Oxidation of LDL by recombinant human 15-lipoxygenase: evidence for α-tocopherol-dependent oxidation of esterified core and surface lipids

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Abstract Various lipoxygenases (LO) oxidize low density lipoprotein (LDL) in vitro and 15-LO has been implicated in the development of atherosclerosis in vivo. Direct oxidation of phospholipids (PL) and cholesteryl esters (CE) by LO has been proposed as a mechanism whereby these enzymes cause or contribute to LDL lipid peroxidation. Herein we show that the extent to which recombinant human 15-LO (rhLO) caused peroxidation of LDL's esterified core and surface lipids depended on, and directly related to, the α-tocopherol (α-TOH) content of the lipoprotein. Thus, CE and PL of in vivo α-TOH-depleted LDL, isolated from a patient with familial isolated vitamin E deficiency, were resistant to oxidation by rhLO, whereas those in α-TOH-containing LDL from the same patient receiving vitamin E supplements readily oxidized. The extent to which rhLO caused oxidation of CE and PL directly and linearly correlated with LDL's content of vitamin E, as demonstrated by studies with in vitro α-TOH-depleted lipoproteins. The geometric isomers of oxidized cholesteryl linoleate formed in LDL during oxidation initiated by rhLO, matched those obtained during non-enzymic, peroxyl radical-initiated oxidation of LDL whilst α-TOH was present. Ascorbate, an efficient co-antioxidant for α-TOH, completely prevented rhLO-initiated oxidation of LDL's CE, but did not inhibit rhLO-mediated oxidation of unesterified linoleate. These results are inconsistent with direct oxidation of LDL esterified lipids by rhLO. Isolated LDL contained free fatty acids (FFA), and its exposure to rhLO caused a rapid formation of linoleate hydroperoxide. To reconcile these data, we propose that during rhLO-initiated oxidation of LDL, enzymic oxidation of FFA preceeds the oxidation of CE and PL, which occurs largely via a tocopherol-dependent process.—**Upston, J. M., J. Neuzil, and R. Stocker.** Oxidation of LDL by recombinant human 15-lipoxygenase: evidence for α-tocopherol-dependent oxidation of esterified core and surface lipids. *J. Lipid Res.* 1996. **37:** 2650–2661.

Supplementary key words antioxidants • atherosclerosis • lipid peroxidation • reactive oxygen species • vitamin E

Low density lipoprotein (LDL) oxidation is thought to play a leading role in the development of atherosclerosis (for review see ref. 1). Evidence for this includes the finding that cells in the presence of transition metals in atherosclerotic lesions can oxidatively modify LDL to a form that is avidly taken up by monocyte-derived macrophages to produce lipid-laden foam cells. Further, antibodies raised against oxidized LDL recognize material in human atherosclerotic lesions (2–6). Although a large body of evidence strongly implicates oxidatively modified LDL in atherogenesis, the exact mechanism(s) involved in LDL oxidation in vivo are not defined. Several processes have been proposed including the participation of various enzymes, primarily 15-lipoxygenase (15-LO) (2, 7–9) and myeloperoxidase (6, 10).

Evidence for a putative role of 15-LO in atherogenesis is based on the finding that human atherosclerotic lesions contain mRNA, protein (11), and lipid oxidation products (7–9) of 15-LO. Also, murine fibroblasts expressing human 15-LO can increase the lipid peroxide level in LDL (12, 13), and transfer of 15-LO gene into rabbit iliac arteries results in the appearance of lipid-protein adducts characteristic of oxidized LDL (14). Furthermore, several LO, including 15-LO, can cause oxidation of LDL's lipids in vitro (15–19). Reticulocyte 15-LO can oxygenate complex substances such as phos-

Abbreviations: apoB, apolipoprotein B-100; AH, ascorbate; AAPH, 2,2′-azo*bis*(2-amindiopropane) hydrochloride; CE, cholesteryl esters; CE-OOH, cholesteryl ester hydroperoxides; Ch18:2, cholesteryl linoleate; Ch18:2-OOH, cholesteryl linoleate hydroperoxides; F_{tOH}, fractional α-TOH content; FFA, free fatty acids; FFA-OOH, free fatty acid hydroperoxides; FFA-OO•, fatty acid peroxyl radicals; FIVE, familial isolated vitamin E deficiency; 13-H(P)ODE, 13-hydro(pero)xy-9*Z*,11*E*-octadecadienoic acid; LDL, low density lipoprotein; LH, lipid containing bisallylic hydrogens; LO, lipoxygenase(s); LOOH, lipid hydroperoxides; NP-HPLC, normal phase high performance liquid chromatography; PBS, phosphate-buffered isotonic saline; PC-OOH, phosphatidylcholine hydroperoxides; PL, phospholipids; R_p, rate of lipid peroxidation; rhLO, recombinant human 15-lipoxygenase; α-TOH, α-tocopherol; TMP, tocopherol-mediated peroxidation; α-TO•, α-tocopheroxyl radical; 18:2-OOH, linoleate hydroperoxide.

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pholipids (PL) and biological membranes (20). This enzyme also oxygenates cholesteryl esters (CE) such as cholesteryl linoleate (Ch18:2, quantitatively the most important oxidizable lipid in LDL) when suspended in phosphate buffer containing 0.1% cholate (21), albeit with substantially lower efficacy than its preferred substrate, unesterified linoleate (18:2) (17, 18). Together, these findings suggest that 15-LO represents a possible agent of LDL oxidation in vivo, and this has resulted in interest in the mechanism of 15-LO-initiated LDL oxidation and its prevention.

Previous work (22–25) has demonstrated that significant amounts of hydroperoxides of CE (CE-OOH) and PL, principally those of phosphatidylcholine (PC-OOH), can be formed in LDL exposed to a variety of oxidizing conditions despite the presence of normal levels of α-tocopherol (α-TOH). Under conditions of low radical flux, and in the absence of co-antioxidants (23, 25, 26), lipid peroxidation proceeds in a chain reaction via tocopherol-mediated peroxidation (TMP), initiated by the formation of α-tocopheroxyl radical (α-TO$^{\bullet}$). The latter propagates lipid peroxidation unless eliminated by reaction with a suitable reducing agent (e.g., ascorbate, AH) (26) or another radical (23). Interestingly, α-TO$^{\bullet}$ has been demonstrated by electron spin resonance studies in LDL undergoing oxidation initiated by soybean 15-LO (27), suggesting that this radical could participate in LDL lipid peroxidation. In this report we show that oxidation of CE and PL of human LDL initiated by recombinant human 15-LO (rhLO) is dependent on the presence of α-TOH. We propose that human 15-LO oxygenates LDL's surface and core esterified lipids largely via initiation of TMP.

