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Lipid peroxidation and susceptibility of low-density lipoprotein to *in vitro* oxidation in hyperhomocysteinaemia

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Abstract. The pathobiochemical mechanism of arteriosclerosis in hyperhomocysteinaemia has not yet been elucidated. *In vitro* studies have shown that the cytotoxic properties of homocysteine can be ascribed to its generation of reactive oxygen species. We studied lipid peroxidation, both *in vivo* and *in vitro*, in 10 homozygous cystathionine synthase-deficient (CSD) patients and in a control group of 10 healthy subjects of comparable age and sex. The susceptibility of low-density lipoprotein (LDL) from hyperhomocysteinaemic patients to oxidation was determined *in vitro* by continuously measuring the conjugated diene production induced by incubation with copper ions. Oxidation resistance (expressed as lag time), maximal oxidation rate, and extent of oxidation (expressed as total diene production) of LDL from CSD patients were not significantly different from those of LDL from controls. Furthermore, the time needed to reach maximal diene production, i.e. t(max), was similar for LDL from patients and controls. In addition, the vitamin E concentrations in LDL of CSD patients and controls were similar. The mean concentration (± SD) of plasma thiobarbituric acid reactive substances (TBARS), an indicator of *in vivo* lipid peroxidation, was 2·2 ± 0·7 μmol L^{-1} in CSD patients, a lower value than that measured in the matched controls (5·0 ± 2·0 μmol L^{-1}). Investigation of *in vivo* and *in vitro* parameters of lipid peroxidation shows that the increased risk of arteriosclerosis in hyperhomocysteinaemia is unlikely to be due to increased lipid peroxidation.

Keywords. Atherosclerosis, cystathionine synthase deficiency, hyperhomocysteinaemia, lipid peroxidation, low-density lipoprotein.

Abbreviations. CSD, cystathionine β-synthase deficiency; LDL, low-density lipoprotein; TBARS, thiobarbituric acid reactive substances.

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Introduction

Severe hyperhomocysteinaemia caused by cystathionine synthase deficiency (CSD) is a rare autosomal recessive inborn error of the methionine transsulphuration pathway. About 50% of untreated patients suffer from arteriosclerosis or thromboembolisms before 30 years of age [1]. Over the last 10 years it has become evident that mild hyperhomocysteinaemia is also an independent risk factor for vascular disease [2–8], and very recently it has been shown to be a risk factor for obstetric complications such as recurrent spontaneous abortion, gross placental infarcts and neural-tube defects [9,10]. Both severe and mild hyperhomocysteinaemia can be effectively treated with simple regimens of vitamin B_6, folate and betaine [3,11]. Mudd *et al.* [1] were able to demonstrate that homocysteine-lowering therapy reduced the vascular risk of patients with severe hyperhomocysteinaemia due to CSD.

The pathobiochemical mechanism of hyperhomocysteinaemia has not yet been elucidated. Endothelial damaging effects of homocysteine have been demonstrated *in vitro* and in animal studies (for reviews *see* [3,6,7]). Cultured endothelial cells are damaged by exposure to high concentrations of homocysteine, most probably because of hydrogen peroxide formation [12]. Furthermore, other thiol-containing amino acids, e.g. cysteine, could induce oxygen radical production, leading to low density lipoprotein (LDL) modification [13]. Oxidative modification of LDL is thought to constitute the link between increased plasma cholesterol concentrations and atherosclerosis [14–16]. Reactive oxygen species, such as superoxide, hydrogen peroxide and the hydroxyl radical, can be formed by auto-oxidation of thiol groups [12,13]. Superoxide and hydrogen peroxide are relatively benign compared to the highly reactive hydroxyl radical. The latter is only generated in the presence of transition metals such as Fe^{3+} and Cu^{2+} [17], which is of interest with regard to the elevated blood levels of copper and other metals in CSD patients [18,19].

Table 1. Homocysteine and methionine concentrations in plasma and homocysteine-lowering therapy in cystathionine β synthase-deficient patients

Patient	Homocysteine	Methionine	Therapy
E51	18	24	B_6, B_{12}
E53	74	20	B_6
U58	16	21	B_6, fol
D66	139	82	B_6, fol, bet
D67	222	113	B_6, fol, bet
B58	195	82	B_6, fol, bet
B61	210	81	B_6, fol, bet
L75	85	57	B_6, fol, bet, B_{12}
K61	244	52	No therapy
M62	172	99	B_6
Control ($n = 10$)			
Mean ± SD	12 ± 2	26 ± 5	

Concentrations are expressed as μmol L^{-1}. B_6, 750 mg pyridoxine per day administered orally; fol, 5 mg folate per day administered orally; bet, 6 g betaine per day administered orally; B_{12}, 1 mg vitamin B_{12} per month administered intramuscularly.

