LDL oxidation by activated monocytes: characterization of the oxidized LDL and requirement for transition metal ions

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Abstract **Monocytes can be activated by incubation with opsonized zymosan (Zop), and under these conditions can oxidize low density lipoprotein (LDL). We have characterized the biochemical changes in the lipoprotein after this oxidation. We found that monocyte-oxidized LDL has increased mobility on agarose gels, increased absorbance at 234 nm, increased content of lysophosphatidylcholine, and fluorescence at 430 nm when excited at 350 nm. All these features were somewhat less pronounced in monocyte-oxidized LDL than in LDL oxidized by 5 μM $CuSO_4$. Under appropriate conditions, Zop-stimulated monocytes oxidized LDL to a form recognized by macrophage scavenger receptors. Monocytes stimulated by Zop produced superoxide and also oxidized LDL, whereas monocytes stimulated by phorbol ester produced slightly more superoxide but did not oxidize LDL. We found that the chelators EDTA and diethylenetriaminepentaacetic acid inhibited LDL oxidation by Zop-stimulated monocytes, implying a requirement for transition metal ions. We found that Zop contained approximately 5 nmol iron per mg, probably as Fe^{3+}. Zop stripped of its iron supported superoxide production by monocytes, but did not support LDL oxidation. Furthermore, Fe^{2+} appeared in the medium when monocytes were incubated with Zop, but not with iron-stripped Zop. Taken together, these results imply that monocytes stimulated by Zop are able to oxidize LDL only because of contaminating iron in the commercial zymosan preparations.—**Xing, X., J. Baffic, and C. P. Sparrow. **LDL oxidation by activated monocytes: characterization of the oxidized LDL and requirement for transition metal ions. *J. Lipid. Res.* 1998.** 39: **2201–2208.**

Supplementary key words scavenger receptor • atherosclerosis • iron • cholesterol • zymosan • superoxide

Low density lipoprotein (LDL) oxidation is believed to be an important feature of the biochemical chain of events leading from elevated cholesterol levels to atherosclerosis (1, 2). Oxidized LDL can be detected in atherosclerotic arterial tissue (3), and certain antioxidants slow the progression of atherosclerosis in animal models (4–7). The importance of oxidized LDL in vascular disease has led to significant research efforts on the causes and effects of LDL oxidation.

LDL oxidation is a complex process. The initial event is probably formation of hydroperoxides in the polyunsaturated fatty acyl chains of LDL lipid(s). Lipid hydroperoxides are conjugated dienes, and their absorbance at 234 nm allows for a simple assay for the early stages of LDL oxidation (8). The hydroperoxides in LDL can decompose to form other oxidizing species, causing a free radical chain reaction within the particle or perhaps between LDL particles. This uncontrolled lipid peroxidation yields many different molecular species (9), some of which can be detected by the thiobarbituric acid assay (10). LDL phospholipids with an oxidized fatty acyl chain at C-2 are substrates for a phospholipase A_2 that converts them to lysolipids (11). The LDL apolipoprotein (apo)B is altered in two ways by oxidation: there is direct oxidative scission of peptide bonds (12), and some of the products of lipid peroxidation react with the free amino groups of apoB, forming covalent adducts (6, 13). These adducts increase the net negative charge of the particle, which can be easily detected by agarose gel electrophoresis (14), and these adducts also have a characteristic fluorescence spectrum (13, 15). Most importantly, it is the presence of these adducts that causes recognition of oxidized LDL by the scavenger receptor of macrophages (15), and macrophage uptake of oxidized LDL may lead to foam cell formation in vivo (1).

Most characterizations of oxidized LDL are from in vitro studies. LDL can be oxidatively modified in vitro by various chemical agents and by certain cultured cell types (reviewed in refs. 1 and 16). To the extent that LDL oxidation occurs in vivo, it is presumably catalyzed by the cells of the artery. Because of the pathophysiological importance of oxidized LDL, it may be useful to understand the mechanism(s) by which cells oxidize LDL.

LDL oxidation by most cultured cells requires a medium containing transition metal ions (17–24). These ob-

Abbreviations: BPS, bathophenanthroline sulfonate; DETAPAC, diethylenetriaminepentaacetic acid; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances; TPA, tetradecanoyl phorbol acetate; Zop, opsonized zymosan; apo, apolipoprotein; LDL, low density lipoprotein(s).

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servations present a puzzle: if LDL oxidation requires free transition metal ions, how can LDL by oxidized in vivo given the normal presence of physiological chelators? Perhaps the in vitro systems that require transition metal ions do not accurately mimic the in vivo situation. It would be interesting to find a cellular mechanism for LDL oxidation that does not require transition metal ions. Monocytes activated with opsonized zymosan (Zop) can oxidize LDL in RPMI medium, which is formulated without transition metal ions (25, 26). This intriguing observation implies that LDL may be oxidized in the absence of metal ions by activated monocyte/macrophages in the subendothelial space.

Given the potential importance of transition metal-independent LDL oxidation, it would be of great interest to understand the mechanism of LDL oxidation by activated monocytes in RPMI medium. It has been demonstrated that LDL oxidation in this system requires cytosolic phospholipase A_2 (27) and protein kinase C (28) but does not require lipoprotein-receptor interactions (29). In the present work, we have characterized LDL oxidation by activated monocytes in RPMI medium: the monocyte-oxidized LDL has been analyzed biochemically, and mechanistic studies have addressed the roles of superoxide and transition metal ions.

MATERIALS AND METHODS

Cells and cell culture

Human peripheral blood monocytes were isolated from heparinized blood using Histopaque-1077 as recommended by the supplier (Sigma, St. Louis, MO). The mononuclear cells were plated into cell culture-treated plastic 35-mm wells (5 million cells per well in 6-well plates to yield 1 million monocytes per well). The monocytes were allowed to adhere for 2 h, and then the non-adherent lymphocytes were removed by washing. This procedure yields a relatively pure culture of monocytes (30). The cells were either used immediately or cultured overnight (no significant differences were seen between immediate or overnight use). HL-60 cells, a human monocyte-like cell line, were obtained from ATCC (Rockville, MD) and grown as recommended. The rabbit aortic endothelial cell line RECB4 (31) was grown as previously described (32). Resident mouse peritoneal macrophages were obtained from female Swiss-Webster mice by peritoneal lavage with phosphate-buffered saline. Macrophages were plated in 6-well cell culture plates at 1 million cells/well (for LDL oxidation) or in 24-well plates at 0.6 million cells/well (for uptake assays) in DMEM containing 10% fetal bovine serum.

