Oxidation of LDL by rabbit and human 15-lipoxygenase: prevalence of nonenzymatic reactions

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Abstract 15-Lipoxygenase (15-LO)-induced oxidation of lipids in human LDL may be pro-atherogenic. However, the extent to which 15-LO promotes enzymatic oxidation of esterified (i.e., major) lipids in LDL may depend on various factors. Here, we show that overall, LDL lipid oxidation was favored with high activity of human 15-LO, that phospholipids were the preferred esterified substrate, and that low temperature maintained a higher proportion of enzymatic product. However, under all conditions, 15-LO induced α-tocopherol consumption and the accumulation of nonenzymatic products that predominated with increasing time of incubation and inactivation of the enzyme. Lysates prepared from cells overexpressing human 15-LO oxidized linoleic acid readily and in an almost exclusive enzymatic manner. In sharp contrast, such lysates failed to oxidize LDL lipids unless linoleic acid was added, in which case nonenzymatic oxidation of LDL lipids occurred. We conclude that although purified 15-LO can oxidize isolated LDL lipids in vitro, such oxygenation always includes nonenzymatic reactions that likely play a major role in the more extensive oxidation of LDL by cell-derived 15-LO.— Heydeck, D., J. M. Upston, H. Viita, S. Ylä-Herttuala, and R. Stocker. **Oxidation of LDL by rabbit and human 15-lipoxygenase: prevalence of nonenzymatic reactions. *J. Lipid Res.* 2001.** 42: **1082–1088.**

Supplementary key words mammalian 15-lipoxygenase • lipid oxidation • vitamin E • lipoprotein

The appearance of lipid-laden macrophages (foam cells) in the subendothelial space is a hallmark of early events in atherogenesis (1), and foam cell lipid is thought to originate from modified LDL such as oxidized LDL. 15-Lipoxygenase (15-LO) is a potentially relevant oxidant, as this enzyme oxygenates complex lipids such as those found in LDL in vitro (2–4), and 15-LO lipid oxidation products, protein and mRNA, are present in rabbit and human atherosclerotic lesions (4–7). Further, atherosclerosis is diminished by disruption of the 12/15-LO gene in apolipoprotein (apo)E-deficient mice (8) or by administration of a specific nonantioxidant 15-LO inhibitor in hypercholesterolemic rabbits (9), and is accelerated by overexpression of human 15-LO in LDL receptor-deficient mice (10).

Original studies suggested that cellular lipoxygenases play an important role in cell-mediated oxidative modification of LDL (11). In vitro, 15-LO can oxidize LDL lipid, although the enzyme is normally cytosolic and the lipoprotein found in extracellular fluid. It has been (3, 12), and continues to be reported (13) that the enzyme primarily affords oxygenation of LDL lipid in vitro by a direct enzymatic mechanism. However, we have previously determined that initial and transient 15-LO activity can be followed by prolonged nonenzymatic LDL lipid oxidation (14, 15). We suggested that the nonenzymatic lipid oxidation represents co-oxidative reactions (16, 17) that may contribute significantly to LDL oxidation in vivo and, hence, atherosclerosis, and that the role of 15-LO-induced nonenzymatic lipid oxidation is currently undervalued, considering the time frame over which this disease develops. Thus, knowledge of the relative contribution of enzymatic versus nonenzymatic oxidation is important for the rational design of an anti-atherogenic strategy aimed at preventing 15-LO-induced LDL oxidation in vivo.

Several groups investigating the action of mammalian 15-LO on LDL used enzymes from different species and various experimental conditions, thus making direct mechanistic comparisons difficult (3, 4, 12–15). We therefore first evaluated LDL oxidation induced by two distinct mammalian 15-LOs, rabbit reticulocyte 15-LO (rLO) and recombinant human 15-LO (hLO). For these studies, different 15-LO activity and reaction temperatures were tested and the relative contribution of enzymatic and nonenzymatic LDL lipid oxidation was assessed by the time-dependent consumption of vitamin E [α-tocopherol, (α-TOH)] and the accumulation of stereospecific lipid

Abbreviations: α-TOH, α-tocopherol; apoB, apolipoprotein B-100; C18:2, cholesterol linoleate; C18:2-O(O)H, C18:2 hydro(pero)xide; CE, cholesterol ester; CP-HPLC, chiral phase-HPLC; hLO, recombinant human 15-LO; HODE, hydroxyoctadecadienoic acid; 15-LO, 15-lipoxygenase; nkat, substrate turnover of 1 nmol/s; NP-HPLC, normal phase-HPLC; PC-O(O)H, phosphatidylcholine hydro(pero)xides; RASMC, rabbit aortic smooth muscle cells; rLO, rabbit reticulocyte 15-LO; RP-HPLC, reverse phase HPLC; 18:2, linoleic acid.

