Lipoxygenase-mediated transformation of human low density lipoprotein to an oxidized and cytotoxic complex[1]

Martha K. Cathcart,[2,*] Amy K. McNally,[*] and Guy M. Chisolm[†]

Departments of Immunology[*] and Vascular Cell Biology,[†] Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195

Abstract We have been studying the mechanisms involved in the oxidative modification of low density lipoprotein (LDL) that lead to its transformation to a cytotoxic complex. Here we examine the direct effect of soybean lipoxygenase (SLO), a 15-lipoxygenase, on normal human LDL. SLO oxidized LDL and rendered it cytotoxic; agents known to interfere with lipoxygenase activity inhibited this reaction. Enhancement of both the SLO-mediated LDL oxidation and the conversion of LDL to a cytotoxin was observed when either superoxide dismutase or copper (II) (3,5,-diisopropylsalicylic acid)$_2$, both of which dismute superoxide anion, were included during the incubation of SLO with LDL. In contrast, catalase inhibited this reaction in the presence or absence of agents that dismute superoxide anion. Thus, purified lipoxygenase can mediate LDL modification and superoxide anion inhibits this reaction. Furthermore, H_2O_2 is essential for SLO-mediated LDL oxidation and conversion of LDL to a cytotoxin. —**Cathcart, M. K., A. K. McNally, and G. M. Chisolm.** Lipoxygenase-mediated transformation of human low density lipoprotein to an oxidized and cytotoxic complex. *J. Lipid Res.* 1991. **32:** 63–70.

Supplementary key words oxidized lipoprotein • cytotoxicity • oxygen free radicals • soybean lipoxygenase • 15-lipoxygenase

Previous studies have shown that normal human low density lipoproteins (LDL) can be readily oxidized by oxygen free radicals generated in a cell-free system or by free radicals emanating from cells (1–5). Once oxidized, the LDL is transformed into a potent cytotoxin (2–4). The mechanisms involved in the oxidation of LDL are of interest from the perspective that it would be beneficial to understand processes by which a normal human serum component becomes a potential mediator of tissue injury. There is mounting evidence that oxidized lipoproteins are present in the vascular lesions of atherosclerosis (6, 7) and in the plasma of diabetic humans and experimental diabetic animals (8–10).

Lipoxygenases (LO) are enzymes that catalyze the incorporation of one oxygen molecule into polyunsaturated fatty acids with a particular 1,4-*cis*, *cis* pentadiene structure yielding a 1-hydroperoxy-2,4-*trans*,*cis*-pentadiene product. Lipoxygenases can oxidize both unesterified fatty acids and esterified fatty acids such as those in phospholipids (11). Soybean lipoxygenase (SLO) is a readily available, purified lipoxygenase that catalyzes the conversion of arachidonate to 15-S-hydroperoxy eicosa-5,8,10,14-tetraenoic acid (15-HPETE). In these studies we used SLO as a model system to determine whether a lipoxygenase enzyme could render LDL toxic to target cells, and to determine the role of superoxide anion generated by LO activity in the modification of LDL. Impetus for these studies arose from several recent findings. Activated monocytes were shown to modify LDL by free radical oxidation, converting it to a potent cytotoxin (2) and this modification appears to involve leukocyte-associated lipoxygenase activity (12) as well as superoxide anion (1). Lipoxygenase has also been reported to be released from activated leukocytes (13). In addition, lipoxygenase acting in conjunction with phospholipase A_2 has been shown to alter LDL to the degree that it becomes a ligand for macrophage scavenger receptors (14).

MATERIALS AND METHODS

SLO reaction conditions

Experiments with soybean lipoxygenase (SLO, Sigma type I, 140,000 U/mg) were performed in RPMI-1640

Abbreviations: LDL, low density lipoprotein; SLO, soybean lipoxygenase; LPDS, lipoprotein-deficient serum; BCS, bovine calf serum; LO, lipoxygenase; PBS, phosphate-buffered saline; TBA, thiobarbituric acid; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive dylcholine; PUFA, polyunsaturated fatty acids; PLA_2, phospholipase A_2; ETYA, 5,8,11,14-eicosatetraynoic acid.

