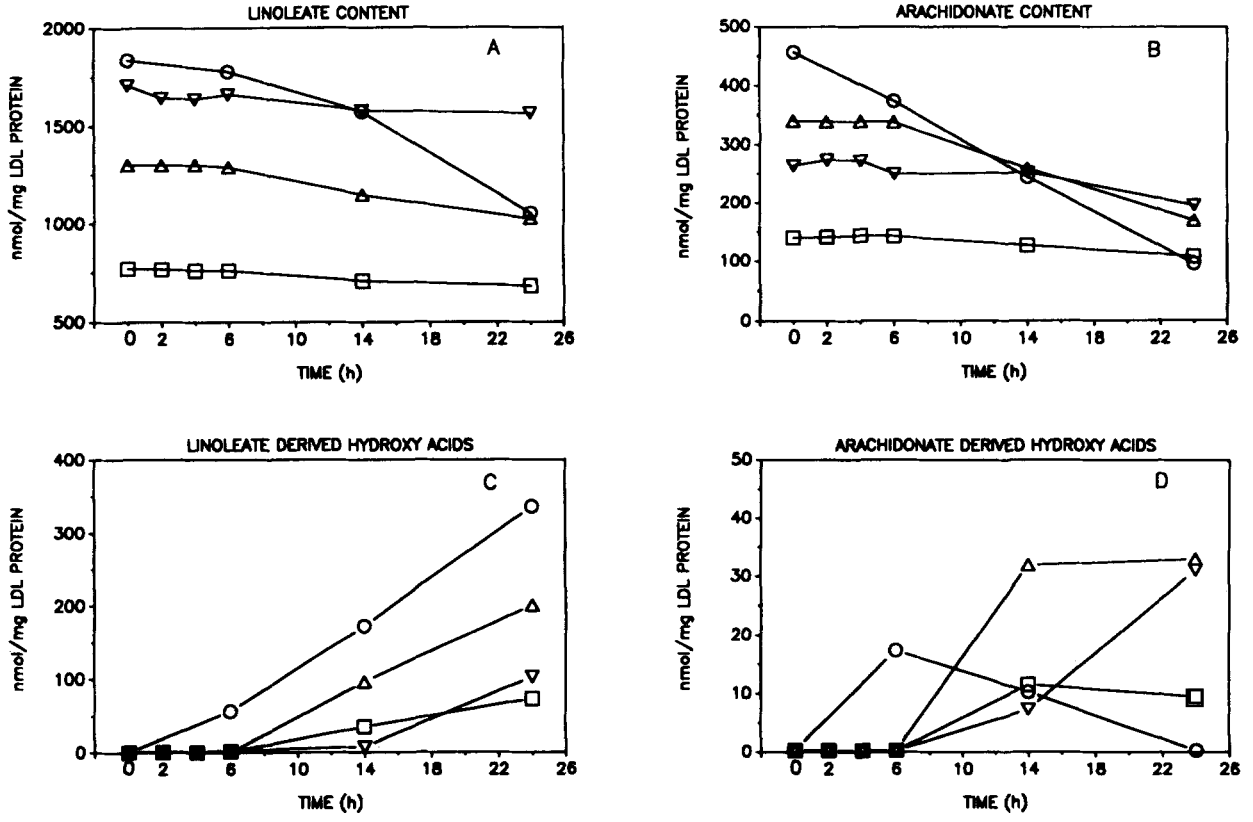

Fig. 1. Polyunsaturated fatty acid and hydroxy derivative content in four samples of LDL oxidized 24 h in the presence of 5 μM $CuSO_4$. Alike symbols represent data from the same LDL sample. Panels A and B show time course of loss of linoleate and arachidonate, respectively. Panels C and D show time course of total linoleate-derived (panel C) and total arachidonate-derived (panel D) hydroxy acid content in oxidized LDL. Samples were analyzed by HPLC after reduction of hydroperoxides with triphenylphosphine. Total linoleate-derived hydroxy acid content shown is the sum of measured 9- and 13-hydroxyoctadecadienoate. Total arachidonate-derived hydroxy acid content shown is the sum of measured 5-, 8-, 9-, 12-, and 15-hydroxyeicosatetraenoate.

