Oxidation of low density lipoprotein by thiols: superoxide-dependent and -independent mechanisms

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Abstract Oxidatively damaged low density lipoprotein (LDL) may cause macrophages to accumulate cholesterol in an unregulated manner, initiating the development of atherosclerotic lesions. Cultured smooth muscle cells oxidize LDL by a superoxide ($O_2^{\cdot-}$)-dependent mechanism that requires L-cystine and redox-active transition metal ions in the incubation medium. To test the hypothesis that cellular reduction of L-cystine to a thiol might be involved, we exposed LDL to L-cysteine, glutathione, and D,L-homocysteine. In a cell-free system each thiol modified LDL by a pathway that required either Cu^{2+} or Fe^{3+}. Thiol- and Cu^{2+}-modified LDL underwent lipid peroxidation and exhibited a number of properties of cell-modified LDL, including increased mobility on agarose gel electrophoresis and fragmentation of apolipoprotein B-100. Superoxide dismutase inhibited modification of LDL by L-cysteine/Cu^{2+}, whereas catalase and mannitol were without effect. In striking contrast, superoxide dismutase had little effect on oxidation of LDL by Cu^{2+} and either homocysteine or glutathione. Moreover, only L-cysteine/Cu^{2+}-modified ^{125}I-labeled LDL was degraded more rapidly than ^{125}I-labeled LDL by human monocyte-derived macrophages: superoxide dismutase in the reaction mixture blocked the facilitated uptake of L-cysteine/Cu^{2+}-modified ^{125}I-labeled LDL, suggesting involvement of $O_2^{\cdot-}$. These results indicate that LDL oxidation by L-cysteine and Cu^{2+} requires $O_2^{\cdot-}$ but not H_2O_2 or hydroxyl radical. The reaction may involve the metal ion-dependent formation of L-cystine radical anion which is oxidized by oxygen, yielding $O_2^{\cdot-}$ and the disulfide. LDL modified by L-cysteine and smooth muscle cells exhibit similar physical and biological properties, indicating that thiol-dependent generation of $O_2^{\cdot-}$ may be the oxidative mechanism in both systems. Thiols also promote lipid peroxidation by $O_2^{\cdot-}$-independent reactions but human macrophages fail to rapidly degrade these oxidized LDLs.—**Heinecke, J. W., M. Kawamura, L. Suzuki, and A. Chait.** Oxidation of low density lipoprotein by thiols: superoxide-dependent and -independent mechanisms. *J. Lipid Res.* 1993. **34:** 2051–2061.

Supplementary key words scavenger receptor • superoxide dismutase • lipid peroxidation • thiyl radical • macrophage • lipoprotein modification • homocystinuria

The pathological hallmark of the atherosclerotic lesion is the accumulation of macrophages, smooth muscle cells, and lipid in the intima of the vascular wall (1, 2). Atherosclerosis is markedly accelerated by genetic and dietary factors that elevate low density lipoprotein (LDL), the major carrier of cholesterol in human blood (3). The earliest cellular event in hypercholesterolemia appears to be adhesion of circulating monocytes to an intact endothelial monolayer (1, 2, 4). The monocyte-derived macrophages migrate into the subendothelial space where they become lipid-laden foam cells through unregulated uptake of lipoprotein-derived cholesterol (2). Native LDL paradoxically fails to induce these events in cultured macrophages, raising the possibility that the only modified lipoprotein is atherogenic. Much evidence suggests that oxidative damage may be one such modification (reviewed in refs. 5–8). Endothelial cells exposed to minimally modified LDL, which exhibits low levels of lipid peroxidation products, express molecules that stimulate monocyte adhesion and chemotaxis (9, 10). Extensively oxidized LDL is rapidly taken up and degraded by cultured mouse and human macrophages (11–13). Epitopes in atherosclerotic lesions in rabbits with genetic hyperlipidemia react with monoclonal and polyclonal antibodies to specific protein adducts of lipid peroxidation products (14–16). Lipoproteins with many properties suggestive of oxidation have been isolated from rabbit and human atheromata (15, 17). Chemically unrelated lipid-soluble antioxidant that inhibits LDL oxidation in vitro (18, 19) slow lesion formation in hypercholesterolemic rabbits (19–21).

Abbreviations: LDL, low density lipoprotein; BHT, butylated hydroxytoluene; BHA, butylated hydroxyanisole; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; SDS-PAGE, sodium dodecylsylfate polyacrylamide gel electrophoresis; $O_2^{\cdot-}$, superoxide.

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Reactive oxygen species are potential initiators of LDL lipid peroxidation by cells of the arterial wall (12, 22). We have shown that cultured human and monkey arterial smooth muscle cells produce extracellular superoxide ($O_2^{\cdot -}$) and modify LDL by a reaction that is inhibited by superoxide dismutase (23). Similar results have been reported for rabbit endothelial cells, rabbit arterial smooth muscle cells, and human skin fibroblasts (24). Both LDL modification and $O_2^{\cdot -}$ production by monkey smooth muscle cells require L-cystine, the disulfide form of L-cysteine, in the incubation medium (25). Because thiols generate $O_2^{\cdot -}$ during oxidation (26–30), we proposed that smooth muscle cells convert L-cystine to a thiol (25). Consonant with this suggestion, LDL incubated with thiols in a cell-free system underwent lipid peroxidation and became recognized by the scavenger receptor (31).

Other pathways for the initiation of LDL modification probably exist. LDL oxidation by cultured endothelial cells and mouse peritoneal macrophages is only partly inhibited by superoxide dismutase (32, 33). Based on studies using inhibitors, Parthasarathy and colleagues (32, 33) suggested that cellular lipoxygenases modify LDL. LDL exposed to purified soybean lipoxygenase and phospholipase A_2 undergoes extensive oxidation (34). Most of the inhibitors used in the original studies also prevented LDL oxidation in the absence of cells (35, 36), however, implicating nonspecific mechanisms. The exact role of lipoxygenases and thiols in LDL oxidation by cells thus remains to be established.

To test further the hypothesis that cells oxidize LDL by reducing L-cystine to a thiol, we have explored the ability of L-cysteine, glutathione, and D,L-homocysteine to oxidatively damage LDL. Our observations indicate that thiols promote lipid peroxidation by both $O_2^{\cdot -}$-dependent and -independent pathways, yielding oxidatively modified lipoproteins with different biological properties.

