Mechanisms of copper- and iron-dependent oxidative modification of human low density lipoprotein

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Abstract Oxidative modification of low density lipoprotein (LDL) has been suggested as a causal step in atherosclerosis, and both redox-active transition metal ions and superoxide ($O_2^{\cdot -}$) have been implicated in this process. In order to determine the mechanisms of metal ion-dependent oxidation of LDL in the presence of $O_2^{\cdot -}$, LDL was exposed to hypoxanthine (HX) and purified xanthine oxidase (XO) without and with added $CuCl_2$ or Fe^{3+}-citrate. Production of $O_2^{\cdot -}$ and hydrogen peroxide (H_2O_2) at pH 7.4 by the HX/XO system in the absence of metal ions was not sufficient to oxidize LDL. Preincubation of LDL with Cu^{2+} or Fe^{3+}-citrate with subsequent removal of metal ions not tightly bound to the lipoprotein did not enable the HX/XO system to oxidize LDL. However, incubation of LDL with HX/XO and Cu^{2+} resulted in extensive modification of LDL. Exposure of LDL to Cu^{2+} alone also led to extensive modification, although the LDL was initially free of detectable amounts of lipid hydroperoxides (LOOH), i.e., <0.005 molecules of LOOH per LDL particle. Although HX/XO and Cu^{2+} did not produce detectable amounts of $O_2^{\cdot -}$ or aqueous hydroxyl radicals ($HO^{\cdot}$), oxidation of LDL under these conditions was partially inhibited by superoxide dismutase, and completely inhibited by the $HO^{\cdot}$ scavenger thiourea. In contrast to Cu^{2+}-mediated oxidation of LDL, oxidation mediated by Fe^{3+}-citrate was strictly dependent upon $O_2^{\cdot -}$, as it was abolished by omission of the HX/XO system or by addition of superoxide dismutase to this system. HX/XO and Fe^{3+}-citrate generated substantial amounts of $HO^{\cdot}$, which were effectively scavenged by mannitol, yet mannitol did not inhibit LDL oxidation under these conditions. Our results show that oxidative modification of LDL by Cu^{2+} can occur in the absence of detectable amounts of preformed LOOH in LDL and is also independent of $O_2^{\cdot -}$, H_2O_2, and aqueous $HO^{\cdot}$ production. The same holds true for LDL oxidation mediated by Fe^{3+}-citrate, except for $O_2^{\cdot -}$, which may be required merely to reduce Fe^{3+}, not because it can drive production of $HO^{\cdot}$ by the Fenton reaction.—**Lynch, S. M., and B. Frei.** Mechanisms of copper- and iron-dependent oxidative modification of human low density lipoprotein. *J. Lipid Res.* 1993. **34:** 1745–1753.

Supplementary key words superoxide • hydroxyl radical • oxygen free radicals • Fenton reaction • oxidized LDL • atherosclerosis

Oxidative modification of low density lipoprotein (LDL) has been suggested to play a causal role in human atherosclerosis (1, 2). All three of the major cell types present in early atherosclerotic lesions (endothelial cells, smooth muscle cells, and monocyte-macrophages) have been shown to oxidize LDL in vitro (3–6). Evidence that LDL oxidation occurs in vivo includes immunochemical demonstration of oxidized LDL (LDL_{ox}) in atherosclerotic lesions (7, 8), the extraction of LDL_{ox} from atherosclerotic lesions (7, 9), and the presence in human plasma of circulating autoantibodies against epitopes of LDL_{ox} (7, 10). In addition to facilitating lipid uptake by monocyte-macrophages, LDL_{ox} has also been shown, inter alia, to stimulate leukocyte adherence to the microvascular endothelium (11), induce monocyte chemotactic protein-1 in endothelial and smooth muscle cells (12), and inhibit endothelium-dependent vasodilation (13, 14).

Despite these extensive data suggesting an important role for oxidative modification of LDL in atherosclerosis, the mechanism of LDL oxidation is presently unclear. The observation that LDL may be oxidatively modified by incubation with soybean lipoxygenase and phospholipase A_2 (15) and data from experiments in which lipoxygenase inhibitors could prevent LDL oxidation by endothelial cells or macrophages (16, 17) suggested that cellular lipoxygenases are involved in oxidative modification of LDL. However, it has been shown that 5- and 15-lipoxygenase activities are not essential for LDL oxidation

Abbreviations: CAT, catalase (EC 1.11.1.6.); DTPA, diethylenetriaminepenta-acetic acid; EDTA, ethylenediaminetetra-acetic acid; GSH, reduced glutathione; HX, hypoxanthine; LDL, low density lipoprotein; LDL_{ox}, oxidized low density lipoprotein; LOOH, lipid hydroperoxide(s); PBS, 10 mM phosphate-buffered saline, pH 7.4; REM, relative electrophoretic mobility; SOD, copper, zinc-containing superoxide dismutase (EC 1.15.1.1.); TBARS, thiobarbituric acid-reactive substances; XO, xanthine oxidase (EC 1.2.3.2.); HPLC, high performance liquid chromatography.

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(18–20). Other experiments suggest that release of superoxide ($O_2^{\cdot -}$) from smooth muscle cells (21, 22) and monocytes (23), and possibly macrophages (24, but see also 17, 25) and endothelial cells (26, but see also 16), or its generation by autooxidation of extracellular thiol compounds (27) are important in LDL oxidation. However, $O_2^{\cdot -}$ at physiological pH induces little lipid peroxidation in LDL (28), consistent with its low reactivity towards unsaturated lipids (29). Only when catalytic quantities of copper or iron are present can $O_2^{\cdot -}$ stimulate LDL oxidation (22, 28). It may be that initiation of lipid peroxidation in LDL by $O_2^{\cdot -}$ and metal ions results from generation of highly reactive hydroxyl radicals ($HO^{\cdot}$) from hydrogen peroxide (H_2O_2) by the $O_2^{\cdot -}$-driven Fenton reaction (Reaction [2]) (30):

$$O_2^{\cdot -} + Me^{(n+1)+} \rightarrow O_2 + Me^{n+} \quad [1]$$

$$H_2O_2 + H^+ + Me^{n+} \rightarrow HO^{\cdot} + H_2O + Me^{(n+1)+} \quad [2]$$

($Me^{(n+1)}$, oxidized metal ion; Me^{n+}, reduced metal ion). However, it has been reported that mannitol, a $HO^{\cdot}$ scavenger, cannot inhibit oxidation of LDL by monocytes, macrophages, smooth muscle cells, or endothelial cells, suggesting that aqueous $HO^{\cdot}$ are not involved (22–24, 26). As copper can bind to LDL (31–33) it may be that $HO^{\cdot}$ are not formed in the aqueous phase but on the LDL particle itself causing damage by a site-specific mechanism (34). Alternatively, metal ions may initiate lipid peroxidation in LDL by a $HO^{\cdot}$-independent mechanism, e.g., by decomposition of preformed lipid hydroperoxides (LOOH) to peroxyl and alkoxyl radicals which subsequently initiate new rounds of radical chain oxidation (35–37).