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[2]To whom reprint requests should be addressed at: Cleveland Clinic Foundation, 9500 Euclid Avenue, Cleveland, OH 44195.

tissue culture medium unless otherwise indicated. SLO was put in solution immediately prior to use. Fatty acids (Sigma) were solubilized in ethanol. Incubations were conducted in a total reaction volume of 1.0 ml at 37°C for 24 h unless otherwise specified. Samples were then assayed for lipid oxidation products and cytotoxicity. Affinity-purified SLO (Sigma type V, 737,000 U/mg) was also tested in numerous experiments similar to those shown in this report and gave comparable results (data not shown). Additionally, an electrophoretically pure SLO isoenzyme with a pH optimum of 9.0, generously provided by Dr. M. Funk, University of Toledo, mediated ETYA-inhibitable LDL oxidation at that pH. In some experiments superoxide dismutase (SOD, 3000 U/mg from bovine erythrocytes, Sigma), catalase (CAT, thymol free, 11,000 U/mg, Sigma C40) or copper(II) [3,5,-diisopropylsalicylic acid]$_2$ (CuDIPS, generously provided by Dr. A. Pezeshk) were added during the incubation period.

Lipoprotein preparation

LDL and lipoprotein-deficient serum (LPDS) were prepared according to previously described methods (15). LDL, prepared and stored in 0.5 mM EDTA, was adjusted to 10 mg cholesterol/ml. Immediately before use, LDL was dialyzed (4°C) against phosphate-buffered saline with calcium and magnesium (PBS). LDL was used at a final concentration of 500 μg cholesterol/ml unless otherwise noted. The LPDS stock solution was adjusted to 40 mg protein/ml, thoroughly dialyzed (4°C) against RPMI-1640, and stored at 4°C until use.

Thiobarbituric acid (TBA) assay for lipid oxidation

The presence of oxidation products was determined by a modification (15) of the assay described by Schuh, Fairclough, and Haschemeyer (16) that detects malondialdehyde (MDA) and MDA-like compounds reacting with thiobarbituric acid (TBA). Although it is not specific for MDA, this assay is widely used as a reliable index of lipid oxidation. Components that react with TBA are referred to as TBA-reactive substances, or TBARS. Briefly, 1.0 ml of 25% trichloroacetic acid was added to 400-μl samples, followed by 1.0 ml of 1% TBA. The samples were then vortexed and incubated for 45 min at 95°C, after which they were centrifuged (1000 *g*) for 15 min. Supernatant TBARS were detected by fluorescence at 515 nm excitation and 553 nm emission. Samples (400 μl) of freshly diluted malondialdehyde bis(dimethyl acetal), i.e., 1,1,3,3-tetramethoxypropane (Sigma), were used as standards (0–10 nmol). Data are the averages of duplicate determinations and are expressed in terms of MDA equivalents (nmol MDA/ml of sample). Differences between experimental treatments were determined by performing two-tailed, paired *t* tests on the means of like groups in several experiments.

Assay for formation of conjugated dienes

SLO and LDL or PUFA substrates were incubated for various times in spectrophotometric cuvettes (1.0 cm light path). Incubations were carried out in 50 mM borate buffer, pH 9.0 (17) at 25°C in a reaction volume of 1.0 ml. Absorbance at 234 nm was periodically recorded after addition of SLO. Results are expressed as an increase in absorbance from the zero time reaction mixture.

Cytotoxicity assay

The HEL 299 human embryonic lung fibroblast cell line (American Type Culture Collection) was used to assess cytotoxicity. The cells were maintained in RPMI-1640 supplemented with 10% BCS; only passages 7–13 were used. Two days before the assay, target cells in culture medium were plated in flat-bottomed 96-well microtiter plates (Costar) at a density of 2×10^3 cells/well. The cells were plated so that they were actively growing during the assay period since the cytotoxicity of oxidized LDL has been shown to be cell cycle-dependent (18). At the start of the assay, the medium was replaced with 100 μl/well of 20% stock LPDS in RPMI, which was subsequently diluted with 100 μl/well of experimental sample. After a 66-h incubation at 37°C, the microtiter plates were washed twice with PBS to remove detached (nonviable) fibroblasts. To assess cell survival, hexosaminidase activity of total target cells remaining per well was measured according to the method of Landegren (19). This assay has previously been shown to correlate with other determinations of target cell survival and cytotoxicity (15). The average hexosaminidase activity (± standard deviation) in quadruplicate wells was calculated and converted to a percent of the control values. The control wells contained RPMI as the sample. Data are thus expressed as a percentage of the control cell survival. Differences between experimental treatments were determined by performing two-tailed, paired *t* tests on data obtained from several experiments.

