Evidence for a dominant role of lipoxygenase(s) in the oxidation of LDL by mouse peritoneal macrophages

Sara M. Rankin, Sampath Parthasarathy, and Daniel Steinberg

Department of Medicine, University of California, San Diego, La Jolla, CA 92093

Abstract It has been suggested that the oxidative modification of low density lipoprotein (LDL) is a key event in atherogenesis. Several mechanisms have been proposed to explain how different types of cells modify LDL. In this study we examine the relative contributions of superoxide anions and cellular lipoxygenase (LO) in the modification of LDL by macrophages. Superoxide dismutase (SOD) inhibited LDL oxidation by macrophages but only by 25%. Under the same conditions, several LO inhibitors (eicosatetraynoic acid (ETYA), piriprost, and A-64077) almost completely inhibited the modification of LDL by macrophages. SOD had a greater inhibitory effect on the modification of LDL by U937 cells and fibroblasts (32% and 64%, respectively) but again LO inhibitors had a much greater effect (79 to 100% inhibition). Incubation of [1-^{14}C]linoleic acid with mouse peritoneal macrophages resulted in its conversion to a single more polar product coeluting with 13- and 9-HODE by reverse phase HPLC. When the cells were preincubated with LO inhibitors, formation of this product was significantly inhibited. It is concluded that the modification of LDL by macrophages is mediated in large part by lipoxygenase-type activity. —**Rankin, S. M., S. Parthasarathy, and D. Steinberg.** Evidence for a dominant role of lipoxygenase(s) in the oxidation of LDL by mouse peritoneal macrophages. *J. Lipid Res.* 1991. **32:** 449–456.

Supplementary key words atherosclerosis • 9/13-HODE • foam cells • superoxide • modified LDL

Recent evidence suggests the involvement of oxidatively modified low density lipoprotein (LDL) in atherogenesis (1). Peroxidation of LDL lipids and the consequent structural and functional modification of the apolipoprotein can be achieved by both cell-dependent and cell-independent means (2). A number of different cell types, including endothelial cells (3), smooth muscle cells (4), fibroblasts (5), and monocyte/macrophages (6–10), have previously been reported to induce peroxidative changes in LDL. Cells could induce peroxidation of LDL lipids in two general ways: *1*) by generating reactive oxygen species, such as superoxide anion, and releasing them into the medium; or *2*) by the action of specific enzymes, such as cyclooxygenases and lipoxygenases (LO), on lipids present either in the cell membrane or in LDL particles themselves. The generation of superoxide anion has been reported to play a major role in LDL oxidation by cultured smooth muscle cells and by activated monocytes (4, 7, 10). On the other hand, LO activity has been suggested to play the primary role in the oxidation of LDL by endothelial cells and activated monocytes (10, 11). It has also been shown that purified soybean LO can act directly on LDL, generating a modified form very similar to that generated by incubation with endothelial cells (12).

Macrophages are the major cell type in the fatty streak lesion (13–16). Mouse peritoneal macrophages (8, 9) as well as macrophage foam cells from arterial lesions (17) are themselves capable of oxidatively modifying LDL. The present study was undertaken to evaluate the importance of LO(s) present in macrophages in the oxidative modification of LDL. The present study, in which mouse peritoneal macrophages were used, shows that several different inhibitors of LO inhibit the ability of macrophages to generate LO products from linoleic acid and to oxidatively modify LDL. Furthermore, it is shown that superoxide dismutase has a much smaller effect on oxidative modification of LDL by these cells, implying that under the conditions used the LO(s) are quantitatively more important than the generation of superoxide anions.

Abbreviations: LDL, low density lipoprotein; SOD, superoxide dismutase; LO, lipoxygenase; HODE, hydroxyoctadecadienoic acid; LPDS, lipoprotein-deficient serum; HETE, hydroxyeicosatetraenoic acid; ETYA, eicosatetraynoic acid; PMSF, phenylmethylsulfonyl-fluoride; DMEM, Dulbecco's modified Eagle's medium; PPACK, D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone; TBA, thiobarbituric acid; MDA, malondialdehyde; PBS, phosphate-buffered saline; FBS, fetal bovine serum; TCA, trichloroacetic acid; HPLC, high performance liquid chromatography.

MATERIALS AND METHODS

Carrier-free Na^{125}I and [1-^{14}C]linoleic acid (56 μCi/mmol) were purchased from Amersham (Arlington, IL). Unlabeled linoleic acid and superoxide dismutase (sp act 3360 U/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). ETYA was from Cayman Chemical Co. (Ann Arbor, MI). Nordihydroguaiaretic acid (NDGA) was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). A-64077 (N-(1-benzo[b]thien-2-ylethyl)-N-hydroxyurea) was a gift from Dr. G. Carter (Abbot Laboratories, Abbot Park, IL). Piriprost (6,9-deepoxy-6,9-(phenylimino)Δ ^{6,8}-prostaglandin I$_1$; [U-60,257]) was a gift from Upjohn (Kalamazoo, MI). 13(s)Hydroxyoctadecadienoic acid (HODE) and 9(s)hydroxyoctadecadienoic acid were purchased from Oxford Biomedical Research Inc. (Oxford, MI). HPLC grade methanol, phosphoric acid, water, and acetonitrile were obtained from Fisher (Fairlawn, NJ). The sources of the cell culture media and supplies were described earlier (2).

