Effect of homocysteine on copper ion-catalyzed, azo compound-initiated, and mononuclear cell-mediated oxidative modification of low density lipoprotein

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Abstract Homocysteine is an independent risk factor for cardiovascular diseases. The mechanisms by which elevated plasma concentrations of homocysteine are related to the pathogenesis of atherosclerosis are not fully understood. To examine whether homocysteine is implicated in atherogenesis through the modification of low density lipoprotein (LDL), the effect of homocysteine on the oxidation of LDL was studied by three different oxidation systems. Thus, LDL was subjected to Cu^{2+}-catalyzed, azo compound-initiated, and peripheral blood mononuclear cell-mediated oxidative modification. The extent of modification was assessed by measuring the formation of conjugated dienes, lipid peroxides, thiobarbituric acid-reactive substances, and the relative electrophoretic mobility. Homocysteine at a normal plasma concentration (6 μM) showed no effect, whereas a concentration corresponding to moderate hyperhomocysteinemia (25 μM) or to concentrations seen in homocystinuria patients (100, 250, and 500 μM) protected LDL from modification of the lipid as well as of the protein moiety. One exception was observed: when the oxidation was initiated by copper ions, homocysteine at concentrations 6 and 25 μM stimulated the lipid peroxidation of LDL to a small, but statistically significant extent. High concentrations of homocysteine showed antioxidative properties as long as the thiol groups were intact, thereby delaying the onset of the oxidation. The 1,1-diphenyl-2-picrylhydracyl radical test demonstrated that homocysteine at concentrations ≥50 μM possessed marked free radical scavenging capacity. Finally, LDL isolated from two patients with homozygous homocystinuria showed similar extent of Cu^{2+}-catalyzed oxidation as LDL from a group of healthy control subjects. Taken together, our data suggest that low concentrations of homocysteine in the presence of copper ions may enhance the lipid peroxidation of LDL, whereas high concentrations of homocysteine may protect LDL against oxidative modification in the lipid as well as in the protein moiety. Thus, homocysteine-induced atherosclerosis may be explained by mechanisms other than oxidative modification of low density lipoprotein.—**Halvorsen, B., I. Brude, C. A. Drevon, J. Nysom, L. Ose, E. N. Christiansen, and M. S. Nenseter.** Effect of homocysteine on copper ion-catalyzed, azo compound-initiated, and mononuclear cell-mediated oxidative modification of low density lipoprotein. *J. Lipid Res.* 1996. **37:** 1591–1600.

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Homocysteine is an independent risk factor for cardiovascular diseases (1–3). Normally the plasma level is about 10 μM. Moderate hyperhomocysteinemia is found in 20–30% of patients with coronary and peripheral vascular diseases. Plasma concentrations up to 500 μM have been reported in patients who suffer from homocystinuria. Homocystinuria is an inborn error of metabolism mainly due to deficiency of the enzyme cystathionine β-synthase, but also deficiency in 5,10-methylenetetrahydrofolate reductase and methionine synthase have been reported (4). Patients with severe homocystinuria may develop atherosclerosis, vascular occlusion, and thromboembolic events at an early age (1–5).

It is established that thiols are able to generate partially reduced oxygen species such as $O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$, and to initiate lipid peroxidation (6). A typical event in the atherosclerotic process in the vessel wall is the formation of fatty streaks. In this process, oxidatively modified low density lipoprotein (LDL) may play an important role in endocytosis via the macrophage scavenger receptor, cholesterol accumulation, and foam cell formation (7, 8). The premature atherogenesis observed in subjects with homocystinuria raises the question whether homocysteine is implicated in atherogenesis through modification of LDL. Studies examining LDL

Abbreviations: AAPH, 2,2′-azobis-(2-amidinopropane hydrochloride); DPPH, 1,1-diphenyl-2-picrylhydracyl; HDL, high density lipoprotein; LDL, low density lipoprotein; TBARS, thiobarbituric acid-reactive substances.

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oxidation in the presence of various thiol compounds in cell-free systems suggested that homocysteine promoted lipid peroxidation of LDL (9–11). However, the finding that elevated plasma concentration of homocysteine did not correlate with the plasma levels of lipid hydroperoxides suggested that lipid peroxidation is not important in the initiation of atherosclerosis among homocystinuric subjects (12, 13).

The aim of the present study was to examine whether homocysteine is implicated in atherogenesis through the modification of LDL. To address this question, we tested the effects of homocysteine on Cu^{2+}-catalyzed, azo-induced, and peripheral blood mononuclear cell-mediated oxidative modification of LDL from healthy volunteers and from patients with homocystinuria.

MATERIALS AND METHODS

Materials

Ham's nutrient mixture F-10 (Ham's F-10) and gentamicin were obtained from Bio Whittaker. Phorbol 12-myristate 13-acetate (PMA), butylated hydroxytoluene (BHT), D,L-homocysteine, and D,L-homocysteine thiolactone hydrochloride were purchased from Sigma Chemical Co., St. Louis, MO. The azo-compound 2,2′-azobis-(2-amidinopropane hydrochloride) (AAPH) was from Polysciences Inc., Warrington, PA, whereas Dynabeads M-450 anti-glycophorin A was purchased from Dynal AS, Oslo, Norway. Polymorphprep was delivered by Nycomed Pharma AS, Oslo, Norway, and tissue culture dishes were supplied by Costar, Cambridge, MA. BCA Protein Assay was obtained from Pierce Laboratories Inc., Rockford, IL. Kits for determination of serum cholesterol and triacylglycerols were purchased from Biodiagnostik, Taunusstein, Germany and Bio Merieux, Marcy-l'Etoile, France, respectively. High density lipoprotein (HDL) cholesterol and lactate dehydrogenase (LDH) were determined by use of kits from Boehringer Mannheim GmbH, Mannheimer, Germany. The kit for determination of lipid peroxides was from Kamiya Biomedical Company, Thousand Oaks, CA, and agarose gels (Paragon lipoprotein electrophoresis) were purchased from Beckman Instruments, Inc., Fullerton, CA.