Thus, although their mechanism of action is not completely understood, redox-active transition metal ions (copper or iron) have been demonstrated to be required for oxidative modification of LDL in vitro (4, 22–26, 28, 35). Interestingly, both copper and iron levels are significantly increased in atherosclerotic, compared with healthy, arterial walls (38), and a recent study (39) has shown that mature atherosclerotic lesions contain detectable iron and copper in a form that promotes lipid peroxidation. Furthermore, epidemiologic studies have found a positive association between serum copper or ferritin levels (the latter a good indicator of body iron stores) and risk for cardiovascular disease (40–42). Therefore, copper and/or iron may play a pivotal role in formation of LDL_{ox} not only in vitro but also in vivo. The experiments reported here were aimed at investigating the mechanisms of metal ion-dependent oxidation of LDL in a cell-free in vitro system utilizing the enzymatic hypoxanthine (HX)/xanthine oxidase (EC 1.2.3.1., XO) system to mimic the previously documented release of $O_2^{\cdot -}$ by arterial wall cells (21–24, 26).

MATERIALS AND METHODS

Materials

Chelex-100 resin was purchased from Bio-Rad (Richmond, CA); copper,zinc-containing superoxide dismutase (EC 1.15.1.1., SOD), catalase (EC 1.11.1.6., CAT), and EDTA-free XO were from Boehringer Mannheim Biochemicals (Indianapolis, IN); and 15-hydroperoxy-eicosatetraen-1-oic acid was from Cayman Chemical Company (Ann Arbor, MI). Ebselen (PZ51) was a gift from Dr. Craig E. Thomas (Marion Merrell Dow Research Institute, Cincinnati, OH). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). Lipo gels for agarose gel electrophoresis were purchased from Beckman (Brea, CA); Acrodisc filters were from Gelman Sciences (Ann Arbor, MI); aprotinin-agarose affinity chromatography columns were from Sigma Chemical Company (St. Louis, MO); and Sephadex G-25M PD-10 columns were from Pharmacia (Uppsala, Sweden).

Purification of XO

Commercial XO preparations may be contaminated with adventitious iron (43), proteinases (44, 45), and phospholipase A_2 activity (46). As each of these contaminants may interfere with the experiments reported here, it was necessary to develop a procedure for their removal. XO was purchased from Boehringer Mannheim Biochemicals (Catalog No. 110 434) as this preparation has been reported to be free of detectable phospholipase A_2 activity (46). To remove adventitious iron and proteinase activity, XO (5 Units/ml) was incubated with 1 mM deferoxamine mesylate at room temperature for 30–60 min with occasional gentle mixing and an aliquot (3.0 ml) was loaded onto a prepacked aprotinin-agarose affinity chromatography column (Sigma Kit No. AP-10) equilibrated with 10 mM phosphate-buffered saline, pH 7.4 (PBS). The sample was allowed to run into the column and the resulting eluate was collected. This eluate (3.0 ml) was loaded onto a second aprotinin column and the eluate was collected as before. This two-step affinity chromatography procedure removed all proteinase activity from the XO preparation as assessed by a hemoglobin degradation assay (47). Proteinase-free XO (2.5 ml) was passed sequentially through three Pharmacia Sephadex G-25M PD-10 columns, equilibrated with PBS, according to the manufacturer's instructions. These gel filtration steps removed low molecular weight contaminants including adventitious iron complexed with deferoxamine and excess deferoxamine. The eluate from the third PD-10 column was used in incubations requiring XO activity. XO activity was determined spectrophotometrically by measuring the increase in absorbance at 295 nm associated with production of uric acid (molar absorption coefficient = 11,000 $M^{-1}cm^{-1}$) from HX (48).

Preparation of unoxidized LDL

LDL was isolated from the plasma of a healthy, normolipidemic volunteer by single vertical spin discontinuous density ultracentrifugation (49). The density of fresh plasma, anticoagulated with sodium heparin, was increased to 1.21 g/ml by addition of solid KBr (0.3265 g/ml plasma). A discontinuous NaCl/KBr gradient was established in Beckman Quick-Seal centrifuge tubes (5.0 ml capacity) by layering density-adjusted plasma (1.5 ml) under 0.154 M NaCl in Chelex-100-treated water (3.5 ml). The samples were centrifuged at 80,000 rpm and 7°C for 45 min in a Beckman Near Vertical Tube 90 rotor (443,000 g_{av}; Beckman L8-80M ultracentrifuge). Slow acceleration and deceleration modes (modes 3) were used to avoid disturbing the gradient. After centrifugation LDL was observed as an orange band in the upper middle portion of the tubes and was removed by puncturing the tube with a needle and withdrawing the LDL into a 1-ml syringe. Contaminating metal ions were removed from isolated LDL by addition of a small quantity of Chelex-100 resin with gentle mixing. The resin was removed by centrifugation (500 g_{av} for 1 min at 7°C) and the supernatant containing LDL was filtered (Gelman 0.2 μm Acrodisc filter). An aliquot (2.0 ml) of LDL was incubated with 20 μM Ebselen (PZ51, added in 12.5 μl ethanol) and 3 mM reduced glutathione (GSH) at 37°C for 15 min (final volume, 2.5 ml) to remove preformed LOOH (37, 50). Ebselen, GSH, and residual KBr were removed from the LDL preparation by sequential passage through three Sephadex G-25M PD-10 columns equilibrated with PBS. The eluate from the third PD-10 column containing LDL was used immediately in experiments. GSH concentrations in LDL preparations were measured (51) and found to be about 5 μM (S. M. Lynch and B. Frei, unpublished data). LDL protein was determined by a modification (52) of the method of Lowry et al. (53).

Measurement of lipid hydroperoxides

Cholesteryl ester hydroperoxides and phospholipid hydroperoxides in LDL were quantitated using an HPLC method with chemiluminescence detection (54, 55) and 15-hydroperoxy-eicosatetraen-1-oic acid as a standard. Alternatively, diene conjugation was measured as absorbance at 234 nm (56), or thiobarbituric acid-reactive substances (TBARS) by a modification of a standard assay (57, 58). For TBARS determination, samples (0.5 ml) were incubated with 1.0% (w/v) 2-thiobarbituric acid in 50 mM NaOH (0.25 ml) and 2.8% (w/v) trichloroacetic acid (0.25 ml) in a boiling water bath for 10 min. After cooling to room temperature the pink chromogen was extracted into *n*-butanol (2.0 ml) and its absorbance at 535 nm was measured. TBARS concentrations were calculated from a calibration prepared using 1,1,3,3-tetramethoxypropane (0–8 μM) as a standard.

