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Enhanced Levels of Lipoperoxides in Low Density Lipoprotein Incubated with Murine Fibroblasts Expressing High Levels of Human 15-Lipoxygenase*

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There is strong experimental evidence that oxidized low density lipoprotein (Ox-LDL) plays an important role in atherosclerosis. However, the mechanisms by which Ox-LDL is formed *in vivo* are unknown. To test whether 15-lipoxygenase (15-LO) could play a role in oxidation of LDL by cells, we expressed 15-LO activity in murine fibroblasts, which do not normally have 15-LO activity, and tested their ability to modify LDL. Using a retroviral vector, we prepared fibroblasts that expressed 2- to 20-fold more 15-LO activity than control fibroblasts infected with a vector containing β-galactosidase (lacZ). Compared with LDL incubated with lacZ cells, LDL incubated with 15-LO-containing cells were enriched with lipid hydroperoxides. When these LDL samples were subsequently subjected to oxidative stress, they were more susceptible to further oxidative modification, as judged by increased conjugated diene formation and by increased ability to compete with ^{125}I-Ox-LDL for uptake by macrophages. These findings establish that cellular 15-LO can contribute to oxidative modification of LDL, but the quantitative significance of these findings to the *in vivo* oxidation of LDL remains to be established.

Oxidatively modified low density lipoprotein (LDL)[1] is potentially much more atherogenic than native LDL (1), and there is strong evidence that it plays a role in experimental atherosclerosis (2). While a causative role in human atherosclerosis has not yet been established, there is indirect evidence to support that possibility (reviewed in Refs. 1, 3). A number of clinical trials to test the effectiveness of antioxidant supplements in the prevention of coronary heart disease are either underway or in the planning stages. These studies are predicated on the introduction into the LDL particle itself of chain-breaking antioxidants (*e.g.* vitamin E) that prevent or limit the extent of LDL oxidation. If we knew the mechanisms involved in oxidation of LDL *in vivo*, it should become possible to design novel forms of intervention in addition to or instead of the introduction of antioxidants into LDL. For example, if, as has been suggested, 15-lipoxygenase (15-LO) plays a role in oxidation of LDL by cells (4, 5), then the use of appropriate 15-LO inhibitors might offer a new complementary mode of treatment. If, on the other hand, oxidation *in vivo* depends on the generation of superoxide anion (6–9), interventions that inhibit NADPH oxidase or other sources of superoxide anion would be of potential benefit, as would interventions that enhance the activity of superoxide dismutase or other systems quenching the action of the superoxide anion. Unfortunately, little or nothing is known about the sites and the mechanisms of LDL oxidation *in vivo*. Studies in cell culture allow us to identify a large number of candidate systems that could participate in oxidative modification of LDL. These include not only 15-LO and NADPH oxidase but also the P450 system, the mitochondrial electron transport chain, myeloperoxidase, cyclooxygenase, and others. From studies *in vitro*, it is already clear that different cells may utilize different systems and that individual cell types probably use multiple systems in the oxidative modification of LDL. For example, monocytes and smooth muscle cells do not express 15-LO (10), but they do oxidatively modify LDL, showing that 15-LO is not *necessary* for oxidative modification of LDL. Similarly, freshly isolated monocytes express little or no NADPH activity, but they can oxidize LDL at a low rate. After activation to induce expression of NADPH oxidase and the accompanying production of superoxide anion, the rate of LDL oxidation is considerably enhanced. Again, the NADPH oxidase system is not *necessary* but it can certainly contribute to oxidative modification. Secretion of H_2O_2 by some cells could also play a role especially if thiols are concurrently secreted (11, 12). We are presently trying to identify which candidate systems are worth further study, especially *in vivo*.

Evidence that supports a role of 15-LO in oxidative modification of LDL can be summarized as follows: 1) purified 15-lipoxygenases can oxidatively modify LDL in cell-free systems (4, 13–14); 2) oxidation of LDL by endothelial cells and by macrophages in culture is suppressed by known inhibitors of lipoxygenase (4, 5); 3) tissue samples from atherosclerotic lesions, but not from normal aortas, exhibit measurable levels of 15-LO mRNA and protein (15, 16), as well as 15-LO enzyme activity (17); 4) transfer of the 15-LO gene into rabbit iliac arteries results in the appearance of epitopes of oxidized LDL (18); 5) most importantly, increased amounts of stereospecific products of the 15-LO reaction have been demonstrated in early atherosclerotic lesions (14) but not in later lesions, where non-enzymatic oxidation reactions due to later steps of the propagation reactions of lipid peroxidation predominate (19).

However, interpretation of studies using LO inhibitors must

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[1] The abbreviations used are: LDL, low density lipoprotein; TBARS, thiobarbituric acid-reactive substances; 15-LO, 15-lipoxygenase; ox-LDL, oxidized LDL; LOOH, lipid hydroperoxide; ETYA, eicosatetraynoic acid; DME, Dulbecco's modified Eagle's medium; kb, kilobase(s); 13-HODE, 13-hydroxy-octadecadienoic acid; PCR, polymerase chain reaction.

be made with caution because most of these inhibitors are also nonspecific antioxidants. Consequently, their presence in the medium could suppress LDL oxidation and yet that inhibition might not be related to inhibition of 15-LO activity. The acetylenic analog of arachadonic acid, eicosatetrayonic acid (ETYA), on the other hand, is devoid of nonspecific antioxidant activity, but it does inhibit 15-LO activity and LDL oxidation in cultured cells (5). Sparrow *et al.* (20) point out, however, that the concentrations needed to inhibit LDL oxidation appear to be much higher than the concentrations needed to inhibit 15-LO activity. Because of this apparent dissociation, they suggested the possibility that the ETYA inhibition of LDL oxidation might be nonspecific, possibly the result of cell toxicity. Indeed, because inhibitors are rarely specific to one and only one target, the use of inhibitors to implicate an enzyme system may yield misleading results. Indeed, ETYA inhibits many other cellular functions as well (21). For these reasons we decided to explore further the role of 15-LO in LDL oxidation by expressing 15-LO activity in murine fibroblasts, which do not normally express 15-LO, and to assess the effect of this overexpression on the ability of these modified cells to oxidize LDL.

EXPERIMENTAL PROCEDURES

Materials—Electrophoresis grade agarose was obtained from Fischer Scientific (Fair Lawn, NJ). Ethidium bromide, Ham's F-10 medium, bovine hematin, and HEPES-free acid were from Sigma. Geneticin (G418) and Dulbecco's modified Eagle's (DME) medium (high glucose) were both obtained in powdered form from Life Technologies, Inc. RPMI 1640 medium was from Irvine Scientific (Santa Ana, CA). Ebselen was a generous gift of Ciba-Geigy. Restriction endonucleases, formamide, and T4 DNA ligase were purchased from Boehringer Mannheim (Mannheim, Germany). 2′,7′-Dichlorofluorescein diacetate and X-OMAT AR film for autoradiography were from Eastman Kodak (Rochester, NY). α-^{32}P-Labeled dCTP (3000 Ci/mmol) and [1-^{14}C]linoleic acid (53.0 mCi/mmol) were from New England Nuclear. Fetal bovine serum was purchased from Hyclone (Logan, UT). The Fast-Track mRNA isolation kit was obtained from Invitrogen (San Diego, CA). The Prime-a-Gene random labeling kit was from Promega (Madison, WI). Magna-Charge nylon transfer membranes were from MSI (Westboro, MA). Human LDL was isolated from normolipidemic donors (22), and ^{125}I-LDL was prepared by the Iodogen method as described previously (22).