MATERIALS AND METHODS

Materials

rhLO, prepared as described (17), was a generous gift from Dr. Elliot Sigal (Syntex Discovery Research, Syntex USA Inc., Palo Alto, CA). The specific activity of the enzyme was 9.5 μmol 13-hydro(pero)xy-9*Z*,11*E*-octadecadienoic acid (13-H(P)ODE(*Z*,*E*)) per mg protein and min, assayed with 100 μM 18:2 in phosphate-buffered saline at 4°C. Plasma from a patient with familial isolated vitamin E (FIVE) deficiency was kindly made available to us by Drs. A. Kohlschütter, B. Finckh, and A. Kontush from the Department of Pediatrics, University of Hamburg, Germany. The water-soluble azo peroxyl radical generator, 2,2′-azo*bis*(2-amidinopropane) dihydrochloride (AAPH) was purchased from Polysciences (Warrington, PA). Ch18:2, 18:2, AH, sodium borohydride ($NaBH_4$) and soybean 15-LO were obtained from Sigma (St. Louis, MO), and D,L-α-TOH was from Kodak (Rochester, NY). Hydroxy 13-(S)(*Z*,*E*) and 9-(S)(*E*,*Z*) Ch18:2 and 13-H(P)ODE were purchased from Cayman Chemicals (Ann Arbor, MI)[2] and authentic standards of racemic cholesteryl linoleate hydroxides (Ch18:2-OH) were prepared by vitamin E-controlled autoxidation of Ch18:2 followed by $NaBH_4$ reduction (28). PD-10 Sephadex G-25M columns were from Pharmacia LKB Biotechnology (Uppsala, Sweden). Organic solvents of HPLC quality were obtained from Mallinckrodt Inc (Clayton, VIC, Australia), except diethyl ether (Fluka, Switzerland). Before use, HPLC grade methanol, phosphate-buffered (50 mM, pH 7.4) isotonic saline (PBS) and all other aqueous solutions were stored over Chelex-100® (Bio-Rad Laboratories, Richmond, CA) to remove any contaminating transition metals. All other chemicals were of the highest available purity. Nanopure water (Millipore Systems, Australia) was used throughout.

LDL preparation

LDL was isolated from fresh human, heparinized whole blood derived from healthy volunteers. The density of plasma was adjusted to 1.2 g/ml with KBr and LDL was isolated by ultracentrifugation for 2 h as described previously (29). The LDL (ρ = 1.06 g/ml) was collected and used immediately or stored under argon on ice for <24 h. Before use, low molecular weight, water-soluble contaminants were removed by passage of the LDL solution through two successive PD-10 columns, equilibrated with PBS.

For in vivo α-TOH depleted and supplemented LDL, plasma was obtained from a FIVE patient after 5 days abstinence from vitamin E supplementation (= in vivo α-TOH-depleted) and again after 3 days vitamin E supplementation (400, 1200, and 1800 mg vitamin E for the 3 successive days). Plasma α-TOH in FIVE patients is typically very low unless a daily vitamin E supplementation regime is implemented (30, 31). The FIVE plasma was supplemented with 0.6% (w/v) sucrose and stored frozen (−80°C) until used. Preliminary experiments demonstrated that supplementation of plasma with sucrose, a cryopreservative for lipoproteins, had no effect on the oxidizability of the plasma lipids initiated by 10 mM AAPH (data not shown), consistent with a recent report (32). LDL was prepared from both in vivo vitamin E-depleted and -supplemented FIVE plasma as described for normal, healthy plasma.

Protein concentrations were determined using the bicinchoninic assay and the LDL concentration was esti-

[2]We note that the geometrical naming of the structure of 13-hydroxy (S)(*Z*,*E*) cholesteryl linoleate shown in the Cayman catalogue (1994, p. 81) is incorrect.

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mated using a molecular weight for apolipoprotein B-100 (apoB) of 500,000 Da. Alternatively, the concentration of LDL was estimated by cholesterol determination and assuming 550 molecules of free cholesterol per lipoprotein particle.

In vitro depletion of α-TOH from LDL

In vitro depletion of α-TOH from LDL was achieved by incubation of the lipoprotein (~1 μM in apoB) with 50 mM AAPH for about 20 min at 37°C (33). Under these conditions of high radical flux, LDL's α-TOH is consumed with little formation of lipid hydroperoxides (23). The precise time taken for total α-TOH consumption in individual LDL samples was predetermined (33). LDL containing different fractional contents of α-TOH (F_{TOH}), i.e., LDL with different amounts of remaining endogenous α-TOH, was obtained by removing aliquots of LDL from the reaction at various times within the 20-min period as previously described (33). The reaction with AAPH was halted by placing the sample on ice. Control LDL was prepared by addition of AAPH to LDL kept on ice, a condition under which formation of peroxyl radicals from the thermo-labile azo-initiator is prevented. Endogenous levels of α-TOH were preserved in these control samples, as verified by HPLC determination. AAPH was removed subsequently from the samples by two successive gel filtration passages (PD-10 columns) using cold PBS as eluant. $NaBH_4$ (50 mM) was then added to LDL to reduce the small amounts of lipid hydroperoxides formed during the brief exposure to AAPH, and the $NaBH_4$ was removed by gel filtration of the lipoprotein (2 passages). The resulting LDL was intact (as indicated by behavior during ultracentrifugation indistinguishable from that of native LDL) and free of lipid peroxides, as verified by HPLC post-column chemiluminescence detection (see below).

In vitro α-TOH enrichment of LDL

Enrichment of LDL with α-TOH in vitro was carried out essentially as described previously (34). Briefly, α-TOH in ethanol (<3% (v/v)) was added to plasma to give a final concentration of 33 μM, the plasma was flushed with argon and incubated at 37°C for 4–5 h protected from light. LDL was then isolated by density gradient ultracentrifugation as outlined above. This procedure enriched the α-TOH content of LDL by 2-fold. Replenishment of in vitro α-TOH-depleted LDL with α-TOH was achieved by incubation of LDL (1 μM in apoB) with lipoprotein-deficient plasma (2:1 v/v) and α-TOH (30 μM) for 3 h at 37°C. LDL was then reisolated and subjected to gel filtration (2 passages) before use.

Nonesterified fatty acid analysis of LDL

The amount of free (nonesterified) fatty acid associated with isolated, human LDL was determined using a NEFA C test kit (Wako Pure Chemical Industries, Osaka, Japan). This enzymic method indirectly measures the acylation of coenzyme A by free fatty acids in the presence of added acyl-coenzyme A synthetase. LDL was prepared as described above, gel filtered by passage through 2 PD-10 consecutive columns, and finally concentrated 2 times using Centricon-30 concentrators (Amicon Inc., Beverly, MA).

Oxidation of LDL, Ch18:2 and 18:2 by 15-LO

Oxidation of LDL by LO was carried out using 0.15–0.90 μM rhLO per 0.36–1.54 μM apoB, as specified in the figure legends, and incubation under aerobic conditions at 37°C. Aliquots (25–100 μl) were removed at various times and added to a mixture of 5 ml hexane and 1 ml methanol (containing 0.1% (v/v) acetic acid) for extraction of CE-OOH, neutral lipids, and α-TOH into the hexane and PC-OOH into the aqueous methanol phase. The hexane phase was evaporated to dryness and the residue was redissolved in isopropanol (200 μl) for reversed phase HPLC analyses or in 100 μl heptane for normal phase (NP)-HPLC. The aqueous methanolic phase was used for PC-OOH determinations. For analyses of free fatty acid hydroperoxides (FFA-OOH), 1-ml aliquots of LDL were added to 4 ml $CHCl_3$–CH_3OH 1:1 (v/v), mixed vigorously, followed by further addition of H_2O and $CHCl_3$ (2 ml each). After phase separation, the $CHCl_3$ layer containing FFA-OOH was evaporated and resuspended in 500 μl hexane. Preliminary experiments determined that this method of extraction resulted in recovery of 75 ± 10% (mean ± SD, n = 5) of authentic 13(S) HPODE added to LDL.

