Secretory Phospholipase A_2 and Lipoprotein Lipase Enhance 15-Lipoxygenase-Induced Enzymic and Nonenzymic Lipid Peroxidation in Low-Density Lipoproteins†

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ABSTRACT: The oxidation of low-density lipoprotein (LDL) is thought to contribute to atherogenesis. 15-Lipoxygenase (15LO) induces LDL oxidation, and phospholipase A_2 enhances this process [Sparrow, C. P., Parthasarathy, S., and Steinberg, D. (1988) *J. Lipid Res. 29,* 745−753]. As the underlying mechanism of the enhancing effect has not been investigated previously, we here show that in the presence of soybean 15LO (SLO) or human 15LO (rhLO), the addition of lipoprotein lipase, porcine pancreatic, or human type IIa secretory phospholipase A_2 (sPLA$_2$) greatly enhanced the accumulation of hydro(pero)xides of all major classes of LDL's lipids. Hydroperoxides of free fatty acids accumulated exclusively as enzymic products with kinetics reflecting both the formation of free fatty acids and the initial 'build-up' of α-tocopheroxyl radical. In contrast, hydroperoxides of cholesteryl esters and phosphatidylcholine accumulated linearly over comparatively longer periods of time and, in the case of rhLO, well beyond inactivation of the oxygenase. With SLO, formation of oxidized esterified lipids occurred nonenzymically, independent of the presence of lipase and despite the oxygenase remaining active until the end of the incubation. Enhancement of rhLO-induced LDL lipid peroxidation by sPLA$_2$ was eliminated by a neutralizing anti-sPLA$_2$ antibody, indicating that lipolytic activity was required for this effect. LDL depleted of α-tocopherol was resistant to oxidation by 15LO alone, whereas lipase overcame this resistance, demonstrating that lipases enhance 15LO-induced enzymic and nonenzymic peroxidation of LDL lipids. This is likely due to provision of free fatty acid substrate, resulting in an enhanced rate of free radical formation which itself causes nonenzymic peroxidation of esterified lipids. As lipases and 15LO are present in atherosclerotic lesions, our findings could be of pathophysiological significance.

Oxidation of low-density lipoprotein (LDL)[1] is thought to be an important early event in atherogenesis (*1*). 15-Lipoxygenase (15LO) is a potential in vivo oxidant for LDL, as the enzyme and oxidized LDL co-localize in atherosclerotic lesions (*2*), '15LO-specific' products are detected in early human lesions (*3*, *4*), transfer of the 15LO gene into rabbit iliac arteries results in the appearance of oxidized lipid−protein adducts characteristic of oxidized LDL (*5*), and administration of a specific, nonantioxidant inhibitor of 15LO reduces atherosclerosis in cholesterol-fed rabbits (*6*). Also, exposure to human fibroblasts overexpressing 15LO results in increased formation of peroxides of esterified lipids in LDL (*7*, *8*), and disruption of 12/15LO activity in macrophages decreases their ability to oxidize LDL in vitro (*9*).

We recently described and verified tocopherol-mediated peroxidation (TMP) as a model for radical-initiated oxidation of lipids in α-tocopherol (α-TOH)-containing LDL (*10*). In this model, α-TOH acts as both a phase- and chain-transfer agent, resulting in pro- or antioxidant activity depending on the radical flux to which LDL is exposed (*10*, *11*) and whether co-antioxidants are present (*12*). A striking feature of TMP is that even in the absence of co-antioxidants and under mild oxidizing conditions, α-TOH-depleted LDL is resistant to oxidation initiated by certain oxidants, including soybean (SLO) (*11*, *13*). It has been proposed (*13*) that these oxygenases initially oxidize free fatty acids (FFA) present in LDL, that FFA peroxyl radicals (FFA-OO•) released from the active site of the enzyme (*14*) convert α-TOH to α-tocopheroxyl radical (α-TO•), and that α-TO• subsequently oxidizes core cholesteryl esters and surface phospholipids via TMP.

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[1] Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; apoB, apolipoprotein B-100; CE-O(O)H, cholesteryl ester hydro(pero)xides; Ch18:2-O(O)H, cholesteryllinoleate hydro(pero)xides; FFA, free fatty acids; FFA-OOH, free fatty acid hydroperoxides; FFA-OH, free fatty acid hydroxides; FFA-OO•, free fatty acid peroxyl radical; 15-H(P)ETE, 15-hydro(pero)xyeicosatetraenoic acid; H(P)ODE, hydro(pero)xyoctadecadienoic acid; LDL, low-density lipoprotein; 15LO, 15-lipoxygenase; LPL, lipoprotein lipase; PC-OOH, phosphatidylcholine hydroperoxides; PLA$_2$, phospholipase A_2; pPLA$_2$, pancreatic (type I) phospholipase A_2; ROO•, peroxyl radical; rhLO, recombinant human 15-lipoxygenase; SLO, soybean lipoxygenase-1; sPLA$_2$, nonpancreatic secretory human (type IIa) phospholipase A_2; TMP, tocopherol-mediated peroxidation; α-TOH, α-tocopherol; α-TO•, α-tocopheroxyl radical.

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This model predicts that the rate and extent of 15LO-induced oxidation of esterified lipids is dependent on LDL's content of FFA. LDL isolated from human plasma by density ultracentrifugation contains between 10 and 50 molecules of FFA per particle (*13*, *15*, *16*), although FFA increase substantially in the presence of lipases. We show here that phospholipase A_2 or lipoprotein lipase (LPL) significantly increases the extent of *nonenzymic* oxidation of LDL's esterified lipids and overcomes the resistance of α-TOH-depleted LDL to 15LO-induced oxidation.

