Redox Status and Protein Binding of Plasma Homocysteine and Other Aminothiols in Patients With Homocystinuria

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Elevations of homocyst(e)ine levels in the blood of patients with homocystinuria may explain the high cardiovascular morbidity. We determined levels of reduced, oxidized, and protein-bound homocyst(e)ine, cyst(e)ine, and cyst(e)inylglycine in plasma from eight patients with homocystinuria. The technique used involved trapping of reduced thiols by collecting blood directly into tubes containing sulfhydryl-reactive reagents. All patients had high levels of homocysteine (range, 1.9 to 91.2 μmol/L), and among the aminothiols investigated, this species showed the most drastic elevation compared with trace levels (<0.4 μmol/L) found in healthy subjects. The ratio between free homocysteine and total homocyst(e)ine (reduced to total ratio) was above normal and positively correlated to the reduced to total ratio for cyst(e)ine, suggesting that an equilibrium exists between these species through sulfhydryl disulfide exchange. The other homocyst(e)ine species (oxidized and protein-bound) were also markedly increased in patients with homocystinuria. Plasma cysteine and cysteinylglycine levels were moderately increased, whereas plasma concentrations of protein-bound cyst(e)ine, protein-bound cyst(e)inylglycine, and free cystine were below normal. Homocysteine in particular and other homocyst(e)ine species are markedly increased in plasma of homocystinurics, and these changes are associated with pronounced alterations in the level and the redox status of other aminothiols. This should be taken into account when considering homocyst(e)ine as an atherogenic agent, and the role of various homocyst(e)ine species in the pathogenesis of homocystinuria.

HOMOCYSTINURIA is a class of metabolic disorders characterized by excretion of large amounts of the sulfur amino acid homocystine in the urine. Cystathionine β-synthase deficiency is the most frequently encountered cause of homocystinuria, but rare forms due to defects of homocyst(e)ine remethylation have also been described.[1]

The major cause of death in patients with cystathionine β-synthase deficiency is premature vascular disease, which may occur in any vessel at any age.[1] Notably, early vascular disease also has occurred in patients with impaired homocyst(e)ine remethylation.[2] This observation suggests that the vascular lesions are caused by homocyst(e)ine itself or a derivative, and has led to the formulation of the homocyst(e)ine theory of arteriosclerosis.[3]

The total homocyst(e)ine level in blood may reach several hundred micromolar in patients with homocystinuria,[1] and this far exceeds the plasma concentration (~10 μmol/L[4]) in healthy subjects. Free and bound forms of homocyst(e)ine have been identified in plasma from both patients with homocystinuria and normal subjects. In healthy persons, about 70% to 80% of total homocyst(e)ine is protein-bound,[5,6] whereas in patients, protein binding seems to be saturated and seldom exceeds 150 μmol/L.[7] Most acid-soluble, free homocyst(e)ine in plasma exists as homocystine or cysteine-homocysteine mixed disulfides.[4]

Knowledge of the species of homocyst(e)ine circulating in vivo is important for evaluating the atherogenic properties of this amino acid. Some effects of elevated homocyst(e)ine level may also result from secondary effects on other thiol components such as cyst(e)ine and cyst(e)inylglycine. The thiol status in plasma in vivo may be difficult to assess because of rapid oxidation of free sulfhydryl groups, and the occurrence of a time-dependent redistribution of free and protein-bound species after blood collection. Therefore, sparse data exist on the presence of homocysteine and other thiol components in blood from healthy subjects[6,8,9] and from patients with homocystinuria.[10,11] The possible relation between the levels of different reduced thiols in plasma has not been evaluated.

We have recently developed a procedure for the determination of levels of reduced, oxidized, and protein-bound homocyst(e)ine and other thiol components in human plasma. The procedure is based on collecting whole blood directly into tubes containing thiol-specific reagents.[6] With this method, we analyzed plasma from eight patients with homocystinuria.