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oxidation products. In addition, we assessed the ability of cell-released human 15-LO to oxidize LDL lipid in a stereospecific manner.

MATERIALS AND METHODS

Materials

Linoleate (18:2), 13-*S*-hydroxyoctadecadienoic acid (13-*S*-HODE), 13-*S*-(*Z*,*E*)- and 9-*S*-(*E*,*Z*)-hydroxycholesterol linoleate (C18:2-OH) were purchased from Cayman Chemicals (Ann Arbor, MI). The different species of 15-LO, human recombinant 15-LO (hLO; 0.54 mg/ml) and rabbit reticulocyte 15-LO (rLO; 1.5 mg/ml), prepared as described (3, 18), were generous gifts from Roche Bioscience (Palo Alto, CA) and Prof. Hartmut Kühn (Berlin, Germany), respectively. The specific activities of rLO and hLO with 18:2 (240 μM) in PBS were 97.3 nkat/mg and 196 nkat/mg, respectively (where 1 nkat is defined as a substrate turnover of 1 nmol/s). PD-10 Sephadex G25 columns were purchased from Pharmacia (Uppsala, Sweden). Organic solvents of HPLC quality were obtained from Mallinckrodt (Clayton, Australia). Before use, aqueous solutions were stored over Chelex-100 (Bio Rad) to remove contaminating transition metals. All other chemicals used were of the highest purity available, and nanopure water (Millipore systems, Sydney, Australia) was used throughout.

Cell culture

Rabbit aortic smooth muscle cells (RASMC), stably transfected with human 15-LO (RASMC-LO) or β-galactosidase (RASMC-LacZ) cDNA (19), were maintained in DMEM containing 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine in 6-well plates (Falcon, Lincoln Park, NJ) at 37°C and 5% CO_2. Cells at 90% confluence were washed in buffer, lysed, and used for LDL oxidation experiments (see below).

Preparation and oxidation of LDL

LDL was isolated from fresh heparin-treated whole blood obtained from healthy volunteers (20). The LDL obtained was used immediately or stored under argon at 4°C for <24 hr and desalted prior to experimentation by gel filtration using a PD-10 column. LDL concentration was determined by its apoB100 (apoB) content. For high enzyme activity experiments, LDL was used at a concentration of 0.48 ± 0.07 mg/ml apoB. For lower enzyme concentration experiments, LDL apoB concentration was 0.82 ± 0.08 mg/ml.

Oxidation of LDL was performed at a rLO-to-LDL apoB ratio of 10 to 1 (mol/mol) and a hLO-to-LDL apoB ratio of 5 to 0.5 (mol/mol). Taking into account the different specific activities of rLO and hLO against 18:2, the above amounts corresponded to 15-LO activity of 70 and 4.3 nkat/nmol LDL apoB for the high and low enzyme concentration experiments, respectively. Incubations were carried out at room temperature or 37°C for up to 120 min. At the time points indicated, aliquots (50 μl) were removed, and α-TOH and neutral nonoxidized and oxidized lipids were extracted with methanol–hexane as described previously (20).

For cell-released hLO lipid oxidation, cells overexpressing hLO (RAMSC-LO) or control cells (RASMC-LacZ) were washed three times with PBS and lysed by freeze-thaw (three cycles) in 1 ml PBS. For free polyunsatured lipid oxidation, 18:2 (100 μM) was added to the lysates (0.5 ml), and oxidation was monitored at 37°C for 30 min. In other experiments, LDL (0.1 μM apoB, 1 ml) in the presence or absence of 18:2 (100 μM) was added to the cell lysates, and lipid oxidation was monitored at 37°C for up to 6 h. Aliquots (400–500 μl) were removed, reduced with $NaBH_4$ (50 mM), and α-TOH and oxidized esterified lipids were extracted with hexane–methanol (5:1 v/v, 6 ml) as described previously (20). Nonesterified lipid was recovered from the methanol phase by addition of chloroform (5 ml). Following vigorous mixing and centrifugation (500 *g*, 5 min, 4°C), 4 ml of the chloroform phase was removed, evaporated to dryness, and reconstituted in methanol for reverse phase (RP)-HPLC or in hexane for normal phase (NP)- and chiral phase (CP)-HPLC (see below).