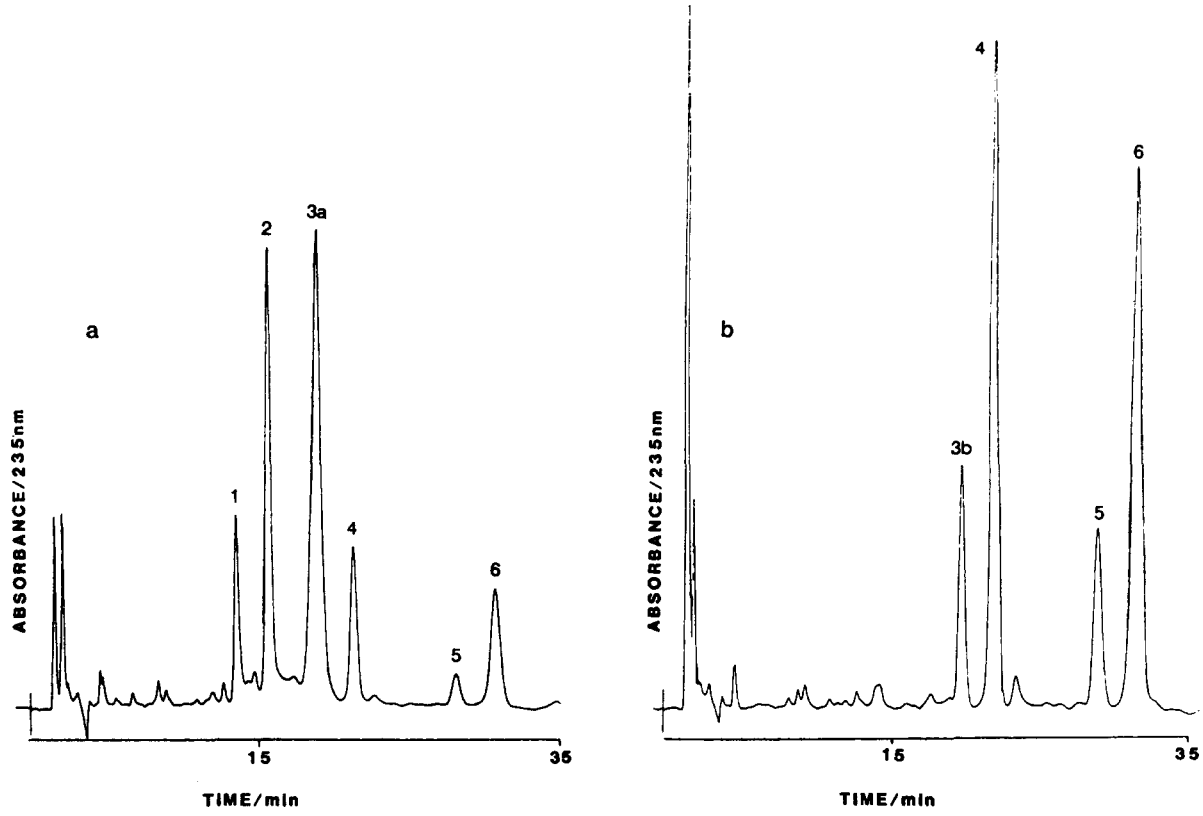

Fig. 2. HPLC separation of hydroperoxy and hydroxy acid methyl esters in LDL oxidized for 24 h with 5 μM Cu^{2+}/O_2. Samples were studied with (panel b) and without (panel a) reduction of hydroperoxides with triphenylphosphine. To confirm the identity of linoleate-derived hydroperoxides in panel a, peaks were collected on HPLC, reduced with triphenylphosphine, recollected on HPLC, and then analyzed by gas chromatography–mass spectrometry. The numbered peaks are: 1) methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate; 2) methyl 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoate; 3a) mixture of methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate, methyl 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate, and methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate, 3b) methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate; 4) methyl 13-hydroxy-*trans*-9,*trans*-11-octadecadienoate; 5) methyl 9-hydroxy-*trans*-10-*cis*-12-octadecadienoate; 6) methyl 9-hydroxy-*trans*-10,*trans*-12-octadecadienoate.