Phospholipase A_2(PLA_2) assay

PLA_2 activity was monitored by incubating radiolabeled phosphatidylcholine (PC) with the enzyme preparations and then separating the free fatty acid from the intact PC by thin-layer chromatography. Incubations included 5 mg/ml dipalmitoyl PC (Sigma, from a stock solution of 10 mg/ml PC sonicated in RPMI with 3 mg/ml deoxycholate), 10 μCi/ml of 2-[9,10-^{3}H(N)]palmitoyl-PC (50 Ci/mmol, New England Nuclear) and PLA_2 (71 U/mg protein, Sigma) and/or SLO as indicated. Reaction volumes were 0.5 ml and the incubation was conducted at 37°C for 6 h. After incubation the reaction mixtures were extracted according to the Bligh and Dyer (20) modification of the extraction procedure of Folch, Lees, and Sloane Stanley (21). The chloroform phase was evapo-

TABLE 1. SLO-mediated oxidation of fatty acids or LDL

Substrate	SLO (5000 U/ml)	Oxidation (TBARS) Expt. A	Expt. B
Stearate (500 μM)	–	0	n.p.
	+	0	n.p.
Linoleate (250 μM)	–	0	0.01
	+	1.50	1.15
Arachidonate (Expt. A: 250 μM, Expt. B: 200 μM)	–	0.25	0.16
	+	4.16	3.35
LDL (500 μg cholesterol/ml)	–	0.17	0.13
	+	2.93	3.00

LDL or fatty acids, as indicated, were incubated in the presence or absence of SLO for 24 h. Oxidation was then determined as described in Materials and Methods; TBARS, thiobarbituric acid reactive substances, expressed in nmol MDA/ml; n.p., not performed.

rated under nitrogen and the samples were spotted onto silica gel thin-layer chromatography plates (20 × 20 cm). The solvent system for chromatography was chloroform–acetone–methanol–acetic acid–water 6:8:2:2:1 (by volume). After development in a pre-equilibrated chromatography tank, the plates were dried and the lanes were scraped and counted in 1.25-cm increments. Substantial PLA_2 activity was observed at 15 U/ml PLA_2 and activity was readily apparent even at 0.5 U/ml. No significant levels of radioactivity were detected on the chromatogram other than those associated with intact PC or free fatty acids.

RESULTS

Soybean lipoxygenase-I (SLO), a plant-derived 15-lipoxygenase involved in arachidonic acid metabolism, has been extensively studied (for reviews, see 22, 23). Experiments were performed with this enzyme to examine the way in which a lipoxygenase could catalyze the oxidation of LDL. Since lipoxygenase activity is conventionally measured with PUFA substrates (24), we tested the effect of SLO on LDL or PUFA using the TBA assay for lipid oxidation. In **Table 1**, data from two experiments are shown in which the SLO-mediated oxidation was compared using linoleate, arachidonate, and LDL as substrates. The saturated fatty acid stearate was included as a negative control in experiment A. Linoleate (18:2) was less oxidized than arachidonate (20:4) and neither substrate was significantly altered unless SLO was present. LDL was significantly oxidized by SLO (see below). Enzymatic inactivation of SLO with 1 mM H_2O_2 prevented the oxidative transformation of LDL by SLO (data not shown; 25).

Experiments were performed to compare the time course of SLO oxidation of LDL to that of PUFA substrates. Conjugated diene formation on PUFA was used as an index of lipoxygenase activity (24), and was monitored spectrophotometrically by the increase in absorbance at 234 nm in borate buffer, pH 9.0, at 25°C. **Fig. 1** illustrates the increase in absorbance associated with PUFA (A) compared to that associated with LDL (B). 5,8,11,14-Eicosatetraynoic acid (ETYA, a gift from Hoffman LaRoche), a competitive inhibitor of lipoxygenase (and cyclooxygenase) by virtue of its struc-

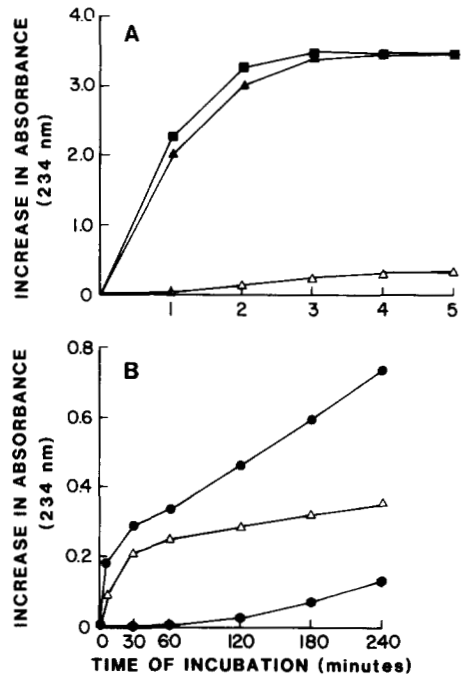

Fig. 1. SLO-mediated formation of conjugated dienes on PUFA or LDL. A: Linoleate (200 μM, closed squares) and arachidonate (200 μM, closed triangles), or arachidonate plus ETYA (100 μM, open triangles) were incubated with 500 U SLO/ml as described in Methods. Absorbance at 234 nm was periodically recorded. Results are expressed as an increase in absorbance from the zero time reaction mixture. B: LDL (500 μg cholesterol/ml) was incubated without SLO (closed hexagons), with 5000 U SLO/ml (closed circles), or with SLO plus 200 μM ETYA (open triangles).