Analysis of LDL lipid oxidation

The aqueous methanol phase of the extract was used directly for analysis of oxidized phospholipids. Briefly, hydroperoxides and hydroxides of phosphatidylcholine [PC-O(O)H] were isolated by RP-HPLC using a Supelcosil LC-NH_2 column as described previously (20), with detection of conjugated dienes at 234 nm.

The hexane phase was collected and dried, and the residue reconstituted in isopropanol for analysis of nonesterified cholesterol, α-TOH, and cholesterol ester hydro(pero) xides (CE-O(O)H) by RP-HPLC using UV and electrochemical detection (20–22). For the analysis of the positional, configurational, and stereoisomers of cholesterol linoleate hydroxide (C18:2-OH), triphenylphosphine (2.5 mg) was added to the hexane phase to reduce hydroperoxides to the corresponding hydroxides. The hexane phase was dried, and the residue reconstituted in hexane. This organic

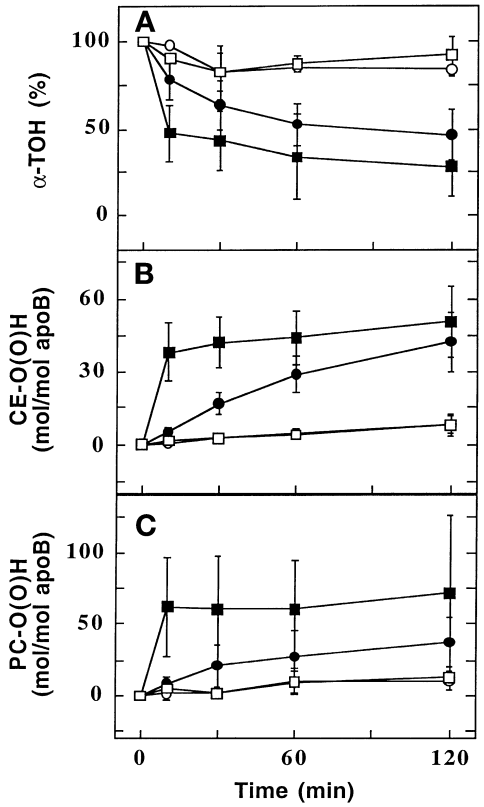

Fig. 1. 15-LO induces surface and core lipid and α-TOH oxidation in LDL. LDL was incubated with hLO (squares) or rLO (circles) at high (70 nkat/nmol LDL, filled symbols) and low (4.3 nkat/nmol LDL, open symbols) 15-LO concentrations. Reactions were carried out at 37°C, and the percentage of α-TOH consumed (A) and CE-O(O)H (B) and PC-O(O)H (C) accumulated over time determined as described in Materials and Methods. Initial α-TOH concentration was 2.7 ± 0.6 mol/mol LDL for high 15-LO activity and 2.8 ± 1.44 mol/mol for low 15-LO activity. The data depict mean values ± SD of three separate experiments.

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extract was subjected to consecutive NP- and CP-HPLC as described previously (14). Briefly, NP-HPLC was carried out using a LC-Si column (25 × 0.46 cm, 5 μm, Supelco) with hexane–isopropanol (100:0.27, v/v) as mobile phase. For CP-HPLC, a CHIRALCEL-ODH column (25 × 0.46 cm, 5 μm, Daicel Chemical Industries, Tokyo, Japan) with hexane–isopropanol (97.5:2.5, v/v) was used. Analysis of nonesterified 18:2 oxidation products including stereospecificity was carried out using RP-, NP-, and CP-HPLC, essentially as described previously (4). The flow rate for all systems was 1 ml/min. Conjugated dienes were detected by $UV_{234\ nm}$ absorbance. All relevant compounds were identified and quantified by peak area comparison with authentic standards.

