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A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein

(atherosclerosis/lipid peroxidation/free radicals/macrophages)

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ABSTRACT **Oxidative modification of low density lipoprotein (LDL) has been implicated as a factor in the generation of macrophage-derived foam cells *in vitro* and *in vivo*. However, the exact mechanism of LDL oxidation has not been established. The present studies show that cellular lipoxygenase activity is involved in endothelial cell-induced oxidation of LDL. Inhibitors of lipoxygenase (but not inhibitors of cyclooxygenase) reduced LDL oxidation by as much as 70–85% under the conditions used. In contrast, the addition of pure (recombinant) superoxide dismutase inhibited by only ≈25% under the same conditions. Oxidation of LDL by smooth muscle cells, on the other hand, was effectively inhibited by superoxide dismutase, as was Cu^{2+}-catalyzed oxidation of LDL. When LDL was added to endothelial cell cultures within a dialysis bag, it did not undergo oxidative modification, suggesting that cell–LDL contact is necessary. We propose that an important element in cell-induced oxidation of LDL depends on (*i*) lipoxygenase oxidation of cellular lipids, followed by their exchange into LDL in the medium; (*ii*) direct lipoxygenase-dependent oxidation of LDL lipids during LDL–cell contact; (*iii*) or both.**

One of the early events in atherosclerosis is the accumulation of cholesterol-laden foam cells in the subendothelial space, and in recent years it has become evident that these cells originate mainly from the monocyte–macrophage (1–5). It is generally accepted that most of the deposited cholesterol is derived from plasma lipoproteins, particularly low density lipoprotein (LDL) (6). However, the monocyte–macrophage expresses only a limited number of classical LDL (B/E) receptors, and so the uptake and degradation of native LDL does not occur at an appreciable rate *in vitro* (7). In contrast, chemically modified negatively charged LDL (e.g., acetyl-LDL) is rapidly taken up by an alternative receptor, the acetyl-LDL or scavenger receptor (8, 9). Thus far, there is no evidence that such chemically modified forms of LDL are made *in vivo*. A biologically modified form of LDL recognized by the scavenger receptor can be generated *in vitro* by incubation of LDL with endothelial cells, smooth muscle cells, or macrophages (10–15). This biological modification depends on trace amounts of metal ions in the medium (13) and is accompanied by a number of other striking changes in the LDL, including a marked increase in electrophoretic mobility and an increase in density (10); hydrolysis of phosphatidylcholine to lysophosphatidylcholine by the action of an intrinsic LDL-associated phospholipase A_2 (14); degradation of apoprotein B-100 (14, 16); generation of thiobarbituric acid-reactive substance (TBARS), indicating peroxidation of the LDL lipids (13, 17); and, of course, generation of a new configuration on apoprotein B that is recognized by the acetyl-LDL receptor (10, 16). This oxidative modification can be mimicked by copper ions in the absence of cells (13). Whether the oxidation is copper-catalyzed or mediated by cells, it can be blocked by the addition of antioxidants such as butylated hydroxytoluene or α-tocopherol (13). Recent evidence strongly suggests that this oxidatively modified LDL plays an important role in experimental atherosclerosis. The studies of Carew *et al.* (18) and of Kita *et al.* (19) show that probucol, acting as an antioxidant, significantly slows the progression of lesions in hypercholesterolemic LDL receptor-deficient rabbits.

Oxidation of LDL may influence the atherogenic process in at least four different ways: (*i*) by recruiting monocytes into the artery wall (20); (*ii*) by inhibiting the motility of tissue macrophages, thus "trapping" them (21); (*iii*) by generating foam cells by uptake via the acetyl-LDL receptor (10–15); and (*iv*) by causing endothelial cell injury (22, 23). It would be important to know the mechanisms involved in the cell-induced oxidation of LDL. Studies in smooth muscle cells and monocytes (24, 25) show that superoxide dismutase (SOD) inhibits oxidation of LDL, suggesting that superoxide anion is responsible. Endothelial cells can also generate superoxide anion (26) and SOD has been reported by some to inhibit endothelial cell-induced oxidation of LDL (27), while others have failed to obtain significant inhibition (28). Endothelial cells, in addition, have two other important means of generating lipid peroxides. They have an efficient prostacyclin-synthesizing system, which is based on cyclooxygenase activity (29, 30); and recent evidence also suggests the presence of lipoxygenases in these cells (31–33). In the present studies, we investigate the effect of inhibitors of the lipoxygenase and cyclooxygenase reactions on the oxidative modification of LDL by cultured endothelial cells.