EXPERIMENTAL PROCEDURES

Materials

SLO (type I, 1.26×10^6 units/mL), porcine pancreatic (type I) phospholipase A_2 ($pPLA_2$; 4.8×10^3 units/mL), bovine milk LPL (2.5 units/mL), and soybean phosphatidylcholine were obtained from Sigma. The specific activity of $pPLA_2$ was 760 units/mg of protein where 1 unit hydrolyzes 1.0 μmol of L-α-phosphatidylcholine to L-α-lysophosphatidylcholine and a free fatty acid moiety per minute at pH 8.0 and 37 °C using soybean L-α-phosphatidylcholine as substrate. The specific activity of LPL was 3800 units/mg of protein where 1 unit releases 1.0 nmol of *p*-nitrophenol per minute at pH 7.2 and 37 °C using *p*-nitrophenyl butyrate as substrate. D,L-α-TOH was purchased from Kodak (Rochester, NY); 13-*(Z,E)*cholesteryl hydroxylinoleate (13-(*Z*,*E*)Ch18:2-OH); 13-(*E*,*E*)-, 9-(*E*,*Z*)-, and 9-(*E*,*E*)Ch18:2-OH, 15-hydroperoxyeicosatetraenoic acid (15-HPETE), 13-*(S)(Z,E)*hydroxyoctadecadienoic acid (13-HODE), and racemic 13-(*Z*,*E*)HODE were from Cayman Chemicals (Ann Arbor, MI). Ebselen was obtained from Biomol (Plymouth Meeting, PA), 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) was from Polysciences (Warrington, PA), and [1-^{14}C]linoleic acid (55 Ci/mol) was from Amersham. rhLO, prepared as described (*17*), was a generous gift from Drs. E. Sigal and M. Mulkins (Roche Biosciences, Palo Alto, CA). Nonpancreatic secretory human (type IIa) PLA_2 ($sPLA_2$) was prepared as described (*18*). The neutralizing monoclonal anti-$sPLA_2$ antibody 10B2 and the control antibody were prepared as described previously (*19*). Briefly, monoclonal antibody 10B2 was raised against recombinant human type IIa PLA_2. Monoclonal antibodies to $sPLA_2$ and control antibody (81193, an isotype-matched murine monoclonal antibody which does not cross-react with human tissue) were purified from hybridoma-induced ascities fluid in BALB/c mice (Bioquest Ltd., Sydney, Australia). 10B2 inhibited human type IIa $sPLA_2$ activity in a mixed micelle system using ^{14}C-labeled phosphatidylethanolamine as substrate (*19*) with an IC_{50} of 2.8 nM at a PLA_2 concentration of 1.4 nM; the control monoclonal antibody had no effect (not shown). Standards of hydroperoxides of cholesteryl ester (CE-OOH) and phosphatidylcholine (PC-OOH) were prepared as described (*20*); for quantitation, the same extinction coefficients were used for the hydroperoxides and hydroxides of cholesteryl esters (CE-O(O)H). Organic solvents were of HPLC quality. Prior to use, all aqueous buffers were treated with Chelex-100 (Bio-Rad) to remove contaminating transition metals. Nanopure water (Millipore) was used throughout.

Methods

LDL Preparation. Native LDL, isolated from fresh plasma of healthy donors (*21*), was used immediately or stored on ice under Ar for <24 h. Immediately prior to use, LDL was gel-filtered through two PD-10 columns (Pharmacia) equilibrated with 50 mM phosphate-buffered saline, pH 7.4. Typically, such LDL was 0.7−1 μM in apolipoprotein B-100 (apoB), and ≤10% of the protein was due to contaminating albumin (*21*). Where indicated, LDL was concentrated (Centriprep-30 units, Pharmacia) to ≈8 μM in apoB prior to gel filtration, or depleted of α-TOH in vitro and lipid hydroperoxides were removed as described in (*11*).

Oxidation of LDL. Native or α-TOH-depleted LDL was incubated with SLO or rhLO at 37 °C in the absence or presence of either $pPLA_2$, $sPLA_2$, or LPL at the concentrations indicated. The amounts of the lipases used were saturating for all experiments (except where the specific activity with LDL as substrate was determined) with regard to the levels of FFA released, as determined by incubating a constant amount of LDL with increasing amounts of $pPLA_2$ and LPL (not shown). To determine the specific activity with LDL as the substrate, LDL (0.38 nmol in apoB) was incubated at pH 7.4 and 37 °C with either 6.3, 18.9, or 44.1 μg of $pPLA_2$ or 2.8, 11, and 22 μg of LPL in 1 mL for periods of up to 20 min. From the kinetics of FFA released, the calculated specific activities for $pPLA_2$ and LPL were 17.3 ± 3.2 and 1.61 ± 0.34 nmol (mean ± SD, $n = 3$) of FFA released per second per milligram of protein, respectively. Where indicated, anti-$sPLA_2$ or control antibody was used at ≈100-fold molar excess over $sPLA_2$. In some experiments, LDL was incubated with SLO as above but in the presence of deoxycholate. For assessment of inactivation of 15LO, LDL (0.8 μM in apoB), previously incubated with SLO (10 μg/mL) or rhLO (40 μg/mL) for varying periods of time, was supplemented with [1-^{14}C]linoleic acid (4.5 μM) and incubated for a further 2 h. The mixture was then treated with sodium dithionite (to reduce lipid hydroperoxides to the corresponding hydroxides), extracted, and analyzed for [1-^{14}C]linoleic acid, [1-^{14}C]13-HODE, and [1-^{14}C]9-HODE (see below).

Electron Paramagnetic Resonance Spectroscopy. This was performed using a Bruker ESP 300 spectrometer fitted with an X-band cavity and using an aqueous flat cell (volume 500 μL; Wilmad Glass Co., Buena, NJ). Spectra were accumulated at power, 20 mW; time constant, 164 ns; modulation frequency, 12.5 kHz; modulation amplitude, 0.1 mT; and averaging the output from seven successive accumulations with a sweep time of 20.9 s. For generation of α-TO$^{\bullet}$, LDL (≈8 μM in apoB) was preincubated at 37 °C in the presence of the lipase indicated or phosphate-buffered saline (control). After 10 min, SLO (≈1×10^5 units/mL) was added, and the reaction mixture was incubated further at 37 °C. Aliquots for both electron paramagnetic spectroscopic (200 μL) and HPLC (20 μL) analyses were removed in parallel, and the former was placed immediately into the EPR cavity for determination of α-TO$^{\bullet}$.

