Oxidative modification of human lipoproteins by lipoxygenases of different positional specificities

Hartmut Kühn,[1,*] Jutta Belkner,[*] Hiroshi Suzuki,[†] and Shozo Yamamoto[†]

Institute of Biochemistry,[*] University Clinicum Charité, Medical Faculty of the Humboldt University Berlin, Hessische Str. 3-4, 10115 Berlin, Germany, and Department of Biochemistry,[†] School of Medicine, Tokushima University, Kuramoto-cho, Tokushima, Japan

Abstract Cellular lipoxygenases have been implicated in foam cell formation during the early stages of atherogenesis. We studied the interaction of lipoxygenases of different positional specificities with human lipoproteins and found that the arachidonate 15-lipoxygenases of rabbit and humans and the arachidonate 12-lipoxygenase of porcine leukocytes oxygenate lipoproteins as indicated by the formation of oxygenated lipids and changes in electrophoretic mobility of low density lipoprotein. The arachidonate 12-lipoxygenase of human platelets, the recombinant arachidonate 5-lipoxygenase of human leukocyte, and the soybean lipoxygenase I were less effective in oxidizing human LDL. As a major oxygenation product, esterified 13S-hydro(pero)xy-9Z,11E-octadecadienoic acid was identified for both the rabbit reticulocyte 15- and the porcine leukocyte 12-lipoxygenase. In addition, esterified 15S-hydro(pero)xy-5,8,11,13(Z,Z,Z,E)-eicosatetraenoic acid (for the rabbit 15-lipoxygenase) and 12S-hydro(pero)xy-5,8,10,14(Z,Z,E,Z)-eicosatetraenoic acid (for the porcine 12-lipoxygenase) as well as small amounts of racemic 9-hydro(pero)xy-10,12-octadecadienoic acid isomers were detected. More than 90% of the oxygenated polyenoic fatty acids were found in the ester lipid fraction, particularly in the cholesteryl esters and in various phospholipid classes (phosphatidylcholine and phosphatidylethanolamine). The possible biological significance of lipoxygenase-induced oxidative modification of lipoproteins in the pathogenesis of atherosclerosis is discussed.—**Kühn, H., J. Belkner, H. Suzuki, and S. Yamamoto.** Oxidative modification of human lipoproteins by lipoxygenases of different positional specificities. *J. Lipid Res.* 1994. **35:** 1749–1759.

Supplementary key words low density lipoproteins • atherogenesis • eicosanoids • lipid peroxidation

Oxidative modification of low density lipoprotein (LDL) and its subsequent uptake by tissue macrophages via scavenger receptor-mediated pathways has been implicated in foam cell formation during early stages of atherogenesis (1, 2). The detection of arachidonate 15-lipoxygenase in atherosclerotic lesions in rabbit (3) and humans (4) and its colocalization with oxidatively modified LDL suggested that cellular lipoxygenases may be involved in the oxidative modification of LDL in vivo. Nonspecific lipoxygenase inhibitors are capable of blocking the oxidative modification of LDL in various cellular in vitro systems (5, 6). However, more quantitative inhibitor studies have indicated that rabbit aortic endothelial cells and mouse peritoneal macrophages are capable of oxidizing LDL even after blockage of the lipoxygenase pathway (7), suggesting that cellular lipoxygenases are not essential for oxidative modification of LDL in these in vitro systems.

The soybean lipoxygenase I, which differs from mammalian lipoxygenases with respect to its protein chemical and enzymatic properties, has been shown to effectively oxidize LDL in the presence of phospholipase A_2 (8). In higher concentrations this enzyme is capable of converting normal LDL to a cytotoxic species, without the preceding action of a lipid-cleaving enzyme, during long term incubations (9). Recently, we reported that the pure arachidonate 15-lipoxygenases of rabbit and humans are capable of oxidatively modifying human LDL into its atherogenic form without the preceding action of lipid-cleaving enzymes (10). In this paper we compare various lipoxygenases with different positional specificities with respect to their capability of oxidizing human lipoproteins. The oxygenation of human LDL by the porcine leukocyte 12-lipoxygenase is characterized in more detail.

MATERIAL AND METHODS

Chemicals

The chemicals used were from the following sources: linoleic acid (9Z,12Z-octadecadienoic acid), arachidonic

Abbreviations: SP-HPLC, normal phase high pressure liquid chromatography; RP-HPLC, reverse-phase high pressure liquid chromatography; 13S-HODE, 13S-hydroxy-9Z,11E-octadecadienoic acid; 15S-HETE, 15S-hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 9-HODE, 9-hydroxy-10E,12Z-octadecadienoic acid; GC–MS, gas chromayography-mass spectrometry; LDL, low density lipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; 1 nkat, 1 nmol substrate turnover/sec.

[1]To whom correspondence should be addressed.

acid (5Z,8Z,11Z,14Z-eicosatetraenoic acid), (D/L)-α-tocopherol, soybean lipoxygenase (grade 1), cholesteryl linoleate, and cholesteryl arachidonate from Serva (FRG), triphenylphosphine from Aldrich, sodium borohydride from Merck (FRG). The Lipidophor electrophoresis system was purchased from Immuno (FRG). All solvents used were of HPLC grade.

Preparations

Rabbit reticulocyte 15-lipoxygenase was partially purified from the lysate supernatant of a reticulocyte-rich blood cell suspension by ammonium sulfate precipitation. For selected experiments the enzyme was further purified to homogeneity (10). The recombinant porcine leukocyte 12-lipoxygenase was expressed in a bacterial expression system (11). From the bacterial lysis supernatant the lipoxygenase was enriched by ammonium sulfate precipitation at 50% saturation. For determination of the turnover rate, the enzyme was further purified to homogeneity by immunoaffinity chromatography (12). The human platelet 12-lipoxygenase was partially purified from human platelet cytosol by ammonium sulfate precipitation at 70% saturation (13). The purified recombinant 5-lipoxygenase was kindly provided by Dr. D. Riendeau (Merck Frosst, Canada).

Very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) were obtained by sequential floating ultracentrifugation in a sodium bromide density gradient (14). The lipoproteins were dialyzed for 24 h against isotonic sodium chloride solution containing 3 mM EDTA. The final EDTA concentration in the assay samples varied between 0.7 and 1.5 mM depending on the dilution of the lipoprotein substrate. Authentic standards of racemic hydroxy fatty acids were prepared by vitamin E-controlled autoxidation of fatty acid methyl esters (15). After triphenylphosphine reduction and alkaline hydrolysis, the resulting free hydroxy fatty acid isomers were separated by SP-HPLC. Chiral standards of 13S-HODE and 15S-HETE were obtained by enzymatic oxygenation (16) of linoleic acid and arachidonic acid, respectively, followed by HPLC preparation of the major oxygenation products. Standards of oxygenated cholesteryl esters were prepared from cholesteryl linoleate and cholesteryl arachidonate with the pure reticulocyte 15-lipoxygenase (17). The chemical structure of all reference compounds prepared was confirmed by ultraviolet spectroscopy, HPLC, including chiral phase HPLC, and gas chromatography–mass spectrometry (GC–MS) of the trimethylsilyl ethers and their hydrogenated derivatives.

