Lipoperoxides in LDL incubated with fibroblasts that overexpress 15-lipoxygenase

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Abstract Oxidative modification of LDL plays an important role in early atherogenesis but the mechanisms, nonenzymatic and/or enzymatic, by which LDL is oxidized in vivo remain to be established. Several lines of evidence suggest that cellular 15-lipoxygenase (arachidonate 15-oxidoreductase, EC.1.13.11.13) (15-LO) may contribute to oxidative modification of LDL, including recent studies demonstrating that murine fibroblasts overexpressing 15-LO have an enhanced capacity to oxidize LDL in the medium. The present studies were undertaken to better understand the mechanisms by which cells expressing 15-LO bring about oxidative modification of LDL. LDL incubated 1-2 h with the 15-LO-enriched cells showed a much higher lipoperoxide (LOOH) content than did LDL incubated with control cells. By far the largest absolute increase occurred in cholesteryl ester hydroperoxide (CE-OOH), a much lesser increase in free fatty acid hydroperoxides (FFA-OOH), and only a very small increase in phospholipid hydroperoxides (PL-OOH). Addition of EDTA to the medium abolished these increases in LDL lipid hydroperoxides. Enrichment of LDL with probucol or vitamin E also prevented CE-OOH accumulation. Incubation of LDL with linoleic acid hydroperoxide in the absence of cells also caused a significant increase in CE-OOH and this was markedly inhibited by EDTA. These findings provide further evidence for the potential of 15-LO to participate in LDL oxidation by way of a mechanism involving introduction of LOOH into the LDL particle followed by metal-catalyzed propagation.

It is well established that oxidative modification of LDL plays a role in early atherogenesis (1-3) but the mechanism(s) by which LDL is oxidized in vivo remains to be established. LDL oxidation in vitro can be catalyzed nonenzymatically by transition metal ions (iron, copper) (4, 5), by hemin (6), by thiols (7, 8), by ceruloplasmin (9), and probably many other catalysts. However, even low concentrations of serum or of nonspecific proteins inhibit such oxidation (4, 10) and thus oxidation of LDL in the plasma compartment is most unlikely. On the other hand, there may be sequestered microenvironments in the extravascular extracellular spaces in which LDL oxidation catalyzed by these agents can take place. LDL oxidation may also be catalyzed as a result of enzyme activities and several candidate enzyme systems have been proposed, including NADPH oxidase (11-13), myeloperoxidase (14), leakage from the mitochondrial electron transport system, peroxidases (15), and lipoxygenase (16, 17). The present studies were undertaken to explore further the possibility that 15-lipoxygenase (arachidonate 15-oxidoreductase, EC.1.13.11.13) (15-LO) might play a role. A role for 15-LO is supported by several lines of evidence. 1) The enzyme is present in endothelial cells and macrophages (17, 18), the major cellular elements in fatty streak lesions. 2) Purified soy bean lipoxygenase (16, 19, 20) and purified mammalian 15-LO (21-24) can oxidatively modify LDL. 3) Inhibitors of 15-LO (but not inhibitors of 5-LO) reduce the extent of LDL oxidation by cultured endothelial cells and macrophages (17, 25-27). 4) 15-LO activity is found in fatty streak lesions (28) and protein and mRNA are found at high levels in fatty streak lesions, colocalizing with histochemically identified oxidized LDL (29). 5) There is a highly significant excess of the stereoisomer of linoleic acid produced by 15-LO (rather than the 1-to-1 ratio expected if the oxidation were nonenzymatic) in fatty streak lesions of cholesterol-fed rabbits (23). 6) Transfer of the human 15-LO gene into rabbit iliac arteries results in the appearance of oxidized-LDL epitopes (30). And, finally 7) introduction of the cDNA for human 15-LO by retroviral techniques enhances the capacity of murine fibroblasts to oxidatively...