Experiments

Only fresh, not stored, LDL prepared as described above was used in experiments. Incubation mixtures in PBS contained, in a final volume of 215 μl, the following reagents at the concentrations indicated: LDL (protein, 0.2 mg/ml), HX (1.5 mM), and purified XO (0.02 Units/ml, 1 Unit defined as converting 0.2 mM HX to uric acid per min) without (control) or with either $CuCl_2$ (10.0 μM) or Fe^{3+}-citrate (10.0 μM, 1:1 complex), the latter used as a physiological iron-chelate. Incubations also contained diethylenetriaminepenta-acetic acid (DTPA; 2.5 μM) to eliminate possible artefactual oxidation of LDL by trace amounts of contaminating metal ions. Incubations were terminated after 24 h by addition of excess DTPA (1.0 mM) and transfer of the samples to an ice bath.

To investigate the requirement for metal ions in the oxidation of LDL by the HX/XO system, excess DTPA (25 μM) or EDTA (100 μM) was added to incubations. SOD (1000 Units/ml) and CAT (1000 Units/ml) were used to determine the importance of $O_2^{\cdot -}$ and H_2O_2, respectively; the $HO^{\cdot}$ scavengers urea, mannitol, and thiourea (50 mM each) were added to determine whether aqueous $HO^{\cdot}$ are involved in LDL oxidation. To investigate the significance of metal ion binding to LDL as a factor in its oxidation, LDL (protein, 0.6 mg/ml) was preincubated at 37°C for 15 min without (control) or with either $CuCl_2$ or Fe^{3+}-citrate (10 μM or 100 μM each). Unbound metal ions were removed by passing LDL preparations through three Sephadex G-25M PD-10 columns equilibrated with PBS. LDL eluted from the third column was incubated (protein, 0.2 mg/ml) at 37°C for 24 h in the standard HX/XO system without (control) or with either $CuCl_2$ or Fe^{3+}-citrate (10 μM).

Increased anodic electrophoretic mobility of LDL on agarose gels was used to assess oxidative modification. LDL was electrophoresed at 100 V for 30 min in 0.5% agarose gels (Beckman Lipo gels) with 0.05 M barbital buffer, pH 8.6, in a Beckman Paragon electrophoresis system. Gels were fixed, stained, and destained according to the manufacturer's instructions.

Generation of $O_2^{\cdot -}$ and $HO^{\cdot}$ by the HX/XO system was determined as SOD-inhibitable reduction of ferricytochrome *c* (59) and formation of TBARS from deoxyribose (57, 58), respectively.

RESULTS

All LDL preparations used in this investigation were isolated by a rapid method (49) and treated with the synthetic peroxidase Ebselen in the presence of GSH (37) immediately before use in experiments. These procedures were used to eliminate LOOH (50) that might have been

formed during LDL isolation, and which would have had a confounding effect on the experimental results. Aliquots of LDL prepared in this way were routinely analyzed for cholesteryl ester hydroperoxides using HPLC with chemiluminescence detection (55) and found to contain no detectable amounts of LOOH (i.e. <10 pmol/mg protein; **Fig. 1A**). Assuming a molecular weight for apolipoprotein B of 500,000 (36), this detection limit corresponds to <0.005 molecules of LOOH per LDL particle.

Characterization of the HX/XO system

Results from analyses of $O_2^{\cdot -}$ and $HO^{\cdot}$ generation by the in vitro systems used in this study are presented in **Table 1** and **Fig. 2,** respectively. Reduction of ferricytochrome *c* by the HX/XO system was shown to result mainly from $O_2^{\cdot -}$ generation as addition of SOD resulted in a 94% inhibition of the reaction (Table 1). While Fe^{3+}-citrate had little effect on $O_2^{\cdot -}$ production by the HX/XO system, no $O_2^{\cdot -}$ could be detected in the presence of Cu^{2+} (Table 1). Uric acid production from HX (1.5 mM) by XO (0.02 Units/ml) was also measured, and occurred at an initial rate of 3.36 μM/min and 3.18 μM/min in the absence and presence of Fe^{3+}-citrate (10 μM), respectively. No uric acid formation could be detected in the HX/XO/Cu^{2+} system, indicating that the enzyme was inactivated by Cu^{2+}.

No detectable $HO^{\cdot}$ were produced by the HX/XO system alone or in the presence of Cu^{2+}. In contrast, the HX/XO/Fe^{3+}-citrate system generated significant quantities of $HO^{\cdot}$, which were effectively scavenged by 50 mM mannitol (Fig. 2).

TABLE 1. Effects of Cu^{2+} and Fe^{3+}-citrate on ferricytochrome *c* reduction by the hypoxanthine (HX)/xanthine oxidase (XO) system

Incubation	A_{550}
	min^{-1}
HX/XO	0.117
HX/XO/SOD	0.007
HX/XO/Cu^{2+}	0.002
HX/XO/Fe^{3+}	0.106

HX (1.5 mM), XO (0.02 Units/ml), and DTPA (2.5 μM) were incubated at 37°C without further addition (standard system) or with superoxide dismutase (SOD; 0.02 Units/ml), Cu^{2+} (10 μM) or Fe^{3+}-citrate (10 μM) in the presence of ferricytochrome *c* (30 μM). Ferricytochrome *c* reduction was measured as the rate of increase in absorbance at 550 nm (A_{550}).