SUBJECTS AND METHODS

Eight nonfasting patients with homocystinuria were enrolled in the study. Their age, sex, cardiovascular disease status, treatment, and biochemical phenotype are listed in Table 1.

Normal values for homocyst(e)ine, cyst(e)ine, and cyst(e)inylglycine in plasma were obtained from 18 nonfasting subjects (eight males and 10 females). These values have been published previously.[6]

Chemicals

N-Ethylmaleimide (NEM), *N*-ethylmorpholine, dithioerythritol, homocysteine, and cysteine were obtained from Sigma Chemical (St Louis, MO), and cystinylglycine was from Serva Chemicals (Heidelberg, Germany). $NaBH_4$ was from Fluka Chemie (Buchs, Switzerland). Dimethyl sulfoxide, hydrogen bromide, 5-sulfosalicylic acid (dihydrate), perchloric acid, acetic acid, phosphoric acid, and acetonitrile (for chromatography) were purchased from Merck (Darmstadt, Germany), and monobromobimane (mBrB) was from Molecular Probes (Eugene, OR). Tetrabutylammonium hydroxide was obtained from Aldrich-Chemie (Steinheim, Germany). ODS

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Table 1. Patient Characteristics

Patient No.	Sex/Age (yr)	Cardiovascular Disease Type	Treatment Agent and Dosing*	Biochemical Parameters		
				Enzyme Deficiency	Plasma Methionine (μmol/L)	Plasma Total Homocyst(e)ine (μmol/L)
1	F/18	CVD	Pyridoxine 160 mg × 3	ND	83	360
2	F/20	VTD	Pyridoxine 200 mg × 3	ND	158	363
3	M/20	VTD	Pyridoxine 450 mg × 3	ND	574	288
4	F/3	None	Folic acid 40 mg × 3	CSD	94	201
5	M/19	CVD	None	CSD	831	319
6	F/14	None	None	ND	320	239
7	F/17	None	Folic acid 5 mg × 3, betaine 12 g × 1	ND	487	127
8	F/4	None	Folic acid 3 mg × 3, betaine 5 g × 4	MRD	21	63

Abbreviations: CVD, cerebrovascular disease; VTD, venous thromboembolic disease; CSD, cystathionine β-synthase deficiency; MRD, 5,10-methylenetetrahydrofolate reductase deficiency; ND, not determined.

*Treatment at the time of blood collection.

Hypersil (3 μm) was obtained from Shandon Southern (Cheshire, UK). Columns for reversed-phase liquid chromatography (3-μm Hypersil, 150 × 4.6) were slurry-packed at 9,000 psi using a Shandon column packer.

Analysis

Blood was routinely collected into three evacuated tubes containing either mBrB or NEM as thiol-derivatizing reagent or no additions. The blood was immediately centrifuged at 10,000 × *g* for 1 minute at room temperature to remove blood cells.

Thiols in blood collected into a tube with mBrB react with this reagent and form fluorescent adducts. Following precipitation of plasma proteins with sulfosalicylic acid, chromatographic analysis of the acid-soluble supernatant is performed, giving the free homocysteine, cysteine, and cysteinylglycine.

Free homocystine, cystine, and cystinylglycine levels and the corresponding mixed disulfide levels were determined after trapping the thiols with NEM and removal of protein-bound thiols by acid precipitation. Protein-bound thiols were determined in acid-precipitated plasma proteins treated with a mixture of $NaBH_4$ and NaOH. Both the oxidized and protein-bound species were reduced with $NaBH_4$ and finally determined as mBrB adducts.