Construction of Retroviral Vector—Human 15-LO cDNA, subcloned into the *Eco*RI site of the Bluescript KS+ plasmid (Stratagene), was kindly provided by Dr. Elliott Sigal (Syntex, Palo Alto, CA) (23). A 2.2-kb *Sal*I-*Bgl*II cDNA fragment from this plasmid was directionally subcloned into the *Sal*I and *Bam*HI (*Bgl*II compatible) sites of the retroviral vector pLDRNL, which has been previously described (24). Restriction analysis confirmed that the LDL receptor cDNA sequence (D) in the retroviral vector was successfully replaced with human 15-LO cDNA (LO), and the new vector was subsequently designated pLLORNL. 15-LO expression in LLORNL is driven from the long terminal repeat of the Molony murine leukemia virus, and expression of the Tn5 neomycin-resistance gene is driven from an internal Rous sarcoma virus long terminal repeat. A second Molony murine leukemia virus long terminal repeat provides transcriptional termination sequences.

A parallel construct which contains the β-galactosidase cDNA (lacZ) instead of 15-LO cDNA was designated pLZRNL (25) and was also used to produce stably transformed PA317 cells (lacZ cells). Because lacZ cells differ from 15-LO cells only in their production of β-galactosidase instead of 15-LO, they were used as controls for the 15-LO cells.

Cell Lines and Culture—Both ecotropic ψ2 and amphotropic PA317 murine fibroblast cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum supplemented with glutamine. Cells were plated in 100-mm^2 tissue culture dishes and grown in humidified incubators with 10% CO_2 tension.

Transfection and Infection—ψ2 cells were transfected with plasmids pLLORNL (or pLZRNL) using the calcium phosphate co-precipitation method as described (24). After allowing 24 h for packaging and release of retroviruses, culture medium was collected, sterile filtered, and applied to PA317 cells in the presence of polybrene (4 μg/ml, Sigma). Stably transduced cells were selected using the neomycin analog G418 (400 μg/ml) and, subsequent to colony or cell line establishment, were maintained permanently in the selection medium. Samples from all populations were stored under liquid nitrogen for future analysis, and several clones were chosen for immediate examination.

Northern Analysis and PCR Quantification of 15-LO mRNA Expression—Polyadenylated RNA samples from PA317 clones were isolated via oligo(dT)-cellulose affinity, using a commercially available kit (Invitrogen, San Diego) and were quantified spectrophotometrically. Aliquots of poly(A)$^+$ RNA ranging from 1 to 10 μg were electrophoresed in 1.0%/2.2 M formaldehyde gels, capillary-blot transferred onto MagnaCharge nylon transfer membranes (MSI), and hybridized overnight with a ^{32}P-labeled human 15-LO cDNA probe (*Mst*II fragment, 1.5 kb) as described (23). Filters were washed sequentially in 1 × SSC, 0.1% SDS, 0.5 × SSC, 0.1% SDS, and 0.1 × SSC, 0.1% SDS (15 min at room temperature, each wash: 1 × SSC is 150 mM NaCl, 15 mM Na_3citrate, pH 7.0), dried, and then exposed to film (Kodak X-OMAT AR) for 16 h.

Quantification of 15-LO mRNA in selected PA317 clonal populations was performed using competitive polymerase chain reaction amplification (26). The oligonucleotide primers used were specific for human reticulocyte 15-LO, as described previously (27). Briefly, 100 ng of poly(A)$^+$ was reverse transcribed, and 10 ng of the product was amplified with the 15-LO primers for 30 cycles in the presence of a range of known concentrations of an internal 15-LO standard which was the 15-LO cDNA with a 115-bp deletion. PCR products were electrophoresed and resolved in 8% polyacrylamide gels and then silver-stained. The destained gels were dried, photographed, and the intensity of the bands quantified on an image processor. When the intensities of both bands in the same lane were equal, the concentration of the unknown was assumed to be equal to the known concentration of the internal standard (26).

Enzymatic Assay of 15-LO Activity—To assay macrophage 15-LO activity, [^{14}C]linoleic acid was added to intact cells and, after 30 min, the medium was harvested, the lipids extracted, and analyzed for [^{14}C]13-hydroxy-octadecadienoic acid (13-HODE), as described (5). Because PA317 cells released almost no 15-LO product to the medium in the absence of LDL or other acceptors, assays were done on broken cell preparations. PA317 cells were frozen (at −70 °C for 30 min) and thawed (at 37 °C for 5 min), and the process was repeated three times. The broken cells were then incubated for 30 min with 20–150 nmol of [^{14}C]linoleic acid (2–4 × 10^3 disintegrations/min/nmol). The medium was aspirated, acidified with 1 N HCl, and the lipids extracted with methanol/chloroform 1:1 (v/v). Samples were dried under nitrogen and resuspended in a minimal volume of methanol/water/acetic acid 90:10:0.01 (v/v) and stored at −20 °C. These samples were then applied to the high performance liquid chromatography for separation and analysis (5). In addition, control standards (linoleic acid and 13-HODE) were run to confirm retention times.

Electrophoretic Assessment of LDL Mobility—LDL (100 μg/ml) was exposed to confluent plates of lacZ or 15-LO cells for varying times, and then the media from like samples were pooled and concentrated by centrifugal ultrafiltration. Agarose gel electrophoresis was then performed using agarose gels (Universal Gel/8) supplied by Ciba Corning Diagnostic Corp as described (22).

Macrophage Degradation—The ability of mouse peritoneal macrophages to degrade LDL modified by the lacZ and 15-LO cell clones was initially measured as described previously (5, 22). ^{125}I-LDL was incubated with cells in Ham's F-10 or in cysteine-free RPMI medium for the indicated times. Then aliquots were incubated with freshly plated mouse peritoneal macrophages for 5 h (final concentration 10 μg of LDL protein in 0.5 ml of DME), and then non-iodide, trichloroacetic acid-soluble degradation products in the media were determined. Because iodination of LDL initiates peroxidation of LDL (22) and complicates interpretation of experiments seeking to assess the ability of cells to modify LDL, we also performed experiments in which unlabeled LDL was used, and the extent of LDL modification was determined by its ability to compete with ^{125}I-ox-LDL for degradation by macrophages. For these experiments, ^{125}I-ox-LDL was incubated with macrophages in the absence or presence of LDL that had been preincubated with lacZ or 15-LO cells and then exposed to further oxidative stress as noted below. LDL (200 μg/ml) was incubated at 37 °C with lacZ cells or with 15-LO cells in cysteine-free RPMI media for 20 h. The media were then diluted 1:1 with RPMI to yield LDL concentrations of 100 μg/ml. Aliquots of these media were incubated with copper (5 μM) for 3.5 h and then 25 μg of LDL from the Cu^{2+} incubations was added to cultures of macrophages containing 2.5 μg of ^{125}I-ox-LDL in DME. The extent of degradation of the ^{125}I-ox-LDL tracer was determined after 20 h of incubation.

In other experiments, aliquots of LDL-containing media that had been preconditioned by incubation with lacZ or 15-LO cells for 20 h were subsequently incubated with cultures of mouse peritoneal macrophages in DME/RPMI/(50:50 mixture) for 20 h to effect an oxidative stress (instead of exposure to copper as described above). Then aliquots con-

taining 25 μg of unlabeled LDL were added to a new set of macrophage cultures in DME containing 2.5 μg of ^{125}I-ox-LDL and extent of degradation of ^{125}I-ox-LDL determined as described above.

Conjugated Diene Assay—In some experiments, aliquots of the LDL preincubated with lacZ or 15-LO cells were subjected to ultracentrifugation to reisolate LDL, dialyzed to remove salts and EDTA, and the time course of conjugated diene formation determined in the presence of 2.5 μM copper as described previously (22).

LOOH and Hydrogen Peroxide Assay—The determination of LOOH/H_2O_2 content in cell-modified LDL was performed according to a modification of the procedure of Cathcart *et al.* (28). Briefly, cultured cells (lacZ and clone 12) at 70–80% confluence were incubated with and without LDL (100 μg/ml) at 37 °C for 0–2 h in Hank's balanced salt solution (pH 7.35) containing 6.5 mM glucose. Aliquots of media (<2.5 μg of protein) were then diluted to final volumes of 1.1 ml with distilled water, and 2.9 ml of the indicator substrate/reagent was subsequently added to all samples.