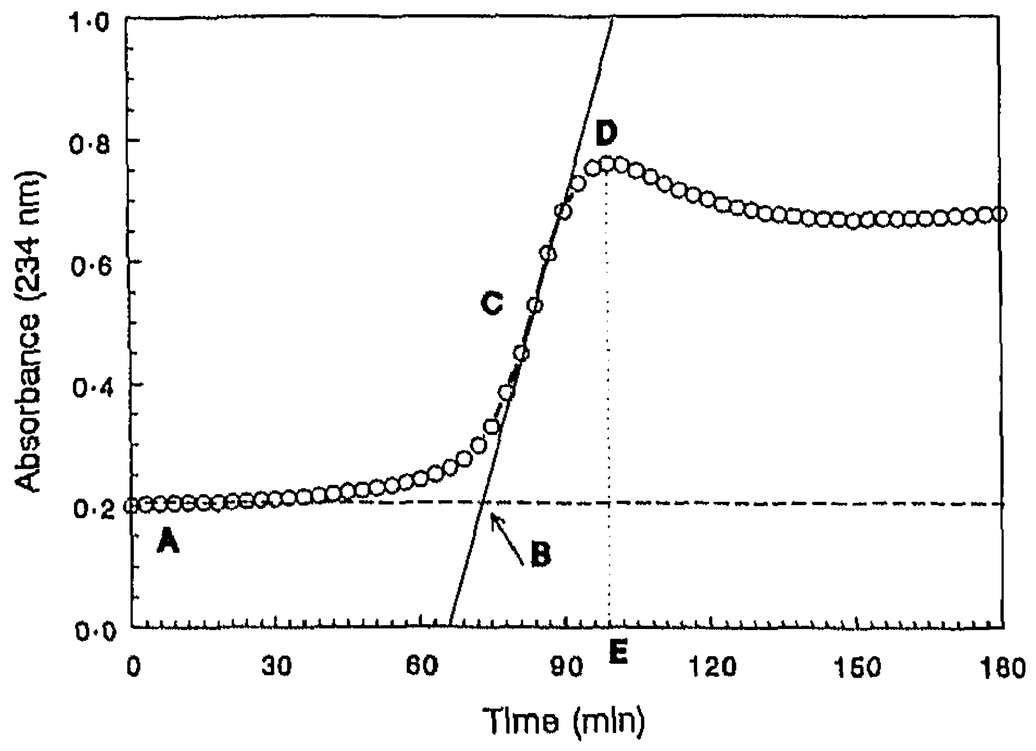

Figure 1. Kinetics of LDL oxidation as determined by measurement of the change in conjugated diene absorbance at 234 nm. The following indexes of LDL oxidizability were determined: diene production (D minus A), lag time (B), maximal rate of oxidation (C), and t(max), i.e. the time needed to reach maximal diene production (E) (from [24]).

In a previous short communication we reported normal concentrations of malondialdehyde and fluorescent lipid peroxidation products in serum of CSD patients [20]. The disadvantage of using serum for measuring lipid peroxidation products has been discussed previously [21,22]. In the present study, we investigated indications of lipid peroxidation *in vivo* and *in vitro* in EDTA-plasma of hyperhomocysteinaemic CSD patients. To our knowledge, studies on the *in vitro* susceptibility to oxidation of LDL from hyperhomocystenaemic patients have not been performed before.

Materials and methods

Patients

Homozygosity for CSD was suspected in the 10 patients (nine males and one female; mean age ± SD, 30·1 ± 6·8 years) on the basis of typical clinical symptoms such as arteriosclerosis, thrombosis, mental retardation, marfanoid features or ectopia lentis, and was confirmed biochemically by severe hyperhomocysteinaemia and (in most cases) elevated methionine and decreased cysteine concentrations in plasma, and by very low cystathionine synthase activity (<10% of that in controls) in cultured fibroblasts. At the time of the present investigation, all patients but one (K61) were on therapy, which included pyridoxine (750 mg daily, administered orally) and in some cases folate (5 mg daily, administered orally), betaine (5 g daily, administered orally) or vitamin B_{12} (1 mg per month, administered intramuscularly) (Table 1). Due to therapy, two patients (E51 and U58) achieved normal homocysteine levels. No other vitamins and no antioxidants were prescribed, and the patients had a normal diet. A control group of 10 healthy subjects (eight males and two females) of mean age (± SD) 34·9 ± 6·0 years was selected for this study. None of the controls used any vitamins or medication. Antecubital venous blood samples were collected in the fasting state in EDTA-containing vials.

