Lipoxygenase treatment renders low-density lipoprotein susceptible to Cu^{2+} -catalysed oxidation

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Oxidative modification of low-density lipoprotein (LDL) has been implicated in foam-cell formation at all stages of atherosclerosis. Since transition metals and mammalian 15-lipoxygenases are capable of oxidizing LDL to its atherogenic form, a concerted action of these two catalysts in atherogenesis has been suggested. Cu^{2+} -catalysed LDL oxidation is characterized by a kinetic lag period in which the lipophilic antioxidants are decomposed and by a complex mixture of unspecific oxidation products. We investigated the kinetics of the 15-lipoxygenase-catalysed oxygenation of LDL and found that the enzyme is capable of oxidizing LDL in the presence of the endogenous lipophilic antioxidants. In contrast with the Cu^{2+} -catalysed reaction, no kinetic lag phase was detected. The pattern of products formed during short-term incubations was highly

specific, with cholesterol-esterified (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid being the major product. However, after long-term incubations the product pattern was less specific. Preincubation with 15-lipoxygenase rendered human LDL more susceptible to Cu^{2+} -catalysed oxidation as indicated by a dramatic shortening of the lag period. Addition of Cu^{2+} to lipoxygenase-treated LDL led to a steep decline in its antioxidant content and to a greatly reduced lag period. Interestingly, if normalized to a comparable hydroperoxide content, autooxidation and addition of exogenous hydroperoxy fatty acids both failed to overcome the lag period. The local peroxide concentrations in various LDL subcompartments will be discussed as a possible reason for this unexpected behaviour.

INTRODUCTION

Oxidative modification of low-density lipoprotein (LDL) is implicated as an early event in atherogenesis [1–3]. Oxidatively modified LDL is rapidly taken up by macrophages and smooth-muscle cells [4] via scavenger-receptor-mediated pathways [5]. In contrast with the LDL-receptor pathway, the scavenger-receptor pathways are not feed-back controlled. Thus cells may take up modified LDL excessively and develop into lipid-loaded foam cells [6] which may accumulate in the subendothelial space of the arteries to form the lipid streaks. Since scavenger receptors do not recognize native LDL but preferentially bind modified LDL, modification of LDL appears to be of major importance for atherogenesis. A substantial body of experimental evidence suggests that oxidative modification is one of the major modifying processes involved in atherogenesis (for a review see ref. [1]). However, the mechanism of LDL oxidation *in vivo* remains unclear. *In vitro*, transition metals such as Cu^{2+} ions [7,8] are capable of oxidizing LDL to its atherogenic form. Among lipid-oxidizing enzymes, mammalian 15-lipoxygenases as well as the leucocyte-type 12-lipoxygenases [9] are capable of oxidizing LDL. Analysis of the composition of atheroma lipids of cholesterol-fed rabbits [10] and of advanced human lesions [11] suggested that lipoxygenases may play a role during early stages of plaque development whereas the non-enzymic lipid peroxidation becomes more relevant in later stages. These data suggest a concerted action of enzymic and non-enzymic lipid peroxidation in atherogenesis.

To study the mechanism of this concerted action we investigated the influence of the rabbit reticulocyte 15-lipoxygenase

(15-Lox) on Cu^{2+} -catalysed LDL oxidation *in vitro*. The data presented here suggest that 15-Lox treatment makes LDL more easily oxidized by Cu^{2+} ions. It is of particular interest that 15-Lox treatment is much more effective than autooxidation or addition of hydroperoxy fatty acids. The possible reasons for this behaviour will be discussed.

MATERIALS AND METHODS

Chemicals

The chemicals used were from the following sources: linoleic acid [(9*Z*,12*Z*)-octadecadienoic acid], arachidonic acid [(5*Z*,8*Z*,11*Z*,14*Z*)-eicosatetraenoic acid], cholesteryl linoleate from Serva; triphenylphosphine from Aldrich; NaBH_4 and D/L- α -tocopherol from Merck; isoluminol (6-amino-2,3-dihydro-1,4-phthalazinedione) and microperoxidase (MP-11) from Sigma; β -carotene from BASF; Chelex-100 from Bio-Rad. All solvents used were HPLC grade (Merck).

Preparations

15-Lox was purified to electrophoretic homogeneity from a 10000 *g* lysate supernatant of a reticulocyte-rich blood cell suspension [12]. LDL was isolated from pooled EDTA/plasma from fasting healthy donors by discontinuous density-gradient ultracentrifugation in a Beckman NVT 65 rotor [13]. Briefly, Beckman quick-seal tubes, containing NaCl/EDTA (density

Abbreviations used: LDL, low-density lipoprotein; 15-Lox, rabbit reticulocyte 15-lipoxygenase; RP(SP)-HPLC, reverse-phase (straight-phase) HPLC; 13-H(P)ODE, (13*S*)-hydro(pero)xy-(9*Z*,11*E*)-octadecadienoic acid; 15-H(P)ETE, (15*S*)-hydro(pero)xy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid; 9-H(P)ODE, (9*S*)-hydro(pero)xy-(10*E*,12*Z*)-octadecadienoic acid; GC/MS, gas chromatography/mass spectroscopy; CE-OOH, cholesteryl-linoleate hydroperoxides.