tural similarity to arachidonate, was included in parallel incubation mixtures in each case. As expected, the ETYA-inhibitable formation of conjugated dienes on arachidonate and linoleate was completed in minutes. Substantial oxidation of LDL was also observed; however, absorbance in the LDL incubation mixture increased at a slower rate and to a lesser extent, with the increase continuing even after several hours.

We then asked whether SLO alteration of LDL rendered the LDL cytotoxic, since it has previously been shown that the oxidation of LDL by a variety of other means results in the conversion of LDL to a potent cytotoxin (1–4, 10, 15, 18, 26). **Fig. 2** shows that the SLO-catalyzed oxidation of LDL and the SLO-mediated conversion of LDL into a cytotoxin increased monotonically with time of incubation. By 24 h the LDL oxidation was quite dramatic. We therefore used this time point for future studies. After 24 h of incubation with SLO, LDL was significantly oxidized as compared to that incubated in the absence of SLO ($P < 0.0001$, n = 12) and was significantly cytotoxic ($P < 0.0001$, n = 8). The concentration of enzyme was varied in the presence or absence of ETYA in the experiment shown in **Fig. 3**. The level of SLO-catalyzed LDL oxidation that developed over a 24-h period could be controlled by varying the concentration of SLO. ETYA was a potent inhibitor of SLO-mediated oxidation of LDL, and was effective at all concentrations of SLO tested. The reduction in oxidation of the LDL incubated for 24 h with SLO (5000 U/ml) in the presence of ETYA (100 μM) as compared to that incubated without ETYA was found to be significant ($P < 0.002$, n = 7). Reduction of LDL oxidation by

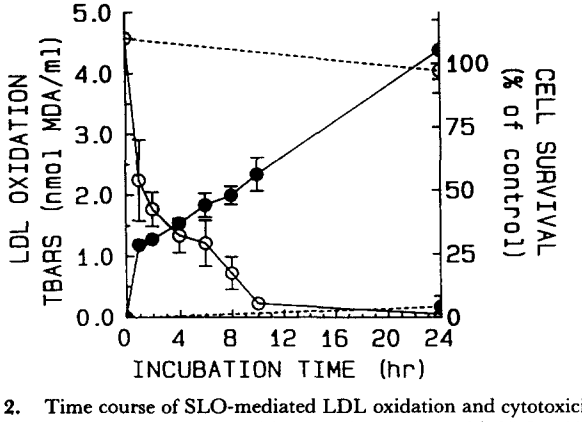

Fig. 2. Time course of SLO-mediated LDL oxidation and cytotoxicity. LDL (500 μg cholesterol/ml) without SLO (------) or with SLO (5000 U/ml) (———) was incubated to the time points indicated. LDL oxidation (●, closed circles) and toxicity to fibroblasts (○, open circles) were then determined as described. These data are from three separate experiments. Data were collected at 2, 4, 6, and 24 h in all three experiments and represent the mean and standard error, whereas 1, 8, and 10 h data points depict the mean and data range from two of these experiments. SLO incubated in the absence of LDL was negative for TBARS and was not cytotoxic to fibroblasts.

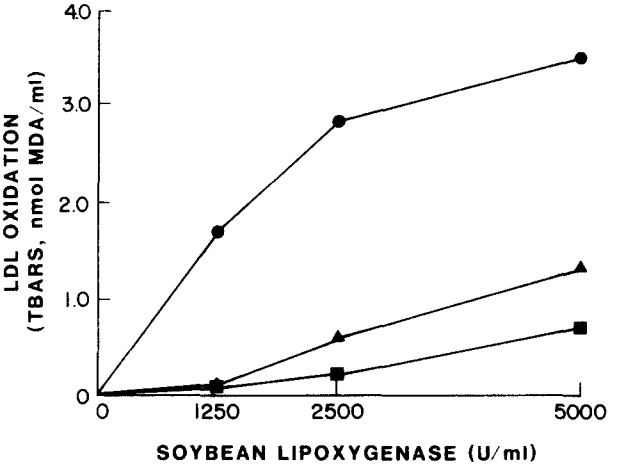

Fig. 3. Effect of concentration of SLO on LDL oxidation in the presence or absence of ETYA. LDL was incubated with varying concentrations of SLO as shown in the absence (circles) or presence of 100 μM ETYA (triangles) or 200 μM ETYA (squares). The incubation period was 24 h. The data are expressed as the average of duplicate determinations and are from an experiment representative of four similar experiments that were performed. The mean standard error between experiments was in the range of ± 10%.

