The Rabbit 15-Lipoxygenase Preferentially Oxygenates LDL Cholesterol Esters, and This Reaction Does Not Require Vitamin E*

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The oxidation of low density lipoprotein (LDL) by mammalian 15-lipoxygenases (15-LOX) was implicated in early atherogenesis. We investigated the molecular mechanism of 15-LOX/LDL interaction and found that during short term incubations, LDL cholesterol esters are oxygenated preferentially by the enzyme. Even when the LDL particle was loaded with free linoleic acid, cholesteryl linoleate constituted the major LOX substrate. In contrast, only small amounts of free oxygenated fatty acid isomers were detected, and re-esterification of oxidized fatty acids into the LDL ester lipid fraction was ruled out. When LDL was depleted from α-tocopherol, specific oxygenation of the cholesterol esters was not prevented, and the product pattern was not altered. Similar results were obtained at low (LDL/LOX ratio of 1:1) and high LOX loading (LDL/LOX ratio of 1:10) of the LDL particle. During long term incubations (up to 24 h), a less specific product pattern was observed. However, when the hydroperoxy lipids formed by the 15-LOX were immediately reduced by the phospholipid hydperoxide glutathione peroxidase, when the reaction was carried out with vitamin E-depleted LDL, or when the assay sample was diluted, the specific pattern of oxygenation products was retained over a long period of time.

These data suggest that mammalian 15-LOX preferentially oxidize LDL cholesterol esters, forming a specific pattern of oxygenation products. During long term incubations, free radical-mediated secondary reactions, which lead to a more unspecified product pattern, may become increasingly important. These secondary reactions appear to be suppressed when the hydroperoxy lipids formed are immediately reduced, when α-tocopherol-depleted LDL was used, or when the incubation sample was diluted. It may be concluded that 15-LOX-initiated LDL oxidation constitutes a dual-type oxygenase reaction with an initial enzymatic and a subsequent nonenzymatic phase. The biological relevance of this dual-type reaction for atherogenesis will be discussed.

The accumulation of lipid-laden foam cells in the sub-endothelial space of arteries is a key event in early atherogenesis (1, 2). Foam cells develop from peripheral monocytes or from smooth muscle cells by taking up modified low density lipoprotein (LDL) via scavenger receptor(s) or oxidized LDL receptor(s)-mediated pathways (3, 4). In vitro LDL oxidation is thought to be a major LDL-modifying process, and the lipid-peroxidizing enzyme 15-lipoxygenase (15-LOX) may be involved. There are several lines of experimental evidence suggesting a patho-physiological role of this enzyme (5). (i) In vitro, the enzyme is capable of oxidizing LDL in the presence (6) or absence of lipid hydrolyzing enzymes (7). (ii) The 15-LOX is expressed in atherosclerotic lesions of various species (8, 9) and the detection of specific 15-LOX products in the lesion lipids suggested the in vivo activity of the enzyme (10, 11, 12). (iii) Fibroblasts transfected with 15-LOX exhibited an augmented LDL oxidizing capability (13). (iv) Somatic gene transfer of the 15-LOX to rabbit iliac arteries induces the formation of epitopes cross-reacting with oxidized LDL antibodies (14). (v) Transgenic mice that overexpress the 15-LOX in endothelial cells develop more lesion area than corresponding controls when cross-bred with LDL receptor-deficient mice (15). (vi) A 15-LOX inhibitor that apparently lacks major antioxidative properties prevented lipid deposition in the aorta of cholesterol-fed rabbits (16). These data are consistent with a proatherogenic activity of the enzyme. On the other hand, transgenic rabbits that overexpress the 15-LOX specifically in monocyte/macrophages (17) develop significantly less atherosclerotic lesions when fed a cholesterol-rich diet or when cross-bred with LDL receptor-deficient Watanabe rabbits (18), suggesting an anti-atherogenic action of the enzyme.

For a better understanding of the processes involved in LDL oxidation in vivo, the in vitro interaction of 15-LOXs with human LDL has been studied. Sparrow et al. (6) have shown that the 15-LOX from soybeans is capable of oxidizing LDL in the presence of phospholipase A₂. Later on, we reported that the native rabbit (19) and the recombinant human 15-LOX (20) specifically oxidize LDL ester lipids in the absence of lipid-hydrolyzing enzymes. Comparison of various LOX isoenzymes indicated that mammalian 15-LOXs and the leukocyte-type 12-LOX show a high LDL-oxidizing capability, whereas the human 5-LOX, the platelet-type 12-LOX, and the soybean LOX-1 were poor catalysts (7). Moreover, it was shown that 15-LOX treatment rendered the LDL particle more susceptible to copper-catalyzed oxidation (21, 22).