Ch18:2 (0.1 mM in PBS containing 0.2% (w/v) sodium cholate) and 18:2 (0.1 mM in PBS alone) were subjected to oxidation by adding 0.05 μM rhLO or 1167 units soybean LO per ml and incubation at 37°C for up to 10 min (both) and 14 h (Ch18:2). The absorbance at 234 nm (conjugated dienes) was recorded using a Hitachi U-3210 Spectrophotometer (Hitachi, Ltd. Tokyo, Japan). The molar extinction coefficient used was 25,000 M^{-1} cm^{-1}. Aliquots (100–200 μl) of the reaction mixture were removed, added to $CHCl_3$–CH_3OH 1:2 (v/v), followed by addition of H_2O and $CHCl_3$ (2 ml each). Phase separation was obtained by centrifugation of the sample at 750 *g* for 10 min (5°C), the $CHCl_3$ phase was collected, evaporated to dryness, and the residue containing linoleate hydroperoxide (18:2-OOH) was redissolved in heptane (100 μl) for NP-HPLC analysis.

HPLC analyses

Unoxidized lipids (free cholesterol and CE), α-TOH, and PC-OOH and CE-OOH (hydroperoxides of Ch18:2 (Ch18:2-OOH) and CE arachidonate) were analyzed by HPLC using UV, electrochemical, and post-column

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chemiluminescence detection, respectively, as described in detail in (29, 35). Standards of CE-OOH and PC-OOH were prepared as described (35). Amounts were quantified by comparison of areas with the appropriate standards. FFA-OOH in LDL was measured by HPLC with post column chemiluminescence detection as described previously (35) using methanol–*tert*-butyl alcohol 1:1 (v/v), 1 ml/min, and an LC-18-DB column (25 × 0.46 cm, 5 μm). The amounts of FFA-OOH were determined using authentic 13(S)-HPODE as a standard.

NP-HPLC was utilized for the analysis of the four geometrical Ch18:2 hydroperoxides (Ch18:2-OOH) and corresponding hydroxides (Ch18:2-OH) produced in either LDL or Ch18:2 samples (36). Saponification of the oxidized CE was not needed, with Ch18:2-OOH and Ch18:2-OH being analyzed directly on a silica column (LC-Si, 25 × 0.46 cm, 5 μm, Supelco) eluted with heptane–diethyl ether–isopropanol 100:0.5:0.175 (v/v/v) at 2 ml/min and monitored at 234 nm using an ABI 1000S diode array detector (Applied Biosystems, Australia). In some LDL samples, CE-OOH were reduced with 50 mM $NaBH_4$ prior to extraction and analysis, as all four Ch18:2-OH isomers were baseline separated whereas the 9-(*E,E*) and 9-(*E,Z*) Ch18:2-OOH isomers co-eluted using the above NP-HPLC method. Slight variation in retention times of the different isomers was observed between batches of solvent. Therefore authentic standards of 13-(S)(*Z,E*) and 9-(S)(*E,Z*) Ch18:2-OH (in heptane) were subjected to chromatography for each set of experiments to allow unambiguous assignment of the different isomers. The different isomers were quantified by peak area comparison with authentic standards. Hydroperoxides and hydroxides of 18:2 were also analyzed in some instances by NP-HPLC with 234 nm detection. The solvent system used for these extracts consisted of heptane–diethyl ether–isopropanol–acetic acid 100:10:0.9:0.1 (v/v/v/v) at 1 ml/min as described previously (37).

RESULTS

The extent of oxidation of isolated human LDL initiated by rhLO was linearly dependent on the amount of enzyme for a given concentration of LDL (1.54 ± 0.35 μM in apoB), as judged by the accumulation of CE-OOH (**Fig. 1**), qualitatively the major lipid oxidation product in peroxidizing LDL. Thus, CE-OOH accumulated linearly with rhLO increasing from 0 to 0.9 μM (*r* value of the fitted line = 0.995; Fig. 1 inset). In samples containing no enzyme (Fig. 1), or containing heat-inactivated rhLO (90°C/10 min; data not shown), significant oxidation of LDL was not observed, as judged by the absence of detectable CE-OOH for at least 8 h.

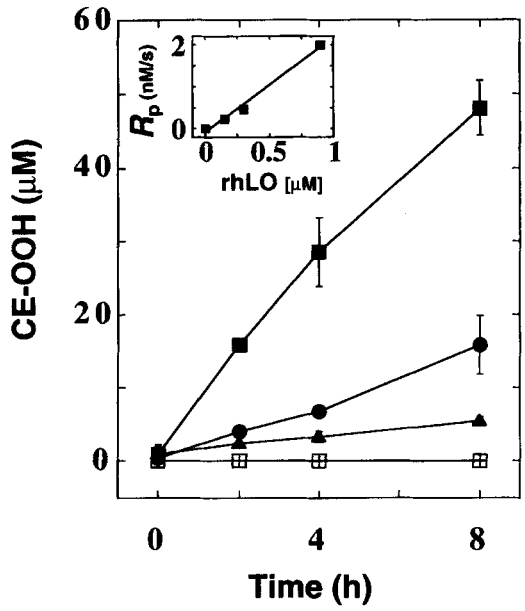

Fig. 1. Oxidation of CE in LDL initiated by rhLO. LDL (1.54 ± 0.35 μM in apoB) was incubated with increasing amounts of rhLO at 37°C for up to 8 h. At various times a 50-μl aliquot of the reaction mixture was removed and analyzed for CE-OOH as described in the Methods section. The amounts of enzyme used were; no enzyme (⊞), 0.15 μM (▲), 0.30 μM (●), and 0.90 μM (■) rhLO. The rate of accumulation of CE-OOH in LDL (R_p) as a function of [rhLO] is shown in the inset. The r-value of the fitted line in the inset = 0.995. The results shown represent mean values ± SD derived from 3 separate experiments using LDL from 3 donors. Where SD cannot be seen, they are smaller than the size of the symbols. The initial α-TOH concentration was 11.1 ± 1.0 μM.

The amount of endogenous α-TOH significantly influenced the extent to which CE-OOH and PC-OOH accumulated upon exposure of LDL to rhLO (Figs. 2–4). Thus, LDL obtained from a FIVE patient whilst abstaining from α-TOH supplements (i.e., in vivo α-TOH-depleted LDL) was substantially depleted of endogenous α-TOH and only small amounts of CE-OOH accumulated when the lipoprotein was incubated with rhLO for up to 8 h (**Fig. 2**). In contrast, LDL isolated from the same patient after 3 ensuing days of vitamin E supplementation contained large amounts of α-TOH, and its CE readily oxidized under the same conditions, as judged by the time-dependent accumulation of CE-OOH (Fig. 2). In vivo supplementation with vitamin E also restored the oxidizability of LDL's surface PL by rhLO, as judged by the time-dependent accumulation of PC-OOH in the supplemented versus vitamin E-deficient LDL (data not shown). As CE-OOH accumulated in rhLO-oxidized LDL, the α-TOH levels of the in vivo supplemented lipoprotein declined linearly to ~70% of the initial value (Fig. 2).