Amino acid analysis

Red cells were separated from the plasma within 30 min and the plasma was stored at −20°C until analysis. Total homocysteine was measured in EDTA-containing plasma as described by Fiskerstrand *et al.* [23], with some minor modifications. Methionine concentrations in plasma were measured by ion-exchange chromatography on an automatic amino-acid analyser (LKB Alpha-plus, Pharmacia, Woerden, The Netherlands).

LDL isolation and in vitro *oxidation*

Plasma was separated within 30 min and stored at −80°C in the presence of 6 g saccharose L^{-1}. Low-density lipoproteins were isolated by a short-run ultracentrifugation method and were thereafter tested for their susceptibility to *in vitro* copper-induced LDL oxidation [24]. Briefly, after isolation, the LDL was dialysed for 24 h in the dark at 4°C against phosphate-buffered saline (pH 7·4), containing 10 μmol L^{-1} EDTA. The buffer was made oxygen-free by vacuum degassing followed by purging with nitrogen. The LDL-containing sample was filtered through a 0·45 μm filter and diluted with dialysis buffer to a final concentration of 0·05 mg LDL-protein mL^{-1} and 10 μmol L^{-1} EDTA. The oxidation was initiated by the addition of a freshly prepared solution (15 μmol L^{-1}) of copper chloride ($CuCl_2$). The kinetics of LDL oxidation were determined by monitoring the change in 234 nm diene absorption on

a UV-spectrophotometer (Lambda 5, Perkin-Elmer, Norwalk, CT, USA), equipped with a six-position automatic sample changer, at 30°C. The change of absorbance at 234 nm vs. time was divided into three consecutive phases, i.e. a lag phase, a propagation phase and a decomposition phase (Fig. 1). Lag time, maximal rate of oxidation, total amount of conjugated dienes formed, and the time needed to reach maximal diene production were calculated as described previously [24], and despite a small increase in the free $CuCl_2$ concentration, identical kinetics of LDL oxidation were observed [24].

In vivo *lipid peroxidation*

The content of thiobarbituric acid reactive substances (TBARS), mainly malondialdehyde (MDA), in plasma was measured by recording the fluorescence spectrum of the thiobarbituric acid-MDA complex between 500 and 600 nm on a Shimadzu RFF-500 fluorescence detector (Shimadzu Corporation, Kyoto, Japan), keeping a constant interval of 14 nm between excitation and emission wavelengths. The fluorescence intensity was measured at 553 nm after subtracting the baseline value (due to the Rayleigh diffusion). This synchronous fluorescence method was found to be free from interfering compounds [25].

Other methods

Cholesterol and triglyceride concentrations in plasma were determined by enzymatic methods (Boehringer-Mannheim, Mannheim, Germany). Plasma HDL-cholesterol was determined by the polyethyleneglycol 6000 method [26]. LDL-cholesterol in plasma was calculated by subtracting the sum of HDL-cholesterol and 0·42 × triglycerides from total plasma cholesterol.

The vitamin E concentrations in LDL were determined by high performance liquid chromatography (HPLC) as described previously [27]. LDL fatty acid composition was measured as described previously [27], and was expressed as a percentage of total fatty acids.

Statistical analysis

The results are expressed as mean values ± SD. Statistical evaluation of the data was performed using Student's *t*-test. Pearson correlation coefficients were computed to determine correlations between homocysteine concentrations and oxidation parameters. A two-tailed *P*-value of less than 0·05 was considered to be statistically significant.

Results

Plasma homocysteine and methionine

The homozygous CSD patients exhibited a wide range of total homocysteine concentrations in plasma, from virtually normal levels up to 244 μmol L^{-1} (Table 1). The mean total homocysteine concentration (± SD) in plasma of the control group was 12 ± 2 μmol L^{-1}. Plasma methionine levels in the patient group ranged from 20 to 161 μmol L^{-1}, and the mean concentration (± SD) for the control group was 26 ± 5 μmol L^{-1}.

Plasma lipids and fatty acid composition of LDL

Plasma total cholesterol (5·2 ± 1·0 mmol L^{-1}), triglycerides (0·93 ± 0·41 mmol L^{-1}), LDL-cholesterol (3·33 ± 1·01 mmol L^{-1}) and HDL-cholesterol (1·31 ± 0·23 mmol L^{-1}) concentrations in the hyperhomocysteinaemic patients were all normal [28]. In the control group the total cholesterol concentration was 6·0 ± 2·1 mmol L^{-1} and the triglyceride concentration was 0·99 ± 0·40 mmol L^{-1}.

