Involvement of 15-lipoxygenase in Early Stages of Atherogenesis

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Summary

The arachidonate 15-lipoxygenase which is expressed in atherosclerotic lesions is implicated in the oxidative modification of low density lipoproteins during atherogenesis. To obtain experimental in vivo evidence for this hypothesis, we analyzed the structure of oxygenated lipids isolated from the aorta of rabbits fed with a cholesterol-rich diet for different time periods and compared the pattern of oxygenation products with that isolated from low density lipoproteins treated in vitro with the pure rabbit 15-lipoxygenase and with oxygenated lipids isolated from advanced human atherosclerotic lesions. In early atherosclerotic lesions (12-wk cholesterol feeding), specific lipoxygenase products were detected whose structure was similar to those isolated from lipoxygenase-treated low density lipoproteins. The appearance of these products did coincide with the lipid deposition in the vessel wall. In later stages of atherogenesis (26-wk cholesterol feeding) the degree of oxidative modification of the tissue lipids did increase but the share of specific lipoxygenase products was significantly lower, suggesting an increasing overlay of the specific lipoxygenase products by nonenzymatic lipid peroxidation. In advanced human atherosclerotic lesions, large amounts of oxygenation products were detected whose structure suggests a nonenzymatic origin. These data suggest that the arachidonate 15-lipoxygenase is of pathophysiological importance during the early stages of atherogenesis. In later stages of plaque development nonenzymatic lipid peroxidation becomes more relevant.

Atherosclerosis is a multifactorial disease with great socioeconomic impact. Although a substantial body of research has been carried out during the last decades, the molecular mechanisms of atherogenesis are not fully understood (1, 2). The development of atherosclerotic lesions, which is characterized by inflammatory and hyperproliferative processes as well as by lipid deposition, may be regarded as a protective response to an injury of the vessel wall (1). A variety of growth factors, cytokines, adhesion molecules, and vasoactive substances are implicated in plaque development. A key process in atherogenesis appears to be the development of lipid-loaded foam cells that accumulate in the subendothelial space to form fatty streaks, that are generally accepted as early atherosclerotic lesions (3, 4). Foam cells may develop from peripheral monocytes/macrophages or from smooth muscle cells by taking up modified low density lipoproteins (LDL)[1] via scavenger receptor–mediated pathways (5, 6). The chemical nature of the modifying processes in vivo is not completely understood. However, the occurrence of oxidatively modified lipoproteins in atherosclerotic lesions (7, 8) and the fact that oxidatively modified LDL is taken up rapidly by macrophages (9) suggested an involvement of oxidative processes in foam cell formation. Copper ions effectively oxidize LDL into its atherogenic form (10, 11). The oxidative modification of LDL in various cellular systems (12–14) was prevented by nonspecific lipoxygenase inhibitors, suggesting a role for the 15-lipoxygenase pathway. This hypothesis was supported by the immunohistochemical detection of the 15-lipoxygenase in atherosclerotic lesions of rabbits and humans (15, 16). Ex vivo studies indicated that the lipoxygenase was enzymatically active in the lesion (17, 18). Further evidence for the implication of the 15-lipoxygenase in atherogenesis was provided by the recent finding that the purified enzyme in vitro is capable of oxidatively modifying human LDL into its atherogenic form (19, 20). On the other hand, more quantitative inhibitor studies indicated that rabbit aortic endothelial cells and mouse peritoneal macrophages are capable of oxidizing LDL

[1] *Abbreviations used in this paper:* CP-HPLC, chiral phase HPLC; GC/MS, gas chromatography/mass spectrometry; HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; LDL, low density lipoprotein; NZW, New Zealand White; RP-HPLC, reverse phase HPLC; SP-HPLC, straight phase HPLC.

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even after blockage of the lipoxygenase pathway (21), which suggests that cellular lipoxygenases are not essential for oxidative modification of LDL. However, these in vitro data do not exclude an implication of the 15-lipoxygenase in the development of atherosclerotic lesions in vivo. The high expression level of this enzyme in lesions of animal atherosclerosis models (15) and humans (16) and its absence in normal vessel wall suggest its involvement in atherogenesis. Hydroxy fatty acids have been detected in the atherosclerotic tissue of humans (22, 23) and rabbits (24) but their exact chemical structure and the mechanism of biosynthesis has not been studied.

Investigations on the in vivo action of the 15-lipoxygenase have been hampered by the lack of specific 15-lipoxygenase inhibitors and of transgenic animals. There is, however, another approach for this problem. In biological samples lipoxygenases leave behind a specific product pattern that can be differentiated from unspecific nonenzymatic oxygenation products. Thus, we analyzed the lipid extracts of the aorta of cholesterol-fed rabbits for the occurrence of specific lipoxygenase products during the time course of plaque development and compared the product pattern with that of the in vitro interaction of the isolated 15-lipoxygenase with human LDL. These experiments, as well as the data on the lipid composition of advanced human atherosclerotic lesions, suggest that the 15-lipoxygenase is of pathophysiological importance in early atherogenesis, whereas nonenzymatic lipid peroxidation is more prominent in later stages.