The in vivo α-TOH-depleted and α-TOH-supple-

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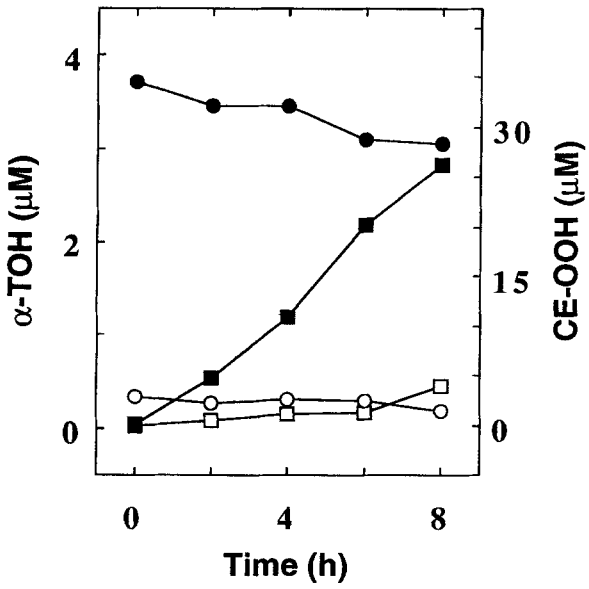

Fig. 2. rhLO readily initiates the oxidation of CE in α-TOH-containing but not in vivo α-TOH-depleted LDL. LDL was obtained from the plasma of a FIVE patient receiving (closed symbols) or not receiving oral supplementation with vitamin E (open symbols). α-TOH-depleted and α-TOH-containing LDL (0.39 ± 0.1 and 0.36 ± 0.13 μM in apoB respectively) were incubated at 37°C with rhLO (0.43 μM). At various times a 25-μl aliquot of the reaction mixture was removed and analyzed for CE-OOH (squares) and α-TOH (circles) as described in the Methods section. The results shown are mean values from 2 separate experiments using two different LDL preparations (with the extent of variation ranging from 11–29% of the values shown). Separate plasma samples containing in vivo α-TOH-depleted and α-TOH-supplemented LDL were obtained from the same FIVE patient in two collection periods ~1 year apart.

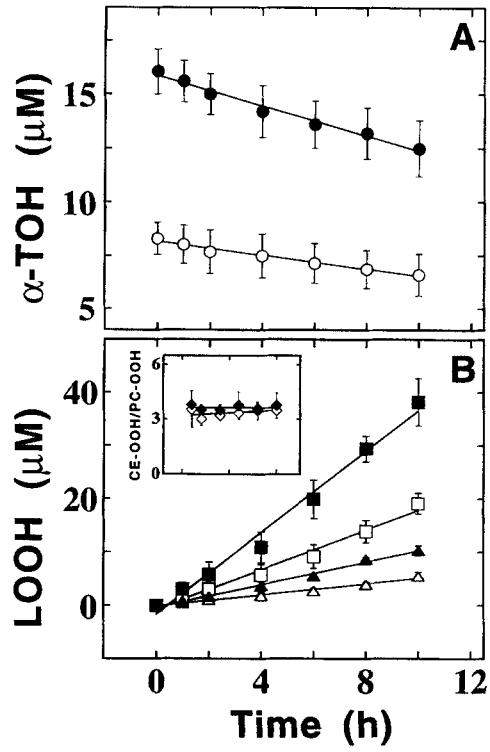

Fig. 3. In vitro enrichment of LDL with α-TOH increases the oxidizability of the lipoprotein's CE and PL by rhLO. Native (open symbols) and α-TOH-enriched (filled symbols) LDL (1.1 μM apoB), obtained as described in the Methods section, were incubated at 37°C with rhLO (0.15 μM) for up to 10 h, and aliquots were analyzed at various times for α-TOH (A, circles), CE-OOH (B, squares), and PC-OOH (B, triangles). The inset shows the ratio of CE-OOH/PC-OOH accumulation in both the enriched (◆) and native (◇) samples. The results show mean values ± SD of 3 separate experiments with 3 different LDL donors and the extent of variation is indicated by the error bars.

mented LDL samples used for the above experiments were obtained from the same patient on two separate occasions, 1 year apart. As the FIVE deficiency syndrome in humans is very rare, plasma and hence LDL from such patients is scarce. We therefore prepared in vitro α-TOH-depleted and α-TOH-enriched LDL (see Methods) to substantiate and extend the above findings. LDL enriched in vitro with α-TOH to ~twice the vitamin E content of native LDL accumulated ~2-fold increased amounts of CE-OOH and PC-OOH compared to native LDL exposed to rhLO (**Fig. 3B**). The increased rates of CE-OOH and PC-OOH accumulation were accompanied by correspondingly increased rates of α-TOH consumption (Fig. 3A). Throughout the incubation, the ratio of accumulating CE-OOH to PC-OOH was 3–4 for both the native and in vitro α-TOH-enriched LDL (Fig. 3B, inset). These results indicate that esterified core and surface lipids oxidized at comparatively relative rates upon exposure of LDL to rhLO; LDL contains ~3-fold more bisallylic hydrogen groups in its CE than PL (see ref. 38 and references therein), and these moieties are the most readily oxidizable lipid substrates of LDL.

The above results suggested a link between α-TOH consumption and formation of hydroperoxides in esterified lipids during rhLO-initiated LDL oxidation. To provide more direct evidence for this, LDL containing differing amounts of endogenous α-TOH was exposed to a constant concentration of rhLO. The extent of lipid peroxidation in LDL decreased with decreasing F_{TOH}, as judged by CE-OOH formation (**Fig. 4**). In fact, the rate of accumulation of CE-OOH in the different LDL populations was directly proportional to F_{TOH} (r-value of the fitted line = 0.998; Fig. 4 inset). Also consistent with the above, CE in LDL totally depleted of α-TOH in vitro were totally resistant to rhLO-initiated oxidation (0.9 μM apoB, 0.32 μM rhLO, 8 h at 37°C), and replenishment of the vitamin to its original level (7.5 μM) restored the oxidizability of CE in such LDL to rhLO (data not shown) and soybean 15-LO (33).

The results presented in Figs. 2–4 are inconsistent with direct oxidation of LDL's CE and PL by rh-LO. By

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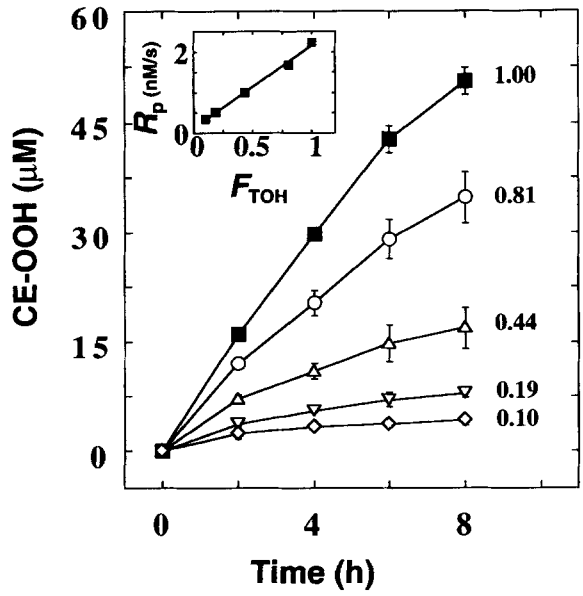

Fig. 4. Oxidation of LDL's CE initiated by rhLO is dependent on and directly proportional to the α-TOH content of the particle. LDL (0.59 ± 0.07 μM in apoB) containing different amounts of endogenous α-TOH were exposed to rhLO (0.32 μM) at 37°C. The initial α-TOH concentration (100% value) was 4.05 ± 0.23 μM. Aliquots were removed at various times up to 8 h and analyzed for CE-OOH and α-TOH. The numbers to the right of the lines represent different F_{TOH}, e.g., 0.81 corresponds to 81% of the initial endogenous α-TOH. The inset shows the dependence of the CE peroxidation rate (R_p) on LDL's F_{TOH}. The r-value of the fitted line in the inset = 0.998. The data (mean values ± SD) were derived from 3 experiments using 3 different sources of LDL.