In some experiments, hydrogen peroxide (H_2O_2) in the media was removed by incubating aliquots for 10 min at 37 °C with gentle shaking (80 revolutions/min) in the presence of catalase (EC 1.11.1.6) immobilized on 4% agarose beads (2.4 units) (Sigma) which selectively destroys H_2O_2 but not LOOH. Aliquots were chilled to 4 °C, centrifuged at 1,500 revolutions/min for 5 min to remove immobilized catalase, and aliquots (<2.5 μg) of LDL obtained for fluorescence study. To measure the LOOH specifically bound to LDL, modified LDL were reisolated from the media as described below.

The indicating reagent was prepared as follows: (i) 2 ml of 0.01 N NaOH was added to 0.5 ml of a solution of 2′,7′-dichlorofluorescein diacetate dissolved in ethanol (1 mM), and after 30 min at room temperature, the reaction solution was diluted with 10 ml of 25 mM sodium phosphate buffer (pH 7.2), yielding 2′,7′-dichlorofluorescein solution; (ii) 1 mg of hematin was dissolved in 0.5 ml of 0.2 N NaOH, diluted with 100 ml of 25 mM sodium phosphate buffer, and a 14-ml aliquot of this hematin solution was boiled for 15 min with 100 ml of 25 mM sodium phosphate buffer, yielding an activated hematin solution; and (iii) the 2′,7′-dichlorofluorescein solution (2 ml) and the activated hematin solution were mixed at 4 °C, yielding the indicator reagent, which was kept under N_2 gas to prevent oxidation.

Reactions between media samples and the indicator reagent were carried out at 50 °C for 50 min and, after cooling in a room temperature water bath, the fluorescence intensity (excitation = 440 nm, emission = 550 nm) of each sample was measured within 30 min. The standard curve for the assay was generated using fluorescence intensities obtained from known quantities (0–2 nmol) of oxidized linoleic acid (1 μmol of linoleic acid was dissolved in 1 ml phosphate-buffered saline, and oxidized with 500-1000 units of soybean lipoxygenase for 60 min at 37 °C). The concentrations of oxidized linoleic acid was confirmed in the standard samples by monitoring absorbance at 234 nm.

In some experiments, LDL was isolated from the media prior to determination of LOOH content according to a modification of the procedure of Lynch and Frei (29). In brief, media were subjected to concentration by centrifugal ultrafiltration (Amicon) and isolation by gel filtration on Sephadex-G25 M columns (PD-10, Pharmacia) equilibrated with phosphate-buffered saline at a flow rate of 1.2–1.5 ml/min. Fractions were collected at 1.5 min and monitored by absorbance at 280 nm. Total lipids in 0.5-ml aliquots were extracted, according to a modified procedure of Bligh and Dyer (using 1 ml of $CHCl_3$, MeOH, and 0.5 ml of 0.03 N HCl) (30), and the $CHCl_3$ layer was dried under N_2, redissolved in 0.1 ml of MeOH and 0.9 ml of distilled H_2O, and used for fluorescence measurement. Another aliquot of media was used for determination of protein, which was measured by the procedure of Lowry *et al.* (31).

Ebselen is a seleno-organic compound (2-phenyl-1,2-benzoisoselenozol-3(2H)-on) that can directly reduce lipoperoxides and can mimic glutathione peroxidase (32–34). To further document that the fluorescence measured in these experiments was indeed LOOH, we pretreated LDL previously incubated with 15-LO cells with ebselen and then measured LOOH fluorescence. Subsequent to its incubation with 15-LO cells (clone 12), LDL was reisolated by gel chromatography using PD-10 columns, concentrated by ultrafiltration, and the protein concentration adjusted to 0.5–1 mg/ml with 0.1 M Tris-HCl, 1 mM EDTA, pH 7.4. Specific additions of ebselen (added in ethanol) were made, and the mixture was then incubated at 37 °C in Tris-HCl/EDTA under nitrogen for indicated times. Ebselen was then removed from the incubation by gel filtration, and LOOH was measured as described above. In some experiments, GSH (3 mM) which enhances the ability of ebselen to reduce LOOH (34) was also added.

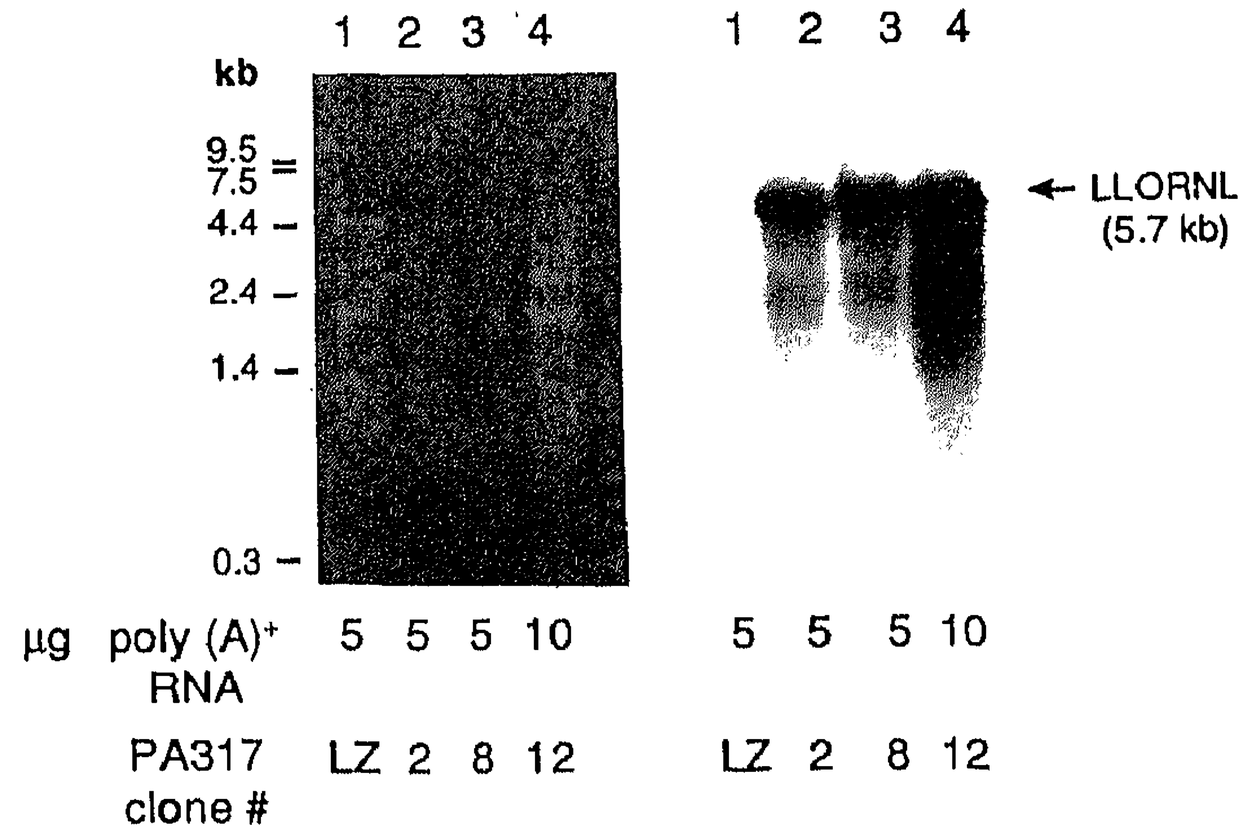

FIG. 1. **Expression of retroviral RNA by stably transformed populations of LLORNL-infected PA317 cells.** *Left panel*, ethidium bromide-stained gel containing poly(A)$^+$ RNA from PA317 cells infected with pLZRNL and pLLORNL, prior to capillary transfer to nitrocellulose membrane. *Right panel*, Northern analysis of samples from *left panel* using a randomly labeled, 1.5-kb cDNA probe (*Mst*II fragment from human 15-LO). *Lane 1*, poly(A)$^+$ RNA from lacZ-infected clone (*LZ*); *lanes 2–4*, 15-LO clones 2, 8, and 12, respectively.