Stock solutions of homocysteine

D,L-Homocysteine thiolactone. A 100-mM stock solution of homocysteine was prepared from D,L-homocysteine thiolactone hydrochloride after alkaline hydrolysis (2 M NaOH) for 5 min at 37°C. Then pH was adjusted to 7.4 (14). All working solutions were made in 0.15 M NaCl and 20 mM sodium phosphate (PBS without EDTA), pH 7.4, and used immediately in the experiments with Cu^{2+}-catalyzed or azo compound-initiated oxidative modification of LDL (see below).

D,L-Homocysteine was dissolved in degassed 0.01 M HCl, and immediately used in the experiments with mononuclear cell-mediated modification of LDL (see below).

Isolation of LDL

LDL was isolated from freshly prepared EDTA–plasma by sequential ultracentrifugation in the density range of 1.019–1.063 g/ml (15) as described before (16). The preparation was dialyzed extensively against 0.15 M NaCl, 20 mM sodium phosphate, and 2 mM EDTA (PBS), pH 7.4. LDL was stored in the presence of EDTA under nitrogen at 4°C and used within 1–2 weeks. Protein concentration was determined by the BCA Protein Assay. Intra-assay coefficient of variation for protein determination was 2.9% (n = 18).

Isolation of peripheral blood mononuclear cells

The cells were isolated from citrated, freshly collected blood from healthy volunteers using Polymorphprep, as described in detail (16). Contaminating erythrocytes were removed by addition of 50 µl suspension of Dynabeads M-450 anti-glycophorin A (16). The erythrocyte-free mononuclear cell fraction was resuspended in Ham's F-10 medium with gentamicin (250 µg/ml). The viability was >97% as determined by exclusion of Trypan Blue, and the purity was >90% as determined by May-Grünwald/Giemsa staining.

Cu^{2+}-catalyzed oxidation of LDL

LDL from 2 to 8 healthy subjects was pooled, dialyzed against EDTA-free PBS (pH 7.4), and subjected to Cu^{2+}-catalyzed oxidative modification (16). LDL (250 µg/ml) was incubated at 37°C for up to 24 h in the presence of 5 µM $CuSO_4$ and 0–500 µM homocysteine. Aliquots were removed at indicated time points, and the oxidation was stopped by addition of EDTA and BHT (final concentrations of 200 µM and 40 µM, respectively) and refrigeration. These aliquots were assayed for lipid peroxides, thiobarbituric acid-reactive substances (TBARS), and relative electrophoretic mobility (see below).

Kinetics of Cu^{2+}-catalyzed oxidation of LDL

The kinetics of Cu^{2+}-catalyzed oxidation of LDL were followed by determining the changes in absorbance at 234 nm in a Shimadzu UV-160A spectrophotometer with six cuvette positions (17). LDL (25 µg/ml) from healthy subjects and the homocystinuria patients was incubated at 37°C in the presence of Cu^{2+} (5 µM). Calculations of lag time (min), formation rates (nmol/mg LDL protein per min), and maximum amount of conjugated dienes formed (nmol/mg LDL protein) were done using a molar extinction coefficient

of E_{234nm} = 2.52 × 10^4 $M^{-1}cm^{-1}$ (18). The intra-assay coefficients of variation were 4.0%, 5.2%, and 2.0% for lag time, formation rate, and maximum amount of conjugated dienes formed, respectively (n = 6).

Azo compound-initiated oxidation of LDL

LDL was dialyzed against EDTA-free PBS (pH 7.4) at 4°C, and immediately subjected to oxidation initiated by AAPH, a water-soluble azo compound that thermally decomposes to produce peroxyl radicals at a constant rate within the water phase (19, 20). The oxidation of LDL (100 μg/ml) was carried out in PBS at 37°C for indicated periods of time in the presence of 4 mM AAPH and 0–500 μM homocysteine. The oxidation was stopped as described above, and LDL was assayed for lipid peroxides and electrophoretic mobility in agarose gel (see below).

Kinetics of azo compound-initiated oxidation of LDL

The kinetics of azo compound-initiated oxidation of LDL were followed by determining the changes in absorbance at 234 nm (17) using a Beckman DU 640 spectrophotometer with a 12 positions micro multicell. LDL (25 μg/ml) was incubated at 37°C in the presence of 1 mM AAPH and 0–500 μM homocysteine. Reagent blanks containing AAPH were measured in parallel. The absorbances of the reagent blanks and samples were measured every 5 min for 240 min. Sample absorbance was corrected according to changes in the absorbance of AAPH in PBS. Lag time (min) and formation rate of conjugated dienes (nmol/mg LDL protein per min) were calculated as above.