Materials and Methods

Chemicals

The chemicals used were from the following sources: 9Z,12Z-octadecadienoic acid (linoleic acid), 5Z,8Z,11Z,14Z-eicosatetraenoic acid (arachidonic acid), (D/L)-α-tocopherol, soybean lipoxygenase (grade 1), cholesteryl linoleate, and cholesteryl arachidonate from Serva (Heidelberg, FRG). All solvents used (Serva) were of HPLC grade.

Preparations

Human LDL was obtained by sequential floating ultracentrifugation in a sodium bromide density gradient (25). The LDL preparation was dialyzed against isotonic sodium chloride solution containing 3 mM EDTA. Rabbit reticulocyte lipoxygenase and the recombinant human 15-lipoxygenase were prepared as described before (19, 20). Authentic HPLC standards of racemic 13-hydroxy-9Z,11E-octadecadienoic acid (13-HODE) and 9-hydroxy-10E,12Z-octadecadienoic acid (9-HODE) were prepared by vitamin E–controlled autoxidation of fatty acid methyl esters (26). After triphenylphosphine reduction and alkaline hydrolysis, the resulting free hydroxy fatty acid isomers were separated by straight phase (SP) HPLC. Chiral standards of 13S-HODE and 15S-HETE (15S hydroxy-5Z,8Z,11Z,13E-eicosatetraenoic acid) were obtained by enzymatic oxygenation (27) of linoleic acid and arachidonic acid, respectively, followed by HPLC preparation of the major oxygenation products. The chemical structure of all reference compounds prepared was confirmed by UV spectroscopy, HPLC including chiral phase (CP) HPLC, and gas chromatography/mass spectrometry (GC/MS) of the trimethylsilyl ethers and their hydrogenated derivatives.

LDL Oxygenation. Human LDL was incubated with the rabbit 15-lipoxygenase or with the recombinant human 15-lipoxygenase in 0.1 M phosphate buffer, pH 7.4 (assay volume 2 ml), for 15 min. The final EDTA concentration in the incubation mixture was adjusted to 1 mM. The reaction was stopped by the addition of sodium borohydride to reduce the hydroperoxy compounds to their corresponding alcohols. 15S-hydroxy-11Z,13E-eicosadienoic acid was added as an internal standard and the lipids were extracted according to the method of Bligh and Dyer (28). After evaporation of the solvents, the lipids were reconstituted in 1 ml of methanol/chloroform (8:2 by vol) and an aliquot (150 μl) was analyzed by reverse phase (RP) HPLC for oxygenated cholesterol esters and for free cholesterol content. 150 μl of 40% KOH was added to the remaining lipid extracts and the lipids were hydrolyzed for 30 min at 60°C under argon atmosphere. As shown earlier, this method completely hydrolyzes the ester lipids and does not lead to a significant formation of autoxidation products (19).

Animal Experiments

Cholesterol Feeding. Male New Zealand White (NZW) rabbits (3.0 ± 0.2 kg body weight) were purchased from Interfauna U.K. Ltd. (Huntington, UK) and housed individually in plastic wire-bottomed cages according to European guidelines for the caging of small rodents and rabbits. The animals were fed a standard chow diet (Sniff Spezialdiäten GmbH, Soest, FRG) and water ad libidum. For the cholesterol feeding experiments, this diet was supplemented with 1% pure cholesterol (cholesterol-rich diet). The animals were categorized into four groups: group 1, 5 rabbits fed for 12 wk with the standard diet (control group); group 2, 10 rabbits fed for 6 wk with the cholesterol-enriched standard diet; group 3, 13 rabbits fed for 12 wk with the cholesterol-enriched standard diet (included two series of experiments, a pilot one with 3 rabbits, a second one with 10 rabbits); and group 4, 9 rabbits fed for 26 wk with the cholesterol-enriched standard diet.

Watanabe Rabbits. 14-wk-old Watanabe rabbits were purchased from Froxfield Farms Ltd. (Hampshire, UK) and fed with a standard diet for 280 d (40 wk) under the same conditions as the NZW rabbits.

Blood and Tissue Samples. For the determination of the serum cholesterol concentration, blood was withdrawn from the ear vein and the cholesterol content was determined by the CHOD-PAP method (Boehringer Mannheim GmbH Diagnostica, Mannheim, FRG). The animals were killed by an overdose injection of phenobarbital; the aortas were removed, shock frozen in liquid nitrogen, and stored at −80°C or in liquid nitrogen until sample work-up.

Human Atherosclerotic Tissue

Samples of human atherosclerotic lesions were removed during thrombaterectomy or endaterectomy because of clinical indications. The tissue samples were shock frozen and stored on dry ice or in liquid nitrogen. Sample work-up was carried out as described for the animal material.