Supplementary key words oxidized LDL • cholesteryl ester hydroperoxide • free fatty acid hydroperoxide • phospholipid hydroperoxide

Abbreviations: LDL, low density lipoprotein; FFA-OOH, free fatty acid hydroperoxide; PL-OOH, phospholipid hydroperoxide; CE-OOH, cholesteryl ester hydroperoxide; LO, lipoxygenase; HPLC, high performance liquid chromatography; EDTA, ethylenediamine tetraacetic acid; AMVN, 2,2'-azobis(2,4-dimethyl)valeronitrile.
modify LDL (31). The present studies were undertaken to better understand the mechanisms by which the murine fibroblasts enriched in 15-LO bring about oxidative modification of LDL in the medium. To that end we utilized an HPLC/chemiluminescence system to quantify the lipoperoxides appearing in LDL incubated with the 15-LO expressing fibroblasts.

MATERIALS AND METHODS

Materials

Cell culture supplies were obtained from the following: Dulbecco's modified Eagle's medium with high glucose (DME), Hanks' balanced salt solution, and Geneticin (G418 sulfate) from Gibco Co. (Grand Island, NY); fetal calf serum from Hyclone (Logun, UT); plastic supplies from Costar Co. (Cambridge, MA); isoluminol (6-amino-2,5-dihydroxy-4-phenylazahidenedione), microperoxidase (MP-11) sodium salt, and soybean lipoxygenase (6.46 × 10^5 units/mg protein) were from Sigma Co. (St. Louis, MO); 2,2'-azoisobis(2,4-dimethyl)valeronitrile (AMVN) from Polyscience Inc. (Warrington, PA); 13-hydroperoxy [S, (E,Z)]-9,11-octadecadienoic acid (13(S)-HpODE) from Cayman Chemical Co. (Ann Arbor, MI).

LDL isolation

LDL (d 1.019–1.063 g/ml) was isolated by density gradient ultracentrifugation from pooled human plasma (32) and always kept in the presence of EDTA (1 mg/ml). For selected experiments, probucol or vitamin E-containing LDL was isolated from plasma of patients taking probucol (1 gm/day) or vitamin E (600 mg IU/day), respectively. After isolation, LDL was usually adjusted to a concentration of 6–9 mg/ml, stored under N2 at 4°C, and used within 2 weeks. EDTA was removed just before the LDL was used by dialysis or by gel-filtration (Sephadex-G25M) (31) with phosphate-buffered saline without added calcium or magnesium (PBS). Protein was determined according to the procedure of Lowry et al. (33).

Cell culture and measurement of 15-LO activity

Murine fibroblasts expressing human 15-LO or β-galactosidase (lacZ) (31, 34) were established by infection with a retroviral vector as previously described (34, 35). Cells were grown in DME containing 10% fetal calf serum and G418 sulfate (50 μg/ml) under 95% air/5% CO2. The activity of 15-LO in cells was determined in terms of the rate of formation of 13-hydroxyoctadecadienoic acid (13-HODE) from added [14C]linoleic acid (17, 31). Subcultured cells at 70–80% confluency were incubated in the presence or absence of LDL (100 μg/ml) at 37°C for 1–2 h in Hanks' balanced salt solution (pH 7.35) containing 6.5 mM glucose. While pathophysiologic concentrations of glucose (25 mM) enhance oxidation significantly (36), the effects at physiologic concentrations are quite small, even after 48 h of incubation (37). In any case, both control and experimental incubations were carried out in the same medium. In some experiments, EDTA (10 μM) was added to the media to determine its effect on lipid hydroperoxide accumulation in LDL. At these concentrations of added EDTA, cells remained attached to the dish. After incubation, cells were immediately rinsed with three changes of PBS and homogenized with 0.2 N NaOH (31). Some aliquots of cell homogenate were used for determination of protein and others were used for analysis of LOOH content.

Oxidative modification by copper

Oxidative modification of LDL by Cu2+ was carried out as reported (31). LDL (250 μg/ml) was incubated for 0–24 h in PBS in the presence of CuSO4 (20 μM). After incubation, the lipids in the modified LDL were extracted as described below.