MATERIALS AND METHODS

Cells. Rabbit aortic endothelial cells were grown in Ham's F-10 medium (Irvine Scientific) supplemented with 15% fetal bovine serum as described (13); rabbit smooth muscle cells were from a primary culture initiated in this laboratory from rabbit carotid artery. The cells were plated in 60-mm plastic culture dishes and used at confluence. Resident peritoneal macrophages were harvested from female Swiss–Webster mice (Simonsen Laboratories, Gilroy, CA) by lavage and 2×10^6 cells were plated on 12-well plastic culture dishes in 2 ml of alpha-MEM medium (GIBCO) containing 10% fetal calf serum and antibiotics. After an overnight incubation at 37°C, nonadherent cells were removed by washing the dishes three times with Dulbecco's modified Eagle's medium (DMEM) (GIBCO). The cells were then used for LDL degradation studies.

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Abbreviations: LDL, low density lipoprotein; NDGA, nordihydroguaiaretic acid; ITYA, 5,8,11,14-icosatetraynoic acid; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substance(s); LPDS, lipoprotein-deficient serum.

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LDL and LDL Modification. LDL (d = 1.019–1.063) was isolated by ultracentrifugation from pooled normal human plasma collected in EDTA (1 mg/ml) (34). At the end of the isolation, LDL was extensively dialyzed against phosphate-buffered saline containing 1 mg of EDTA per ml. LDL was radioiodinated by using 1,3,4,6-tetrachloro-3,6-diphenylglycouril (Iodo-Gen, Pierce) and carrier-free ^{125}I (35). Protein was determined by the Lowry method (36) using serum albumin as a standard. ^{125}I-labeled LDL (100 μg/ml) was incubated in 60-mm culture dishes in 2 ml of serum free Ham's F-10 medium at 37°C in the presence of either endothelial cells or 5–10 μM Cu^{2+} for 24 hr. LDL modification was estimated by measuring lipid peroxidation in terms of TBARS expressed as malondialdehyde equivalents (37) and measuring macrophage degradation. For the latter, 10 μg of labeled LDL (native or modified) was added to macrophages in 12-well dishes in 1 ml of DMEM and incubated at 37°C for 5 hr. The medium was then analyzed for trichloroacetic acid-soluble noniodide radioactivity as described (13).

Effects of Inhibitors and of SOD. 5,8,11,14-Icosatetraynoic acid (ITYA) was purchased from Cayman Chemical (Ann Arbor, MI). Nordihydroguaiaretic acid (NDGA), aspirin, and indomethacin were purchased from Sigma and were added in ethanol (20 μl) to the LDL incubations (final concentration, 1% ethanol). ITYA was added as sodium salt. Control dishes were incubated under identical conditions without the inhibitors present. In addition, controls without cells or copper ions were run.

Recombinant human Mn and Cu/Zn SOD were a generous gift from Amnon Gonenne (Bio-Technology General Corp., New York) (3800 units/mg) (38). SOD samples were also purchased from Sigma, ICN, and Calbiochem. SOD activity was measured by monitoring the inhibition of reduction of ferricytochrome c (39).

RESULTS

Inhibitory Effects of NDGA. NDGA has been shown to inhibit lipoxygenase activity both in human umbilical vein endothelial cells (31) and in a purified system of soybean lipoxygenase plus fatty acids (40). In the endothelial cell system, inhibition of generation of 15-lipoxygenase products was 85% at 30 μM and in the soybean lipoxygenase system inhibition was 38% at 10 μM. As shown in Table 1, the generation of TBARS during incubation of LDL in the presence of rabbit endothelial cells was strongly inhibited by NDGA. Even at 0.5 μM, inhibition was almost 40% and at 1.0 μM, inhibition was ≈90% complete. The NDGA effect on biological modification (i.e., the enhanced rate of degradation by macrophages of LDL previously incubated with endothelial cells) was even more striking. Even at the lowest concentration tested (0.5 μM), NDGA almost completely blocked biological modification. To show that the inhibitor does not affect the TBARS measurement or the subsequent assay for rates of degradation of modified LDL in the macrophage system, NDGA was added at the end of the incubation (7.5 μM). As shown in Table 1, this had no effect. The NDGA concentrations used for the studies in Table 1 had no effect on oxidation of LDL induced by copper ions in the absence of cells (data not shown). However, slightly higher concentrations (2.5 μM) did inhibit copper-induced oxidation. Therefore, we turned to an inhibitor that should have no nonspecific antioxidant activity—ITYA.