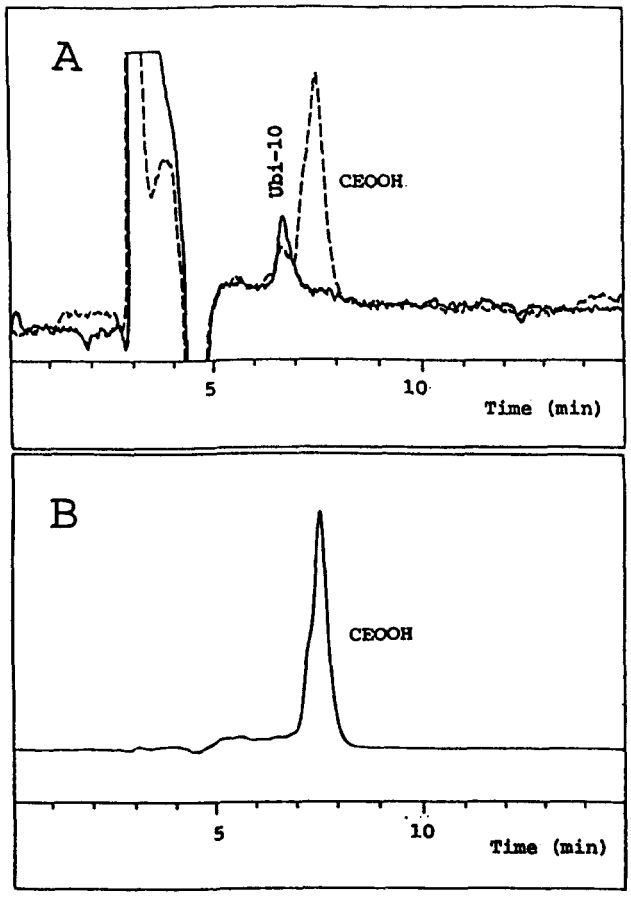

Fig. 1. Detection of cholesteryl ester hydroperoxides (CEOOH) in LDL samples by HPLC. Chemiluminescence elution profiles of LDL before (——) and after (----) incubation with Fe^{3+}-citrate (10 μM) for 15 min at 37°C are shown in panel A. Ubiquinol-10 (Ubi-10) eluted at *t* = 6.7 min while CEOOH eluted at *t* = 7.6 min. Note the absence of detectable CEOOH in fresh (unincubated) LDL. Panel B shows the chemiluminescence elution profile of LDL incubated with Cu^{2+} (10 μM) for 15 min at 37°C. Detector sensitivity was 30-fold lower in panel B (ordinate scale = 0–450 mV), compared with panel A (ordinate scale = 0–15 mV). CEOOH in LDL incubated with Fe^{3+}-citrate and Cu^{2+} were 139 pmol/mg protein and 4850 pmol/mg protein, respectively.

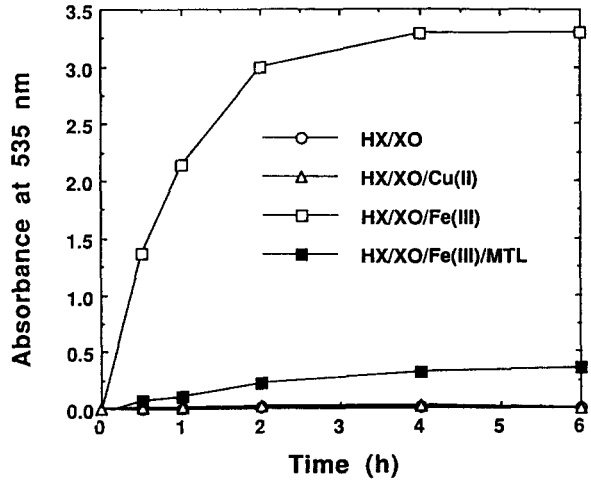

Fig. 2. Measurement of hydroxyl radical ($HO^{\cdot}$) formation by the hypoxanthine (HX)/xanthine oxidase (XO) system in the absence and presence of metal ions. HX (1.5 mM) and XO (0.02 Units/ml) were incubated at 37°C with deoxyribose (4.0 mM) and DTPA (2.5 μM) in the absence or presence of either Cu^{2+} or Fe^{3+}-citrate (10 μM). Generation of $HO^{\cdot}$ is reported as absorbance at 535 nm of the resulting thiobarbituric acid reactive substances. Fifty mM mannitol (MTL) was added to the standard HX/XO/Fe^{3+}-citrate system to confirm that the observed increase in absorbance at 535 nm resulted from $HO^{\cdot}$ formation.

Oxidative modification of LDL by the HX/XO system and Cu^{2+} or Fe^{3+}-citrate

Incubation of LDL with the HX/XO system in the absence of added metal ions did not result in oxidative modification of LDL (**Table 2** and **Fig. 3**). However, a striking increase in relative electrophoretic mobility (REM) was observed when LDL was exposed to Cu^{2+}, irrespective of whether HX and/or XO were present during the incubation (Table 2 and Fig. 3). The increase in REM was preceded by rapid formation of LOOH as assessed by HPLC with chemiluminescence detection (Fig. 1B) or measurement of diene conjugation as absorbance at 234 nm (data not shown). In contrast, Fe^{3+}-citrate increased REM of LDL only in the presence of both HX and XO (Table 2 and Fig. 3), and the rate of lipid peroxidation was much slower in the presence of Fe^{3+}-citrate than in the presence of Cu^{2+} (Fig. 1A; data for diene conjugation not shown). Concentrations of TBARS were also measured as an indicator of lipid peroxidation and found to be 1.4 ± 0.7, 35.9 ± 1.8, and 20.3 ± 2.8 nmol/mg protein (n = 3) after incubation of LDL for 24 h with HX/XO alone or in the additional presence of Cu^{2+} or Fe^{3+}-citrate, respectively; thus TBARS measurements were in good agreement with the data on REM.

Although REM exhibited some inter-experimental variations, it was always significantly less for LDL incubated with HX/XO/Fe^{3+}-citrate (1.9–3.3) than for LDL incubated with HX/XO/Cu^{2+} (5.8–8.1) (see Tables 2–4, and Figs. 3–4). Increasing the DTPA concentration in all incubations described in Table 2 from 2.5 μM to 25 μM completely inhibited LDL oxidation (REM = 1.0 for all incubations); addition of the metal ions to LDL 15 min before (to allow for maximal binding; see below) or after addition of DTPA (25 μM) produced the same result, i.e. REM = 1.0. Likewise, premixing the metal ions with a tenfold excess of EDTA (100 μM final concentration) completely prevented LDL oxidation under all experimental conditions, in agreement with inhibition of endothelial cell-mediated oxidation of LDL by 50 μM EDTA (4). These data show that metal ions are strictly required for oxidative modification of LDL, and that the metal ions mediating oxidation of LDL are not tightly bound to the lipoprotein.

TABLE 2. Relative electrophoretic mobility of human low density lipoprotein (LDL) incubated with the hypoxanthine (HX)/xanthine oxidase (XO) system without or with added metal ions

Incubation	–	Cu^{2+}	Fe^{3+}
		relative electrophoretic mobility	
LDL	1.0	7.7	1.1
LDL/HX	1.2	7.6	1.2
LDL/XO	1.2	8.2	1.3
LDL/HX/XO	1.2	8.1	3.3

LDL (protein, 0.2 mg/ml) was incubated at 37°C for 24 h with HX (1.5 mM), XO (0.02 Units/ml) and DTPA (2.5 μM) with no further addition, or Cu^{2+} (10 μM) or Fe^{3+}-citrate (10 μM). Control incubations contained LDL alone, or LDL incubated with Cu^{2+} or Fe^{3+}-citrate in the absence of HX and/or XO. Electrophoretic mobility was measured relative to that of native LDL (4–5 mm) in 0.5% agarose gels after electrophoresis at 100 V for 30 min in 0.05 M barbital buffer, pH 8.6. Data were pooled from two independent experiments and varied by <16%.