Incubation of LDL with linoleic acid hydroperoxide

Incubation of LDL with linoleic hydroperoxide (18:2-OOH) was performed according to a modification of the procedure of Wu, Stein, and Mead (38). In brief, LDL (100 μg/ml) was incubated for 1 h in PBS containing varying concentrations of 13(S)-HpODE in the absence or presence of 10 μM EDTA. After incubation, the lipids in the modified LDL were extracted as described below.

Determination of lipid hydroperoxide content in LDL by HPLC-post-column method

Lipid extraction was performed according to a modification of the procedures of Holley and Slater (39). Lipids in modified LDL were extracted with ten volumes of hexane in the presence of HCl (pH 3) and after centrifugation (1500 g, 10 min), the hexane phase was evaporated under nitrogen and the lipids were re-dissolved in MeOH–tert-BuOH 1:1. Samples were filtered through 0.45-micron filters (3 mm length, Gelman Sciences, Ann Arbor, MI), kept on ice (protected from light), and immediately injected into the HPLC system. When lipids were extracted from cell homogenates, samples were immediately acidified with 1 N HCl (pH about 3) and hexane was added to the cell homogenate.

Lipid hydroperoxide levels in LDL were determined in terms of the induction of isoluminol-derived chemiluminescence using a high performance liquid chromatography (HPLC) post-column method, using modifications of the procedures of Yamamoto et al. (40) and Sattler, Mohr, and Stocker (41). Lipids were separated
TABLE 1.  Lipid hydroperoxides in LDL incubated with 15-LO- and lacZ-expressing murine fibroblasts

<table>
<thead>
<tr>
<th>LDL Sample</th>
<th>LOOH Content (nmol/mg LDL prot)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FFA-OOH</td>
</tr>
<tr>
<td>Native LDL</td>
<td>0.47 ± 0.33</td>
</tr>
<tr>
<td>LDL incubated in the absence of</td>
<td>0.62 ± 0.39</td>
</tr>
<tr>
<td>cells</td>
<td></td>
</tr>
<tr>
<td>LDL incubated with LacZ cells</td>
<td>0.78 ± 0.35</td>
</tr>
<tr>
<td>LDL incubated with 15-LO cells</td>
<td>2.48 ± 0.77**</td>
</tr>
</tbody>
</table>

LDL was incubated for 1 h in Hanks' glucose media with 15-LO cells or lacZ cells (ca 0.5–0.8 mg cell protein per dish) or control dishes containing no cells. Lipids were extracted from media and analyzed by HPLC-chemiluminescence assay as described in Methods. Each value was determined in duplicate or triplicate and results reported are mean ± SD of 3–4 experiments; ND, not detected.

*Different from lacZ cells at P < 0.001 by non-paired Student's t-test.

**Different from lacZ cells at P < 0.03 by non-paired Student's t-test.

with an HPLC analytical column (SSODSB, 250 × 4.6 mm, Phase-Separations Ltd, Norwalk, CT) using as solvent system MeOH-tert-BuOH 1:1. The outflow was monitored by absorbance at 210 nm (Hitachi, Model L4250). The resolved lipids were then directed into a mixing coil (dead-volume 3.1 μl, Upchurch Scientific, Temecula, CA) where they were mixed with isoluminol (1 mM) and microperoxidase (25 μg/ml) and the outflow then passed through a Hitachi Model F-1080 continuously recording fluorometer equipped with a 12 μl flow cell with the excitation source turned off. The emitted light was measured at 430 nm. The flow rates of the two different pumps (L6200A, Hitachi, and Acuflow series II pump) were 1.0 and 1.5 ml/min., respectively.

Standard lipid hydroperoxides were prepared according to Holley and Slater (39) and Yamamoto et al. (40). 18:2-OOH was prepared by incubation of linoleic acid (1 μmol) with soybean lipoxygenase (1000–1500 units/ml) for 30–60 min. Phosphatidylcholine hydroperoxide (PC-OOH) and cholesteryl ester hydroperoxide (CE-OOH) were prepared from soybean phosphatidylcholine and cholesteryl linoleate oxidized with a lipophilic free radical generator AMVN (10 mM, 37°C, 30 min) in ethanol. The retention times for 18:2-OOH and CE-OOH were, respectively, 2.8–2.9 and 5.8–5.9 min. PC-OOH eluted as two peaks with retention time of 4.3–4.4 and 5.1–5.2 min, respectively. The data were analyzed with chromatography data station software (Hitachi D-6000 HPLC manager).