Lipoproteins

Blood from normolipidemic donors fasted overnight was collected directly into syringes containing benzamidine (2 mM), PPACK (1 μM), and EDTA (1 mg/ml). PMSF (0.5 mM in DMSO), chloramphenicol (50 μg/ml), and gentamicin (100 μg/ml) were then added to the combined plasmas. LDL (d 1.019–1.057 g/ml) was isolated by ultracentrifugation (18). The LDL was dialyzed against phosphate-buffered saline (PBS) containing 0.01% EDTA at 4°C. Radioiodination of LDL was performed as described earlier (19). The LDL was diluted to a specific activity of about 30 cpm/ng protein with unlabeled LDL.

Cells

Mouse peritoneal macrophages were used in these studies for three different purposes.

For the oxidative modification of LDL. Macrophages were harvested by peritoneal lavage of female Swiss-Webster mice (2 to 3 months of age, weighing 25–35 g, from Simonsen Lab., Gilroy, CA) with ice-cold PBS. The cells were plated in 12-well dishes (Costar, Cambridge, MA) at 2 × 10^6 cells/well in RPMI 1640 media, containing 10% (v/v) heat-inactivated fetal bovine serum (FBS). After overnight incubation, nonadherent cells were removed by washing. Cells were incubated with ^{125}I-labeled LDL (100 μg protein/ml) in 0.6 ml Ham's F-10 medium containing 50 μg/ml gentamicin and 0.01% (w/v) LPDS at 37°C for 24 h. This ^{125}I-labeled LDL is referred to below as cell-incubated LDL. ^{125}I-labeled LDL was also incubated under identical conditions in the absence of cells. Inhibitors were included in this incubation as indicated. After the incubation the medium was removed and an aliquot was taken for measuring the thiobarbituric acid-reactive substances (TBARS). The remaining LDL was diluted to 10 μg protein/ml in DMEM and 0.5 ml was added to a fresh set of macrophages to measure its rate of degradation (as described in the following section). Parallel incubations were always carried out in which the inhibitors were added directly to the second incubation (macrophage degradation) along with cell-incubated ^{125}I-labeled LDL from the first incubation (oxidative modification). We found that none of the inhibitors had a direct effect on the degradation of cell-incubated ^{125}I-labeled by macrophages.

For the assessment of rates of degradation of ^{125}I-labeled LDL. A fresh set of mouse peritoneal macrophages was harvested as described in the previous section. The cells were plated at 1.5 × 10^6 cells/well in 24-well dishes in RPMI 1640 medium containing 10% (v/v) heat-inactivated FBS. After overnight incubation the cells were washed and used for the degradation assay. These fresh macrophages (or cell-free dishes) were incubated for 5 h at 37°C with ^{125}I-labeled LDL (10 μg protein/ml), that had been previously incubated in the presence or absence of other macrophages (as described above). After 5 h the noniodide, TCA-soluble degradation products in the media were determined (2).

For the measurement of the conversion of ^{14}C-labeled linoleic acid to oxidation products. A fresh set of mouse peritoneal macrophages was harvested as described above. The cells were plated at 4 × 10^6 cells/dish in 60-mm dishes in RPMI 1640 medium containing 10% (v/v) heat inactivated FBS. After overnight incubation the cells were washed and used. Macrophages or cell-free dishes were preincubated in the presence or absence of either NDGA (15 μM) or A-64077 (5 μM) in 1 ml of DME medium for 60 min at 37°C in a humidified atmosphere of 95% O_2/5% CO_2. Then 20 nmol of [^{14}C]linoleic acid was added, giving a final concentration of 20 μM, and the incubation was continued for a further 20 min (fatty acid was added in 5 μl ethanol; sp act 2220–3000 dpm/nmol). The incubation was terminated by the removal of the medium. The cells were then washed once with 0.8 ml PBS. The combined medium and wash solution was acidified to pH 4.0 using 0.5 N HCl. The acidified medium was then extracted with chloroform-methanol 1:1 (v/v) by the method of Bligh and Dyer (20). The chloroform phase was filtered and then evaporated under nitrogen and the lipids were resuspended in acetonitrile-water 1:1 (v/v) and stored at −20°C until analyzed.

The lipid extracts of the medium were fractionated by reverse phase HPLC using a Rainin HPLC system equipped with a 4.2 × 250 mm, 5 μm Dynamax ODS column. The solvent system consisted of water adjusted to pH 3.4 with phosphoric acid and acetonitrile. An elution gradient starting with 27% acetonitrile and increasing linearly to 100% acetonitrile over 42 min was used to separate the metabolites, as described by Kaduce et al.

