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### Research Article

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# Superoxide-mediated Modification of Low Density Lipoprotein by Arterial Smooth Muscle Cells

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## Abstract

Extracellular superoxide was detected in cultures of monkey and human arterial smooth muscle cells as indicated by superoxide dismutase inhibitable reduction of cytochrome c. Superoxide production by these cells in the presence of Fe or Cu resulted in modification of low density lipoprotein (LDL). The degree of LDL modification was directly proportional to the rate of superoxide production by cells. Superoxide dismutase (100 µg/ml), and the general free radical scavengers butylated hydroxytoluene and butylated hydroxyanisole (50 µM), inhibited Fe- and Cu-mediated modification of LDL by monkey smooth muscle cells, while catalase (100 µg/ml) and mannitol (25 mM) had no effect. The chelators desferrioxamine and diethylenetriamine pentaacetic acid completely inhibited Fe- and Cu-promoted modification of LDL, while EGTA had no inhibitory effect. EDTA stimulated Fe-promoted modification in the 1–100 µM range while inhibiting Cu-mediated modification of LDL. LDL modified by smooth muscle cells in the presence of 10 µM Fe or Cu stimulated [<sup>14</sup>C]oleate incorporation into cholesteryl ester by human macrophages and murine J774 cells to a degree comparable to that produced by acetylated LDL. LDL incubated with smooth muscle cells and metal ions in the presence of superoxide dismutase failed to enhance macrophage cholesteryl ester accumulation.

Thus, arterial smooth muscle cells in culture generate superoxide and modify LDL by a superoxide-dependent, Fe or Cu catalyzed free radical process, resulting in enhanced uptake of the modified LDL by macrophages. Neither hydroxyl radicals nor H<sub>2</sub>O<sub>2</sub> are likely to be involved. Superoxide-dependent lipid peroxidation may contribute to biological modification of LDL, resulting in foam cell formation and atherogenesis.

## Introduction

Low density lipoprotein (LDL) that has been chemically modified by acetylation (1) or malondialdehyde treatment (2) can be taken up in an unregulated manner via the scavenger receptor on macrophages, resulting in cellular accumulation of cholesteryl esters. Biological modification of LDL by endothelial or arterial smooth muscle cells also stimulates cholesteryl ester formation by macrophages in culture (3, 4). Previous studies have demonstrated that modification of LDL by endothelial cells and human arterial smooth muscle cells was promoted by micromolar concentrations of copper in the incubation medium (5, 6).

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Smooth muscle cell modification of LDL also was promoted by iron in the medium (6). Modification of LDL by arterial smooth muscle and endothelial cells in culture is accompanied by lipid peroxidation as indicated by increased thiobarbituric acid reacting substances (TBARS)<sup>1</sup> content of the modified LDL. LDL modification by both these cell types could be inhibited by chelators of metal ions and by the free radical scavenger butylated hydroxytoluene (5–7). Since transition metals are known to facilitate free radical reactions (8), these findings suggest that biological modification of low density lipoprotein occurs via a free radical-mediated process involving lipid peroxidation.

Superoxide, the one electron-reduced form of molecular oxygen, is a reactive oxygen species able to initiate free radical attack and lipid peroxidation. Transition metal complexes may facilitate free radical processes by converting superoxide to more reactive chemical species or by promoting lipid hydroperoxide breakdown to form further free radicals.

The present study was undertaken to define the mechanism by which arterial smooth muscle cells modify LDL and to determine whether these cells are able to generate superoxide. Unstimulated arterial smooth muscle cells in culture produced superoxide as indicated by superoxide dismutase (SOD) inhibitable reduction of cytochrome c. Cellular modification of LDL was inhibited by SOD. LDL modification also required the presence of the transition metals, iron or copper, in the incubation medium, and was inhibited by general free radical scavengers. These findings suggest that the mechanism by which arterial smooth muscle cells in culture modify LDL is a superoxide-dependent, metal catalyzed free radical process.