meric forms of methyl 13-HODE, the *trans,trans* isomer eluting later on HPLC (28). The 9-hydroxy isomers (peaks 5 and 6) could be distinguished from 13-HODE by mass spectrometry by virtue of the low intensity (<10%) of the ion at *m/z* 311 relative to that at *m/z* 225. Additional products isolated from oxidized LDL by HPLC were identified as arachidonate-derived hydroxyeicosatetraenoates by GC–MS analyses (22). Specifically, 5-, 8-, 9-, 12-, and 15-hydroxyeicosatetraenoates were quantitated (**Table 1**). Neither hydroxyeicosatetraenoates nor HODEs were detectable in the surrounding dialysis buffer by HPLC analysis at any time during LDL oxidation.

The linoleate- and arachidonate-derived hydroperoxide and hydroxy acid content remained less than 0.5 nmol/mg

TABLE 1. Lipid hydroxy acids in LDL oxidized 24 hours

	Linoleate Products				Arachidonate Products			
Time	13-OH *cis,trans*	13-OH *trans,trans*	9-OH *cis,trans*	9-OH *trans,trans*	5-OH	8,9-OH	12-OH	15-OH
h	*nmol/mg LDL protein*							
0	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
2	0.3 ± 0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
4	0.4 ± 0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3	<0.3
6	7.5 ± 6.3	1.0 ± 0.8	6.3 ± 6.0	0.8 ± 0.6	0.9 ± 0.6	2.3 ± 2.0	1.0 ± 0.7	1.1 ± 0.8
14	26.3 ± 10.7	15.1 ± 12.6	22.7 ± 8.4	13.5 ± 11.5	3.0 ± 1.0	5.6 ± 1.9	3.0 ± 1.4	3.7 ± 1.4
24	45.0 ± 7.6	46.6 ± 28.2	40.1 ± 7.1	46.2 ± 28.6	4.3 ± 2.3	5.9 ± 2.7	4.5 ± 2.1	4.1 ± 1.5

Linoleate- and arachidonate-derived lipid hydroxy acids in LDL oxidized 24 h with 5 μM $CuSO_4$; mean ± SEM.

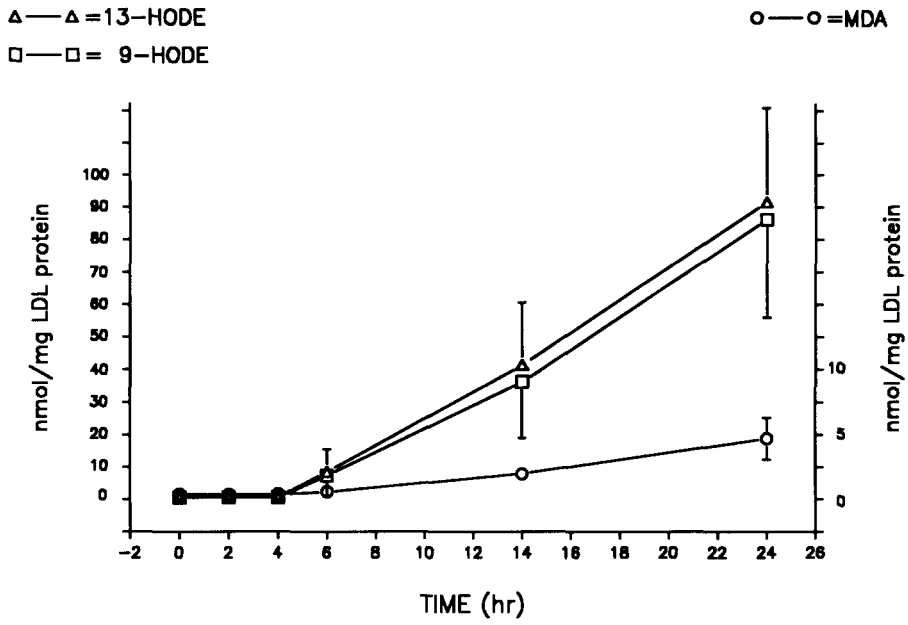

Fig. 3. Comparison of MDA measured as TBARS and the linoleate-derived lipid hydroxy acids 13- and 9-HODEs during LDL oxidation over 24 h with 5 μM Cu^{2+}/O_2. Results are from four experiments; mean ± SEM. Hydroperoxides of lipid extracts were reduced with triphenylphosphine prior to HPLC analysis of the HODEs.