Cell-mediated oxidation of LDL

Immediately after dialysis, LDL was subjected to cell-mediated oxidation by freshly isolated peripheral blood mononuclear cells, as described in detail (16). The oxidation was carried out for 6 h at 37°C (in a 95% air and 5% CO_2 atmosphere) in the presence of LDL (100 μg/ml), PMA (100 ng/ml), Cu^{2+} (5 μM), and 0–1000 μM homocysteine. In separate experiments we found that LDL lipid peroxidation was reduced by about 60% in the absence versus the presence of 5 μM Cu^{2+} in Ham's F-10 medium. Furthermore, the amount of lipid peroxides formed during cell-mediated oxidation was about 2-fold higher than that formed during Cu^{2+}-induced oxidation in Ham's F-10 without cells. Thus, to provide a suitable and effective oxidation system, Cu^{2+} (5 μM) was added to the media. The oxidation was stopped as described above, and LDL in the medium was assayed for lipid peroxides and electrophoretic mobility in agarose gel (see below). To test for cytotoxicity, LDH leakage to the media was measured by a colorimetric assay (see Materials).

Extent of LDL modification

Lipid peroxides. The amount of lipid peroxides was determined by a colorimetric endpoint method (see Materials), as described (16). Reagent blanks containing copper ions, the azo compound AAPH, or Ham's F-10 medium were included in the assay. The intra-assay coefficient of variation was 1.6% (n = 10 for Cu^{2+}-oxidized LDL).

TBARS. The amount of TBARS was measured in microtiter plates (21). Briefly, LDL was mixed with 50% TCA and 1.3% thiobarbituric acid in 0.3% NaOH in a microtiter plate and incubated at 60°C for 40 min. After incubation, 20% SDS was added, and the absorbance at 530 nm and 590 nm was measured using a Titertek Multiscan Plus MK II Spectrophotometer. The results are expressed as nmole equivalents of malondialdehyde, using freshly diluted 1,1,3,3-tetramethoxypropane as the standard. The intra-assay coefficient of variation was 3.1% (n = 15 for Cu^{2+}-oxidized LDL).

Electrophoretic mobility. The net negative surface charge of LDL was measured by agarose gel electrophoresis (Paragon) in 0.05 M barbital buffer, pH 8.6, and stained with Sudan Black B (22). Relative electrophoretic mobility was calculated as the mobility of oxidized LDL relative to that of native LDL. The intra-assay coefficient of variation was <1% (n = 7 for Cu^{2+}-oxidized LDL).

Thiol assay procedure

The changes in the concentration of thiol during Cu^{2+}-catalyzed oxidation of LDL in the presence of homocysteine were measured using the thiol-specific reagent 5,5′-dithio-*bis*(2-nitrobenzoic acid) (23, 24). Briefly, LDL was Cu^{2+}-oxidized as described above. Aliquots were removed at indicated time points, and the concentrations of thiol were measured immediately. For thiol determination, 900 μl solution containing LDL and homocysteine in EDTA-free PBS was mixed with 100 μl of 1 mM 5,5′-dithio-*bis*(2-nitrobenzoic acid) in 200 mM NaP_i (pH 8), and the absorbance at 412 nm was measured. Thiol concentrations were calculated using a molar extinction coefficient of E_{412nm} = 1.36 × 10^4 $M^{-1}cm^{-1}$ for thiol adduct (25).

Scavenging of 1,1-diphenyl-2-picrylhydracyl (DPPH) radical

Scavenging of free radicals was measured as decline in DPPH radical concentration by continuously monitoring the loss of absorbance at 517 nm (26) using a Beckman DU 640 spectrophotometer with a 12 positions micro multicell, as described in detail before (16).

Patients with homocystinuria

Patients. LDL was isolated from two patients (18-year-old female and 14-year-old male) with homozygous homocystinuria. The male patient also suffered from familial hypercholesterolemia. Therefore, one patient (10-year-old male) with familial hypercholesterolemia was also included in the study as a control. The homocystinuria patients were currently on treatment with betaine, folic acid, and pyridoxine. However, plasma concentrations of homocysteine were never within the normal range for healthy individuals (134 and 33 μmol/l for the female and male patient, respectively). The plasma concentration of homocysteine was 4.9 μM for the familial hypercholesterolemic control patient. Serum cholesterol, HDL cholesterol, and triacylglycerol concentrations were 4.1, 1.6, and 0.6 mmol/l, respectively, for the female patient, 6.0, 0.8, and 1.2 mmol/l, respectively, for the male patient, and 9.5, 1.3, and 1.1 mmol/l, respectively, for the control patient with familial hypercholesterolemia. The oxidizability parameters of LDL from the patients were compared with those of LDL from the healthy control subjects used in the present study (females and males, 25–50 years old).

Serum lipid concentrations. Determination of serum concentrations of cholesterol and triacylglycerols was performed by enzymatic methods (see Materials). The coefficients of variation were 3.5% and 3% for assay of cholesterol and triacylglycerols, respectively. HDL cholesterol was measured after precipitation of apolipoprotein B-containing lipoproteins with a standard heparin-manganese solution. HDL cholesterol was then determined in the supernatant with an enzymatic method (see Materials).

Plasma homocysteine concentrations. Determination of plasma homocysteine concentrations was kindly performed by Professor Helga Refsum, Haukeland Hospital, Bergen, Norway, using high-performance liquid chromatography (27, 28).

Statistical analysis

The results are presented as means ± SD. Student's two-sample, two-tailed *t*-test was used for calculation of statistical significance of the difference between groups. The level of significance was set at $P < 0.05$.