RESULTS

The overall extent of LDL lipid oxidation induced by 15-LO at 37°C was investigated. At high 15-LO activity (70 nkat/nmol LDL apoB), the extent of oxidation was greater with hLO compared with rLO, as judged by the initial rates of α-TOH consumption (**Fig. 1A**) and the accumulation of CE-O(O)H (Fig. 1B) and PC-O(O)H (Fig. 1C). At this enzyme concentration, hLO-induced oxidation reached a plateau within the first time point studied (10 min). The preferred substrates in LDL appeared to be phospholipids, as assessed by the amount oxidized versus cholesterol ester (CE) per mol LDL. Although up to 70% of α-TOH in LDL was consumed, even after 2 h, each LDL particle still contained approximately 1 mol of the vitamin (Fig. 1). In contrast to hLO, rLO induced a more linear oxidation of LDL lipid, as assessed by all three oxidation parameters (Fig. 1).

When 15-LO was employed at a lower activity (4.3 nkat/nmol LDL), lipid oxidation proceeded more linearly for both enzyme species (Fig. 1). Compared with the higher enzyme concentration, the overall extent of lipid oxidation and α-TOH consumption was decreased for both 15-LO species. Further, there was no significant difference between the human and rabbit enzymes, in terms of the extent of oxidation of surface (phospholipids) and core lipid (CE; Fig. 1). Similar results were obtained when incubations were carried out at room temperature (data not shown).

The relative contribution of enzymatic versus nonenzymatic lipid oxidation was also determined. For 18:2-containing lipid, the 13-*Z,E* positional and "*S*" chiral isomer formed by r-LO is ⩾90% during direct enzymatic oxidation (23). Thus, the accumulation of 13-*Z,E*-C18:2-O(O)H as a percentage of the four possible positional and configurational isomers (13-*Z,E*, 9-*E,Z*, 13-*E,E*, and 9-*E,E*) was determined for LDL oxidation using the two enzyme species at high and low 15-LO concentrations and different temperature conditions. In addition, we also assessed the stereospecificity (chirality) of 13-*Z,E*-C18:2-O(O)H to determine the overall extent of enzymatic oxidation of CE.

The percentage of the positional isomer, 13-*Z,E*-C18:2-O(O)H, formed during LDL oxidation at 37°C induced by both species of 15-LO ranged from 51–63% at the earliest time point (10 min) for the high enzyme activity experiments (**Table 1**), and was 65% for the low activity study (**Table 2**). These values decreased time dependently (Tables 1 and 2) and were similar to those found for LDL oxidized nonspecifically using aqueous alkyl peroxyl radicals (Table 1). Compared with 37°C, oxidation performed at room temperature appeared to favor 13-*Z,E*-C18:2-O(O)H accumulation with both 15-LO at low enzyme activity (Table 2) and hLO at high activity (Table 1). Overall, low enzyme activity appeared to maintain a higher percentage of the 13-*Z,E*-C18:2-O(O)H isomer for times <120 min (Tables 1 and 2).

"*S*" stereoisomer formation, as a percentage of total 13-*Z,E*-C18:2-O(O)H, is normally accepted to represent the true extent of 15-LO action on CE. The percentage of the "*S*" isomer was high, ranging from 77–95% at the earliest time point (10 min; Tables 1 and 2). At high enzyme concentrations, the percentage of the "*S*" isomer was higher

TABLE 1. Positional and stereoisomers of C18:2-O(O)H generated in LDL in the presence of high 15-LO activity

	rLO		hLO		AAPH/LDL[a]
Isomer/Incubation Time	37°C	RT	37°C	RT	1,000 mol/mol
min					
13-*Z,E*-C18:2-O(O)H (%)					
10	51 (5)	42 (9)	63 (6)	71 (1)	
30	48 (1)	53 (4)	55 (4)	68 (7)	
60	45 (6)	50 (5)	50 (5)	60 (5)	47
120	54 (14)	55 (2)	43 (2)	54 (4)	48
"*S*" isomer (%)					
10	77 (6)	ND	88 (1)	92 (4)	
30	71 (6)	65 (4)	85 (5)	85 (4)	
60	63 (17)	84(6)	71 (4)	79 (8)	ND
120	52 (13)	61 (2)	59 (11)	69 (16)	ND

LDL was incubated at room temperature (RT) or 37°C with rLO or hLO at 70 nkat/nmol LDL apoB. Positional and stereoisomers of C18:2-O(O)H were isolated and quantified by HPLC as described in Materials and Methods. The data represent the mean value (SD) of three separate experiments with LDL from different donors. AAPH, 2,2′-azo*bis*(2-amidinopropane) hydrochloride; ND, not determined.