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Figure 1 Time course of LDL oxidation by 15-Lox

LDL samples ($0.1 \mu\text{M}$) in 10 mM chelexed PBS were incubated in 1 cm quartz cuvettes in the absence and presence (1, 2, 4, 5, 6 μM) of 15-Lox. The kinetics of oxidation were followed by measuring the increase in A_{234} in a thermostatically controlled (28°C) Beckman DU 640 spectrophotometer equipped with a six-cuvette holder. The trace for 1 μM 15-Lox represents the original curve; all other traces were smoothed. The inset shows the dependence of initial oxygenation rates on 15-Lox concentration; the data points represent means $\pm$ S.D. of three independent experiments. Rates were calculated from the initial linear part of the progress curves using a molar absorption coefficient of $29500 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

1.006 g/ml) underlaid with EDTA/plasma (density 1.24 g/ml adjusted with KBr) were ultracentrifuged at $100000 g$ (60000 rev./min) for 2 h at 10°C in a Beckman L-70 M ultracentrifuge. The LDL band was removed, sterile-filtered through a $0.45 \mu\text{m}$ filter (Corning), stored under vacuum in the dark at 4°C and used for the experiments within 1 week. LDL stored under such conditions for up to 2 months shows no signs of oxidation [13]. Before use, the native LDL was desalted into PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4) by gel filtration on an Econo-Pac 10 DG desalting column (Bio-Rad). *In vitro* loading of LDL with α -tocopherol was performed by the addition of $40 \mu\text{l}$ of an ethanolic D/L- α -tocopherol solution to 4 ml of EDTA/plasma [8]. After incubation of the plasma under argon for 4 h at 37°C in a shaking water bath, LDL was isolated as described above. This treatment led to an increase in the α -tocopherol content of LDL of up to 4-fold.

The LDL concentration was determined by enzymic cholesterol determination using the Chol MPR 2 kit (Boehringer-Mannheim). An average LDL cholesterol content of 31.6% (total cholesterol) and an LDL molecular mass of 2.5 MDa was used for the calculations of molar concentrations. The PBS used for the incubations was stored over Chelex-100 at 4°C for at least 24 h. Before use, the chelexed PBS was filtered through a $0.2 \mu\text{m}$ filter (Corning).

Authentic standards of racemic hydroxy fatty acids were

prepared by vitamin E-controlled autoxidation of fatty acid methyl esters [14]. After triphenylphosphine reduction and alkaline hydrolysis, the resulting free hydroxy fatty acid isomers were separated by straight-phase (SP)-HPLC. Chiral standards of (13*S*)-hydroxy-(9*Z*,11*E*)-octadecadienoic acid (13-HODE), (13*S*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (13-HPODE) and (15*S*)-hydroxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid (15-HETE) were obtained by enzymic oxygenation [15] of linoleic acid and arachidonic acid respectively, followed by HPLC preparation of the major oxygenation products. The chemical structure of all HPLC reference compounds was confirmed by UV spectroscopy, HPLC including chiral-phase HPLC, and gas chromatography/mass spectrometry (GC/MS) of the trimethylsilyl ethers and their hydrogenated derivatives. Cholesteryl linoleate hydroperoxide (CE-OOH) used as standard for quantification was prepared as described previously [16].

Assay systems

The oxygenase activity of purified 15-Lox was assayed spectrophotometrically, recording the increase in A_{234} . The assay mixture consisted of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.2% sodium cholate and 0.24 mM linoleic acid as substrate. The molar concentration of the enzyme was estimated using an average linoleic acid-turnover rate of 20 nmol/s per nmol of enzyme.

Human LDL was treated with 15-Lox in 10 mM chelexed PBS at 28°C . Control experiments were carried out by incubating LDL under the same conditions without 15-Lox. During the incubations the formation of conjugated dienes was measured by recording the A_{234} with a temperature-controlled spectrophotometer (Beckman DU 640 or Shimadzu UV 2100). For the kinetic studies the 15-Lox concentration was varied between 0 and 6 μM and LDL concentration between 0.025 and 1 μM .

Cu^{2+} -catalysed LDL oxidation was carried out by incubating 0.1 μM LDL in chelexed PBS with 1.67 μM CuSO_4 at 28°C . The lag period of this reaction [17] was used as an indicator for the oxidation resistance of the LDL. To investigate the impact of lipoxygenase pretreatment on the Cu^{2+} -induced oxidation, LDL (0.1 μM) was preincubated with 15-Lox for various time periods. Then CuSO_4 (1.67 μM final concentration) was added to yield an LDL/ Cu^{2+} ratio of 1:16, and the duration of the lag period was determined. For LDL autoxidation, LDL (0.1 μM) was incubated in the absence of 15-Lox for various time periods and subsequently Cu^{2+} -catalysed oxidation was initiated by the addition of 1.67 μM CuSO_4 . In another set of experiments, LDL was preincubated with 13-HPODE for 5 min at room temperature and then 1.67 μM CuSO_4 was added.