## Methods

Materials were purchased from the following sources: bovine erythrocyte SOD (superoxide:superoxide oxidoreductase, EC 1.15.1.1) from Miles Laboratories, Inc. (Elkhart, IN); horse heart type VI cytochrome c, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), EGTA, diethylenetriamine pentaacetic acid (DTPA), and sodium xanthine from Sigma Chemical Co. (St. Louis, MO); FeSO<sub>4</sub>, CuSO<sub>4</sub>, and mannitol from Mallinckrodt, Inc. (Paris, KY); bovine milk xanthine oxidase (activity, 1.04 U/mg) from Calbiochem-Behring Corp. (La Jolla, CA); EDTA from MC/B Manufacturing Chemists (Norwood, OH); FeCl<sub>3</sub> from J. T. Baker Chemical Co. (Phillipsburg, NJ); Dulbecco's modified Eagle's medium (DME), minimal essential medium (MEM), and RPMI 1640 from Gibco (Grand Island, NY); desferrioxamine mesylate from Ciba Pharmaceutical (Summit, NJ); 35-mm tissue culture dishes from Corning Glassworks (Corning, NY); 16-mm tissue culture dishes from Falcon Labware, Becton-Dickinson & Co. (Oxnard, CA); catalase (type CTR) from Worthington Diagnostics Div., Millipore Corp. (Freehold, NJ). All chemicals were reagent grade.

1. *Abbreviations used in this paper:* BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; DME, Dulbecco's modified Eagle's medium; DTPA, diethylenetriamine pentaacetic acid; MEM, minimal essential medium; SOD, superoxide dismutase; TBARS, thiobarbituric acid reacting substances.

**Cell culture.** Arterial smooth muscle cells were derived from intimal-medial explants of monkey (*Macaca nemestrina*) or human aorta (9). The cells had typical smooth muscle cell morphology by light microscopy, were positive by immunocytochemistry for the smooth muscle cell marker antigens recognized by antibodies HHF and CGA7 (10), did not react with antibody T-200 (a pan-leukocyte marker), and lacked the endothelial marker, Factor VIII. Subcultures were used between passages 3 to 10. Cells were plated at  $5 \times 10^4$  cells/ $35 \times 10$  mm dish, fed three times weekly with DME supplemented with 10% calf serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and were used for experiments within 1 d of reaching confluency.

Human monocytes were isolated by a combination of density gradient and counterflow centrifugation as previously described (11). They were plated at  $4 \times 10^5$  cells/ $16 \times 10$  mm dish and were fed twice weekly with RPMI 1640 medium supplemented with 20% autologous serum. Monocyte-derived macrophages were used within 7–14 d of plating. J774 cells, an established line of murine macrophage-like cells, were purchased from the American Type Culture Collection (Rockville, MD). They were plated at  $2.5 \times 10^4$  cells/ $16 \times 10$  mm dish in DME supplemented with 10% fetal calf serum and used within 7 d. For measurement of superoxide production, LDL modification, and [ $^{14}$ C]oleate incorporation into cholesteryl ester, cells were incubated at 37°C in humidified air in MEM without phenol red, buffered at pH 7.4 with 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes).

**Lipoproteins.** Human LDL (density, 1.019–1.063 g/ml) was prepared by ultracentrifugation as previously described (6). The isolated LDL was dialyzed against 150 mM NaCl and 1 mM EDTA at pH 7.4, stored at 4°C under N<sub>2</sub>, and used within 2 wk. The final concentration of EDTA in experiments employing LDL was 16–32  $\mu$ M. For inhibitor studies involving chelating agents, LDL was freshly dialyzed overnight at 4°C against N<sub>2</sub>-saturated 150 mM NaCl buffered at pH 7.4 with 4 mM Hepes. LDL was acetylated by the method of Basu et al. (12).

Arterial smooth muscle cell-modified LDL was prepared as follows. Cultured monkey or human arterial smooth muscle cells were washed once with serum-free MEM and incubated with 1 ml of MEM containing 300  $\mu$ g LDL protein. At the end of 24 h of incubation, the medium was collected, centrifuged at 1,000 g for 10 min, stored at 4°C under nitrogen, and assayed within 1 d. Cell-free controls were incubated under identical conditions. FeSO<sub>4</sub> and CuSO<sub>4</sub> in distilled H<sub>2</sub>O were added as freshly prepared solutions. In experiments comparing Fe(II) and Fe(III), stock solutions of FeCl<sub>3</sub> were prepared in equimolar EDTA. BHT and BHA were added in 95% ethanol and compared with ethanol-treated controls. Catalase was dialyzed against distilled H<sub>2</sub>O before use. The order of medium additions was metal ion, LDL, and inhibitor.