[a] Data are derived from ref. (26).

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TABLE 2. Positional and stereoisomers of C18:2-O(O)H generated in LDL in the presence of low 15-LO activity

Isomer/Incubation Time	RLO 37°C	RLO RT	hLO 37°C	hLO RT
min	%		%	
13-*Z*,*E*-C18:2-O(O)H				
10	65 (10)	68 (24)	65 (6)	79 (1)
30	58 (7)	73 (6)	57 (2)	72 (2)
60	56 (5)	66 (7)	57 (4)	71 (1)
120	53 (4)	69 (12)	53 (2)	65 (4)
"*S*" isomer				
10	88 (3)	84 (16)	84 (13)	95 (8)
30	78 (11)	95 (5)	81 (11)	94 (7)
60	77 (13)	89 (3)	68 (8)	90 (10)
120	60 (7)	80 (8)	59 (3)	89 (16)

LDL was incubated at room temperature or 37°C with rLO or hLO at 4.3 nkat/nmol LDL apoB. Positional and stereoisomers of C18:2-O(O)H were isolated and quantified by HPLC as described in Materials and Methods. The data represent the mean value (SD) of three separate experiments with LDL from different donors.

under room temperature conditions only for the human enzyme (Table 1). The extent of "*S*" isomer formation under low enzyme activity was comparable with the different 15-LO and temperature conditions and favored by lower temperature (Table 2). However, compared with high activity, low enzyme activity appeared to favor "*S*" isomer generation only for rLO (Tables 1 and 2). In addition, the overall proportion of "*S*" isomer decreased time dependently for all conditions. Importantly, even under optimal conditions where 95% of the "*S*" isomer was formed, at most, 75% of the total product was enzymatic. At physiological temperature and low enzyme activity conditions, enzymatic products comprised only 55–57%. Thus, the data show that even under conditions that favor enzymatic oxidation, a substantial amount of CE-O(O)H is formed nonenzymatically, and that nonenzymatic oxidation takes place under all conditions tested (Tables 1 and 2).

15-LO is a potential in vivo oxidant for LDL, even though it is a cytosolic enzyme. Cell necrosis in developing atherosclerotic lesions may lead to the release of active 15-LO and subsequent oxidation of extracellular lipoproteins. We therefore tested whether cell-released 15-LO induces oxidation of extracellular lipid in a stereospecific manner.

We first tested whether lysates of RASMC-LO oxidized free polyunsaturated fatty acid. Lysates of hLO-cells induced substantial 18:2 oxidation compared with lysates prepared from RAMSC-LacZ (**Table 3**). The major 18:2 oxidation product (91%) detected was 13-*Z*,*E*-HODE, >95% of which comprised the "*S*" stereoisomer (Table 3). In addition, stereospecific 18:2 oxidation by hLO-transfected cell lysates was blocked by 5, 8, 11, 14-eicosatetraynoic acid, an inhibitor of 15-LO (data not shown).

Oxidation of LDL lipid by cell-released hLO was similarly tested. In contrast to the situation with 18:2, lysates of hLO-transfected cells failed to oxidize LDL lipids unless the reaction mixture was supplemented with 18:2 (**Fig. 2**). In the presence of added 18:2, LDL lipid oxidation occurred over a relatively long period of time (Fig. 2A) that

TABLE 3. Enzymatic oxidation of 18:2 by lysates of cells overexpressing hLO

Cell Type	HODE	13-*Z*,*E*-HODE	13-*Z*,*E*-HODE "*S*" isomer
	nmol/mg	%	%
RASMC-LO	56 (8)	91 (1)	96 (1)
RASMC-LacZ	3 (1)	54 (5)	47 (4)

Cell lysates in PBS, prepared from RASMC cells overexpressing either hLO or β-galactosidase (LacZ), were incubated with 100 μM 18:2 for 30 min at 37°C. The HODE formed was first separated by RP-HPLC, and the configurational and stereoisomers were then isolated by NP- and CP-HPLC, respectively, as described in Materials and Methods. The data shown are mean values (SD) derived from four individual experiments.