Identification of oxygenation products

Lipid hydroperoxides were determined as described by Yamamoto et al. [18]. Briefly, 0.5 ml of the samples (about 125 μg of LDL) were mixed with butylated hydroxytoluene and EDTA (final concentrations 20 μM and 2 mM respectively) and extraction was performed with 2 ml of methanol containing 0.02% acetic acid and 10 ml of water-washed *n*-hexane. The hexane phase was recovered and the solvent was evaporated. The remaining lipids were dissolved in 200 ml of ice-cold *t*-butanol/methanol (1:2, v/v), and aliquots were immediately injected on to an S5 ODS2 column (Spheris) for reverse-phase (RP)-HPLC. Compounds were eluted with a solvent system consisting of *t*-butanol/methanol (1:2, v/v) and a flow rate of 1 ml/min. The eluate was mixed on-line with a 1:1 mixture of 100 mM sodium borate buffer (pH 10) and methanol (containing 8 mM isoluminol

Figure 2 Dependence of the initial oxygenation rate on the LDL concentration

The kinetics of LDL oxidation were measured as described in the legend to Figure 1. (A) Progress curves for oxygenation of LDL (concentration ranging from 0.025 to 0.5 μM) by 15-Lox (1 μM). (B) Initial rates of LDL oxidation with 1.0 and 0.5 μM 15-Lox. The data points are means of duplicate experiments; standard deviation was never larger than $\pm 10\%$.

and 0.64 mM microperoxidase), and lipid hydroperoxides were detected by post-column chemiluminescence. Quantification of the various lipids was performed by comparing peak areas with those of the corresponding standards.

Measurement of LDL antioxidants

Lipophilic antioxidants were determined by extracting 1 ml of the incubation mixture (250 μg of LDL, containing 20 μM butylated hydroxytoluene and 2 mM EDTA) with a mixture of 1 ml of ethanol and 2 ml of *n*-hexane [19]. The organic phase was recovered, the solvent was evaporated and the residue was dissolved in 100 μl of ethyl acetate/ethanol (1:10, v/v). Aliquots were analysed by HPLC with the solvent system acetonitrile/methanol/ethanol/water (50:60:20:2, by vol.) containing 0.01% ammonium acetate. Tocopherols were detected by their fluorescence (excitation wavelength 292 nm, emission wavelength 335 nm), and carotenoids were detected by recording A_{450} . Identification and quantification of the tocopherols were performed with D/L- α -tocopherol and D/L- γ -tocopherol, and carotenoids were quantified with all-*trans*- β -carotene as standard.

Analysis of oxygenated fatty acid isomers

For analysis of their chemical structure, the hydroperoxy lipids formed during 15-Lox- and Cu^{2+} -catalysed reaction were reduced to the corresponding hydroxy derivatives by the addition of NaBH_4 . After neutralization, the lipids were extracted [12], the solvent was evaporated and the residue was redissolved in 0.85 ml of methanol. Then 0.15 ml of 40% KOH was added, and the ester lipids were hydrolysed for 30 min at 60 $^\circ\text{C}$ under argon atmosphere. The hydrolysates were acidified with 0.15 ml of acetic acid and the hydroxy fatty acids were analysed by HPLC. RP-HPLC was carried out on a Nucleosil C_{18} column (Macherey/Nagel; KS-system; 250 mm $\times$ 4 mm; 5 μm particle size). A solvent system of methanol/water/acetic acid (82:18:0.1, by vol.) and a flow rate of 1 ml/min were used. SP-HPLC of hydroxy fatty acid isomers was carried out on a Zorbax SIL column (DuPont; 250 mm $\times$ 4.6 mm; 5 μm particle size) with a solvent

system of *n*-hexane/propan-2-ol/acetic acid (100:2:0.1, by vol.) and a flow rate of 1 ml/min. The enantiomer composition of the hydroxy fatty acids was analysed by chiral-phase HPLC on a Chiralcel OD column (Diacel Chemical Industries; 250 mm $\times$ 4.6 mm; 5 μm particle size) with a solvent system of hexane/propan-2-ol/acetic acid (100:5:0.1, by vol.) and a flow rate of 1 ml/min.

RESULTS

Kinetics of 15-Lox-catalysed LDL oxidation

The kinetics of LDL oxidation by 15-Lox was measured by monitoring the increase in conjugated diene (A_{234}). Figure 1 shows an experiment where 0.1 μM LDL was incubated in the absence and presence of 1–6 μM 15-Lox. A_{234} increased at low 15-Lox concentration (1 and 2 μM) more or less linearly over a time period of 300 min. At higher concentrations, the increase slowed down with incubation time. The oxidation rates, determined from the initial linear part of the progress curves, were 21 and 70 mM diene/min for 1 and 6 μM 15-Lox respectively. The respective concentrations of conjugated dienes at 300 min were 6.4 and 15.2 μM . The oxidation curves shown in Figure 1 were reproducible, and the means $\pm$ S.D. for initial rates obtained in three independent experiments are shown in the inset of the Figure. The initial oxidation rates increased linearly with 15-Lox concentration, according to the equation $y = 0.11x + 0.0095$ ($r^2 = 0.09$, $P < 0.001$). In the absence of 15-Lox (curve 0 in Figure 1) or in the presence of heat-inactivated 15-Lox (not shown) only a slow increase in A_{234} was observed over a period of 300 min. To ensure that the A_{234} was due to the formation of conjugated dienes, UV spectra (220–350 nm) were recorded at intervals of 60 min, for each 15-Lox concentration. The difference spectra obtained by subtracting the time-zero spectrum from the spectra of the various time points indicated characteristic conjugated diene chromophores with an absorbance maximum between 234 and 236 nm.