Lipoprotein oxygenation

Aliquots of the various lipoprotein classes were incubated with the lipoxygenases in 0.1 M phosphate buffer, pH 7.4 (assay volume, 2 ml). After the times indicated the reaction was stopped by the addition of sodium borohydride which reduces the hydroperoxy compounds to their corresponding alcohols and 15S-hydroxy-11Z,13E-eicosadienoic acid was added as an internal standard. The lipids were extracted as described previously (18). Briefly, 5 ml methanol and 2.5 ml chloroform were added to the incubation mixture and vortexed for about 3 min. Then 2.5 ml water and 2.5 ml chloroform were added, vortexed for 2 min, and phase separation was obtained by centrifugation. The lower (chloroform) phase was recovered and after evaporation of the solvents the lipids were reconstituted in 1 ml chloroform. Aliquots (100 μl) were analyzed for oxygenated cholesteryl esters and phospholipids by SP-HPLC. The remaining lipid extracts were dried and the residue was reconstituted in 0.85 ml methanol–chloroform 8:1 (v/v). KOH (40%, 0.15 ml) was added and the samples were hydrolyzed by incubating for 30 min at 60°C under an argon atmosphere. As shown earlier (19), this method completely hydrolyzes the ester lipids and does not lead to a significant formation of autoxidation products. Samples were generally stored under argon at −20°C not longer than a week. We found that the addition of antioxidants was not necessary as the product composition did not change during storage.

Assay

The arachidonate oxygenase activities of the various lipoxygenase preparations were assayed as follows. Rabbit reticulocyte 15-lipoxygenase (ammonium sulfate precipitate), recombinant porcine leukocyte 12-lipoxygenase (ammonium sulfate precipitate), human platelet 12-lipoxygenase (ammonium sulfate precipitate), and soybean lipoxygenase I: 1 ml 0.1 M phosphate buffer, pH 7.4, containing 100 μM arachidonic acid as substrate, incubation time 15 min. Recombinant human 5-lipoxygenase: 1 ml 0.1 M phosphate buffer, pH 7.4, containing 0.1 mM ATP, 0.1 mM EDTA, 2 mM $CaCl_2$, 12 μg/ml phosphatidylcholine, and 20 μM arachidonic acid as substrate (20), incubation time 15 min. After 15 min the hydroperoxy fatty acids formed were reduced with sodium borohydride, 1 ml methanol was added, and aliquots of the samples were directly analyzed by HPLC without any lipid extraction. The conversion of arachidonic acid was quantified in relation to a control incubation (no lipoxygenase). One nkat of arachidonate oxygenase activity corresponds to the conversion of 1 nmol of arachidonic acid/sec.

HPLC analysis

High pressure liquid chromatography was carried out on a Shimadzu instrument coupled with a Hewlett-Packard diode array detector 1040 A. Reverse-phase HPLC (RP-HPLC) was performed on a Nucleosil C-18 column (Macherey/Nagel, KS-system, 250 × 4 mm, 5 μm particle size). The solvent system was methanol–water–acetic acid 82:18:0.1 (v/v) and the flow rate was

1 ml/min. Straight phase HPLC (SP-HPLC) of the hydroxy fatty acid isomers was carried out on a Zorbax SIL column (DuPont, 250 × 4.6 mm, 5 μm particle size) with a solvent system of n-hexane–2-propanol–acetic acid 100:5:0.1 (v/v) and a flow rate of 1 ml/min. The enantiomer composition of the hydroxy fatty acids was analyzed by chiral phase HPLC (CP-HPLC) on a Chiralcel OD column (Diacel Chem. Industries, 250 × 4.6 mm, 5 μm particle size) with a solvent system of hexane–2-propanol–acetic acid 100:5:0.1 (v/v) and a flow rate of 1 ml/min. Oxygenated cholesteryl esters were analyzed by RP-HPLC with a solvent system of 2-propanol–acetonitrile 25:75 (v/v) and a flow rate of 1 ml/min at 45°C. Phospholipid classes were separated by SP-HPLC with a solvent system consisting of acetonitrile–2-propanol–methanol–water–trifluoracetic acid 135:40:10:6.7:0.85 (v/v) and a flow rate of 1 ml/min. After the phosphatidylethanolamine peak was eluted, the flow rate was increased to 2 ml/min. Compounds were generally identified by coinjections with authentic standards. Chromatograms were quantified by peak areas. Calibration curves (5 point measurements) were established for linoleic acid, arachidonic acid, 13-HODE, and free cholesterol.

RESULTS

Interaction of various lipoxygenases with human lipoproteins

The purified arachidonate 15-lipoxygenase from rabbits and humans (10) as well as the recombinant arachidonate 12-lipoxygenase from porcine leukocytes are capable of oxygenating human lipoproteins as indicated by RP-HPLC. Analysis of the hydrolyzed lipid extracts of human LDL treated with the partially purified porcine leukocyte 12-lipoxygenase indicated the formation of oxygenated polyenoic fatty acids that co-chromatographed with an authentic standard of 13-HODE[2] (**Fig. 1, upper trace**). The UV spectrum of these compounds was characterized by a conjugated diene chromophore with an absorbance maximum at 235 nm and by a conjugated ketodiene chromophore with an absorbance maximum at 270 nm (inset to Fig. 1). Similar chromatograms were obtained when the LDL was incubated with the rabbit reticulocyte and the recombinant human 15-lipoxygenases under identical conditions (data not shown). In control incubations of the LDL without lipoxygenases and with the heat-denatured enzymes (not shown), only small amounts (less than 10% of the samples with the native enzymes) of oxygenated

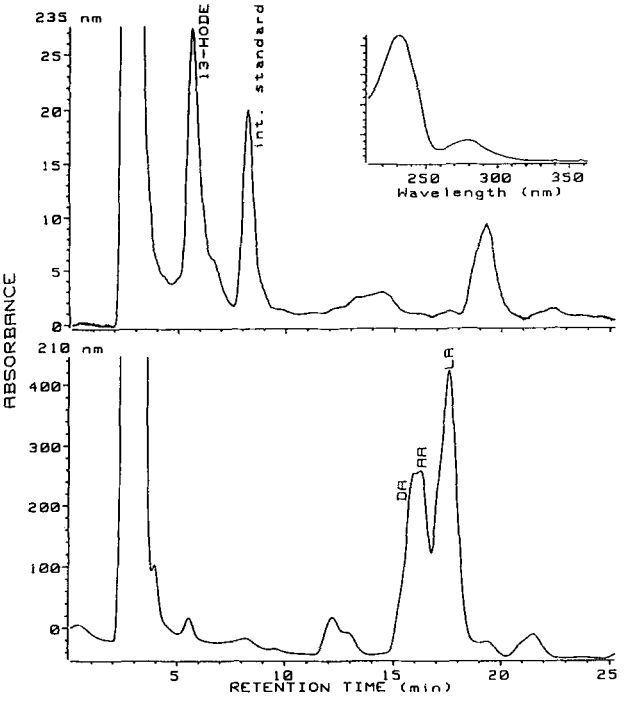

Fig. 1. RP-HPLC of hydrolyzed lipid extracts obtained from human LDL treated with the porcine leukocyte 12-lipoxygenase. Human LDL (1.1 mg protein/ml) was incubated with the recombinant porcine leukocyte 12-lipoxygenase (9.3 nkat/ml arachidonate oxygenase activity) in PBS, pH 7.4, for 15 min; reaction volume 2 ml. After reduction of the hydroperoxy compounds formed with sodium borohydride, lipid extraction, and alkaline hydrolysis, the extract was analyzed by RP-HPLC (see Material and Methods). The absorbances at 210 nm (lower panel, detection of non-oxygenated polyenoic fatty acids) and 235 nm (upper panel, detection of oxygenated polyenoic fatty acids) were recorded simultaneously. As an internal standard 15S-hydroxy-11Z,13E-eicosadienoic acid was used. Inset: Ultraviolet spectrum of the hydroxy fatty acids. The chemical structure of the compounds was deduced from coinjections with authentic standards. DA, docosahexaenoic acid; AA, arachidonic acid; LA, linoleic acid.

polyenoic fatty acids were detected. RP-HPLC of the non-hydrolyzed lipid extracts and comparison with the hydrolyzed samples indicated that more than 85% of the oxygenation products were located in the ester lipid fraction. The non-oxygenated polyenoic fatty acids were analyzed at 210 nm (Fig. 1, lower trace). It can be seen that linoleic acid, a major polyenoic fatty acid in LDL, is baseline-separated from arachidonic acid and from docosahexaenoic acid. On the other hand, docosahexaenoic acid is not well separated from arachidonic acid. Quantification of the chromatograms at 235 nm and 210 nm allowed the calculation of the oxygenated polyenoic fatty acid/polyenoic fatty acid ratio which appears to be a suitable measure for the oxidative modification of the LDL lipids.