HPLC Analyses. Aliquots (20−200 μL) of the lipoprotein samples were extracted with acidified methanol and hexane, and the lipid phase was analyzed for α-TOH, unoxidized lipids (cholesterol and cholesteryl esters), and CE-O(O)H using reverse-phase HPLC with electrochemical and ultra-

violet detection, as described (*21*). The UV absorbance-based method detects both hydroperoxides and hydroxides of cholesteryl esters. The methanolic layer of the biphasic LDL extracts was used for the detection of PC-OOH and fatty acid hydroperoxides (FFA-OOH) using an NH_2 column (250 × 4.6 mm, 5 μm; Supelco) eluted with methanol containing 4% (v/v) Na_2HPO_4, with postcolumn chemiluminescence detection (*20*). The retention times for FFA-OOH and PC-OOH were ≈4.5 and 7.5 min, respectively.

HPLC Analysis of Positional and Regio- and Stereospecific Isomers of Lipid Hydroxides. A combination of normal-phase and chiral HPLC was employed to resolve the regio- and stereoisomers of Ch18:2-OH, as described previously (*13*, *22*). As applied, the method fully resolves the *S* and *R* stereoisomers of 13-(*Z*,*E*)-, 13-(*E*,*E*)-, and 9-(*E*,*Z*)Ch18:2-OH, whereas those of 9-(*E*,*E*)Ch18:2-OH elute as a single peak. Analysis of free fatty acid hydroxides (FFA-OH) was performed by reverse-phase HPLC (*23*). For regio- and stereospecific analyses, FFA-OH were collected, reextracted, and analyzed by consecutive normal-phase and chiral HPLC (*23*). Authentic racemic and stereospecific standards of 13-(*Z*,*E*)- and 9-(*E*,*Z*)HODE were used for quantitation. For analysis of [1-^{14}C]HODE, aliquots of [1-^{14}C]linoleic acid-supplemented LDL samples (see above) were treated with sodium dithionite and extracted with hexane−methanol, and the methanolic layer was extracted with chloroform. The chloroform phase was dried in vacuo, and the residue was resuspended in hexane and analyzed by normal-phase HPLC using an LC-Si column (25 × 0.26 cm, 5 μm) eluted with heptane, diethyl ether, propan-2-ol, acetic acid (100:10:0.9:0.1; v/v) (*24*) at 1.5 mL/min with $UV_{234\ nm}$ and on-line radiometric detection (Radiomatic Flo-One Beta detector, Packard) using Ultima-FLQ M scintillation cocktail at 1.5 mL/min. The elution times for [1-^{14}C]linoleic acid, [1-^{14}C]-13-HODE, and [1-^{14}C]9-HODE were ≈4.5, 11.5, and 16.5 min, respectively.

Other Methods. FFA in LDL samples were determined using a nonesterified fatty acid (NEFA) test kit (Boehringer Mannheim) (*13*). Triglycerides and phosphatidylcholine in LDL were determined in the absence and presence of lipases by specific enzymatic kits (Boehringer Mannheim), while phospholipase activity was measured using an $sPLA_2$ kit (Cayman). The neutralizing activity of the monoclonal antibody was also verified in a mixed-micelle assay using [^{14}C]phosphatidylethanolamine as substrate (*19*). Protein was determined by the bicinchoninic acid assay (Sigma) using bovine serum albumin as standard. The concentration of LDL was calculated from cholesterol determination, assuming a protein molecular mass of 500 kDa and 550 molecules of unesterified cholesterol per LDL particle.

RESULTS

Previous studies by others have shown that $pPLA_2$ enhances SLO-induced oxidation of LDL lipids, as assessed by increased rates of accumulation of material absorbing at 234 nm (*25*), although the underlying mechanism has not been investigated. The addition of $pPLA_2$ or LPL to SLO and LDL resulted in the rapid release of relatively large amounts of FFA, particularly in the case of $pPLA_2$ (Figure 1A). In the presence of either lipase and SLO, FFA-OOH were formed immediately and at levels relatively high and proportional to those of FFA; subsequently, the concentration of FFA-OOH increased at comparatively lower rates (Figure 1B).

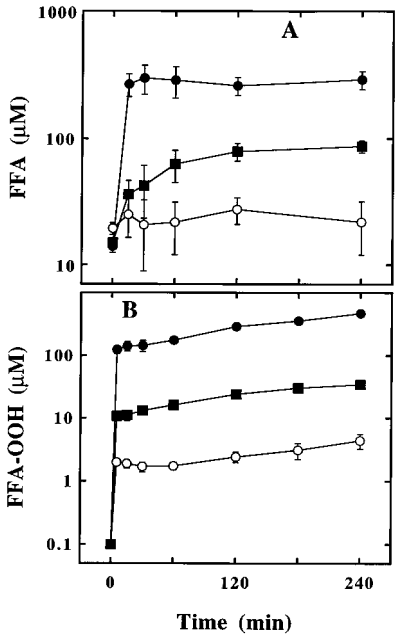


FIGURE 1: Lipases release FFA and enhance accumulation of FFA-OOH in LDL exposed to SLO. LDL (7.2 μM in apoB) was incubated with SLO (0.15 mg/mL) at 37 °C in the absence (○) or presence of $pPLA_2$ (63 μg/mL) (●) or LPL (11 μg/mL) (■); the final activity of either $pPLA_2$ or LPL was ≈70 units/μM apoB. At the times indicated, an aliquot was analyzed for FFA (A) and FFA-OOH (B) as described under Methods. The data shown are mean values ± SD derived from three separate experiments. Note the difference in scale between panels A and B.