(21). Absorbance at 234 nm was monitored and 1-ml fractions were collected. The fractions were mixed with 10 ml Scintiverse and radioactivity was measured.

U937 cells (a gift from Dr. D. Hudig, University of California, San Diego) were maintained in suspension cultures in RPMI 1640 containing 10% heat-inactivated FBS. Cells were initially cultured at 0.2×10^6/ml and passaged every 3 days (reaching a maximal density of 1×10^6/ml). After counting, (using a Coulter counter) 10^7 cells were taken into 15-ml sterile plastic tubes, spun at 250 *g* and washed with Ham's F-10 media. These cells were resuspended in media to study their ability to oxidize LDL.

Human skin fibroblasts were grown in monolayers from a preputial biopsy of a normal infant. Cultures were maintained in DME media containing 10% FBS. To study the oxidation of LDL, cells were seeded in 12-well dishes at a density of 4×10^4 cells/well and used at confluency (after 4–5 days).

For the oxidation of LDL, U937 cells or human skin fibroblasts were incubated with ^{125}I-labeled LDL (100 μg protein/ml) in 0.6 ml Ham's F-10 media containing 50 μg/ml gentamicin and 0.01% (w/v) LPDS at 37°C for 24 h. An aliquot of this cell-incubated LDL was taken to measure its rate of degradation by mouse peritoneal macrophages, as described above.

The degree of lipid peroxidation was determined by measuring at 532 nm the amount of thiobarbituric acid-reactive substances that were generated (22). Tetramethoxypropane was used as standard.

Cell protein was determined according to the method of Lowry et al. (23) using bovine serum albumin as standard.

RESULTS

The modification of LDL was assessed by measuring the generation of TBARS and by determining the rate of degradation of the modified ^{125}I-labeled LDL by a fresh set of mouse peritoneal macrophages. In order to minimize cell-independent modification, incubations were carried out in the presence of a low concentration of LPDS (0.01% w/v), which reduces the extent of metal-catalyzed oxidation occurring in the medium but does not prevent the cell-induced oxidation.

As shown in **Table 1**, when LDL was incubated with macrophages there was a significant increase in TBARS and in the rate of its degradation in a subsequent incubation with a fresh set of macrophages. In previous studies we have demonstrated that the enhanced rate of degradation of macrophage-modified LDL is due to uptake mediated by receptor(s) recognizing acetyl- and endothelial-modified LDL (8, 9). Superoxide dismutase (SOD) at 10 to 100 μg/ml had very little effect on the modification of LDL. For example, at a concentration of 100 μg/ml, SOD produced only a partial inhibition (less than 25%) of the TBARS generated and the biological modification of LDL (as measured by the enhanced rate of degradation by a fresh set of macrophages) (Table 1). The same SOD preparations were found to be effective inhibitors of superoxide anion production when tested in the hypoxanthine/xanthine oxidase system (data not shown).

ETYA was earlier shown to inhibit the modification of LDL by endothelial cells (11). When macrophages were incubated with ETYA (25 μM) substantial inhibition of the modification of LDL (70% inhibition in TBARS and

TABLE 1. Effect of superoxide dismutase (SOD) on the modification of LDL by macrophages

	TBARS (nmol/mg LDL protein)	Degradation (μg ^{125}I-LDL protein/mg cell protein in 5 h)
Unincubated LDL	1.23 ± 0.7	1.57 ± 1.2
LDL incubated without cells	12.31 ± 1.9	1.31 ± 0.8
LDL incubated without cells + 100 μg/ml SOD	12.98 ± 0.8	1.18 ± 0.9
LDL incubated with macrophages	56.25 ± 2.4	10.26 ± 3.5
LDL incubated with macrophages + 100 μg/ml SOD	45.47 ± 4.3	9.01 ± 4.2

^{125}I-labeled LDL (100 μg/ml) was incubated with mouse peritoneal macrophages (2×10^6 cells/well in 12-well culture dishes) in 0.6 ml Ham's F-10 media containing 50 μg/ml gentamicin and 0.01% (w/v) LPDS for 24 h. One aliquot (250 μl) of the medium was taken for determination of TBARS. A second aliquot (50 μl) was diluted to 500 μl with DME media and added to a fresh set of macrophages (1.5×10^6 cells/well in 24-well culture dishes) or cell-free dishes and incubated for 5 h. The noniodide, TCA-soluble degradation products present in the media were then determined. To control for possible direct effects of SOD on the ability of macrophages to degrade LDL, parallel incubations were carried out in which an equal concentration of SOD (10% that in the first incubation) was added along with LDL that had been incubated with macrophages in the absence of inhibitors during the first incubation. There was no effect. The results represent the mean ± standard error of the mean (SEM) of four experiments, each with duplicate determinations.