Workup of the Tissue Samples

After thawing of the tissue samples, areas with macroscopically visible atherosclerotic alterations (fatty streaks and advanced plaques) were prepared. Typically, samples of 200–1,000 mg wet weight were prepared and the lipids were extracted (28). Briefly, the tissue samples were homogenized in 4.75 ml of a mixture of PBS/methanol/chloroform (1:2.5:1.25 by vol) with an Ultraturrax microhomogenizer (Janke-Kunkel GmbH & Co. KG, Staufen, FRG) three times for 30 s with a 2-min break between the homogenization steps. To

avoid artificial lipid peroxidation during the work-up procedure, homogenization was carried out under argon atmosphere on ice. After addition of 15S-hydroxy-11Z,13E-eicosadienoic acid as an internal standard, the homogenate was kept on ice for 30 min. 1.25 ml of water and 1.25 ml of chloroform were then added, and the mixture was vortexed for 60 s and centrifuged for phase separation for 10 min at 10,000 *g*. The lower chloroform phase which contained the total tissue lipids was recovered, the solvent was evaporated, the lipids were reconstituted in 1 ml of a mixture of chloroform/methanol (2:8, by vol), and aliquots (150 μl) were analyzed for oxidized cholesterol esters and for the free cholesterol content by RP-HPLC. The remaining extracts were subjected to alkaline hydrolysis (addition of 0.15 ml of 40% KOH and incubation for 30 min at 60°C under argon atmosphere). Afterwards, the samples were acidified by the addition of 0.1 ml of glacial acetic acid and aliquots were directly injected to RP-HPLC for the determination of the degree of oxidation of the tissue lipids.

Analytics

HPLC was carried out on a Shimadzu instrument (Tokyo, Japan) coupled with a diode array detector (model 1040 A; Hewlett-Packard Co., Palo Alto, CA). The lipoxygenase reaction differs from the nonenzymatic lipid peroxidation with respect to its high reaction specificity. This specificity can be quantified by various methods the most suitable of which appears to be the determination of the enantiomer composition (S-isomer/R-isomer ratio [28a]) of the major oxygenation product (13-hydroxy-9Z,11E-octadecadienoic acid). The lipoxygenase reaction is characterized by a strong preponderance of the S-isomer whereas nonenzymatic reactions lead to a racemic mixture (S/R-ratio, 1:1). According to our experience with post-mortem samples of advanced human atherosclerotic lesions (23) five consecutive steps of HPLC are necessary (Table 1) in order to analyze the enantiomer composition of the 13-hydroxy-9Z,11E-octadecadienoic acid present in the lesions. By analytic RP-HPLC (step 1) the degree of oxidative modification of the tissue lipids (hydroxy polyenoic fatty acid/polyenoic fatty acid ratio) was determined by simultaneous measurement of the concentration of hydroxy polyenoic fatty acids and nonoxygenated polyenoic fatty acids in the hydrolyzed lipid extracts. As step 2 of the HPLC protocol, a preparative RP-HPLC was introduced to prepare the oxygenated polyenoic fatty acids which constitute a mixture of various positional and geometric isomers of hydroxy and keto polyenoic fatty acids. By analytic SP-HPLC (step 3) this mixture was separated into its constituents and the percentage of the 13-HODE, which was shown to be the major oxygenation product, was determined. This product was prepared by SP-HPLC (step 4) and subsequently analyzed for its enantiomer composition by CP-HPLC (step 5). RP-HPLC was performed on a Nucleosil C-18 column (Macherey/Nagel, Düren, FRG, KS-system, 250 × 4 mm, 5-μm particle size). A solvent system of methanol/water/acetic acid (82:18:0.1 by vol) and the flow rate of 1 ml/min were used. The fractions containing the oxygenated polyenoic fatty acids were collected, the solvent was evaporated, the residue was reconstituted in a mixture of *n*-hexane/2-propanol/acetic acid (100:2:0.1 by vol) and injected for SP-HPLC purification of the 13-HODE(Z,E). SP-HPLC was carried out on a Zorbax SIL column (DuPont, Wilmington, DE; 250 × 4.6 mm, 5-μm particle size) with a solvent system of *n*-hexane/2-propanol/acetic acid (100:2:0.1 by vol) and a flow rate of 1 ml/min. Fractions containing 13-hydroxy-9Z,11E-octadecadienoic acid were collected, concentrated under vacuum, and the enantiomer composition was determined by CP-HPLC which was carried out on a Chiralcel OD column (Diacel Chem. Industries, Kyoto, Japan; 250 × 4.6 mm, 5-μm particle size) with a solvent system of hexane/2-propanol/acetic acid (100:5:0.1 by vol) and a flow rate of 1 ml/min. Oxygenated cholesterol esters were analyzed by RP-HPLC with a solvent system of 2-propanol/acetonitrile (25:75 by vol) and a flow rate of 1 ml/min at 45°C. Compounds were generally identified by coinjections with authentic standards. Chromatograms were quantified by peak areas. Calibration curves (5-point measurements) were established for free cholesterol, linoleic, arachidonic and 13-hydroxy-9Z,11E-octadecadienoic acid.

GC/MS. GC/MS was performed on a GC/MS system (Hewlett-Packard) equipped with a capillary RSL-150 column (polydimethylsiloxane, 0.25-μm coating thickness; 30 m × 0.32 mm; Research Separation Labs., Eke, Belgium). The hydroxy fatty acids prepared by SP-HPLC were methylated with diazomethan, silylated with bis-(trimethylsilyl)-trifluoroacetamide and than injected to CG/MS. For more informative mass spectra hydrogenation of the hydroxy fatty acid methyl esters was carried out in some cases.