RESULTS

Expression of 15-LO mRNA in Infected PA317 Cells—Northern analysis, using a radiolabeled 15-LO cDNA probe, detected an abundance of intact retroviral poly(A)$^+$ RNA in cells infected with LLORNL (15-LO cells) (Fig. 1). The size of the predominant transcript in all clones examined was 5.7 kb, in agreement with prediction. Smaller hybridizing transcripts were also visible. Poly(A)$^+$ RNA isolated from LZRNL infected cells (lacZ cells) failed to give a detectable signal (*lane 1*). Quantitative PCR, utilizing an internal standard, demonstrated that clone 12 expressed approximately 300 copies/cell and clones 2 and 8 approximately 163 and 150, respectively.

Expression of 15-LO Activity in Infected PA317 Cells—To detect 15-LO activity in macrophages, we previously used a method in which radiolabeled linoleic acid was added to the culture medium, and the medium was subsequently assayed for the presence of radioactive 13-HODE, a specific product of 15-LO (5). However, when the 15-LO-infected PA317 cells were assayed in this way, little or no 13-HODE was detected in the media (Table I). This could be either because the added linoleic acid substrate itself was too rapidly converted into esterified lipids or because the 15-LO product was rapidly esterified in the cells. Indeed, analysis of lipids from cells incubated with [^{14}C]linoleic acid confirmed that almost all of the added free fatty acid taken up was rapidly esterified. Therefore, we turned to assaying 15-LO activity in broken cell preparations (see "Experimental Procedures"). We found a 10–20-fold higher level of enzyme activity in the 15-LO-infected cell populations than in the lacZ-infected control cells (Fig. 2). This high activity could be reduced 70–90% by the addition of ETYA (1–5 μM), a potent inhibitor of LO activity (data not shown). The differences in enzyme activity in the three different 15-LO clones paralleled the differences in mRNA expression, as determined through quantitative PCR analysis (see above). Comparison of these results with those previously observed for mouse peritoneal macrophages is difficult because of the difference in assay conditions (*i.e.* use of *intact* macrophages). Keeping that limitation in mind, clones 2 and 8 demonstrated 30–50% as much 15-LO activity, and clone 12 about 2–3-fold more activity than peritoneal macrophages.

Increased Content of Lipid Hydroperoxides in 15-LO Clones—The different 15-LO fibroblast clones were incubated

TABLE I

Measurement of 15-LO activity in lacZ and 15-LO cells (clone 12)

Murine fibroblasts were grown to confluence in DME with 10% fetal calf serum and glutamine. Cells were washed three times with Ham's F-10. One set of cultures was exposed to three cycles of freezing (−70 °C, 15 min) and thawing (37 °C, 5 min). Then, 1 ml of F-10 containing 20 nmol of [^{14}C]linoleic acid was added to intact and broken cells. After 30 min, the media were collected, centrifuged to remove cellular debris, and supernatants extracted for determination of [^{14}C]linoleic acid conversion to 13-HODE as described under "Experimental Procedures."

PA 317 clone	15-LO activity[a]	
	Intact cells	Broken cells
lacZ	1.13	0.82
15-LO (clone 12)	1.71	11.58

[a] Nanomoles of 13-HODE/30 min/confluent dish.

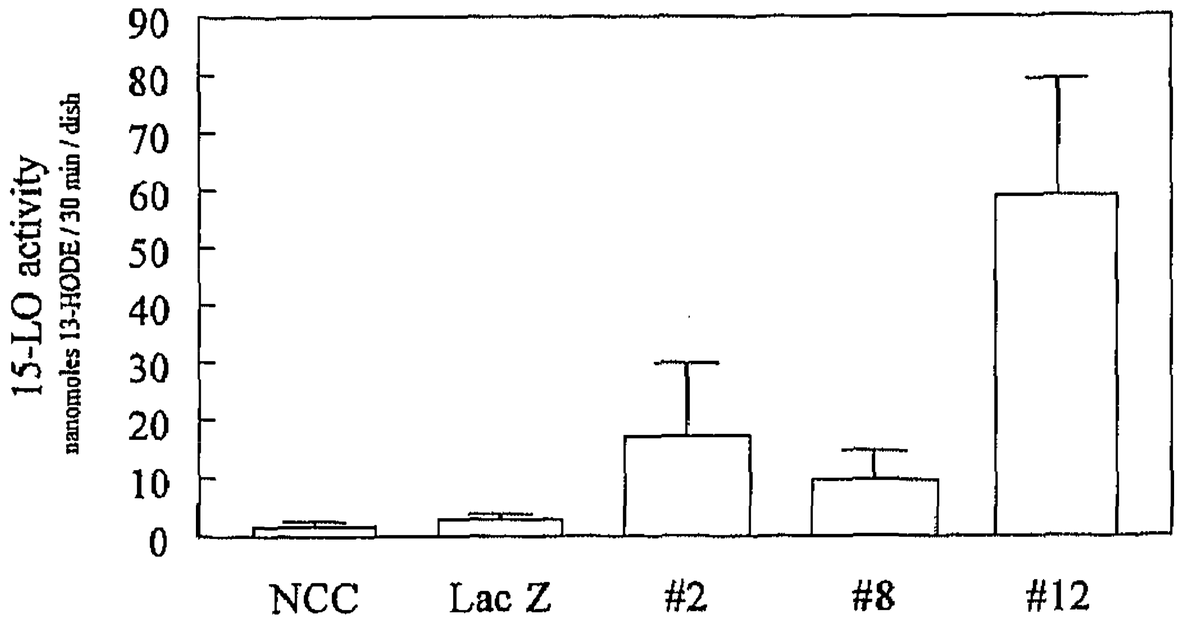

FIG. 2. **15-LO activity in murine fibroblast clones.** 15-LO activity (in nanomoles of 13-HODE/30 min/dish) was determined in broken cell preparations of lacZ and 15-LO cells (*clones 2, 8,* and *12*) and in control dishes with no cells (*NCC*). Results shown are 24 ± S.D. of six separate experiments. By analysis of variance, values for lacZ were not different from NCC; values of clones 2 and 8 were greater than lacZ ($p < 0.04$); and values for clone 12 greater than clones 2 and 8 ($p < 0.0001$). Assay of 15-LO activity in *intact* macrophages usually yields values of 20–30 nmol of 13-HODE/30 min/dish.

TABLE II

Total LOOH recovered in cells and in medium after incubation of lacZ and 15-LO clone 12 cells for 1 h in the absence or in the presence of LDL

Cells were incubated for 1 h at 37 °C in 4 ml of Hanks' balanced salt solution in the absence or in the presence of LDL (100 μg/ml). Lipids were extracted from media and from cells (washed three times with phosphate-buffered saline) and LOOH determined as described under "Experimental Procedures." Results expressed in nanomoles LOOH/well (~0.4–0.7 mg cell protein/well).

	Total LOOH recovered (nmol)				
	In cells		In medium		
	−LDL	+LDL	−LDL	+LDL	Corrected for no cell controls
No cell control			ND[a]	4.64	
Lac Z	ND	ND	ND	6.16	1.52
15-LO (clone 12)	0.26	2.32	1.23	15.5	10.9

[a] ND, not detected.

TABLE III

Lipoperoxides recovered in medium after 1 and after 2 h of incubation of various cell lines, without and with LDL in the medium

Cells were incubated in Hanks' balanced solution with or without 100 μg/ml LDL.