Total amounts of homocyst(e)ine, cyst(e)ine, and cyst(e)inylglycine in plasma were determined with a procedure involving reduction of disulfides in whole plasma with $NaBH_4$, and derivatization of the free thiols with mBrB. The thiol-mBrB adducts were separated by ion-paired liquid chromatography on a ODS-Hypersil column. Details on the construction and performance of these assays have been described previously.[6]

Plasma methionine level was determined in deproteinized plasma with an assay based on derivatization with *o*-phthaldialdehyde and fluorescence detection.[12]

RESULTS

Patient Characteristics

We studied eight patients with homocystinuria (Table 1). The total plasma homocyst(e)ine level was high (range, 163 to 363 μmol/L) and plasma methionine level was elevated (range, 83 to 831 μmol/L) in seven patients. This metabolic profile is consistent with cystathionine β-synthase deficiency,[1] which was confirmed by enzymic analysis in two patients. One patient (no. 8) had a moderately elevated total plasma homocyst(e)ine level (63 μmol/L) and a subnormal plasma methionine level (21 μmol/L), and 5,10-methylenetetrahydrofolate reductase deficiency had been established by enzymic analysis.[13] Four patients (no. 1, 2, 3, and 5) had experienced at least one episode of vascular disease, and all patients except no. 5 and 6 received treatment with vitamins and/or betaine (Table 1).

Reduced, Oxidized, and Protein-Bound Homocyst(e)ine

The mean plasma homocysteine level was 0.24 μmol/L in one population of healthy nonfasting subjects[6] and even lower in young fasting subjects.[9,14] In patients with homocystinuria, the increase in the plasma concentration relative to physiologic concentrations was highest for homocysteine (up to 91 μmol/L, 400-fold above normal; patient no. 2), followed by homocystine (up to 173 μmol/L, 90-fold above normal) and the protein-bound form (up to 201 μmol/L, 20-fold above normal) in that order. Notably, the values for protein-bound homocyst(e)ine in the seven patients with cystathionine β-synthase deficiency were centered around 150 μmol/L (Fig 1).

Reduced, Oxidized, and Protein-Bound Cyst(e)ine and Cyst(e)inylglycine

In homocystinurics, the most dramatic change in the plasma content of cyst(e)ine species as compared with that in healthy subjects is a pronounced decrease in the protein-bound fraction (from 164 μmol/L to between 3.7 and 93.3 μmol/L). The amount of cystine was also markedly decreased (from 82.6 μmol/L to between 15.5 and 59.7 μmol/L), whereas cysteine level was increased twofold to threefold (from 9.3 μmol/L to between 16.4 and 36.8 μmol/L; Fig 1). In patients with homocystinuria, the sum of these species, which is designated total cyst(e)ine (52 to 167 μmol/L), is far below normal (~ 250 μmol/L[6]).

The changes in plasma cyst(e)inylglycine content in patients with homocystinuria are less pronounced, but resembled those observed with cyst(e)ine. Cystinylglycine and total cyst(e)inylglycine levels were not significantly different from values found in healthy subjects (Fig 1).

Covariations

There was a linear relationship between reduced to total ratios (ie, ratio between free reduced thiol and the total

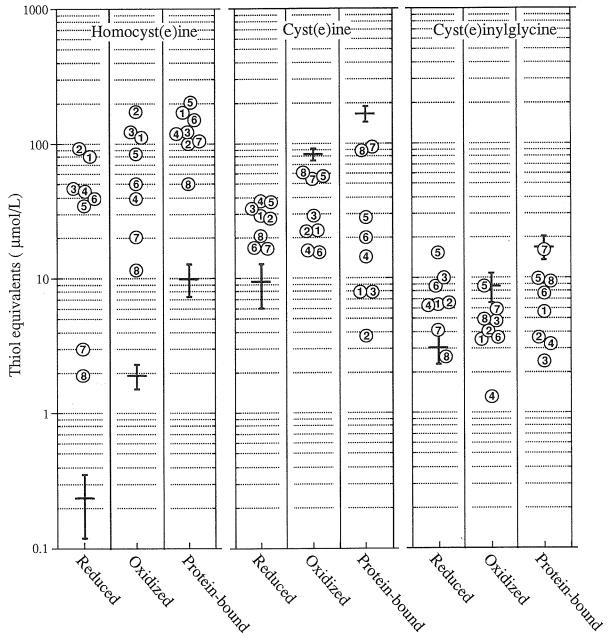

Fig 1. Thiol components in plasma of eight patients with homocystinuria. Patients are identified by the numbers placed on the data points, which correspond to the coding of the patients in Table 1. Means ± SD for normal nonfasting subjects are represented by the bold bars; data from Mansoor et al.[6]