**Measurement of superoxide production.** Superoxide production was measured as the SOD inhibitable reduction of cytochrome c (13) or acetylated cytochrome c (14). Arterial smooth muscle cells were preincubated in MEM for 15 min at 37°C, washed once with MEM containing cytochrome c, and incubated with 1 ml cytochrome c (1 mg/ml) with or without SOD (25  $\mu$ g/ml) in MEM in humidified air on a shaking table. At the indicated time the medium was removed from the cells, placed on ice, and the absorbance was immediately read at 550 nm against a distilled H<sub>2</sub>O blank. Dishes used for cell-free control experiments were incubated overnight with DME plus 10% calf serum and assayed in parallel. Superoxide specific reduction of cytochrome c was expressed as the difference in absorbance between cells incubated with or without SOD using an extinction coefficient of 21 mM<sup>-1</sup> cm<sup>-1</sup> (15). Xanthine oxidase (1.5 mU/ml) model system production of superoxide was measured under identical conditions, using Na xanthine (100  $\mu$ M) as substrate (16).

**Thiobarbituric acid reacting substances (TBARS).** The TBARS assay of Buege and Aust (17) was used in modified form. 60  $\mu$ g of LDL protein was present in a final volume of 1.5 ml. To ensure that metal ion content of the assay did not vary significantly, CuSO<sub>4</sub> and FeSO<sub>4</sub> were added to final concentrations of 87  $\mu$ M before heating. TBARS were expressed as malondialdehyde equivalent content (nanomoles MDA per milligram LDL protein) using an extinction coefficient determined from a MDA

tetramethyl acetal-generated standard curve (18). Protein was measured by the method of Lowry (19) using bovine serum albumin as the standard.

**Other assays.** The uptake of control and smooth muscle cell-modified LDL by human monocyte-derived macrophages or J774 cells was measured by incorporation of [ $^{14}$ C]oleate acid into cholesteryl ester (20). The final concentration of lipoprotein to which macrophages were exposed was 100  $\mu$ g protein/ml.

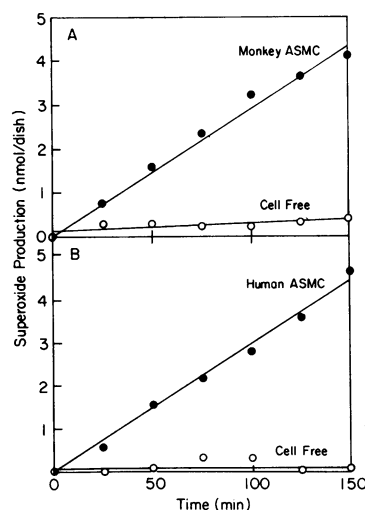
LDL electrophoresis was carried out at pH 8.6 in barbital buffer on agarose gel. Electrophoretic mobility was expressed as relative mobility (the ratio of distance migrated of modified to control LDL).

The Fe and Cu concentrations of MEM were determined by inductively coupled atomic emission spectroscopy using the method of standard additions (21).

## Results

Extracellular superoxide was detected in cultures of both monkey and human arterial smooth muscle cells as indicated by SOD inhibitable reduction of cytochrome c (Fig. 1). Superoxide generation was linear over the entire assay period. For monkey cells, acetylated cytochrome c detected 91±3% ( $n = 3$ ) of the superoxide detectable by native cytochrome c. A similar fraction (79±10%) of superoxide was detected by acetylated cytochrome c when a xanthine oxidase model system was used as the source of superoxide. Freeze thaw lysates of smooth muscle cells and medium preincubated with viable cells showed no greater superoxide production than did cell-free controls. The rate of superoxide production was not affected by the addition of Fe or Cu to the medium. Detection of superoxide in the presence of LDL was 81±8% ( $n = 3$ ) of LDL-free controls, suggesting either a modest inhibition of superoxide production or a competitive reaction of LDL with superoxide.