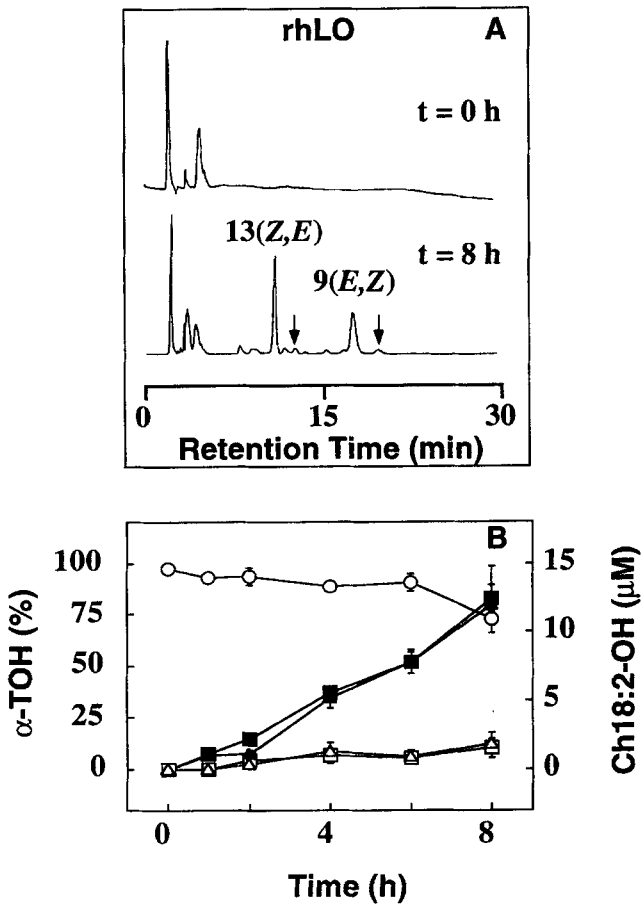

Fig. 5. Analysis of geometric isomers of Ch18:2-OH from LDL oxidation initiated by rhLO. Native LDL (1.30 ± 0.38 μM in apoB) was incubated with rhLO (0.30 μM) for up to 8 h at 37°C. Aliquots of the reaction mixtures were removed at various times then reduced with $NaBH_4$ and the lipids were extracted and analyzed by NP-HPLC as described in the Methods section. Treatment with $NaBH_4$ resulted in reduction of the Ch18:2-OOH isomers to the corresponding Ch18:2-OH isomers. A: chromatographic profile of LDL's Ch18:2-OH isomers present at 0 and 8 h, with the major oxidation products being the 13-(*Z,E*) and 9(*E,Z*)-Ch18:2-OH, and the minor products (13- and 9(*E,E*)Ch18:2-OH) indicated by the arrows. B: Time-dependent accumulation of 13-(*Z,E*) (■) and 9(*E,Z*)-(▲) and 13(*E,E*)-(□) and 9(*E,E*)Ch18:2-OH (△) isomers during rhLO-initiated oxidation of LDL. α-TOH (○) was present initially at 9.5 ± 1.9 μM. The data shown in B are mean values ± SD of 4 separate experiments using 4 individual LDL preparations. Note that although the area of the 13(*Z,E*)Ch18:2-OH in the 8 h trace shown in A is 16% larger than that of the 9(*E,Z*)-isomer, the SD of the corresponding time point shown in B is 19.7%, so that there are no significant differences in the levels of the two isomers.

contrast, the results are consistent with a role for TMP (23; see Introduction), i.e., that a substantial part of the lipid peroxidation process in LDL is mediated by α-TOH. We therefore determined the geometrical isomers of CE-OOH produced by rhLO in α-TOH-containing LDL and compared them to those found in LDL oxidized by a low flux of aqueous peroxyl radicals, where TMP is the predominant mechanism responsible for lipid peroxidation (22, 23, 39). For this we analyzed LDL's geometrical isomers of Ch18:2-OH, derived from the Ch18:2-OOH isomers, formed over time in the presence of either rhLO or AAPH (see Methods). As can be seen, the NP-HPLC method clearly separated all four Ch18:2-OH isomers (**Fig. 5** and **Fig. 6**). The patterns of isomer formation were comparable for AAPH and rhLO as long as LDL contained α-TOH: similar amounts of the 9-(*E,Z*)Ch18:2-OH and 13-(*Z,E*)Ch18:2-OH isomers were detected for each oxidant, with only traces of 9- and 13-(*E,E*)Ch18:2-OH (Figs. 5B and 6B). Oxidation of LDL's CE by AAPH (1 mM) proceeded faster than that initiated by rhLO (0.3 μM), as seen by the increased rate of α-TOH consumption in the former, and the resulting shorter period of time during which the (*E,Z*) and (*Z,E*) isomers were detected in preference over the (*E,E*) isomers. Only after complete α-TOH consumption in AAPH-oxidizing LDL did we observe larger amounts of 9- and 13-(*E,E*)Ch18:2-OH (Fig. 6B). This similarity in the Ch18:2-OH isomer patterns in vitamin E-containing lipoprotein was suggestive of a common pathway of oxidation of LDL's CE with both AAPH and rhLO as the oxidants which

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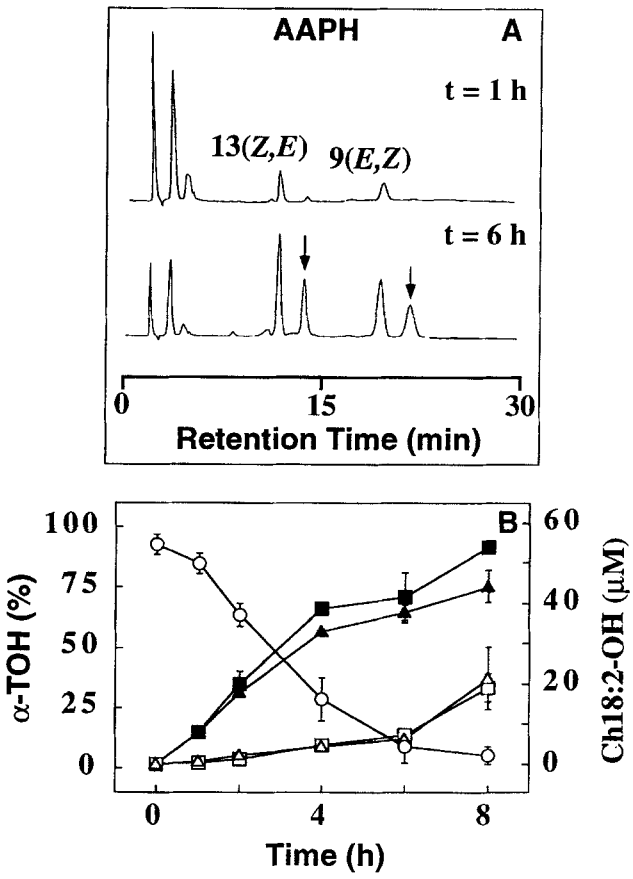