Cell line	LDL in medium	Lipoperoxides (nmol/mg cell protein[a])	
		1 h	2 h
Lac Z	−	5.5 ± 1.2 (10)	5.4 ± 1.6 (6)
	+	6.4 ± 0.7 (28)	17.7 ± 2.1 (30)
Clone 12	−	7.6 ± 0.9 (6)	10.0 ± 0.8 (6)
	+	34.0 ± 3.3 (22)	52.4 ± 5.2 (22)
Clone 2	+	14.3 ± 6.3 (12)	11.9 ± 2.0 (12)
Clone 8	+	24.0 ± 1.8 (12)	19.1 ± 3.6 (12)
Mouse peritoneal macrophages	+	239.4 ± 19.2 (6)	431.7 ± 38.1 (6)

[a] Results are expressed as nanomoles of total lipoperoxides in the medium at 1 or 2 h. Data presented are experimental values obtained after subtraction of no-cell control dishes run in parallel. Values are mean ± S.E.; numbers in parentheses indicate the number of individual cell incubations conducted.

in Hank's medium in the absence or presence of LDL for 1 h. Media were removed and lipid extracts prepared from both the media and the washed cells, and LOOH content determined (Table II). In the absence of LDL there was no LOOH detectable in the media. Only small amounts of LOOH were detected in 15-LO cell extracts (clone 12) and none in the lacZ cells. With LDL in the medium, LOOH was now readily detectable in the 15-LO cell extracts, but there was still no detectable LOOH in the lacZ cells. The greater abundance of LOOH in 15-LO cells incubated with LDL could be due either to provision of substrate or stimulation of 15-LO activity (35), although this latter mechanism would have to be of a post-transcriptional nature (as the cDNA contains only the coding region of 15-LO).

Increased Content of LOOH in LDL Incubated with 15-LO Clones—One mechanism by which increased 15-LO activity within cells might lead to enhanced oxidative modification of LDL in the medium would be to seed the LDL with lipid hydroperoxides. To test this idea we measured lipid hydroperoxides in LDL incubated with lacZ or with 15-LO cells. As shown in Table II, LDL incubated with clone 12 contained 2.5-fold more LOOH than did LDL incubated with lacZ cells and up to four times more than the no-cell control. In Table III, we show similar studies with all three 15-LO clones and, for comparison, with mouse peritoneal macrophages. In these studies, values for no-cell controls have been subtracted in every case (as in the right column of Table II). After a 1-h incubation with LDL, the LOOH in the medium incubated with clone 12 cells was about five times greater than that in the medium incubated with lacZ cells. The value at 2 h (52.4) was less than twice that at 1 h (34.0). The apparent release from mouse peritoneal macrophages was more nearly linear, but in the clones with lower 15-LO activity (no. 8 and no. 2) the values at 2 h were actually lower than the 1-h values, implying reuptake or decomposition of LOOH. For this reason, the 1-h values may better reflect the initial rate of LOOH release. Note that the initial rate of release of LOOH from peritoneal macrophages was more than six times that from the clone 12 cells. In several experiments, we passed the media over a PD-10 column to isolate the LDL (see "Experimental Procedures") and demonstrated that when LDL was present, more than 95% of the measured LOOH in the medium was associated with LDL.

The fluorometric method used to measure LOOH in these studies also detects H_2O_2. However, when LDL-containing media were preincubated with catalase bound to agarose, the fluorescence yield was not decreased, indicating that almost all of the measured fluorescence represented LOOH rather than H_2O_2 (data not shown). Frequently, the apparent LOOH after catalase treatment was paradoxically modestly *increased*, for reasons not known. To test whether some of the fluorescence might be nonspecific, LDL isolated after incubation with 15-LO cells was pretreated with ebselen, an agent known to reduce LOOH content (32–34). Ebselen was then removed by gel filtration, and the content of LOOH was determined. As shown in Table IV, nearly all of the fluorescence was eliminated by pretreatment with 25 μM of ebselen. When ebselen and GSH were added together, the elimination of LOOH was greatly accelerated (see legend to Table IV).

Oxidative Modification of LDL Assessed in Several Ways

TABLE IV
Reduction of LOOH content in LDL by ebselen

LDL was incubated with 15-LO cells (clone 12) for 2 h and then reisolated by gel filtration using 0.1 M Tris, 1 mM EDTA. Indicated amounts of ebselen (in absolute ETOH) were added to LDL which was then incubated for 5 min at 37 °C and then further incubated under N_2 for the times indicated in the table. At the end of the reactions, ebselen was removed by gel filtration and LOOH content determined. Values represent the average of two to four determinations for each sample. In other experiments, the addition of GSH in addition to the ebselen was shown to greatly accelerate LOOH decomposition. Thus, incubation of ebselen (25 μM) plus GSH (3 mM) for only 10 min yielded an LDL with only 2.62 nmol of LOOH/mg LDL protein.

	LOOH in LDL (nmol/mg LDL protein)		
Incubation time	None	Ebselen 12.5	25.0
min		*μM*	
0	34.45	34.45	38.69
3		25.26	15.23
5	30.52	23.21	7.18
10	29.26	18.31	4.68
20			3.30
30	24.22	12.63	2.17

after Incubation with 15-LO Cell—Initially, we incubated LDL with the 15-LO cells and lacZ control cells for varying periods up to 20 h and then performed agarose gel electrophoresis. LDL incubated with lacZ cells showed greater mobility than LDL incubated in the absence of cells. However, LDL incubated with the 15-LO clones did not show any greater increase in mobility than lacZ-conditioned LDL (data not shown). We also measured thiobarbituric acid-reacting substances (TBARS) and found similar results, *i.e.* there was a small increase in TBARS for LDL incubated with lacZ cells compared to no-cell control, but there was no greater increase in LDL exposed to the different 15-LO clones, including clone 12.

We next tested the ability of the different clones to oxidatively modify LDL as measured by enhanced uptake in macrophages. In this set of experiments, ^{125}I-LDL was incubated with lacZ cells or with clone 12 cells for 20 h in either Ham's F-10 or in cysteine-free RPMI and then the rate of degradation of the conditioned ^{125}I-LDL by mouse peritoneal macrophages was determined (Fig. 3). LDL conditioned by lacZ cells was consistently degraded more rapidly than native LDL or LDL incubated in the absence of cells. There was a wide range of values from experiment to experiment, probably reflecting in part the degree of preexisting oxidation of the LDL preparations used. This in turn is determined importantly by the "age" of the LDL preparation and the time between iodination and use in the experiment (22). However, the *difference* between clone 12 cells and lacZ cells was highly consistent, being in the same direction in every one of 20 separate experiments. Using Student's paired t test, clone 12 displayed a statistically highly significant ($p < 0.003$), nearly 2-fold greater ability to modify LDL than did the lacZ cells (Fig. 3).

Consequences of Increased Seeding of LDL with LOOH by 15-LO Cells—Although the LDL incubated with clone 12 cells showed a nearly 2-fold increase in rates of degradation by macrophages, this was not nearly proportional to the nearly 20-fold increase in their 15-LO activity. One possibility is that a rate-limiting step is the transfer of LOOH from the cells to the LDL and not the rate of LOOH generation within the cells. Indeed, the data in Tables II and III show that the initial rate of appearance of LOOH in LDL incubated with clone 12 cells was only two to three times that for LDL incubated with lacZ cells and not 20-fold greater. An LDL with a 2–3-fold increased level of LOOH should be more susceptible to further oxidative modification when subjected to an additional oxidative stress. To test this possibility, we examined the susceptibility of the LOOH-enriched LDL (harvested and refloated after a 24-h incubation) to copper-induced lipid peroxidation in the absence of cells as measured by rate of conjugated diene formation (Fig. 4). Compared to LDL incubated with lacZ cells, LDL incubated with clone 12 cells had the shortest lag time, while LDL from clones 2 and 8 had intermediate values, results compatible with the observed differences in "seeding" with LOOH (Table III).