EXPERIMENTAL PROCEDURES

Materials

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), L-cysteine, glutathione, D,L-homocysteine, L-cystine, glutathione disulfide, D,L-homocystine, catalase (dialyzed against H_2O prior to use), butylated hydroxytoluene (BHT; 2,[6]-di-tert-butyl-*p*-cresol), butylated hydroxyanisole (BHA; 2,[3]-tert-butyl-4-hydroxyanisole), aprotinin, leupeptin, soybean trypsin inhibitor (type II), molecular weight standards, and mannitol were obtained from Sigma Chemical Co. (St. Louis, MO). Benzamidine was from Aldrich Chemical Co. (Milwaukee, WI). All other materials were obtained from sources described previously (25).

Methods

Lipoproteins. Human plasma anticoagulated with EDTA (final concentration 4 mM) prepared from fasting, normolipidemic individuals was treated immediately after separation from red blood cells with phenylmethylsulfonyl fluoride (final concentration 1 μM). LDL (d 1.019–1.063 g/ml) was isolated from pooled plasma by discontinuous density gradient ultracentrifugation in a Beckman VTi 50 vertical rotor by the method of Chung et al. (37) and extensively dialyzed versus EDTA-saline (1 mM EDTA, 150 mM NaCl, pH 7.3). The LDL (11–14 mg protein/ml, pH 7.4) was stored at 4°C under N_2 in the dark and used within 2 weeks. LDL was acetylated by the method of Basu et al. (38) and iodinated by the method of Bilheimer, Eisenberg, and Levy (39) using $Na^{125}I$.

LDL modification. All experiments were carried out in buffer A (NaCl 116 mM, KCl 5.36 mM, $CaCl_2$ 1.80 mM, $MgSO_4$ 0.81 mM, $NaH_2PO_4 \cdot H_2O$ 1 mM, 25 mM HEPES, pH 7.4). One ml of buffer A containing LDL (300 μg protein/ml), EDTA (final concentration 20–30 μM), and the indicated additions was incubated in 13 × 100 mm borosilicate glass test tubes for 24 h at 37°C in air. Thiols were added to the reaction mixture after metal ions and inhibitors. Because of the high affinity of EDTA for transition metals, under these conditions essentially all Cu^{2+} or Fe^{3+} should be present as an EDTA–metal ion complex (40). Stock solutions of $CuSO_4 \cdot 5H_2O$ (1 mM) and $FeCl_3$ (1 mM) were prepared in H_2O and 1 mM EDTA, respectively. Stock solutions of L-cysteine, reduced glutathione, and D,L-homocysteine (100 mM) were prepared in ice-cold, nitrogen-saturated, distilled H_2O immediately prior to use and the pH was adjusted to 7.0 using 6 N NaOH before addition of the thiol to buffer A. Stock solutions of L-cystine, glutathione disulfide, and L-homocystine (10 mM) were prepared by addition of 6 N NaOH to a rapidly stirring solution of disulfide until the pH was 11.2. As soon as the disulfide was in solution, the pH was adjusted to 7.0 using 6 N HCl and the disulfide was added to buffer A.

Thiobarbituric acid reacting substances. The lipid peroxide content of LDL was measured using the thiobarbituric acid-reacting substances assay of Buege and Aust (41) as modified (23). Results are expressed as malondialdehyde (MDA) equivalent content (nmol MDA per mg LDL protein) using an extinction coefficient determined from MDA prepared by acid hydrolysis of malondialdehyde tetramethyl acetal (42).

SDS polyacrylamide gel electrophoresis (SDS-PAGE). Apolipoprotein B-100 was prepared for electrophoresis by lyophilization of buffer A containing LDL. After solubilization of apolipoprotein B-100 in sample buffer containing 1% SDS and 5% 2-mercaptoethanol, electrophoresis was performed in buffer supplemented with 1% SDS using a linear 4–12.5% gradient polyacrylamide gel in a

vertical slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco) (25). The gels were stained with 0.25% Coomassie brilliant blue R and destained with water–isopropanol–acetic acid 4:5:5 (v/v/v). The molecular weight standards used were myosin (M_r 205,000), β-galactosidase (M_r 116,000), phosphorylase b (M_r 97,400), bovine serum albumin (M_r 66,000), ovalbumin (M_r 45,000), and carbonic anhydrase (M_r 29,000).

Cell culture. Human skin fibroblasts were cultured as described (25). Prior to use in experiments the cells were incubated for 48 h in 10% lipoprotein-deficient serum to up-regulate LDL receptor activity. Human monocytes were isolated by density gradient centrifugation using the method of Böyum (43). The cells were plated at 5×10^5 monocytes per 16 mm dish (Falcon Labware, Becton, Dickinson and Company, Oxnard, CA) in RPMI 1640 medium supplemented with L-glutamine (Whittaker M.A. Bioproducts, Walkersville, MD), 100 U/ml penicillin, and 100 μg/ml streptomycin. After a 2-h incubation at 37°C in 5% CO_2, non-adherent cells were removed by 3 washes with medium. The cells were placed in fresh medium containing 20% autologous serum, fed twice weekly, and used after 10–14 days in culture. Uptake and degradation of ^{125}I-labeled LDL by fibroblasts and macrophages was measured as the appearance of trichloroacetic acid-soluble radioactivity (non-iodide) in the medium (44). Degradation rates were corrected for cell-free controls incubated in parallel. Delivery of LDL cholesterol to cells was measured by the incorporation of [^{14}C]oleic acid into ^{14}C-labeled cholesteryl ester (45).

Other assays. LDL electrophoresis was carried out at pH 8.6 in barbital buffer on 0.5% agarose gel (12). Protein was measured by the method of Lowry et al. (46) using bovine serum albumin as the standard. The distilled water used to prepare all solutions contained less than 10^{-9} M Cu and Fe (12). The specific activity of superoxide dismutase and catalase were 5.4 U/μg (47) and 58.2 U/μg (48), respectively.