RESULTS

Cu^{2+}-catalyzed oxidation of LDL

LDL from normolipidemic volunteers were subjected to Cu^{2+}-catalyzed lipid peroxidation in the absence and presence of homocysteine. Homocysteine at concentrations of 6 and 25 μM significantly enhanced the amounts of lipid peroxides and TBARS generated during oxidation for 1 and 6 h, whereas higher concentrations (≥100 μM) significantly reduced the amounts of lipid peroxides and TBARS (**Fig. 1** and **Fig. 2**). Similarly, LDL oxidized in the presence of 250 and 500 μM homocysteine for 1 h (data not shown), or in the presence of 500 μM for 6 h (**Table 1**) showed reduced relative electrophoretic mobility as compared to LDL oxidized without homocysteine. However, after 24 h oxidation, no effect of homocysteine was observed on any of the oxidizability parameters measured (data not shown).

In separate experiments, thiol concentrations were determined during the Cu^{2+}-catalyzed oxidation of LDL in the presence of homocysteine (**Fig. 3**). The thiol concentrations decreased with time at all homocysteine concentrations tested. The absorbance at 234 nm increased with increasing incubation time as the thiol concentration decreased. Furthermore, homocysteine delayed the onset of oxidation as long as the thiol groups were intact.

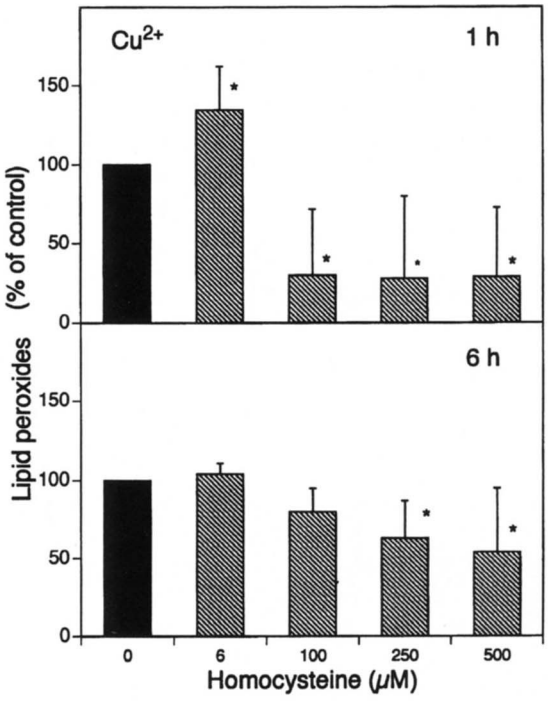

Fig. 1. Amount of lipid peroxides formed in LDL during Cu^{2+}-catalyzed oxidation in the absence or presence of homocysteine. LDL (250 μg/ml) was oxidized at 37°C with 5 μM Cu^{2+} in the absence (filled bar) and in the presence of 6–500 μM homocysteine (hatched bars) for 1 h (upper panel) and 6 h (lower panel). Lipid peroxides were determined as described in Materials and Methods. The data are presented as % of control with no addition of homocysteine and show means ± SD from 4 separate experiments. Absolute values for control LDL (100%) were 35 ± 8, 110 ± 25, and 812 ± 78 nmol lipid peroxides per mg LDL protein after 0, 1, and 6 h, respectively; *, $P < 0.05$.

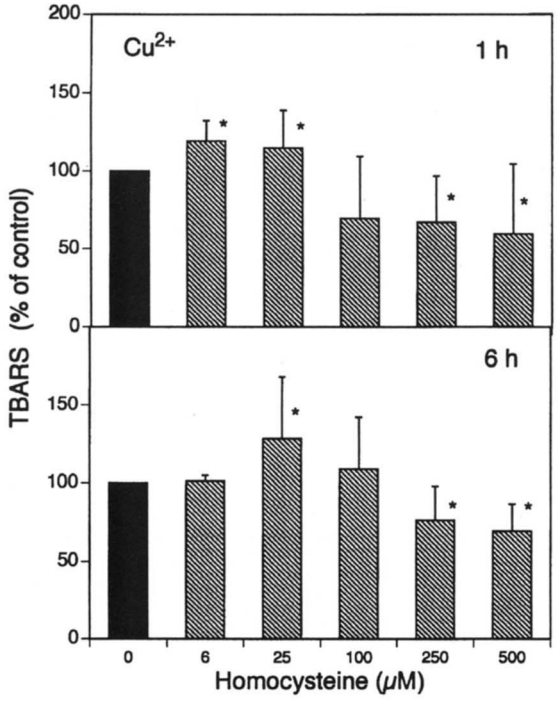

Fig. 2. Amount of TBARS formed in LDL during Cu^{2+}-catalyzed oxidation in the absence or presence of homocysteine. LDL (250 μg/ml) was oxidized at 37°C in the presence of 5 μM Cu^{2+} in the absence (filled bar) and in the presence of 6–500 μM homocysteine (hatched bars) for 1 h (upper panel) and 6 h (lower panel). TBARS were determined as described in Materials and Methods. The data are presented as % of control with no addition of homocysteine and show means ± SD from 5 separate experiments. Absolute values for control LDL (100%) were 10.2 ± 6.6, 28.6 ± 8.9, and 106 ± 16 nmol malondialdehyde equivalents per mg LDL protein after 0, 1, and 6 h, respectively; *, $P < 0.05$.