TABLE 2. Effect of ETYA on the modification of LDL by macrophages

	TBARS (nmol/mg LDL protein)	Degradation (μg ^{125}I-LDL protein/mg cell protein in 5 h)
Unincubated LDL	1.86 ± 1.9	0.24 ± 0.1
LDL incubated without cells	19.31 ± 2.9	0.85 ± 0.5
LDL incubated without cells + 25 μM ETYA	15.02 ± 2.8	0.83 ± 0.6
LDL incubated with macrophages	51.48 ± 3.5	4.13 ± 0.7
LDL incubated with macrophages + 25 μM ETYA	18.26 ± 1.3	0.92 ± 0.4

^{125}I-labeled LDL (100 μg/ml) was incubated with mouse peritoneal macrophages (2 × 10^6 cells/well in 12-well culture dishes) in 0.6 ml Ham's F-10 media containing 50 μg/ml gentamicin and 0.01% (w/v) LPDS for 24 h. Where indicated 25 μM ETYA was added to the media in 6 μl ethanol; 6 μl ethanol was added to control dishes. One aliquot (250 μl) of the medium was taken for determination of TBARS. A second aliquot (50 μl) was diluted to 500 μl with DME media and added to a fresh set of macrophages (1.5 × 10^6 cells/well in 24-well culture dishes) or cell-free dishes and incubated for 5 h. The noniodide, TCA-soluble degradation products present in the media were then determined. The control for possible effects of inhibitors carried over from the first to the second incubation showed no effect (see legend to Table 1). The results represent the mean ± SEM of four experiments, each with duplicate determinations.

an 83% inhibition of the biological modification) were observed (**Table 2**). At the concentration used there was no evidence that ETYA was toxic to the macrophages.

To further test the possibility that LOs may be involved in the modification of LDL, two other LO inhibitors, piriprost and A-64077, were tested. Piriprost (50 μM) and A-64077 (5 μM) inhibited the formation of TBARS by 70% and 86% respectively (**Table 3** and **Table 4**). The biological modification of LDL was also inhibited over 90% by both piriprost and A-64077 (Tables 3, 4).

The modification of LDL by both unstimulated U937 cells, a human histiocytic cell line, and by human skin fibroblasts was examined (**Table 5**). It was found that when LDL was incubated with either U937 cells or human skin fibroblasts, its rate of degradation was increased in a subsequent incubation with mouse peritoneal macrophages, i.e., it was biologically modified. ETYA (25 μM) and piriprost (50 μM) inhibited the biological modification of LDL by U937 cells by 80% and 79%, respectively. Piriprost (50 μM) and A-64077 (5 μM) inhibited the biological modification of LDL by fibroblasts by 86% and 100%, respectively. It was found that SOD produced a 64 and 32% inhibition of fibroblast and U937 cell modification, respectively. These data suggest a possible role for both lipoxygenases and superoxide anion in the modification of LDL by these cell types.

In addition, we studied the conversion of [1-^{14}C]linoleic acid to 13-HODE or 9-HODE by mouse peritoneal macrophages. When resident mouse peritoneal macrophages were incubated with ^{14}C-labeled linoleic acid at 37°C, a proportion of the linoleic acid was converted into more polar products, as detected by reverse phase HPLC of the lipid extracted from the media. With a relatively short incubation period (20 min), a single distinct peak (retention time 29–30 min) was observed in addition to that due to the substrate (40–42 min) (**Fig. 1**). When the incubation

TABLE 3. Effect of piriprost on the modification of LDL by macrophages

	TBARS (nmol/mg LDL protein)	Degradation (μg ^{125}I-LDL protein/mg cell protein in 5 h)
Unincubated LDL	4.22	0.28
LDL incubated without cells	20.54	0.56
LDL incubated without cells + 50 μM piriprost	16.8	0.42
LDL incubated with macrophages	61.62	5.49
LDL incubated with macrophages + 50 μM piriprost	21.79	0.65

^{125}I-labeled LDL (100 μg/ml) was incubated with mouse peritoneal macrophages (2 × 10^6 cells/well in 12-well culture dishes) in 0.6 ml Ham's F-10 media containing 50 μg/ml gentamicin and 0.01% (w/v) LPDS for 24 h. Where indicated 50 μM piriprost was added to the media in 6 μl ethanol; 6 μl ethanol was added to control dishes. One aliquot (250 μl) of the medium was taken for determination of TBARS. A second aliquot (50 μl) was diluted to 500 μl with DME media and added to a fresh set of macrophages (1.5 × 10^6 cells/well in 24-well culture dishes) or cell-free dishes and incubated for 5 h. The noniodide, TCA-soluble degradation products present in the media were then determined. The control for possible effects of inhibitors carried over from the first to the second incubation showed no effect (see legend to Table 1). The results are averages of four independent values.