RESULTS

Studies of LDL oxidation in a cell-free system generally use lipoprotein that has been extensively dialyzed to remove EDTA and then incubated with high concentrations of free Cu^{2+}. The oxidative mechanism is poorly understood but probably involves the Cu^{2+}-dependent breakdown of endogenous lipid hydroperoxides formed during dialysis. To explore possible pathways of cell-promoted LDL oxidation, it is thus necessary to prevent adventitious lipid peroxidation. We accomplished this by using non-dialyzed LDL. This leaves EDTA in the reaction mixture to inhibit chain-propagating radical reactions catalyzed by free metal ions. A simple physiological salt solution buffered at neutral pH was used to eliminate possible oxidants present in tissue culture medium.

Oxidative modification of LDL by thiols

LDL incubated for 24 h in buffer A (Earles' salts buffered at pH 7.4 with 25 mM HEPES) containing 27 μM EDTA in the presence of air showed little evidence of lipid peroxidation as measured by the appearance of malondialdehyde-like oxidation products reactive with thiobarbituric acid (**Fig. 1**). Similarly, when LDL was incubated in buffer A supplemented with 3 μM Cu^{2+} alone or thiol alone, no significant lipid peroxidation occurred. In contrast, when LDL was incubated with L-cysteine, glutathione or D,L-homocysteine in buffer A supplemented with 3 μM Cu^{2+}, lipid peroxidation was substantial (Fig. 1). Replacement of Cu^{2+} with equimolar Fe^{3+}

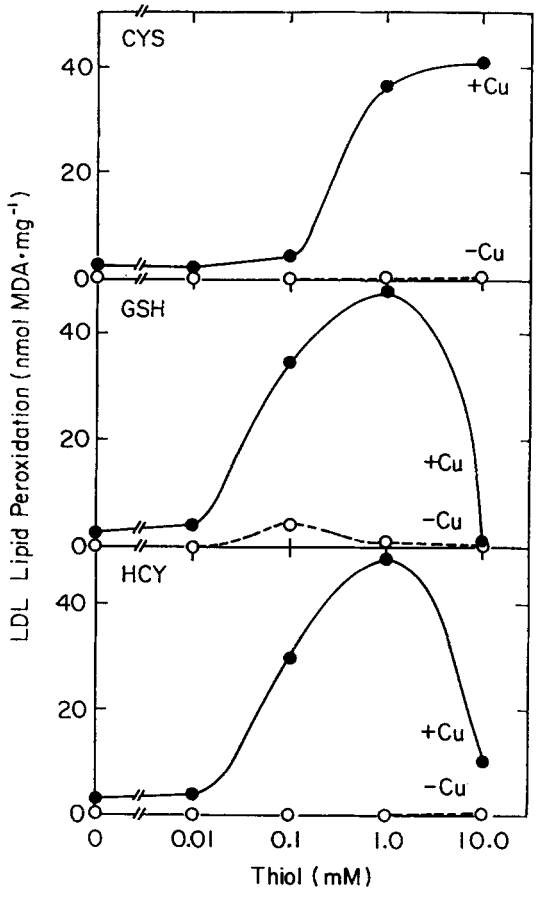

Fig. 1. Effect of L-cysteine (CYS), glutathione (GSH), and D,L-homocysteine (HCY) on LDL lipid peroxidation. The indicated final concentration of thiol was added to buffer A (pH 7.4) supplemented with 3 μM Cu^{2+}, LDL (300 μg/ml protein), and 27 μM EDTA. After incubation for 24 h at 37°C under air, the degree of LDL modification was assessed as malondialdehyde-like oxidation products (●) using the thiobarbituric acid reacting substances assay as described in Methods. Cu^{2+} was omitted where indicated (○). Control LDL (incubated in buffer A alone for 24 h at 4°C under N_2) contained 1.3 nmol MDA per mg protein.

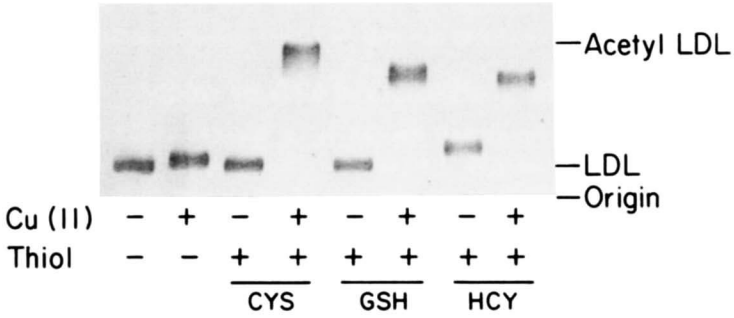

Fig. 2. Mobility on agarose gel electrophoresis of LDL modified by incubation with thiols. Conditions of LDL modification were as described in the legend to Fig. 1. The thiols (1 mM) added were: L-cysteine (CYS), lanes 3–4; glutathione (GSH), lanes 5–6; D,L-homocysteine (HCY), lanes 7–8.

gave similar results (data not shown), though Fe^{3+} promoted LDL oxidation only when it was prepared in equimolar EDTA (23). The degree of lipid peroxidation depended on the concentration of added thiol and was maximal at 1 mM for all three thiols. It decreased at high concentrations (10 mM) of glutathione and D,L-homocysteine and, in approximately half of the experiments, at 10 mM L-cysteine. The time course of LDL modification was similar to that reported for LDL modification by human arterial smooth muscle cells (12); an initial lag phase of 4–8 h was followed by a rapid increase in lipid peroxidation that peaked at 16–24 h. As previously demonstrated for LDL modified by cells (11–13, 22), LDL became more mobile on agarose gel electrophoresis after oxidation (**Fig. 2**), indicating that the lipoprotein was more negatively charged. This altered electrophoretic mobility closely paralleled the degree of lipid peroxidation. When LDL was incubated with the disulfide form of each amino acid in buffer A supplemented with 3 μM Cu^{2+}, there was little evidence of LDL lipid peroxidation or change in mobility on agarose gel electrophoresis (data not shown). Thus, under our experimental conditions, LDL modification required both a reduced sulfur-containing amino acid and a redox-active transition metal.