Figure 2(A) shows experiments in which 1 μM 15-Lox was incubated with various concentrations of LDL ranging from 0.025 to 0.5 μM . Up to an LDL concentration of 0.1 μM , A_{234} increased linearly with time indicating a constant oxidation rate.

Figure 3 Kinetics of antioxidant consumption during oxidation of LDL by 15-Lox

LDL ($0.1 \mu\text{M}$) was oxidized with $1 \mu\text{M}$ 15-Lox as described in Figure 1. Conjugated dienes were measured on-line. At different time points, aliquots were taken from the cuvettes and analysed for antioxidants. Values obtained are expressed as percentage of the initial value. The data represent means $\pm$ S.D. of three independent experiments. The antioxidant contents of the LDL preparation (mol of antioxidant/mol of LDL) used in this experiment were as follows: α -tocopherol, 5.06 ± 0.14 ; γ -tocopherol, 0.34 ± 0.01 ; zeaxanthin + lutein, 0.086 ± 0.007 ; cryptoxanthin, 0.075 ± 0.005 ; lycopene, 0.215 ± 0.044 ; α -carotene, 0.043 ± 0.002 ; β -carotene, 0.263 ± 0.016 . (A) Consumption of α -tocopherol ($\square$) and γ -tocopherol ($\blacktriangle$), and formation of conjugated dienes by 15-Lox ($\blacksquare$) and by autooxidation ($\bullet$). (B) Loss of the other antioxidants. Standard deviation [not shown in (B)] was similar to that in (A), the mean standard deviation being 7.1 %.

At higher LDL concentrations, the oxidation rate slowed down with time. At a 15-Lox concentration of $1 \mu\text{M}$ (Figure 2B), we observed a linear dependence of the initial oxygenation rates on LDL concentration ($y = 0.47x$; $r^2 = 0.99$; $P < 0.001$). This indicates that the enzyme was not saturated with substrate. To investigate whether substrate saturation can be reached at a lower 15-Lox concentration, the dependence of oxidation rate on LDL concentration was also determined for $0.5 \mu\text{M}$ 15-Lox. Here again (Figure 2B) the initial rates depended linearly on the substrate concentration ($y = 0.36x$; $r^2 = 0.99$; $P < 0.001$). The linear dependence of initial rates on both the 15-Lox and LDL concentration indicates that under these experimental conditions the reaction follows the Michaelis–Menten equation for $S \ll K_m$ according to $v = k[15\text{-Lox}][\text{LDL}]$. The proportionality factor k was constant ($0.72 \pm 0.05 \mu\text{M}^{-1} \cdot \text{min}^{-1}$ equal to $1.2 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$) for all oxidations where 15-Lox was in a molar excess not larger

Figure 4 Structure of the 15-Lox-derived LDL oxygenation products

LDL ($0.1 \mu\text{M}$) was incubated in chelexed PBS with $1 \mu\text{M}$ 15-Lox at 28°C for 30 min. Lipid extraction, alkaline hydrolysis, RP-HPLC purification of the oxygenated fatty acids and SP-HPLC analysis were performed as described in the Materials and methods section. The major oxygenation product was characterized by a typical conjugated diene UV chromophore (right inset). The enantiomer composition (left inset) of 13-HODE was determined by chiral-phase HPLC. The chemical structure of the major product has been confirmed by GC/MS of the native and hydrogenated trimethylsilyl derivatives of the hydroxy fatty acid methyl esters.

than 2. At higher 15-Lox/LDL ratios the k value decreased and was $0.118 \mu\text{M}^{-1} \cdot \text{min}^{-1}$ at a 15-Lox/LDL ratio of 60:1.

Vitamin E and carotenoids do not inhibit 15-Lox-catalysed LDL oxidation

In contrast with Cu^{2+} -mediated LDL oxidation, 15-Lox oxygenation did not show a kinetic lag phase but commenced immediately after addition of the enzyme. In Cu^{2+} -catalysed oxidation LDL is depleted of antioxidants before the onset of rapid lipid oxidation. Figure 3 shows that this is not the case in 15-Lox-catalysed oxygenation. During a 180 min incubation period the oxidation proceeded at an almost constant rate but the major antioxidants were not (γ -tocopherol, β -carotene, α -carotene) or only slowly (α -tocopherol, cryptoxanthin, zeaxanthin, lutein, lycopene) consumed. Three independent experiments with duplicate analyses indicated a mean $\pm$ S.D. for the rates of α -tocopherol consumption and diene formation of $1.1 \pm 0.05 \text{ nM/min}$ and $31.1 \pm 1.4 \text{ nM/min}$ respectively (ratio 1:30). After 180 min $2.1 \pm 0.16 \text{ mol}$ of α -tocopherol/mol of LDL were consumed (40 %) and the LDL contained $56 \pm 3 \text{ mol}$ of dienes/mol of LDL (ratio 1:25). In control experiments (absence of 15-Lox but otherwise identical conditions) only slow diene formation was measured reaching a maximum of $8.0 \pm 0.4 \text{ mol}$ of dienes/mol of LDL after 180 min. This diene formation was not accompanied by a loss of antioxidants; an exception was lycopene which was slightly but statistically significantly decreased (20 %) after 180 min.