Lipoprotein

Plasma was obtained from fasted normal volunteers, and LDL were isolated by standard procedures (33). Concentrations of LDL are expressed on the basis of protein, measured using the micro BCA (bicinchoninic acid) protein reagent (Pierce Chemical Co., Rockford, IL), with bovine serum albumin as the standard. LDL was iodinated with $Na^{125}I$ using the "trapped label" tyramine cellobiose (34). No significant differences were found in preliminary experiments comparing the oxidative modification of LDL labeled with ^{125}I-tyramine cellobiose and LDL labeled conventionally (data not shown).

Preparation of opsonized zymosan (Zop) and stripping of iron from zymosan

Zymosan was purchased from Sigma (St. Louis) and United States Biochemical (Cleveland); similar results were obtained with zymosan from the two different sources. Zymosan was opsonized using human serum as described (30). During the course of these studies, we considered the possibility that commercial zymosan preparations contained Fe^{3+}. We therefore developed a method to simultaneously strip zymosan of iron and quantitate the iron removed. Our method used ascorbate to reduce Fe^{3+} to Fe^{2+}, and *o*-phenanthroline to chromogenically bind Fe^{2+} (35). Specifically, iron was removed from zymosan by suspending 0.5 g zymosan in 40 ml of stripping solution, which contained 50 mm HEPES (pH 7.4), 1 mm diethylenetriaminepentaacetic acid (DETAPAC), 100 μm *o*-phenanthroline, and 10 mm ascorbate (all solutions used were sterile filtered). The suspension, in a sterile 50-ml conical tube, was rotated slowly at room temperature for 2–3 days with 4 or 5 changes of stripping solution. The last two treatments also contained 20% ethanol. Zymosan is particulate, so the material can be easily sedimented to change solutions. The stripping solutions removed from the zymosan were pooled, their volume was measured, and their absorbance was measured at 510 nm against standards prepared from *o*-phenanthroline and $FeSO_4$ (35). This allowed quantitation of the iron originally present in the zymosan: we found zymosan contained approximately 5 nmol of iron per mg. The iron bound to the zymosan was probably Fe^{3+}, because solutions without ascorbate liberated no Fe^{2+}. After the treatments with ascorbate and *o*-phenanthroline, the zymosan was washed twice with 10 mm HEPES containing 1 mm DETAPAC, and then boiled for 10 min, as is standard for preparing Zop (30). The zymosan was then opsonized using human serum that had been treated with chelex resin (Bio-Rad) to remove any transition metal ions. Finally, the Zop was washed with 10 mm HEPES and stored at 4°C until it was used. The "control" preparation of Zop was produced by treating a second aliquot of zymosan exactly in parallel, except all chelators and ascorbate were omitted.

Assays of oxidative modification of LDL

LDL oxidation was carried out in 35-mm wells (6-well plates) using 1 million cells per well in serum-free RPMI medium containing 200 μg/ml LDL, in a final volume of 1 ml. In some incubations, Zop was present at a final concentration of 2 mg/ml (25, 26). The incubations, usually in duplicate or triplicate, were performed at 37°C in 95% air/5% CO_2. After 24 h, media were harvested and centrifuged to remove cells and/or Zop. Some experiments used LDL radioiodinated with tyramine cellobiose (20–50 cpm/ng). Small amounts of EDTA that were present in the LDL solution carried over into the incubations (<3 μm); this level of EDTA had no effect on the results, as judged by experiments in which LDL was dialyzed against RPMI prior to incubation with cells (data not shown). Controls for LDL oxidation assays were performed by incubating LDL in RPMI in the absence of cells. Oxidative modification of LDL was routinely assayed by measurement of thiobarbituric acid-reactive substances (TBARS) as previously described (36), measuring fluorescence (excitation at 515 nm, emission at 553 nm).

Other assays

Superoxide production was measured as described (30). Briefly, 1 million cells were incubated in 1 ml of Earles salts containing cytochrome c, in the presence and absence of superoxide dismutase (SOD). After 60 min, the medium was removed, and the SOD-inhibitable reduction of cytochrome c was measured spectrophotometrically. Results are expressed as μm superoxide produced in 1 h. Iron (as Fe^{2+}) released into cell culture media was quantitated with bathophenanthroline sulfonate (BPS) as described (37).

TABLE 1. Cell-type specificity of LDL oxidation stimulated by Zop

Cell Type	LDL Oxidation (TBARS)	
	Without Zop	With Zop
	nmol/mg LDL	
Human peripheral blood monocytes (7)	0.61 ± 0.2	11.2 ± 2.1
HL-60 human monocyte-like cell line (6)	0.56 ± 0.2	15.0 ± 2.9
Mouse resident peritoneal macrophages (3)	0.70 ± 0.3	15.2 ± 2.0
Rabbit aortic endothelial cells (2)	0.48	0.53
LDL incubated without cells (5)	0.23 ± 0.1	2.0 ± 0.5

Various cell types were incubated with LDL in RPMI medium with or without Zop (see Materials and Methods for details). After 24 h at 37°C, media were harvested and LDL oxidation was assayed by TBARS. Results are presented as the mean and SEM of results from independent experiments, except only means are given for endothelial cells. Numbers in parentheses indicate the number of independent experiments. Duplicate incubations were performed for each experiment.

RESULTS

Characterization of LDL oxidized by Zop-stimulated monocytes

Human peripheral blood monocytes and mouse peritoneal macrophages oxidized LDL in RPMI medium when stimulated by Zop, but not when unstimulated (**Table 1**). Our data are similar to data in previous reports (25, 26). There was cell-type specificity to this oxidation because rabbit aortic endothelial cells were unable to oxidize LDL in RPMI whether or not Zop was present (Table 1).

LDL oxidized by activated monocytes was similar to copper-oxidized LDL with respect to a number of biochemical changes that are characteristic of oxidized LDL. In most cases, the characteristics of monocyte-oxidized LDL were intermediate between native LDL and copper-oxidized LDL, indicating that activated monocytes oxidize LDL, but not as heavily as does 5 μm $CuSO_4$. The characteristics analyzed included migration on agarose gels (**Fig. 1**), UV absorbance and fluorescence (**Fig. 2**), and recognition by the macrophage scavenger receptor (**Table 2**). Lysophosphatidylcholine content of LDL was also measured, following standard procedures (38, 39). We found that native LDL contained 38 nmol lysophosphatidylcholine per mg LDL protein, and this increased when the LDL was oxidized by 5 μM $CuSO_4$ (388 nmol/mg) or by Zop-activated monocytes (121 ± 2; mean and range of duplicates). In contrast, lysophosphatidylcholine content of LDL did not significantly increase after incubation of LDL with unactivated monocytes (36 ± 8 nmol/mg) nor with Zop alone in the absence of cells (47 ± 3).