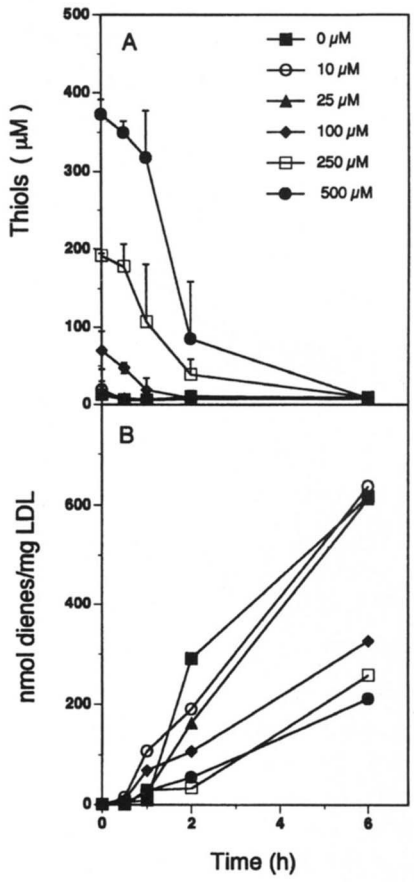

Fig. 3. Changes in the concentration of thiol (panel A), and in the absorbance at 234 nm (panel B), during Cu^{2+}-catalyzed oxidation of LDL in the absence or presence of homocysteine. LDL (250 μg/ml) was incubated at 37°C in PBS containing 5 μM Cu^{2+} and 0–500 μM homocysteine. Aliquots were withdrawn at indicated time points and assayed for thiol concentrations and absorbance at 234 nm as described in Materials and Methods. The data show means ± SD from 3 separate experiments.

Azo compound-initiated oxidation of LDL

To further examine the mechanism by which homocysteine influenced the oxidative modification, LDL was subjected to metal ion-independent oxidation, initiated by the water-soluble azo compound AAPH. The azo compound induces oxidation by a temperature-dependent generation of peroxyl radicals, independent of preformed lipid peroxides in the LDL particles (20). When AAPH was used to initiate oxidation, the changes in optical density at 234 nm showed striking effects of homocysteine (**Fig. 4** and **Table 2**). Thus, the lag time for conjugated diene formation increased with increasing concentrations of homocysteine, from 82.3 min without homocysteine to >200 min in the presence of 100 μM homocysteine. In the presence of 250 and 500 μM homocysteine, the onset of oxidation was totally blocked for at least 240 min (data not shown). Homocysteine at 6 and 10 μM had no significant effect on the rate of formation of conjugated dienes, whereas 25 μM increased the rate from 6.5 ± 1.4 nmol/mg per min to 16.0 ± 6.4 nmol/mg per min. Consistant with the in-

TABLE 1. Effect of homocysteine on the relative electrophoretic mobility (REM) of LDL after Cu^{2+}-, azo compound-, or cell-mediated oxidation for 6 h at 37°C

Homocysteine	Relative Electrophoretic Mobility		
	Cu^{2+}	Azo	Cells
μM			
0	3.2 ± 0.5	3.0 ± 0.6	1.9 ± 0.7
2.5	ND	ND	2.2 ± 0.2
6	3.3 ± 0.4	2.8 ± 0.5	2.0 ± 1.1
25	2.9 ± 0.3	2.6 ± 0.5	1.4 ± 0.4
100	2.8 ± 0.4	2.3 ± 0.3	ND
125	ND	ND	1.1 ± 0.0[a]
250	2.7 ± 0.4	1.0 ± 0.3[a]	1.0 ± 0.0[a]
500	2.4 ± 0.2[a]	1.0 ± 0.3[a]	1.0 ± 0.1[a]

Relative electrophoretic mobility was determined as described in Materials and Methods. Values are given as means ± SD (n = 5, n = 3, and n = 4 for Cu^{2+}-, azo compound-, and cell-mediated oxidation, respectively; ND, not determined.

[a] $P < 0.05$.

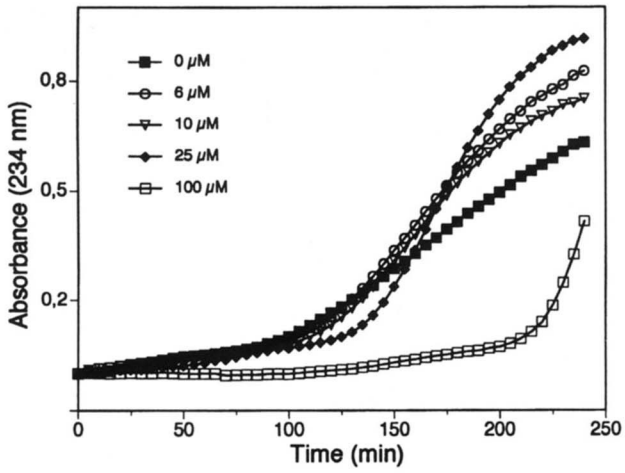

Fig. 4. Formation of conjugated dienes during azo compound-initiated oxidation of LDL in the absence or presence of homocysteine. LDL (25 μg/ml) was incubated at 37°C in PBS containing 1 mM AAPH and 0–100 μM homocysteine. Changes in absorbance at 234 nm were measured every 5 min for 240 min as described in Materials and Methods. The data are means of duplicates from one of 3 representative experiments.