Incubation of LDL with arterial smooth muscle cells in MEM alone (Fe  $\leq 0.5$   $\mu$ M, Cu  $\leq 0.5$   $\mu$ M) produced no LDL modification. As demonstrated with human arterial smooth muscle cells (6), incubation of LDL with monkey arterial smooth muscle cells and 10  $\mu$ M Fe or Cu resulted in modification of LDL as indicated by increases in the TBARS content and electrophoretic



**Figure 1.** Superoxide production by monkey (A) and human (B) arterial smooth muscle cells (ASMC). Cells were preincubated for 15 min in MEM, washed once, and incubated in MEM with cytochrome c (1 mg/ml). Cell-free dishes were preincubated overnight in medium with 10% calf serum and were treated identically to cell-containing dishes. Superoxide-specific reduction of cytochrome c was determined as the difference in absorbance at 550 nm between dishes incubated with or without superoxide dismutase (SOD, 25  $\mu$ g/ml).  $\epsilon = 21$  cm<sup>-1</sup> mM<sup>-1</sup> was used. Average protein per dish was 102  $\mu$ g for the monkey cells and 140  $\mu$ g for the human cells. Each data point represents the mean of duplicate determinations. ●, smooth muscle cells; ○, cell-free control.

**Table I. Effect of Superoxide Dismutase (SOD) on LDL Modification**

Medium addition	TBARS*		Electrophoretic mobility‡	
	(-) SOD	(+) SOD	(-) SOD	(+) SOD
<b>Smooth muscle cell modified</b>				
None	2.4±0.2	-1.7±0.1	1.0	1.1
Cu (10 μM)	58±1	1.5±0.2	4.3	1.1
Fe (10 μM)	49±1	17±1	4.2	1.7
<b>Cell-free dishes</b>				
None	0.9±0.3	0.5±0.1	1.1	1.2
Cu (10 μM)	13±1	0.3±0.1	1.4	1.0
Fe (10 μM)	10±1	5.6±0.5	1.8	1.1

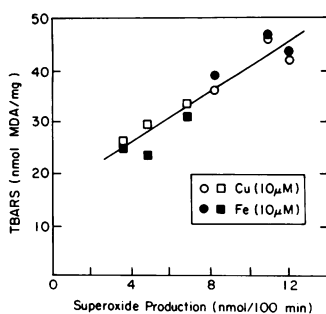
Cells were washed once with MEM and incubated with 1 ml MEM (300 μg LDL protein/ml) alone or supplemented with 10 μM Cu or Fe for 24 h. SOD was added at 100 μg/ml. TBARS and electrophoretic mobility were determined as described in Methods. Results are the mean±SD of triplicates (TBARS) or mean of duplicates (electrophoretic mobility) of a single experiment.

\* nmol MDA/mg.

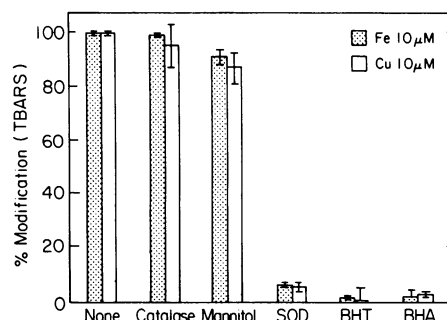
‡ ratio of migration of modified to control LDL.

mobility (Table I). Fe(III) added as FeCl<sub>3</sub> in EDTA promoted LDL modification by cells as effectively as Fe(II) (FeSO<sub>4</sub> freshly prepared in H<sub>2</sub>O), with almost identical TBARS formation (108±11% of FeSO<sub>4</sub> control, *n* = 3). Addition of SOD (100 μg/ml) inhibited modification of LDL in the presence of either 10 μM Fe or Cu. Similar findings were observed with human smooth muscle cells (data not shown). Extensively dialyzed SOD retained full inhibitory activity, while heat inactivated SOD had <4% of the inhibitory effect of native enzyme. In a cell free system, Fe and Cu (10 μM) promoted LDL modification to a much smaller degree than was observed in the presence of cells. LDL modification as measured by the TBARS assay was directly proportional to superoxide production (Fig. 2).

To further define the mechanism of modification of LDL by arterial smooth muscle cells in culture, inhibitor studies were carried out (Fig. 3). The general free radical scavengers BHT and BHA (50 μM) completely inhibited modification of LDL by monkey smooth muscle cells at Fe and Cu 10 μM with no increase in TBARS reactivity. Catalase (100 μg/ml) and mannitol (25 mM) were without effect. The chelating agents desferriox-