amount) for homocyst(e)ine and cyst(e)ine. The slope of the linear regression curve was 1.97, showing that a higher fraction of cyst(e)ine compared with homocyst(e)ine existed in the reduced form (Fig 2). A similar relation seems to exist between homocyst(e)ine and cyst(e)inylglycine, but the correlation was weaker (Fig 2).

DISCUSSION

Evaluation of the Method

This study is based on a recently developed procedure for the determination of reduced, oxidized, protein-bound, and total homocyst(e)ine, cyst(e)ine, and cyst(e)inylglycine levels in human plasma.[6] The method is based on reduction of disulfides with $NaBH_4$, derivatization of free thiols with mBrB, and blocking free thiol groups with NEM. The sequential combination of these reagents allows the separate determination of all of these sulfur compounds in plasma. The total amount of each compound assayed directly fits with the sum of the separate species.[6]

Homocysteine and Other Thiols

The relation between various species of homocyst(e)ine and cyst(e)ine in plasma from homocystinurics has not been investigated previously. Two studies have reported on homocysteine trapping with iodoacetic acid in plasma from a patient with homocystinuria.[10,11] Whether lack of specificity of this reagent[15] or rapid oxidation of homocysteine preclude quantitative determination was not evaluated.

We found that among the thiol components in plasma, homocysteine showed the most substantial increase compared with the level in healthy subjects. Thus, in homocystinurics, homocysteine is by far the most abundant sulfhydryl species in plasma, and the concentration exceeds that of cysteine and cysteinylglycine (Fig 1). This contrasts with plasma from healthy subjects, which contains only trace amounts of homocysteine but significant amounts of both cysteine[16] (mean, 9.3 μmol/L) and cysteinylglycine[6] (Fig 1; mean, 3.1 μmol/L).

There was a striking linear relationship between the reduced to total ratios for homocyst(e)ine and cyst(e)ine (Fig 2). A similar relationship has been demonstrated during the transient hyperhomocysteinemia obtained in healthy subjects given a peroral dose of homocysteine.[14] When the concentration of total homocyst(e)ine is elevated, a substantial fraction exists in the reduced form, which in turn increases the reduced to total ratio of other aminothiols such as cyst(e)ine and cyst(e)inylglycine.[14]

It is conceivable that homocyst(e)ine and cyst(e)ine in plasma undergo redox cycling. The position of the equilibrium of the thiol-disulfide exchange reactions involving these aminothiols is probably determined by their chemical structure and acidity,[17] by the turnover of homocyst(e)ine or cyst(e)ine in plasma, and by the levels of these thiol components and other antioxidants[18] in plasma.

A high level of homocysteine and the associated changes in the redox status of other aminothiols may play a role in atherogenesis, and there are experimental data supporting this possibility. The reduced forms of sulfur-containing amino acids, including homocysteine, have been shown to oxidize low-density lipoprotein in vitro, and thiol-induced