Superoxide production does not correlate with LDL oxidation

It has been previously suggested that superoxide causes LDL oxidation (26). Both Zop and tetradecanoyl phorbol acetate (TPA) stimulate monocytes to produce superoxide. We found that the extent and time course of superoxide production was similar for monocytes stimulated with Zop or with TPA (**Fig. 3A**). In contrast, LDL oxidation occurred when monocytes were stimulated with Zop, but not when monocytes were stimulated with TPA (Fig. 3B). Therefore, superoxide alone did not cause LDL oxidation under these conditions.

Nitric oxide production is not required for LDL oxidation by activated monocytes

It has been reported that nitric oxide can oxidize LDL (40), and that the combination of nitric oxide plus superoxide is a potent oxidant (41). Because of the physiological importance of nitric oxide, we tested whether nitric oxide was required for LDL oxidation, even though human monocytes probably do not produce nitric oxide (42). We measured TBARS production during LDL oxidation by activated monocytes in the presence or absence of N-methyl arginine, which inhibits nitric oxide synthase (43), or dexamethasone, which blocks production of the inducible form of nitric oxide synthase (44). In this experiment, control activated monocytes oxidized LDL and produced 9.9 ± 0.9 nmol/mg TBARS; neither N-methyl arginine (11.7 ± 1.0) nor dexamethasone (8.7 ± 0.2) significantly blocked LDL oxidation. We conclude that nitric oxide production is not required for LDL oxidation by Zop-activated monocytes.

Inhibition of LDL oxidation by various compounds

To further characterize LDL oxidation by activated monocytes, we tested the ability of various compounds to

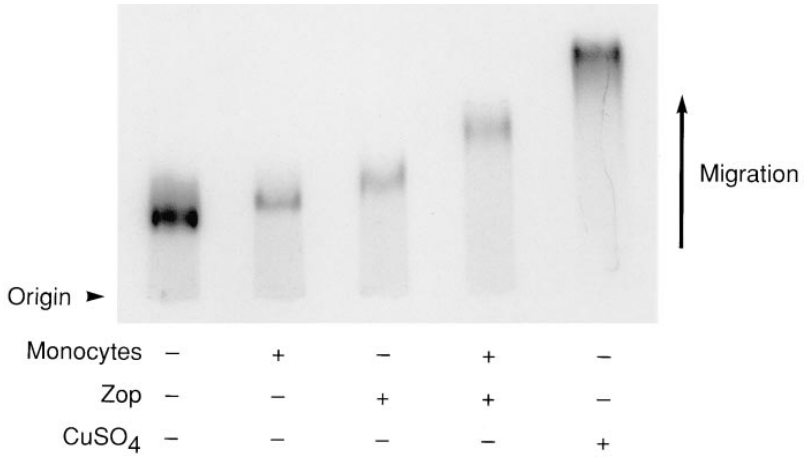

Fig. 1. Agarose gel electrophoresis of LDL oxidized by stimulated monocytes. ^{125}I-labeled LDL was incubated with monocytes and/or Zop in RPMI under usual conditions (see Materials and Methods), and electronegativity of the samples was compared to native ^{125}I-labeled LDL and copper-oxidized ^{125}I-labeled LDL. Samples were loaded into pre-cast agarose gels and electrophoresed as recommended (Ciba Corning Lipoprotein Electrophoresis System, Palo Alto, CA). The electrophoretogram was subjected to autoradiography. The treatment of the ^{125}I-labeled LDL samples is indicated beneath the lanes; the lane on the far left contained native (unincubated) ^{125}I-labeled LDL. The general pattern of these results was confirmed using Fat Red B staining of non-radioactive LDL preparations that were re-isolated by ultracentrifugal flotation after incubation with monocytes and/or Zop (data not shown).

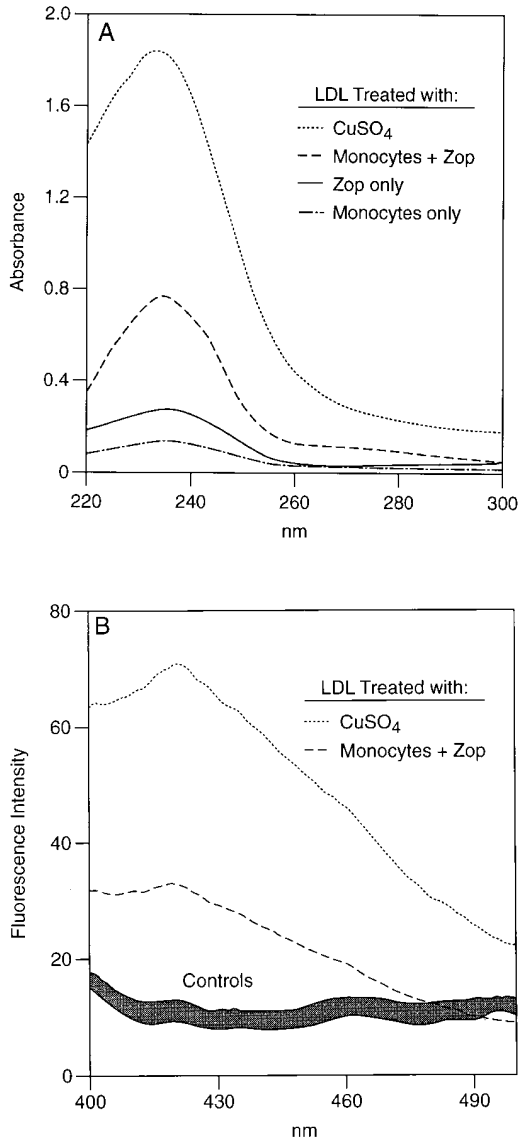

Fig. 2. UV and fluorescence spectra of LDL oxidized by stimulated monocytes. LDL was incubated with monocytes and/or Zop in RPMI under usual conditions (see Materials and Methods). The LDL samples were re-isolated by ultracentrifugal flotation, dialyzed, and sterile filtered, and then spectra were recorded at room temperature. A: UV spectra were measured at LDL concentrations of 66 μg/ml as difference spectra versus native (unincubated) LDL. B: Fluorescence emission spectra were measured using LDL samples at 270 μg/ml, with excitation at 350 nm (350 nm was the peak of the excitation spectrum; data not shown). The shaded band indicates the ranges of fluorescence spectra of three different control LDL samples: native LDL, LDL incubated with unstimulated monocytes, and LDL incubated with Zop alone.

inhibit the oxidation. As shown in **Table 3**, classic chain-terminating antioxidants such as butylated hydroxytoluene and N,N′-diphenyl-phenylenediamine (5) were effective inhibitors. The chelators EDTA and DETAPAC were found to be potent inhibitors of LDL observation by Zop-activated monocytes. We also found that SOD inhibited LDL oxidation in this system, as previously reported (26).