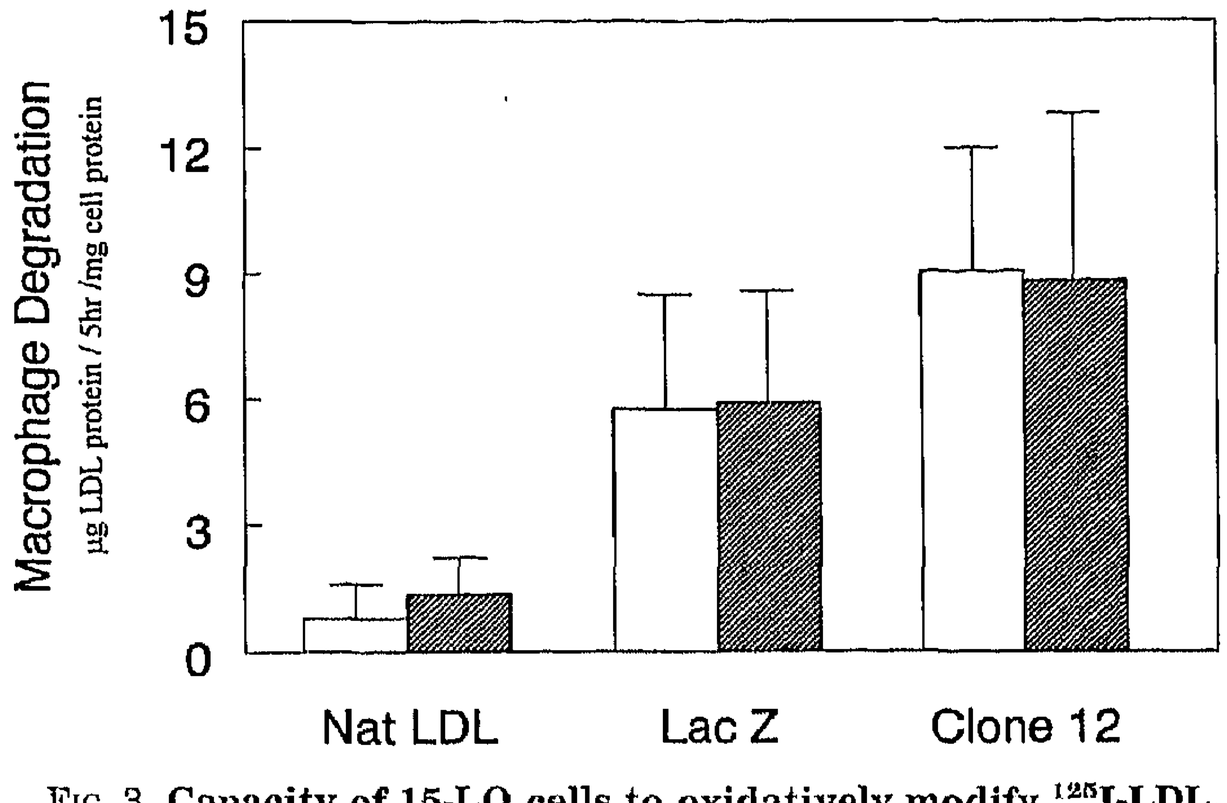

FIG. 3. **Capacity of 15-LO cells to oxidatively modify ^{125}I-LDL.** ^{125}I-LDL was preincubated with lacZ cells or clone 12 for 20 h, and then its rate of degradation by macrophages was determined. Shown are the mean values (± S.D.), in μg LDL degraded/5 h/mg cell protein, of results obtained in 11 separate experiments conducted in Ham's F-10 medium (□) and 9 separate experiments conducted in cysteine-free RPMI (■). Shown also is the rate of degradation by macrophages of native ^{125}I-LDL. For all experiments the values were corrected by subtraction of degradation products formed in the absence of cells. Macrophage degradation of ^{125}I-LDL modified by 15-LO clone no. 12 is different from that of lacZ in both types of media ($p < 0.003$, Student's t test).

Another possibility is that the ^{125}I-labeled LDL used in the experiments shown in Fig. 3 had already undergone oxidation secondary to the radiation (22), and this pro-oxidant effect masked small differences in the abilities of cells to further oxidize LDL. To test this idea, we incubated *unlabeled* LDL for 20 h with lacZ cells and with the three different clones of 15-LO cells and then subjected each of these LDL samples to a subsequent oxidative stress by incubation with mouse peritoneal macrophages (cultured in DME/RPMI at 1:1 ratio) for 20 h. Each LDL was then tested for its ability to *compete* with ^{125}I-ox-LDL for uptake by a different set of macrophages (Table V). In this experiment, under the experimental conditions used, 25 μg of unlabeled copper-oxidized LDL was able to compete for 70% of the uptake of 2.5 μg of ^{125}I-ox-LDL. Twenty-five μg of LDL initially incubated with lacZ cells inhibited degradation of ox-LDL by only 8%, but 25 μg of LDL previously incubated with clone 12 inhibited ^{125}I-ox-LDL uptake by 71%, *i.e.* it was just as effective as copper-oxidized LDL. Twenty-five μg of LDL conditioned by incubation with clones 2 and 8 yielded intermediate values of inhibition.

In another set of experiments, we took unlabeled LDL preconditioned by a 20-h incubation with lacZ cells and 15-LO cells and then exposed the conditioned LDL samples to a brief oxidative stress by exposure to copper for 3.5 h in the absence of cells. These LDL were then tested for their ability to compete with ^{125}I-ox-LDL for uptake by macrophages (Table VI). In this experiment, unlabeled copper-oxidized LDL inhibited uptake of ^{125}I-ox-LDL by 61%, and clone 12-conditioned LDL inhibited uptake by 44%, whereas LDL conditioned by incubation with clone 2 and 8 inhibited uptake by only 15 and 12%, respectively. LDL from lacZ cells did not inhibit degradation at all. Similar results were seen in four other experiments.

It is of interest that despite the obvious enhanced susceptibility of LDL conditioned by incubation with clone 12 cells to

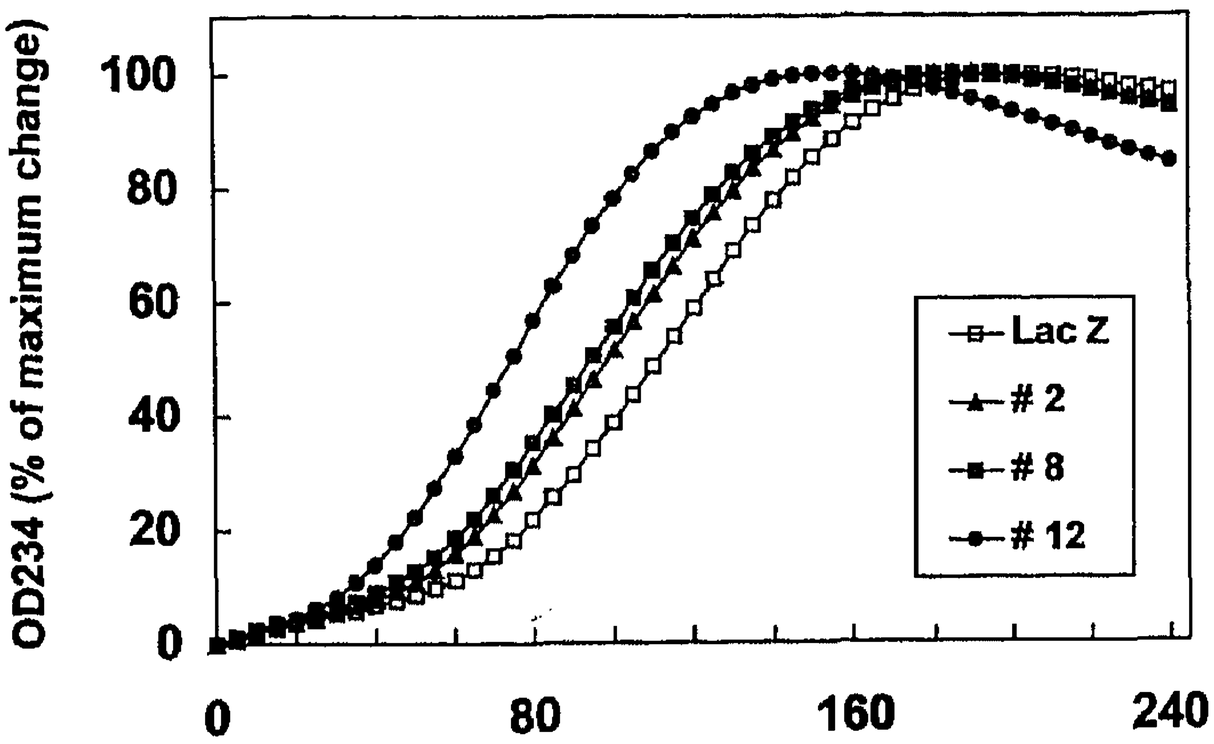

FIG. 4. **Time course of conjugated diene formation of LDL preincubated with murine fibroblasts.** LDL were preincubated with lacZ cells or 15-LO cells (*clones 2, 8,* and *12*) for 24 h in cysteine-free RPMI. LDL was then isolated from the medium by ultracentrifugation and conjugated diene formation measured in the presence of 2.5 μM copper in a continuously recording spectrophotometer as described (22).