More recently, the long term interaction of the recombinant human 15-LOX with isolated LDL was investigated (23, 24). It was concluded that the enzyme initiates LDL oxidation by preferentially oxygenating the free fatty acids present in the

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The abbreviations used are: LDL, low density lipoprotein; RP-HPLC, reverse phase high performance liquid chromatography; SP-HPLC, straight phase HPLC; 15-LOX, 15-lipoxygenase; 13S-HODE, (13R,9Z,11E)-13-hydroxyoctadeca-9,11-dienoic acid; 9S/6-HODE, (9S/ R,10E,12Z)-9-hydroxyoctadeca-10,12-dienoic acid; PUFA, polyunsaturated fatty acids; OH-FA/PUFA, ratio hydroxy fatty acid/polyenoic fatty acid; PH-GPx, phospholipid hydroperoxide glutathione peroxidase.
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LDL particle. Radical intermediates formed during this reaction may subsequently induce oxidation of LDL ester lipids via α-tocopherol-mediated lipid peroxidation. This hypothesis was supported by the finding that α-tocopherol-depleted LDL appeared to be resistant toward 15-LOX-initiated oxidation (23). However, these mechanistic conclusions were not consistent with the specific pattern of oxidation products described in previous reports (7, 19, 22).

Because of this discrepancy, we re-investigated the interaction of a mammalian 15-LOX with native and vitamin E-depleted LDL and found that the rabbit 15-LOX preferentially oxidizes LDL cholesterol esters when incubated with native or with linoleic acid-loaded LDL and that the α-tocopherol content of the LDL particle was not of major importance for the enzymatic reaction.

MATTERIALS AND METHODS

Chemicals—The chemicals were from the following sources: tri-α-tocopherol, (9Z,12Z)-octadec-9,12-dienoic acid (linoleic acid), chiral (15S,9Z,11E)-13-hydroxyoctadec-9,11-dienoic acid (13S-HODE), racemic (15S/R,9Z,11E)-13-hydroxyoctadeca-9,11-dienoic acid (13R/S-HODE), racemic (9S/R,10E,12Z)-9-hydroxyoctadec-10,12-dienoic acid (9S/R-HODE), free cholesterol, and cholesterol linolate from Serva (Heidelberg, Germany); sodium borohydride and 2,2'-azobis-(2-methylpropionamide) dihydrochloride were from Aldrich; triphenylphosphine and EDTA were from Merck; sodium bromide was from Sigma; [1-14C]linoleic acid (specific radioactivity of 58 mCi/mmol) was from Amersham Pharmacia Biotech. Econo-Pac 10 DG columns were obtained from Bio-Rad. All solvents were of HPLC-grade and purchased from J. T. Baker Inc.

Preparations—Rabbit reticulocyte 15-lipoxygenase was purified from the hemolsate of anemic rabbits as described before (19). The final enzyme preparation, which exhibited a molecular turnover rate of linoleic acid oxygenation of 20 s⁻¹, showed a single band in SDS-polyacrylamide gel electrophoresis and was proved to be pure when analyzed by RP-HPLC. Phenolphosphodiesterase glutathione peroxidase (PH-GPx) was prepared from pig hearts according to Ursini et al. (25). Briefly, the 20,000 × g supernatant of beef heart homogenate was used for fractionated ammonium sulfate precipitation, and the 30–60% fraction was dialyzed and injected to fast protein liquid chromatography on a Mono-Q column. The fractions exhibiting the highest PH-GPx activity were pooled and used as enzyme preparation. LDL was isolated from EDTA plasma of healthy donors by sequential floating ultracentrifugation in sodium bromide solutions (26). It was depleted from vitamin E by incubation with 2,2'-azobis-(2-methylpropionamide)-dihydrochloride (50 mM) for about 60 min at 37 °C. To follow the depletion kinetics, aliquots were taken at different time points, and the α-tocopherol content was assayed by HPLC. After complete depletion from vitamin E the excess of 2,2'-azobis-(2-methylpropionamide) dihydrochloride was removed by two consecutive passages through Econo-Pac 10 DG desalting columns. Afterward, sodium borohydride was added to reduce the hydroperoxides formed during 2,2'-azobis-(2-methylpropionamide) dihydrochloride treatment, and the excess of sodium borohydride was also removed by two consecutive steps of desalting. The last desalting step was carried out with a 0.1 M phosphate buffer, pH 7.4.

LDL was loaded with free fatty acid by adding 1 μl of a methanolic stock solutions of [1-14C]linoleic acid (different linoleic acid concentrations) to 0.3 ml of a LDL preparation (6.6 nmol of apoB/ml). The mixture was incubated at 32 °C for 150 min, then mixed with 10 ml of a KBr solution (density of 1.19 g/ml) and the LDL was repurified by ultracentrifugation for 20 h. Measurement of the radioactivity in the top, yellow-colored LDL layer and in the KBr solution indicated that 63–75% of the fatty acid added was associated with the LDL. Since free fatty acids exhibit strong detergent-like effects, we avoided excessive linoleic acid loading to prevent structural alterations of the LDL particle. Thus, we prepared two LDL species in which the linoleic acid concentration was moderately increased (2.5-fold in species I, 4.5-fold in species II) and the specific linoleic acid content was 20-fold higher in the LDL species III, as in the native LDL. HPLC analysis with radioactivity detection indicated that the linoleic acid added was incorporated as free fatty acid. We did not obtain any evidence for its esterification into the LDL ester lipids.