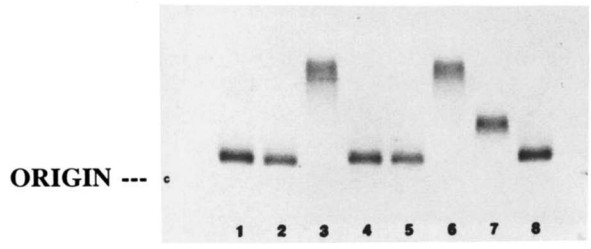

Fig. 3. Agarose gel electrophoresis of native LDL (lanes 1 and 8) and LDL incubated at 37°C for 24 h with hypoxanthine (HX, 1.5 mM), xanthine oxidase (XO, 0.02 Units/ml), and DTPA (2.5 μM) with no further addition (lane 5) or Cu^{2+} (10 μM; lane 6) or Fe^{3+}-citrate (10 μM; lane 7). Control incubations without HX and XO contained LDL and DTPA (lane 2) with Cu^{2+} (lane 3) or Fe^{3+}-citrate (lane 4). Samples were electrophoresed in 0.5% agarose gels at 100 V for 30 min in 0.05 M barbital buffer, pH 8.6. LDL bands were visualized by staining with Sudan Black B.

Involvement of $O_2^{\cdot -}$, H_2O_2, and aqueous $HO^{\cdot}$ in metal ion-dependent LDL oxidation

Incubation of LDL with SOD afforded complete protection from oxidation mediated by HX/XO/Fe^{3+}-citrate, and partial protection from HX/XO/Cu^{2+}-induced oxidation (**Fig. 4**). SOD inhibited by sodium azide (10 mM)

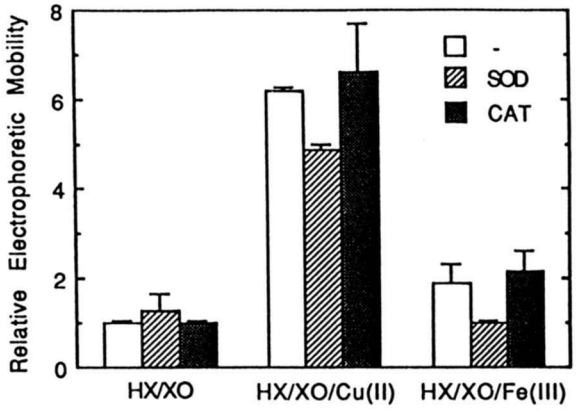

Fig. 4. Effects of superoxide dismutase (SOD) and catalase (CAT) on relative electrophoretic mobility of human low density lipoprotein (LDL) incubated with the hypoxanthine (HX)/xanthine oxidase (XO) system without or with added metal ions. Experimental conditions were identical to those reported in the legend to Table 2 except that SOD or CAT (1000 Units/ml each) were added to the standard LDL/HX/XO incubation without or with metal ions. Data represent mean ± standard deviation from three independent experiments.

and CAT did not affect LDL oxidation by either Fe^{3+}-citrate or Cu^{2+} and the HX/XO system (not shown, and Fig. 4, respectively). Heat-inactivated SOD promoted LDL oxidation (data not shown), most likely due to release of Cu^{2+} from the active site of the enzyme (25).

The $HO^{\cdot}$ scavengers urea and mannitol (50 mM) failed to protect LDL from oxidation by either Cu^{2+} or Fe^{3+}-citrate in the presence of HX and XO (**Table 3**), although mannitol very effectively scavenged $HO^{\cdot}$ generated by the HX/XO/Fe^{3+}-citrate system (Fig. 2). Thiourea (50 mM), the most effective $HO^{\cdot}$ scavenger used in this study, completely protected LDL from both Cu^{2+}- and Fe^{3+}-citrate-mediated shifts in REM (Table 3). Lipid peroxidation was also completely inhibited by thiourea as no phospholipid or cholesteryl ester hydroperoxides could be detected by HPLC with chemiluminescence detection (data not shown). However, thiourea also completely inhibited XO activity as no uric acid was formed from HX (data not shown).

Dependence of LDL oxidation on metal ion binding

To investigate whether binding of metal ions to LDL increases its susceptibility to oxidation by the HX/XO system, we used incubation conditions shown previously to result in maximal binding of Cu^{2+} to LDL (33). Thus, LDL was preincubated with either 10 μM or 100 μM Cu^{2+} or Fe^{3+}-citrate for 15 min before removal of unbound or loosely associated metal ions from LDL by gel filtration. Regardless of the metal ion concentration used in the preincubation, similar results were obtained for the subsequent incubation of LDL with the HX/XO system, and data from two experiments were pooled (**Table 4**). Preincubation of LDL with Cu^{2+} or Fe^{3+}-citrate resulted in no effect or only a marginally increased susceptibility of LDL to oxidation by the HX/XO system, respectively. Similarly, no effect was seen of preincubation conditions on HX/XO/Cu^{2+}-mediated oxidation of LDL (Table 4).

TABLE 3. Effect of hydroxyl radical ($HO^{\cdot}$) scavengers on relative electrophoretic mobility of human low density lipoprotein (LDL) incubated with the hypoxanthine (HX)/xanthine oxidase (XO) system without or with added metal ions

$HO^{\cdot}$ Scavenger	k[a]	–	Cu^{2+}	Fe^{3+}
		relative electrophoretic mobility		
–	–	1.0	6.0	3.1
Urea	<0.001	1.0	5.7	3.0
Mannitol	2.7	1.1	6.6	2.8
Thiourea	4.7	1.0	1.1	1.0

Experimental conditions were identical to those reported in the legend to Table 2 but with urea, mannitol, or thiourea (50 mM) added to the standard LDL/HX/XO incubation without or with metal ions. Data were pooled from at least two independent experiments for each incubation (<23% variation).

[a]Second order rate constant for reaction with $HO^{\cdot}$ ($M^{-1}s^{-1}$; $\times 10^{-9}$).

TABLE 4. Effect of metal ion binding to human low density lipoprotein (LDL) on relative electrophoretic mobility after incubation with the hypoxanthine (HX)/xanthine oxidase (XO) system without or with metal ions

Preincubation (15 min)	Incubation (24 h)		
	HX/XO	HX/XO/Cu^{2+}	HX/XO/Fe^{3+}
	relative electrophoretic mobility		
LDL	1.1	5.8	2.4
LDL/Cu^{2+}	1.0	5.7	3.2
LDL/Fe^{3+}	1.4	5.8	2.7

LDL (protein, 0.6 mg/ml) was preincubated at 37°C for 15 min without or with either Cu^{2+} or Fe^{3+}-citrate (10 and 100 μM each). Unbound metal ions were removed from LDL preparations by gel filtration and the resulting LDL (protein, 0.2 mg/ml) was incubated at 37°C for 24 h with HX (1.5 mM), XO (0.02 Units/ml), and DTPA (2.5 μM) without or with metal ions (see legend to Table 2). Results from two independent experiments using preincubations with Cu^{2+} or Fe^{3+}-citrate concentrations of 10 μM and 100 μM yielded similar results (<19% variation) and were pooled.