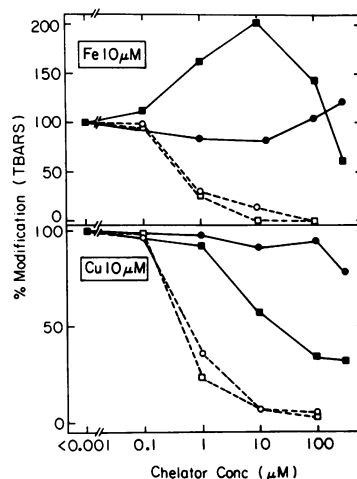
**Figure 2. Relationship between superoxide production and LDL modification by monkey smooth muscle cells.** Superoxide production was measured at the beginning of each experiment. Conditions were as in Fig. 1 and Table I. Each data point represents the results of a single experiment. Two strains of monkey smooth muscle cells (M405 and M406) were used. M405, □; M406, ○; Fe, 10 μM, ●; Cu, 10 μM, ■.



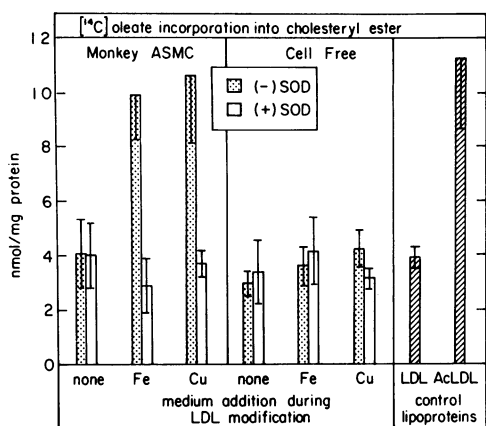
**Figure 3. Effect of inhibitors of reactive oxygen species and general free radical scavengers on LDL modification by monkey arterial smooth muscle cells.** Conditions were as indicated in Table I. The concentration of inhibitors added was catalase, 100 μg/ml; mannitol, 25 mM; SOD, 100 μg/ml; BHT, 50 μM; and BHA, 50 μM. Data are the mean±SD of two experiments with triplicate determinations per experiment.

amine and DTPA inhibited Fe- or Cu-dependent modification of LDL by arterial smooth muscle cells by >50% when present at ≥1 μM concentration (Fig. 4). In contrast, EGTA showed no inhibitory effect at concentrations up to 300 μM. EDTA at ≥100 μM inhibited Cu-promoted modification of LDL, while actually increasing Fe-promoted modification in the 1–100 μM concentration range. A close correlation between TBARS and electrophoretic mobility was observed in all inhibitor studies.

LDL modified by monkey smooth muscle cells in the presence of 10 μM Fe or Cu stimulated cholesteryl ester formation by human monocyte-derived macrophages to an extent similar to acetyl LDL (Fig. 5). Addition of SOD during incubation of LDL with the smooth muscle cells reduced cholesteryl ester formation by human macrophages to the level found with native LDL. Similar results were observed using J774 cells, a murine macrophage-like cell line. Addition of SOD to human smooth muscle cells also inhibited the increased cholesteryl ester formation stimulated by modified LDL in human macrophages (data not shown). LDL modified with 10 μM Fe or Cu in a cell-free system did not stimulate cholesteryl ester formation by macrophages. Addition of SOD to the medium after LDL mod-



**Figure 4. Effect of chelating agents on LDL modification by monkey smooth muscle cells.** Before use, LDL was extensively dialyzed at 4°C against N<sub>2</sub>-saturated 150 mM NaCl. Chelators were added at the concentration indicated. Control LDL is represented by the <0.001 μM EDTA point. Other conditions were as indicated in Table I. Data are the mean of five experiments with triplicate determinations per experiment. EDTA, ■; EGTA, ●; desferrioxamine, □; DTPA, ○.



**Figure 5.** Uptake of modified LDL by human monocyte-derived macrophages. Macrophages were washed twice with MEM and incubated with 100  $\mu\text{g}/\text{ml}$  lipoprotein and [ $^{14}\text{C}$ ]oleate for 24 h. Data are the mean  $\pm$  SD of triplicate values of a single experiment.

ification by arterial smooth muscle cells did not inhibit cholesteryl ester formation by human macrophages.