Assay Systems—Linoleic acid oxygenase activity of the purified reticulocyte lipoxygenase was assayed spectrophotometrically by measuring the increase in absorbance at 235 nm (molar extinction coefficient of conjugated dienes 25,000 (m × cm)⁻¹). The assay mixture was a 0.1 M phosphate buffer, pH 7.4, containing 0.2% sodium cholate and 0.26 mM linoleic acid as substrate. For 15-LOX-catalyzed LDL oxidation, aliquots of native or depleted LDL were incubated in room temperature with the purified rabbit reticulocyte 15-LOX in 0.1 M KBr in a small volume (0.1–0.5 ml) of isopropanol. The samples were stored at –20 °C under argon atmosphere.

Analytical—HPLC analysis was performed on a Shimadzu SPD-M10A VP instrument coupled with a diode array detector and a CTO-10A column oven. RP-HPLC was accomplished on a Nucleosil C-18 column (Macherey/Nagel, Düren, Germany; RS-system, 250 × 4 mm, 5 μm particle size). SP-HPLC was performed on a Zorbax SIL column (DuPont, Wilmington, DE; 250 × 4.6 mm, 5 μm particle size). Oxidized and nonoxidized cholesterol esters were analyzed by RP-HPLC at 45 °C with the solvent system acetonitrile:2-propanol (75:25, v/v) and a flow rate of 1 ml/min. The absorbencies at 235 nm (detection of oxidized cholesterol esters) and at 210 nm (detection of nonoxidized cholesterol esters) were recorded. For detection, methanol was used instead of acetonitrile. Free fatty acid derivaties were analyzed by RP-HPLC with the solvent system methanol/water/actic acid (80:20:0.1, v/v/v) at a flow rate of 1 ml/min. Here again the absorbencies at 235 nm (detection of oxidized fatty acids) and at 210 nm (detection of polyenoic fatty acids) were recorded. SP-HPLC of the hydroyoxy fatty acid isomers was performed with the solvent system n-hexane/2-propanol/acetic acid (100:5:0.1, by volume) and a flow rate of 1 ml/min. The enantiomer composition of the hydroxylated fatty acids was analyzed on a Chiralcel OD column (Diacel Chem. Industries, Tokyo, Japan; 250 × 4.6 mm, 5 μm particle size) with the solvent system n-hexane/2-propanol/acetic acid (100:5:0.1, by volume) recording the absorbance at 235 nm. The α-tocopherol content of the LDL particle was completely inactivated within 15 min. The oxidation products were determined by RP-HPLC using a combination of RP- and SP-HPLC.

RESULTS

15-LOXs Are Rapidly Inactivated during LDL Interaction at 37 °C—Mammalian 15-LOXs are capable of oxidizing LDL, but the kinetics of this reaction have not been studied in detail. There are reports on short term interactions (up to 30 min) of 12- and 15-LOXs with LDL (7, 19), and recently 15-LOX-initiated LDL oxidation was followed over longer time periods (23, 24). In the latter reports it was shown that 15-LOX-initiated LDL oxidation proceeds almost linearly over an incubation period of up to 24 h. This finding was rather surprising since purified LOXs are rather unstable. In addition, most mammalian LOX undergo rapid suicidal inactivation during the oxygenase reaction. To optimize the assay system, we followed the use of enzyme inactivated during its interaction with LDL. For this purpose the purified 15-LOX was incubated with LDL under different conditions and the residual linoleic acid oxygenase activity was assayed (Fig. 1). We found that at 37 °C and at low LOX loading (LDL/15-LOX ratio of 1:1), the enzyme was completely inactivated within 15 min. In contrast, when LDL oxidation was carried out at room temperature (20 °C) and...
higher 15-LOX concentrations (LDL/15-LOX ratio of 1:10), the enzyme survived longer incubation periods. These data indicate that at low enzyme concentrations and 37 °C 15-LOX-catalyzed oxidation of LDL can only be measured during a rather narrow time window after the addition of the enzyme. At later time points when the enzyme is catalytically inactive, mainly nonenzymatic secondary reactions will be measured. To minimize suicidal enzyme inactivation, most of our mechanistic studies were carried out at room temperature and at higher LOX loading of the LDL particle (LDL/15-LOX ratio of 1:10).