LDL preincubated with Cu^{2+} was somewhat more susceptible to subsequent oxidative modification by the HX/XO/Fe^{3+}-citrate system than LDL preincubated with Fe^{3+}-citrate or without metal ions (Table 4).

DISCUSSION

In the experiments reported here we have used the HX/XO system to investigate the molecular mechanism of metal ion-dependent oxidative modification of human LDL. XO in the presence of HX generates $O_2^{\cdot -}$ and H_2O_2, but no $HO^{\cdot}$ (this study, 43, 60). Addition of Fe^{3+}-citrate did not affect $O_2^{\cdot -}$ production by the HX/XO system and led to formation of substantial amounts of $HO^{\cdot}$, most likely via the Fenton reaction. In contrast, no $O_2^{\cdot -}$ and $HO^{\cdot}$ could be detected when Cu^{2+} was added to the HX/XO system, because XO was inactivated by Cu^{2+}. The latter explains why the results obtained in this study on Cu^{2+}-dependent oxidation of LDL were the same whether HX and XO were present or not.

Remarkably, Cu^{2+} induced extensive oxidative modification of LDL even though no detectable amounts of preformed LOOH were present in our LDL preparations, and breakdown of preformed LOOH is thought to be the predominant mechanism by which Cu^{2+} stimulates oxidative modification of LDL (35–37). It may be that minute amounts of LOOH below the detection limit of our monitoring assay (<10 pmol/mg protein, i.e., <0.005 molecules of LOOH per LDL particle) are sufficient to initiate a chain reaction of lipid peroxidation resulting from Cu^{2+}-catalyzed breakdown of LOOH to reactive peroxyl

(LOO·) and alkoxyl (LO·) radicals according to the scheme below.

$$LOOH + Cu^{2+} \rightarrow LOO^{\cdot} + H^{+} + Cu^{+} \quad [3]$$

$$Cu^{+} + LOOH \rightarrow LO^{\cdot} + {}^{-}OH + Cu^{2+} \quad [4]$$

However, it has been suggested that the reduction of Cu^{2+} by LOOH (Reaction [3]) is not favored thermodynamically (36) and, therefore, the relevance of this reaction mechanism to Cu^{2+}-induced LDL oxidation is not clear. Cu^{+}, on the other hand, is highly reactive and readily reduces LOOH to alkoxy radicals (Reaction [4]). Perhaps some unidentified agent associated with LDL reduces Cu^{2+} to Cu^{+}. Indeed, we have observed that Cu^{2+} added to LDL is readily reduced to Cu^{+} (S. M. Lynch and B. Frei, unpublished results).

Unlike the HX/XO/Cu^{2+} system, the HX/XO/Fe^{3+}-citrate system generated substantial amounts of $O_2^{\cdot -}$ and HO·. Furthermore, Fe^{3+}-citrate-mediated oxidation of LDL occurred only in the presence of both HX and XO, in contrast to Cu^{2+}-mediated oxidation. The lack of significant oxidative modification of LDL by Fe^{3+} alone at concentrations of $<10\ \mu M$ is in agreement with published data (36). The complete inhibition of HX/XO/Fe^{3+}-citrate-mediated LDL oxidation by SOD demonstrates the dependence of this reaction on $O_2^{\cdot -}$ and suggests that HO· production by classical Fenton chemistry is involved (see Reactions [1] and [2]). However, aqueous HO· do not appear to play an important role in LDL oxidation, as mannitol efficiently scavenged HO· generated by the HX/XO/Fe^{3+}-citrate system but did not inhibit oxidative modification of LDL by this system. These findings are consistent with the lack of an effect of mannitol on LDL oxidation mediated by arterial wall cells in culture (22–24, 26), and the concept that Fe-citrate-dependent lipid peroxidation is not mediated by HO· (61).

The strong inhibitory effect of the HO· scavenger thiourea against oxidative modification of LDL mediated by HX/XO/Fe^{3+}-citrate can be readily explained by inactivation of XO by thiourea, not HO· scavenging. Further evidence against the Fenton reaction being important for LDL oxidation is the finding that CAT did not inhibit HX/XO/Fe^{3+}-citrate-dependent LDL oxidation, although H_2O_2 is required for the Fenton reaction (Reaction [2]). Thus, $O_2^{\cdot -}$ and Fe^{3+}, but not H_2O_2 or aqueous HO·, are required for LDL oxidation. It may be that iron ion–oxygen complexes such as perferryl ($Fe^{2+}O_2 \leftrightarrow Fe^{3+}O_2^{\cdot -}$), ferryl ($FeO^{2+}$), or $Fe^{2+}/Fe^{3+}/O_2$ complexes (30, 62) initiate lipid peroxidation in LDL. Hence, an Fe^{2+}:Fe^{3+} complex may be the initiating species of lipid peroxidation, with $O_2^{\cdot -}$ functioning as a reductant of Fe^{3+} (Reaction [1]) (61–63). Likewise, a Cu^{+}:Cu^{2+} complex may initiate LDL oxidation, with LDL acting as a Cu^{2+} reductant (see above). Interestingly, we have recently found that Fe^{3+}-citrate, unlike Cu^{2+}, is not reduced by incubation with LDL; only in the presence of the HX/XO system is there formation of Fe^{2+} from Fe^{3+} (S. M. Lynch and B. Frei, unpublished results).

The observations that SOD partially and thiourea completely inhibited Cu^{2+}-dependent LDL oxidation in the presence of HX and XO is surprising as neither $O_2^{\cdot -}$ nor HO· were detected in the HX/XO/Cu^{2+} system. It is possible that SOD and thiourea act as Cu^{2+}-chelators, preventing the metal ion from participating in free radical reactions. Interestingly, it has recently been shown that SOD also partially inhibits LDL oxidation by Cu^{2+} alone (16, 64), and that this is most likely due to nonspecific binding of Cu^{2+} to SOD in a redox-inactive form (25, 64). Our observations that SOD, but not CAT, can inhibit metal ion-dependent LDL oxidation by the HX/XO system are consistent with data on cell-mediated oxidation of LDL. It has been reported that oxidative modification of LDL by smooth muscle cells (22), monocytes (23), and endothelial cells (26) is inhibited by SOD, but not, or only partially (23), by CAT, although some studies have not been able to demonstrate a consistent and substantial inhibitory effect of SOD on LDL oxidation mediated by endothelial cells (16) or macrophages (17, 24, 25).