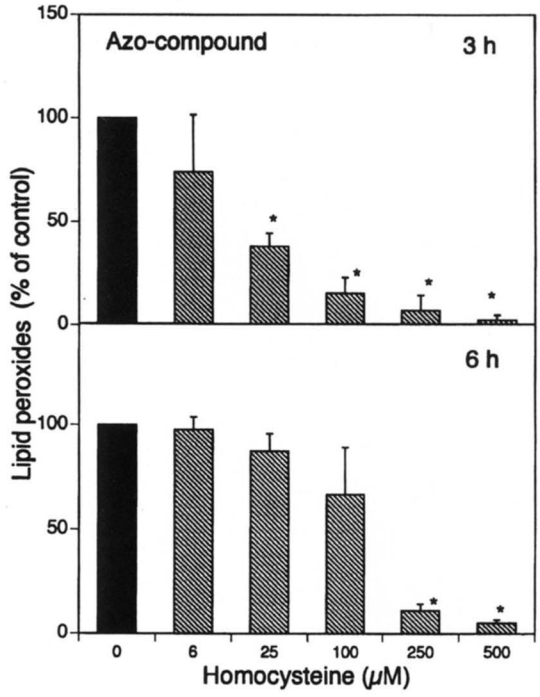

Fig. 5. Amount of lipid peroxides formed in LDL during azo compound-initiated oxidation in the absence or presence of homocysteine. LDL (100 μg/ml) was oxidized at 37°C in the presence of 4 mM AAPH in the absence (filled bar) and in the presence of 6–500 μM homocysteine (hatched bars) for 3 h (upper panel) and 6 h (lower panel). Lipid peroxides were determined as described in Materials and Methods. The data are presented as % of control without homocysteine and show means ± SD from 3 separate experiments with LDL from different donors. Absolute values for control LDL (100%) were 58 ± 5, 560 ± 102, and 1685 ± 122 nmol/mg after 0, 3, and 6 h, respectively; *, $P < 0.05$.

creased lag time, the amounts of lipid peroxides generated during AAPH-induced oxidation were reduced by homocysteine in a concentration-dependent manner (**Fig. 5**). Concentration of 6 μM had no significant effect, whereas 25 and 100 μM reduced the lipid peroxidation after 3 h of incubation. Concentrations of 250 and 500 μM abolished the oxidation in the lipid moiety of LDL after 6 h of incubation. Furthermore, homocysteine at 250 and 500 μM blocked modification in the surface charge of LDL (Table 1). These altered relative electrophoretic mobilities closely paralleled the changes in amounts of lipid peroxides generated.

Cell-mediated oxidation of LDL

To examine the effect of homocysteine in a more physiological system, freshly isolated human peripheral blood mononuclear cells were used to modify LDL for 6 h. Homocysteine at concentrations of 2.5 and 6 μM showed no effect on the oxidation of LDL, whereas concentrations ≥25 μM significantly reduced the generation of lipid peroxides in a dose-dependent manner (**Fig. 6**). Again, homocysteine at concentrations ≥250 μM blocked the modification in the protein moiety of LDL (Table 1). Homocysteine at 1 mM reduced the amount of lipid peroxides formed by >90%, and totally abolished changes in electrophoretic mobility of LDL (data not shown).

Elevated concentrations of homocysteine have been shown to injure endothelial cells through copper-catalyzed generation of H_2O_2 (29, 30). The mononuclear cells were tested for homocysteine-induced cytotoxicity by measuring LDH released to the media during the 6 h oxidation time. LDH in media containing homocysteine at all the concentrations shown in Fig. 6 (2.5–500 μM) ranged from 100.2 ± 5.8% to 103.2 ± 6.6% (n = 3) of that of control media without homocysteine added, whereas homocysteine at a concentration of 1 mM increased the LDH released to 113.0 ± 5.7% (n = 3; $P > 0.05$) of that of control. Thus, it is unlikely that the observed inhibition of cell-mediated LDL oxidation in

TABLE 2. Effect of homocysteine on the lag time and the rate of conjugated dienes formed in LDL during azo compound-induced oxidation

Homocysteine	Lag Time	Formation Rate
μM	*% of control*	
0	100	100
6	119 ± 3[a]	146 ± 36
10	128 ± 2[a]	154 ± 30
25	156 ± 8[a]	240 ± 50[a]

LDL (25 μg/ml) was incubated at 37°C in PBS containing 1 mM AAPH and indicated concentrations of homocysteine. Values are given as the means ± SD from three experiments.
[a] $P < 0.05$.

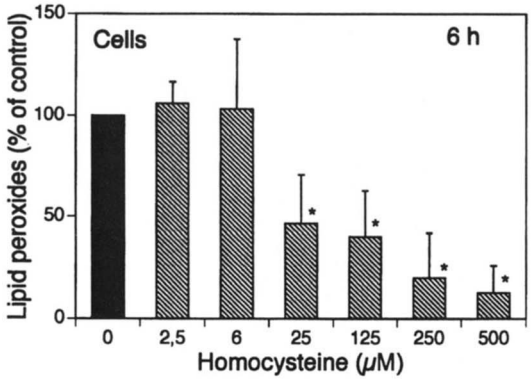

Fig. 6. Formation of lipid peroxides in LDL during human mononuclear cell-mediated oxidation in the absence or presence of homocysteine. LDL (100 μg/ml) and cells (2×10^6/ml) were incubated at 37°C for 6 h in the absence (filled bar) and in the presence of 2.5–500 μM homocysteine (hatched bars). Lipid peroxides were determined as described in Materials and Methods. The data are presented as % of control LDL oxidized in the absence of homocysteine and are given as means ± SD of triplicate cultures from three separate experiments with cells and LDL from different donors. Absolute values for control LDL (100%) were 27 ± 44 and 714 ± 408 nmol/mg after 0 and 6 h, respectively; *, $P < 0.05$.

the presence of homocysteine can be explained by cytotoxic effects of homocysteine.