**LDL Cholesterol Esters Are Directly Oxygenated by the 15-LOX**—When the lipids of 15-LOX-treated LDL (1 μM LDL, 10 μM 15-LOX, 30-min incubation at room temperature) were extracted under reducing conditions and subsequently analyzed by RP-HPLC, compound(s) that co-migrated with an authentic standard of oxidized cholesterol esters were detected (Fig. 2). These compounds, which were characterized by a conjugated diene chromophore, were not detected when the incubation was carried out with the heat-denatured enzyme. For more detailed information on the chemical structure of the reaction products, additional HPLC was carried out. When the lipid extracts obtained under nonreducing conditions were analyzed by RP-HPLC with post-column chemiluminescence detection, we found that the oxidized cholesterol esters showed a strong chemiluminescence signal, indicating the presence of a hydroperoxy group (Fig. 3). In addition, the oxidized cholesterol esters extracted under reducing conditions were prepared by RP-HPLC, hydrolyzed, and analyzed by SP-HPLC. From Fig. 4 it can be seen that 13-HODE was the major oxygenation product. Other product isomers, such as (10E,12Z)-9-hydroxyoctadec-10,12-dienoic acid (9-HODE) and the two all-E isomers, were formed only in small amounts. Analysis of the enantiomer composition of 13-HODE indicated a strong preponderance of the S isomer (S/R ratio > 9:1; inset to Fig. 4). These data strongly suggest that the oxidized cholesterol esters are formed predominantly via direct 15-LOX-catalyzed oxygenation of LDL cholesterol esters. Moreover, the fair stoichiometry between the formation of conjugated dienes and cholesterol ester hydroperoxides observed under comparable experimental conditions (22) suggested that oxidized cholesterol esters are the major products of the 15-LOX reaction and that free oxidized polyenoic fatty acids may only contribute a small share to the pattern of oxygenation products. This conclusion was further supported by the fact that only trace amounts of free oxygenated fatty acid derivatives were found when the nonhydrolyzed lipid extracts of 15-LOX-treated LDL were analyzed by HPLC.

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**Fig. 1. Inactivation of 15-LOX during incubation with human LDL.** A solution of human LDL (1 μM apoB in 0.1 M phosphate buffer, pH 7.4) was incubated with the rabbit 15-LOX (LDL/15-LOX ratio 1:10 and 1:1) at 20 and 37 °C. At the times indicated, aliquots were taken, and the residual linoleic acid oxygenase activity was assayed in the standard assay system (see "Materials and Methods"). The initial activity was set 100%.

**Fig. 2. Formation of oxygenated cholesterol esters during short-term incubation of human LDL with the rabbit 15-LOX.** A solution of human LDL (1 μM apoB in 0.1 M phosphate buffer, pH 7.4) was incubated with the rabbit 15-LOX (LDL/15-LOX ratio of 1:10) at room temperature for 30 min. The lipids were extracted (26) under reducing conditions, the solvent was evaporated, and the residue was reconstituted in 0.2 ml of 2-propanol/acetonitrile (25:75, by volume). Aliquots were injected to RP-HPLC of the cholesterol derivatives as described under "Materials and Methods." The absorbencies at 210 and 235 nm were recorded simultaneously. A, incubation with heat-denatured 15-LOX; B, incubation with native 15-LOX.

**Fig. 3. Formation of cholesterol ester hydroperoxides during 15-LOX/LDL interaction.** A solution of human LDL (1 μM apoB in 0.1 M phosphate buffer, pH 7.4) was incubated with the rabbit 15-LOX (LDL/15-LOX ratio of 1:10) at room temperature for 30 min. After lipid extraction, the solvent was removed, the remaining lipids were reconstituted in 0.2 ml of HPLC solvent, and aliquots were injected to RP-HPLC with post-column chemiluminescence detection (38). The chromatogram was developed with the solvent system methanol/2-propanol (75:25, by volume) and a flow rate of 1 ml/min. The negative peaks indicate the elution of chemiluminescence-quenching compounds. HPODE, hydroperoxylinoic acid.
The oxygenated cholesterol esters formed during 15-LOX/LDL interaction were prepared as indicated in Fig. 2. After evaporation of the solvent, the lipids were hydrolyzed, and the resulting free hydroxy fatty acid derivatives were prepared by RP-HPLC (see Fig. 6). Aliquots of the hydroxy fatty acids were further analyzed by SP-HPLC as described under "Materials and Methods." 

Inset, enantiomer analysis of 13-HODE(Z,E) prepared by SP-HPLC.

(Fig. 5). In contrast, large amounts of oxygenated fatty acids were detected in the hydrolyzed lipid extracts.

In additional experiments we quantified the hydroxy fatty acid/polyenoic fatty acid ratio (19) of the LDL particle before and after 15-LOX treatment. This ratio, which was determined for the hydrolyzed lipid extracts, appears to be a suitable measure for the degree of oxidation of the LDL lipids. Under the reaction conditions specified in the legends to Fig. 2, this ratio varied between 2.5 and 7.5%, depending on the LDL preparation (4.4 ± 1.7, n = 5), suggesting that about 1 of 20 polyenoic fatty acid residues present in the LDL particle was oxygenated. Since 1 nmol of LDL particles contains about 1300 nmol of esterified polyunsaturated fatty acids, one can calculate that after 30 min of incubation, 65 nmol of oxygenated polyenoic fatty acid residues are present per nmol of LDL particles. In contrast, very small amounts (less than 1 nmol/nmol of apoB) of free oxygenated polyenoic fatty acids were found in the nonhydrolyzed lipid extracts. Thus, oxygenated free fatty acids appear to contribute less than 1% to the oxidized lipids formed under our experimental conditions.