## Discussion

Arterial smooth muscle cells in culture produced extracellular superoxide as indicated by the inhibition of ferricytochrome c reduction by low concentrations of superoxide dismutase. Unstimulated monkey arterial smooth muscle cells produced superoxide at rates of 0.3–3.0 nmol/min per mg protein (1–13% of the rate for phorbol myristate acetate-stimulated human monocytes [22]). Until recently, detection of significant amounts of extracellular superoxide anion has been a phenomenon associated with the metabolism of activated phagocytes. Stimulated phagocytic cells undergo a burst of superoxide production as a consequence of the activation of a nonmitochondrial (cyanide-insensitive) electron transport system with NADPH as electron donor and  $\text{O}_2$  as the acceptor. The source and regulation of superoxide production by arterial smooth muscle cells is currently unknown. Unlike the burst of superoxide produced by stimulated neutrophils and monocytes, superoxide production by arterial smooth muscle cells was continuous, with considerable cumulative production of superoxide.

As previously shown for human cells (6), LDL modified by monkey arterial smooth muscle cells exhibited increased lipid peroxide content and increased electrophoretic mobility. Micromolar concentrations of Fe and Cu were required for arterial smooth muscle cell-mediated LDL modification, suggesting that transition metal-mediated oxidation was involved in the lipoprotein modification. Fe promoted LDL modification effectively regardless of the oxidation state of the added Fe. However, superoxide may have reduced Fe(III) to Fe(II). SOD completely inhibited Cu promoted modification and largely inhibited Fe promoted modification of LDL as indicated by changes in TBARS and electrophoresis. The degree of LDL modification was directly proportional to the rate of superoxide production by the cells. Thus, the superoxide generated by the arterial smooth muscle cells contributed to the oxidation of LDL lipids and played a central role in the modification of LDL.

LDL incubated with monkey arterial smooth muscle cells in the presence of Cu or Fe 10  $\mu\text{M}$  stimulated cholesteryl ester

formation by human macrophages and the murine macrophage-like J774 cell line to the same extent as acetyl LDL. However, LDL that was incubated with monkey smooth muscle cells at 10  $\mu\text{M}$  Cu or Fe in the presence of SOD did not stimulate cholesteryl ester formation to any greater extent than native LDL by both macrophage cell types. Thus, superoxide generation by arterial smooth muscle cells seems to be important in mediating both LDL modification and the facilitated uptake of the modified LDL by macrophages. LDL modified by the cell-free system in the presence of Fe or Cu failed to stimulate cholesterol formation by macrophages, despite the presence of low levels of lipid peroxides and change in electrophoretic mobility. It is conceivable that the LDL that was modified by this cell-free system failed to reach the threshold of modification required for uptake to occur via scavenger receptors on macrophages (23).

To further elucidate the nature of the reactive oxygen species involved in LDL modification by arterial smooth muscle cells, inhibitor studies were performed. LDL modification by monkey smooth muscle cells was completely inhibited by the general free radical scavengers BHA and BHT. The hydroxyl radical scavenger mannitol was without effect, which suggests the hydroxyl radical was not a major reactive species. However, since mannitol would only scavenge aqueous hydroxyl radicals, a lipid phase hydroxyl radical-mediated process cannot be excluded. The lack of inhibition by catalase suggests an  $\text{H}_2\text{O}_2$ -independent mechanism. Thus, a Haber-Weiss type mechanism involving Fe-catalyzed hydroxyl radical formation seems unlikely.

The chelator effects probably relate to the completeness with which the chelators sequester the metal ion from water. Fe-catalyzed free radical formation requires that the iron be chelated with an open site in its coordination sphere, accessible to water (24). EDTA and EGTA both chelate Fe with an open site in their coordination sphere, while DTPA and desferrioxamine completely sequester Fe from water (25). The complete inhibition of LDL modification by DTPA and desferrioxamine but not by EDTA or EGTA, suggests that inhibition of LDL modification by chelation requires complete sequestration of the metal ion from aqueous solution.

These findings suggest that smooth muscle cells in culture can generate superoxide and modify LDL by a superoxide-initiated Cu- or Fe-catalyzed free radical process. Lipid peroxidation occurs, probably via free radical attack on the unsaturated fatty acid component of the lipoprotein. Free radical oxidation of LDL, initiated by a variety of one-electron transfer reactions, may represent a general mechanism for biological modification of lipoprotein. Such oxidative events could occur in the arterial wall, where smooth muscle cells are abundant and surrounded by small amounts of interstitial fluid. The concentrations of extracellular oxidants there may be sufficient to convert LDL to a form that promotes accumulation of cholesteryl esters by macrophages with subsequent foam cell formation.

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