was accompanied by the formation of HODE (Fig. 2B). The stereospecificity of LDL lipid (C18:2-O(O)H) and 18:2 oxidation products (HODE) was assessed under conditions where LDL oxidation was observed. Similar to the experiments above, the major 18:2 oxidation product was 13-*S*-*Z*,*E*-HODE (**Table 4**). Although nonesterifed 18:2 oxidation by cell-released hLO was enzymatic, LDL lipid oxidation was completely nonenzymatic, as stereorandom lipid oxidation was observed (Table 4). Analogous to the experiments carried out with recombinant enzyme, LDL

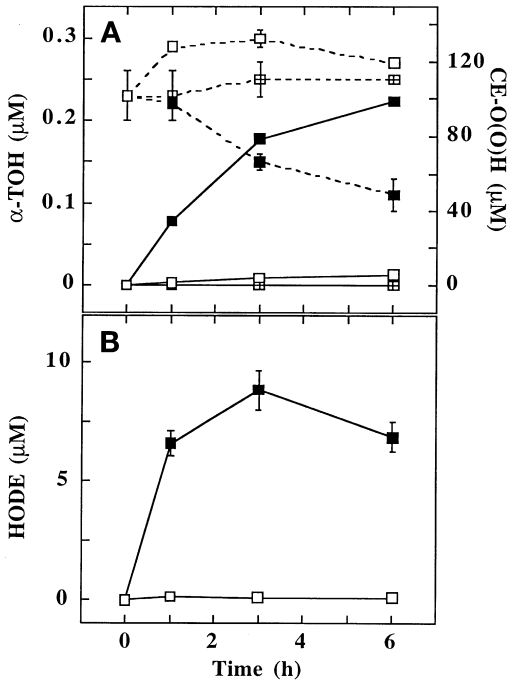

Fig. 2. Cell-derived hLO induces LDL oxidation in the presence of 18:2. Lysates from RASMC-LO-overexpressing (closed squares) or RASMC-LacZ-transfected control (open squares) cells were incubated with LDL (0.04 μM apoB) and 100 μM 18:2 for 6 h at 37°C. At specified times, aliquots (400 μl) were removed and CE-O(O)H accumulation (solid lines) and α-TOH consumption (dashed lines) (A) and HODE accumulation (B) determined by HPLC as described in Materials and Methods. CE-O(O)H accumulation and α-TOH consumption were also determined for hLO-overexpressing cell lysates incubated with LDL, but in the absence of added 18:2 (hatched squares). The data shown are mean values ± SD from two individual experiments carried out in duplicate.

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TABLE 4. Cell-derived hLO induces nonenzymatic lipid oxidation in LDL in the presence of 18:2

Time	13-*Z,E*-C18:2-OH	13-*Z,E*-C18:2-OH "*S*" isomer	13-*Z,E*-HODE	13-*Z,E*- HODE "*S*" isomer
min	%	%	%	%
10	49 (1)	55 (2)	ND	ND
60	51 (1)	58 (1)	95 (2)	99 (1)
180	51 (1)	58 (4)	86 (2)	98 (1)
360	50 (1)	57 (5)	90 (1)	98 (1)

Lysates in PBS, prepared from RASMC cells overexpressing hLO, were incubated with LDL (0.04 μM in apoB) in the absence and presence of 100 μM 18:2 for 6 h at 37°C. At specified times, aliquots (400 μl) were removed and C18:2-OH and HODE configurational isomers were determined by NP-HPLC as described in the Materials and Methods. The data shown are mean values (SD) derived from two experiments performed in duplicate. ND, not determined.

lipid oxidation under such conditions was accompanied by α-TOH consumption (Fig. 2A).