We also explored the effect of thiols on LDL dialyzed to remove EDTA. In the absence of added Cu^{2+}, L-cysteine, glutathione and D,L-homocysteine all stimulated LDL lipid peroxidation, although to a lesser degree than observed in the presence of Cu^{2+}-EDTA. Oxidation was maximal at 0.1 mM for all three thiols. Variable results were obtained when Cu^{2+} and thiol were included in the reaction mixture: depending on their concentrations, LDL oxidation was either stimulated or inhibited. A problem with interpreting these experiments was the large increase in lipid peroxidation of LDL exposed to Cu^{2+} alone. As we were interested in studying mechanisms potentially employed by smooth muscle cells to modify LDL, all subsequent experiments were performed with EDTA present to minimize nonspecific oxidation.

During spontaneous oxidation of LDL during dialysis (49) or modification during incubation with cells (13, 25, 50), apolipoprotein B-100 fragmentation accompanies LDL lipid peroxidation. When LDL was incubated in buffer A containing 1 mM thiol and 3 μM Cu^{2+}, loss of intact apolipoprotein B-100 of LDL was apparent on SDS-PAGE (**Fig. 3**). Fragmentation of apolipoprotein B-100 required the presence of both Cu^{2+} and thiol. Discrete low molecular-weight bands derived from oxidized apolipoprotein B-100 were not well visualized after staining with Coomassie blue. Similar results have been noted for endothelial cell-modified LDL (50).

Cell-modified LDL was originally proposed to play a role in atherogenesis by virtue of its ability to promote macrophage foam cell formation (11). The unregulated uptake of oxidized LDL is mediated, at least in part, by the scavenger receptor (11, 51). It was therefore of interest to explore the uptake and degradation of thiol-modified LDL by human monocyte-derived macrophages. ^{125}I-labeled LDL incubated with 1 mM L-cysteine and 3 μM

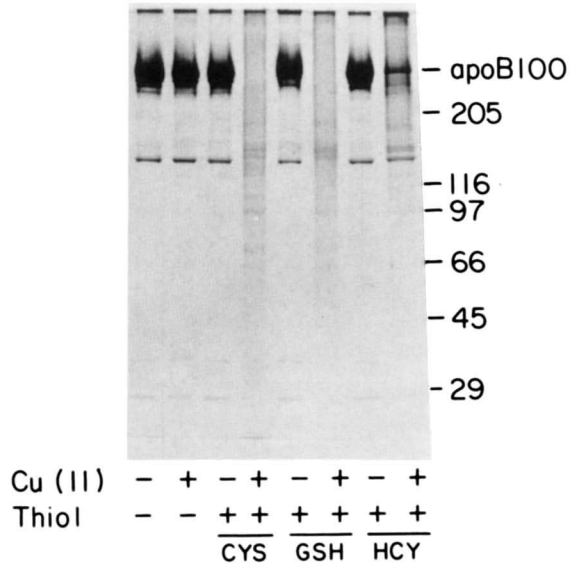

Fig. 3. Fragmentation of apolipoprotein B-100 of LDL modified by incubation with thiols. Conditions of LDL modification were as described in the legend to Fig. 1. The thiols (1 mM) added were: L-cysteine (CYS), lanes 3–4; glutathione (GSH), lanes 5–6; D,L-homocysteine (HCY), lanes 7–8. LDL protein (30 μg) solubilized with 1% SDS and 5% 2-mercaptoethanol was subjected to electrophoresis on a 4–12.5% linear gradient polyacrylamide gel in the presence of 1% SDS as described in Methods. Molecular weight standards ($M_r \times 10^{-3}$) are indicated.

Cu^{2+} for 24 h was degraded more rapidly than native LDL by human monocyte-derived macrophages (**Fig. 4**), consistent with recognition of the modified lipoprotein by the scavenger receptor (2, 11, 51, 52). The modification reaction required Cu^{2+}: in the absence of metal ion, L-cysteine-modified ^{125}I-labeled LDL and ^{125}I-labeled LDL were degraded by the cells at similar rates (Fig. 4). In striking contrast, ^{125}I-labeled LDL modified by Cu^{2+} together with either glutathione or D,L-homocysteine was degraded by human macrophages at rates similar to that of ^{125}I-labeled LDL. In two independent experiments with thiol/Cu^{2+}-modified ^{125}I-labeled LDL, similar results were obtained using J774 cells, a murine macrophage-like cell line (data not shown). Passage of L-cysteine/Cu^{2+}-modified ^{125}I-labeled LDL over a desalting column did not affect its rate of degradation by macrophages, indicating that the changes in receptor recognition of LDL modified by L-cysteine are unlikely to involve free thiol or Cu^{2+}. When ^{125}I-labeled LDL was incubated with 3 μM Cu^{2+} and L-cystine, the disulfide form of L-cysteine, rates of degradation by human monocyte-derived macrophages were similar to those observed with control ^{125}I-labeled LDL. Thus, lipid peroxidation, apolipoprotein B-100 fragmentation, and altered mobility on agarose gel electrophoresis all required Cu^{2+} and the reduced form of the sulfur-containing amino acid. Each thiol induced LDL oxidation, but only L-cysteine/Cu^{2+}-modified ^{125}I-labeled LDL was degraded more rapidly than ^{125}I-labeled LDL by human monocyte-derived macrophages.

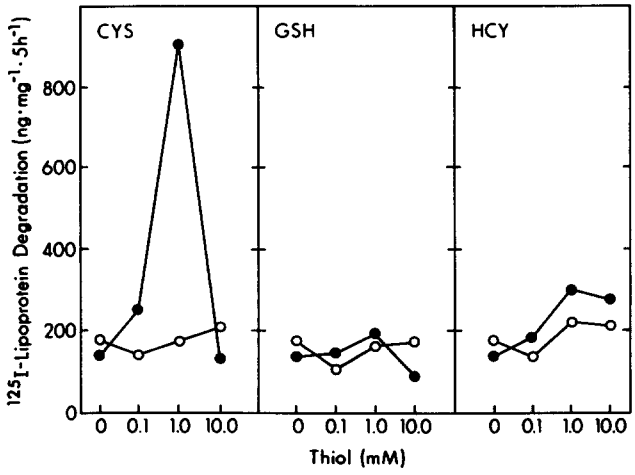

Fig. 4. Degradation of thiol-oxidized ^{125}I-labeled LDL by human monocyte-derived macrophages. ^{125}I-labeled LDL (300 μg/ml) was modified by incubation with the final indicated concentration of L-cysteine (CYS), glutathione (GSH), or D,L-homocysteine (HCY) in buffer A for 24 h at 37°C under air in the presence (●) or absence (○) of 3 μM Cu^{2+} as described in the legend to Fig. 1. The modified lipoprotein was then added to the medium of the macrophages at a final concentration of 10 μg LDL protein/ml. After a 5-h incubation, the appearance of acid-soluble ^{125}I activity in the medium (non-iodide) was determined as described in Methods. The rate of uptake and degradation of native LDL was 0.273 $\mu g \cdot mg^{-1} \cdot 5\ h^{-1}$ and of acetylated LDL was 1.81 $\mu g \cdot mg^{-1} \cdot 5\ h^{-1}$.