Fig. 6. Formation of geometric isomers of Ch18:2-OH during LDL oxidation initiated by AAPH. Native LDL (1.24 ± 0.46 μM) was incubated as for Fig. 5, except that AAPH (1 mM) was used as the oxidant. A: Representative chromatograms of Ch18:2-OH isomers produced while α-TOH was present (i.e., 1 h) and after α-TOH depletion (6 h). The different isomers are indicated as per the legend for Fig. 5. B: Time-dependent accumulation of the various Ch18:2-OH isomers during AAPH-initiated oxidation of LDL. α-TOH was present initially at 9.7 ± 1.4 μM. Symbols are as described in the legend to Fig. 5. The data represent the mean values ± SD of 3 separate experiments. LDL was obtained from 3 different donors.

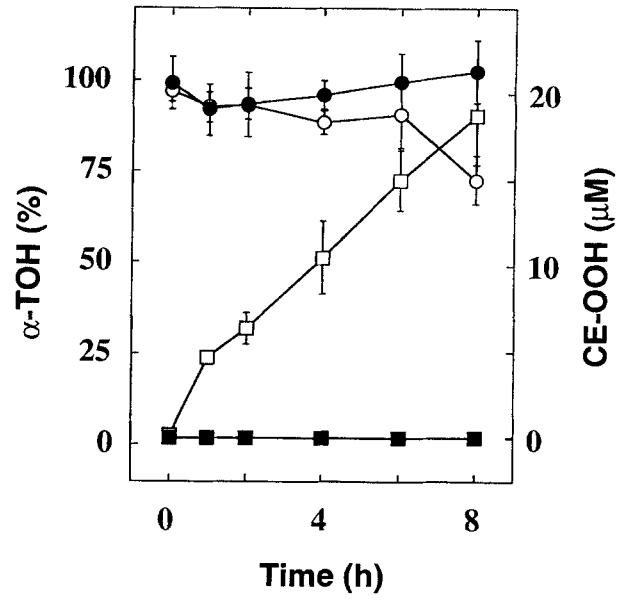

Fig. 7. Ascorbate (AH) efficiently inhibits rhLO-initiated peroxidation of LDL's CE. LDL (1.25 ± 0.29 μM in apoB) oxidation initiated by rhLO (0.33 μM) was carried out at 37°C in the presence (closed symbols) or absence (open symbols) of 50 μM AH. At various times, aliquots (50 μl) of the reaction mixture were removed and CE-OOH formation (squares) and the consumption of α-TOH (circles), initially present at 8.9 ± 2.0 μM, followed as described in the Methods section. The results depicted represent mean determinations and SD from 3 separate experiments using 3 different LDL preparations.

could be explained if α-TO• was the propagating radical for lipid peroxidation in both circumstances. In contrast to the situation with LDL's CE, and in support of previous work by others (40), oxidation of 18:2 with rhLO in the absence of LDL gave 13-(*Z,E*) 18:2-OOH as the predominant and typical enzymic product, with comparatively much smaller amounts of the 9-(*E,Z*)18:2-OOH and other isomers formed (data not shown).

To further test whether oxidation of LDL's CE by rhLO could be largely mediated via a free radical (α-TO•) mechanism rather than via a direct reaction, we examined the effect of ascorbate (AH) on this process. AH is known to reduce α-TO• to α-TOH in oxidizing LDL (26), thereby inhibiting TMP (22). The results in **Fig. 7** show that 50 μM AH completely prevented both α-TOH consumption and CE-OOH accumulation in LDL exposed to rhLO. In contrast, AH did not inhibit the enzyme, as indicated by identical rates of conjugated diene formation during the oxidation of 18:2 by rhLO **(Fig. 8)** or soybean LO (data not shown), in both the presence or absence of AH. The product responsible for the absorbance increase at 234 nm during these reactions was 13-(*Z,E*) 18:2-OOH, as confirmed by NP-HPLC (data not shown). Up to 18 μM 13-(*Z,E*) 18:2-OOH were formed in 10 min at 37°C under the conditions described in Fig. 8. Thereafter, no further change in absorbance at 234 nm was detected, most likely due to suicide inactivation of the enzyme (17, 18). The rapidity of this enzymic reaction was in contrast to the slow reaction of soybean LO (data not shown) or rhLO with isolated Ch18:2 (Fig. 8) or LDL's Ch18:2 (Figs. 1–5 and 7) (33). There was no detectable increase in 234 nm absorbance for up to 10 min when Ch18:2 in PBS containing 0.2% (w/v) cholate was used as substrate (Fig. 8); after 14 h at 37°C only 0.8 μM Ch18:2-OOH was formed. Thus, rhLO did not readily oxidize isolated Ch18:2.

LDL may contain free (nonesterified) fatty acids (41) and these could act as preferred substrates. We therefore determined the content of nonesterified fatty acids in isolated, human LDL and found it to be 9.1 ± 3.2 μM/μM apoB (mean value ± SD, n = 4). We next deter-

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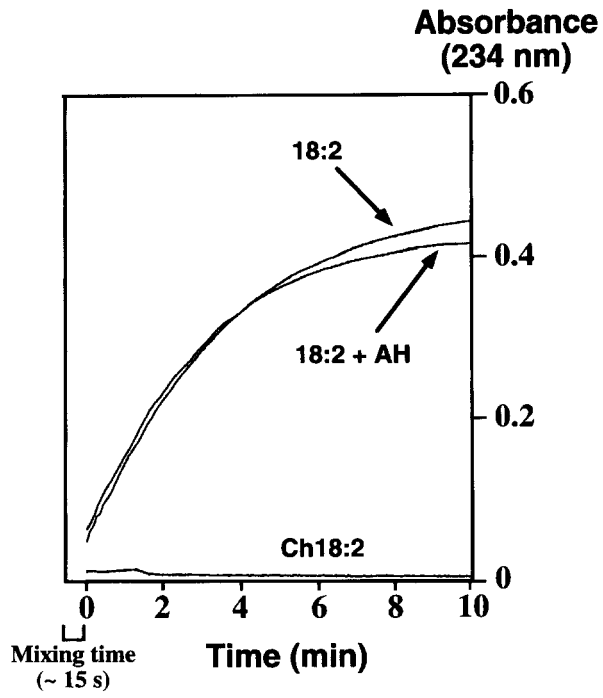

Fig. 8. Oxidation of linoleic acid (18:2) and cholesteryl linoleate (Ch18:2) by rhLO: Effect of AH on 18:2 oxidation. Suspensions of 18:2 (0.1 mM in PBS) and Ch18:2 (0.1 mM in PBS containing 0.2% (w/v) sodium cholate) were prepared in 1 ml quartz cuvettes maintained at 37°C. The reactions were started by addition of rhLO (0.05 μM) and the absorbance at 234 nm followed for 10 min as an indication of conjugated diene production. Where indicated, 50 μM AH was included before addition of the enzyme; AH was also added to the blank. Note that conjugated dienes were not detected over the time period examined when Ch18:2 was used as substrate. The lines shown are representative of 3 separate experiments.