In **Fig. 2** the dependence of the oxygenation rate on the LDL concentration is shown for the rabbit 15-lipoxygenase. It can be seen that the amount of hydroxy fatty

[2]Under our chromatographic conditions, 13-HODE, 15-HETE, and 12-HETE had similar retention times. Thus the chemical structure of the oxygenation products, in particular the positional isomerism, could not be concluded from these data (see below).

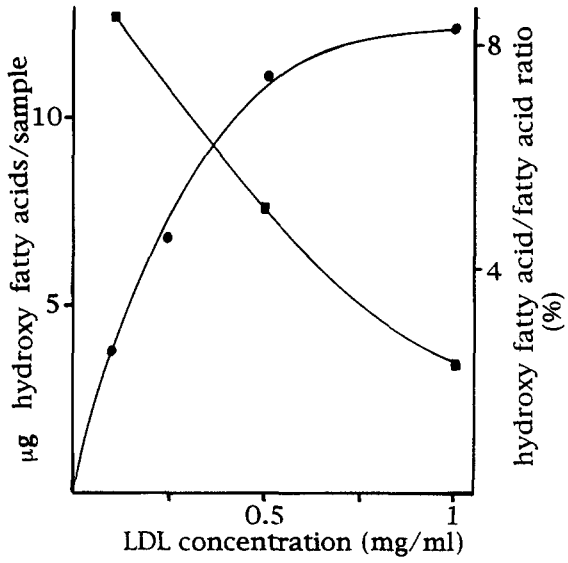

Fig. 2. Dependence of LDL oxygenation on substrate concentration. The pure rabbit reticulocyte lipoxygenase (4 nkat/ml arachidonate oxygenase activity) was incubated with various concentrations of LDL for 15 min at room temperature. After reduction, lipid extraction, and alkaline hydrolysis, the extracts were analyzed for hydroxy polyenoic fatty acids and non-oxygenated polyenoic fatty acids by RP-HPLC. Circles, amounts of hydroxy fatty acids formed/sample; squares, hydroxy fatty acid/fatty acid ratio (%).

acids formed during a 15-min incubation did increase at higher LDL concentrations. From the Lineweaver-Burk plot (not shown) an apparent K_m value of 0.3 mg LDL protein/ml and a V_{max} of 16.6 µg hydroxy fatty acids/15 min were calculated. For the porcine enzyme an apparent K_m of 1.1 mg LDL protein/ml and a V_{max} of 4.2 µg hydroxy fatty acids/15 min were obtained. These data suggest that LDL is a better substrate for the rabbit 15-lipoxygenase than for the porcine enzyme. Furthermore it can be seen from Fig. 2 that the hydroxy polyenoic fatty acid/polyenoic fatty acid ratio is lower at higher substrate concentrations. This may be explained by the fact that the lipoxygenase loading of the LDL particles (lipoxygenase/LDL ratio) is lower at higher LDL concentrations, which leads to a lower degree of oxidative modification of the single LDL particle. The lipoxygenase loading in the experiment shown in Fig. 2 varied between 0.8 nmol lipoxygenase/nmol apoB (0.8 nmol lipoxygenase/nmol LDL particle) and 8 nmol lipoxygenase/nmol apoB. Similar data were obtained for the porcine leukocyte 12-lipoxygenase (not shown).

For comparison of the LDL oxygenase activity of various lipoxygenases, the same arachidonate oxygenase activity of the various enzyme preparations was adjusted. After a 15-min incubation with human LDL, the hydrolyzed lipid extracts were analyzed by RP-HPLC and the oxygenated polyenoic fatty acids formed were quantified. The data shown in **Table 1** indicate that the rabbit 15-lipoxygenase was more effective in oxidizing LDL than the porcine leukocyte 12-lipoxygenase. The human leukocyte 5-lipoxygenase, the soybean lipoxygenase I, and the human platelet 12-lipoxygenase were less effective under identical conditions.

In additional experiments we investigated whether lipoxygenases are also capable of oxygenating other lipoproteins such as VLDL and HDL. For better comparison of the different lipoprotein classes, a comparable polyenoic fatty acid concentration (about 250 nmol/sample) was adjusted in the assay mixture. It can be seen from **Table 2** that both the rabbit reticulocyte 15-lipoxygenase and the porcine leukocyte 12-lipoxygenase are capable of oxygenating VLDL, LDL and HDL. The higher hydroxy polyenoic fatty acid/polyenoic fatty acid ratios suggest that LDL is the best substrate for the rabbit enzyme whereas HDL was preferred by the porcine lipoxygenase.

Structure elucidation of the oxygenation products

For more detailed information on the chemical structure of the oxygenation products and on the reaction specificity, the oxygenated fatty acids were prepared by

TABLE 1. LDL oxygenation by various lipoxygenases

Lipoxygenase	Amount	Arachidonate Oxygenase Activity Added/Sample	LDL Oxygenase Activity Assayed/Sample	Arachidonate Oxygenase Activity/LDL Oxygenase Activity Ratio
	µg protein/sample	*nmol oxygenated arachidonate/15 min*	*nmol oxygenated fatty acids/15 min*	
Rabbit reticulocytes	52	2636	10.0	264
Porcine leukocytes	2625	2530	4.7	538
Soybean I	13	2636	2.3	1146
Human platelets	7800	2636	1.7	1550
Human 5-lipoxygenase	54	2636	2.9	909

Human LDL (0.52 mg protein/ml) was incubated with various mammalian lipoxygenase preparations in 0.1 M phosphate buffer for 15 min at room temperature. After the incubation period, sodium borohydride was added to reduce the hydroperoxy lipids formed to the corresponding hydroxy derivatives and the lipids were extracted (18). The hydrolyzed lipid extracts (alkaline hydrolysis) were analyzed by RP-HPLC as described in Material and Methods, and the quantity of oxygenated polyenoic fatty acids formed is given in the table. The assay samples for arachidonate oxygenase activities are described in Material and Methods.

TABLE 2. Oxygenation of various lipoprotein classes by mammalian lipoxygenases

Lipoprotein Class	Degree of Oxygenation: Hydroxy Polyenoic Fatty Acid/Polyenoic Fatty Acid Ratio	
	Rabbit Reticulocyte 15-Lipoxygenase	Porcine Leukocyte 12-Lipoxygenase
	%	
VLDL	1.1	0.9
LDL	1.8	1.2
HDL	1.6	5.2

Human VLDL (236 nmol polyenoic fatty acids/sample), human LDL (255 nmol polyenoic fatty acids/sample), and human HDL (250 nmol polyenoic fatty acids/sample) were incubated with the rabbit reticulocyte 15-lipoxygenase (5.6 nkat/ml arachidonate oxygenase activity) and with the recombinant porcine leukocyte 12-lipoxygenase (5.6 nkat/ml arachidonate oxygenase activity) for 15 min at room temperature. Sample workup and RP-HPLC analysis as described in the legend of Fig. 2. The hydroxy polyenoic fatty acid/polyenoic fatty acid ratio was determined in the hydrolyzed lipid extracts as a measure for the oxidative modification of the lipoprotein lipids.