Scavenging of DPPH-radicals

Free radicals are involved in all the three oxidation systems used in the present study. To examine whether scavenging of free radicals could be one of the mechanisms by which homocysteine exerted its protective effect, the DPPH-radical test was performed, whereby scavenging of free radicals can be followed spectrophotometrically as the non-radical form is produced. A decline in the radical concentration occurred in the presence of homocysteine at concentrations from 50–500 μM (**Fig. 7**). Concentrations of 250 and 500 μM showed nearly complete scavenging after 1 min. In contrast, homocysteine at concentrations of 10 and 25 μM showed no significant radical scavenging properties.

Susceptibility of LDL to lipid peroxidation in patients with homocystinuria

To examine whether the present findings were relevant for patients with homocystinuria, experiments were performed in which oxidation parameters of LDL isolated from two patients with homozygous homocystinuria were compared with those of LDL from our group of healthy controls. In addition, LDL from the male homocystinuria patient, also suffering from familial hypercholesterolemia, was compared with LDL from a subject with familial hypercholesterolemia. Cu^{2+}-catalyzed oxidation of LDL from the patients showed that the lag time, formation rate, and amount of conjugated dienes formed were within the range of mean ± 2 SD for control LDL (**Table 3**). Similarly, the amounts of lipid peroxides and TBARS generated and the relative electrophoretic mobility were within the range of mean ± 2 SD for control LDL (**Table 4**). Also, all the oxidizability parameters measured in LDL from the control patient with familial hypercholesterolemia were similar to those from the male homocystinuria patient. These data suggest that LDL isolated from the patients with homocystinuria was not more susceptible to lipid peroxidation in vitro, as compared to LDL from the healthy control individuals.

DISCUSSION

The present data suggest that homocysteine at concentrations corresponding to normal plasma concentrations has no significant effects on the lipid peroxidation of LDL, whereas moderately elevated and high concentrations of homocysteine may protect LDL against in vitro oxidative modification. One exception was observed; when copper-ions were used to initiate oxidation, homocysteine at concentrations of 6 and 25 μM stimulated the generation of lipid peroxides to a small, but statistically significant extent. This supports the

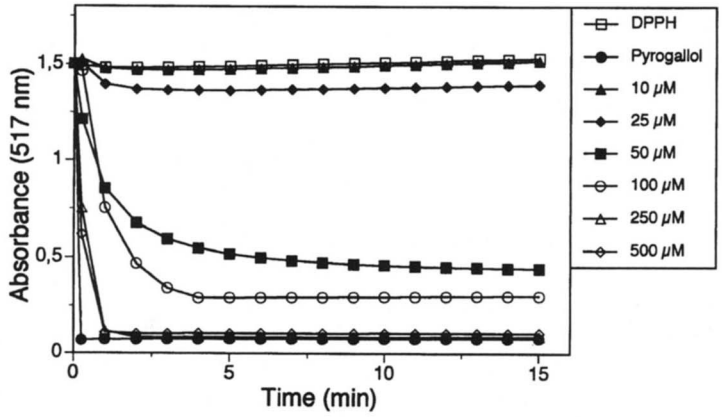

Fig. 7. DPPH radical scavenging by homocysteine. DPPH (45 μg/ml) dissolved in methanol and homocysteine (10–500 μM) were mixed in the cuvettes. Scavenging of DPPH radicals by homocysteine was followed spectrophotometrically by measuring the absorbance at 517 nm every minute for 15 min. DPPH (□) indicates that the absorbance of DPPH without addition of homocysteine was stable for at least 15 min. Pyrogallol, 2% (●) was used as control for 100% scavenging. The data are presented as absorbance at 517 nm, and show means of duplicates from one of three representative experiments.

TABLE 3. Lag time, formation rate, and amount of conjugated dienes formed in LDL during Cu^{2+}-catalyzed oxidation

Subjects		Formation Rate	Conjugated Dienes
	min	*nmol/min/mg LDL*	*nmol/mg LDL*
Patient (female)	22	16	518
Patient (male)	35	23	736
FH patient (male)	41	16	540
Controls	36 ± 7	20 ± 8	643 ± 318

LDL was isolated from a patient with homocystinuria (female), a patient with homocystinuria and FH (male), a patient with FH (male), and from healthy control individuals. LDL (25 μg/ml) was incubated at 37°C in PBS containing 5 μM Cu^{2+}. Values for the patients are given as the means of duplicates from one experiment performed immediately after LDL isolation. Values for controls are given as means ± SD (n = 6). FH, familial hypercholesterolemia.

notion that thiol compounds in the presence of transition metal ions are able to generate free radicals and initiate lipid peroxidation (6). The observed protective effect of homocysteine was shown with all the three oxidation systems used. Furthermore, our data suggest that homocysteine protected LDL against lipid peroxidation as long as the thiol groups were intact, and thereby delayed the onset of the oxidation. The antioxidant effect of homocysteine at high concentrations may be explained by the free radical scavenging properties of homocysteine, as demonstrated by the DPPH radical test and by the finding that homocysteine effectively inhibited the peroxyl radical-initiated lipid peroxidation. Thus, in the presence of copper ions and low concentrations of homocysteine (≤25 μM), the free radicals formed promoted lipid peroxidation, whereas at high concentrations (≥100 μM), homocysteine had the capacity to scavenge the radicals released and, in turn, inhibit lipid peroxidation. Similar concentration-dependent pro-oxidant and antioxidant activities toward LDL oxidation in cell-free systems have recently been reported for aminoguanidine (31). Whether the pro-oxidant nature of low homocysteine concentrations in the presence of transition metal ions is relevant for initiation of LDL oxidation in vivo remains unclear.