Table 1: Regio- and Stereospecific FFA-OH Accumulate in Chemically Reduced LDL Previously Exposed to SLO in the Presence of $pPLA_2$ or LPL[a]

time (min)	lipase	HETE:HODE ratio[b]	13-(*Z*,*E*)HODE[c] (% of total HODE)	13-(*Z*,*E*)HODE (*S*:*R* ratio)
10	$pPLA_2$	0.2 ± 0.02	81.5 ± 2.3	28.4 ± 0.7
120	$pPLA_2$	0.08 ± 0.01	88.3 ± 2.3	25.3 ± 0.4
240	$pPLA_2$	0.04 ± 0.01	86.5 ± 0.9	33.5 ± 0.2
10	LPL	0	95.0 ± 2.1	19.4 ± 1.0
120	LPL	0	92.1 ± 2.5	18.6 ± 0.5
240	LPL	0	87.5 ± 5.1	17.9 ± 0.3

[a] LDL (≈8 μM in apoB) was incubated with SLO (0.1 mg/mL) and $pPLA_2$ (200 μg/mL) or LPL (9 μg/mL) at 37 °C. At the times indicated, 30 μL aliquots were removed and treated with $NaBH_4$ to reduce FFA-OOH to the corresponding hydroxides, and the regioisomers of all FFA-OH isomers and the stereospecificity of the 13-(*Z*,*E*)HODE isomer were determined as described under the Methods. The data shown represent mean values ± SD of three separate experiments using pooled LDL obtained from three different donors. [b] Ratio of all HETE to HODE isomers comprising total FFA-OH. HETE were not detected in extracts of LDL incubated with LPL and SLO. [c] For the nomenclature of fatty acid oxidation product isomers, see, e.g., (*37*).

The major oxidized FFA found in LDL exposed to SLO in the presence of $pPLA_2$ or LPL and treated with $NaBH_4$ was 13-(*S*)(*Z*,*E*)HODE (Table 1). For both lipases, the enzymic *S* stereoisomer comprised ≥95% of the total isomers detected, demonstrating that SLO-induced oxidation of FFA in LDL in the presence of either lipase occurs via an enzymic process. In the presence of $pPLA_2$, but not LPL, 15-HETE was also formed as a minor product (Table 1), indicating that linoleic acid was the major FFA released from LDL by either lipase and that some arachidonate was released by $pPLA_2$.

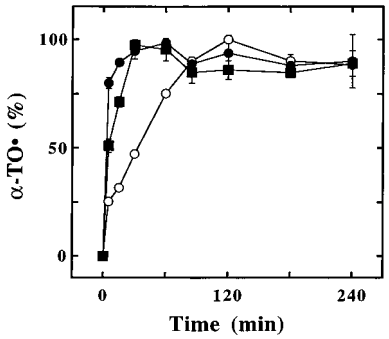


FIGURE 2: Lipases enhance the rate of accumulation, but not the steady-state concentration, of α-TO• in LDL exposed to SLO. LDL (7.8 ± 0.3 μM in apoB) was incubated with SLO in the absence (○) or presence of $pPLA_2$ (●) or LPL (■) using identical conditions as given for Figure 1. At the times indicated, an aliquot was taken and α-TO• measured by EPR as described under Methods. The data shown are mean values ± SD derived from three separate experiments. The results for 0−60 min obtained with $pPLA_2$ and LPL are significantly different from each other as well as from the control with $p < 0.001$ as evaluated by ANOVA using the MYSTAT statistical software package. The 100% value for α-TO• was 0.36 ± 0.03 μM.

The presence of $pPLA_2$ or LPL significantly enhanced the rate of SLO-induced α-TO• accumulation, although it did not affect its steady-state level (Figure 2). This effect was somewhat more pronounced with $pPLA_2$ than with LPL. A similar enhancement is seen when exogenous linoleic acid is added to LDL exposed to SLO (*22*) and reflects the effect of the lipases on the rates both of FFA release and of formation of FFA-OOH (cf. Figure 1). In addition, $pPLA_2$ or LPL also increased the rates of α-TOH consumption and accumulation of CE-O(O)H and PC-OOH (Figure 3). However, in contrast to FFA-OOH, oxidized esterified lipids accumulated and α-TOH was consumed linearly over time (compare Figure 1 vs Figure 3), clearly indicating different mechanism(s) for the oxidation of FFA vs cholesteryl esters, phospholipids, and α-TOH.

PLA_2 treatment may change the physicochemical properties of lipoproteins (*26*). However, treatment of LDL with either $pPLA_2$ or LPL, at lipase-to-LDL ratios used in experiments reported herein, had no effect on the physical properties of LDL, as both the density and size of the lipoprotein remained unaltered as assessed by gradient ultracentrifugation and gel filtration, respectively (data not shown). As expected, however, treatment with lipase resulted in changes in LDL's lipid composition that reflected the activities of the enzyme used (Table 2). Thus, while little change occurred in the levels of unesterified cholesterol and cholesteryl esters in the presence of lipases, exposure of LDL to $pPLA_2$ or LPL led to decreased phosphatidylcholine or triglyceride levels, respectively. These changes were accompanied by corresponding increases in the levels of FFA (Table 2).