RP-HPLC and further analyzed by SP-HPLC, chiral phase-HPLC, and GC-MS (data not shown). From **Fig. 3** it can be seen that 13-HODE(Z,E) and 12-HETE were the major oxygenation products formed during the oxygenation of HDL by porcine leukocyte 12-lipoxygenase. Smaller amounts of 9-HODE(E,Z), 9-HODE(E,E), and 13-HODE(E,E) were also detected. Analysis of the enantiomer composition of the major oxygenation products indicated that both 12-HETE and 13-HODE were predominantly the S-isomer (inset to Fig. 1). For the rabbit reticulocyte 15-lipoxygenase, 13S-HODE and 15S-HETE were identified as major oxygenation products (**Table 3**). Furthermore, it can be seen from Table 3 that the same oxygenation products were formed during the oxygenation of all major lipoprotein classes. Quantitative differences in the product composition might be due to the different fatty acid composition of various lipoprotein classes and/or to differences in the apolipoprotein content of the particles. Chiral phase HPLC (data in parentheses in Table 3) indicated that the major reaction products (13-HODE and 12- and 15-HETE) are predominantly the S-isomer, whereas the minor products (9-HODE) are racemic mixtures.

SP-HPLC indicated that esterified hydroxy linoleate isomers are the major oxygenation products formed during LDL oxidation (Table 3). This is not surprising as linoleate is the major polyenoic fatty acid in lipoproteins. However, it should be stressed that the overall fatty acid composition of the lipoproteins may not be of major importance for the lipoxygenase reaction. The core lipids may be protected from the lipoxygenase attack because it appears unlikely that the enzyme penetrates the outer shelf of the particles to reach the core. In **Table 4** the HETE/arachidonate and HODE/linoleate ratios for the oxygenation of various lipoprotein classes by the rabbit and the porcine lipoxygenase are summarized. For LDL and HDL, the HODE/linoleate ratio is higher than the HETE/arachidonate ratio, suggesting that linoleate is a better substrate for both enzymes than arachidonate when presented in these lipoproteins. For VLDL, the HODE/linoleate and HETE/arachidonate ratios are close to each other.

It is remarkable that the product specificity of lipoprotein oxygenation by both the rabbit reticulocyte 15- and the porcine leukocyte 12-lipoxygenase is lower than that of the oxygenation of free polyenoic fatty acids. With linoleic acid or arachidonic acid as substrate, only very small amounts of racemic side products were detected (less than 5% of the total product formation). However, with lipoproteins the specific lipoxygenase products (13S-HODE and 12S- or 15S-HETE) were accompanied by a share of stereo-random oxygenation products [13R-HODE, 15R- or 12R-HETE, 9(R/S)-HODE(E,Z), 13-HODE(E,E),

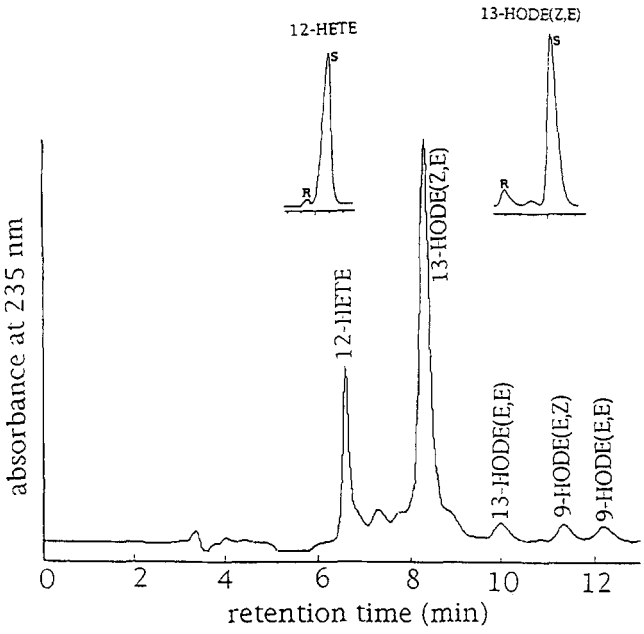

Fig. 3. SP-HPLC of hydroxy fatty acids obtained from human HDL treated with the recombinant porcine leukocyte 12-lipoxygenase. Human HDL (6.2 mg/ml) was oxygenated with the recombinant porcine leukocyte 12-lipoxygenase (9.3 nkat/ml arachidonate oxygenase activity) for 15 min at room temperature. Sample workup, lipid extraction, and RP-HPLC preparation of the hydroxy fatty acids were as described in the legend of Fig. 1. SP-HPLC analysis was carried out with the solvent system hexane–2-propanol–acetic acid 100:5:0.1 (v/v). The absorbance at 235 nm was recorded. The structure of the major oxygenation products was deduced from co-chromatography with authentic standards and was confirmed by GC-MS of the fatty acid methyl esters and their hydrogenated derivatives. Inset: The enantiomer composition of the major oxygenation products was determined by HPLC (R, R-isomer; S, S-isomer).

TABLE 3. Composition of the oxygenated polyenoic fatty acids formed during the oxidation of human VLDL, LDL, and HDL by mammalian lipoxygenases (LOX)

Lipoprotein	Lipoxygenase	12-HETE	15-HETE	13-HODE(Z,E)	13-HODE(E,E)	9-HODE(E,Z)	9-HODE(E,E)
				% of the sum of hydroxy fatty acids formed			
VLDL	rabbit 15-LOX	-	14	57 (79/21)	11	11	7
VLDL	porcine 12-LOX	21	–	53 (85/15)	10	9	7
LDL	rabbit 15-LOX	–	13	51 (77/23)	13	22	1
LDL	porcine 12-LOX	27 (77/23)	–	48 (70/30)	6 (48/52)	14	5
HDL	rabbit 15-LOX	–	31	43 (66/34)	9	16	1
HDL	porcine 12-LOX	28 (97/3)	–	62 (91/9)	4	4	2

Human VLDL (1 mg/ml), LDL (2.5 mg/ml), and HDL (5 mg/ml) were incubated with the rabbit reticulocyte 15-lipoxygenase (5 nkat/ml arachidonate oxygenase activity) and with the recombinant porcine leukocyte 12-lipoxygenase (5 nkat/ml arachidonate oxygenase activity) for 15 min at room temperature. Sample workup, RP-HPLC preparation of the hydroxy fatty acids, and SP-HPLC analysis as described in Material and Methods. The enantiomer composition of the major products (numbers in parentheses) was determined by chiral phase HPLC.

and 9-HODE(E,E)]. It is likely that the chemical composition of the substrate, particularly the lipid/protein interaction in the lipoproteins, may influence the substrate binding so that the lipoxygenase reaction is not completely controlled by the enzyme. A partial lack of steric control should lead to the formation of free radicals which subsequently may induce nonspecific oxygenation reactions. A similar share of stereo-random oxygenation products has been observed for the oxygenation of biomembranes by the rabbit 15-lipoxygenase (19).