Statistics. Significance calculations (Students *t* test) were performed with the StatWorks 1.2. software package on a Macintosh SE personal computer.

Results

Oxygenation of Human LDL by Pure Mammalian 15-lipoxygenases. The 15-lipoxygenases of rabbits and humans

Table 1. *Scheme of Consecutive Steps of HPLC for the Quantification of the Share of Specific Lipoxygenase Products in Tissue Lipid*

Step	HPLC	Purpose	Information obtained
1	RP-HPLC	Analytical	Determination of the hydroxy linoleic acid/linoleic acid ratio (%) as a measure for the degree of oxidative modification of tissue lipids
2	RP-HPLC	Preparative	Preparation of the sum of the oxygenated polyenoic fatty acids (hydroxy and keto derivatives)
3	SP-HPLC	Analytical	Separation of the positional (9- and 13-HODE) and geometric isomers (Z,E- and E,E- isomers) of the hydroxy and keto fatty acids
4	SP-HPLC	Preparative	Isolation of pure 13(R/S)-HODE(Z,E) for enantiomer analysis
5	CP-HPLC	Analytical	Analysis of the enantiomer composition of 13(S/R)-HODE(Z,E)

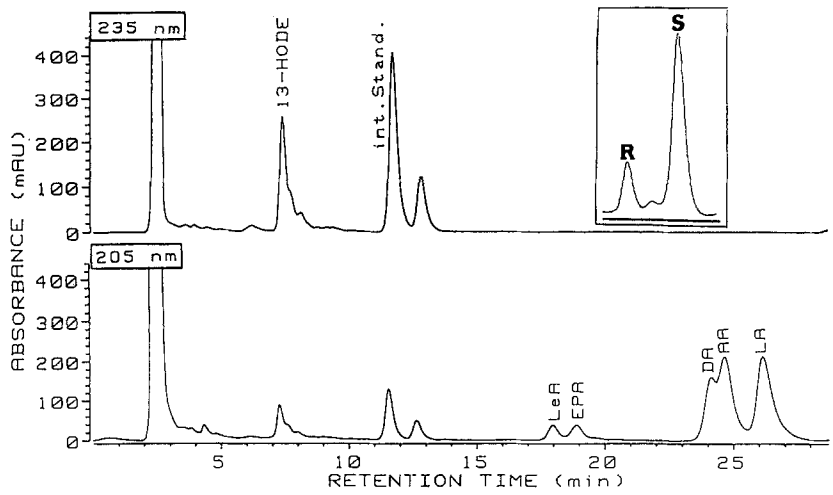

Figure 1. RP-HPLC of oxygenated polyenoic fatty acids of lipoxygenase-treated human LDL. Human LDL (2 nmol apoprotein B) was incubated with the pure rabbit reticulocyte lipoxygenase (4 nkat/ml linoleate oxygenase activity) which corresponds to 2 nmol of the enzyme (molar lipoxygenase/apoprotein B ratio of 1:1) for 15 min at 25°C. After addition of sodium borohydride the sample was acidified to pH 3, the lipids were extracted, and the extracts were hydrolyzed under alkaline conditions. RP-HPLC was carried out as described in Materials and Methods. (*Top*) Absorbance at 235 nm for analysis of the conjugated dienes (hydroxy polyenoic fatty acids); (*bottom*) absorbance at 210 nm for analysis of the nonoxygenated polyenoic fatty acids. (*LeA*) Linolenic acid; (*EPA*) eicosapentaenoic acid; (*DA*) docosahexaenoic acid; (*AA*) arachidonic acid; (*LA*) linoleic acid. (*Inset*) Determination of the enantiomer composition of 13-hydroxy-9Z,11E-octadecadienoic acid (*13-HODE*) isolated by RP- and SP-HPLC.

are capable of oxygenating human LDL in vitro (19, 20). RP-HPLC of the hydrolyzed lipid extracts indicate the formation of oxygenated polyenoic fatty acids comigrating with an authentic standard of 13-hydroxy-9Z,11E-octadecadienoic acid (Fig. 1, *top*). The UV-spectrum of these compound(s) showed a characteristic conjugated diene chromophore with an absorbance maximum at 234 nm. Control incubation of LDL with the heat-denatured enzyme or without lipoxygenase showed only a small peak at this retention time (<10% of the peak detected in lipoxygenase-treated LDL). Recording the chromatogram at 210 nm (Fig. 1, *bottom*), the nonoxygenated polyenoic fatty acids were analyzed. Quantification of the chromatograms at both wavelengths (235 and 210 nm) allowed the calculation of the hydroxy polyenoic fatty acid/polyenoic fatty acid ratio, which is a suitable measure for the oxidative modification of the LDL lipids. For LDL oxygenation, this ratio varied between 0.5 and 4%, depending on the incubation time, on the amount of lipoxygenase added (lipoxygenase loading of the LDL particle), and also on the LDL concentration. As major oxygenation product, esterified 13S-hydroxy-9Z,11E-octadecadienoic acid was identified by straight (data not shown) and chiral phase-HPLC (Fig. 1, *inset*) as well as by GS/MS. These compounds were mainly located in the cholesterol ester fraction, but also in various phospholipid classes of the LDL particle (19).