When the experiment was carried out at a lower 15-LOX loading (LDL/15-LOX ratio of 1:1), we also observed a specific product pattern. However, the hydroxy fatty acid/PUFA ratio was somewhat smaller (2.10 ± 0.08, n = 3). Although we have not precisely determined the contribution of free oxygenated fatty acids to the sum of the oxidation products under these experimental conditions, comparative analysis of the hydrolyzed and nonhydrolyzed lipid extracts suggests that free fatty acids contribute only a small share (Fig. 5).

**Esterification of Free Hydroxy Fatty Acids to Cholesterol Esters Does Not Occur**—The strong preponderance of specifically oxidized cholesterol esters over free hydroxy fatty acids suggests that cholesterol esters may constitute the preferred 15-LOX substrates in the LDL particle. However, there may be an alternative explanation. The 15-LOX may preferentially oxygenate free fatty acids, and the resulting oxygenated derivatives may be rapidly incorporated into the cholesterol ester fraction. In exchange, nonoxidized fatty acids may be released and thus presented for the 15-LOX reaction. If such a reesterification cycle would be involved in 15-LOX-catalyzed LDL oxidation, radioactively labeled exogenous 13-HODE should also be incorporated into the cholesterol ester fraction. We incubated human LDL with labeled 13-HODE in the presence of 15-LOX for 30 min at room temperature (Fig. 6) and found that the oxidized cholesterol esters were not enriched in radioactivity, suggesting that re-esterification of free oxygenated fatty acids may be minimal. Similar results were obtained with other HODE isomers.

**Loading of LDL with Free Linoleic Acid Does Not Increase Its Oxidizability**—It has been reported that the rabbit 15-LOX oxidizes human LDL more effectively when the incubation was carried out in the presence of free polyenoic fatty acids or in the presence of phospholipase A2 (24). However, these data do not suggest that the endogenous free polyenoic fatty acids of the LDL particle constitute the preferred LOX substrate. When free linoleic acid is added to an LDL preparation, not the entire amount of the fatty acid is incorporated into the lipoprotein. Instead, there is a distribution steady state of the linoleic acid between the LDL particle and the surrounding medium. In other words, there will be different pools of linoleic acid, and the susceptibility of these pools toward 15-LOX attack may be different. To find out whether the free fatty acids incorporated into the LDL particle constitute a suitable 15-LOX substrate, we carried out linoleic acid-loading studies in which the excess linoleic acid not incorporated into the lipoprotein was removed. Before starting this experiment, we analyzed the free linoleic acid content of this LDL preparation and found about 2 nmol of free linoleic acid/nmol of apoB. When 1 nmol of LDL particles (1 nmol of apoB/ml containing 2 nmol of free linoleic acid) was loaded with 6.5 nmol of exogenous [1-14C]linoleic acid and the LDL was repurified (loaded LDL species I), we found 63% of the radioactivity associated with the LDL, whereas 37% was recovered in the KBr solution. When fatty acid loading was carried out with larger amounts of linoleic acid (loaded LDL species II and III) a similar distribution pattern was observed (Table I).
To check whether the exogenously added linoleic acid was incorporated into the LDL particle as free fatty acid or whether esterification took place during the loading procedure, we analyzed the nonhydrolyzed and hydrolyzed lipid extracts of the loaded LDL species by HPLC and found that more than 95% of the incorporated into the LDL particle as free fatty acid or whether esterification took place during the loading procedure, we analyzed the nonhydrolyzed and hydrolyzed lipid extracts of the loaded LDL species by HPLC and found that more than 95% of the incorporated linoleic acid was minimal.

To investigate the impact of linoleic acid loading on the oxidizability of LDL, the loaded LDL species I, II, and III were incubated with the 15-LOX for 30 min at room temperature. From Table II it can be seen that comparable amounts of specifically oxidized cholesterol esters were formed irrespective of the different concentrations of free linoleic acid. When the lipid extracts of the loaded LDL species were analyzed for the occurrence of oxygenated fatty acids, we detected large amounts of these compounds in the hydrolyzed extracts but very small amounts in the nonhydrolyzed extracts (Table II). These data suggest that even with the linoleic acid-loaded LDL species, the cholesterol esters are the preferred 15-LOX substrates. However, the free fatty acids present in the LDL particle appeared to be shielded from oxidation. Only under the conditions of excessive free fatty acid loading (20-fold increase in the free linoleic acid content) small amounts of specific hydroxy fatty acids were detected, but even under these conditions the cholesterol esters were the preferred LOX substrates.