As indicated above, only small amounts of free hydroxy fatty acids were detected after lipoxygenase-catalyzed lipoprotein oxygenation. Therefore, we investigated different ester lipid classes for the occurrence of oxygenation products. Analyzing the nonhydrolyzed lipid extracts of porcine leukocyte 12-lipoxygenase-treated LDL in a suitable HPLC system, compounds comigrating with an authentic standard of 13-HODE-cholesteryl ester were detected (**Fig. 4, upper trace**). Among all the peaks

TABLE 4. Hydroxy fatty acid/fatty acid ratios of various lipoprotein classes after lipoxygenase treatment

	HODE/Linoleate		HETE/Arachidonate	
Lipoprotein	Rabbit	Porcine	Rabbit	Porcine
	%		%	
VLDL	1.1	1.0	1.2	1.3
LDL	1.7	2.4	0.4	0.4
HDL	1.5	4.6	0.7	3.2

Incubation and lipid extraction were as described in Table 2. The amount of hydroxy polyenoic fatty acids formed and the linoleate and arachidonate content were determined by RP-HPLC. The HODE and HETE content was quantified by SP-HPLC.

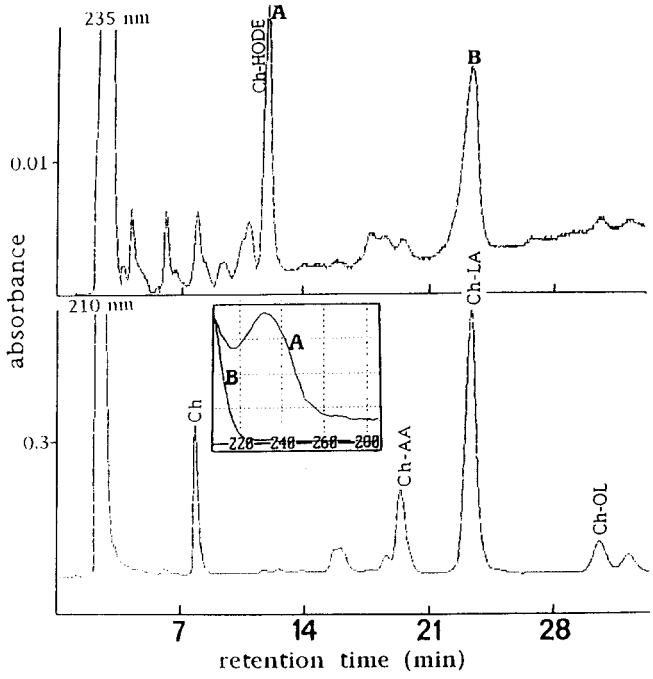

Fig. 4. Detection of oxygenated cholesterol esters in lipoxygenase treated LDL. The unhydrolyzed lipid extract of LDL treated with the recombinant porcine leukocyte 12-lipoxygenase (see legend to Fig. 1) was analyzed by SP-HPLC with a solvent system 2-propanol-acetonitrile 25:75 (v/v) and a flow rate of 1 ml/min. The temperature was kept at 45°C. The absorbances at 235 nm (upper panel, detection of oxygenated cholesteryl esters) and 210 nm (lower panel, detection of non-oxygenated cholesteryl esters) were recorded. Inset: Ultraviolet spectra of the major compounds absorbing at 235 nm were recorded at time points A and B. Ch-HODE, HODE-cholesteryl esters; Ch-AA, cholesteryl arachidonate; Ch-LA, cholesteryl linoleate; Ch-OL, cholesteryl oleate; Ch, free cholesterol. UV spectrum of the oxygenated cholesteryl esters, which is characterized by the conjugated dienes chromophore.

monitored at 235 nm, only that (peak A) comigrating with the cholesteryl-13-HODE standard was characterized by the conjugated dienes chromophore (inset to Fig. 4). Peak B, which does not show a conjugated diene chromophore, may be due to non-oxygenated cholesteryl linoleate which has a small absorbance at 235 nm. After alkaline hydrolysis of product A, free cholesterol and 13-HODE were detected in SP-HPLC indicating the chemical structure of this compound. The lower trace in Fig. 4 showed the analysis of the non-oxygenated cholesteryl esters of lipoxygenase-treated LDL. It can be seen that cholesteryl linoleate is the major cholesteryl ester in human LDL. It should be stressed that integration of the peak areas is not a direct measure for the amount of these compounds because their molar absorption coefficients are quite different. Quantification of the chromatogram and comparison with the RP-HPLC of the hydrolyzed lipid extracts indicated that about 60–70% of the oxygenation products were located in the cholesteryl ester fraction. As only small amounts (<5%) of free hydroxy fatty acids were detected, 30–40% of the oxygenation products should be located in the phospholipid fraction. SP-HPLC separation of the major phospholipid classes and UV spectroscopy with the diode array detector indicated the presence of oxygenated fatty acid in the major phospholipid classes, particularly in phosphatidylcholine and phosphatidylethanolamine (data not shown). For the rabbit enzyme, similar data were obtained.

Time course and temperature dependence of lipoprotein oxidation

It has been shown before (21, 22) that mammalian lipoxygenases undergo rapid suicidal inactivation during the oxygenation of free polyenoic fatty acids. This suicidal inactivation is much higher at 37°C as compared with 20°C. At 2°C it is largely suppressed (22). We observed that the rabbit reticulocyte 15- and the porcine leukocyte 12-lipoxygenases were almost completely inactivated after a 15-min incubation with arachidonic acid (data not shown). In contrast, the oxygenation of LDL by the porcine 12-lipoxygenase (**Fig. 5**) and by the rabbit 15-lipoxygenase (data not shown) did continue even after much longer incubation periods. However, the shape of the progress curve (Fig. 5) suggests a suicidal inactivation that is much slower than with free polyenoic fatty acids. Substrate exhaustion as a possible reason for the decrease in activity with time is unlikely as HPLC analysis indicated the presence of large amounts polyenoic fatty acids even after long-term incubations. The lack of rapid suicidal inactivation during LDL oxygenation may be of biological relevance as the lipoxygenase remains active for longer time periods, which may contribute to a more efficient oxidative modification of the LDL in vivo. Analysis of the product composition after long- and short-term incubation indicated no major differences in the product

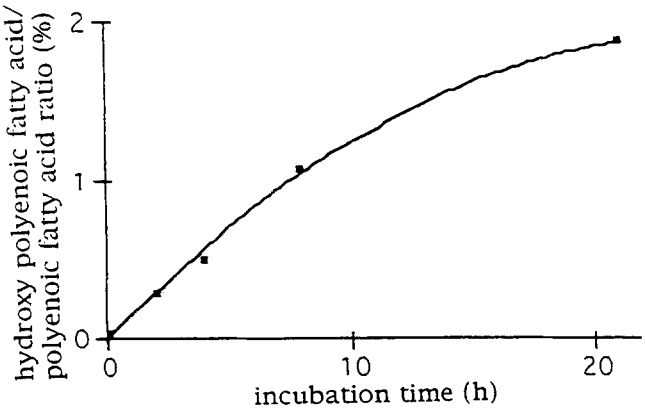

Fig. 5. Time-dependence of LDL oxygenation by the recombinant porcine leukocyte 12-lipoxygenase. Human LDL (2 mg/ml) was incubated with the recombinant porcine leukocyte 12-lipoxygenase (18.2 nkat arachidonate oxygenase activity/ml) at room temperature for the times indicated. The formation of oxygenated polyenoic fatty acids was analyzed by RP-HPLC as shown in Fig. 2. The degree of oxygenation of the LDL lipids (hydroxy fatty acid/fatty acid ratio) for each time point is given.

specificity or in the distribution of the oxygenation products among different lipid classes, in particular among phospholipids and cholesteryl esters.