α -Tocopherol is not an effective inhibitor of 15-Lox-catalysed oxygenation of linoleic acid. We tested this antioxidant in the concentration range 0.1 – $100 \mu\text{M}$ and did not see any significant inhibition of linoleate oxygenation in our standard system. In

Table 1 Composition of hydro(pero)xy fatty acids formed during 15-Lox- and Cu²⁺-catalysed LDL oxidation

The *S/R* ratios are given in parentheses. The sum of the hydroxy fatty acids was set at 100%. The experiment was repeated twice with different 15-Lox and different LDL preparations. Results of a representative experiment are given.

Catalyst	Incubation time (min)	Products (%)			
		13-HODE(<i>Z,E</i>)	13-HODE(<i>E,E</i>)	9-HODE(<i>E,Z</i>)	9-HODE(<i>E,E</i>)
15-Lox	30	90.0 (91:9)	3.4	4.2 (49:51)	2.4
Cu ²⁺	30	44.4 (51:49)	9.0	36.8 (48:52)	9.8
15-Lox	240	65.3 (65:35)	4.2	26.2 (50:50)	4.3
Cu ²⁺	240	30.8 (50:50)	26.2	17.3 (49:51)	25.9

order to find out whether α -tocopherol inhibits 15-Lox-catalysed LDL oxygenation, LDL samples with different α -tocopherol content but otherwise identical composition were prepared by preincubation of human plasma without (control) and with α -tocopherol before LDL isolation. LDL samples containing 5.7 (control), 10, 12, 14 or 17 mol of α -tocopherol/mol of LDL were oxidized with 15-Lox as described in the legend to Figure 3. The rates of diene formation determined in triplicate experiments in these five LDL samples were 22.7 ± 2.4 (control), 19.9 ± 2.1 , 20.1 ± 1.0 , 23.9 ± 3.3 and 22.6 ± 2.2 nM/min. These findings indicate that α -tocopherol is not capable of inhibiting 15-Lox-catalysed oxygenation of LDL.

Characterization of oxygenation products of 15-Lox- and Cu²⁺-catalysed reactions

As shown previously [12] short-term incubations of human LDL with 15-Lox leads to a specific pattern of lipoxygenase products. Analysing the hydrolysed lipid extracts of 15-Lox-treated LDL under our experimental conditions we identified 13-H(P)ODE (*Z,E*) as the major product (90%) after a 30 min incubation period (Figure 4). The other positional and geometric isomers of hydro(pero)xylinoleic acid [13-H(P)ODE(*E,E*), 9-H(P)ODE(*E,Z*) and 9-H(P)ODE(*E,E*)] were identified as minor products (Figure 4, Table 1). Chiral-phase HPLC analysis revealed an enantiomer ratio (*S/R* ratio) of 91:9 for 13-H(P)ODE(*Z,E*). With prolonged incubations (240 min), the product pattern became less specific as indicated by the lower *S/R* ratio of 13-H(P)ODE and a change in the share of the positional and geometric isomers (Table 1). Analysis of the non-hydrolysed lipid extracts indicated only small amounts of free hydroxy fatty acids (less than 5% compared with the hydrolysed extract). These data indicate that the oxygenated fatty acids are predominantly localized in the ester lipids. In LDL oxidized for 30 min with Cu²⁺, equal amounts of esterified 13- and 9-H(P)ODE with an *S/R* ratio of both positional isomers close to 1:1 were found (Table 1). Interestingly about 80% of both 13- and 9-H(P)ODE were the *cis,trans*-isomers and only about 20% of the products were in the *trans,trans*-configuration. After 240 min of Cu²⁺-catalysed oxidation, the isomer distribution became more randomly distributed with 48% of the products in *cis,trans*- and 52% in the *trans,trans*-configuration.