As the substrate concentration can affect the extent of suicide inactivation of SLO (*27*, *28*), we next tested 15LO activity during LDL oxidation in the absence and presence of $pPLA_2$ (see Methods). As shown in Figure 4A, [1-^{14}C]-linoleic acid was converted to [1-^{14}C]13-HODE by SLO at similar rates, independent of the lipase and whether added at the onset or up to 60 min after the initiation of LDL oxidation. Even when added after 2 h of oxidation, significant formation of [1-^{14}C]13-HODE was still observed,

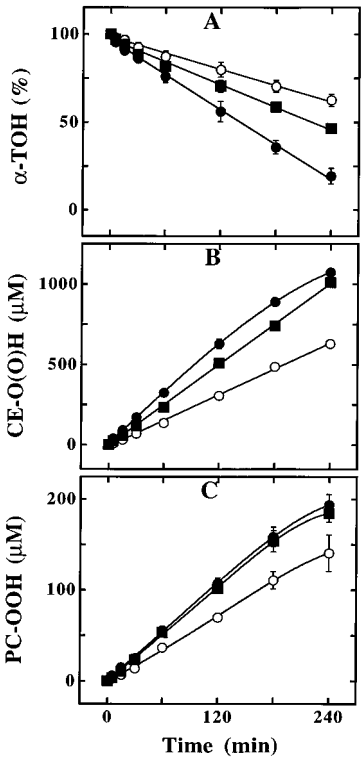


FIGURE 3: Lipases enhance the rates of α-TOH consumption and esterified lipid hydroperoxide accumulation in LDL exposed to SLO. LDL (7.2 μM in apoB) that was incubated with SLO in the absence (○) or presence of $pPLA_2$ (●) or LPL (■) using identical conditions as described in Figures 1 and 2 was also analyzed for α-TOH (A), CE-O(O)H (B), and PC-OOH (C) as described under Methods. The data shown are mean values ± SD derived from three separate experiments. The data arising from $pPLA_2$ and LPL incubations are significantly different from the control with $p < 0.001$ as evaluated by ANOVA using the MYSTAT statistical software package. The initial α-TOH concentration was 56.2 ± 6.8 μM.

although to a lesser extent. The [1-^{14}C]13-HODE formed were largely enzymic products, as judged by the ratio of [1-^{14}C]13- to [1-^{14}C]9-HODE: 8.5 ± 1.2 and 5.1 ± 0.2 when the substrate was added at 0 and 120 min, respectively. In contrast, corresponding experiments using rhLO at an oxygenase:apoB ratio similar to that used with SLO showed such high [1-^{14}C]13- to [1-^{14}C]9-HODE ratios only when [1-^{14}C]linoleic acid was added at the beginning of the oxidation. When added at 15 min or later, oxidation of [1-^{14}C]linoleate was no longer observed, independent of the presence of $pPLA_2$ (Figure 4B). These results demonstrate that in the presence of LDL and $pPLA_2$, rhLO but not SLO becomes inactivated rapidly.

Similar to $pPLA_2$, addition of recombinant human $sPLA_2$ to LDL also enhanced the rates of rhLO-induced CE-O(O)H accumulation and α-TOH depletion (Figure 5). This enhancement was prevented by a neutralizing anti-$sPLA_2$ antibody (*19*) but not by the control, nonneutralizing antibody (Figure 5), indicating that the enzymic activity of $sPLA_2$ was responsible for the enhancing effect.

Depletion of α-TOH renders LDL lipids resistant to peroxidation induced by SLO (*11*). The results in Figure 6 confirm this. However, when α-TOH-depleted LDL was exposed to SLO in the presence of either $pPLA_2$ or LPL, CE-O(O)H accumulated at a substantial and sustained rate (Figure 6). Thus, the presence of lipases overcomes the

Table 2: Changes in Lipid Composition during Incubation of LDL with $pPLA_2$ and LPL[a]

time (min)	cholesterol (mM)	cholesteryl esters[b] (mM)	triglycerides (μM)	phosphatidylcholine (mM)	FFA (μM)
			Control LDL		
0	0.41 ± 0.03	1.22 ± 0.14	58.8 ± 2.0	0.47 ± 0.03	7.8 ± 1.9
10	0.41 ± 0.04	1.25 ± 0.11	59.0 ± 6.2	0.44 ± 0.02	7.9 ± 0.5
360	0.46 ± 0.03	1.31 ± 0.11	58.3 ± 3.9	0.46 ± 0.03	8.7 ± 1.5
			+$pPLA_2$		
10	0.41 ± 0.04	1.24 ± 0.10	60.3 ± 7.9	0.36 ± 0.01	138.6 ± 10.7
360	0.43 ± 0.02	1.17 ± 0.05	61.0 ± 7.8	0.33 ± 0.02	152.6 ± 19.5
			+LPL		
10	0.41 ± 0.02	1.21 ± 0.03	45.5 ± 4.5	0.42 ± 0.02	18.3 ± 3.4
360	0.43 ± 0.02	1.33 ± 0.10	36.9 ± 5.0	0.44 ± 0.02	45.7 ± 8.2

[a] LDL (0.75 μM in apoB) was incubated at 37 °C in the absence (control) or presence of $pPLA_2$ (75 μg/mL) or LPL (28 μg/mL). At the times indicated, aliquots were analyzed by HPLC for unesterified cholesterol and cholesteryl esters, and for triglycerides, phosphatidylcholine, and FFA using enzymic kits as described under Methods. Data shown are mean values ± SD of three separate experiments, except for $t = 0$ min ($n = 9$). Three separate LDL preparations from the same donor were used. [b] Values shown represent the sum of cholesteryl arachidonate, cholesteryl linoleate, and cholesteryl oleate levels comprising the majority of cholesteryl esters in LDL.