**Product Specificity of 15-LOX-initiated LDL Oxidation Is Altered during the Time Course of Reaction—Short term incubation of the rabbit 15-LOX with human LDL led to a specific pattern of oxidized cholesterol esters. However, after long term incubations but rendered more unspecific during longer incubation periods. To test this hypothesis, LDL oxidation was carried out in the presence of the PH-GPx, an enzyme capable of effectively reducing hydroperoxy ester lipids (30–32). In the absence of PH-GPx, the product pattern of 15-LOX-initiated LDL oxidation was highly specific after short term incubations but rendered more unspecific during longer incubation periods (Table IV). However, in the presence of PH-GPx, the product pattern remained specific even after 20 h of incubation. Moreover, we found that in the presence of PH-GPx, the LDL oxidation rate was slowed down (Table IV). A similar partial inhibition was observed when submitochondrial particles were used as 15-LOX substrates (30).

The specificity of the product pattern does also depends on the LDL concentration (Table V). At 1 μM LDL and a LDL/15-LOX-catalyzed oxygenation of free linoleic acid and cholesterol esters in linoleic acid-supplemented LDL

| LDL species | Linoleic acid in LDL after loading | Oxygenated cholesterol esters | Free oxygenated cholesterol isomers |
|-------------|----------------------------------|-------------------------------|-------------------------------------|
|             | nmol nmol of apoB                | nmol nmol of apoB             | nmol nmol of apoB                   |
| Native LDL  | 2.0                              | 1.5 (50:50)                   | <0.1                                |
| Native LDL  | 2.0                              | 33.5 (78:24)                  | <0.1                                |
| Loaded LDL I| 5.3                              | 36.3 (73:27)                  | <0.1                                |
| Loaded LDL II| 9.1                             | 34.2 (73:27)                  | <0.1                                |
| Loaded LDL III| 39.4                           | 42.3 (73:27)                  | 3.9 (75:25)                         |

* No 15-LOX treatment; linoleic acid loading was carried out as described under "Materials and Methods."

* The LDL species were named according to this procedure.

**FIG. 6.** Free 13-HODE is not esterified to the LDL cholesterol esters. A solution of human LDL (1 μM apoB in 0.1 M phosphate buffer) was incubated with the rabbit 15-LOX (LDL/15-LOX ratio 1:10) at room temperature for 30 min in the presence of 7 nmol of radioactively labeled [1-14C]13-HODE. The lipids were extracted under reducing conditions, and the extracts were analyzed for cholesterol derivatives as described in the legend to Fig. 2. Fractions of 1 ml were collected and assayed for radioactivity by liquid scintillation counting. ox, oxidized; chol, cholesterol.

**TABLE I**

*Incorporation of exogenous linoleic acid into the LDL particle*

| LDL species | LDL Linoleic acid added | Radioactivity recovered in the LDL layer | KBr solution |
|-------------|-------------------------|------------------------------------------|--------------|
|             | nmol | nmol | % |
| I           | 2    | 8.7  | 63 | 37 |
| II          | 2    | 17.5 | 70 | 30 |
| III         | 2    | 97.2 | 75 | 25 |
LOX ratio of 1:10 we detected a rather unspecific product pattern after long term incubation. However, if the incubation mixture was diluted, keeping the LDL/15-LOX ratio, the product pattern remained specific over a much longer incubation period.

**Table III**

**Product specificity of 15-LOX-initiated LDL oxidation**

| LDL/15-LOX ratio | Incubation time | OH-FA/PUFA ratio/mol/mol | S/R ratio |
|------------------|-----------------|--------------------------|----------|
| 1:1              | 10              | 2.2                      | 82.18    |
|                  | 120             | 2.2                      | 58.42    |
|                  | 1200            | 8.0                      | 53.47    |
| 1:10             | 10              | 4.8                      | 88.12    |
|                  | 120             | 5.4                      | 77.23    |
|                  | 1200            | 26.8                     | 58.42    |

**Vitamin E Is Not Involved in 15-LOX-catalyzed Oxidation of LDL Cholesterol Esters**—To find out whether α-tocopherol may be of importance for 15-LOX-catalyzed oxidation of the LDL cholesterol esters, we incubated native and vitamin E-depleted LDL (partial and complete depletion) with the purified 15-LOX at 25° C for 30 min and 23 h at two different LDL concentrations (Table VI). It can be seen that the degree of oxygenation of the LDL lipids after 30 min of incubation was comparable for native, partially depleted, and completely depleted LDL at both LDL concentrations. In these cases specific patterns of oxygenation products were formed (S/R ratios of 90:10). At 23 h incubation, the OH-FA/PUFA ratio was increased in the case of native LDL but was less much augmented with partially depleted and completely depleted LDL. Under these conditions the specificity of the reaction products dropped down when high concentrations of native LDL were used but was retained at low LDL concentrations. Surprisingly, the drop in product specificity was not observed when vitamin E-depleted LDL was used. These data suggest that in addition to the specific 15-LOX-catalyzed oxygenation of cholesterol esters, there are 15-LOX-independent secondary oxygenation reactions that appear to be vitamin E-dependent. These secondary reactions, which lead to an unspecific pattern of oxygenation products, are not of major relevance for short incubation periods but may become particularly important during long term incubations.