In contrast to the specific product pattern of the lipoxygenase-catalyzed LDL oxidation, the nonenzymatic copper–catalyzed oxidation of human LDL led to unspecific oxygenation products with 13-hydroxy-9Z,11E-octadecadienoic acid and 9-hydroxy-10E,12Z-octadecadienoic acid (ratio 1:1) being the major oxygenation products (data not shown). Both compounds were racemic mixtures with an equal distribution of the S- and R-enantiomer (S/R-ratio 1:1). Thus, analyzing the enantiomer composition of the major oxygenated lipids of in vivo samples one may differentiate between lipoxygenase catalyzed and nonenzymatic LDL oxygenation.

Oxygenated Lipids in Advanced Human Atherosclerotic Lesions. Arachidonate 15-lipoxygenase is expressed in foamy macrophages of human atherosclerotic lesions (16), but the question of whether this enzyme acts in vivo on endogenous lipids including LDL has not been investigated so far. Since enzymatic oxidation of LDL can be differentiated from nonenzymic oxidation processes by its specific product pattern, we analyzed atherosclerotic lesions obtained from 10 different patients during endaterectomy for the occurrence of oxidized lipids and determined the specificity of these compounds. We found that advanced human lesions (cholesterol content of the lesions: mean 25.4 mg/g wet weight, SD 9.9 mg/g wet weight) contain large amounts of oxygenated polyenoic fatty acids, mainly 9- and 13-hydroxy linoleic acid isomers (Fig. 2), and in smaller amounts the corresponding keto derivatives. The degree of oxidation of the lesion lipids varied between 3 and 10% (mean 5.5%, SD 2.7%), e.g., in some cases up to 10% of all linoleic acid residues in the lesion were present as oxygenated derivatives. CP-HPLC (Fig. 2, *inset*) of the 13-hydroxy-9Z,11E-octadecadienoic acid isolated from advanced lesions indicated an equal distribution of the S- and the R-isomer (share of the S-isomer: mean 50.0%, SD 1.6%) suggesting a nonenzymatic origin. These results which are in line with our earlier findings on the structure elucidation of oxygenated lipids in *post-mortem* samples of human atherosclerotic lesions (23) suggest that the majority of oxygenation products are formed by nonenzymatic lipid peroxidation.

Oxygenated Lipids in the Time Course of Plaque Development in Animal Models of Atherosclerosis. The deposition of lipids in developing human atherosclerotic lesions is a long-lasting process that takes years or even decades. During this time the lipids may undergo autooxidation after being deposited in the plaque. This nonspecific autooxidation may overlap the specific lipoxygenase reaction that is implicated in the initial phase of plaque development. To get information on

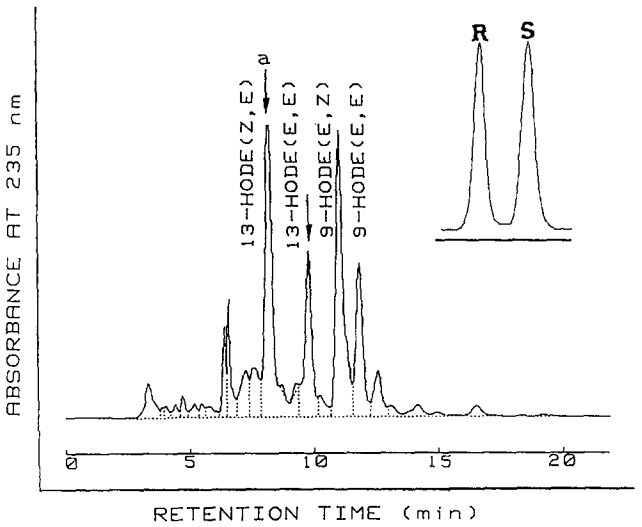

Figure 2. SP-HPLC of the oxygenated polyenoic fatty acids isolated from advanced human atherosclerotic lesions. A human atheroma was removed during thromboaterectomy from a 35-yr-old patient suffering from severe stenosis of the right *A. carotis*. After removal, the tissue was shock frozen and stored in liquid nitrogen. Sample work-up, RP-HPLC purification, and SP-HPLC analysis of the oxygenated polyenoic fatty acids were described in Materials and Methods. The chemical structure of the compounds eluted is given above the traces. (*Inset*) CP-HPLC analysis of the 13-hydroxy-9Z,11E-octadecadienoic acid (*a*) isolated by SP-HPLC.