RESULTS

The 15-LO activity of the retrovirally infected cells used in most of these studies (clone 12, ref. 31) was approximately 19-times that of the control lacZ cells. The 15-LO activity remained constant at least through passage 12 and the cells were used in the present studies between passages 8 and 12.

As shown in Table 1, LDL incubated with clone 12 showed a much higher lipoperoxide content than did LDL incubated with the lacZ control cells. By far the largest absolute increase occurred in cholesteryl ester hydroperoxides (CE-OOH), a much lesser increase in free fatty acid hydroperoxides (FFA-OOH), and only a very small increase in absolute terms in phospholipid hydroperoxides (PL-OOH). Slightly over 70% of the total lipoperoxide recovered was in the CE-OOH fraction, 24% in the FFA-OOH fraction, and only about 5% in the PL-OOH fraction. Cholesteryl ester hydroperoxides were also the major lipid peroxide fraction in the native LDL and in the LDL incubated in the absence of cells. It should be noted that low values were found in the no-cell control LDL and in the LDL incubated with lacZ.

To focus on the cell-induced increment in LDL-hy-
TABLE 2. Lipid hydroperoxides in LDL in the medium after 1 h incubation with clone 12 or with two clones expressing 20% to 30% the 15-LO activity of clone 12 (clones 2 and 8).

<table>
<thead>
<tr>
<th>Lipid Hydroperoxides (nmol/mg cell protein)</th>
<th>Cell Type</th>
<th>FFA-OOH</th>
<th>PLOOH</th>
<th>CE-OOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lacz</td>
<td>0.09 ± 0.05</td>
<td>ND</td>
<td>0.74 ± 0.44</td>
<td></td>
</tr>
<tr>
<td>Clone 2</td>
<td>0.19 ± 0.07</td>
<td>ND</td>
<td>1.59 ± 1.33</td>
<td></td>
</tr>
<tr>
<td>Clone 8</td>
<td>0.08 ± 0.05</td>
<td>ND</td>
<td>2.70 ± 1.25</td>
<td></td>
</tr>
<tr>
<td>Clone 12</td>
<td>2.07 ± 0.41</td>
<td>0.16 ± 0.25</td>
<td>5.93 ± 2.51</td>
<td></td>
</tr>
</tbody>
</table>

LDL was incubated for 1 h in Hanks' glucose media with 15-LO-enriched cells (clones 2, 8, and 12) or lacZ cells or control dishes containing no cells. Lipid was then extracted from media and analyzed by HPLC-chemiluminescence assay. Each value was determined in duplicate or triplicate and results represented are mean ± SD; ND, not detected.

*Data presented are experimental values after subtraction of values from no-cell control dishes run in parallel.

We then asked whether the pattern of lipoperoxides found in LDL incubated with clone 12 differed from that found when LDL was oxidized nonenzymatically in the presence of copper. LDL was incubated with 20 μM copper and samples were analyzed after time intervals as shown in Fig. 4. Here again the dominant lipoperoxide that accumulated was CE-OOH, with much smaller amounts of PL-OOH and FFA-OOH being formed. The values peaked at 1 or 2 h and then fell progressively.
Fig. 3. Inhibition of LOOH appearance in LDL by EDTA. LDL (100 μg/ml) was incubated in Hanks' glucose for 1 h with 15-LO cells (ca. 0.5–0.8 mg cell protein per well) in the presence or the absence of EDTA (10 μM). After incubation, media were harvested and lipids were extracted and analyzed as described in Methods. Results are expressed as mean ± SD. Each value was determined from 2–5 observations.

during the rest of the incubation, a pattern previously observed by others (42).

One of the possible mechanisms by which 15-LO-enriched cells could induce oxidation of LDL is by a transfer of lipoperoxides from the cell to the LDL. Therefore, we determined the nature of lipoperoxides generated in LDL incubated with linoleic acid hydroperoxide in the absence of cells. As shown in Fig. 5, CE-OOH was the dominant lipoperoxide generated.