The fatty acid composition of LDL in the hyperhomocysteinaemic patients was also normal: arachidonic acid (20 : 4), 5·1 ± 1·2%; linoleic acid (18 : 2). 40·2 ± 4·9%; oelic acid (18 : 1), 21·1 ± 3·4%; stearic acid (18 : 0), 9·0 ± 3·3%; and palmitic acid (16 : 0), 24·5 ± 1·3%. Control values were as follows: arachidonic acid (20 : 4), 6·2 ± 1·4%; linoleic acid (18 : 2), 40·0 ± 3·7%; oleic acid (18 : 1), 19·3 ± 3·0%; stearic acid (18 : 0), 8·4 ± 1·8%; and palmitic acid (16 : 0), 26·1 ± 2·3%.

Susceptibility to oxidation of low-density lipoprotein and LDL vitamin E content

The susceptibility to oxidation of LDL from hyperhomocysteinaemic patients was determined *in vitro* by continuously measuring the conjugated diene production induced by incubation with copper (Fig. 1). Oxidation resistance (expressed as lag time), maximal oxidation rate, and extent of oxidation (expressed as total diene production) of LDL from CSD patients were not significantly different from control values (Table 2). In addition, the time taken to reach maximal diene production, t(max), was similar for LDL from patients and controls. No correlations were observed between plasma homocysteine concentrations and biochemical LDL oxidizability indexes.

The vitamin E content of LDL was also determined, since vitamin E is quantitatively and qualitatively the most important endogenous antioxidant in LDL. Decreased levels of vitamin E might be indicative of increased *in vivo* exposure to oxidative stress. However, the vitamin E concentrations of LDL did not differ significantly between controls and CSD patients (Table 2). No significant correlation was observed between the concentration of vitamin E in LDL and the plasma homocysteine concentration.

Lipid peroxidation

TBARS are products of peroxidation of unsaturated

Table 2. Individual low-density lipoprotein (LDL) oxidizability indexes and LDL vitamin E concentrations in cystathionine synthase-deficient patients and controls

Patient number	Lag time (min)	Oxidation rate (nmol min^{-1} mg^{-1} LDL-protein)	Diene production (nmol mg^{-1}) LDL-protein)	t(max) (min)	Vitamin E ($mg\,g^{-1}$) LDL-protein)
E51	133	11·75	654	213	5·32
E53	125	8·81	528	210	5·92
U58	112	9·49	464	183	4·29
D66	134	9·72	533	213	4·70
D67	126	9·27	532	207	4·93
B58	133	5·20	297	204	2·91
B61	121	5·88	292	192	3·04
L75	148	9·49	574	237	5·59
K61	119	10·62	580	198	5·06
M61	141	9·04	536	225	5·81
Mean	129	8·9	499	208	4·76
SD	9	2·0	118	17	1·06
Control ($n = 10$) ($n = 10$)	129	9·8	540	209	4·04
SD	16	1·9	68	21	1·34

fatty acids. The mean plasma TBARS concentration (± SD) in CSD patients was 2·2 ± 0·7 μmol ($n = 11$). The TBARS concentration in the matched controls was significantly higher (5·0 ± 2·0 μmol L^{-1}; $n = 12$, $P < 0·001$). No significant correlation was observed between the concentrations of homocysteine and TBARS in plasma of the hyperhomocysteinaemic patients.

Discussion

The susceptibility to *in vitro* oxidation of LDL from patients with hyperhomocysteinaemia due to CSD was not increased compared to that of LDL from controls. Furthermore, the LDL vitamin E content was not decreased in these CSD patients, and the plasma TBARS concentration, indicative of *in vivo* lipid peroxidation, was even lower in the CSD patients than in controls. Even in patients with very high homocysteine concentrations (patients D67, B61 and K61), LDL oxidizability and plasma TBARS concentration were not increased, and the vitamin E content of LDL was not decreased. These data are in agreement with our previous data on malondialdehyde and fluorescent lipid peroxidation products in the serum of hyperhomocysteinaemic patients [20], and argue against the view that lipid peroxidation acts as a mediator in the vascular damaging effect of homocysteine. Many *in vitro* studies (for reviews, *see* [3,6,7]) have described endothelial damaging properties of homocysteine mediated through the production of reactive oxygen species. In virtually all of these studies, applied very high and non-physiological concentrations of 1 to 10 mM homocysteine, i.e. the reduced form. This is extremely unphysiological because almost all homocysteine in the blood is present in its oxidized disulphide form, either as homocystine or in combination with other thiol-group-containing biomolecules, such as cysteine and proteins. Homocysteine in its reduced form is only present in small amounts (typically 1% or even less of the total pool of homocysteine present in the blood [29]), which argues against homocysteine-mediated lipid peroxidation in hyperhomocysteinaemia. Our observation that plasma TBARS concentrations are even decreased in hyperhomocysteinaemic patients is in agreement with the recent findings of Dudman *et al.* [30]. They also reported in CSD patients decreased concentrations of lipid peroxidation products, in their case reduced levels of high-density lipoprotein cholesteryl ester hydroperoxides, and an elevated ubiquinol-10/ubiquinone-10 ratio. Taking these results together, it seems unlikely that hyperhomocysteinaemia induces lipid peroxidation in the bloodstream of CSD patients.