Using HPLC with post-column chemiluminescence detection we measured the kinetics of the formation of cholesteryl-linoleate hydroperoxides (CE-OOH) during oxygenation of LDL by 15-Lox. The chromatograms showed a major peak (> 93%) with the retention time of cholesteryl linoleate hydroperoxide (CE-OOH) and several minor peaks probably representing the corresponding hydroperoxides of arachidonic acid and docosahexanoic acid. During the first 30 min of incubation, the increase

Figure 5 Formation of conjugated dienes and CE-OOH during 15-Lox-catalysed LDL oxidation

LDL (0.1 μ M) was incubated either with 1 μ M 15-Lox (●, ▲) or without enzyme (○, △), and the formation of conjugated dienes was measured by recording A_{234} . The concentration of dienes formed was calculated using a molar absorption coefficient of $29500 \text{ M}^{-1} \cdot \text{cm}^{-1}$. At different time points, aliquots were taken and the cholesteryl linoleate hydroperoxides (CE-OOH) were measured by HPLC with chemiluminescence detection. The data are means $\pm$ S.D. of three independent experiments. In one of these experiments, CE-OOH and dienes were measured (triplicate analysis) at 5 min intervals over the first 35 min of incubation. The mol/mol of LDL ratio on the y-axis is valid for CE-OOH as well as for the diene content of the 15-Lox-treated LDL. The inset shows the correlation between CE-OOH and dienes.

in conjugated dienes was almost exclusively due to the formation of CE-OOH (inset of Figure 5) as indicated by linear-regression analysis ($[\text{CE-OOH}] = 1.01[\text{dienes}] + 0.65$; $r^2 = 0.93$; $P < 0.01$). However, after longer incubation times, the two curves deviated from each other and dienes increased faster than CE-OOH (Figure 5). In principle, there are two possible explanations for this deviation: preferential oxygenation of non-cholesteryl ester lipids or formation of non-peroxide conjugated dienes. We analysed the major phospholipid subclasses but could not detect any disproportional increase in the degree of oxygenation of these lipids after long-term incubation. It should be noted in this context that, in a previous study [12] performed under different experimental conditions (different preparations of 15-Lox and LDL, different concentrations of LDL and 15-Lox, different 15-Lox/LDL ratio), we also detected oxygenated polyenoic fatty acids in the phospholipids of Lox-treated LDL.

Hydroperoxy lipids may undergo decomposition to secondary products some of which contain a conjugated diene system such as hydroxy fatty acids and fatty acid dimers [20]. As indicators

Figure 6 Preincubation of LDL with 15-Lox shortens the lag period of Cu²⁺-catalysed LDL oxidation

(A) LDL (0.1 μ M) was incubated with 1 μ M 15-Lox for 30 min at 28 °C. Then (time point 0) 1.67 μ M CuSO₄ was added to induce Cu²⁺-catalysed LDL oxidation, and the formation of dienes was recorded (left trace). As a control, LDL was incubated for 30 min in the absence of 15-Lox before addition of CuSO₄ (right curve). (B) Decrease in antioxidant content during Cu²⁺-catalysed oxidation of 15-Lox-pretreated LDL. Antioxidants were measured as described in the Materials and methods section. Mean values of triplicate analyses are given; standard deviation was as in Figure 3. For analysis of the specificity of the reaction products, aliquots of the incubation mixture were taken after the preincubation period, after 35 min and at 75 min. The S/R ratio of 13-HODE is given in the boxes as a measure of the specificity.

for such hydroperoxidase reactions we measured the formation of conjugated ketodienes by SP-HPLC as previously described [21]. After short-term incubations, ketodienes were hardly detectable in the product mixture whereas after long-term incubations (30–180 min) conjugated ketodienes could clearly be detected. The partial decomposition of CE-OOH to products still containing a conjugated diene chromophore would lead to a decrease in peroxide concentration whereas the conjugated diene content would keep increasing. This may at least in part explain the deviation of the diene and CE-OOH traces at later time points (Figure 5).

During the time course of LDL autooxidation (absence of 15-Lox) formation of small amounts of CE-OOH occurred. In this case the content of CE-OOH was always lower than that of the conjugated dienes (Figure 5).

Interaction of 15-Lox- and Cu²⁺-catalysed reactions

The ability of 15-Lox to oxidize LDL in the presence of endogenous antioxidants suggested that lipoxygenase treatment may render LDL more susceptible to Cu²⁺-catalysed oxidation because the hydroperoxy lipids provided by the enzyme may be

Figure 7 Correlation between hydroperoxide content and duration of lag period of 15-Lox-treated LDL, autoxidized LDL and LDL supplemented with 13-HPODE

(A) LDL ($0.1 \mu\text{M}$) was incubated with $1 \mu\text{M}$ 15-Lox, and diene formation was measured on-line. CE-OOH content was calculated from the diene/CE-OOH ratio indicated in Figure 5. At different time points, which correspond to different CE-OOH concentrations, $1.67 \mu\text{M}$ Cu^{2+} was added and the lag time of Cu^{2+} -catalysed LDL oxidation was determined. (B) Autoxidation was carried out by incubating $0.1 \mu\text{M}$ LDL in PBS (absence of 15-Lox). After various time periods aliquots were taken, the A_{234} was measured and CE-OOH content was calculated using a CE-OOH/conjugated diene ratio of 0.7. This value was determined in five independent autoxidation experiments in which the formation of conjugated dienes (UV absorbance) and CE-OOH (HPLC with chemiluminescence detection) was measured simultaneously. Linear regression of the data points led to the equation $[\text{CE-OOH}] = 0.7 [\text{dienes}] - 0.06$ ($r^2 = 0.96$; $P < 0.001$). (C) $0.1 \mu\text{M}$ LDL was preincubated with various concentrations of 13-HPODE for 5 min at 28°C before the addition of Cu^{2+} . For all lag-time measurements shown in (A), (B) and (C) the Cu^{2+} concentration was $1.67 \mu\text{M}$, the oxidation was carried out at 28°C and the lag times were determined as indicated in Figure 6. The lag time is expressed as a percentage of that observed with untreated LDL (120 min) and plotted against the lipid hydroperoxide (i.e. CE-OOH, 13-HPODE) content of the LDL.