ETYA also resulted in decreased cytotoxicity (data not shown).

In further attempts to link the oxidation and toxicity directly to the lipoxygenase activity, reduced glutathione (GSH) and other inhibitors of lipoxygenases were also tested for their ability to inhibit SLO-mediated oxidation of LDL. In **Fig. 4**, the data show that complete inhibition of LDL oxidation and toxicity to target fibroblasts was found with several agents that have been reported to be LO inhibitors. SLO alone did not develop increased TBARS or become cytotoxic, and neither indomethacin (30 μM) nor ibuprofen (30 μM) was effective in inhibiting the LDL oxidation by SLO (data not shown).

Interpretation of results from studies using inhibitors of lipoxygenase is complicated by the fact that some of these inhibitors are general antioxidants. To determine whether the particular inhibitors used modulated LDL oxidation through a specific effect on lipoxygenase activity or by their action as general free radical scavengers, the antioxidant properties of the inhibitors were evaluated for their effects on cell-free, $CuSO_4$-mediated LDL oxidation (5). The concentration of $CuSO_4$ was adjusted to 2 μM, to yield a level of LDL oxidation similar to that observed in the SLO-mediated oxidation of LDL. The LO inhibitors nordihydroguaiaretic (NDGA), quercetin, phenidone, and propyl gallate were found to inhibit $CuSO_4$-mediated oxidation of LDL. ETYA, at 50 μM, did not affect LDL oxidation mediated by 2 μM $CuSO_4$, yet SLO-mediated LDL oxidation was significantly inhibited by this concentration of ETYA ($P < 0.05$, n = 3) and ETYA at 100 μM only caused a 15% inhibition of $CuSO_4$-mediated LDL

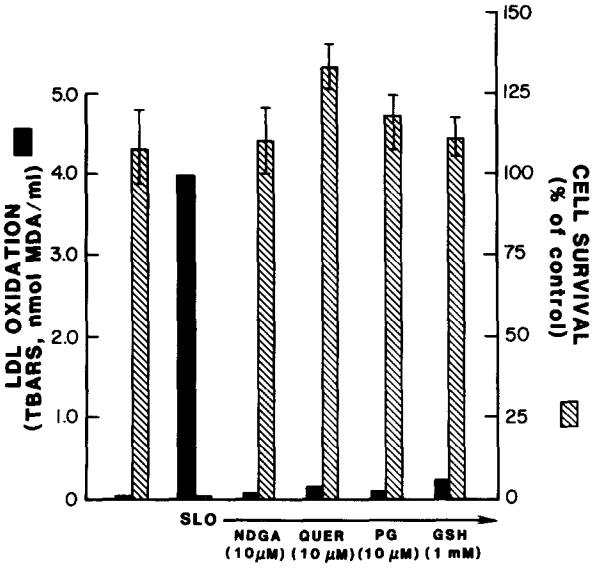

Fig. 4. Effects of lipoxygenase inhibitors on SLO-mediated LDL oxidation and cytoxicity. LDL, in the presence of 5000 U SLO/ml, was incubated for 24 h with the indicated inhibitors: NDGA (nordihydroguaiaretic acid), QUER (quercetin), PG (propyl gallate), or GSH (reduced glutathione). LDL incubated without SLO is also shown. LDL oxidation (solid bars) and toxicity to fibroblasts (cell survival, hatched bars) were determined as described. The data are the average of duplicates (LDL oxidation) or mean of quadruplicate determinations ± SD (cell survival). Data from a second experiment gave similar results.

oxidation. Furthermore, GSH at 1 mM did not reduce $CuSO_4$-catalyzed LDL oxidation, indicating that it was not serving as a general antioxidant in this system. Thus, both ETYA and GSH appeared to interfere with LDL oxidation by inhibiting SLO.