**Discussion**

15-LOXs are lipid-peroxidizing enzymes that are capable of oxygenating the lipids of biomembranes (33–35) and lipoproteins (7, 19, 22). In LDL, the cholesterol esters are preferentially oxygenated. This finding was somewhat surprising since the majority of the cholesterol esters are localized in the hydrophobic core of the LDL particle, and the enzyme may not be capable of penetrating into this micro environment. However, a certain percentage of the cholesterol ester is also present in the LDL shell and thus may be attacked by the 15-LOX (19, 22). Although substantial amounts of cholesterol arachidonate are present in LDL particle, we did not find significant amounts of the corresponding hydroxy derivatives. One reason for the lack of these compounds may be the fact that they can be further metabolized to double and/or triple oxygenation products, which have actually been observed in RP-HPLC. The free fatty acids present in LDL may also be oxidized, but LOX catalysis is not restricted to them. When we analyzed the PUFA content of the LDL particle, we detected 1–2 nmol of free linoleic acid/nmol of apoB. If only free polyenoic fatty acids would have been oxygenated by the LOX, a hydroxy fatty acid/PUFA ratio of about 0.1% would be expected. However, we found hydroxy fatty acid/PUFA ratios of 1% up to 10%. Moreover, HPLC analysis of the ester lipid fraction of 15-LOX-treated LDL indicated large amounts of specifically oxidized cholesterol esters (Fig. 2). Since esterification of free oxygenated polyenoic fatty acids into the LDL cholesterol esters does not occur (Fig. 6), cholesterol esters appear to be the preferred 15-LOX substrates in human LDL. Even under the conditions of strong linoleic acid loading (20-fold increase in the free linoleic acid content of the LDL particle), the relative share of the oxygenation products originating from free fatty acids was less than 10% of the amount of oxygenated cholesterol esters. It may be possible that incorporation of fatty acid molecules into an LDL particle may impair the availability for the 15-LOX.

The vitamin E content of the LDL may not be of major importance for 15-LOX-catalyzed oxidation of LDL cholesterol esters. At short term incubations, when the 15-LOX reaction can be measured best, there was no difference in the oxygenation rate of native and α-tocopherol-depleted LDL. However, at long term incubations, native LDL shows a higher sensitivity toward oxidation than the vitamin E-depleted lipoprotein (Table IV). These data suggest that at longer incubation periods, vitamin E-dependent secondary oxidation reactions may take place. It has been reported before that in the presence of hydroperoxides, vitamin E may be capable of mediating peroxidation of unsaturated lipids via the formation of an α-tocopherol radical (36, 37), and this reaction has recently been implicated in 15-LOX-initiated LDL oxidation (23, 24). According to our results, the specific 15-LOX-catalyzed oxidation of LDL cholesterol esters is not influenced by vitamin E. However, the cholesterol ester hydroperoxides formed by the enzyme may serve as starters for α-tocopherol-mediated lipid peroxidation. When the lipid hydroperoxides are quickly reduced to their corresponding hydroxy derivatives, these secondary reactions may not take place anymore. These data suggest that 15-LOX-initiated LDL oxidation appears to be a dual process: (i) 15-LOX-catalyzed oxidation of LDL cholesterol esters, leading to the formation of specific oxygenation products; (ii) nonenzymatic vitamin E-dependent secondary reactions, forming an unspecific product pattern. At short term incubations, at low LDL concentrations, with vitamin E-depleted LDL, and when the incubation is carried out in the presence of a peroxide-reducing enzyme, vitamin E-dependent secondary reactions are largely suppressed. However, during long term incubations of native LDL, vitamin E-mediated secondary reactions may become increasingly important with time. If one translates these findings into the in vivo situation, one may conclude that 15-LOX-catalyzed oxidation of LDL ester lipids may always take place when a functional 15-LOX and LDL get in physical contact. In contrast, vitamin E-dependent LDL oxidation may only take place when the oxidative processes in the micro environment exceed the reductive capacity so that the 15-LOX-derived peroxides survive and may initiate vitamin E-dependent secondary reactions. Thus, the reductive capacity in the developing atherosclerotic plaque appears to be of major importance. It may be speculated that at early stages of lesion development when the LDL concentration in the arterial wall is rather low and when the reductive capacity exceeds the oxidative processes, 15-LOX-catalyzed oxygenation of LDL ester lipids may prevail. During these stages of plaque development, specific 15-LOX products may be detectable (10, 11). At later stages, however, when the LDL concentration in the
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Human LDL was incubated with the purified rabbit 15-LOX (LDL/15-LOX ratio 1:5) at room temperature for the times indicated in the presence or absence of PH-GPx (885 picokatal/ml) and glutathione (10 mM). The reaction was stopped by the addition of sodium borohydride. After acidification to pH 3, the lipids were extracted and hydrolyzed under alkaline conditions. The OH-FA/PUFA ratio was determined as a measure of the degree of oxidation of the LDL lipids by RP-HPLC (see “Materials and Methods”). The 13-HODE formed during 15-LOX/LDL interaction was prepared by consecutive RP- and SP-HPLC, and its enantiomer composition was determined by chiral phase HPLC (see “Materials and Methods”).