used by Cu^{2+} ions for rapid decomposition of the endogenous antioxidants. From Figure 6(A) it can be seen that pretreatment of LDL with 15-Lox shortened the lag period of the Cu^{2+} -catalysed reaction. After the addition of Cu^{2+} ions the rapid decrease in the content of the major endogenous antioxidants is accompanied by a large increase in the formation of conjugated dienes (Figure 6B). Analysis of the chemical structure of the reaction products indicated the formation of specific oxygenation products during the preincubation period (30 min). After the addition of Cu^{2+} this product pattern became less specific, indicating that the increase in A_{234} is due to massive non-enzymic lipid peroxidation which overlays the enzymic oxidation products.

For more quantitative information on the correlation between hydroperoxide content and degree of lag-period shortening, LDL was preincubated with 15-Lox for various time periods. The hydroperoxide content was calculated from the absorbance at 234 nm (Figure 5), and the length of the lag period of the Cu^{2+} -catalysed oxidation was measured. From Figure 7(A) it can be seen that, with 15-Lox treatment, 5 mol of CE-OOH/mol of LDL was sufficient to reduce the lag period by about 70% (note that up to 15 mol of CE-OOH/mol of LDL, peroxides other than CE-OOH are not present, or only in small amounts, in 15-Lox-treated LDL; Figure 5). In contrast, CE-OOH formed by LDL autoxidation did not effectively reduce the lag period (Figure 7B). At a 5:1 molar ratio (CE-OOH/LDL) only a 20% reduction in the lag period was observed. Even a 10-fold higher CE-OOH concentration led only to a 60% reduction in the lag period. Similarly, the addition of exogenous hydroperoxylinoleic acid (13-HPODE) did not effectively reduce the lag period (Figure 7C). At a molar 13-HPODE/LDL ratio of 5:1 a 30% reduction in the lag period was observed. In order to find out what percentage of 13-HPODE added was bound to the LDL particle, radioactively labelled 13-HPODE was incubated with LDL for 15 min at 28°C at LDL/13-HPODE ratios of 1:1 and

1:20. After desalting, the radioactivity in the LDL particle and in the protein-free column wash was counted. We found that under these conditions, 81% of the radioactivity added was associated with the LDL particle and 19% was found in the protein-free supernatant.

It is interesting that the dose-response curves for 15-Lox treatment and addition of exogenous 13-HPODE show a break around 5 mol of CE-OOH/mol of LDL. In the case of 15-Lox treatment, increasing the CE-OOH content led to a further decrease in the lag period which approached zero at about 90 mol of CE-OOH/mol of LDL. In contrast, loading LDL with an excess of exogenous 13-HPODE up to 100 mol/mol of LDL had no further effect on the lag period of Cu^{2+} -mediated oxidation.

DISCUSSION

The spectrophotometric on-line measurement of the formation of conjugated dienes (Figures 1 and 2) is a useful method of measuring the kinetics of 15-Lox-catalysed LDL oxygenation. All progress curves lack a kinetic lag period. At higher 15-Lox concentrations ($> 0.1 \mu\text{M}$) the initial rate gradually slowed down with time. The reasons for this decrease have not been studied in detail. However, self-inactivation of the enzyme [22] as well as limited accessibility of oxidizable lipids to 15-Lox may be put forward. In fact, we found that the majority of esterified polyenoic fatty acids in LDL appear not to be accessible to 15-Lox. Assuming that an LDL particle contains about 1300 polyenoic fatty acid residues [8], the maximum oxidation observed in this study (Figure 1; $6 \mu\text{M}$ 15-Lox) was about 13%. In separate experiments we found that the enzyme was still active after long-term incubations but no longer oxidized LDL, suggesting that substrate accessibility may be the limiting factor.

Our kinetic studies included extensive investigation of the effect of LDL and 15-Lox concentrations on the oxidation rate.

The measured initial rates (v) changed proportionally with both 15-Lox and LDL concentration: $v = k [15\text{-Lox}][\text{LDL}]$. These data suggest that, under our experimental conditions, the enzyme was not saturated but operated under condition ($S \ll K_m$) for which the Michaelis–Menten equation predicts that the rate increases proportionally with the enzyme and substrate concentrations. For free linoleic acid a K_m -value of $7 \mu\text{M}$ has been determined [23]. In our assays the maximal LDL concentration was $1 \mu\text{M}$ which corresponds to about $1300 \mu\text{M}$ polyenoic fatty acid residues. Thus the apparent K_m for oxygenation of polyenoic fatty acid residues in LDL by 15-Lox must be higher than 1.3 mM .