The oxygenation of LDL by the rabbit reticulocyte 15-lipoxygenase shows an unusual temperature dependence. At 20°C more lipoxygenase products (115 nmol oxygenated polyenoic fatty acids/sample) were formed than at higher temperatures (68 nmol oxygenated polyenoic fatty acids/sample at 30°C and 58 nmol oxygenated polyenoic fatty acids/sample at 37°C). The reasons for this unusual behavior have not been clarified, but an increase in suicidal inactivation at higher temperatures and the thermolability of enzymes may contribute to this phenomenon. Furthermore, there may have been a more rapid decomposition of the hydroperoxy lipids that were formed.

Cooxidative modification of apolipoproteins

Oxidation of LDL by transition metals leads to an increase in electrophoretic mobility which was postulated to be due to the oxidation of lysine residues of apoB-100. Incubation of LDL with rabbit reticulocyte 15- and the porcine leukocyte 12-lipoxygenase did also lead to an increase in electrophoretic mobility. It can be seen from **Fig. 6** that the shift in electrophoretic mobility was higher with the rabbit lipoxygenase as compared with the porcine enzyme when comparable amounts of lipoxygenase were added. This result is in line with the fact that LDL is a better substrate for the rabbit reticulocyte lipoxygenase (Table 1). It is of mechanistic importance that changes in electrophoretic mobility were only detected with incubation times longer than 2 h. It appears likely that secondary

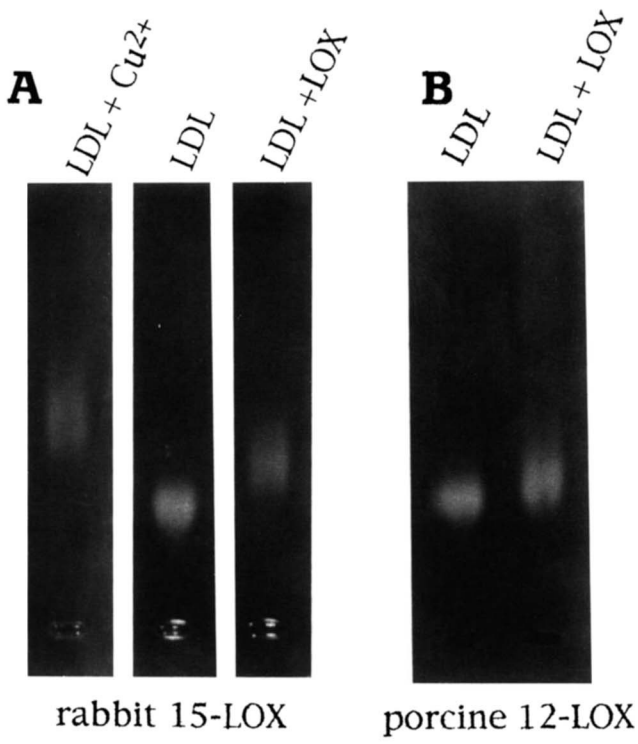

Fig. 6. Changes in electrophoretic mobility of LDL after lipoxygenase treatment. A) Human LDL (0.5 mg/ml) was incubated with the rabbit reticulocyte 15-lipoxygenase (11 nkat/ml arachidonate oxygenase activity) and with 30 μM $CuCl_2$ for 20 h at room temperature. Aliquots of the samples were directly applied to lipidophor electrophoresis system (2-h electrophoresis). For the copper-catalyzed oxidation, EDTA was removed from the LDL preparation by gel filtration prior to incubation. For the lipoxygenase-catalyzed reactions, the EDTA concentration in the incubation mixtures was 1 mM. B) Human LDL (0.5 mg/ml) was incubated with the recombinant porcine leukocyte 12-lipoxygenase (9.5 nkat/ml arachidonate oxygenase activity) for 20 h at room temperature. Aliquots of the samples were directly applied to lipidophor electrophoresis system (3-h electrophoresis).

decomposition of the hydroperoxy lipids formed by the enzyme via radical mediated hydroperoxidase reactions (23, 24) may be involved in this process. A similar combination of oxygenase and hydroperoxidase reaction has been proposed to be responsible for the changes in the passive electric properties of biomembranes after lipoxygenase treatment (25). To obtain experimental evidence for this hypothesis, we analyzed the lipid extracts of porcine leukocyte 12-lipoxygenase-treated LDL for the occurrence of ketodienoic fatty acids as indicator for hydroperoxidase reactions. The keto fatty acids cochromatograph with the hydroxy fatty acids under our RP-HPLC conditions, but can be differentiated from the hydroxy compounds by their UV spectrum. The hydroxy fatty acids with their conjugated diene chromophore have an absorbance maximum at 235 nm whereas the keto fatty acids absorb at 270 nm. From **Fig. 7** it can be seen that the hydroxy fatty acid/keto fatty acid ratio did increase during the time course of the reaction indicating a time-dependent increase in hydroperoxidase product formation. Ketodienes are proposed to be formed from hydroperoxy lipids via homolytic cleavage of the O–O bond of the hydroperoxide forming an alkoxy and a hydroxy radical (23). These radical intermediates may induce cooxidative modification of amino acid residues of the apolipoproteins, thus leading to an increase in electrophoretic mobility.

DISCUSSION

Rabbit and human reticulocyte 15-lipoxygenases (10) as well as the 12-lipoxygenase from porcine leukocytes are capable of oxygenating the major human lipoprotein classes without the preceding action of a lipid-cleaving enzyme such as phospholipase and/or cholesterol esterase. The human platelet 12-lipoxygenase, the human leukocyte 5-lipoxygenase, and the soybean I lipoxygenase are less effective. These data are in line with the earlier observations that the mammalian 15-lipoxygenases (19) and the leukocyte type 12-lipoxygenase oxygenate pure phospholipids and biomembranes whereas the platelet type 12-lipoxygenase does not react with complex substrates (26). The soybean lipoxygenase I has been reported to oxidize phospholipids only in the presence of detergents (27) and effectively modifies LDL in combination with phospholipase A_2 (8). It is, however, much less effective without lipid-cleaving enzymes. We detected small amounts of oxygenation products after the incubation of LDL with the soybean lipoxygenase I, with the human platelet 12- and the recombinant human 5-lipoxygenase. These data were somewhat surprising as these enzymes do not effectively oxygenate esterified substrates. Because we analyzed the hydrolyzed lipid extracts for these enzymes, it might be possible that the oxygenation products originate, in part, from the free polyenoic fatty acids present in the LDL. The capability of the porcine leukocyte 12-lipoxygenase to oxygenate lipoproteins is further evidence for the close relation of this enzyme to the mammalian 15-lipoxygenases. Nucleotide sequence data, amino acid homology, genomic structure, as well as enzymatic properties (28, 29) indicate that the porcine 12-lipoxygenase is evolutionarily more closely related to the rabbit and human 15-lipoxygenases than to the human platelet type 12-lipoxygenase (30).