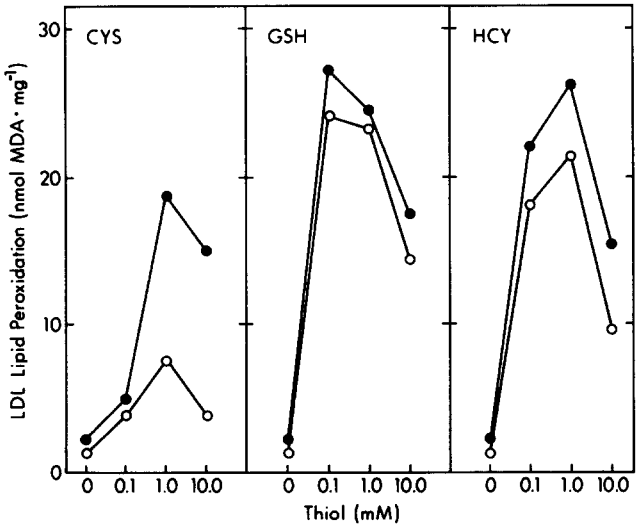

Fig. 5. Influence of superoxide dismutase on thiol-mediated LDL lipid peroxidation. The indicated final concentration of L-cysteine (CYS), glutathione (GSH), or D,L-homocysteine (HCY) was added to buffer A (pH 7.4) supplemented with 3 μM Cu^{2+}, LDL (300 μg/ml protein), and 25 μM EDTA. After 24 h incubation at 37°C under air, the degree of LDL modification was assessed as malondialdehyde-like oxidation products (●) using the thiobarbituric acid reacting substances assay described in Methods. Where indicated (○), superoxide dismutase (30 μg/ml) was included in the reaction mixture. Values are the mean of duplicate determinations from two independent experiments. Control LDL (incubated in buffer A alone for 24 h at 4°C under N_2) contained 0.2 nmol MDA per mg protein.

Role of superoxide in LDL oxidation by thiols

LDL modification by cultured smooth muscle cells (23, 25), endothelial cells (24), and fibroblasts (24) is inhibited by superoxide dismutase, which catalyzes the breakdown of $O_2^{\cdot -}$ (47). To test the possibility that oxidation by thiols involves this reactive intermediate, we explored the effect of superoxide dismutase on LDL oxidation. Only L-cysteine-induced LDL lipid peroxidation (measured as thiobarbituric acid reactive material) was inhibited significantly by superoxide dismutase (**Fig. 5**). Monitoring the increases in electrophoretic mobility of thiol-modified LDL gave similar results: superoxide dismutase prevented the increase in negative charge of L-cysteine/Cu^{2+}-modified LDL (**Table 1**), but not glutathione- or D,L-homocysteine/Cu^{2+}-modified LDL (data not shown).

Inhibition of L-cysteine/Cu^{2+}-mediated LDL lipid peroxidation by superoxide dismutase suggested that $O_2^{\cdot -}$ might play a role in altering the lipoprotein to a form recognized by the scavenger receptor. We therefore examined the effect of superoxide dismutase on the uptake of thiol-modified ^{125}I-labeled LDL by human monocyte-derived macrophages. These cells degraded ^{125}I-labeled LDL modified by incubation with 1 mM L-

TABLE 1. Ability of scavengers of reactive oxygen species to inhibit L-cysteine/Cu^{2+}-dependent LDL modification

Inhibitor	LDL Modification	
	Lipid Peroxidation[a]	Mobility[b]
None	47.9	3.2
Superoxide dismutase (SOD) (20 μg/ml)	15.6	1.5
Catalase (20 μg/ml)	39.6	3.0
Mannitol (25 mM)	47.1	3.3
Heat-inactivated SOD[c]	49.6	3.0
Heat-inactivated catalase[d]	44.3	3.3
BHT (10 μM)	2.7	1.1
BHA (10 μM)	3.3	1.1
Ethanol (0.2%)	48.3	3.1

LDL was modified in the presence of 3 mM L-cysteine, 3 μM Cu^{2+}, and 21 μM EDTA as described in Fig. 1. Inhibitors were added at the indicated final concentrations prior to addition of L-cysteine. BHA and BHT were added in 95% ethanol (final concentration 0.2% ethanol).

[a]nmol MDA/mg LDL protein.

[b]Mobility on agarose gel electrophoresis relative to native LDL.

[c]Autoclaved for 10 min.

[d]Boiled for 10 min.

cysteine and 3 μM Cu^{2+} three times more rapidly than control ^{125}I-labeled LDL; inclusion of 30 μg/ml superoxide dismutase in the reaction mixture prevented the increase (**Fig. 6**). In contrast, oxidation of ^{125}I-labeled LDL with Cu^{2+} and either glutathione or D,L-homocysteine failed to enhance degradation by human macrophages (Fig. 6). Superoxide dismutase had little influence on the degradation rates of LDL modified by either thiol. Thus, only the L-cysteine-mediated oxidative changes in LDL appeared to involve $O_2^{\cdot -}$.

Characterization of L-cysteine/Cu^{2+}-modified LDL

The modification of LDL by smooth muscle cells and by L-cysteine thus exhibit certain similarities: in both systems, LDL is oxidized by a superoxide dismutase-inhibited reaction to a form that is rapidly degraded by human macrophages. To search for further evidence that smooth muscle cells might convert L-cystine to an L-cysteine-like molecule that promotes LDL oxidation, we investigated the reaction pathway and the cellular metabolism of L-cysteine/Cu^{2+}-modified LDL.