In summary, the results reported in this paper demonstrate that generation of $O_2^{\cdot -}$ and H_2O_2 at pH 7.4 does not lead to oxidative modification of LDL, consistent with previous findings (28). Our data also confirm the strict requirement of metal ions for LDL oxidation to occur. Fe^{3+}-citrate-mediated oxidation of LDL is dependent upon $O_2^{\cdot -}$, but requires neither H_2O_2 nor production of aqueous HO· by the Fenton reaction. Cu^{2+}-mediated oxidation of LDL can occur without $O_2^{\cdot -}$ production and is also independent of H_2O_2 and aqueous HO·. Cu^{2+}- and Fe^{3+}-citrate-dependent LDL oxidation can take place in the absence of detectable amounts of preformed LOOH (<0.005 molecules of LOOH per LDL particle), and the metal ions mediating oxidation of LDL appear not to be tightly bound to the lipoprotein. The significance of Cu^{2+} and Fe^{3+} reduction and the redox ratio of the metal ions for the initiation of oxidative modification of LDL is currently being investigated in our laboratory.

The authors are grateful to Dr. Craig E. Thomas (Marion Merrell Dow Research Institute, Cincinnati, OH) for the gift of Ebselen (PZ51). Financial support for this project was provided by Grant No. 1968 from the Milton Fund, Harvard University, and Grant No. 3412 from the Council for Tobacco Research—USA, Inc.

Manuscript received 29 December 1992 and in revised form 26 April 1993.

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. Engl. J. Med.* **320:** 915–924.
2. Schwartz, C. J., A. J. Valente, E. A. Sprague, J. L. Kelley, and R. M. Nerem. 1991. The pathogenesis of atherosclerosis: an overview. *Clin. Cardiol.* **14:** I 1–16.
3. Morel, D. W., P. E. DiCorleto, and G. M. Chisolm. 1984. Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis.* **4:** 357–364.
4. 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.
5. Cathcart, M. K., D. W. Morel, and G. M. Chisolm, III. 1985. Monocytes and neutrophils oxidize low density lipoproteins making it cytotoxic. *J. Leukocyte Biol.* **38:** 341–350.
6. Parthasarathy, S., D. J. Printz, D. Boyd, L. Joy, and D. Steinberg. 1986. Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis.* **6:** 505–510.
7. Palinski, W., M. E. Rosenfeld, S. Ylä-Herttuala, G. C. Gurtner, S. S. Socher, S. W. Butler, S. Parthasarathy, T. E. Carew, D. Steinberg, and J. L. Witztum. 1989. Low density lipoprotein undergoes oxidative modification in vivo. *Proc. Natl. Acad. Sci. USA.* **86:** 1372–1376.
8. Rosenfeld, M. E., W. Palinsky, S. Ylä-Herttuala, S. Butler, and J. L. Witztum. 1989. Distribution of oxidation specific lipid-protein adducts and apolipoprotein B in atherosclerotic lesions of varying severity from WHHL rabbits. *Arteriosclerosis.* **10:** 336–349.
9. Ylä-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.
10. Salonen, J. T., S. Ylä-Herttuala, R. Yamamoto, S. Butler, H. Korpela, R. Salonen, K. Nyyssonen, W. Palinski, and J. L. Witztum. 1992. Autoantibody against oxidised LDL and progression of carotid atherosclerosis. *Lancet.* **339:** 883–887.
11. Lehr, H. A., C. Hubner, D. Nolte, B. Finckh, U. Beisiegel, A. Kohlschutter, and K. Messmer. 1991. Oxidatively modified human low-density lipoprotein stimulates leukocyte adherence to the microvascular endothelium in vivo. *Res. Exp. Med.* **191:** 85–90.
12. Cushing, S. D., J. A. Berliner, A. J. Valente, M. C. Territo, M. Navab, F. Parhami, R. Gerrity, and C. J. Schwartz. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein-1 in human endothelial cells and smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* **87:** 5134–5138.
13. Galle, J., A. Mulsch, R. Busse, and E. Bassenge. 1991. Effects of native and oxidized low density lipoproteins on formation and inactivation of endothelium-derived relaxing factor. *Arterioscler. Thromb.* **11:** 198–203.
14. Tanner, F. C., G. Noll, C. M. Boulanger, and T. F. Luscher. 1991. Oxidized low density lipoproteins inhibit relaxations of porcine coronary arteries. Role of scavenger receptor and endothelium-derived nitric oxide. *Circulation.* **83:** 2012–2020.
15. 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.
16. Parthasarathy, S., E. Wieland, and D. Steinberg. 1989. A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **86:** 1046–1050.
17. Rankin, S. M., S. Parthasarathy, and D. Steinberg. 1991. Evidence for a dominant role of lipoxygenase(s) in the oxidation of LDL by mouse peritoneal macrophages. *J. Lipid Res.* **32:** 449–456.
18. Jessup, W., V. Darley-Usmar, V. O'Leary, and S. Bedwell. 1991. 5-Lipoxygenase is not essential in macrophage-mediated oxidation of low density lipoprotein. *Biochem. J.* **278:** 163–169.
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. Folcik, V. A., and M. K. Cathcart. 1993. Assessment of 5-lipoxygenase involvement in human monocyte-mediated LDL oxidation. *J. Lipid Res.* **34:** 69–79.
21. Heinecke, J. W., 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.
22. Heinecke, J. W., H. Baker, H. Rosen, and A. Chait. 1986. Superoxide-mediated modification of low density lipoprotein by human arterial smooth muscle cells. *J. Clin. Invest.* **77:** 757–761.
23. Hiramatsu, K., H. Rosen, J. W. Heinecke, G. Wolfbauer, and A. Chait. 1987. Superoxide initiates oxidation of low density lipoprotein by human monocytes. *Arteriosclerosis.* **7:** 55–60.
24. Wilkins, G. M., and D. S. Leake. 1990. Free radicals and low-density lipoprotein oxidation by macrophages. *Biochem. Soc. Trans.* **18:** 1170–1171.
25. 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.
26. Steinbrecher, U. P. 1988. Role of superoxide in endothelial-cell modification of low density lipoproteins. *Biochim. Biophys. Acta.* **959:** 20–30.
27. 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.
28. Bedwell, S., R. T. Dean, and W. Jessup. 1989. The action of defined oxygen-centered free radicals on human low-density lipoprotein. *Biochem. J.* **262:** 707–712.
29. Gebicki, J. M., and B. J. H. Bielski. 1981. Comparison of the capacities of the peroxyl and superoxide radicals to initiate chain oxidation of linoleic acid. *J. Am. Chem. Soc.* **103:** 7020–7022.
30. Kappus, H. 1985. Lipid peroxidation: mechanisms, analysis, enzymology and biological relevance. *In* Oxidative Stress. H. Sies, editor. Academic Press, London. 273–310.
31. Esterbauer, H., M. Dieber-Rotheneder, G. Waeg, G. Striegl, and G. Jürgens. 1990. Biochemical, structural and functional properties of oxidized low-density lipoprotein. *Chem. Res. Toxicol.* **3:** 77–92.
32. Sattler, W., G. M. Kostner, G. Waeg, and H. Esterbauer. 1991. Oxidation of lipoprotein Lp[a]. A comparison with low-density lipoproteins. *Biochim. Biophys. Acta.* **1081:** 65–74.
33. Kuzuya, M., K. Yamada, T. Hayashi, C. Funaki, M. Naito, K. Asai, and F. Kuzuya. 1992. Role of lipoprotein-copper complex in copper-catalyzed peroxidation of low-density lipoprotein. *Biochim. Biophys. Acta.* **1123:** 334–341.
34. Chevion, M. 1988. A site-specific mechanism for free