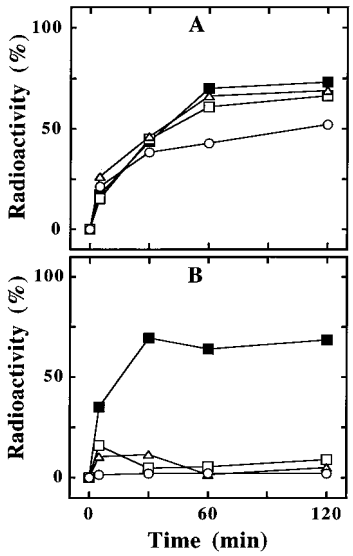


FIGURE 4: Differences in the inactivation of SLO vs rhLO during incubation with LDL. LDL (≈0.8 μM in apoB) was incubated with SLO (A, 10 μg/mL or 0.1 μM) or rhLO (B, 40 μg/mL or 0.55 μM) at 37 °C; [1-^{14}C]linoleic acid (0.25 μCi/mL ≈ 0.45 μM) was added at 0 (■), 15 (□), 60 (△), and 120 min (○), before the mixture was incubated for an additional 2 h. Aliquots were taken during this 2 h incubation at the times indicated and analyzed by normal-phase HPLC with radiometric detection for [1-^{14}C]linoleic acid, [1-^{14}C]-13HODE, and [1-^{14}C]9HODE as described under Methods. The percentage of the two latter analytes relative to the total radioactivity detected is represented. The data shown are representative of two independent experiments with different pools of LDL showing comparable results.

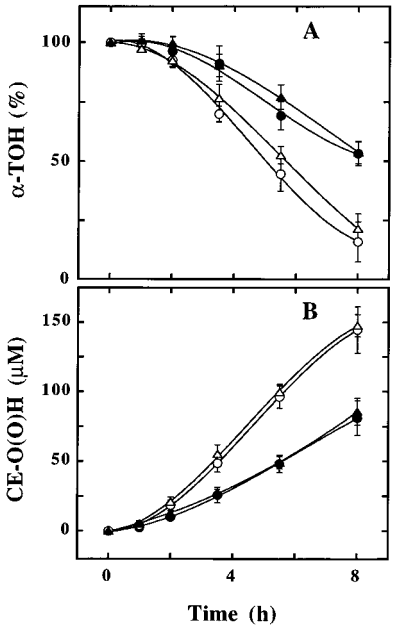


FIGURE 5: Oxidation of LDL induced by rhLO is enhanced by $sPLA_2$. LDL (1.5 μM in apoB) was incubated with rhLO (40 μg/mL) in the absence (●) or presence of $sPLA_2$ (○) (70 μg/mL). To some samples was also added a neutralizing anti-$sPLA_2$ antibody (▲) or a control antibody (△) at 100-fold molar excess over $sPLA_2$. At the times indicated, α-TOH consumption (A) and CE-O(O)H accumulation (B) were determined. The data shown are mean values from three or two experiments in the absence or presence of the antibodies, respectively, and the extent of variation is indicated by error bars. The initial α-TOH concentration was 13.8 ± 1.1 μM.

resistance of α-TOH-depleted LDL to SLO-induced lipid oxidation.

To test whether LDL's cholesteryl esters peroxidized enzymically in the presence of SLO and lipase, we first determined the ratio of 13-(*Z*,*E*)- to 9-(*E*,*Z*)CH18:2-O(O)H and of 13-(*E*,*E*)- to 9-(*E*,*E*)Ch18:2-O(O)H. For a given regioisomer (i.e., *Z*,*E* or *E*,*E*), both the 13- and 9-Ch18:2-O(O)H positional isomers accumulated at similar rates, independent of the presence of $pPLA_2$ and whether native or α-TOH-depleted LDL was used (Table 3). In addition, the 13-(*Z*,*E*)- and 9-(*E*,*Z*)Ch18:2-OH formed were racemic mixtures at all time points examined. These results show that SLO oxidized LDL's core lipids nonenzymically, regardless of the presence of $pPLA_2$. As SLO can directly oxidize phospholipids in the presence of high concentrations of deoxycholate (*29*), we also added this detergent to LDL prior to exposure to SLO. At <1 mM, deoxycholate was without effect, whereas at 10 mM it enabled SLO to directly oxidize LDL's cholesteryl esters ≈100-fold faster in the first (15 min) time point of the incubation when compared to control samples (data not shown). Such oxidation was largely enzymic, as 13-(*S*)(*Z*,*E*)Ch18:2-O(O)H was the major product

DISCUSSION

Two features were observed in our investigation of the effect of lipases on 15LO-induced peroxidation of LDL lipids. First, $pPLA_2$ or LPL exposure to LDL accelerated

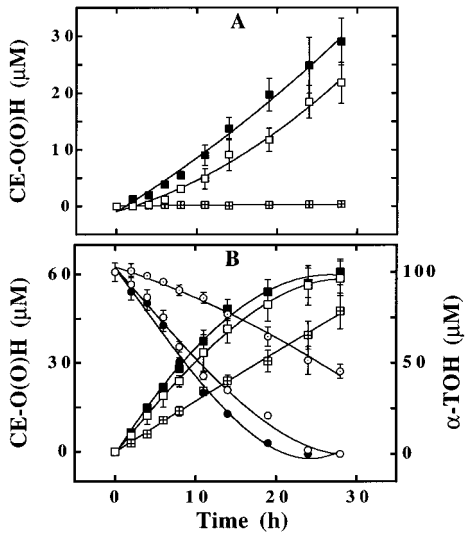


FIGURE 6: Resistance of α-TOH-depleted LDL to SLO-induced oxidation is overcome by lipases. LDL (0.4 μM in apoB), either depleted of its endogenous α-TOH (A) or native (B), was incubated with SLO (4 μg/mL) in the absence (⊞, ⊙) or presence of $pPLA_2$ (40 μg/mL; ■, ●) or LPL (15 μg/mL; □, ○) at 37 °C. At the indicated times, an aliquot was extracted and analyzed for CE-O(O)H (squares) and, where applicable, for α-TOH (circles). The data shown are mean values from three experiments, and the variation is indicated by the error bars. The initial α-TOH concentrations were 0 μM and 3.4 ± 0.4 μM for α-TOH-depleted and native LDL, respectively.

esterified lipid oxidation and α-TOH consumption. Second, α-TOH-depleted LDL, which is resistant to 15LO-induced peroxidation, oxidized readily in the presence of either lipase. Since 15LO (*30*) and lipases are present and may co-localize in the arterial wall (*31*, *32*) where LDL oxidation takes place (*30*), our results are of potential biological relevance.