This implies that when linoleic acid hydroperoxide initiates a propagation reaction, cholesteryl esters may, for some reason, be a favored substrate and/or that CE-OOH accumulates because it has greater stability than PL-OOH. As shown in Fig. 5, the addition of EDTA to the medium markedly inhibited the increases in CE-OOH and in PL-OOH, implying again the need for metal ions to break down the added FFA-OOH to peroxy or alkoxy radicals followed by chain reactions oxidizing other classes of lipids.

DISCUSSION

The critical point in these studies is the use of murine fibroblasts expressing high levels of 15-LO activity. These retrovirally infected cells differ from the control infected cells only in that the cDNA for human 15-lipoxygenase was used in the construct to infect the 15-LO cells (clone 12) and the cDNA for β-galactosidase in the construct was used to infect the control (lacZ) cells (31). The results show that LDL incubated with the 15-LO-expressing cells contained significantly more cholesteryl ester hydroperoxides (CE-OOH), free fatty acid hydroperoxides (FFA-OOH), and phospholipid hydroperoxides (PL-OOH) than did LDL incubated with the control cells (Fig. 1). It should be noted that LDL incubated with the lacZ cells contained only minimally more CE-OOH than did LDL incubated in the absence of cells (Table 1), making it unlikely that other systems in these cells...
play much of a role in the appearance of lipoperoxides in LDL. Two additional clones of infected cells that expressed much lower enzyme activities than clone 12 were also studied (Table 2). The 15-LO activity in these cells was only 20–30% that in clone 12 (31), and the extent of accumulation of CE-OOH was accordingly much reduced. This “dosage” effect further strengthens the inference that the observed increment in lipid hydroperoxides in LDL incubated with 15-LO cells is, in fact, due to the expression of 15-LO activity.

The action of the 15-LO-enriched cells to modify LDL could be direct or indirect. The possibilities include the following: 1) the 15-LO is acting on endogenous cell membrane lipids (43) and the peroxidized forms of these lipids (or peroxides derived from them) are being transferred into LDL in the medium; 2) cellular 15-LO has access to and acts upon LDL particles that make contact with the cell membrane; 3) there is “leakage” of reactive oxygen species (generated in association with enhanced 15-LO activity) that enter the medium and act directly or indirectly to increase LDL oxidation (27); and 4) there are secondary changes in the cells overexpressing 15-LO that lead to release of factors that accelerate oxidation of LDL in the medium (e.g., sulfhydryl compounds). Presumably propagation reactions within the LDL could be involved in any of these. We cannot definitively assess the roles of these relative potential mechanisms but a few relevant points can be made. 15-LO appears to be a cytosolic enzyme and there is, to our knowledge, no evidence that it can act directly on substrates in the medium. Moreover, when [14C]linoleic acid was added to intact 15-LO cells we could not measure any appearance of [14C]linoleoyl-13-HODE in the medium, strongly suggesting that 15-LO activity did not escape to the medium (31). Studies by Cathcart et al. (44) and by McNally et al. (27) suggest that release of superoxide anion and increased activity of lipoxygenases may be linked, making the third option listed above viable. On the other hand, recent studies by Garner, Dean, and Jessup (45) show that LDL incubated with human monocyte-derived macrophages (6 days in culture) accumulate cholesteryl ester hydroperoxides but that this is probably independent of superoxide anion production. The authors concluded that superoxide anion production by these cells was not an important contributor to LDL oxidation, if it contributed at all. These findings are in contrast to those reported by other laboratories in which the respiratory burst production of superoxide anion has been found to correlate with an increase in oxidation of LDL (11, 27, 44, 46). The reason for the differences in findings remains to be elucidated.

Whatever the source of the initiating lipoperoxides in the LDL, our data suggest that propagation is essential to explain the levels of lipoperoxides that accumulate. Thus, the addition of EDTA markedly inhibited the appearance of lipoperoxides both in LDL incubated with the 15-LO cells (Fig. 3) and also the build up of lipoperoxides in LDL incubated directly with linoleic acid hydroperoxide (Fig. 5). Furthermore, probucol or vitamin E enrichment of LDL strongly inhibited the accumulation of CE-OOH in LDL incubated with the 15-LO cells.