Amino acids or peptides with a free thiol moiety, including cysteine and glutathione, are able to generate reactive oxygen species through an auto-oxidation process [31]. Thiol auto-oxidation is likely to occur in the presence of trace amounts of Fe^{3+} or Cu^{2+}, resulting in a Fenton-type reaction, with ensuing radical production [17]. Despite this, thiol compounds are not known to cause vascular damage *in vivo*. Compounds with a free thiol function, such as N-acetylcysteine and other glutathione precursors, are even thought to increase glutathione concentrations, thereby providing increased availability of the substrate for the intracellular enzyme glutathione peroxidase, which detoxifies hydrogen peroxide and lipid peroxides [32]. Clearly, thiol functions have ambivalent characteristics with regard to scavenging and generation of reactive oxygen species, as we have recently shown for N-acetylcysteine [33].

The absence of a correlation between plasma homocysteine concentration and indices of lipid peroxidation suggests that other pathobiochemical

mechanisms are responsible for the association between hyperhomocysteinaemia and atherosclerosis. The enhanced binding of lipoprotein(a) to fibrin, induced by sulphydryl compounds, including homocysteine, might be an alternative explanation linking thrombosis and atherosclerosis with hyperhomocysteinaemia [34]. Also intriguing are the recently described effects of homocysteine on the endothelium-derived relaxing factor nitric oxide [35], and the relationship between homocysteine and disturbance of fibrinolysis [36].

In conclusion, although several parameters of lipid peroxidation, both *in vivo* and *in vitro*, were measured, no evidence was obtained that the increased risk of arteriosclerosis in hyperhomocysteinaemia can be explained by increased lipid peroxidation. It seems more likely that another mechanism, unrelated to lipid peroxidation, is involved in vascular disease due to hyperhomocysteinaemia.

Acknowledgments

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Abbreviations. CSD, cystathionine β-synthase deficiency; LDL, low-density lipoprotein; TBARS, thiobarbituric acid reactive substances.

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Introduction

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Mean ± SD	12 ± 2	26 ± 5	

Concentrations are expressed as μmol L^{-1}. B_6, 750 mg pyridoxine per day administered orally; fol, 5 mg folate per day administered orally; bet, 6 g betaine per day administered orally; B_{12}, 1 mg vitamin B_{12} per month administered intramuscularly.