TABLE V

Ability of LDL conditioned by incubation with PA317 clones and then further oxidized by macrophages to compete with ^{125}I-ox-LDL for macrophage degradation

Unlabeled LDL was subjected to oxidative modification either by incubation with copper (5 μM) for 20 h in RPMI in the absence of cells or by incubation for 20 h in cysteine-free RPMI with the lacZ control clone or with clones 2, 8, and 12. The cell-conditioned LDL preparations were subsequently incubated for an additional 20 h with mouse peritoneal macrophages in DME/RPMI (50:50) to effect a further oxidative stress. Each of these unlabeled, oxidized LDL preparations (25 μg/ml) was then tested for its ability to competitively inhibit the degradation of ^{125}I-copper-oxidized LDL (2.5 μg/ml) by mouse peritoneal macrophages in DME (20-h incubation). Each value is the average of duplicate determinations.

Competitor present	^{125}I-ox-LDL degraded	% inhibition
	μg/mg cell protein/20 h	
None	20.1	
1. Copper-oxidized LDL	6.1	70
2. LacZ-conditioned LDL	18.6	8
3. Clone 12-conditioned LDL	5.9	71
4. Clone 2-conditioned LDL	13.5	33
5. Clone 8-conditioned LDL	12.2	39

copper-mediated oxidation, as measured by conjugated diene formation, and by its greater ability to compete with ^{125}I-ox-LDL for macrophage uptake, we could not demonstrate enhanced agarose gel electrophoretic mobility after 3, 6, or even *18* h of copper exposure (data not shown), as compared to the lacZ conditioned LDL exposed to similar conditions. The reason for this dissociation is not known but it implies that a greater increase in net negative charge is not a *sine qua non* for recognition by macrophage scavenger receptors.

DISCUSSION

These studies clearly show that when LDL was incubated with a cell line overexpressing 15-LO there was significantly greater seeding of that LDL with LOOH compared to LDL incubated with control cell line lacZ (Tables II and III). At the end of the first hour, the medium incubated with clone 12 showed five to six times more LOOH than was found in medium incubated with lacZ cells. However, the LOOH content of the medium did not increase linearly. Even in the incubations with clone 12, which showed the highest rate of LOOH buildup, the medium content of LOOH did not double between the end of the first hour and the end of the second hour (Table III). In

TABLE VI

Ability of LDL conditioned by incubation with PA317 clones and then further oxidized by incubation with copper to compete with ^{125}I-ox-LDL for macrophage degradation

Unlabeled LDL was oxidized in various ways and then tested for its ability to compete with ^{125}I-ox-LDL for degradation by mouse peritoneal macrophages. The unlabeled competitors include: 1) LDL incubated with 5 μM copper for 20 h in cysteine-free RPMI; 2–5) LDL preincubated with lacZ or with clones 12, 2, and 8, respectively, in RPMI for 20 h and then oxidized further by exposure to copper (5 μM) in RPMI for 3.5 h. ^{125}I-ox-LDL was prepared by incubation in the presence of 5 μM copper for 20 h in cysteine-free RPMI. It was added at 2.5 μg/ml to mouse peritoneal macrophages along with 25 μg/ml of the unlabeled competitor for 20 h in DME. Values are average of duplicate determinations and representative of four other experiments.

Competitor present	^{125}I-ox-LDL degraded	% inhibition
	μg/mg cell protein/20 h	
None	6.53	
1. Copper-oxidized LDL	2.53	61
2. LacZ-conditioned LDL	7.2	0
3. Clone 12-conditioned LDL	3.0	44
4. Clone 2-conditioned LDL	5.58	15
5. Clone 8-conditioned LDL	5.77	12

the case of the other two clones expressing 15-LO, there was actually *less* LOOH in the medium at the end of the second hour. The reason for this decided non-linearity is not clear, but it has a great deal of relevance to the interpretation of the studies in which LDL was incubated for longer times with these cell lines. One would anticipate that the higher content of LOOH in LDL incubated with clone 12 would lead to a greater rate of subsequent oxidative modification. Indeed, LDL conditioned by incubation with clone 12 had significantly greater rates of macrophage degradation than LDL conditioned by incubation with lacZ cells ($p < 0.003$) (Fig. 3). This was true despite the fact that the absolute rate of degradation of the LDL conditioned by the lacZ mouse fibroblasts was surprisingly high. In some experiments it approached that seen with LDL modified by incubation with endothelial cells or macrophages. Thus, LDL incubated for 24 h with monolayers of mouse peritoneal macrophages is generally degraded at about 7.5 μg/mg macrophage protein/5 h, and the rate for LDL conditioned by endothelial cells is only slightly less than that. In fact, it is unusual to see degradation at a rate greater than 10 μg/mg/5 h, even with copper-oxidized LDL. Thus, the values seen here with PA317 cells may be approaching a "ceiling." In retrospect, the choice of these cells was not optimal for the present studies. Even though the wild-type cells contain no 15-LO activity, they clearly contain other systems that can oxidize LDL at a brisk rate. Despite this, the addition of 15-LO increased the capacity of the cells to seed LDL in the medium with LOOH and make the LDL more susceptible to oxidative modification. O'Leary *et al.* (36) demonstrated directly that enrichment of LDL with lipid peroxides resulting from 15-LO activity led to propagation of LDL oxidation in the presence of transition metals. Similarly, we demonstrate here that an enhanced content of LOOH in LDL incubated with 15-LO cells also made these LDL more susceptible to modification when subsequently challenged with an oxidative stress.

Different LDL preparations can differ widely in their susceptibility to oxidative modification (37, 38). Many factors contribute to that variability, including the progressive oxidation of LDL, even when stored at low temperatures. Khouw *et al.* (22) have recently called attention to the way in which radioiodination of LDL accentuates this oxidation during storage. LDL that has already been "seeded" with lipoperoxides (as a result of storage and/or iodination) will undergo significantly more rapid modification when subjected to oxidative stress, whether it be oxidation catalyzed by copper or oxidation catalyzed by

cells. The fact that EDTA can inhibit macrophage-induced modification of LDL in F-10 medium (39) tells us that metal-catalyzed propagation in the medium is essential for maximum biological modification of LDL and that this probably rests on the presence of lipoperoxides seeded in the LDL itself. We believe that in our first studies (Fig. 3), in which the 15-LO cells had only a 2-fold greater ability to modify LDL, the results were strongly affected by artifactual oxidation of the substrate ^{125}I-LDL, *i.e.* the radiation-induced oxidation minimized differences that resulted from seeding by 15-LO cells. This is supported by comparison with the data in Tables V and VI showing results from later studies in which unlabeled LDL was used rather than ^{125}I-LDL. The ability of such conditioned LDL to compete with copper-oxidized ^{125}I-LDL was studied rather than directly measuring the rate of degradation of conditioned ^{125}I-LDL. Now the contrast between LDL conditioned by clone 12 or by lacZ cells was much greater. In these studies, the unlabeled LDL was first seeded with hydroperoxides by incubation with the PA317 clones, then subjected to an oxidative stress by incubation with macrophages, and finally tested for competition with ^{125}I-ox-LDL. LacZ-conditioned unlabeled LDL showed only an 8% inhibition of the degradation of oxidized ^{125}I-LDL while clone 12-conditioned LDL inhibited by 71%, a value similar to that achieved by unlabeled LDL oxidized by exposure to copper (Table V). As shown in Table VI, similar results were obtained when the cell-conditioned unlabeled LDL was incubated with copper for 3.5 h (instead of the preliminary macrophage incubation) before testing its ability to compete with ^{125}I-ox-LDL. In this instance, the lacZ-conditioned LDL showed no detectable inhibition while the clone 12-conditioned LDL inhibited by 44% (compared with 61% for competition by unlabeled copper-oxidized LDL).