TABLE 2. LDL oxidation by Zop-stimulated monocytes leads to macrophage uptake via scavenger receptors

Incubation Conditions		Macrophage Uptake		
Additions	[LDL]	Total	+ Poly-I	SR-specific[a]
	μg/ml	*μg LDL taken up/mg cell protein*		
Cells + Zop	50	2.8 ± 0.1	0.97 ± 0.1	1.8
Cells + Zop	100	2.4 ± 0.03	1.0 ± 0.04	1.4
Zop alone	50	0.76 ± 0.04	0.59 ± 0.5	0.17
Cells alone	50	0.71 ± 0.04	1.09 ± 0.06	0
5 μm $CuSO_4$	100	3.2 ± 0.1	1.06 ± 0.16	2.1
Unincubated LDL	—	0.85 ± 0.15	0.95 ± 0.03	0

LDL was radioiodinated using the "trapped ligand" tyramine cellobiose (see Materials and Methods). The concentration of ^{125}I-labeled LDL given in the table was incubated with monocytes and/or Zop in RPMI medium. After 24 h, the media were harvested and Zop was removed by centrifugation. The ^{125}I-labeled LDL in the supernatants was diluted to 5 μg/ml in DMEM containing 1 mg/ml of lipoprotein-deficient serum and incubated with mouse peritoneal macrophages in the presence and absence of 5 μg/ml polyinosinic acid, which is a potent inhibitor of scavenger receptors (32). After 5 h, the media were removed and the macrophages were washed in saline, then dissolved in NaOH. Aliquots of the dissolved macrophages were assayed for ^{125}I and protein and uptake is expressed as μg of LDL per mg of macrophage protein during the 5 h incubation. Values given are the mean and range for duplicate determinations. Similar results were obtained in an independent experiment using a different batch of ^{125}I-labeled LDL.

[a] Scavenger receptor-specific uptake was calculated by subtracting uptake in the presence of polyinosinic acid from uptake in the absence of polyinosinic acid. Values less than zero are given as zero.

Zop contains iron, which is necessary for LDL oxidation

The ability of the chelators EDTA and DETAPAC to inhibit LDL oxidation by Zop-activated monocytes implied that transition metal ions are required in this system. The transition metal ions might come from either the Zop or the monocytes. Using *o*-phenanthroline and ascorbate (see Materials and Methods), we found that zymosan contains approximately 5 nmol of Fe per mg, probably as Fe^{3+}. This result was confirmed by inductively coupled plasma atomic emission spectroscopy, which gave a value of 4.2 nmol of Fe per mg of zymosan (analysis done at the Soil Testing Laboratories, University of Minnesota, St. Paul, MN). Copper was also present at 0.14 nmol per mg. The relative significance of the iron and copper is discussed below.

To determine whether the iron associated with the zymosan was important for the biochemical activity of zymosan, we compared monocytes treated with Zop to monocytes treated with Zop that had been stripped of its iron. Zymosan was stripped of iron using ascorbate plus *o*-phenanthroline (see Materials and Methods). The iron-stripped Zop supported superoxide production by monocytes and macrophages, but did not support LDL oxidation (**Table 4**).

Monocytes cause release of Fe^{2+} from Zop

We hypothesized that iron present in the Zop preparations was released as Fe^{2+} during incubation with cells, and that the Fe^{2+} then participated in LDL oxidation. To quantitate release of Fe^{2+} from Zop, cells were incubated in RPMI with Zop stripped of its iron or with control Zop, in the presence and absence of the Fe^{2+} reagent batho-

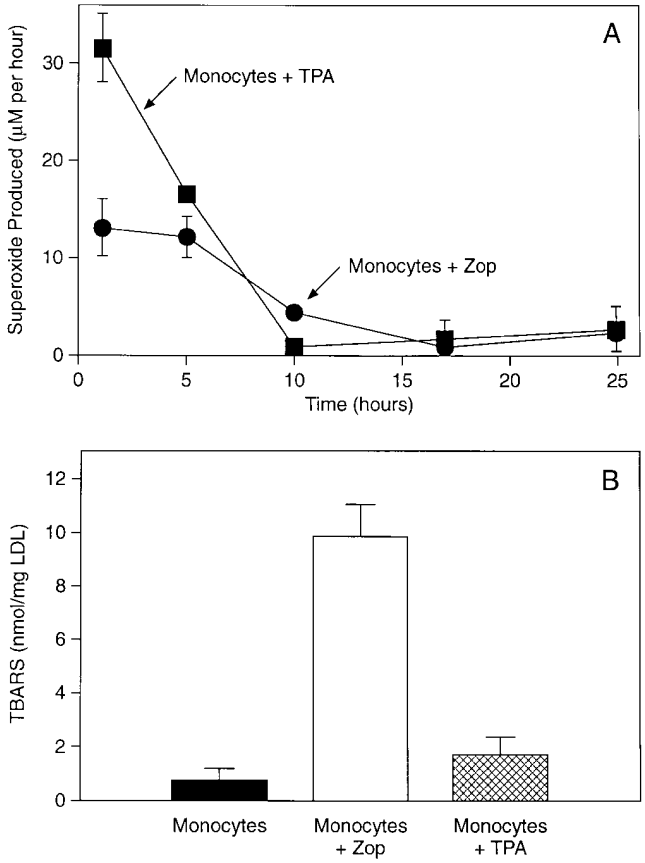

Fig. 3. Superoxide production does not correlate with LDL oxidation. A: Time course of superoxide production by human monocytes incubated with TPA or Zop. The activating agent was added to the monocytes at time zero, and superoxide production was measured at various times. At each time point, superoxide production was measured for 1 h; values represent the production of superoxide over the preceding hour. Unstimulated monocytes produced 1.9 ± 0.5 μm superoxide per hour. B: LDL oxidation by human monocytes incubated with TPA, Zop, or with no activator. Incubations were for 24 hours, as described in Materials and Methods.

phenanthroline sulfonate (BPS). After a 24 h incubation, the media were harvested, centrifuged, and the iron–BPS complex was quantitated. As shown in **Table 5**, both HL-60 cells and human peripheral blood monocytes cause the release of iron from Zop but not from Zop that had been stripped of iron. Based on the iron content of zymosan measured by atomic emission spectroscopy, the cells caused the release of approximately one-third of the total zymosan-associated iron (2.7 μm iron in 1 ml conditioned medium divided by 8.4 nmol iron present in 2 mg zymosan in the incubation = 32%). Our assay detected no iron when monocytes or Zop were incubated in the absence of the other (data not shown). Comparison of Tables 4 and 5 suggest that iron released from Zop is required for LDL oxidation by activated monocytes.