The reactivity of mammalian lipoxygenases with lipoproteins is much lower than with free polyenoic fatty acids. As indicated in Table 1, arachidonic acid oxygenase/LDL oxygenase ratios of various lipoxygenases vary between about 1:250 and 1:1600. In other words, for comparable product formation from LDL, 100 times as much lipoxygenase is necessary as for arachidonate oxy-

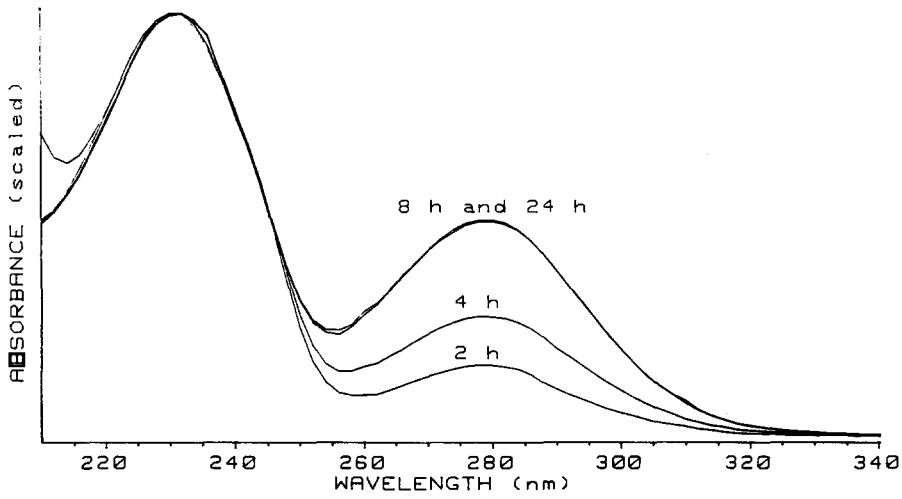

Fig. 7. Formation of hydroperoxidase products (ketodienes) during LDL oxidation by the recombinant porcine leukocyte 12-lipoxygenase. Human LDL (2.0 mg/ml) was incubated with the recombinant porcine leukocyte 12-lipoxygenase (18.2 nkat arachidonate oxygenase activity) at room temperature for the times indicated. The formation of oxygenated polyenoic fatty acids was analyzed by RP-HPLC as shown in Fig. 1. The UV spectra of the oxygenated fatty acids eluting at 5.32 min indicate the presence of conjugated dienes (oxygenase products) and conjugated ketodienes (hydroperoxidase products).

genation. As the concentration of free polyenoic fatty acids in LDL is very low, they may not serve as lipoxygenase substrate to a sizeable extent in our in vitro system in which no lipid-cleaving enzyme is present. However, in atherosclerotic lesions, the situation might be different. Here lipid-cleaving enzymes may hydrolyze the LDL ester lipids leading to an increase in the free polyenoic fatty acid content of the LDL. These free fatty acids are preferentially oxygenated by the lipoxygenase forming free hydroperoxy fatty acids.

Despite these low oxygenation rates, the rabbit and the porcine lipoxygenases are capable of converting LDL into its atherogenic form during long-term incubations. The degree of oxidative modification of LDL depends on the lipoxygenase loading of the LDL particle or on the molar lipoxygenase/apoB ratio and on the incubation time. For the experiment shown in Table 1, the lipoxygenase loading of the LDL particle by the porcine 12-lipoxygenase can be calculated as follows. The pure porcine 12-lipoxygenase (data not shown) exhibits a specific arachidonate oxygenase activity of 4.5 μmol 12-HETE/mg enzyme min (1 ml assay volume). In other words, 1 nmol of 12-lipoxygenase produces 338 nmol of 12-HETE/min. The amount of porcine 12-lipoxygenase we added to the incubation mixture (Table 1) exhibited an arachidonate oxygenase activity of 960 nmol/15 min or 64 nmol/min. This corresponds to 0.2 nmol of the pure enzyme. As the assay sample contained 0.5 nmol of LDL particles, a molar lipoxygenase/apoB ratio of 1:2.5 can be calculated.

As discussed before for the oxygenation of biomembranes (19), the lipoxygenase-catalyzed oxidative modification of LDL is a biphasic process. During the initial phase (the oxygenase reaction) esterified polyenoic fatty acids are converted to their corresponding hydroperoxy derivatives. This process appears to be restricted to the lipid compartment but does not affect the apolipoproteins. These LDL species containing a modified lipid compartment but an unmodified apoB may be regarded as minimally modified LDL (no increased electrophoretic mobility). During the second phase, the hydroperoxidase reaction, the unstable hydroperoxy lipids may be broken down via free radical-mediated secondary reactions. The hydroperoxidase reactions may be catalyzed in vivo by special enzymes, by transition metals, but also by the lipoxygenases themselves. Under certain conditions such as reduced oxygen tension or lack of oxygenase substrate pure lipoxygenases have been shown to exhibit a hydroperoxidase activity (31, 32). The radical intermediates formed during the secondary decomposition of the hydroperoxy lipids may induce the cooxidative modification of the apolipoproteins leading to an increase in electrophoretic mobility, thus converting minimally oxidized LDL into its completely oxidized form.

The susceptibility of LDL towards lipoxygenase-catalyzed oxidation and the fatty acid composition of the LDL varied when lipoprotein preparations obtained from different donors were used as substrate. Thus, it is impossible to compare the data of experiments carried out with different lipoprotein preparations. The reasons for these interindividual differences have not been investigated in detail, but it appears likely that nutritional differences may contribute to the different fatty acid composition and

to variations in the antioxidative capacity of the LDL. A similar phenomenon has been reported for the copper-catalyzed reaction (33).

The role of 15-lipoxygenase in the pathogenesis of atherosclerosis is not yet clarified. The high level expression of the enzyme in atherosclerotic lesions but its absence in normal vessel wall (3, 4) suggests its involvement in atherogenesis. The colocalization of this lipid-oxidizing enzyme with oxidatively modified lipoproteins in the lesion suggests that this enzyme is implicated in the oxidative modification of LDL. However, for verification of this hypothesis one has to answer the question: how is the intracellular lipoxygenase capable of oxidizing extracellular LDL, rendering it prone for binding to the scavenger receptor. In principle, two mechanisms may be discussed. *i)* There may be release of lipoxygenase from foamy macrophages into the extracellular space where the enzyme may catalyze a direct oxygenation of LDL. Despite the fact that there are no indications for an active transport of the intracellular enzyme across the cell membrane, there is immuno-electron-microscopic evidence for an extracellular localization of the enzyme in human liver (H. Robenek, unpublished data). *ii)* There may be intracellular action of the lipoxygenase on cellular membranes and subsequent release of reactive lipid hydroperoxides into the extracellular space where they may induce non-enzymatic oxidative modification of the LDL. The oxygenation of membrane phospholipids by a lipoxygenase has been reported before in rabbit reticulocytes (34) and IL-4-treated human peripheral monocytes (35).

The implication of 15-lipoxygenase in atherogenesis may not be limited to oxidative modification of LDL. Alternatively, the enzyme may play a role in the inflammatory reaction in the atherosclerotic lesion. The specific induction of a 15-lipoxygenase in monocytes by the pro-inflammatory cytokine IL 4 (35) supports this hypothesis. As inflammation may be regarded as a defense mechanism against endothelial injury in the early stages of plaque development, the intracellular action of the lipoxygenase might be involved in a rather beneficial process in early stages of the plaque formation. In later stages, however, the intracellular lipoxygenase may be released from the foamy macrophages and then may oxidize extracellular LDL. This oxidation converts the lipoprotein into its atherogenic form, but may also lead to the formation of free radicals. The share of stereo-random oxygenation products described here suggests the formation of free radicals during lipoxygenase-lipoprotein interaction.

The authors wish to thank Drs. D. Riendeau and A. Ford-Hutchinson for the kind gift of the recombinant human leukocyte 5-lipoxygenase. This work was supported in part by a grant from Bayer AG to H. K., and a grant from EC (PL93-1790).

Manuscript received 17 August 1993, in revised form 16 December 1993, and in re-revised form 10 March 1994.