The enhancement of SLO-induced LDL oxidation by $pPLA_2$ has been reported previously to mimic cell-mediated oxidative modification of the lipoprotein (*25*), although the underlying mechanism(s) was (were) not studied. We investigated whether this enhancement can also be observed with rhLO and other lipases, and determined enzymic vs nonenzymic peroxidation of both unesterifed and esterified lipids in LDL. Regio- and stereospecific analyses of the lipid oxidation products (Tables 1 and 3) revealed enzymic oxidation of FFA, independent of whether SLO or rhLO was used as the oxidation-inducing enzyme, and $pPLA_2$, human $sPLA_2$, or LPL as the peroxidation-enhancing enzyme. The observation that a neutralizing anti-$sPLA_2$ antibody completely blocked the enhancing activity of $sPLA_2$ (Figure 5) indicated that the latter process was enzymic in nature. In sharp contrast to these enzymic processes, the SLO-induced oxidation of cholesteryl esters (and probably also phospholipids) was clearly nonenzymic. Thus, at no time did we observe preferential accumulation of the enzymic product 13-(*S*)(*Z*,*E*)Ch18:2-OH, independent of the presence of lipases and despite SLO remaining active throughout the oxidation period studied. Enzymic oxidation of cholesteryl linoleate in LDL by SLO could only be demonstrated in the presence of nonphysiological concentrations of deoxycholate, similar to the previously reported bile salt-dependent oxidation of polyunsaturated fatty acids esterified in phospholipids (*29*). It is not known at present whether this is due to a 'solubilization' of LDL's cholesteryl esters and/or a 'widening' of the substrate pocket. From its crystal structure (*33*), we would predict that the latter is required to accommodate the bulky cholesterol moiety of cholesteryl linoleate in the narrow and 40 Å long substrate cavity of SLO to correctly position the linoleate moiety close to the end of the cavity. In any case, the present results clearly argue against the previously implied enzymic oxidation of LDL's lipids by SLO (*23*).

The present studies employing human $sPLA_2$ and rhLO confirmed the results with nonhuman lipases and nonmammalian 15LO, although notable differences are observed between SLO and rhLO. Thus, rhLO was rapidly inactivated during LDL oxidation, independent of the presence of lipases, whereas SLO remained active. Such differences are not observed when solubilized FFA is used as substrate [see, e.g., (*17*)] where both enzymes undergo rapid suicide inactivation. Also, rhLO (but not SLO) directly oxidizes a proportion of LDL's cholesteryl esters (*22*). These differences indicate that care should be taken in using SLO as a model of human 15LO, although this is commonly done [see, e.g., (*33*)].

A striking and novel observation presented here is that the presence of lipases overcomes the previously reported (*11*) resistance to peroxidation of phospholipids and cholesteryl esters in α-TOH-depleted LDL (Figure 6). This observation can be rationalized readily by the model proposed (*13*) to explain enzymic and nonenzymic oxidation of LDL's FFA and esterified lipids, respectively. Accordingly, FFA-OO•, released as a byproduct of SLO-catalyzed peroxidation of FFA (*14*), oxidize LDL's α-TOH to α-TO•, which itself initiates the peroxidation of LDL's esterified lipids via TMP (*13*, *22*) (reaction 5 in Scheme 1). As lipases substantially elevate the concentration of FFA in LDL (Figure 1 and Table 2), the rate of FFA-OO• formation is also expected to increase, as supported indirectly by the enhanced rate of α-TO• accumulation (Figure 2). As with SLO, α-TOH-depleted LDL is also resistant to oxidation initiated by AAPH-derived peroxyl radicals, although the extent of this resistance decreases with increasing rates of radical fluxes (*11*). As the chemical reactivity of AAPH-derived peroxyl radicals is comparable to that of FFA-OO•, enhanced fluxes of FFA-OO• can therefore readily explain why in the presence of lipase even α-TOH-depleted LDL is oxidized by SLO.

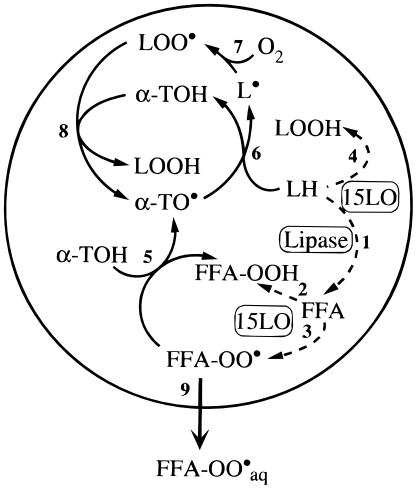
Incubation of LDL with rhLO in the presence of $sPLA_2$ resulted in inactivation of the enzyme within 15 min (see above), yet LDL lipid peroxidation continued for hours (Figure 5). This may be explained by reactions 6−8, whereby α-TO•, once formed, continues to propagate lipid peroxidation even in the absence of active rhLO. However, this scenario *alone* cannot readily explain why ≥80% of LDL's α-TOH was consumed over a period of 8 h after inactivation of rhLO (Figure 5). Thus, oxidizing LDL represents a lipid emulsion in which there is only one radical per oxidizing lipoprotein particle, and radical reactions in one oxidizing particle are assumed to be physically segregated from other (oxidizing and nonoxidizing) particles [for details, see (*10*, *12*, *34*)]. Also, even under conditions where radicals are generated continuously, ≤50% of the LDL particles contain a radical (α-TO•) [see Model 1A in (*10*)]. Furthermore, under the conditions used (i.e., molar ratio of rhLO:apoB ≈ 0.3, and inactivation of rhLO within 15 min),