the oxidative reaction in early atherogenesis, we analyzed the specificity of the oxidation products during the time course of plaque development in cholesterol-fed rabbits. For this purpose, NZW rabbits were categorized into four groups according to the duration of cholesterol feeding (see Materials and Methods). At the beginning of the feeding period, the serum cholesterol content of all rabbits was determined. No significant differences in the serum cholesterol level were found between the various groups. After killing the animals, the aortas were removed, areas with atherosclerotic lesions were prepared, the lipids were extracted, and the extracts were analyzed by HPLC for the following three parameters: (*a*) cholesterol content of the aorta lipid extracts (RP-HPLC) as indicator for the lipid deposition in the vessel wall; (*b*) degree of oxidative modification of the tissue lipids (calculation of the hydroxy linoleic acid/linoleic acid ratio from RP-HPLC analysis); and (*c*) enantiomer composition (S/R ratio) of the major oxygenation product (13-hydroxy-9Z,11E-octadecadienoic acid) reflecting the specificity of the oxygenation reaction (CP-HPLC). In addition, the serum cholesterol level at the end of the feeding period was determined. Representative HPLC chromatograms of the enantiomer analysis (Fig. 3) are shown. Table 2 demonstrates that cholesterol feeding led to a drastic increase in the serum cholesterol level after a 6-wk feeding period, but did not further increase if the rabbits were fed with the cholesterol-rich diet for a longer time period. In contrast, the cholesterol content of the aorta did not increase during the first 6 wk of cholesterol feeding. These data are in line with earlier observations that no sudanophil areas were detectable after a 6-wk feeding period under our experimental conditions. After a 12-wk feeding period, the cholesterol content of the aorta strongly increased and large sudanophil areas were detected (data not shown). These results indicate that under our experimental conditions the lipid deposition in the vessel wall started between 6 and 12 wk of cholesterol feeding and suggest that the atherosclerotic changes observed after a 12-wk feeding period may be regarded as early developing lesions. After 26 wk of feeding, the cholesterol content of the aorta did further increase, indicating a continuation of lipid deposition.

The degree of oxygenation of the aorta lipids in the control animals of about 0.16% was somewhat higher than that of other rabbit tissues (lung, kidney, liver, and skeletal muscle) where 0.1% of all linoleate residues were found to be oxygenated. Cholesterol feeding tended to lead to an increase in the degree of oxygenation of the tissue lipids which was not significant after 12 wk of feeding because of the large interindividual differences. After 26 wk, a further increase in the degree of oxygenation was detected which was significant ($p = 0.02$) when compared with the 12-wk feeding period. Summarizing these data, one may conclude that there are subtle but no drastic changes in the degree of oxidation of tissue lipids in early stages of plaque development. Thus, the hydroxy polyenoic fatty acid/polyenoic fatty acid ratio does not appear to be a suitable parameter to differentiate between various stages of plaque development in early atherogenesis.

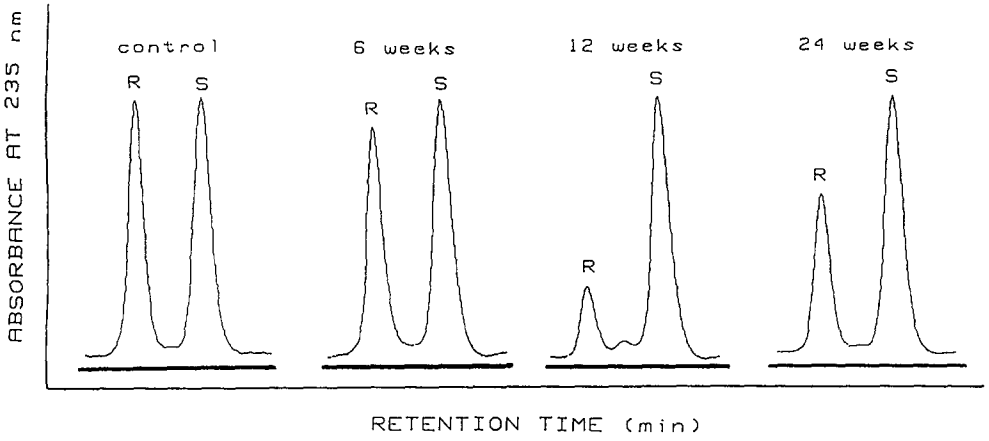

Figure 3. Enantiomer composition of 13-hydroxy-9Z,11E-octadecadienoic acid during the time course of cholesterol feeding. 13-HODE was isolated from the aorta of cholesterol-fed rabbits by consecutive steps of RP- and SP-HPLC. CP-HPLC was carried out as described in Materials and Methods. The cholesterol feeding period is indicated above the traces. Representative chromatograms for the different groups are shown. The enantiomers (*R*- and *S*-isomer) were identified by coinjections with authentic chiral and racemic standards.

Table 2. *Time-dependent Changes of the Lipid Composition of the Peripheral Blood and the Aorta during Feeding of Rabbits with a Cholesterol-rich Diet*

Group	No. of animals	Serum cholesterol content (mg/ml serum)			Aorta cholesterol content (mg/g wet weight)			Degree of oxidative modification of tissue lipids (hydroxy linoleic acid/linoleic acid ratio)			Product specificity (share of the S-isomer of 13-HODE)		
		Mean	SD	Significance	Mean	SD	Significance	Mean	SD	Significance	Mean	SD	Significance
									%			%	
1/control	5	22.4	5.1		2.8	0.3		0.166	0.05		51.0	2.0	
2/6-wk				<0.001*			0.01*						0.003*
feeding	7	1,500	385		1.8	0.6			ND		54.1	1.9	
3/12-wk				0.228			<0.001			0.303			<0.001
feeding	13	1,790	622		8.0	4.0		0.276	0.22		73.7	10.6	
4/26-wk				0.070			0.030			0.020			0.003
feeding	9	1,300	460		12.2	3.0		0.589	0.42		60.2	6.8	

NZW rabbits were categorized into four groups and fed with a cholesterol-enriched standard diet as described in Materials and Methods. After killing the animals, the aortas were recovered, pieces with atherosclerotic lesions were prepared, and the total tissue lipids were extracted (24). Analysis of the parameters was carried out as described in Materials and Methods.
*Significance calculations were carried out in comparison with the data of the next shorter feeding period.