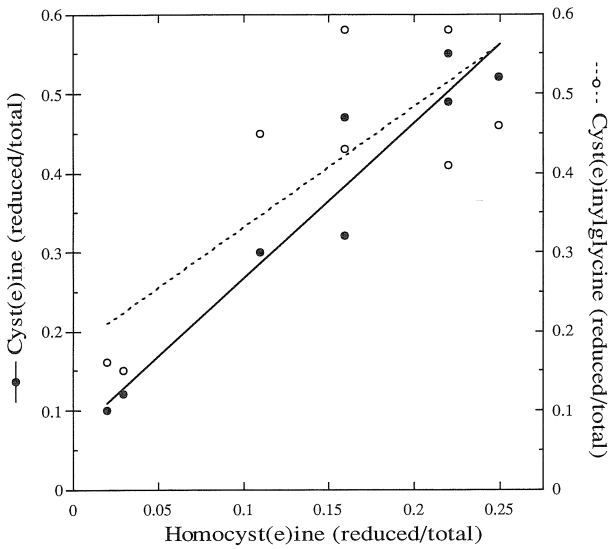

Fig 2. Relationship between different species of homocyst(e)ine, cyst(e)ine, and cyst(e)inylglycine in plasma from eight patients with homocystinuria. The fraction existing in the reduced form is calculated as the amount of reduced thiol divided by the total amount of the particular thiol, ie, the reduced to total ratio. Linear regression analysis of the relation between homocyst(e)ine and cyst(e)ine is given by the equation y = 1.971x + 0.070, *R* = .963, and the relation between homocyst(e)ine and cyst(e)inylglycine by y = 1.528x + 0.179, *R* = .801. These lines are shown in the graph.

modification of low-density lipoprotein has been implicated in atherogenesis.[19,20] Another biochemical link between elevated levels of homocysteine, other sulfhydryl compounds, and atherogenesis is suggested by the recent finding that these compounds enhance the binding of lipoprotein(a) to fibrin.[21]

Protein-Bound Aminothiols and Disulfides

In plasma from seven patients with homocystinuria, we found high levels of all homocyst(e)ine species, a moderate decrease in cystine levels, and a marked decrease in protein-bound cyst(e)ine levels as compared with plasma levels in healthy subjects (Fig 1). These findings confirm data published by others.[7,22,23]

Both experimental[24,25] and clinical studies[7] demonstrate the presence in plasma of binding sites for aminothiols, which preferentially interact with homocyst(e)ine. Binding of homocyst(e)ine seems to be saturable, and maximal binding capacity is about 150 μmol/L.[7] This agrees with the data presented in Fig 1.

The present investigation is the first to report cyst(e)inylglycine species in plasma from patients with homocystinuria. The changes observed in these patients resembled those found with plasma cyst(e)ine, but some differences were noted. A marked decrease in protein-bound cyst(e)inylglycine levels was observed in seven patients (Fig 1). This could be explained by displacement of protein-bound cyst(e)inylglycine [and cyst(e)ine] by high concentrations of homocyst(e)ine. Normal plasma levels of free cyst(e)inylglycine (reduced plus oxidized form) suggest normal formation of cyst(e)inylglycine from glutathione in homocystinurics, whereas low free cyst(e)ine levels can be explained by impaired formation of cyst(e)ine via the transsulfuration pathway.

Plasma Aminothiols in Healthy Subjects

We have recently investigated the alterations in the concentrations of plasma thiol components in healthy persons subjected to methionine[9] or homocysteine[14] loading, and the resulting hyperhomocyst(e)inemia induced changes resembling those observed in homocystinurics. The transient hyperhomocyst(e)inemia was associated with increased amounts of homocysteine and decreased amounts of protein-bound cyst(e)ine.[9,14] Thus, both in normal persons and in homocystinurics, an elevation of plasma homocyst(e)ine level has profound effects on the level and distribution of other aminothiols.

Summary and Conclusion

A new method based on immediate trapping of sulfhydryl compounds in blood by mBrB and NEM allows the quantitation of reduced, oxidized, and protein-bound homocyst(e)ine and other aminothiols in human plasma. This technique reveals the presence of large amounts of homocysteine in plasma from patients with homocystinuria. In these patients, this parameter shows the most drastic changes when compared with trace levels found in healthy subjects. The metabolic profile also includes altered level, redox state, and protein binding of other plasma aminothiols such as cyst(e)ine and cyst(e)inylglycine (Figs 1 and 2). Both the increase in plasma homocysteine level and the secondary changes in other aminothiols should be considered as pathogenic factors in future research on homocyst(e)ine and vascular disease.

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