Since SLO activity has been shown to generate O_2^- as a by-product (27), we added superoxide dismutase (SOD) during SLO-catalyzed LDL oxidation to examine a potential contribution by O_2^-. Surprisingly, SOD enhanced the oxidation of LDL as compared to SLO plus LDL alone. In the experiment depicted in **Fig. 5A**, the concentration of LDL was varied with a constant amount of SLO in the presence or absence of SOD. The enhancing effect of SOD was observed particularly at the higher concentrations of LDL. This effect of SOD at LDL concentrations of 500 μg cholesterol/ml was found to be statistically significant ($P < 0.0001$, n = 6). In Fig. 5B, cell survival results are shown from the experiment in Fig. 5A and demonstrate that the SOD-enhanced oxidation of LDL is associated with an increase in toxicity of the lipoprotein to target cells. This effect was statistically significant as well ($P < 0.04$, n = 5, LDL = 500 μg cholesterol/ml).

Lipoxygenase enzymes are known to contain an iron catalytic center which alternately cycles from the active ferric (Fe^{3+}) to the inactive ferrous (Fe^{2+}) state during fatty acid oxygenation (17, 23). We considered that the side-product O_2^- might inactivate Fe^{3+}-SLO by simple reduction. If this were the case, one would expect that LDL oxidation would be more vigorous in the presence of SOD. This effect was observed at LDL concentrations above about 100 μg cholesterol/ml (see Fig. 5A). An additional effect of the added SOD would be to enhance the formation of hydrogen peroxide. We therefore examined whether the H_2O_2, formed by this action, might be mediating the enhanced LDL oxidation (28). To test this idea, we included CAT (1000 U/ml) during the 24-h incubation of LDL (500 μg cholesterol/ml) with SLO in the presence or absence of SOD. CAT inhibited the majority of SLO-catalyzed LDL oxidation and cytotoxicity (see open triangle and open square at 500 μg cholesterol/ml in Figs. 5A and 5B), including the SOD enhancement of these parameters. Several similar experiments were conducted using LDL at 500 μg cholesterol/ml and significant

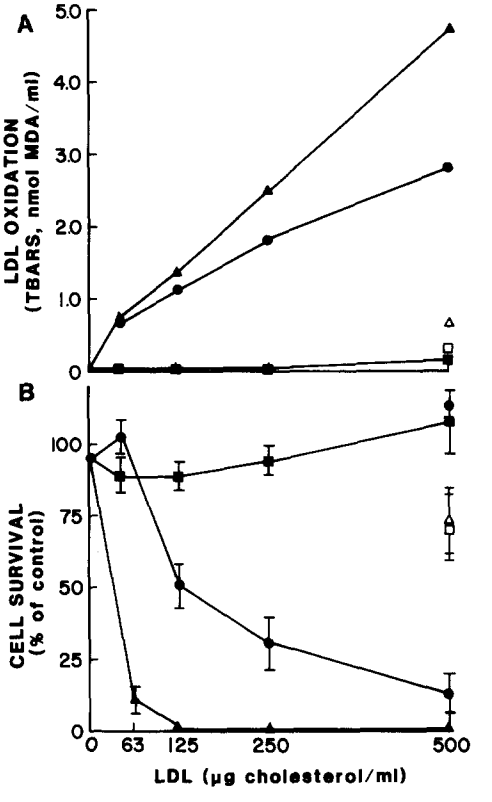

Fig. 5. Effect of SOD on SLO-mediated LDL oxidation and cytotoxicity. A: Various concentrations of LDL were incubated for 24 h with 5000 U SLO/ml in the absence (closed circles) or presence (closed triangles) of 30 U SOD/ml. LDL alone (no SLO) is represented by closed squares. In parallel incubation mixtures, catalase (1000 U/ml) was added to LDL at 500 μg cholesterol/ml with (open triangle) or without (open square) SOD (30 U/ml). The data are the average of duplicate determinations. B: Survival of fibroblasts exposed to the preparations in (A) are shown. The data are expressed as the mean ± SD of quadruplicate determinations. LDL alone (without SLO, closed squares) is compared to LDL with SLO (closed circles) or LDL plus SLO and SOD (closed triangles). Catalase results are shown as in (A). The data are from a representative experiment of four that were performed.

inhibition of SLO-mediated oxidation ($P < 0.0001$, n = 4) and cytotoxicity ($P < 0.0002$, n = 4) by CAT was observed, whereas boiled CAT was without effect (data not shown). Even at a tenfold lower concentration (100 U/ml), CAT substantially inhibited LDL oxidation and cytotoxicity in the presence or absence of SOD (data not shown).