The specificity of the oxygenation products, which is an indicator for the in vivo action of the 15-lipoxygenase, showed an unusual time course. In the control group the 13-HODE isolated from the aorta was a racemic mixture with an equal distribution of S- and R-isomers indicating the absence of lipoxygenase activity. After 6 wk of cholesterol feeding, there was a slight but significant increase in the product specificity, suggesting a small but detectable share of the lipoxygenase in the biosynthesis of the oxygenation products. After 12 wk of feeding, the product pattern became much more specific. The S/R ratio of the 13-hydroxy-9Z,11E-octadecadienoic acid of about 7:3 differs significantly from that of the control group and from that of the 6-wk feeding group, indicating the in vivo action of the 15-lipoxygenase in the developing lesion. It may be of pathophysiological importance that the in vivo action of the 15-lipoxygenase coincided with the beginning of the lipid deposition into the vessel wall. After 26 wk of cholesterol feeding the product pattern again became more unspecific, suggesting an overlay of the specific lipoxygenase products by nonenzymatic lipid peroxidation products.

In an additional set of experiments, the aortas of 9-mo-old Watanabe rabbits (n = 9) were analyzed for oxygenated polyenoic fatty acids. Both the serum cholesterol level (mean 347.8 mg/ml blood, SD 44.5 mg/ml) and the cholesterol content of the aorta (mean 11.3 mg/g wet weight, SD 2.3 mg/g wet weight) were significantly increased as compared with control rabbits fed with the same standard diet. The specificity of the oxygenation products measured as the share of the S-enantiomer of 13-HODE (mean 58.6%, SD 5.2%) was comparable with that of the rabbits fed with a cholesterol-rich diet for 26 wk (mean 60.2%, SD 6.8%). However, the specificity was significant higher (p = 0.01) than in the control group but lower (p = 0.003) than in the 12-wk feeding group. These data suggest action of a 15-lipoxygenase in the lesions of 9-mo-old Watanabe rabbits. Here again, the specific share of lipoxygenase products is strongly overlaid by unspecific oxygenation products which probably originate from nonenzymatic lipid peroxidation.

Discussion

The arachidonate 15-lipoxygenase is implicated in atherogenesis because it is expressed in foamy macrophages (15, 16) and is capable of oxidatively modifying LDL into its atherogenic form (19, 20). However, since no specific 15-lipoxygenase inhibitors and no transgenic animals are available so far, investigations on the role of the 15-lipoxygenase in atherogenesis have focused on in vitro experiments with cellular (12–14) and molecular model systems (19). As with any model, these systems may not necessarily reflect the events going on in developing atherosclerotic plaque. The detection of specific lipoxygenase products in early atherosclerotic lesions of cholesterol-fed rabbits is not a direct proof for their lipoxygenase origin. However, the formation of these products can be explained by the action of the lipoxygenase which is present in the lesion in an enzymatically active form (15–18). In later stages of plaque development, unspecific nonenzymatic oxygenation reactions become more prominent. These data indicate that the pathogenesis of atherosclerosis involves both enzymatic and nonenzymatic lipid peroxidation. It may be hypothesized that, by providing hydroperoxy lipids, the lipoxygenase may act as an initiator of an oxidative cascade in

atherosclerotic lesions. The hydroperoxy lipids may function as source for free radical–mediated propagation reactions which lead to a more unspecific product pattern. Under physiological conditions, hydroperoxy lipids are rapidly reduced in cellular systems to hydroxy derivatives by glutathione-dependent hydroperoxidases, such as the phospholipid hydroperoxide glutathione peroxidase (29, 30). This reduction does not involve the formation of free radicals (31) and therefore may counteract free radical–mediated propagation reactions. However, if the reductive capacity of the cells is exceeded by the cellular hydroperoxide content, radical-mediated hydroperoxidase reactions become dominating. These radical-mediated hydroperoxidase reactions, which are usually initiated by a homolytic cleavage of the hydroperoxy group leading to the formation of free alkoxy and hydroxy radicals, may be catalyzed by transition metals but also by hemoproteins (32). The radical intermediates may induce secondary lipid peroxidation leading to an unspecific pattern of oxygenation products.

According to our data, the formation of the specific lipoxygenase products coincides with the beginning of the lipid deposition in the vessel wall during the time course of cholesterol feeding. Thus a causal relationship between the action of the 15-lipoxygenase and the lipid deposition may be discussed. Our data, of course, do not prove that the in vivo action of the lipoxygenase is the reason for the lipid deposition. It may even be the other way around. However, since oxidative modification of LDL into its atherogenic form is implicated in early atherogenesis (33, 34) the in vivo action of the lipoxygenase may contribute to this process.