TABLE 3. Inhibition of monocyte-mediated LDL oxidation by various agents

Compound	IC_{50}
N,N′-diphenyl-phenylenediamine (DPPD)	0.59 ± 0.25 μm
Butylated hydroxytoluene (BHT)	13.4 ± 3.7 μm
Diethylenetriaminepentaacetic acid (DETAPAC)	6.1 ± 2.8 μm
EDTA	17.9 ± 2.1 μm
Superoxide dismutase (SOD)	0.2 ± 0.1 U/ml

LDL was incubated with monocytes activated by Zop. Compounds were tested at various concentrations and the concentration required to inhibit 50% of the production of TBARS (IC_{50}) was determined graphically. For the experiments involving EDTA and DETAPAC, the LDL was pre-dialyzed against RPMI overnight at 4°C to remove all traces of EDTA from the LDL preparation. Values given are the mean and range for duplicate determinations from independent experiments.

TABLE 4. Iron-stripped Zop supports superoxide production but not LDL oxidation

	Monocytes	Macrophages	HL-60 Cells
Superoxide production (μM/h)			
Control Zop	11.1 ± 0.9	4.9 ± 0.3	n.d.
Iron-stripped Zop	11.0 ± 0.7	5.9 ± 0.4	n.d.
LDL oxidation (nmol/mg LDL)			
Control Zop	13.4 ± 2.9	17.3 ± 3.5	16.9 ± 4.3
Iron-stripped Zop	1.5 ± 0.6	1.7 ± 0.3	0.89 ± 0.3

See Materials and Methods for details of the preparation of iron-stripped Zop. Zop preparations were incubated with human peripheral blood monocytes, mouse peritoneal macrophages, or HL-60 cells and superoxide production or LDL oxidation (TBARS) was measured (see Materials and Methods). Values given are means ± SEM of results from 3 independent experiments, using 3 different batches of Zop. Each experiment used duplicate incubations; n.d., not determined.

Replenishing the iron allows stripped Zop to support LDL oxidation

To determine whether iron was the critical factor lost from zymosan during stripping, iron was added back to incubations with stripped Zop. Reagent $FeSO_4$ was added to achieve the same amount that was found to be released from the commercial zymosan by the cells. Reagent $CuSO_4$ was also tested, because the presence of 0.14 nmol copper per mg of zymosan had been detected by inductively coupled plasma atomic emission spectroscopy. In the experiment, HL-60 cells incubated with iron-stripped Zop and LDL produced 0.8 ± 0.1 nmol/mg of TBARS. The addition of 0.15 μM $CuSO_4$ did not increase the production of TBARS (0.9 ± 0.1), whereas the addition of 3 μm $FeSO_4$ significantly increased TBARS production to 9.5 ± 0.2 nmol/mg. The addition of 3 μm $FeSO_4$ to RPMI medium did not enhance LDL oxidation in the absence of cells. This last observation is simi-

TABLE 5. Monocytes cause release of Fe^{2+} from Zop

	HL-60 Cells	Monocytes
	μM Fe^{2+} in medium	
Control Zop	2.67 ± 0.63	2.78 ± 0.52
Iron-stripped Zop	0.36 ± 0.27	0.59 ± 0.31

Cells (HL-60 cells or human peripheral blood monocytes) were incubated in RPMI (without phenol red) with iron-stripped Zop or control Zop, in the presence and absence of the iron reagent bathophenanthroline sulfonate (BPS) at 50 μM. After 24 h, the media were harvested, centrifuged, and the supernatants' absorbances at 530 nm were compared to a standard curve of Fe^{2+}. The samples without BPS were used as the blank in the assay to correct for media absorbance. Values given are means ± SEM of results from 3 independent experiments, using 3 different batches of Zop. Each experiment used triplicate incubations.

TABLE 6. Contact between Zop and the cells is not required for LDL oxidation

Additions to Cells in Well	Additions to Insert	LDL Oxidation (TBARS)
		nmol/mg
None	none	0.3 ± 0.1
TPA	none	0.4 ± 0.2
Zop	none	21.9 ± 3.7
None	Zop	6.9 ± 0.8
TPA	Zop	23.0 ± 2.1

HL-60 cells were incubated in 6-well plates into which were placed Trans-well inserts (Costar, Cambridge, MA). The inserts have a microporous membrane with 0.25 micron pores such that soluble molecules pass through freely but particulates are held in place approximately 1 mm above the cells. Zop was added directly to the cells as usual, or to the inserts, or not added at all. Other conditions were as usual (see Materials and Methods). After 24 h, media were harvested and LDL oxidation was measured as TBARS. Data are given as mean ± SEM from triplicate experiments, each with duplicate incubations.

lar to data published by Lamb and Leake (18). Taken together, these data imply that the iron present in the zymosan is important for LDL oxidation, and also that the procedure used to strip the zymosan did not introduce an antioxidant that prevents LDL oxidation by monocytes.

Contact between Zop and the cells is not required for LDL oxidation

To test whether contact between Zop and the cells was required for iron release we used Trans-well inserts. These inserts fit inside the wells of 6-well plates, and are made of a microporous membrane with 0.25-micron pores such that soluble molecules pass through freely, but particulates are held in place approximately 1 mm above the cells. When HL-60 cells were placed in the wells and Zop was placed in the insert such that there was no contact, very little LDL oxidation occurred (**Table 6**). When TPA was also added, however, there was significant LDL oxidation, even though cells plus TPA alone caused no LDL oxidation (Table 6). This suggests that activated monocytes secrete a factor that causes iron release from Zop, leading to LDL oxidation. This factor is released by cells stimulated by the Zop itself or by TPA.