The precise mechanism(s) linking the increased cellular expression of 15-LO to the increased seeding of LDL with lipoperoxides is not established by these studies. One possibility is that 15-LO acts on endogenous lipids in the cell and that these are subsequently transferred to LDL in the medium. Additional possibilities are not ruled out, however. For example, while 15-LO is an intracellular enzyme, it might conceivably be able to act directly on LDL making contact with the cell's surface or there might be increased generation of reactive oxygen species linked to the action of 15-LO, as suggested by the studies of Cathcart and co-workers (40).

These studies establish that cellular 15-LO has the *potential* to play a role in oxidative modification of LDL. Several lines of evidence compatible with such a role were briefly listed in the introduction. Perhaps the strongest evidence that it plays a role *in vivo* comes from the studies of Kühn *et al.* (14, 19), showing that the lipids in fatty streak lesions of cholesterol-fed rabbits contain a disproportionately high percentage of stereospecifically derived hydroxy fatty acids. After 12 weeks of feeding, 73.7% of the 13-HODE was present in the S form (instead of the theoretical 50% if no enzymatic activity were involved). These findings, together with the previously reported finding that the 15-LO gene and its protein product are present at high levels in early lesions (15, 16), are compatible with a role of 15-LO *in vivo* but do not establish it. Ultimately, it may require studies in transgenic mice to assign the proper weight to 15-LO in the atherogenic process.

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REFERENCES

1. Steinberg, D., Parthasarathy, D., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989) *N. Engl. J. Med.* **320,** 915–924
2. Carew, T. E., Schwenke, D. C., and Steinberg, D. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84,** 7725–7729
3. Witztum, J. L. (1994) *Lancet* **344,** 793–795
4. Sparrow, C., Parthasarathy, S., and Steinberg, D. (1988) *J. Lipid Res.* **29,** 745–753
5. Rankin, S. M., Parthasarathy, S., and Steinberg, D. (1991) *J. Lipid Res.* **32,** 449–456
6. Heinecke, J. W., Baker, L., Rosen, H., and Chait, A. (1986) *J. Clin. Invest.* **77,** 757–761
7. Hiramatsu, K., Rosen, H., Heinecke, J. W., Wolfbauer, G., and Chait, A. (1987) *Arteriosclerosis* **7,** 55–60
8. Cathcart, M. K., McNally, A. K., Morel, D. W., and Chisolm, G. M., III (1989) *J. Immunol.* **142,** 1963–1969
9. Steinbrecher, U. P. (1988) *Biochim. Biophys. Acta* **959,** 20–30
10. Sigal, E., Laughton, C. W., and Mulkins, M. A. (1994) *Annals N. Y. Acad. Sci.* **714,** 211–224
11. Parthasarathy, S. (1987) *Biochim. Biophys. Acta* **917,** 337–340
12. Heinecke, J. W., Rosen, H., Suzuki, L. A., and Chait, A. (1987) *J. Biol. Chem.* **262,** 10098–10103
13. O'Leary, V., Graham, A., Darley-Usmar, V., and Stone, D. (1993) *Biochem. Soc.* **21,** 89S
14. Kühn, H., Belkner, J., Zaiss, S., Fährenklemper, T., and Wohlfeil, S. (1994) *J. Exp. Med.* **179,** 1903–1911
15. Ylä-Herttuala, S., Rosenfeld, M. E., Parthasarathy, S., Glass, C. K., Sigal, E., Witztum, J. L., and Steinberg, D. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6959–6963
16. Ylä-Herttuala, S., Rosenfeld, M. E., Parthasarathy, S., Sigal, E., Sarkioja, T., Witztum, J. L., and Steinberg, D. (1991) *J. Clin. Invest.* **87,** 1146–1152
17. Simon, T. C., Makheja, A. N., and Bailey, J. M. (1989) *Thromb. Res.* **55(2),** 171–178
18. Ylä-Herttuala, S., Luoma, J., Myllyharju, H., Hiltunen, T., Sisto, T., and Nikkari, T. (1994) *Circulation* **90,** I-135 (Abstract)
19. Kühn, H., Belkner, J., Wiesner, R., Schewe, T., Lankin, V., and Tikhaze, A. K. (1992) *Eicosanoids* **5,** 17–22
20. Sparrow, C. P., and Olszewski, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 128–131
21. Anderson, K. M., Ondrey, F., and Harris, J. E. (1992) *Clin. Biochem.* **25,** 1–9
22. Khouw, A. S., Parthasarathy, S., and Witztum, J. L. (1993) *J. Lipid Res.* **34,** 1483–1496
23. Sigal, E., Craik, C. S., Highland, E., Grunberger, D., Costello, L. L., Dixon, R. A. F., and Nadel, J. A. (1988) *Biochem. Biophys. Res. Commun.* **157,** 457–464
24. Miyanohara, A., Sharkey, M. F., Witztum, J. L., Steinberg, D., and Friedmann, T. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 6538–6542
25. Sharkey, M. F., Miyanohara, A., Elam, R. L., Friedmann, T., and Witztum, J. L. (1990) *J. Lipid Res.* **31,** 2167–2178
26. Gilliland, G., Perrin, S., Blanchard, K., and Bunn, F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 2725–2729
27. Funk, C. D., and FitzGerald, G. A., (1991) *J. Biol. Chem.* **266,** 12508–12513
28. Cathcart, R., Schwiers, E., and Ames, B. N. (1983) *Anal. Biochem.* **134,** 111–116
29. Lynch, S. M., and Frei, B. (1993) *J. Lipid Res.* **34,** 1745–1753
30. Bligh, E. G., and Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–917
31. Lowry, O. H., Rosebrough, N. J., Farr, A., and Randall, R. J. (1991) *J. Biol. Chem.* **193,** 265–275
32. Noguchi, N., Yoshida, Y., Kaneda, H., Yamamoto, Y., and Niki, E. (1992) *Biochem. Pharmacol.* **44,** 39–44
33. Thomas, C. E., and Jackson, R. C. (1991) *J. Pharmacol. Exp. Ther.* **256,** 1182–1188
34. Sattler, W., Maiorino, M., and Stocker, R. (1994) *Arch. Biochem. Biophys.* **309,** 214–221
35. Derian, C. K., and Lewis, D. F. (1992) *Prostaglandins Leukotrienes Essent. Fatty Acids* **45,** 49–57
36. O'Leary, V. J., Darley-Usmar, V. M., Russell, L. J., and Stone, D. (1992) *Biochem. J.* **282,** 631–634.,
37. Jialal, I., Freeman, D. A., and Grundy, S. M. (1991) *Arterioscler. Thromb.* **11,** 482–488
38. Philis-Tsimikas, A., Parthasarathy, S., Picard, S., Palinski, W., and Witztum, J. L. (1995) *Arterioscler. Thromb.*, in press
39. Leake, D. S., and Rankin, S. M. (1990) *Biochem. J.* **270,** 741–748
40. McNally, A. K., Chisolm, G. M., III, Morel, D. W., and Cathcart, M. K. (1990) *J. Immunol.* **145,** 254–259