The simultaneous measurement of conjugated dienes and CE-OOH indicated that the first 10–15 polyenoic fatty acid residues oxidized by the 15-Lox appear to be only cholesteryl linoleate molecules (Figure 5). The observation that 15-Lox first selects cholesteryl linoleate molecules from among the large number of oxidizable lipids is a remarkable new finding because the majority of cholesteryl esters are located in the LDL core, through to which the 15-Lox is unlikely to penetrate. However, a small proportion of cholesteryl linoleate may be located in the outer monolayer of the LDL particle [24], perhaps within a micro-environment that is easily accessible to 15-Lox. In addition, there may be some shell-to-core exchange of the oxidized cholesteryl esters or trans-esterification of the oxidized polyenoic fatty acids.

The role of endogenous LDL antioxidants in lipid peroxidation is still a matter of discussion [25]. During Cu^{2+} -catalysed LDL oxidation in our standard assay system, they appear to protect the LDL lipids from being oxidized. As indicated in Figure 3, 15-Lox is capable of oxidizing LDL in the presence of antioxidants. Although the lipoxygenase reaction is suggested to involve the formation of lipid radicals, these intermediates remain enzyme-bound and might not be readily accessible to lipophilic antioxidants. Long-term treatment of LDL with 15-Lox does not only lead to oxidation of the esterified polyenoic fatty acids but also to a co-oxidative modification of the apoprotein [9,12]. The co-oxidation of apoprotein B is probably initiated by radical intermediates originating from the secondary decomposition of hydroperoxy lipids but not from the lipoxygenase reaction itself. It has previously been reported that lipoxygenases are capable of decomposing hydroperoxy lipids via the homolytic cleavage of the hydroperoxy group forming radical intermediates [21,26,27]. These radicals may induce co-oxidation of the apoprotein. Alternatively, the radicals may be trapped by lipophilic antioxidants causing their slow time-dependent decrease (Figure 2). One may conclude that the lipophilic antioxidants of the LDL particle are not capable of inhibiting the lipoxygenase-catalysed oxidation of LDL lipids but they may protect other LDL components from being co-oxidatively modified. The inability of lipophilic antioxidants to prevent 15-Lox-catalysed oxidation of the LDL lipids may be of importance for the interpretation of pharmacological-intervention studies. Although 15-Lox-catalysed formation of hydroperoxy lipids *in vivo* may not be inhibited by α -tocopherol, the antioxidant may scavenge radicals formed during the secondary decomposition of the hydroperoxy lipids and thus may have a protective function.

Preincubation of LDL with 15-Lox shortens the lag period of Cu^{2+} -catalysed LDL oxidation (Figures 6 and 7). The most plausible explanation for this phenomenon is that the 15-Lox-derived hydroperoxy lipids are utilized by Cu^{2+} for initial non-enzymic lipid peroxidation. These reactions are accompanied by a rapid breakdown of lipophilic antioxidants which counteract the initiation of Cu^{2+} -catalysed oxidation. Already 5 mol of 15-Lox-derived CE-OOH/mol of LDL are sufficient to reduce the lag period by more than 70 % (Figure 7). However, CE-OOH

formed by autooxidation was much less effective in reducing the lag period. A possible explanation for this phenomenon may be different compartmentation of the hydroperoxy lipids. Since the lipoxygenase is unlikely to penetrate into the core, the CE-OOH formed by the enzyme may accumulate in the shell and thus may be more accessible to Cu^{2+} ions for induction of the oxidation reaction. During autooxidation, the hydroperoxy lipids may be distributed more randomly in the LDL particle. Since the concentration of polyenoic fatty acids is high in the cholesteryl ester fraction and since the cholesteryl esters are predominantly located in the core of the LDL, the majority of CE-OOH formed during autooxidation should be located in the core. Thus they may be sterically protected from Cu^{2+} ions which are probably not capable of penetrating easily into the core. Furthermore the addition of exogenous 13-HPODE was less effective in reducing the lag period. This observation is consistent with a report by O'Leary et al. [28], who studied the effects of various hydroperoxy fatty acids (13-HPODE, 15-HPETE, 9-HPODE, 5-HPETE) and found that they decrease the lag time of Cu^{2+} -initiated LDL oxidation by about 14–44 %, depending on the peroxide/LDL ratio. It was found that the extent was maximal at about 25–50 mol of HPODE/mol of LDL [28].

Oxidation of LDL during atherogenesis may involve the concerted action of transition metals and 15-lipoxygenases. It has been reported previously that lipoxygenases are capable of oxidizing LDL with [29] and without [9,12] the preceding action of an ester-lipid-cleaving enzyme. We have investigated the interplay between the two catalysts in a reconstituted model system and found that, with respect to the specificity of the oxidation products our *in vitro* model consisting of a pure 15-Lox and Cu^{2+} ions reflects the oxidation events that occur in developing atherosclerotic lesions. In the early stages of plaque development, a specific product pattern was detected in cholesterol-fed rabbits suggesting the action of a 15-lipoxygenase *in vivo* [10]. This period may correspond to the preincubation period of the LDL with 15-Lox in our *in vitro* studies. In later stages of plaque development the pattern of oxidation products *in vivo* was less specific [11]. This stage of plaque development may correspond to the metal-catalysed propagation phase in our reconstituted model system.

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