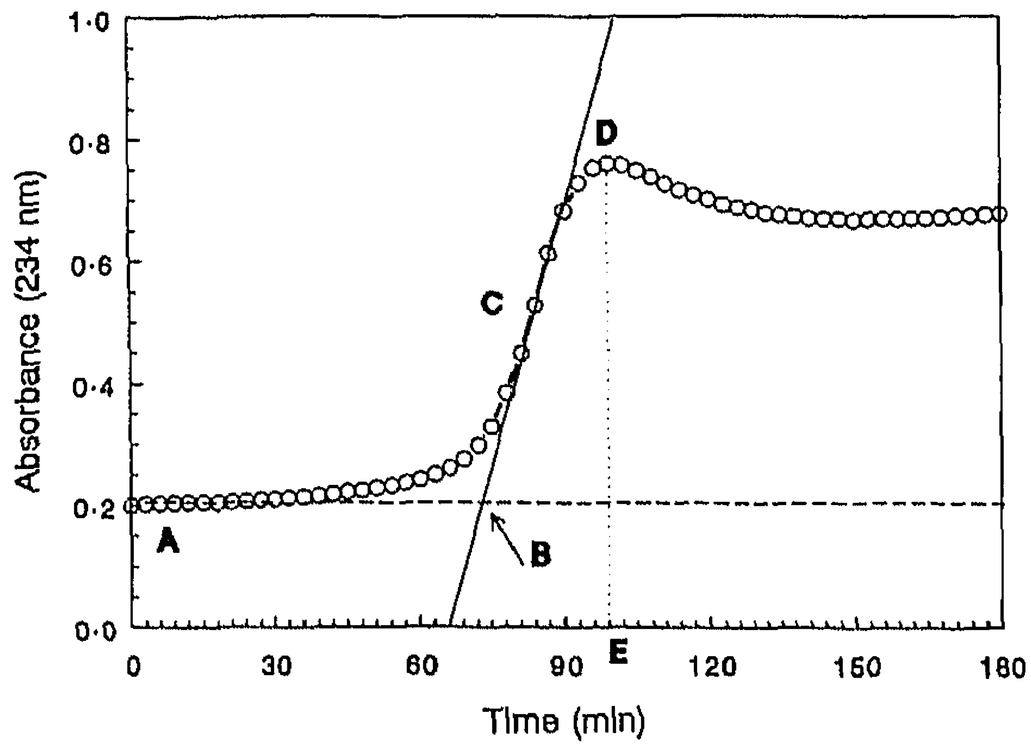

Figure 1. Kinetics of LDL oxidation as determined by measurement of the change in conjugated diene absorbance at 234 nm. The following indexes of LDL oxidizability were determined: diene production (D minus A), lag time (B), maximal rate of oxidation (C), and t(max), i.e. the time needed to reach maximal diene production (E) (from [24]).

In a previous short communication we reported normal concentrations of malondialdehyde and fluorescent lipid peroxidation products in serum of CSD patients [20]. The disadvantage of using serum for measuring lipid peroxidation products has been discussed previously [21,22]. In the present study, we investigated indications of lipid peroxidation *in vivo* and *in vitro* in EDTA-plasma of hyperhomocysteinaemic CSD patients. To our knowledge, studies on the *in vitro* susceptibility to oxidation of LDL from hyperhomocystenaemic patients have not been performed before.

Materials and methods

Patients

Homozygosity for CSD was suspected in the 10 patients (nine males and one female; mean age ± SD, 30·1 ± 6·8 years) on the basis of typical clinical symptoms such as arteriosclerosis, thrombosis, mental retardation, marfanoid features or ectopia lentis, and was confirmed biochemically by severe hyperhomocysteinaemia and (in most cases) elevated methionine and decreased cysteine concentrations in plasma, and by very low cystathionine synthase activity (<10% of that in controls) in cultured fibroblasts. At the time of the present investigation, all patients but one (K61) were on therapy, which included pyridoxine (750 mg daily, administered orally) and in some cases folate (5 mg daily, administered orally), betaine (5 g daily, administered orally) or vitamin B_{12} (1 mg per month, administered intramuscularly) (Table 1). Due to therapy, two patients (E51 and U58) achieved normal homocysteine levels. No other vitamins and no antioxidants were prescribed, and the patients had a normal diet. A control group of 10 healthy subjects (eight males and two females) of mean age (± SD) 34·9 ± 6·0 years was selected for this study. None of the controls used any vitamins or medication. Antecubital venous blood samples were collected in the fasting state in EDTA-containing vials.

Amino acid analysis

Red cells were separated from the plasma within 30 min and the plasma was stored at −20°C until analysis. Total homocysteine was measured in EDTA-containing plasma as described by Fiskerstrand *et al.* [23], with some minor modifications. Methionine concentrations in plasma were measured by ion-exchange chromatography on an automatic amino-acid analyser (LKB Alpha-plus, Pharmacia, Woerden, The Netherlands).

LDL isolation and in vitro *oxidation*

Plasma was separated within 30 min and stored at −80°C in the presence of 6 g saccharose L^{-1}. Low-density lipoproteins were isolated by a short-run ultracentrifugation method and were thereafter tested for their susceptibility to *in vitro* copper-induced LDL oxidation [24]. Briefly, after isolation, the LDL was dialysed for 24 h in the dark at 4°C against phosphate-buffered saline (pH 7·4), containing 10 μmol L^{-1} EDTA. The buffer was made oxygen-free by vacuum degassing followed by purging with nitrogen. The LDL-containing sample was filtered through a 0·45 μm filter and diluted with dialysis buffer to a final concentration of 0·05 mg LDL-protein mL^{-1} and 10 μmol L^{-1} EDTA. The oxidation was initiated by the addition of a freshly prepared solution (15 μmol L^{-1}) of copper chloride ($CuCl_2$). The kinetics of LDL oxidation were determined by monitoring the change in 234 nm diene absorption on

a UV-spectrophotometer (Lambda 5, Perkin-Elmer, Norwalk, CT, USA), equipped with a six-position automatic sample changer, at 30°C. The change of absorbance at 234 nm vs. time was divided into three consecutive phases, i.e. a lag phase, a propagation phase and a decomposition phase (Fig. 1). Lag time, maximal rate of oxidation, total amount of conjugated dienes formed, and the time needed to reach maximal diene production were calculated as described previously [24], and despite a small increase in the free $CuCl_2$ concentration, identical kinetics of LDL oxidation were observed [24].