DISCUSSION

Previous studies have indicated that the extent to which 15-LO may directly oxidize lipid in LDL in vitro may be influenced by the ratio of 15-LO to LDL employed, the mammalian species of 15-LO utilized, and the reaction temperature (12). Using in vitro experiments with authentic enzyme, we show herein that hLO was a more effective oxidant for CE and phospholipids in LDL, but only under high enzyme-to-LDL ratios (≥5 mol/mol hLO:LDL apoB). Despite this, the extent of enzymatic oxidation of CE at physiological temperature (37°C) was similar for both high and low enzyme-to-LDL ratios. Enzymatic oxidation of CE at 37°C was preferred with low enzyme activity for rLO, as assessed by accumulation of stereospecific products. The human enzyme generated more enzymatic product at room temperature, and phospholipids appeared to be a preferred substrate compared with CE. For all conditions with isolated 15-LO, α-TOH was consumed, and the percentage of C18:2-O(O)H generated enzymatically was ≤75%, suggesting that even under the most favored conditions, oxygenation of lipid was always partially nonenzymatic. Nonenzymatic lipid oxidation induced by 15-LO increased time dependently and became extensive. Cell-released hLO oxidized free 18:2 in a highly stereospecific manner, yet LDL lipids were oxidized nonspecifically, via nonenzymatic reactions. Even when employing 15-LO-overexpressing cells, oxidation of LDL by cell-released 15-LO required free polyunsaturated fatty acid. Together, these findings suggest that although 15-LO may trigger the LDL oxidation process, nonenzymatic lipid oxidation is likely the predominant path by which the enzyme generates substantially oxidized LDL under conditions that mimic the in vivo environment.

For lipoxygenases, conversion of unsaturated fatty acid is stereospecific, initiated by a stereoselective hydrogen abstraction (24). Thus, we first assessed, with isolated 15-LO and LDL, the positional and configurational isomers of C18:2-O(O)H (derived from C18:2, the major oxidizable lipid in LDL), as these products can initially indicate whether hydrogen abstraction was exclusively enzymatic. We also compared the formation of these products with the generation of the expected stereospecific product, 13-*S*-*Z,E*-C18:2-O(O)H. During complete enzymatic oxidation of 18:2-containing lipid, the proportion of the 13-*Z,E* positional and configurational isomer is ≥96%, with an equivalent proportion of this in the "*S*" configuration (23). Thus, if oxidation were exclusively enzymatic, the enzymatic products are expected to represent ≥92% of the total products. We did not observe such values under any condition tested with the authentic 15-LO enzymes. Although oxidation appeared to be initially enzymatic (≤10 min), the percentage of enzymatic product was in fact ≤65%. Self-inactivation of 15-LO appeared to terminate enzymatic activity after the earliest time point as described previously by others (4, 12, 13).

In atherosclerotic lesions, oxidized LDL is found extracellularly, and 15-LO is thought to be largely an intracellular enzyme. When considering the potential role of 15-LO for LDL oxidation in vivo, cell necrosis could result in conditions where the enzyme and lipoproteins come into contact, assuming that the released 15-LO retains activity. Whether cell-released 15-LO is capable of oxidizing lipid, however, is unknown. We show herein that cell-released hLO oxidizes free 18:2 in a stereospecific manner. We show further that at low enzyme-to-LDL ratio, which may be expected in vivo, LDL lipid oxidation by 15-LO requires the presence of nonesterified polyunsaturated fatty acid, and that such lipid oxidation is nonenzymatic and accompanied by α-TOH consumption.

For both the isolated enzyme and cell-released hLO experiments, nonenzymatic CE oxidation products were formed at all times and increasingly over the incubation period. In addition, α-TOH in LDL was consumed at all times. Together, these results clearly show that 15-LO induces nonenzymatic oxidation of LDL. This is fully consistent with earlier literature describing the co-oxidation of nonlipid substrates by 15-LO (17) and the release of free radicals from active 15-LO (16). Similarly, we have previously demonstrated that tocopherol-mediated peroxidation promotes and drives extensive nonenzymatic LDL lipid peroxidation during and beyond 15-LO activity (14, 15, 25). Furthermore, we (26) and others (27) have demonstrated that unless α-TOH in LDL is fully consumed, free radical-mediated lipid oxidation generates significant amounts of 13-*Z,E*-C18:2-O(O)H. Considering that α-TOH was not fully consumed under any of the conditions tested herein, tocopherol-mediated peroxidation of LDL lipid

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(28) would account for CE oxidation at extended incubation times and beyond inactivation of 15-LO. That such oxidation is controlled by α-TOH is also consistent with the observation that lipid in LDL devoid of the vitamin is highly resistant to oxidation induced by 15-LO (25). It follows from these considerations that compounds such as ascorbic acid that inhibit tocopherol-mediated peroxidation of lipoprotein lipids [i.e., co-antioxidants (29)] are expected to effectively inhibit this type of the 15-LO-induced nonenzymatic component of LDL lipid oxidation.