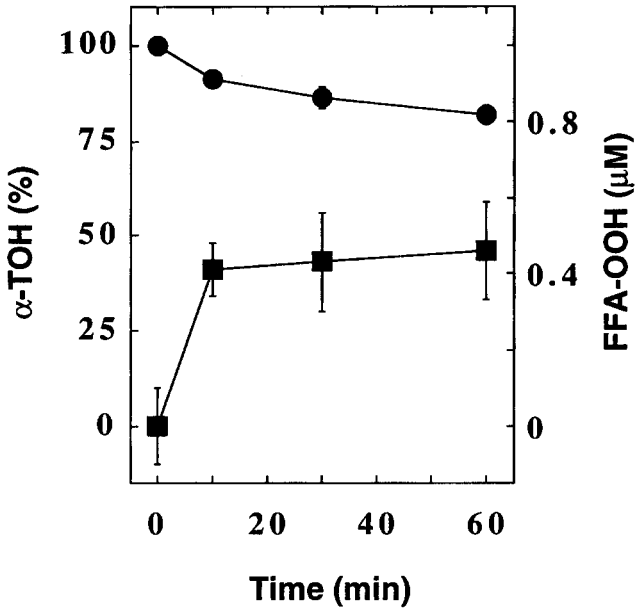

Fig. 9. Accumulation of FFA-OOH in native LDL during oxidation initiated by rhLO. Aliquots (1 ml) of isolated, human LDL (0.8 ± 0.1 μM in apoB) were incubated with rhLO (1.5 μM) at 37°C for various periods of time. Lipids were extracted and analyzed for α-TOH (●) and FFA-OOH (■) as described in the Methods section. α-TOH was initially present at 6.8 ± 1.2 μM. The results show mean values ± SD and represent data from 4 separate experiments using LDL from 4 individual donors.

mined whether oxidation of LDL by rhLO caused formation of FFA-OOH (see Methods). Incubation of LDL with rhLO indeed resulted in formation of FFA-OOH (**Fig. 9**). As expected, only small amounts of these hydroperoxides were detected, and relatively large amounts of rhLO were required for their detection. Also FFA-OOH accumulated in LDL non-linearly, with ~90% being formed within the first 10 min of oxidation (Fig. 9). The FFA-OOH formed in rhLO-oxidizing LDL co-eluted with authentic 13(S) HPODE, as verified by NP-HPLC analysis (data not shown), suggesting that isolated, native LDL contained 18:2, the preferred substrate for rhLO. The amount of 18:2-OOH which accumulated over 1 h represented ~7% of the total free fatty acids present in isolated LDL.

DISCUSSION

There is increasing evidence in the literature that closely associates 15-LO activity with in vivo LDL oxidation and the development of atherosclerosis (7–9, 11, 14). Oxidation of LDL's PL and CE by 15-LO has been previously suggested to occur via a direct action of the enzyme on the esterified core and surface lipids (16–19). We report herein, however, that the extent to which CE-OOH and PC-OOH were formed in LDL exposed to rhLO depended on and directly related to the α-TOH content of the lipoprotein. Also, the pattern of positional isomers of oxidized Ch18:2 formed during the initial stages of LDL oxidation was virtually identical to that found in non-enzymic, peroxyl radical oxidizing LDL, that proceeds via TMP. Furthermore, rhLO-initiated oxidation of LDL's CE was inhibited completely by AH, which is a known anti-TMP reagent (23, 26, 42), and which itself did not inhibit the enzyme. These findings are inconsistent with a direct oxidation of LDL's esterified lipids by rhLO, whilst supporting a role of α-TOH and TMP in the oxidation of these lipids.

We have shown previously that α-TOH is a pro-oxidant for LDL under conditions where α-TO• is formed and allowed to react with the lipoprotein's polyunsaturated lipids (22–25, 33, 39). The observation that CE and PL of naturally or chemically α-TOH-depleted LDL were largely resistant to oxidation initiated by rhLO (this study) or soybean LO (33), and further that in vivo or in vitro supplementation of such LDL with vitamin E restored lipid oxidizability, strongly suggested that 15-

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LO-induced oxidation of LDL's esterified lipids was largely dependent on α-TOH. Interestingly, biomembranes also show an enhanced susceptibility to oxygenation by rabbit reticulocyte 15-LO in the presence of α-TOH (43). That is, extraction of α-TOH from beef heart submitochondrial particles almost totally abolished lipid oxygenation initiated by 15-LO, whereas replenishment of the membranes with α-TOH fully restored their oxidizability (43). This suggests that the requirement of 15-LO for α-TOH to oxidize esterified lipids is not restricted to LDL, but may extend to other complex biological materials, such as membranes.

Previous studies have established that α-TO$^{\bullet}$ is formed upon exposure of LDL to LO (27). We now demonstrate that the relative peroxidizability of surface and core lipids are comparable (Fig. 3B, inset) in rhLO-oxidizing LDL. Together, these findings are consistent with α-TO$^{\bullet}$ being the lipid peroxidation-propagating radical (23, 39). They do not support a direct oxidation of LDL's esterified lipids by rhLO, in which case we would have expected PL on the surface of LDL to be oxidized at relatively higher rates than CE, the great majority of which are present in the core of LDL, and hence likely inaccessible to the enzyme.

The above findings appear to be in contrast to a recent report by Ezaki, Witztum, and Steinberg (13), where CE-OOH formation in LDL exposed to fibroblasts over-expressing 15-LO was inhibited by enrichment of the particle with α-TOH. However, this apparent difference may be explained by the different experimental conditions used. For example, in the more complex, cellular study of Ezaki et al. (13), the LDL may have acquired free fatty acids from the 15-LO-transfected cells. This is supported by the fact that the relative contribution of FFA-OOH to the total lipid hydroperoxides detected in (13) was at least 10-fold higher than that observed in the present study. A higher LDL content of free fatty acids is expected to result in a higher flux of radicals in LDL incubated with 15-LO (see below). Indeed, in vitro enrichment of LDL with 18:2, or inclusion of phospholipase A_2, greatly accelerates rhLO-induced loss of LDL's α-TOH (J. Neuzil, J. M. Upston, and R. Stocker, unpublished observations), indicative of an increased radical flux. Unfortunately, α-TOH consumption was not reported in (13), so that the radical fluxes used are not known. What is known, however, is that when LDL is exposed to a high flux of radicals, the lipid peroxidation chain transfer part of TMP is prevented and α-TOH acts as an antioxidant (so that little lipid hydroperoxides accumulate as long as it or other phenolic compounds are present) (23, 33). In contrast, the radical fluxes used in the present study are mild, as judged by the rate of α-TOH loss.