LDL protein during the first 4–6 h of oxidation (Fig. 1C and 1D). After this period, however, a dramatic increase in the lipid peroxidation products was observed. Linoleate-derived hydroperoxides and hydroxy acids continued to increase through the 24 h of oxidation used in the study. A similar pattern was observed for arachidonate-derived products; however, in two LDL samples product concentrations peaked and then were decreased at 24 h indicating decomposition or further oxidation of arachidonate derivatives. The 9- and 13-HODEs accounted for 67 ± 8% (mean ± SEM) of the linoleate consumed at 24 h. The hydroxyeicosatetraenoates accounted for 48 ± 17% (mean ± SEM) of the arachidonate consumed at 14 h and 25 ± 10% (mean ± SEM) at 24 h. Malondialdehyde, measured as TBARS, was determined in LDL samples and in the surrounding dialysis buffer. **Fig. 3** demonstrates that the total amount of TBARS measurable after oxidation of LDL for 24 h was less than 3% that of the amount of linoleate-derived HODEs.

Similar to results reported by others (15), LDL solutions were yellow prior to oxidation, but became pale yellow or colorless after 24 h exposure to oxidative conditions, presumably due to oxidation of β-carotene. Oxidation of LDL led to increased electronegativity as demonstrated by increased electrophoretic mobility on agarose gel electrophoresis. LDL oxidized for 24 h migrated 2.3 ± 0.4 (mean ± SEM, n = 4) times as rapidly as did unoxidized LDL (data not shown).

DISCUSSION

Numerous investigations of LDL oxidation have used the measurement of TBARS, quantified as malondialdehyde (MDA) equivalents, to serve as an index of lipid peroxidation in oxidized LDL. This colorimetric assay is relatively sensitive for MDA but is not specific, as many other compounds will react with TBA to give the pink chromophore. Since the measurement of TBARS establishes an upper limit for MDA levels, our results indicate that MDA is a minor product of copper-catalyzed LDL oxidation, accounting for at most 2% of the polyunsaturated fatty acid consumed. On the other hand, linoleate-derived hydroperoxides and hydroxy acids accumulate in much greater amounts and are the major lipid peroxidation products. In the present study they accounted for 67% of the linoleate consumed. These results are in accord with previous work demonstrating that TBA reactivity underestimates total lipid hydroperoxide and that only about 5% of linoleic acid hydroperoxides and 10% of arachidonic acid hydroperoxides yield TBARS (29). Since the most abundant polyunsaturated fatty acid in LDL is linoleate, measurement of its hydroperoxides as hydroxyoctadecadienoates may serve as a useful indicator of lipid peroxidation during oxidation of LDL.

Arachidonate-derived hydroxyeicosatetraenoates were also identified in oxidized LDL but represented a small percentage (25%) of the loss of this fatty acid at 24 h. The

formation of cyclic endoperoxides and other products in the oxidation of arachidonate may explain the smaller proportion of hydroperoxy and hydroxy acids observed from arachidonate compared to linoleate. Arachidonate's four homoconjugated double bonds, in contrast to the two in linoleate, may lead to greater instability of its primary products of peroxidation and render its primary products more susceptible to further oxidation.

The reasons for the marked variability (Table 1 and Fig. 1) in the extent of oxidation between LDL samples are not known at this time, but may relate to differences in vitamin E content or to other properties of LDL that may affect its rate or extent of oxidation. It appears that the presence of transition metal ions is necessary for significant oxidation of LDL to occur (15, 26, 30). Therefore, much of the variability in oxidation could be related to differences in the types and amounts of copper chelates formed in different samples of LDL. Although BHT was added to plasma to prevent oxidation during the isolation procedure and BHT concentrations were not monitored in the isolated LDL samples, it is doubtful that differences in the amount of BHT retained would account for variability in oxidation as it has been demonstrated previously that dialysis effectively removes BHT from LDL (15). In addition, differences in concentrations of chain-breaking antioxidants such as BHT or vitamin E would be expected to be reflected predominantly in the length of the lag phase of the oxidation (31), whereas the interindividual differences we observe appear to be more complex.