In LDL, phospholipids, rather than CE, appeared to be the preferred substrate for both authentic rLO and hLO (Fig. 1). Whether this is due to steric considerations (surface vs. core lipid) or active site preference is not known. 15-LO action on LDL phospholipids may be relevant in vivo, as oxidized phospholipids have potent pro-atherogenic effects (30). In addition to direct attack of phospholipids, 15-LO induces oxidative fragmentation of linoleate-containing phospholipid hydroperoxides to form novel platelet-activating factor-like lipids (31). Interestingly, this type of oxidized lipid is generated largely via a nonstereoselective mechanism, fully consistent with the results shown here and in our previous work (14, 26).

Modified and pro-atherogenic LDL are thought to be present in the extracellular space of atherosclerotic lesions (5), and we demonstrate herein that cell-released hLO oxidizes the esterified lipids of LDL almost exclusively via nonenzymatic reactions (Table 4). Alternatively, and perhaps more relevant for atherosclerosis, 15-LO within intact cells may induce extracellular lipoprotein lipid peroxidation. Original studies of cellular 15-LO-induced LDL oxidation proposed "seeding" of LDL with oxidized lipid, thereby rendering the lipoprotein more susceptible to oxidation in vivo (11, 32, 33). Indeed, subsequent studies demonstrated that mouse fibroblasts overexpressing 15-LO induced extracellular LDL oxidation (34, 35), and that LDL enriched with lipid hydroperoxides via incubation with 15-LO-overexpressing cells was more susceptible to further oxidation in transition metal ion-containing medium (35). However, these studies did not directly determine whether the oxidized lipids detected in LDL were enzymatic or nonenzymatic products. Potentially important however, the concept of seeding LDL with lipid hydroperoxides does not necessarily predict that such hydroperoxides are enzymatic (15-LO) products. This is in sharp contrast to the proposal that 15-LO directly oxidizes LDL lipid (3, 4). Interestingly, if the seeding involved enzymatic lipid hydroperoxides, their formation would not be expected to be inhibited by co-antioxidants (see above).

Cytokine-mediated up-regulation of 15-LO activity in human monocytes induces LDL lipid oxidation, and such oxidation has been shown to be nonenzymatic (36). Recently, specific 15-LO oxidation products were demonstrated in hydrolyzed lipid extracts from 15-LO-transfected mouse macrophage (J774) cells incubated with LDL (37). However, in this study, the ratio of enzyme to substrate used was very high and unlikely to be achieved in vivo. Thus, the concentration of active 15-LO in tissue and the mechanism whereby extracellular LDL undergoes oxidation by a cytosolic enzyme needs to be elucidated. Concerning the latter, we have preliminary data indicating that LDL can undergo oxidation while physically separated (by a membrane) from isolated 15-LO (J. Neuzil et al., unpublished observations).

We have evaluated herein the relative contribution of enzymatic and nonenzymatic LDL lipid oxidation by 15-LO by assessing the time-dependent consumption of α-TOH and the accumulation of stereospecific products under various experimental conditions. With isolated 15-LO, reactions carried out at physiological temperature (37°C) yielded a greater proportion of nonenzymatic products for both 15-LO species, particularly under low enzyme activity. The human enzyme also displayed a significantly increased affinity for LDL esterified lipid when standardized for free 18:2 oxygenation activity compared with the rabbit enzyme. The two enzymes share 82% homology in the amino acid sequence (38). The species differences observed may be due to different rates of suicide inactivation by the respective enzymes (Fig. 1). The data herein agrees with previous work showing a higher LDL oxygenation by the human enzyme (3) and a slower rate of inactivation of the rabbit enzyme with LDL as a substrate (4). Nonetheless, nonenzymatic LDL lipid oxidation, induced by 15-LO but mediated by α-TOH, was prominent under all conditions and, especially, for cell-released hLO. Our data show that even under conditions where nonenzymatic oxidation products predominate, enzyme (15-LO) activity is still implicated in the overall oxidative mechanism. We conclude that both enzymatic and nonenzymatic lipid peroxidation likely are relevant when considering the role of 15-LO in atherosclerosis in which oxidized LDL may cause and prolong the disease (39).

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