TABLE 4. Effect of A-64077 on the modification of LDL by macrophages

	TBARS (nmol/mg LDL protein)	Degradation (μg ^{125}I-LDL protein/mg cell protein in 5 h)
Unincubated LDL	9.44 ± 4.2	0.65 ± 0.2
LDL incubated without cells	13.01 ± 2.5	0.99 ± 0.2
LDL incubated without cells + 5 μM A-64077	16.88 ± 3.8	1.82 ± 0.8
LDL incubated with macrophages	48.88 ± 5.4	12.37 ± 2.9
LDL incubated with macrophages + 5 μM A-64077	14.93 ± 7.3	1.37 ± 0.5

^{125}I-labeled LDL (100 μg/ml) was incubated with mouse peritoneal macrophages (2 × 10^6 cells/well in 12-well culture dishes) in 0.6 ml Ham's F-10 media containing 50 μg/ml gentamicin and 0.01% (w/v) LPDS for 24 h. Where indicated 5 μM A-64077 was added to the media in 6 μl ethanol; 6 μl ethanol was added to control dishes. One aliquot (250 μl) of the medium was taken for determination of TBARS. A second aliquot (50 μl) was diluted to 500 μl with DME media and added to a fresh set of macrophages (1.5 × 10^6 cells/well in 24-well culture dishes) or cell-free dishes and incubated for 5 h. The noniodide, TCA-soluble degradation products present in the media were then determined. The control for possible effects of inhibitors carried over from the first to the second incubation showed no effect (see legend to Table 1). The results represent the mean ± SEM of three experiments, each with duplicate determinations.

period was extended, several additional more polar products were observed (data not shown). The product formed after 20 min coeluted with both 9- and 13-hydroxyoctadecadienoic acid standards.

To evaluate the role of LO in the oxidation of linoleic acid, macrophages were preincubated for 1 h with the LO inhibitors NDGA (15 μM) or A-64077 (5 μM). Both NDGA and A-64077 produced a significant inhibition of the conversion of linoleic acid into its more polar product (Fig. 1, **Table 6**). We have also found that NDGA (15 μM) and ETYA (25 μM) inhibit the oxidation of exogenously added ^{14}C-labeled arachidonic acid by macrophages into 12- and 15- HETE (data not shown). By contrast, in preliminary trials we have found that SOD (100 μg/ml) had little or no effect on the oxidation of linoleic acid by macrophages.

DISCUSSION

This study focuses on determining the mechanism by which macrophages modify LDL. Three different mechanisms were considered: *1*) superoxide anion-induced lipid peroxidation; *2*) oxidation mediated by cyclooxygenase; and *3*) LO-catalyzed lipid peroxidation. In the present study we show that SOD produced only a small, but consistent, inhibition of the biological modification of LDL by resting peritoneal macrophages. We propose that, in addition to the generation of superoxide anions, other mechanism(s) may be involved in the biological modification of LDL by these cells. In contrast, the generation of superoxide anions has previously been reported to play a major role in the oxidation of LDL to a cytotoxin by activated monocytes (7, 10). In those studies, however, the uptake of cell-incubated LDL by macrophages was not measured. A recent paper by McNally et al. (10) suggests that superoxide anions involved in the oxidation of LDL by activated monocytes are generated via lipoxygenase. This would be compatible with our findings (Table 5) in U937 cells and fibroblasts. The relative importance of superoxide anions in modification may depend on which cells are used to modify LDL. It is also possible that different mechanisms are responsible for the oxidation of LDL to a cytotoxin as compared to its biological modification, i.e., modification to a form recognized by scavenger receptors. The relative contributions of superoxide anion-generating systems and of LO in the artery wall remain to be determined. As discussed below, we now know that LO mRNA is strongly expressed in macrophage-rich lesions. Nevertheless, superoxide anion could play a role in vivo, possibly in the generation of

TABLE 5. Effect of SOD and lipoxygenase inhibitors on the modification of LDL by U937 cells and fibroblasts

	% Inhibition of the Biological Modification of LDL			
Cell Type	SOD (100 μg/ml)	ETYA (25 μM)	Piriprost (50 μM)	A64077 (5 μM)
U937 cells	32	80	79	n.d.
Fibroblasts	64	n.d.	86	100

^{125}I-labeled LDL (100 μg/ml) was incubated with either U937 cells or human skin fibroblasts in 0.6 ml Ham's F-10 medium and 0.01% (w/v) LPDS for 24 h. Where indicated either 25 μM ETYA, or 50 μM piriprost, or 5 μM A-64077 was added in 6 μl ethanol; 6 μl ethanol was added to control dishes. After the incubation an aliquot of the medium (50 μl) was diluted to 500 μl with DME media and added to a fresh set of macrophages for degradation studies. The results are expressed as % inhibition as compared to the cell incubations that had no inhibitors. The degradation rates for U937 cell- and fibroblast-incubated LDL were 3.22 and 11.93 μg/mg macrophage protein in 5 h, respectively. The values were corrected for unincubated LDL degradation (0.24 and 1.10 μg/mg macrophage protein in 5 h, for experiments with U937 cells and fibroblasts, respectively). The values represent means from at least two experiments with duplicate determinations; n.d., not determined.