Inhibitory Effects of the Acetylenic Analog of Arachidonic Acid. The acetylenic analog of arachidonic acid has been extensively utilized as an inhibitor of lipoxygenase and cyclooxygenase (41, 42). As a free fatty acid, it exhibits cytotoxicity unless added in the presence of proteins and so our studies using it were done in 0.2% lipoprotein-deficient serum (LPDS). The use of LPDS somewhat reduced the extent of oxidative modification by endothelial cells but did not prevent it, as shown in Table 2. ITYA inhibited the biological modification of LDL by ≈70% at 20 μM. TBARS values were also correspondingly reduced (data not shown). To rule out cytotoxic effects, it was shown that the inhibition was reversible. The ITYA-containing medium was removed and the cells were washed and then incubated a second time with LDL but without LPDS and without ITYA. As shown in Table 2, the extent of biological modification during the second incubation was fully as great as that during the first. In contrast to its inhibition of endothelial cell-induced modification, ITYA had absolutely no effect on oxidative modi-

Table 1. Effect of NDGA on LDL modification by endothelial cells

Incubation conditions	TBARS, nmol of MDA per mg of LDL	Macrophage degradation, μg per 5 hr per mg of protein
Unincubated native LDL	1.0	0.5
LDL incubated in the absence of cells	1.5	0.5
LDL incubated in the presence of cells	67.0	8.1
LDL incubated in the presence of cells plus		
0.5 μM NDGA	26.0	1.1
1.0 μM NDGA	7.0	0.5
LDL incubated in the presence of cells minus NDGA and then with 7.5 μM NDGA added at the end of the incubation	75.0	10.1

^{125}I-labeled LDL (100 μg/ml) was incubated with endothelial cells in Ham's F-10 medium (2 ml) for 24 hr in 60-mm plastic culture dishes. NDGA was added in 20 μl of ethanol; ethanol only was added to control dishes. One aliquot (500 μl) of medium was used for measurement of TBARS. A second aliquot (corresponding to 10 μg of LDL) was added to murine macrophages (2×10^6 per dish) in 12-well dishes for measurement of degradation over 5 hr. Results are averages of duplicate determinations from a representative experiment. MDA, malondialdehyde.

Table 2. Effects of ITYA on oxidative modification of LDL by endothelial cells

Addition to medium	Increase in rate of degradation by macrophage, μg per 5 hr per mg: After first 24-hr incubation (with or without ITYA)	Increase in rate of degradation by macrophage, μg per 5 hr per mg: After washing to remove ITYA and then incubating for an additional 24 hr in F-10 medium without inhibitors
None	9.81	11.16
0.2% LPDS only	7.01	11.72
Plus 20 μM ITYA	3.10	11.14
Plus 30 μM ITYA	2.01	9.39

^{125}I-labeled LDL (100 μg/ml) was incubated with endothelial cells in 2 ml of Ham's F-10 medium in the presence of LPDS and ITYA as indicated above. After 24 hr, LDL in the medium was tested for macrophage degradation (after first incubation). The endothelial cells were then washed twice with 2 ml of F-10 medium. The cells were then incubated with 2 ml of serum-free F-10 medium containing 100 μg of ^{125}I-labeled LDL per ml without LPDS or ITYA for another 24 hr. LDL contained in this medium was then tested for macrophage degradation (after second incubation). The values for rates of degradation of unincubated LDL have been subtracted to show the change during the incubations as indicated. The control values were 1.31 μg per 5 hr per mg for the first incubation and 1.48 μg per 5 hr per mg for the LDL used in the second incubation. Results are averages of duplicate determinations of a representative experiment.

fication of LDL induced by incubation with 5 μM copper sulfate (data not shown), confirming that the compound lacks intrinsic nonspecific antioxidant activity.

Effects of Aspirin and Indomethacin. Aspirin and indomethacin are well-known inhibitors of the cyclooxygenase system. However, even at levels well above those at which they inhibit cyclooxygenase activity, they had no effect on the modification of LDL induced by endothelial cells (Table 3). It appears that the cyclooxygenase system plays little role, if any, in oxidative modification of LDL, a conclusion in concordance with that of van Hinsbergh *et al.* (28) using much lower concentrations of the inhibitors.