Table 3: Regio- and Stereospecific Isomers of Ch18:2-OH in Native and α-TOH-Depleted LDL Exposed to SLO in the Presence or Absence of $pPLA_2$[a]

time (h)	CE-O(O)H (μM)	Ch18:2-OH 13-(*Z*,*E*):9-(*E*,*Z*)	13-(*Z*,*E*)Ch18:2-OH (*S*:*R*)	9-(*E*,*Z*)Ch18:2-OH (*S*:*R*)	Ch18:2-OH 13-(*E*,*E*):9-(*E*,*E*)
			Native LDL		
0	0	–	–	–	–
3.25	14.8 ± 3.2	1.02 ± 0.03	1.03 ± 0.08	1.02 ± 0.09	1.04 ± 0.07
6.25	28.8 ± 4.1	1.01 ± 0.05	1.01 ± 0.05	1.04 ± 0.06	1.01 ± 0.08
11.25	95.2 ± 6.7	1.01 ± 0.05	0.99 ± 0.06	1.02 ± 0.07	0.97 ± 0.05
3[b]	235 ± 45	23.4 ± 1.5	8.4 ± 0.8		
			Native LDL plus $pPLA_2$		
0	0	–	–	–	–
3.25	33.8 ± 3.1	1.03 ± 0.08	0.97 ± 0.05	0.98 ± 0.09	0.96 ± 0.08
6.25	59.9 ± 7.1	1.04 ± 0.03	0.97 ± 0.01	1.06 ± 0.12	0.99 ± 0.06
11.25	116.4 ± 7.2	0.99 ± 0.03	0.94 ± 0.01	1.05 ± 0.08	0.99 ± 0.05
			α-TOH-Depleted LDL plus $pPLA_2$		
0	0	–	–	–	–
3.25	13.3 ± 3.1	1.02 ± 0.03	1.05 ± 0.07	0.98 ± 0.04	1.0 ± 0.03
6.25	37.8 ± 4.8	1.05 ± 0.04	1.01 ± 0.05	1.02 ± 0.03	1.0 ± 0.05
11.25	78 ± 7.2	1.03 ± 0.02	0.98 ± 0.03	1.03 ± 0.06	0.98 ± 0.06

[a] Native or α-TOH-depleted LDL (1 μM in apoB) was incubated with SLO (10 μg/mL) alone or plus $pPLA_2$ (46 μg/mL) at 37 °C. At the times indicated, aliquots (500 μL) were removed and treated with sodium dithionite, and the regio- and stereospecific isomers of Ch18:2-OH were determined as described under Methods. The data shown represent mean values ± SD of 3 individual experiments. For native LDL exposed to SLO in the absence and presence of $pPLA_2$, 40.8 ± 5.4 and 2.1 ± 2.3%, respectively, of endogenous α-TOH remained at 11.25 h. [b] In the presence of 10 mM deoxycholate.

Scheme 1: Proposed Model for the Enzymic and Nonenzymic Peroxidation of LDL's Lipids Induced by 15LO and Enhanced by Lipases[a]



[a] rhLO preferrentially, and SLO exclusively, acts on FFA, converting them to the corresponding FFA-OOH (reaction 2), with some FFA-OO• being released from the active site (reaction 3) as 'by-products' (*14*). FFA-OO• are scavenged rapidly by α-TOH, the most redox-active component in LDL, resulting in the formation of FFA-OOH and α-TO• (reaction 5), the latter initiating TMP (reactions 6−8) (*10*). In contrast to SLO, rhLO can also directly oxidize appropriate esterfied lipids (LH) of LDL (*22*) (reaction 4), giving rise to some enzymic oxidized esterified lipids. Lipases greatly increase LDL's content of FFA (reaction 1), which results in increased levels of FFA-OO•, some of which may 'escape' the lipoprotein particle (reaction 9), thereby potentially 'transferring' the free radical chain reactions to another lipoprotein particle. Solid and dashed lines refer to nonenzymic and enzymic reactions, respectively.

we would expect that oxidation was initiated in $<^1/_3$rd of the LDL particles. Therefore, the simplest explanation for the observed consumption of >80% α-TOH is that the physical segregation of radicals no longer holds true. The implied radical diffusion between particles must be due to a radical species which diffuses relatively slowly and does not readily decompose to nonradical products; otherwise, TMP would be inhibited (*10*, *12*). The most likely species that fulfills these criteria is FFA-OO•. Thus, we speculate that in the presence of lipases, FFA-OO• formed in an oxidizing particle 'escapes' (*14*) (reaction 9 in Scheme 1), and the resulting 'aqueous' FFA-OO• reacts with either α-TO• in another oxidizing particle (resulting in consumption of α-TOH) or α-TOH in a nonoxizing particle, thereby 'transferring' lipid peroxidation from one to another LDL particle (not shown in Scheme 1). Preliminary results obtained from experiments using oxidizing LDL particles physically separated from nonoxidizing ones fully support the possibility of such radical transfer in the presence but not absence of lipase (J. Neuzil, unpublished results).

Lipases have been suggested to participate in pro- and antiatherogenic events via remodeling of circulating lipoproteins, anchoring lipoproteins to the extracellular matrix, and/or by releasing bioactive lipids such as lysophosphatidylcholine (*31*, *32*, *35*). In particular, $sPLA_2$ has proinflammatory activities (*36*) that may affect atherogenesis, and active enzyme has been isolated from atherosclerotic arteries (*31*). The present results suggest that enhancement of LDL oxidizability by 15LO may be such an effect, although it is assumed that most of the intimal 15LO is intracellular, excluding a direct action of the oxygenase on LDL. However, in analogy to the above proposed radical transfer between lipoprotein particles, we speculate that in the presence of *both* active 15LO (located at the endofacial side of plasma membranes of cells) and a lipase (located either intra- or extracellularly), some of the FFA-OO• formed 'escapes' from the cell and initiates extracellular LDL oxidation. This could provide a mechansm by which intracellular LO could cause oxidation of extracellular LDL. We are currently investigating this possibility.

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