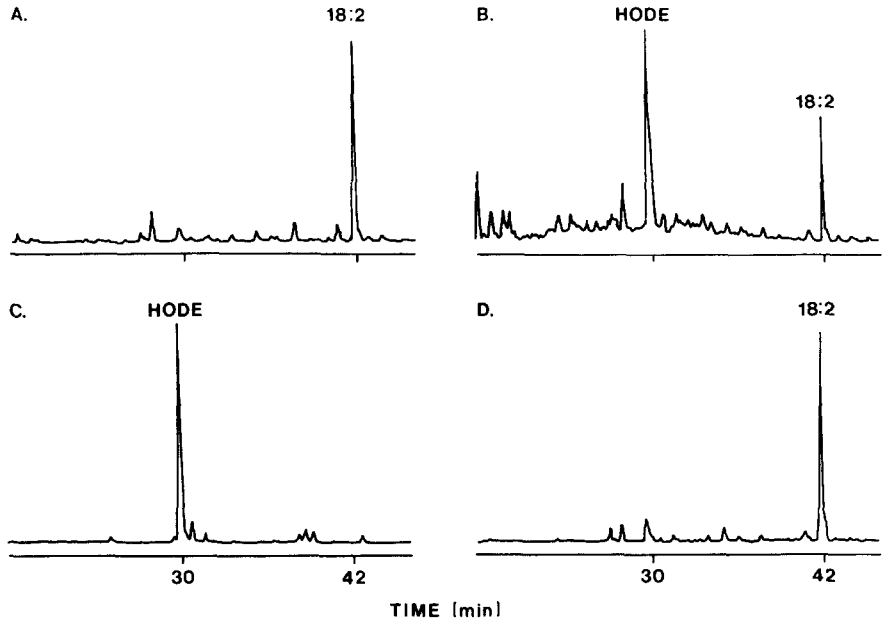

Fig. 1. Oxidation of [1-^{14}C]linoleic acid by mouse peritoneal macrophages. Twenty nmol of [1-^{14}C]linoleic acid was incubated with mouse peritoneal macrophages for 20 min in DME. Where indicated, cells were pretreated with 15 μM NDGA for 60 min. Medium containing labeled fatty acid products was acidified and extracted by the method of Bligh and Dyer (18) and analyzed by HPLC as described in the Methods section. The figure represents absorption at 234 nm. A: Linoleic acid incubated without cells. B: Linoleic acid incubated with mouse peritoneal macrophages. C: 13-HODE standard. D: Cells incubated with linoleic acid after preincubation with 15 μM NDGA. These chromatograms are representative of those obtained from at least three experiments.

cytotoxic products. Macrophages have the capacity to oxidize arachidonic acid by cyclooxygenase and LO reactions (24, 25). Earlier experiments with aspirin and indomethacin had ruled out the involvement of cyclooxygenase in the oxidation of LDL (9). We show that macrophages are able to oxidize linoleic acid to 15- or 12-LO products. In the reverse phase HPLC separation, as described, these products could not be separated clearly. As the focus of these experiments was to define the role of superoxide anion and lipoxygenase reactions in the modification of LDL by macrophages, no further attempt was made to determine the exact identity of the lipoxygenase product. Macrophages are also capable of generating 5-LO products when incubated with arachidonic acid (24). We tested the effect of several LO inhibitors on the macrophage LO activity and the modification of LDL. All these inhibitors inhibited the modification of LDL. More importantly, these inhibitors also inhibited the conversion of linoleic acid into LO products at comparable concentrations. These data taken together strongly support our hypothesis that LO is involved in the modification of LDL by macrophages.

TABLE 6. Effect of lipoxygenase inhibitors on the oxidation of ^{14}C-labeled linoleic acid by mouse peritoneal macrophages

Incubation Conditions	% Total cpm as 13- or 9-HODE
Set 1:	
[^{14}C]linoleic acid incubated without cells	7.38 ± 1.92
[^{14}C]linoleic acid incubated with macrophages	40.9 ± 8.47
[^{14}C]linoleic acid incubated with macrophages + A-64077 (5 μM)	16.7 ± 2.48
Set 2:	
[^{14}C]linoleic acid incubated without cells	1.2 ± 0.45
[^{14}C]linoleic acid incubated with macrophages	38.06 ± 9.54
[^{14}C]linoleic acid incubated with macrophages + NDGA (15 μM)	7.36 ± 1.59

One ml HPLC fractions were mixed with 10 ml scintillation fluid and the radioactivity in the fractions was determined. The radioactivity in the fractions eluting from 29 through 33 min corresponded to 13- or 9-HODE. The radioactivity in the HODE fractions was calculated as a percentage of the total collected off the column. The results represent the means ± SEM of at least three separate experiments.