We also performed a control experiment to show that there was no leakage of the Zop from the insert onto the cells. Zop is particulate and is prepared via repeated low-speed sedimentations (30), so it would be expected that the membrane pores would retain the Zop in the insert. To confirm this, we measured superoxide production by HL-60 cells incubated with empty inserts or with inserts containing Zop. HL-60 cells alone produced 1.6 ± 0.4 μm superoxide per h, and with Zop in the inserts, the cells produced 1.8 ± 0.3 μm/h (mean ± SEM of triplicate incubations). Superoxide production increased to 15.8 ± 2.1 μM/hr when the Zop was placed directly onto the cells in the standard method. This experiment showed that Zop in the insert does not leak through to the cells.

Zop alone, but not iron-stripped Zop, can oxidize LDL

We noticed that control incubations of LDL with Zop in the absence of cells gave slightly higher TBARS than LDL incubated in medium alone. We therefore tested whether Zop alone can oxidize LDL over many days at 37°C. LDL was incubated in the absence of cells at 37°C in RPMI medium with or without Zop or iron-stripped Zop. After 48 h, TBARS (nmol/mg) produced were 0.3 ± 0.03 without Zop, 0.5 ± 0.05 with iron-stripped Zop, and 7.0 ± 0.2 with Zop. The TBARS increased after 96 h to 0.5 ± 0.04 , 0.8 ± 0.07, and 11 ± 0.1 , respectively. These data suggest that the iron carried by Zop is capable of causing limited LDL oxidation in the absence of cells.

DISCUSSION

LDL oxidation by cultured cells has been intensively studied (1, 11–29). Transition metal ions are usually required for this process (14–24). Free transition metal ions may not be available in vivo, however, due to the presence of endogenous chelators. Thus it would be of great interest if there existed an in vitro cellular mechanism for LDL oxidation that was independent of transition metal ions. Activated monocytes are known to secrete oxidizing agents (30), and it was reported that monocytes activated by Zop could oxidize LDL in RPMI medium, which is formulated without transition metal ions (25, 26). This was an intriguing candidate for a possible physiologically relevant mechanism for cellular LDL oxidation. We have further characterized this system and found that the oxidized LDL produced is similar to copper-oxidized LDL. The monocyte-oxidized LDL contained lysophosphatidylcholine, had increased mobility on agarose gels, displayed increased fluoresence, and was a ligand for scavenger receptors. In general, the absolute extent of LDL oxidation by activated monocytes was less than the oxidation achieved by copper ions.

We have also addressed the mechanism of LDL oxidation by activated monocytes. We found that, despite the absence of transition metal ions in the formulation for RPMI medium, the LDL oxidation required transition metal ions, as judged by the fact that chelators inhibited the oxidation. We discovered that small amounts of iron were present in the commercial zymosan used to prepare the Zop. The ability of Zop to stimulate LDL oxidation was completely lost when the Zop was subjected to a procedure that stripped it of iron, even though iron-stripped Zop still activated the cells. Commercial zymosan preparations apparently are contaminated with sufficient iron to support LDL oxidation by activated monocytes. Thus activated monocytes require transition metal ions to oxidize LDL, as do other cell types (14–24).

If Zop carries iron, why doesn't Zop alone cause LDL oxidation? Although Zop alone does catalyze minimal LDL oxidation, Zop plus monocytes oxidize LDL to a much greater extent. Our data suggest that activated monocytes secrete a factor that reduces Zop-bound Fe^{3+} to Fe^{2+}, and the Fe^{2+} is released from the Zop and participates in LDL oxidation. This factor is likely to be superoxide, given the known ability of superoxide to reduce Fe^{3+} in ferritin, followed by iron-catalyzed lipid peroxidation

(37, 45). This action of superoxide would explain the ability of SOD to inhibit LDL oxidation (Table 3 and reference 26). In our experiments, superoxide alone did not directly oxidize LDL, as judged by the fact that stimulation of monocytes with TPA, which caused substantial superoxide production, did not cause LDL oxidation. Stimulation of monocytes with Zop, however, caused superoxide production and also caused LDL oxidation. The ability of Zop to stimulate LDL oxidation was completely lost when the Zop was subjected to a procedure that stripped it of iron, even though iron-stripped Zop still supported superoxide production by the cells. Thus we suggest that the superoxide, or some other factor released by activated monocytes, liberates contaminating iron in the commercial zymosan preparations, which is followed by iron-catalyzed LDL oxidation. The liberated iron may interact with superoxide molecules to cause LDL oxidation, e.g., via Haber-Weiss reactions. Hiramatsu et al. (46) have previously shown that cellular superoxide production can cause LDL oxidation in the presence of iron. It is also worth noting that Garner et al. (47) have shown that macrophages can reduce transition metal ions by superoxide-independent mechanisms.

There is disagreement in the literature as to whether superoxide alone can cause LDL oxidation. Production of superoxide by radiolysis can lead to LDL oxidation, although this oxidation is efficient only at pH below physiological levels (48). Lynch and Frei (49) used xanthine plus xanthine oxidase to produce superoxide and showed that superoxide only oxidizes LDL in the presence of transition metal ions. The elegant work of Hiramatsu et al. showed (46) that TPA-stimulated human monocytes could oxidize LDL only if they could produce superoxide. A critical observation in this study was that 10 μm EDTA inhibited 85% of the LDL oxidation catalyzed by activated monocytes (46), implying that transition metal ions were required. Transition metal ions, especially iron, are common contaminants in commercial reagents and even in distilled water stored in glass; their presence may, however, vary among different laboratories. To summarize, it is clear that superoxide plus transition metal ions can cause LDL oxidation and lipid peroxidation in general (21, 37, 45, 46, 49–51 and the present work). It is unlikely that superoxide alone is capable of oxidizing LDL at physiological pH.

Which of the many proposed mechanisms for LDL oxidation are most likely to be relevant in vivo? In the presence of transition metal ions, cells can cause LDL oxidation by secretion of superoxide, as discussed above. Superoxide production occurs in vivo, and superoxide can spontaneously dismutate to H_2O_2. These molecules are oxidants, but as discussed above, their ability to significantly oxidize LDL appears to be dependent on transition metal ions. Free transition metal ions may or may not be available in vivo. Total body iron was shown to be correlated with carotid artery atherosclerosis in humans (52), but iron overload did not increase atherosclerosis in hypercholesterolemic rabbits (53). Atherosclerotic "gruel" has been reported to contain reactive iron that is competent for lipid peroxidation (54). LDL oxidation has been achieved in the absence of transition metal ions by enzymatic systems using peroxidase (55), lipoxygenase (36, 56), or myeloperoxidase (57). Atherosclerotic lesions have been shown to contain both lipoxygenase (58) and myeloperoxidase (59). Future research may clarify the important issue of whether LDL oxidation in vivo requires free transition metal ions, or if enzymes present in the lesion can oxidize LDL without free transition metal ions.