Scavengers of H_2O_2 and the hydroxyl radical fail to affect LDL oxidation by monkey arterial smooth muscle cells (23), rabbit endothelial cells (24), and human skin fibroblasts (24). The potential role of these reactive oxygen species in L-cysteine-mediated LDL modification was investigated using catalase and mannitol. In contrast to superoxide dismutase (20 μg/ml), which suppressed the L-cysteine-dependent modification of LDL that occurred in the presence of 3 μM Cu^{2+} (Table 1), neither catalase (20 μg/ml) nor mannitol (25 mM) was inhibitory. If the superoxide dismutase in the buffer was previously heat-inactivated, LDL lipid peroxidation was not inhibited (Table 1). Similar results were obtained with the inhibitors when LDL oxidation was assessed by apolipoprotein B-100 fragmentation (**Fig. 7**). Catalase, mannitol, and urea (10 mM) also failed to inhibit LDL lipid peroxidation catalyzed by Cu^{2+} and either glutathione or homocysteine.

The lipid-soluble phenols BHT and BHA block lipid peroxidation by terminating peroxy and alkoxy radical chain propagating reactions. Both antioxidants inhibit LDL modification by cultured smooth muscle cells (12, 23). When added at 10 μM final concentration to the LDL prior to incubation with 1 mM L-cysteine and 3 μM Cu^{2+}, BHT and BHA completely blocked LDL lipid peroxidation and changes in electrophoretic mobility (Table 1) as well as apolipoprotein B-100 fragmentation (Fig. 7). In control experiments the addition of ethanol (used to solubilize BHT and BHA) did not inhibit apolipoprotein B-100 fragmentation or LDL lipid peroxidation (Table 1 and Fig. 7). As previously shown for endothelial cell-modified LDL (50), a variety of proteolytic enzyme inhibitors failed to block apolipoprotein B-100 fragmentation at concentrations that did not inhibit LDL lipid peroxidation (data not shown). The proteolytic inhibitors tested were soybean trypsin inhibitor (1 mg/ml), benzamidine (2 mM), aprotinin (100 μg/ml), and leupeptin (100 μM).

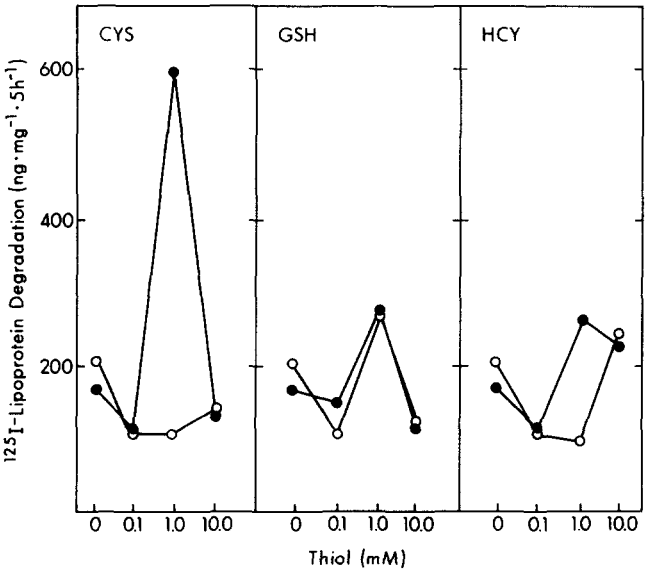

Fig. 6. Effect of superoxide dismutase on the degradation of thiol-oxidized ^{125}I-labeled LDL by human monocyte-derived macrophages. ^{125}I-labeled LDL was modified at the indicated final concentration of L-cysteine (CYS), glutathione (GSH), or D,L-homocysteine (HCY) as described in the legend to Fig. 1 in the presence of 3 μM Cu^{2+} (●). Superoxide dismutase (30 μg/ml) was included in the reaction mixture where indicated (○). After a 24-h incubation at 37°C under air, the modified lipoprotein was added to the medium of the macrophages at a final concentration of 10 μg LDL protein/ml. After 5 h incubation, the appearance of acid-soluble ^{125}I activity in the medium (non-iodide) was determined as described in Methods. The rate of uptake and degradation of control ^{125}I-labeled LDL was 0.256 μg·mg^{-1}·5 h^{-1} and of acetylated ^{125}I-labeled LDL was 1.16 μg·mg^{-1}·5 h^{-1}.

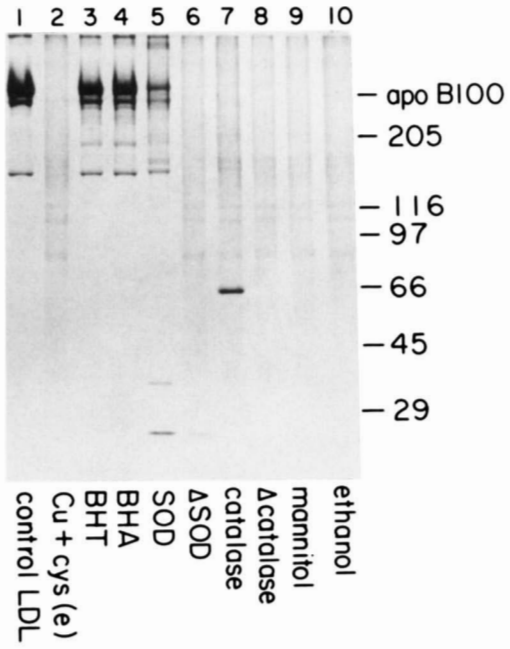

Fig. 7. Inhibition of apolipoprotein B-100 fragmentation by scavengers of reactive oxygen species. LDL was modified by incubation in buffer A supplemented with the indicated additions for 24 h as described in the legend to Fig. 1. SDS-PAGE of apolipoprotein B-100 was performed as described in the legend to Fig. 3. Lane 1, LDL incubated under N_2 at 4°C in buffer A (Control LDL). Lane 2, LDL incubated for 24 h under air at 37°C in buffer A supplemented with 3 mM L-cysteine and 3 μM Cu^{2+} (Cu+cys(e)). Lanes 3–10, LDL modified as described in lane 2 with: lane 3, 10 μM BHT; lane 4, 10 μM BHA; lane 5, 20 μg/ml superoxide dismutase (SOD); lane 6, heat-inactivated superoxide dismutase (Δ SOD); lane 7, 20 μg/ml catalase; lane 8, heat-inactivated (Δ) catalase; lane 9, 25 mM mannitol; lane 10, 0.2% ethanol. Stock solutions of BHT and BHA were prepared in 95% ethanol, yielding a final concentration of 0.2% alcohol in the reaction mixture. Superoxide dismutase and catalase were heat-inactivated as described in the legend to Table 1.