### Table IV

**Impact of PH-GPx on 15-LOX-initiated oxidation of LDL**

| LDL | Incubation time | OH-FA/PUFA ratio | S/R ratio of 13-HODE |
|-----|----------------|------------------|----------------------|
|     | µM h            | -PH-GPx (%)      | +PH-GPx (%)          | -PH-GPx (%)      | +PH-GPx (%) |
| 0.5 | 0.5             | 11.9             | 5.0                  | 91.9             | 96.4        |
| 0.5 | 1               | 11.0             | 4.6                  | 87.13            | 96.4        |
| 0.5 | 4               | 10.7             | 4.7                  | 78.22            | 94.6        |
| 0.5 | 24              | 20.0             | 7.0                  | 64.36            | 92.8        |

### Table V

**Impact of LDL concentration on product specificity of 15-LOX-initiated LDL oxidation**

Different concentrations of human LDL were incubated with the purified rabbit 15-LOX (LDL/15-LOX ratio of 1:10) at room temperature for 20 h. The lipids were extracted under reducing conditions, and the extracts were hydrolyzed. The OH-FA/PUFA ratio was determined by RP-HPLC as a measure of the degree of oxidation of the LDL lipids (see “Materials and Methods”). SP-HPLC was carried out to analyze the relative share of the positional (9- and 13-HODE) and geometric (Z,E and E,E) isomers of hydroxy linoleic acid (HODE). The 13-HODE(Z,E) formed was prepared, and its enantiomer composition was determined by chiral phase HPLC (see “Materials and Methods”).

| LDL concentration | OH-FA/PUFA ratio | 13-HODE/ZE HODE ratio | 13-HODE(Z,E) + 9-HODE(13-Z,E) | S/R ratio of 13-HODE(Z,E) |
|-------------------|------------------|------------------------|-------------------------------|--------------------------|
| µM apoB (%)       |                  |                        |                               |                          |
| 1                 | 26.8             | 1.2:1                  | 4.1:1                         | 58.42                    |
| 0.5               | 24.5             | 1.7:1                  | 7.8:1                         | 67.33                    |
| 0.1               | 17.7             | 21.0:1                 | 8.3:1                         | 74.26                    |

### Table VI

**15-LOX-initiated oxidation of native and α-tocopherol-depleted LDL**

Human LDL (0.1 or 0.8 µM apoB) was incubated with the purified rabbit 15-LOX at room temperature for the times indicated. The lipids were extracted under reducing conditions, and the extracts were hydrolyzed. The OH-FA/PUFA ratio was determined by RP-HPLC as a measure of the degree of oxidation of the LDL lipids (see “Materials and Methods”). The 13-HODE formed during 15-LOX/LDL interaction was prepared by consecutive RP- and SP-HPLC, and its enantiomer composition was determined by chiral phase HPLC (see “Materials and Methods”).

| LDL concentration | 0.1 µM LDL | 23 h | 0.8 µM LDL | 23 h |
|-------------------|------------|------|------------|------|
|                   | OH-FA/PUFA | S/R  | OH-FA/PUFA | S/R  |
|                   | ratio (%)  | ratio| ratio (%)  | ratio|
| Native            | 6.4        | 90:10| 17.7       | 74:26|
| Vitamin E-depleted| 6.2        | 97:3 | 8.9<sup>a</sup> | 90:10<sup>a</sup>|
| (57% depletion)   |            |      |            |      |
| Vitamin E-depleted| 6.5        | 92:8 | 7.6        | 89:11|
| (100% depletion)  |            |      |            |      |
|                   |            |      |            |      |
| 0.5 h             |            |      |            |      |
| 23 h              |            |      |            |      |

* 8 h of incubation.

It is well established that oxidized LDL is taken up by macrophages via scavenger receptors and/or oxidized LDL receptor-mediated pathways. However, little is known about the metabolic fate of the internalized oxidized lipids. Recently, it has been shown that free oxygenated linoleic acid derivatives (13-HODE, 9-HODE) activate the transcription of peroxisome proliferation activation receptor γ-dependent genes (40), which are involved in monocyte/macrophage differentiation (41). Interestingly, the oxidized cholesterol esters did not activate gene transcription (40). These data suggest that the oxidized ester lipids must be hydrolyzed before acting as peroxisome proliferation activation receptor γ ligands. Several intracellular lipid-hydrolyzing enzymes such as phospholipases and/or triglyceride lipases as well as the hormone-sensitive neutral cholesterol ester hydrolase (42, 43) may be involved. We carried out a series of in vitro experiments with the neutral cholesterol ester hydrolase of murine macrophages and found that oxidized cholesterol ester are well hydrolyzed by this enzyme. In fact, when oxidized and nonoxidized cholesterol linolate were compared, the oxidized lipids turned out to be a better substrate.<sup>2</sup>

<sup>2</sup> J. Belkner and H. Kühn, unpublished data.
in progress in our laboratory to characterize the lipid-hydrolyzing enzymes of human macrophages.

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