Mode of Action of Cellular Lipoxygenase in Endothelial Cell-Induced Oxidation of LDL. Cellular lipoxygenase activity could contribute to oxidation of LDL in the medium in at least two distinct ways. First, it might oxidize cellular lipids, which then exchange into the LDL in the medium. Second, as reported by Kukreja *et al.* (43), it could generate superoxide anion as a byproduct of its activity. To the extent that the former mechanism is operative, cell–LDL contact would probably be necessary since there are no transfer proteins in the medium. We therefore placed ^{125}I-labeled LDL in a small dialysis bag and incubated the bag with endothelial cell monolayers in F-10 medium in the usual way. The LDL within the dialysis bag did not undergo oxidative modification, either as judged by the development of TBARS or by the rate of its degradation in macrophages. However, when the experiment was conducted in exactly the same way but with labeled LDL outside of the dialysis bag, that LDL did undergo oxidative modification to an extent comparable to that seen in experiments without the dialysis bag, showing that the latter did not interfere with oxidative modification.

The absence of oxidation of LDL separated from the cells by a dialysis membrane might suggest that superoxide anion released from the endothelial cells did not play a major role, but it is unclear whether or not superoxide anion can cross the dialysis membranes used rapidly enough.

Evaluation of the Role of Superoxide Anion. As shown in Table 4, human recombinant Cu/Zn SOD in some studies had almost no inhibitory effect on endothelial cell-induced modification of LDL. The SOD-induced inhibition of generation of TBARS and of the enhanced macrophage degradation was in this case <10%. In striking contrast, the oxidation induced by incubation with 5 μM copper ion in the absence of cells was almost totally inhibited. In this same experiment, SOD inhibited smooth muscle cell-induced oxidation by ≈60%, in agreement with the findings of Heinecke *et al.* (24). Almost identical results were obtained with human recombinant Mn SOD at concentrations from 5 to 200 μg/ml.

In a long series of experiments utilizing various commercially available SOD preparations, we have observed on average only 20–30% inhibition of endothelial cell-induced oxidative modification of LDL even at very high concentrations (Table 5). In every experiment, the inhibition of copper-induced modification was much greater, often exceeding 90% at high SOD concentrations, emphasizing the importance of superoxide anion in the latter. In five experiments utilizing recombinant human SOD, the mean inhibition of TBARS generation was 21% and the inhibition of macrophage degradation of LDL was 23%.

A further test of the effectiveness of superoxide anion was carried out by incubating LDL in the presence of xanthine oxidase and xanthine or acetaldehyde in F-10 medium. Regardless of the rate of superoxide generation, there was very little oxidation of LDL and almost no increase in macrophage degradation after such treatment, evidence against the hypothesis that superoxide anion generated by cells is directly responsible for LDL modification.

Table 3. Lack of effect of cyclooxygenase inhibitors on LDL modification by endothelial cells

Addition to medium	Increase in TBARS, nmol of MDA per mg of LDL	Increase in macrophage degradation, μg per 5 hr per mg
None	84.3	5.0
Acetylsalicylic acid (1 mM)	76.9	5.5
Indomethacin (50 μM)	79.0	4.7

Experimental protocol was as described in the legend to Table 1. The values for unincubated LDL have been subtracted; those values were for TBARS and macrophage degradation (8.3 nmol per mg of LDL and 0.89 μg per 5 hr per mg of cell protein, respectively). Results are averages of duplicate determinations from a representative experiment. MDA, malondialdehyde.

Table 4. Effects of recombinant SOD on oxidative modification of LDL

Treatment of LDL	Increase in TBARS, nmol/mg	Increase in macrophage degradation μg per 5 hr per mg
Incubated with		
Endothelial cells	50.6	4.47
Endothelial cells + SOD	44.6	4.29
5 μM Cu^{2+}	68.0	6.55
5 μM Cu^{2+} + SOD	4.4	0.00
Smooth muscle cells	22.0	1.11
Smooth muscle cells + SOD	8.1	0.39
Smooth muscle cells and 10 μM Cu^{2+}	90.2	7.58
Smooth muscle cells and 10 μM Cu^{2+} + SOD	25.6	3.37

The native LDL used in these studies had a TBARS value of 6.2 nmol/mg and was degraded by macrophages at a rate of 0.63 μg per 5 hr per mg. These values were subtracted from the observed values in each experiment. Incubations were for 24 hr in Ham's F-10 medium. Where used, SOD (Cu/Zn human recombinant enzyme) was added at 100 μg/ml.