The present findings are in contrast to the previously reported pro-oxidant effects of homocysteine on copper- or iron-catalyzed LDL oxidation in cell-free systems (9–11). The reasons for the apparently contradictory data may depend partly on the different experimental conditions applied. Thus, Parthasarathy (9) oxidized LDL in the presence of a very high concentration of homocysteine (1 mM) for 24 h. The oxidation was carried out in Ham's F-10 medium, which contains transition metal ions as well as free cysteine (9). In comparison, we oxidized LDL in the presence of 6–500 μM homocysteine for 0–6 h. When we initiated oxidation by copper ions or the azo compound, the oxidation took place in PBS without EDTA, and for cell-mediated oxidation we used Ham's F-10 medium. Heinecke et al. (10) oxidized LDL in the presence of EDTA (27 μM), to inhibit chain-propagating radical reactions catalyzed by free metal ions. The degree of lipid peroxidation was dependent on the homocysteine concentration (0.01, 0.1, 1.0, or 10 mM), and was maximal at 1 mM (10). In the study from Hirano et al. (11), 0.02, 0.2, and 2 mM homocysteine promoted iron-catalyzed oxidation of LDL in the presence of 5 μM EDTA, whereas no oxidation occurred in the absence of homocysteine. Despite these different experimental conditions described, we

TABLE 4. Formation of lipid peroxides (LPO), thiobarbituric acid-reactive substances (TBARS), and the relative electrophoretic mobility of LDL oxidized for 0, 1, 6, and 24 h at 37°C

	Oxidation Time			
	0 h	1 h	6 h	24 h
LPO (nmol/mg LDL)				
Patient (female)	44	58	517	651
Patient (male)	20	45	714	670
FH patient (male)	10	27	715	751
Controls	35 ± 8	110 ± 25	812 ± 78	643 ± 78
TBARS (nmol MDA/mg LDL)				
Patient (female)	5.8	7.1	48.5	50.8
Patient (male)	8.2	ND	78.4	51.6
FH patient (male)	6.3	ND	77.4	53.4
Controls	10.2 ± 6.6	28.6 ± 8.9	106 ± 16	81.0 ± 10.8
Relative electrophoretic mobility (REM)				
Patient (female)	1.0	1.2	2.1	4.5
Patient (male)	1.0	1.7	2.9	4.1
FH patient (male)	1.0	1.4	2.8	5.4
Controls	1.0	1.3 ± 0.2	3.2 ± 0.5	5.2 ± 0.7

LPO, TBARS, and relative electrophoretic mobility were determined as described in Materials and Methods. For patient identification, see Table 3. Values for the patients are given as the means of duplicates from one experiment run immediately after LDL isolation. Values for controls are given as means ± SD (n = 4, n = 5, and n = 4 separate experiments for LPO, TBARS, and relative electrophoretic mobility, respectively); FH, familial hypercholesterolemia; ND, not determined; MDA, malondialdehyde.

still cannot fully explain the apparently conflicting results.

Cellular oxidation of LDL by arterial smooth muscle cells (32), macrophages, and endothelial cells (24) was dependent on cystine in media containing transition metal ions. Cystine was required for thiol production, giving rise to thiyl radicals and/or active oxygen species (24, 32). Cystine-induced modification of LDL also occurred in Ham's F-10 medium in the absence of cells (9). In contrast, Santanam and Parthasarathy (33) recently reported that cellular cysteine generation did not contribute to initiation of LDL oxidation. However, cysteine enhanced the rate of oxidation of LDL that contained peroxides. They suggested that the use of LDL preparations containing preformed lipid peroxides could account for the previously observed pro-oxidant effects of cysteine and cystine (33). The present antioxidant effect of homocysteine is consistent with this potent inhibitory effect of cysteine on LDL oxidation (33).

After our study was completed, Blom et al. (34) reported that the susceptibility of LDL from hyperhomocysteinemia patients to Cu^{2+}-catalyzed oxidation was similar to that of control subjects, as assessed by measuring conjugated diene production. Blom et al. (12) and Dudman, Wilcken, and Stocker (13) previously concluded that high serum concentrations of homocysteine were not associated with increased lipid peroxidation products, and suggested that homocystinuria could represent a non-lipid model for initiation of atherosclerosis. Alternative mechanisms linking hyperhomocysteinemia with cardiovascular diseases might be vascular endothelial damage, promotion of vascular smooth muscle cell growth, and involvement in a variety of thrombotic complications, like stimulation of procoagulant activities and impairment of endothelial cell thromboresistance (1, 4, 29, 30, 35).

In conclusion, the present in vitro studies suggest that homocysteine at concentrations corresponding to those in patients with homocystinuria may scavenge free radicals, thereby protecting LDL against attacks of free radicals. LDL from two patients with homozygous homocystinuria was not more susceptible to lipid peroxidation than LDL from our group of control subjects. The data do not support the hypothesis that homocysteine is implicated in the premature atherosclerosis via oxidative modification of LDL. The knock-out mice recently generated with moderate and severe homocysteinemia (36) may be a useful animal model for studies on the role of elevated plasma levels of homocysteine in the pathogenesis of atherosclerosis and thrombosis.

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