The LO inhibitors used in this study have a diverse specificity. For example, NDGA is an antioxidant that inhibits all the LOs (26). Likewise, ETYA inhibits all the LOs as well as cyclooxygenase reactions, but not as a result of an antioxidant property (27, 28). Piriprost has been reported to be a specific 5-LO inhibitor (29). It was reported that it did not inhibit superoxide anion production by neutrophils (30) However, it did inhibit other enzymes, such as glutathione S-transferase (31), suggesting that it may have other mechanisms of action. A-64077 was reported to be a specific inhibitor of 5-LO based on its inability to inhibit platelet (12-LO) and soybean (15-LO) LO (32, 33). In the present studies we show that A-64077 prevents the conversion of linoleate to 12- and 15-LO products. As linoleic acid is not acted upon by 5-LO, it suggests that A-64077 can inhibit other LOs. The specificity of the LOs involved, while relevant when considering inhibitors and assay systems, may not be of great importance in the modification of LDL. The position of the hydroperoxy group in the polysaturated fatty acid may not much affect the propagation of lipid peroxidation that it initiates, after it gains entry into LDL.

The actual involvement of LO activity in the modification of LDL in vivo remains to be established. In a recent study it has been shown (34) that antibody generated against human leukocyte 15-LO showed strong immunoreactivity in macrophage-foam cells of the atherosclerotic lesion. These immunoreactive sites corresponded to similar areas that reacted positively with antibodies against oxidized LDL and macrophages. Using probes specific for 15-LO mRNA and in situ hybridization techniques, Ylä-Herttuala et al. (34) also showed specific hybridization of the riboprobes in the macrophage-rich lesion. In addition, there is recent evidence to suggest that macrophage-rich lesions from cholesterol-fed and WHHL rabbits contain increased levels of 15-LO products (13-HODE and 15-HETE) and actively convert arachindonic and linoleic acid into 15-LO products (35, 36). Based on the above discussion, LO activity (perhaps 15-LO) of macrophages may be an important initiator of the peroxidative modification of LDL.

We thank Lorna Joy for her expert technical assistance with cell cultures. This work was supported by the grant HL-14197 (SCOR) from the National Heart, Lung, and Blood Institute. SMR is a postdoctoral fellow supported by the American Heart Association, California Affiliate.

Manuscript received 12 July 1990 and in revised form 25 September 1990.

REFERENCES

1. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witzum. 1989. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320:** 915–924.
2. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witzum, and D. Steinberg. 1984. Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA.* **81:** 3883–3887.
3. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1981. Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: recognition by receptors for acetylated low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **78:** 6499–6503.
4. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1986. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest.* **74:** 1890–1894.
5. Steinbrecher, U. P. 1988. Role of superoxide in endothelial-cell modification of low density lipoproteins. *Biochim. Biophys. Acta.* **959:** 20–30.
6. Cathcart, M. K., D. W. Morel, and G. M. Chisolm. 1985. Monocytes and neutrophils oxidize low density lipoprotein making it cytotoxic. *J. Leukocyte Biol.* **38:** 341–350.
7. Hiramatsu, K., H. Rosen, J. W. Heinecke, G. Wolfbauer, and A. Chait. 1987. Superoxide initiates oxidation of low density lipoprotein by human monocytes. *Arteriosclerosis.* **7:** 55–60.
8. Parthasarathy, S., D. J. Printz, D. Boyd, L. Joy, and D. Steinberg. 1986. Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis.* **6:** 505–510.
9. Rankin, S. M., and D. S. Leake. 1988. The modification of low density lipoproteins by macrophages by oxidation or proteolysis. *Agents Actions.* **26:** 233–239.
10. McNally, A. M., G. M. Chisolm, D. W. Morel, and M. K. Cathcart. 1990. Activated human monocytes oxidize low-density lipoprotein by a lipoxygenase-dependent pathway. *J. Immunol.* **145:** 254–259.
11. Parthasarathy, S., E. Wieland, and D. Steinberg. 1989. A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **86:** 1046–1050.
12. Sparrow, C. P., S. Parthasarathy, and D. Steinberg. 1988. Enzymatic modification of low density lipoprotein by purified lipoxygenase and phospholipase A_2 mimics cell-mediated oxidative modification. *J. Lipid Res.* **29:** 745–753.
13. Fowler, S., H. Shio, and N. J. Hayley. 1979. Characterisation of lipid-laden aortic cells from cholesterol-fed rabbits. IV. Investigation of macrophage-like properties of aortic cell populations. *Lab. Invest.* **41:** 372–378.
14. Schaffner, T., K. Taylor, E. J. Bartucci, K. Fisher-Dzoga, J. H. Beeson, S. Glagov, and R. W. Wissler. 1980. Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. *Am. J. Pathol.* **100:** 57–73.
15. Gerrity, R. G. 1981. The role of the monocyte in atherogenesis. I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* **103:** 181–190.
16. Rosenfeld, M. E., T. Tsukada, A. M. Gown, and R. Ross. 1987. Fatty streak initiation in Watanabe Heritable Hyperlipidemic and comparably hypercholesterolemic fat-fed rabbits. *Atherosclerosis.* **1:** 9–23.
17. Rosenfeld, M. E., J. C. Khoo, E. Miller, S. Parthasarathy, W. Palinski, and J. L. Witzum. 1990. Macrophage-derived foam cells freshly isolated from rabbit atherosclerotic lesions degrade modified lipoproteins, promote oxidation of LDL, and contain specific lipid-protein adducts. *J. Clin. Invest.* **87:** 90–99.

18. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. Distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
19. Naruszewicz, M., T. E. Carew, R. C. Pittman, J. L. Witzum, and D. Steinberg. 1984. A novel mechanism by which probucol lowers LDL levels demonstrated in the LDL receptor-deficient rabbit. *J. Lipid Res.* **25:** 1206–1213.
20. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
21. Kaduce, T. L., P. H. Figard, R. Leifur, and A. A. Spector. 1989. Formation of 9-hydroxyoctadecadienoic acid from linoleic acid in endothelial cells. *J. Biol. Chem.* **264:** 6823–6830.
22. Schuh, J., G. F. Fairclough, and R. H. Haschemeyer. 1978. Oxygen-mediated heterogeneity of apo-low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **75:** 3173–3177.
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
24. Rabinovich, H., J. Durand, M. Rigaud, F. Mendy, and J-C. Breton. 1981. Transformation of arachidonic acid into monohydroxyeicosatetraenoic acids by mouse peritoneal macrophages. *Lipids.* **16:** 518–524.
25. Scott, W. A., N. A. Pawlowski, M. Andreach, and Z. A. Cohn. 1982. Resting macrophages produce distinct metabolites from exogenous arachidonic acid. *J. Exp. Med.* **155:** 535–547.
26. Tappel, A. L., W. O. Lundberg, and P. D. Boyer. 1953. Effect of temperature and antioxidants upon the lipoxidase-catalyzed oxidation of sodium linoleate. *Arch. Biochem. Biophys.* **42:** 293–304.
27. Hamberg, M., and B. Samuelsson. 1974. Prostaglandin endoperoxides. Novel transformations of arachidonic acid in human platelets. *Proc. Natl. Acad. Sci. USA.* **71:** 3400–3404.
28. Sun, F. F., J. C. McGuire, D. R. Morton, J. E. Pike, H. Sprecher, and W. H. Kunau. 1981. Inhibition of platelet arachidonic acid 12-lipoxygenase by acetylenic compounds. *Prostaglandins.* **21:** 333–343.
29. Sun, F. F., and J. C. McGuire. 1983. Inhibition of human neutrophil arachidonate 5-lipoxygenase by, 6,9-dëepoxy-6,9 (phenylimino)-delta^{6,8} prostaglandin I_1 (U 60,257). *Prostaglandins.* **26:** 211–221.
30. Ozaki, Y., T. Ohashi, and Y. Niwa. 1986. A comparative study on the effects of inhibitors of the lipoxygenase pathway on neutrophil function: inhibitory effects on neutrophil function may not be attributed to inhibition of the lipoxygenase pathway. *Biochem. Pharm.* **35:** 3481–3488.
31. Bach, M. K., J. O'Brian, J. R. Brashler, M. A. Johnson, and D. R. Morton, Jr. 1985. Inhibition of rat liver glutathione S-transferase by piriprost: kinetics of the inhibition and preliminary evidence that piriprost may be a poor alternative substrate for these enzymes. *Res. Commun. Chem. Pathol. Pharmacol.* **49:** 361–386.
32. Carter, G. W., P. R. Young, D. Albert, J. Bouska, R. D. Dyer, P. E. Malo, R. L. Bell, J. B. Summers, D. E. Brooks, P. Rubin, and J. Kesterson. 1989. A64077, a new, potent, orally active inhibitor of 5-lipoxygenase (5-LO). *3rd Interscience World Conference on Inflammation.* Monte Carlo. 67.
33. Malo, P. E., Y. D. Wiley, T. K. Shaughnessy, R. Bell, J. B. Summers, D. W. Brooks, and G. W. Carter. 1989. Inhibition of antigen-induced contractions of guinea-pig tracheal strips (GPTS) by lipoxygenase (5-LO) inhibitors. *Am. Rev. Resp. Dis.* **139:** A92.
34. Ylä-Herttuala, S., M. E. Rosenfeld, S. Parthasarathy, C. K. Glass, E. Sigal, J. L. Witzum, and D. Steinberg. 1990. Colocalisation of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA.* **87:** 6959–6963.
35. Simon, T. C., A. N. Makheja, and J. M. Bailey. 1989. Formation of 15-hydroxyeicosatetraenoic acid (15-HETE) as the predominant eicosanoid in aortas from Watanabe Heritable Hyperlipidemic and cholesterol-fed rabbits. *Atherosclerosis.* **75:** 31–38.
36. Henriksson, P., M. Hamberg, and U. Diczfalusy. 1985. Formation of 15-HETE as a major hydroxyeicosatetraenoic in the atherosclerotic vessel wall. *Biochim. Biophys. Acta.* **834:** 272–274.