To verify the effects of SOD as being due to the dismutation of O_2^- rather than a non-specific effect, we examined the effects of another O_2^--specific scavenger on SLO-mediated LDL oxidation. The compound chosen was a small (503 daltons) SOD mimetic, termed CuDIPS [copper (II) (3,5,-diisopropylsalicylic acid)$_2$] (29, 30). **Fig. 6** illustrates the data from two experiments in which two concentrations of CuDIPS were tested with various concentrations of LDL and a constant concentration of SLO. CuDIPS was found to enhance SLO-mediated LDL oxidation in a manner analogous to SOD. Analysis of data from four experiments with SLO and LDL (500 μg cholesterol/ml) incubated in the presence or absence of 1 μg/ml CuDIPS revealed that CuDIPS significantly enhanced SLO-catalyzed LDL oxidation ($P < 0.0002$, n = 4). In the experiment in which 0.5 μg CuDIPS/ml was added, CAT was included in a parallel incubation

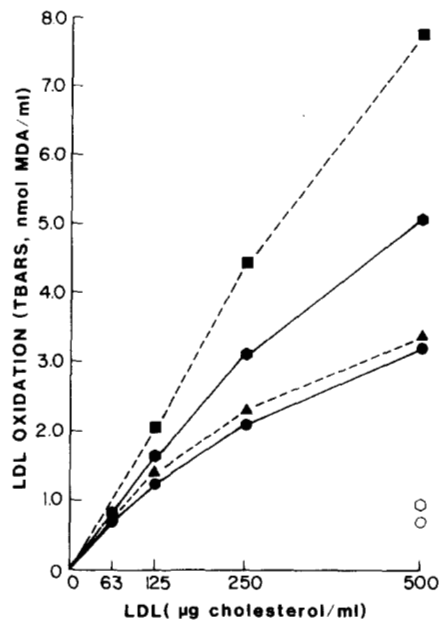

Fig. 6. Effect of CuDIPS on SLO-mediated LDL oxidation. Oxidation of LDL is shown from an experiment (———) in which the various concentrations of LDL (as indicated) were incubated for 24 h with SLO (5000 U/ml) in the absence (closed circles) or presence (closed hexagons) of 0.5 μg CuDIPS/ml. A similar experiment (------) performed in the absence (triangles) or presence (squares) of 1.0 μg CuDIPS/ml is also presented. In parallel incubation mixtures, catalase (1000 U/ml) was added to LDL (500 μg cholesterol/ml) with (open hexagon) or without (open circle) CuDIPS (0.5 μg/ml). Data points represent the average of duplicate determinations. Similar observations were made in many experiments.

TABLE 2. Effect of ETYA on CuDIPS enhancement of LDL oxidation

Conditions	LDL Oxidation (TBARS[a])
CuDIPS[b] + LDL + 2500 U SLO/ml	7.63
CuDIPS[b] + LDL + 2500 U SLO/ml + ETYA[c]	2.58
CuDIPS[b] + LDL + 1250 U SLO/ml	7.74
CuDIPS[b] + LDL + 1250 U SLO/ml + ETYA[c]	0

[a]nmol MDA/ml. The data shown are typical of results obtained in repeated experiments. The incubation time was 24 h.
[b]LDL oxidation due to 1.0 μg CuDIPS/ml (0.89 nmol MDA/ml) was subtracted as background such that the values shown represent only SLO-mediated LDL oxidation.
[c]100 μM.

with LDL. CAT was again observed to block enhancement of LDL oxidation by the O_2^-scavenger. CuDIPS alone did not display TBA reactivity and was not cytotoxic at the concentrations used in these studies (data not shown). In the presence of LDL without SLO, CuDIPS mediated a background oxidation that may represent Cu-catalyzed LDL oxidation via the Haber-Weiss reaction (5). The TBARS values obtained in both experiments in the absence of SLO increased with the concentration of LDL from 0.45 to 1.11 nmol MDA/ml. These were subtracted from the results shown in Fig. 6 such that only SLO-catalyzed oxidation is represented.

Additionally, the effects of ETYA on CuDIPS-enhanced LDL oxidation were examined. In **Table 2**, background oxidation due to 1.0 μg CuDIPS/ml was again subtracted as described for the data in Fig. 6, such that only SLO-catalyzed LDL oxidation is represented. ETYA effectively blocked CuDIPS enhancement of LDL oxidation indicating that the enhancement was indeed mediated through SLO. The inhibition was optimized by decreasing the concentration of enzyme.

Other investigators have reported that certain SLO-mediated LDL alterations are dependent on the presence of PLA_2 (14). Since we observed oxidation of LDL in the presence of SLO alone, we conducted experiments to determine whether our SLO preparation was contaminated with PLA_2. The assay used here was capable of detecting 0.5 U/ml of PLA_2 activity. The fact that no PLA_2 was detected in 5.0×10^4 U/ml of the SLO used in our experiments indicates that this preparation had less than 1 U of activity per 10^5 U of SLO (**Table 3**). Coincubation of PLA_2 and SLO was also examined to verify that SLO did not inhibit the detection of PLA_2.