In vivo *lipid peroxidation*

The content of thiobarbituric acid reactive substances (TBARS), mainly malondialdehyde (MDA), in plasma was measured by recording the fluorescence spectrum of the thiobarbituric acid-MDA complex between 500 and 600 nm on a Shimadzu RFF-500 fluorescence detector (Shimadzu Corporation, Kyoto, Japan), keeping a constant interval of 14 nm between excitation and emission wavelengths. The fluorescence intensity was measured at 553 nm after subtracting the baseline value (due to the Rayleigh diffusion). This synchronous fluorescence method was found to be free from interfering compounds [25].

Other methods

Cholesterol and triglyceride concentrations in plasma were determined by enzymatic methods (Boehringer-Mannheim, Mannheim, Germany). Plasma HDL-cholesterol was determined by the polyethyleneglycol 6000 method [26]. LDL-cholesterol in plasma was calculated by subtracting the sum of HDL-cholesterol and 0·42 × triglycerides from total plasma cholesterol.

The vitamin E concentrations in LDL were determined by high performance liquid chromatography (HPLC) as described previously [27]. LDL fatty acid composition was measured as described previously [27], and was expressed as a percentage of total fatty acids.

Statistical analysis

The results are expressed as mean values ± SD. Statistical evaluation of the data was performed using Student's *t*-test. Pearson correlation coefficients were computed to determine correlations between homocysteine concentrations and oxidation parameters. A two-tailed *P*-value of less than 0·05 was considered to be statistically significant.

Results

Plasma homocysteine and methionine

The homozygous CSD patients exhibited a wide range of total homocysteine concentrations in plasma, from virtually normal levels up to 244 μmol L^{-1} (Table 1). The mean total homocysteine concentration (± SD) in plasma of the control group was 12 ± 2 μmol L^{-1}. Plasma methionine levels in the patient group ranged from 20 to 161 μmol L^{-1}, and the mean concentration (± SD) for the control group was 26 ± 5 μmol L^{-1}.

Plasma lipids and fatty acid composition of LDL

Plasma total cholesterol (5·2 ± 1·0 mmol L^{-1}), triglycerides (0·93 ± 0·41 mmol L^{-1}), LDL-cholesterol (3·33 ± 1·01 mmol L^{-1}) and HDL-cholesterol (1·31 ± 0·23 mmol L^{-1}) concentrations in the hyperhomocysteinaemic patients were all normal [28]. In the control group the total cholesterol concentration was 6·0 ± 2·1 mmol L^{-1} and the triglyceride concentration was 0·99 ± 0·40 mmol L^{-1}.

The fatty acid composition of LDL in the hyperhomocysteinaemic patients was also normal: arachidonic acid (20 : 4), 5·1 ± 1·2%; linoleic acid (18 : 2). 40·2 ± 4·9%; oelic acid (18 : 1), 21·1 ± 3·4%; stearic acid (18 : 0), 9·0 ± 3·3%; and palmitic acid (16 : 0), 24·5 ± 1·3%. Control values were as follows: arachidonic acid (20 : 4), 6·2 ± 1·4%; linoleic acid (18 : 2), 40·0 ± 3·7%; oleic acid (18 : 1), 19·3 ± 3·0%; stearic acid (18 : 0), 8·4 ± 1·8%; and palmitic acid (16 : 0), 26·1 ± 2·3%.

Susceptibility to oxidation of low-density lipoprotein and LDL vitamin E content

The susceptibility to oxidation of LDL from hyperhomocysteinaemic patients was determined *in vitro* by continuously measuring the conjugated diene production induced by incubation with copper (Fig. 1). Oxidation resistance (expressed as lag time), maximal oxidation rate, and extent of oxidation (expressed as total diene production) of LDL from CSD patients were not significantly different from control values (Table 2). In addition, the time taken to reach maximal diene production, t(max), was similar for LDL from patients and controls. No correlations were observed between plasma homocysteine concentrations and biochemical LDL oxidizability indexes.

The vitamin E content of LDL was also determined, since vitamin E is quantitatively and qualitatively the most important endogenous antioxidant in LDL. Decreased levels of vitamin E might be indicative of increased *in vivo* exposure to oxidative stress. However, the vitamin E concentrations of LDL did not differ significantly between controls and CSD patients (Table 2). No significant correlation was observed between the concentration of vitamin E in LDL and the plasma homocysteine concentration.