The cellular incorporation of [^{14}C]oleic acid into ^{14}C-labeled cholesteryl ester measures the free cholesterol released by the uptake and degradation of lipoprotein (2, 45). L-cysteine/Cu^{2+}-oxidized LDL was a 2- to 3-fold more potent stimulator of this reaction in human monocyte-derived macrophages than was LDL (data not shown).

Apolipoprotein B-100 of LDL is the ligand recognized by the LDL receptor on cells (53). Human fibroblasts fail to take up and degrade smooth muscle cell-modified ^{125}I-labeled LDL, perhaps due to apolipoprotein B-100 fragmentation and loss of LDL receptor recognition (25). After ^{125}I-labeled LDL was modified by incubation with L-cysteine and 3 μM Cu^{2+} in buffer A for 24 h, it was degraded by human skin fibroblasts at a lower rate than native LDL (data not shown), consistent with loss of recognition by the LDL receptor. The degradation rate of L-cysteine/Cu^{2+}-modified ^{125}I-labeled LDL by fibroblasts depended on the concentration of L-cysteine: at 1 mM L-cysteine, which induced maximal lipid peroxidation (Fig. 1), there was little uptake and degradation of the modified lipoprotein. Similar findings were obtained using the stimulation of [^{14}C]oleic acid incorporation into cholesteryl ester as an index for the delivery of lipoprotein cholesterol to fibroblasts. Loss of uptake and degradation of the modified lipoprotein by fibroblasts was paralleled by loss of intact apolipoprotein B-100 of LDL on SDS-PAGE (**Fig. 8**). LDL modified in the presence of thiol alone or 3 μM Cu^{2+} alone was degraded by fibroblasts or stimulated cholesteryl ester formation by fibroblasts at rates similar to those observed with control LDL. Minimal fragmentation of apolipoprotein B-100 occurred under these conditions (Fig. 8). When ^{125}I-labeled LDL was incubated with Cu^{2+} and L-cystine, the disulfide form of L-cysteine, there was little change in its degradation rate by human fibroblasts (data not shown), indicating that the reduced form of the amino acid was necessary for modification of the lipoprotein to a form with altered receptor-mediated uptake. Thus, L-cysteine/Cu^{2+}-modified LDL shares many properties with smooth muscle cell-modified LDL: its formation is blocked by the same scavengers of reactive oxygen, it has similar physical and chemical features, and it is metabolized in a comparable manner by cultured fibroblasts and macrophages.

DISCUSSION

The current studies demonstrate that L-cysteine, glutathione, and D,L-homocysteine modified LDL by reactions requiring redox-active transition metal ions; the

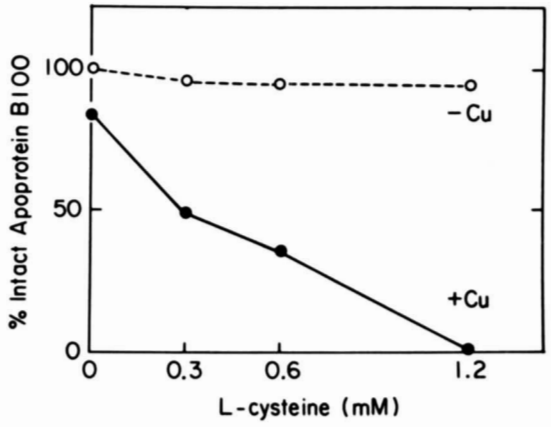

Fig. 8. Effect of L-cysteine concentration on loss of intact apolipoprotein B-100 of modified LDL. LDL was modified by incubation in buffer A supplemented with L-cysteine and 3 μM Cu^{2+} (●) for 24 h at 37°C under air as described in the legend to Fig. 1. Cu^{2+} was omitted during LDL modification where indicated (○). Apolipoprotein B-100 was analyzed by SDS-PAGE under reducing conditions as described in the legend to Fig. 3. The amount of apolipoprotein B-100 was then determined from the peak area of intact protein by densitometric scanning of the Coomassie brilliant blue R-stained band. Results are expressed as percent of intact apolipoprotein B-100 of control LDL (LDL incubated in buffer A alone at 4°C under N_2 for 24 h).

general free radical scavengers BHT and BHA were inhibitory. Thiol/Cu^{2+}-modified LDL exhibited lipid peroxidation, increased negative charge on agarose gel electrophoresis, and apolipoprotein B-100 fragmentation. Collectively these results indicate that thiols modify LDL by an oxidative pathway.

The mechanism for LDL modification by thiols was investigated using scavengers of reactive oxygen species. Superoxide dismutase inhibited LDL modification by L-cysteine and Cu^{2+}, monitored as changes in lipid peroxidation, apolipoprotein B-100 fragmentation, and increased negative charge. In contrast, catalase (which catalyzes the breakdown of H_2O_2) and mannitol (an aqueous phase hydroxyl radical scavenger) had little effect. Neither superoxide dismutase, catalase, nor mannitol inhibited LDL oxidation by Cu^{2+} and either glutathione or D,L-homocysteine. Thus, the oxidative modification of LDL by L-cysteine, but not glutathione or D,L-homocysteine, requires $O_2^{\cdot -}$.

LDL oxidation by thiols probably involves the metal ion-dependent generation of thiyl radical ($RS^{\cdot}$). This species can react with thiolate anion ($RS:^-$) to form the disulfide radical anion ($RSSR^{\cdot -}$), which is oxidized by oxygen, yielding $O_2^{\cdot -}$ and the disulfide (27–30):

$$RS:^- + M^{n+} \rightarrow RS^{\cdot} + M^{n-1}$$
$$RS^{\cdot} + RS:^- \rightarrow RSSR^{\cdot -}$$
$$RSSR^{\cdot -} + O_2 \rightarrow RSSR + O_2^{\cdot -}$$

This may be the preferred pathway for autoxidation by L-cysteine since superoxide dismutase inhibited LDL oxidation by this thiol.