REFERENCES

1. Steinberg, D., and J. L. Witztum. 1990. Lipoproteins and atherogenesis. Current concepts. *J. Am. Med. Assoc.* **246:** 3047–3052.
2. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol. Modification of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
3. Ylä-Herttuala, S., M. E. Rosenfeld, S. Parthasarathy, E. Sigal, T. Särkioia, J. L. Witztum, and D. Steinberg. 1991. Gene expression in macrophage-rich human atherosclerotic lesions. *J. Clin. Invest.* **87:** 1146–1152.
4. Ylä-Herttuala, S., M. E. Rosenfeld, S. Parthasarathy, C. K. Glass, E. Sigal, J. L. Witztum, and D. Steinberg. 1990. Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* **87:** 6959–6963.
5. Parthasarathy, S., E. Wieland, and D. Steinberg. 1989. A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **86:** 1046–1050.
6. McNally, A. K., G. M. Chisolm, M. T. Morel, and M. K. Cathcart. 1990. Activated human monocytes oxidize low-density lipoprotein by a lipoxygenase-dependent pathway. *J. Immunol.* **145:** 254–259.
7. Sparrow, C. P., and J. Olszewski. 1992. Cellular oxidative modification of low density lipoprotein does not require lipoxygenase. *Proc. Natl. Acad. Sci. USA.* **89:** 128–131.
8. Sparrow, C. P., S. Parthasarathy, and D. Steinberg. 1988. Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase A_2 mimics cell-mediated oxidative modification. *J. Lipid Res.* **29:** 745–753.
9. Cathcart, M. K., A. K. McNally, and G. M. Chisolm. 1991. Lipoxygenase mediated transformation of human low density lipoprotein to an oxidized cytotoxic complex. *J. Lipid Res.* **32:** 63–70.
10. Belkner, J., R. Wiesner, J. Rathman, J. Barnett, E. Sigal, and H. Kühn. 1993. Oxygenation of lipoproteins by mammalian lipoxygenases. *Eur. J. Biochem.* **213:** 251–261.
11. Yoshimoto, T., H. Suzuki, S. Yamamoto, T. Takai, C. Yokoyama, and T. Tanabe. 1990. Cloning and expression of arachidonate 12-lipoxygenase cDNA from porcine leukocytes. *Adv. Prostaglandin Thromboxane Leukotriene Res.* **21A:** 29–32.
12. Yokoyama, C., F. Shinjo, T. Yoshimoto, S. Yamamoto, J. A. Oates, and A. R. Brash. 1986. Arachidonate 12-lipoxygenase purified from porcine leukocytes by immunoaffinity chromatography and its reactivity with hydroperoxyeicosatetraenoic acids. *J. Biol. Chem.* **261:** 16714–16721.
13. Hada, T., N. Ueda, Y. Takahashi, and S. Yamamoto. 1991. Catalytic properties of human platelet 12-lipoxygenase as compared with the enzymes of other origins. *Biochim. Biophys. Acta.* **1083:** 89–93.
14. Havel, R. J., H. A. Eder, and H. J. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
15. Peers, K. E., and D. T. Coxon. 1983. Controlled synthesis of monohydroperoxy fatty acids by α-tocopherol. *Chem. Phys. Lipids.* **32:** 49–56.
16. Iacazio, G., G. Langrand, J. Baratti, G. Bouno, and C. Triantaphylides. 1990. Preparative, enzymatic synthesis of linoleic acid (13S)-hydroperoxide using soybean lipoxygenase I. *J. Org. Chem.* **55:** 1690–1691.

17. Belkner, J., R. Wiesner, and H. Kühn. 1991. The oxygenation of cholesterol esters by the reticulocyte lipoxygenase. *FEBS Lett.* **279:** 110–114.
18. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Biophysiol.* **37:** 911–917.
19. Kühn, H., J. Belkner, R. Wiesner, and A. R. Brash. 1990. Oxygenation of biological membranes by the pure reticulocyte lipoxygenase. *J. Biol. Chem.* **265:** 18351–18361.
20. Percival, M. D., D. Denis, D. Riendeau, and M. J. Gresser. 1992. Investigation of the mechanism of non-turnover-dependent inactivation of purified human 5-lipoxygenase. *Eur. J. Biochem.* **210:** 109–117.
21. Schewe, T., S. M. Rapoport, and H. Kühn. 1986. Enzymology and physiology of reticulocyte lipoxygenase. *Adv. Enzymol.* **58:** 191–271.
22. Härtel, B., P. Ludwig, T. Schewe, and S. M. Rapoport. 1982. Self-inactivation by 13-hydroperoxylinoleic acid and lipohydroperoxidase activity of the reticulocyte lipoxygenase. *Eur. J. Biochem.* **126:** 353–357.
23. Frankel, E. N. 1991. Recent advances in lipid oxidation. *J. Sci. Food Agric.* **54:** 495–511.
24. Gardner, H. W., and P. A. Jursinic. 1981. Degradation of linoleic acid hydroperoxides by a cysteine $FeCl_3$ catalyst as a model for similar biological reactions. *Biochim. Biophys. Acta.* **665:** 100–112.
25. Kühn, H., F. Pliquett, S. Wunderlich, T. Schewe, and W. Krause. 1983. Reticulocyte lipoxygenase changes the passive electric properties of bovine submitochondrial particles. *Biochim. Biophys. Acta.* **735:** 283–290.
26. Takahashi, Y., W. C. Glasgow, H. Suzuki, Y. Taketani, S. Yamamoto, M. Anton, H. Kühn, and A. R. Brash. 1993. Investigation of the oxygenation of phospholipids by the porcine leukocyte and human platelet arachidonate 12-lipoxygenase. *Eur. J. Biochem.* **218:** 165–171.
27. Brash, A. R., C. D. Ingram, and T. M. Harris. 1987. Analysis of a specific reaction of soybean lipoxygenase I with fatty acids esterified in phospholipids. *Biochemistry.* **26:** 5465–5471.
28. Yoshimoto, T., H. Suzuki, S. Yamamoto, T. Takai, C. Yokoyama, and T. Tanabe. 1990. Cloning and sequence analysis of cDNA for the arachidonate 12-lipoxygenase of porcine leukocytes. *Proc. Natl. Acad. Sci. USA.* **87:** 2142–2146.
29. Yamamoto, S. 1992. Mammalian lipoxygenases: molecular structures and functions. *Biochim. Biophys. Acta.* **1128:** 117–131.
30. Toh, H., C. Yokoyama, T. Tanabe, T. Yoshimoto, and S. Yamamoto. 1992. Molecular evolution of cyclooxygenases and lipoxygenases. *Prostaglandins.* **44:** 291–315.
31. Gardner, H. W. 1992. Recent investigations into the lipoxygenase pathway in plants. *Biochim. Biophys. Acta.* **1084:** 221–239.
32. Kühn, H., U. Salzmann-Reinhardt, P. Ludwig, K. Pönicke, T. Schewe, and S. M. Rapoport. 1986. The stoichiometry between oxygen uptake and conjugated diene formation during the oxygenation of linoleic acid by the pure reticulocyte lipoxygenase. *Biochim. Biophys. Acta.* **876:** 187–193.
33. Esterbauer, H., M. Diebner-Rothenerder, G. Waeg, G. Striegl, and G. Jürgens. 1990. Biochemical, structural, and functional properties of oxidized low density lipoproteins. *Chem. Res. Toxicol.* **3:** 77–92.
34. Kühn, H., and A. R. Brash. 1990. The occurrence of lipoxygenase products in membranes of rabbit reticulocytes. *J. Biol. Chem.* **265:** 1454–1458.
35. Conrad, D. J., H. Kühn, M. Mulkins, E. Highland, and E. Sigal. 1992. Specific inflammatory cytokines regulate the expression of human monocyte 15-lipoxygenase. *Proc. Natl. Acad. Sci. USA.* **89:** 217–221.