Lipid peroxidation

TBARS are products of peroxidation of unsaturated

Table 2. Individual low-density lipoprotein (LDL) oxidizability indexes and LDL vitamin E concentrations in cystathionine synthase-deficient patients and controls

Patient number	Lag time (min)	Oxidation rate (nmol min^{-1} mg^{-1} LDL-protein)	Diene production (nmol mg^{-1}) LDL-protein)	t(max) (min)	Vitamin E ($mg\,g^{-1}$) LDL-protein)
E51	133	11·75	654	213	5·32
E53	125	8·81	528	210	5·92
U58	112	9·49	464	183	4·29
D66	134	9·72	533	213	4·70
D67	126	9·27	532	207	4·93
B58	133	5·20	297	204	2·91
B61	121	5·88	292	192	3·04
L75	148	9·49	574	237	5·59
K61	119	10·62	580	198	5·06
M61	141	9·04	536	225	5·81
Mean	129	8·9	499	208	4·76
SD	9	2·0	118	17	1·06
Control ($n = 10$)	129	9·8	540	209	4·04
($n = 10$)					
SD	16	1·9	68	21	1·34

fatty acids. The mean plasma TBARS concentration (± SD) in CSD patients was 2·2 ± 0·7 μmol ($n = 11$). The TBARS concentration in the matched controls was significantly higher (5·0 ± 2·0 μmol L^{-1}; $n = 12$, $P < 0·001$). No significant correlation was observed between the concentrations of homocysteine and TBARS in plasma of the hyperhomocysteinaemic patients.

Discussion

The susceptibility to *in vitro* oxidation of LDL from patients with hyperhomocysteinaemia due to CSD was not increased compared to that of LDL from controls. Furthermore, the LDL vitamin E content was not decreased in these CSD patients, and the plasma TBARS concentration, indicative of *in vivo* lipid peroxidation, was even lower in the CSD patients than in controls. Even in patients with very high homocysteine concentrations (patients D67, B61 and K61), LDL oxidizability and plasma TBARS concentration were not increased, and the vitamin E content of LDL was not decreased. These data are in agreement with our previous data on malondialdehyde and fluorescent lipid peroxidation products in the serum of hyperhomocysteinaemic patients [20], and argue against the view that lipid peroxidation acts as a mediator in the vascular damaging effect of homocysteine. Many *in vitro* studies (for reviews, *see* [3,6,7]) have described endothelial damaging properties of homocysteine mediated through the production of reactive oxygen species. In virtually all of these studies, applied very high and non-physiological concentrations of 1 to 10 mM homocysteine, i.e. the reduced form. This is extremely unphysiological because almost all homocysteine in the blood is present in its oxidized disulphide form, either as homocystine or in combination with other thiol-group-containing biomolecules, such as cysteine and proteins. Homocysteine in its reduced form is only present in small amounts (typically 1% or even less of the total pool of homocysteine present in the blood [29]), which argues against homocysteine-mediated lipid peroxidation in hyperhomocysteinaemia. Our observation that plasma TBARS concentrations are even decreased in hyperhomocysteinaemic patients is in agreement with the recent findings of Dudman *et al.* [30]. They also reported in CSD patients decreased concentrations of lipid peroxidation products, in their case reduced levels of high-density lipoprotein cholesteryl ester hydroperoxides, and an elevated ubiquinol-10/ubiquinone-10 ratio. Taking these results together, it seems unlikely that hyperhomocysteinaemia induces lipid peroxidation in the bloodstream of CSD patients.

Amino acids or peptides with a free thiol moiety, including cysteine and glutathione, are able to generate reactive oxygen species through an auto-oxidation process [31]. Thiol auto-oxidation is likely to occur in the presence of trace amounts of Fe^{3+} or Cu^{2+}, resulting in a Fenton-type reaction, with ensuing radical production [17]. Despite this, thiol compounds are not known to cause vascular damage *in vivo*. Compounds with a free thiol function, such as N-acetylcysteine and other glutathione precursors, are even thought to increase glutathione concentrations, thereby providing increased availability of the substrate for the intracellular enzyme glutathione peroxidase, which detoxifies hydrogen peroxide and lipid peroxides [32]. Clearly, thiol functions have ambivalent characteristics with regard to scavenging and generation of reactive oxygen species, as we have recently shown for N-acetylcysteine [33].

The absence of a correlation between plasma homocysteine concentration and indices of lipid peroxidation suggests that other pathobiochemical

mechanisms are responsible for the association between hyperhomocysteinaemia and atherosclerosis. The enhanced binding of lipoprotein(a) to fibrin, induced by sulphydryl compounds, including homocysteine, might be an alternative explanation linking thrombosis and atherosclerosis with hyperhomocysteinaemia [34]. Also intriguing are the recently described effects of homocysteine on the endothelium-derived relaxing factor nitric oxide [35], and the relationship between homocysteine and disturbance of fibrinolysis [36].

In conclusion, although several parameters of lipid peroxidation, both *in vivo* and *in vitro*, were measured, no evidence was obtained that the increased risk of arteriosclerosis in hyperhomocysteinaemia can be explained by increased lipid peroxidation. It seems more likely that another mechanism, unrelated to lipid peroxidation, is involved in vascular disease due to hyperhomocysteinaemia.

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