It has been reported in several systems now that the predominant hydroperoxide appearing in oxidized LDL is CE-OOH, as in the present study, although the ratio of CE-OOH to FFA-OOH varied considerably from experiment to experiment. This occurs whether the oxidation is catalyzed by incubation with lipid hydroperoxides, by incubation with copper ion or free radical generators (47–50), or by treatment with purified lipoxygenases (24). Even a lipophilic free radical generator yields predominantly CE-OOH in LDL (whereas it generates comparable quantities of CE-OOH and PL-OOH when the lipids are dissolved in an organic solvent along with the free radical generator) (49). In preliminary studies we have collected the CE-OOH peak from HPLC and either saponified (6% KOH, 30 min, 60°C) (25) or treated with cholesterol esterase (EC 3.1.1.13; sterol-ester acyl hydrolase) (IU/ml, 60 min, 37°C). On HPLC, the re-extracted lipids showed only FFA-OOH and no detectable free cholesterol hydroperoxide (data not shown).

Linoleic acid is the major polyunsaturated fatty acid target for peroxidation in LDL, exceeding the amount of arachidonic acid peroxidized by about 8 to 1 on a molar basis (51). It has been suggested that the predominance of CE-OOH simply reflects the predominance of linoleic acid in LDL cholesteryl esters relative to that in phospholipids and that therefore no selectivity with regard to priming or propagation is necessary to explain the finding. Actually the PL-OOH recovered here is much lower than would be predicted by the concentration of polyunsaturated fatty acids in phospholipids relative to that in cholesteryl esters. However, if we assume that all of the FFA-OOH is derived from breakdown of PL-OOH and pool the values for FFA-OOH and PL-OOH for comparison with those for CE-OOH, there is good agreement. Thus, calculating from the data of Esterbauer et al. (52) on lipid class composition of LDL and the fatty acid composition of each lipid class as reported by Lecerf et al. (53), LDL phospholipids would contribute 291 moles of polyunsaturated fatty acid per mole of LDL and LDL cholesteryl esters would contribute 949 moles/mole. Our data show the PL-OOH content less than 10% that of CE-OOH after 1 or 2 h of incubation (instead of the 30% expected if generation of peroxides were random). Some or all of this discrepancy could reflect preferential degradation of...
PL-OOH. We know that LDL does contain phospholipase activity (54), and that phospholipases, especially the PAF hydrolase associated with LDL (55, 56), are much more active against oxidized, truncated fatty acids in the 2-position. A strong case can be made for the proposition that most if not all of the phospholipid hydrolysis is attributable to the PAF hydrolase (56).

The present results support the conclusion that 15-LO can play a role in LDL oxidation, but leave open the question of whether 15-LO activity contributes importantly to the oxidation of LDL in developing atherosclerotic lesions. 15-LO enzyme activity is found in lesions (28) and 15-LO mRNA and protein are strongly expressed in lesions (29, 57) and colocalize with macrophages, cells that have been shown previously to express 15-LO (18). Perhaps the best in vivo evidence that 15-LO contributes to atherogenesis comes from the recent studies of Kühn et al. (23). They have shown that the hydroxylinoleic acid in the lesion lipids of cholesterol-fed rabbits, particularly at an early stage, is not a racemic mixture of the S- and the R-forms. If oxidation were triggered by superoxide anion, hydroxyl radical, or any other non-enzymatic oxidative system, an equimolar mixture of R- and S-forms would be expected. Kühn and colleagues (23) found that in the lesion lipids of rabbits fed cholesterol for 6 weeks, fully 72% of the hydroxylinoleic acid present was in the S-form. Thus we can say that 15-LO is not only present in the lesion, but also that it is actively oxidizing the lipids being deposited. Quantifying its contribution to the rate of progression of the lesion will require in vivo intervention studies with specific antagonists or genetic engineering studies in which 15-LO is either knocked out or overexpressed.

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