DISCUSSION

Previous studies from this laboratory showed that LDL can undergo oxidative modification at a reasonable rate by incubation with purified soybean lipoxygenase and at a much higher rate by a combination of soybean lipoxygenase and phospholipase A_2 (44). The present studies provide evidence that cellular lipoxygenase activity or activities play an important role in endothelial cell-induced oxidative modification of LDL. On the other hand, the negative results with aspirin and indomethacin rule against any significant role for cyclooxygenase, in agreement with the conclusions of others (28). The inhibitors of lipoxygenases used are not highly

Table 5. Effect of various commercial SOD preparations on LDL modification by endothelial cells and by copper ion

Addition to medium	% inhibition			
	TBARS		Macrophage degradation	
	Endothelial cells	Cu^{2+} ion	Endothelial cells	Cu^{2+} ion
Low SOD (5–50 μg/ml)	25 ± 12	56 ± 22	27 ± 14	69 ± 14
High SOD (100–200 μg/ml)	28 ± 12	78 ± 15	28 ± 12	83 ± 14

The table summarizes the results of 14 or more experiments with various SOD preparations (see *Materials and Methods*) at the concentrations indicated.

specific; ITYA in fact inhibits both cyclooxygenase and lipoxygenase (41, 42) and is not positionally specific. Consequently, we cannot conclude which lipoxygenase(s) are involved.

Cellular lipoxygenases could play a role in oxidative modification in a number of ways. One possibility is that the lipoxygenase somehow acts directly on the lipids of LDL in the medium, presumably requiring LDL–cell contact. The fact that most lipoxygenases appear to be cytosolic makes this unlikely. However, there is the possibility of leakage of the enzyme under conditions of cell culture and the possibility that the endothelial cells have some plasma membrane enzyme activity. A second possibility is that the lipoxygenase system acts first on endogenous cell lipids, generating peroxy radicals and/or peroxides, which are then transferred to LDL by lipid exchange. Both of these mechanisms would depend, of course, on cell–LDL contact, something that is apparently essential according to the studies reported above. An attractive possibility, as discussed by Sparrow *et al.* (44), is that a two-step process is involved: (*i*) generation of oxidized lipids by the action of a cell lipoxygenase system with the subsequent transfer of the lipid to LDL; (*ii*) a propagation reaction within the LDL in the medium catalyzed by transition metals and/or sulfhydryl compounds (45). Lipid peroxidation, even if initiated by a site-specific enzyme, would tend to become random due to metal-catalyzed propagation reactions. The lengthy incubations and metal ion requirement of cell-induced LDL modification would tend to mask any specificity in the initial lipoxygenase attack. Any such specific initial products would be decomposed to yield additional reactive intermediates that may be essential for propagation of lipid peroxidation and, ultimately, for the modification of the apoprotein.

The present studies show that under appropriate conditions, SOD has very little effect on oxidation of LDL induced by the line of rabbit aortic endothelial cells used here. On the other hand, we confirm the findings of Heinecke *et al.* (24) that, under other conditions, smooth muscle cell-induced oxidation of LDL can be effectively inhibited by SOD (Table 4). Cell-induced oxidation of LDL proceeds optimally only in the presence of metal ions in the medium, presumably because these are essential for propagation reactions. In the studies of Heinecke *et al.* (24), a very high concentration of copper or iron ion was added to the medium. Under those conditions, much of the oxidation occurring might be related to metal-catalyzed oxidation in the medium, masking the contribution of the cell. As shown here, SOD can completely inhibit copper-induced autooxidation in the absence of cells (Table 4). Conceivably, the extent of inhibition by SOD in cell culture studies will depend on the extent to which conditions allow generation of lipoperoxides within the cell, on the one hand, and the rate at which autooxidation occurs in the medium (possibly facilitated by compounds secreted from the cells), on the other hand. Until more is known about the extracellular environment *in vivo*, it will not be possible to establish the relative importance of these various mechanisms.

The present findings implicate lipoxygenases as one contributor to cell-induced oxidation of LDL. Whatever the precise molecular mechanisms by which lipoxygenases act, if further studies confirm that they play a role *in vivo* it would open the way to unusual interventions that could inhibit atherogenesis as does antioxidant (probucol) treatment (18, 19). Further studies should be done to determine whether inherited or acquired abnormalities in the functional levels of lipoxygenase can account for some of the regional differences in susceptibility to atherosclerosis along the arterial tree, differences in species susceptibility, and differences in individual susceptibility.

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