Thiols also initiate lipid peroxidation by other mechanisms. Liposomes incubated with thiols and ADP-chelated iron underwent lipid peroxidation by a superoxide dismutase- and oxygen-insensitive reaction (54). Thiyl radicals abstract $H^{\cdot}$ from polyunsaturated fatty acids with a rate constant in the range of 10^7 $dm^3mol^{-1}s^{-1}$ (55). It is noteworthy that the promotion of LDL oxidation by glutathione and D,L-homocysteine was little affected by superoxide dismutase, catalase, and hydroxyl radical scavengers. Perhaps these thiols form sulfur-centered radicals that directly oxidize LDL lipids. Thus, under our experimental conditions, thiols can promote lipid peroxidation by both $O_2^{\cdot -}$-dependent and -independent pathways. The mechanism used will likely depend on factors such as the chelation state of the metal ion, the reduction potential of the thiol, and the substrate for oxidation. Cu^{2+} chelated by L-cysteine and glutathione may form different complexes due to the gamma carboxyl group in glutathione tripeptide (56). The additional methylene carbon in D,L-homocysteine might similarly result in a Cu^{2+} complex with altered redox properties (57).

LDL oxidation by thiols may involve additional reactions. Peroxidation of brain synaptosomes (58) and phospholipid liposomes (59) requires both oxidized and reduced iron. Observing that the optimum ratio of Fe^{3+} to Fe^{2+} is approximately 1 (59), Minotti and Aust (59, 60) have proposed that a Fe^{3+}–O–O–Fe^{2+} complex initiates lipid peroxidation. This may explain the decrease in LDL oxidation we observed at high concentrations of thiol: under these conditions, most of the Cu^{2+} is presumably reduced to Cu^{1+}, inhibiting lipid peroxidation. Peroxidation of microsomal lipids is likewise inhibited at high concentrations of L-cysteine (54). Alternatively, either the thiol or its disulfide may have directly scavenged a reactive intermediate (61).

A hallmark of modified LDL is altered recognition by macrophage cell surface receptors (2, 11, 51, 52). ^{125}I-labeled LDL oxidized by L-cysteine/Cu^{2+} was degraded more rapidly than ^{125}I-labeled LDL by human monocyte-derived macrophages but more slowly by human skin fibroblasts. These changes may reflect a gain of scavenger receptor recognition and a loss of LDL-receptor recognition, respectively (2, 11, 51–53). In striking contrast, neither glutathione- nor homocysteine/Cu^{2+}-modified ^{125}-labeled LDL was degraded more rapidly than ^{125}I-labeled LDL by human monocyte-derived macrophages. These results show that thiols can promote LDL oxidation by both $O_2^{\cdot -}$-dependent and -independent pathways, to yield modified lipoproteins with different biological properties. Using Ham's F10 medium as the reaction mixture, Parthasarathy (31) obtained different results: glutathione, homocysteine, and cysteine all modified LDL to a form apparently recognized by the scavenger receptor on mouse peritoneal macrophages. We have obtained similar results with cultured human macrophages. Perhaps other components in the tissue culture medium affected the pathway for LDL oxidation.

Modification of LDL by L-cysteine and smooth muscle cells exhibit remarkable similarities. In both systems, LDL oxidation requires transition metal ions, is inhibited by superoxide dismutase but not catalase or mannitol, is associated with lipid peroxidation and apolipoprotein B-100 fragmentation, and alters lipoprotein degradation by fibroblasts and macrophages. These observations are support for our proposal that smooth muscle cells modify LDL by converting L-cystine to a thiol (25), which promotes LDL oxidation by a $O_2^{\cdot -}$-dependent pathway (23, 25). The production of extracellular H_2O_2 by P815 mastocytoma cells, perhaps by the direct two-electron reduction of oxygen by thiol, also requires L-cystine (62). Steinbrecher (24) found that superoxide dismutase inhibits LDL oxidation by rabbit endothelial cells, rabbit arterial smooth muscle cells, and human skin fibroblasts; LDL lipid peroxidation was proportional to the rate of $O_2^{\cdot -}$ generation. Thus, the generation of reactive oxygen species by secretion of cellular thiols may be widespread.

Thiols play a central role in protecting cells from oxidative stress by regulating the intracellular redox status and

serving as substrates for enzymatic scavengers of reactive oxygen species (63, 64). Thiols are also potentially deleterious. In the presence of oxygen and metal ions, thiols are oxidized, generating $O_2^{\cdot -}$, H_2O_2, and hydroxyl radical (26–30, 65, 66). This raises the prospect that regulation of the oxidation state of sulfur-containing amino acids may protect cells from oxidative damage. Intracellular thiols, which exist in an environment where free metal ions are presumably deficient, protect against oxidant stress (63, 64). Extracellular thiols might execute a variety of oxidative insults in the presence of redox catalysts. Although the plasma concentrations of cysteine and homocysteine (67) are much lower than those used in our experiments, the cumulative exposure of lipoproteins to thiol would be considerable because LDL may have a long residence time in the artery wall (1, 2, 7). It is noteworthy that vascular thrombosis and coronary artery disease are frequent in patients suffering from homocystinuria, a genetic disorder of the transsulfuration pathway that greatly elevates plasma levels of homocystine (67). Primates continuously infused with homocysteine exhibit endothelial denudation, suggesting that the thiol or its oxidation products directly injure the artery wall (68). Recent studies implicate the heterozygous state for homocystinuria as a significant risk factor for atherosclerosis (69, 70). Our results suggest that homocysteine may not convert LDL to a form recognized by the scavenger receptor. Homocysteine promotes LDL lipid peroxidation, however, and oxidized LDL has been suggested to play a role in vascular disease by many other pathways (5–8). The role of thiols in the pathogenesis of vascular disease clearly warrants further study.

Note added in proof. Sparrow and Olszewski (71) have recently shown that LDL oxidation by cultured rabbit endothelial cells and mouse peritoneal macrophages is L-cystine-dependent. Most importantly, they demonstrated that the cellular generation of a thiol mediates LDL oxidation. These results offer strong support for our hypothesis that LDL oxidation by arterial smooth muscle cells involves the reduction of L-cystine to a thiol, and raise the possibility that cellular thiol production is a general pathway for the oxidative damage of lipoproteins.

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