In the present study, total lipid extracts from oxidized LDL were transesterified to methyl esters prior to analysis. The products measured thus represented both hydroxylated free fatty acids and hydroxylated derivatives esterified in LDL lipid. The finding that lipid hydroperoxy and hydroxy derivatives are major products in oxidized LDL indicates that their potential roles in atherogenesis should be considered. Nakao et al. (32) have demonstrated that both 12- and 15-hydroxyeicosatetraenoic acid (HETE) stimulate rat aortic smooth muscle cell migration in vitro. In addition, 5- and 12-HETE have chemoattractant activity for human mononuclear leukocytes, although the potency of these arachidonic acid-derived hydroxy acids to stimulate mononuclear cell movement is significantly less than their effects on neutrophil chemotaxis (33, 34). Migration of both smooth muscle cells and monocyte-macrophages to the intimal layer of arteries is thought to be an important early stage in the development of atherosclerotic plaques (35, 36) and chemotactic lipid products in oxidized LDL could be involved in these processes. The work of Palinski et al. (37), demonstrating immunochemical evidence for the presence of oxidized LDL in early fatty streaks as well as in more advanced atherosclerotic lesions, provides circumstantial evidence to support this hypothesis.

Oxidized lipids produced by LDL oxidation could also play a role in the development of the necrotic core of atherosclerotic plaques. Linoleic acid hydroperoxides have been shown to be cytotoxic to fibroblasts and endothelial cells (38, 39). Interestingly, Harland et al. (40) identified cholesteryl esters of 9- and 13-HODE in human atherosclerotic lesions and noted a positive correlation between the concentrations of these oxidized linoleate products and the severities of atherosclerotic lesions. It is therefore reasonable to suspect that lipid hydroperoxides and hydroxy acids, formed in the arterial wall, could contribute to the cellular organization of atheromas by chemoattractant activities, and upon further accumulation could exert toxic effects on resident cells leading to necrosis and plaque ulceration.

The finding that lipid hydroperoxy and hydroxy derivatives are present in circulating LDL in, at most, very low levels (<0.5 nmol/mg LDL protein) suggests that the concentrations of these products are indeed low in healthy individuals. It is not yet known whether plasma levels of these specific oxidized lipid products are higher in LDL of patients with atherosclerosis or if such an occurrence would promote this disease. Using monoclonal antibody techniques, Haberland, Fong, and Cheng (41) have found no evidence for MDA-modified LDL in plasma of Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model of atherosclerosis. Their demonstration of MDA-altered protein that co-localized with extracellular apoB-100 in atheroma from WHHL rabbits suggests that lipoprotein modification by peroxidative products occurs in vivo, but that the arterial wall is the site of LDL oxidation.

Recently, Parthasarathy, Wieland, and Steinberg (42) have reported evidence that endothelial cell lipoxygenase activity is capable of oxidatively modifying LDL. In these experiments a lipoxygenase inhibitor reduced endothelial cell-mediated LDL oxidation by 70 to 85% but did not affect modification of LDL in the absence of cells. Superoxide dismutase effectively inhibited Cu^{2+}-catalyzed oxidation of LDL but only modestly inhibited endothelial cell-mediated oxidation of LDL, thus suggesting a more critical role for superoxide in the cell-free system. The rather surprising stability of lipid hydroperoxides in LDL particles that we observed in the present studies suggests that products characteristic of lipoxygenase activity might be identifiable in early atheroma. The formation of lipid hydroperoxides or hydroxy acids in cell-mediated oxidation and in early atheroma needs to be examined. Of particular interest is whether the product distributions are characteristic of free radical autoxidation such as we have observed in this model study, or characteristic of one or more lipoxygenase or cyclooxygenase enzymes. Chemically specific methods of analysis are essential for such studies.

Supported in part by the National Institutes of Health Grant #GM-34120 and HL-34111 and The Methodist Hospital Foundation, Houston, Texas. Dr. Lenz was a recipient in the Burroughs Wellcome Clinical Pharmacology Supplemental Stipend Program during performance of this work. Special thanks to Barbara Tornay for her help in preparation of the manuscript.

Manuscript received 6 October 1989 and in revised form 2 January 1990.

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