The authors wish to thank Dr. Hugh Rosen for many helpful discussions about activated monocytes. We thank Dr. Steve Grant for advice on cellular nitric oxide production. The authors are grateful to Dr. Yu-sheng Chao for his support and advice.

Manuscript received 10 April 1998 and in revised form 8 July 1998.

REFERENCES

1. Steinberg, D., S. Parthasarathy, T. E. Carew, J. C. Khoo, and J. L. Witztum. 1989. Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N. Eng. J. Med.* **320**: 915–924.
2. Heinecke, J. W. 1997. Mechanisms of oxidative damage of LDL in human atherosclerosis. *Curr. Opin. Lipidol.* **8:** 268–274.
3. Yla-Herttuala, S., W. Palinski, M. E. Rosenfeld, S. Parthasarathy, T. E. Carew, S.Butler, J. L. Witztum, and D. Steinberg. 1989. Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84:** 1086–1095.
4. Carew, T. E., D. C. Schwenke, and D. Steinberg. 1987. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc. Natl. Acad. Sci. USA.* **84:** 7725–7729.
5. Björkhem, I., A. Henriksson-Freyschuss, O. Breuer, U. Diczfalusy, L. Berglund, and P. Henriksson. 1991. The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arterioscler. Thromb.* **11:** 15–22.
6. Sparrow, C. P., T. W. Doebber, J. Olszewski, M. S. Wu, J. Ventre, K. A. Stevens, and Y-S. Chao. 1992. Low density lipoprotein is protected from oxidation and the progression of atherosclerosis is slowed in cholesterol-fed rabbits by the antioxidant N,N′-diphenylphenylenediamine. *J. Clin. Invest.* **89:** 1885–1891.
7. Wu, Y., C. Hong, S. Lin, P. Wu and M. Shiao. 1998. Increase of vitamin E content in LDL and reduction of atherosclerosis in cholesterol-fed rabbits by a water-soluble antioxidant-rich fraction of *Salvia miltiorrhiza*. *Arterioscler. Thromb. Vasc. Biol.* **18:** 481–486.
8. Esterbauer, H., H. Puhl, M. Dieber-Rotheneder, G. Waeg and H. Rabl. 1991. Effect of antioxidants on oxidative modification of LDL. *Ann. Med.* **23:** 573–581.
9. Esterbauer, H., G. Jurgens, O. Quehenberger, and E. Koller. 1987. Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J. Lipid Res.* **28:** 495–509.
10. Yagi, K. 1976. A simple fluorometric assay for lipoperoxide in blood plasma. *Biochem. Med.* **15:** 212–216.
11. Parthasarathy, S., U. P. Steinbrecher, J. Barnett, J. L. Witztum, and D. Steinberg. 1985. Essential role of phospholipase A_2 activity in endothelial cell-induced modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **82:** 3000–3004.
12. Fong, L. G., S. Parthasarathy, J. L. Witztum, and D. Steinberg. 1987. Nonenzymatic oxidative cleavage of peptide bonds in apoprotein B-100. *J. Lipid Res.* **28:** 1466–1477.
13. Steinbrecher, U. P. 1987. Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. *J. Biol. Chem.* **262:** 3603–3608.
14. Henriksen, T., E. M. Mahoney, and D. Steinberg. 1983. Enhanced

macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis.* **3:** 149–159.
15. Steinbrecher, U. P., M. Lougheed, W. C. Kwan, and M. Dirks. 1989. Recognition of oxidized low density lipoprotein by the scavenger receptor of macrophages results from derivatization of apolipoprotein B by products of fatty acid peroxidation. *J. Biol. Chem.* **264:** 15216–15223.
16. Chait, A., and J. W. Heinecke. 1994. Lipoprotein modification: cellular mechanisms. *Curr. Opin. Lipidol.* **5:** 365–370.
17. Steinbrecher, U. P., S. Parthasarathy, D. S. Leake, J. L. Witztum, 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.
18. Lamb, D. J., and D. S. Leake. 1992. The effect of EDTA on the oxidation of low density lipoprotein. *Atherosclerosis.* **94:** 35–42.
19. Sparrow, C. P., and J. Olszewski. 1992. Cellular oxidative modification of low density lipoprotein does not require lipoxygenases. *Proc. Natl. Acad. Sci. USA.* **89:** 128–131.
20. Kugiyama, K., S. A. Kerns, J. D. Morrisett, R. Roberts, and P. D. Henry. 1990. Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature.* **344:** 160–162.
21. Jessup, W., J. A. Simpson, and R. T. Dean. 1993. Does superoxide radical have a role in macrophage-mediated oxidative modification of LDL? *Atherosclerosis.* **99:** 107–120.
22. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait, 1984. Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest.* **74:** 1890–1894.
23. Heinecke, J. W., H. Rosen, L. A. Suzuki, and A. Chait. 1987. The role of sulfur-containing amino acids in superoxide production and modification of low density lipoprotein by arterial smooth muscle cells. *J. Biol. Chem.* **262:** 10098–10103.
24. Sparrow, C. P. and J. Olszewski. 1993. Cellular oxidation of low density lipoprotein is caused by thiol production in media containing transition metal ions. *J. Lipid Res.* **34:** 1219–1228.
25. Cathcart, M. K., D. W. Morel, and G. M. Chisolm. 1985. Monocytes and neutrophilis oxidize low density lipoprotein making it cytotoxic. *J. Leukocyte Biol.* **38:** 341–350.
26. Cathcart, M. K., A. K. McNally, D. W. Morel, and G. M. Chisolm III. 1989. Superoxide anion participation in human monocyte-mediated oxidation of low density lipoprotein and conversion of low density lipoprotein to a cytotoxin. *J. Immunol.* **142:** 1963–1969.
27. Li, Q., and M. K. Cathcart. 1997. Selective inhibition of cytosolic phospholipase A_2 in activated human monocytes. Regulation of superoxide anion production and LDL oxidation. *J. Biol. Chem.* **272:** 2404–2411.
28. Li, Q., and M. K. Cathcart. 1994. Protein kinase C is required for lipid oxidation of LDL by activated human monocytes. *J. Biol. Chem.* **269:** 17508–17515.
29. Cathcart M. K., Q. Li, and G. M. Chisolm III. 1995. Lipoprotein receptor interactions are not required for monocytes oxidation of LDL. *J. Lipid Res.* **36:** 1857–1865.
30. Johnston, R. B., D. O. Adams, P. J. Edelson, and H. Koren. 1981. Secretion of superoxide anion. *In* Methods for Studying Mononuclear Phagocytes. Academic Press, New York. 489–498.
31. Buonassisi, V., and J. C. Venter. 1976. Hormone and neurotransmitter receptors in an established vascular endothelial cell line. *Proc. Natl. Acad. Sci. USA.* **73:** 1612–1616.
32. Sparrow, C. P., S. Parthasarathy, and D. Steinberg. 1989. A macrophage receptor that recognizes oxidized low density lipoprotein but not acetylated low density lipoprotein. *J. Biol. Chem.* **264:** 2599–2604.
33. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
34. Pittman, R. C., and C. A. Taylor, Jr. 1986. Methods for assessment of tissue sites of lipoprotein degradation. *Methods Enzymol.* **129:** 613–628.
35. Brumby, P. E., and V. Massey. 1967. Determination of nonheme iron, total iron, and copper. *Methods Enzymol.* **10:** 463–474.
36. Sparrow, C. P., S. Parthasarathy, and D. Steinberg. 1988. Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase A_2 mimics cell-mediated oxidative modification. *J. Lipid Res.* **29:** 745–753.
37. Bolann, B. J., and R. J. Ulvik. 1987. Release of iron from ferritin by xanthine oxidase. *Biochem. J.* **243:** 55–59.
38. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.
39. Marinetti, G. V. 1962. Chromatographic separation, identification, and analysis of phosphatides. *J. Lipid. Res.* **3:** 1–30.
40. Jessup, W., D. Mohr, S. P. Gieseg, R. T. Dean, and R. Stocker. 1992. The participation of nitric oxide in cell free- and its restriction of macrophage-mediated oxidation of low-density lipoprotein. *Biochim. Biophys. Acta.* **1180:** 73–82.
41. Darley-Usmar, V. M, N. Hogg, V. J. O'Leary, M. T. Wilson, and S. Moncada. 1992. The simultaneous generation of superoxide and nitric oxide can initiate lipid peroxidation in human low density lipoprotein. *Free Radical Res. Commun.* **17:** 9–20.
42. Nathan, C. 1992. Nitric oxide as a secretory product of mammalian cells. *FASEB J.* **6:** 3051–3064.
43. Gross, S. S., D. J. Stuehr, K. Aisaka, E. A. Jaffe, R. Levi and O. W. Griffith. 1990. Macrophage and endothelial cell nitric oxide synthesis: cell-type selective inhibition by N-aminoarginine, N-nitroarginine and N-methylarginine. *Biochem. Biophys. Res. Commun.* **170:** 96–103.
44. O'Connor, K. J., and S. Moncada. 1991. Glucocorticoids inhibit the induction of nitric oxide synthase and the related cell damage in adenocarcinoma cells. *Biochim. Biophys. Acta.* **1097:** 227–231.
45. Thomas, C. E., L. A. Morehouse, and S. D. Aust. 1985. Ferritin and superoxide-dependent lipid peroxidation. *J. Biol. Chem.* **260:** 3275–3280.
46. Hiramatsu, K., H. Rosen, J. W. Heinecke, G. Wolfbauer, and A. Chait. 1987. Superoxide initiates oxidation of LDL by human monocytes. *Arteriosclerosis.* **7:** 55–60.
47. Garner, B., D. van Reyk, R. T. Dean, and W. Jessup. 1997. Direct copper reduction by macrophages: its role in LDL oxidation. *J. Biol. Chem.* **272:** 6927–6935.
48. Bonnefont-Rousselot, D., M. Gardes-Albert, S. Lepage, J. Delattre, and C. Ferradini. 1992. Effect of pH on LDL oxidation by superoxide free radicals produced by gamma radiolysis. *Radiat. Res.* **132:** 228–236.
49. Lynch, S. M., and B. Frei. 1993. Mechanisms of copper- and iron-dependent oxidative modification of human low density lipoprotein. *J. Lipid. Res.* **34:** 1745–1753.
50. Steinbrecher, U. P. 1988. Role of superoxide in endothelial cell modification of LDL. *Biochim. Biophys. Acta.* **959:** 20–30.
51. Heinecke, J. W., L. Baker, H. Rosen, and A. Chait. 1986. Superoxide-mediated modification of LDL by arterial smooth muscle cells. *J. Clin. Invest.* **77:** 757–761.
52. Kiechl, S., F. Aichner, F. Gerstenbrand, G. Egger, A. Mair, G. Rungger, F. Spogler, E. Jarosch, F. Oberhollenzer, and J. Willeit. 1994. Body iron stores and presence of carotid atherosclerosis. *Arterioscler. Thromb.* **14:** 1625–1630.
53. Dabbagh, A. J., G. T. Shwaery, J. F. Keaney, and B. Frei. 1997. Effect of iron overload and iron deficiency on atherosclerosis in the hypercholesterolemic rabbit. *Arterioscler. Thromb. Vasc. Biol.* **17:** 2638–2645.
54. Smith C., M. J. Mitchinson, O. I. Aruoma, and B. Halliwell. 1992. Stimulation of lipid peroxidation and hydroxyl-radical generation by the contents of human atherosclerotic lesions. *Biochem. J.* **286:** 901–905.
55. Wieland, E., S. Parthasarathy, and D. Steinberg. 1993. Peroxidase-dependent metal-independent oxidation of low density lipoprotein in vitro: a model for in vivo oxidation? *Proc. Natl. Acad. Sci. USA.* **90:** 5929–5933.
56. Ezaki M., J. L. Witztum, and D. Steinberg. 1995. Lipoperoxides in LDL incubation with fibroblasts that overexpress 15-lipoxygenase. *J. Lipid Res.* **36:** 1996–2004.
57. Savenkova M. L., D. M. Mueller, and J. W. Heinecke. 1994. Tyrosyl radical generated by myeloperoxidase is a physiological catalyst for the initiation of lipid peroxidation in low density lipoprotein. *J. Biol. Chem.* **269:** 20394–20400.
58. Yla-Herttuala, S., M. E. Rosenfeld, S. Parthasarathy, C. K. Glass, E. Sigal, J. L. Witztum, and D. Steinberg. 1990. Colocalization 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.
59. Daugherty, A. J. L. Dunn, D. L. Rateri, and J. W. Heinecke. 1994. Myeloperoxidase, a catalyst for lipoprotein oxidation, is expressed in human atherosclerotic lesions. *J. Clin. Invest.* **94:** 437–444.