Previous work on the oxidation of LDL by LO focused on rabbit reticulocyte 15-LO (16–19) and showed that during the earliest stages of LDL oxidation 13-H(P)ODE(*Z,E*) was the predominant lipid oxidation product, of which the S enantiomer was produced at higher levels than the corresponding R stereoisomer. As both the regio- and stereo-specificity in the oxidation products are the typical and necessary characteristics of LO-mediated lipid oxidation (44), Belkner et al. (16), Kühn and colleagues (17, 18), and Lass et al. (19) concluded that the initial oxidation of LDL's CE and PL was enzymic. Interestingly however, the regio- and stereo-specificity of LDL oxygenation in each case was notably lower than that of free polyenoic fatty acids even at the earliest time points taken. By contrast, we observed equal amounts of 13- and 9-hydro(pero)xy Ch18:2 from the onset of LDL oxidation by rhLO (Fig. 5). As Belkner et al. (16) reported similar specificity for rabbit reticulocyte LO and rhLO, differences in the enzyme used appear unlikely to explain this discrepancy. However, we directly analyzed the positional isomers of Ch18:2-O(O)H, whereas Belkner et al. (16) saponified LDL lipids before analysis, thereby allowing for the possibility of FFA to contribute to the oxidation products measured. This could be important as isolated LDL contains FFA (41, this study), some of which are preferred substrates for LO (21). For example, in one study (18), Kühn et al. reported that after 15 min of oxidation of LDL, 59% of the oxidized linoleate were present as 13-HODE(*Z,E*) and up to 15% of the oxidized fatty acids detected were derived from non-esterified lipids in LDL. Assuming that all of these non-esterified lipids contributed to the 13-HODE detected, all of the regiospecific oxidation products detected can be accounted for by FFA, as during these early stages of LDL oxidation only two positional isomers are formed (see below). Similar arguments can be put forward to at least partly explain the observed stereospecificity during the early stages of 15-LO-induced LDL oxidation (16). Also, where oxidized CE produced in 15-LO-exposed LDL was isolated and characterized (16), the collected fraction clearly contained several products, making unambiguous assignment of product specificity difficult.

The above considerations suggest that our findings do not necessarily contradict previous reports (16–19), and that a potential contribution of FFA to the enzymic lipid oxidation products in LO-oxidized LDL requires careful re-evaluation. What is clear is that the proposed direct oxidation of LDL's CE and PL cannot explain several of the findings reported here (see above). An additional argument against direct oxidation of esterified fatty acids is that the length of the substrate cavity of soybean LO-1, a homolog of rhLO, corresponds to ≈1.5 times the length of all *trans* arachidonate (45). Thus, the fatty acid (moiety) may need to "penetrate"

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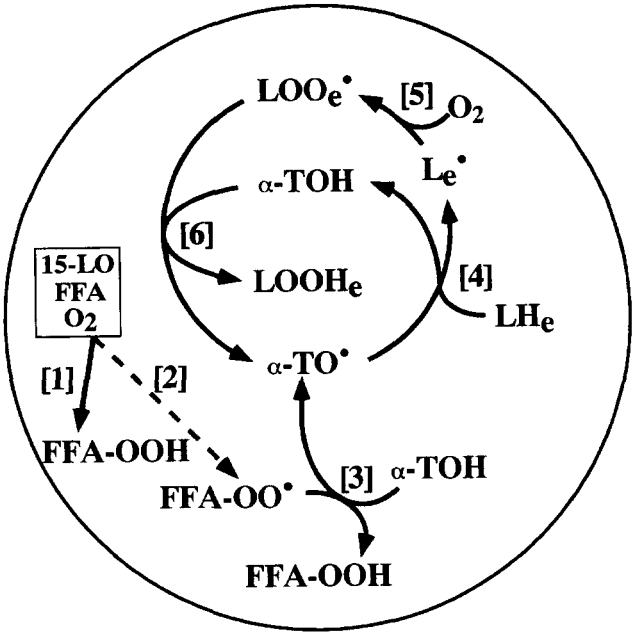

Fig. 10. Proposed model for the involvement of α-TOH and free fatty acids in 15-LO-initiated oxidation of LDL's esterified lipid, CE and PL. The initial oxidation by 15-LO of free fatty acids (FFA) associated with LDL leads to the stereospecific production of FFA-OOH (reaction [1]). FFA peroxyl radicals (FFA-OO•) are released as by-products (reaction [2], broken line) of this enzymic reaction (48). As the most reactive component of LDL, α-TOH rapidly scavenges FFA-OO•, leading to the formation of FFA-OOH and α-TO• (reaction [3]). The latter initiates the peroxidation of esterified lipid containing bisallylic hydrogens (LH_e, where "e" represents esterified) with production of the corresponding carbon-centered lipid radical ($L_e^{\bullet}$) and regeneration of α-TOH (reaction [4]). In the presence of O_2, lipid peroxyl radicals are formed ($LOO_e^{\bullet}$) (reaction [5]) which regenerate the peroxidation chain carrying α-TO• whilst $LOOH_e$ accumulate (reaction [6]). If a second radical enters the LDL particle, it will rapidly combine with α-TO• resulting in termination of TMP and a loss of α-TOH (not shown in model). Reactions [4–6] form part of TMP (23) and provide an explanation for the observed relationship between α-TOH content and esterified core and surface lipid peroxidation in LDL exposed to 15-LO.

the enzyme to reach the iron of the active site. If so, it does not seem likely to us that CE or PL are oxidized readily and directly by rhLO if they are presented in an intact lipoprotein particle that contains free fatty acids.

The pattern of Ch18:2-O(O)H isomers formed during rhLO-induced LDL oxidation is identical to that for α-TOH-controlled autoxidation of polyunsaturated lipids (28). That is, in triglyceride and arachidonic acid autoxidation, the presence of α-TOH causes exclusive production of *E,Z*-conjugated diene monohydroperoxides, while effectively suppressing the formation of *E,E* isomers and secondary oxidation products. This activity of α-TOH is based on its ability to efficiently donate a hydrogen atom to a lipid peroxyl radical, thereby preventing β-scission which gives rise to the *E,E* isomers of lipid hydroperoxides (46). The fact that 13-(*Z,E*) Ch18:2-O(O)H and 9-(*E,Z*) Ch18:2-O(O)H isomers were produced in equimolar amounts during both rhLO- and non-enzymic LDL oxidation is in accordance with a recent finding of Kenar et al. (47).

In an attempt to reconcile the present findings and previous literature reports, we propose the following model (**Fig. 10**): upon exposure to LDL, rhLO acts on FFA, leading to the formation of stereospecific FFA-OOH (Fig. 9). It has been demonstrated previously that FFA-derived peroxyl radicals (FFA-OO•) are formed and released as by-products during the enzymic action of LO (48). In addition or alternatively, lipid-derived radicals could be formed from interaction of rhLO with FFA-OOH (49; not shown in model). In any case, we propose that these secondary radicals are scavenged rapidly by α-TOH, thereby giving rise to α-TO• (Fig. 10), which has been detected in 15-LO-oxidizing LDL (27). Once formed, the α-TO• is responsible for the formation of CE-OOH and PL-OOH, by initiating and carrying the chain of lipid peroxidation of LDL's esterified lipids (23, 39, Fig. 10).

From the above model we predict that bolstering the antioxidant defences against TMP, such as those described previously (26, 50), may represent a useful approach to inhibit 15-LO-induced LDL oxidation, in addition to the direct inhibition of the enzyme. This could contribute to the prevention of atherogenesis if 15-LO participates in the in vivo oxidation of LDL.

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