DISCUSSION

Normal human low density lipoprotein can be modified by free radical oxidation to become oxidized and the ox-

TABLE 3. SLO has no detectable PLA_2 activity

Culture Components (U/ml)		Fatty Acid Ratio Nonesterified/Esterified
PLA_2	SLO	
–	–	0.03
15	–	1.47
5×10^{-1}	–	0.17
5×10^{-2}	–	0.03
–	500	0.01
15	500	1.65
5×10^{-1}	500	0.27
5×10^{-2}	500	0.03
–	500	0.01
–	5.0×10^4	0.01

Incubations were conducted in 0.5 ml volumes including phosphatidylcholine (dipalmitoyl, 5 mg), [^{3}H]phosphatidylcholine (10 μCi), and PLA_2 and/or SLO as indicated above. Mixtures were incubated for 6 h at 37°C. The details of the methods followed for reagent preparation and for determination of the fatty acid ratios are described in Materials and Methods. These results are from a representative experiment of two performed.

idized lipid fraction has been shown to be toxic to target cells (26). Since lipoxygenases are involved in the oxidative modification of fatty acids, we examined the ability of soybean lipoxygenase to oxidize LDL and to render it cytotoxic.

We have demonstrated that SLO alone can mediate the transformation of LDL to a cytotoxin. The results presented in this report extend our understanding of the mechanisms that can participate in LDL modification. The mechanism highlighted here is one that relies on H_2O_2 as an essential mediator. This process is distinct from that mediated by activated human monocytes suggesting that extracellular monocyte-derived lipoxygenase is not involved in monocyte-mediated LDL oxidation. In the latter system, extracellular scavengers of O_2^- that function to dismute O_2^-, totally block LDL modification (1), whereas the removal of O_2^- and/or the promotion of peroxide formation by these agents increase the oxidation of LDL by SLO. Catalase-sensitive mechanisms predominate in the SLO system, whereas CAT alone has no inhibitory effect in the monocyte system (M. K. Cathcart, A. K. McNally, and G. M. Chisolm, unpublished observations). Inhibition of SLO-mediated LDL oxidation by CAT, whether or not SOD was present, suggests that the mechanism is peroxidative. The hydroperoxide product of lipoxygenase is known to be required for reactivation of the catalytic iron center by oxidation (22, 23). Thus, CAT could alternatively interfere with the reactivation of SLO and its ability to oxidize LDL.

Sparrow, Parthasarathy, and Steinberg (14) recently compared the oxidative modification of LDL that occurs during incubation with endothelial cells (4, 5) to LDL alteration after its incubation with SLO plus phospholipase A_2. They observed a moderate increase in LDL TBARS after incubation with SLO alone, not unlike that observed in the present study. However, other endothelial cell-dependent LDL alterations, such as transforming LDL into a ligand recognized by macrophage scavenger receptors, were not observed unless phospholipase A_2 was added to the LDL-SLO mixture. They did not address the issue of whether the modified LDL was cytotoxic. It is possible that while moderately oxidized LDL is clearly cytotoxic further LDL oxidation is required to observe scavenger receptor uptake. It should be noted that some differences in reaction conditions exist between our studies and those reported by Sparrow et al. (14). For the most part, our studies were conducted in RPMI-1640 tissue culture medium, pH 7.4, as opposed to borate buffer, pH 9.0. We have observed an approximate 50% reduction in LDL-associated TBARs induced by SLO (Sigma, Type V) in borate buffer, pH 9.0, as compared to that in RPMI 1640 at physiologic pH (data not shown) and products formed at this pH may differ from those formed at pH 9.0 (22). To address whether there was a contribution of PLA_2 in our system, we measured PLA_2 activity in the purified SLO. No PLA_2 activity was detected in the SLO preparations used in our studies (Table 3). We also repeated selected experiments with an even more highly purified preparation of SLO (affinity purified, 7.4 = 10^5 U/mg) and confirmed our findings (data not shown).

In summary, lipoxygenase can alter LDL to become both oxidized and cytotoxic, and these alterations are inhibited by agents known to inhibit lipoxygenase activity. We found that this enzyme-mediated modification is modulated by oxygen free radicals. SOD, an enzyme capable of dismuting O_2^- to H_2O_2, enhanced the modification of LDL by lipoxygenase; catalase blocked the modification in the presence or absence of SOD. These results implicate H_2O_2 as an essential component for this modification and suggest that conversion of O_2^- to H_2O_2 facilitates the transformation of LDL to a cytotoxin by SLO.

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