In vitro, the 15-lipoxygenase is capable of oxidizing both lipoproteins and cellular membranes without the preceding action of any lipid-cleaving enzyme such as phospholipase or cholesterol ester hydrolase. Analyzing the hydrolyzed lipid extracts of atherosclerotic aortas it is not possible to tell whether the lipoxygenase in the lesion oxygenates cellular membranes or LDL ester lipids. However, the detection of specific lipoxygenase products in the cholesterol ester fraction, which are not normal constituents of biomembranes indicates that the enzyme in vivo catalyzes at least in part a direct oxygenation of LDL. Alternatively, the lipoxygenase may act intracellularly, oxygenating the plasma membrane phospholipids of macrophages. Such a lipoxygenase-catalyzed membrane oxidation has been reported for rabbit reticulocytes (35) and IL-4–treated human peripheral monocytes (36). The hydroperoxy lipids may then exchange with LDL lipids and induce secondary oxygenation of the LDL. Recent studies on the oxidative modification of LDL by J-774.A1 cells suggested that LDL oxidation requires binding of the LDL particle to its membrane receptor (37). Such a binding is expected to facilitate the exchange process of oxidized lipids between the cell membrane and LDL.

A direct oxygenation of the LDL by the lipoxygenase requires a physical contact between the enzyme and the LDL particle. It remains to be investigated how the intracellular enzyme comes in contact with the extracellular LDL. Two possible pathways may be discussed. The first is internalization of the native LDL via the classical LDL receptor–mediated pathway and subsequent intracellular oxygenation as part of the metabolic breakdown of the particle. In this case, the oxidative modification of LDL would depend on the expression of the classical LDL receptor at the cell membrane. It has been shown before that arachidonic acid which is taken up by cells with the native LDL may be metabolized via 5-lipoxygenase and the cyclooxygenase pathway after being released from the LDL ester lipids in the lysosomes (38, 39). The second is extracellular localization of the 15-lipoxygenase. In animal cells the 15-lipoxygenase is synthesized as intracellular protein. In rabbit reticulocytes, there are no indications for an active transport of the enzyme across the cell membrane. However, we have immunohistochemical evidence (immunogold technique) of an extracellular localization of the 15-lipoxygenase in human liver (Robenek, H., and H. Kühn, unpublished data). Investigations of the subcellular localization of the 15-lipoxygenase in atherosclerotic lesions of humans and rabbit are currently being carried out in our laboratory.

The oxygenation of free polyenoic fatty acids by lipoxygenases leads to a highly specific product pattern. However, the in vitro oxygenation of complex substrates such as biomembranes and lipoproteins is characterized by a share of stereorandom oxygenation products (19, 40) which sometimes accounts for up to 40% of the sum of oxygenation products. With biomembranes this stereorandom share is strongly inhibited by radical scavengers such as BHT which is no inhibitor of the lipoxygenase reaction itself. These data suggested that free radicals may be involved in the formation of the unspecific products (40). Thus, the interaction of lipoxygenase with complex substrates, particularly with LDL, may be regarded as source of lipid radicals. When formed in vivo, these radicals may induce damage to healthy tissue (41) and hence may contribute to the progression of atherosclerotic lesions. Radical scavengers which have been reported to slow down the progression of atherosclerosis (42, 43) may exhibit their beneficial effect by trapping these radicals. The lipoxygenase or, more precisely, the hydroperoxy lipids formed during the lipoxygenase reaction are certainly not the only source for noxious radicals in atherosclerotic lesions. Radicals may also be generated during cell death and necrosis which are characteristic of advanced atherosclerotic plaques. The unspecific products of these reactions may swamp out the specific products formed during the initial stages of plaque development.

The physiological role of the 15-lipoxygenase in mammalian cells is still unclear. Because of its capability to oxygenate biomembranes the enzyme is implicated in membrane remodeling and organel breakdown during the maturation of red blood cells (44, 45). Similarly, the enzyme may be involved in monocyte-macrophage transition or in the maturation of tissue macrophages. Coming in contact with LDL outside the cell the 15-lipoxygenase may catalyze the oxidative modification of LDL rendering these particles prone to macrophage uptake via scavenger receptor–mediated pathways. On the other hand, the role of 15-lipoxygenase in the pathogenesis of atherosclerosis may not be limited to oxidative modification of LDL. Alternatively, the enzyme may be im-

portant for inflammatory reactions in the lesion. The specific induction of the 15-lipoxygenase in human monocytes by the proinflammatory IL-4 (36) supports this hypothesis. Since inflammation may be regarded as a defense mechanism against endothelial injury in the early stages of plaque development, the action of the lipoxygenase might be involved in repair reactions.

The degree of oxygenation of the tissue lipids of developing atherosclerotic lesions turned out to be an unsuitable measure to characterize the stage of plaque development in early atherogenesis for two reasons: (*a*) the hydroxy linoleic acid/linoleic acid ratio ranging from 0.1 to 0.8% is usually very low in early atherosclerotic lesions and sometimes come close to the detection limit of our HPLC diode array detector. (*b*) There are considerable interindividual differences that severely hamper statistical analysis with limited n-numbers. In later stages of atherogenesis and in particular in advanced human atherosclerotic lesions, the hydroxy linoleic acid/linoleic acid ratio is much higher and may be used as reliable parameter to quantify the degree of oxidative modification (23).

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