radical-induced biological damage: the essential role of redox-active transition metals. *Free Radical Biol. Med.* **5:** 27–37.
35. Halliwell, B. 1989. Current status review: free radicals, reactive oxygen species and human disease. A critical evaluation with special reference to atherosclerosis. *Br. J. Exp. Pathol.* **70:** 737–757.
36. Esterbauer, H., J. Gebicki, H. Puhl, and G. Jürgens. 1992. The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radical Biol. Med.* **13:** 341–390.
37. Thomas, C. E., and R. L. Jackson. 1991. Lipid hydroperoxide involvement in copper-dependent and independent oxidation of low density lipoprotein. *J. Pharmacol. Exp. Ther.* **256:** 1182–1188.
38. Dubick, M. A., G. C. Hunter, S. M. Casey, and C. L. Keen. 1987. Aortic ascorbic acid, trace elements, and superoxide dismutase activity in human aneurysmal and occlusive disease. *Proc. Soc. Exp. Biol. Med.* **184:** 138–143.
39. 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.
40. Salonen, J. T., R. Salonen, K. Seppanen, M. Kantola, S. Suntioinen, and H. Korpela. 1991. Interactions of serum copper, selenium, and low density lipoprotein cholesterol in atherogenesis. *Br. Med. J.* **302:** 756–760.
41. Salonen, J. T., R. Salonen, H. Korpela, S. Suntioinen, and J. Tuomilehto. 1991. Serum copper and the risk of acute myocardial infarction: a prospective population study in men in eastern Finland. *Am. J. Epidemiol.* **134:** 268–276.
42. Salonen, J. T., K. Nyyssonen, H. Korpela, J. Tuomilehto, R. Seppanen, and R. Salonen. 1992. High stored iron levels are associated with excess risk of myocardial infarction in eastern Finnish men. *Circulation.* **86:** 803–811.
43. Britigan, B. E., S. Pou, G. M. Rosen, D. M. Lilleg, and G. R. Buettner. 1990. Hydroxyl radical is not a product of the reaction of xanthine oxidase and xanthine. The confounding problem of adventitious iron bound to xanthine oxidase. *J. Biol. Chem.* **265:** 17533–17538.
44. Ager, A., D. J. Wenham, and J. L. Gordon. 1984. Stimulation of endothelial cells by protease activity in commercial preparations of xanthine oxidase. *Thromb. Res.* **35:** 43–52.
45. Ager, A., and J. L. Gordon. 1984. Cellular effects of proteinase activity in commercial xanthine oxidase. *Biochem. J.* **222:** 839.
46. Gamache, D. A., L. J. Kornberg, M. Bartolf, and R. C. Franson. 1986. Contamination of commercial preparations of xanthine oxidase by a Ca^{2+}-dependent phospholipase A_2. *Biochim. Biophys. Acta.* **858:** 217–220.
47. Rick, W. 1974. Trypsin. *In* Methods of Enzymatic Analysis. Volume 2. H. U. Bergmeyer, editor. Academic Press, New York. 1013–1018.
48. Fridovich, I. 1989. Xanthine oxidase. *In* CRC Handbook of Methods for Oxygen Radical Research. R. A. Greenwald, editor. CRC Press Inc., Boca Raton, FL. 51–53.
49. Chung, B. H., J. P. Segrest, M. J. Ray, J. D. Brunzell, J. E. Hokanson, R. M. Krauss, K. Beaudrie, and J. T. Cone. 1984. Single vertical spin density gradient ultracentrifugation. *Methods Enzymol.* **128:** 181–209.
50. Maiorino, M., A. Roveri, and F. Ursini. 1992. Antioxidant effect of Ebselen (PZ 51): peroxidase mimetic activity on phospholipid and cholesterol hydroperoxides vs. free radical scavenger activity. *Arch. Biochem. Biophys.* **295:** 404–409.
51. Griffith, O. W. 1980. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* **106:** 207–212.
52. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* **83:** 346–356.
53. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
54. Frei, B., Y. Yamamoto, D. Niclas, and B. N. Ames. 1988. Evaluation of an isoluminol chemiluminescence assay for the detection of hydroperoxides in human blood plasma. *Anal. Biochem.* **175:** 120–130.
55. Yamamoto, Y., B. Frei, and B. N. Ames. 1990. Assay of lipid hydroperoxides using high-performance liquid chromatography with isoluminol chemiluminescence detection. *Methods Enzymol.* **186:** 371–380.
56. Esterbauer, H., G. Striegl, H. Puhl, and M. Rotheneder. 1989. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radical Res. Commun.* **6:** 67–75.
57. Gutteridge, J. M. C. 1981. Thiobarbituric acid-reactivity following iron-dependent free-radical damage to amino acids and carbohydrates. *FEBS Lett.* **128:** 343–346.
58. Halliwell, B., and J. M. C. Gutteridge. 1981. Formation of a thiobarbituric-acid-reactive substance from deoxyribose in the presence of iron salts. *FEBS Lett.* **128:** 347–352.
59. Green, M. J., and H. A. O. Hill. 1984. Chemistry of dioxygen. *Methods Enzymol.* **105:** 3–22.
60. Link, E. M., and P. A. Riley. 1988. Role of hydrogen peroxide in the cytotoxicity of the xanthine/xanthine oxidase system. *Biochem. J.* **294:** 391–399.
61. Minotti, G., and S. D. Aust. 1987. An investigation into the mechanism of citrate-Fe^{2+}-dependent lipid peroxidation. *Free Radical Biol. Med.* **3:** 379–387.
62. Minotti, G., and S. D. Aust. 1987. The role of iron in the initiation of lipid peroxidation. *Chem. Phys. Lipids.* **44:** 191–208.
63. Miller, D. M., T. A. Grover, N. Nayini, and S. D. Aust. 1993. Xanthine oxidase- and iron-dependent lipid peroxidation. *Arch. Biochem. Biophys.* **301:** 1–7.
64. Thomas, C. E. 1992. The influence of medium components on Cu^{2+}-dependent oxidation of low-density lipoproteins and its sensitivity to superoxide dismutase. *Biochim. Biophys. Acta.* **1128:** 50–57.