Dynamic Relation between Reduced, Oxidized, and Protein-Bound Homocysteine and Other Thiol Components in Plasma during Methionine Loading in Healthy Men

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We used a newly developed procedure to determine reduced, oxidized, and protein-bound forms of homocysteine, cysteine, cysteinylglycine, and glutathione to measure the plasma concentrations of these species during methionine loading in six young healthy men with normal fasting concentrations of plasma homocysteine and cysteine. The methionine loading induced a transient increase in total homocysteine, which peaked after ~6–8 h. All six subjects showed a concurrent significant increase in reduced homocysteine and cysteine, which peaked 2 h after loading, and a rapid decrease in protein-bound cysteine and cysteinylglycine. The concentration of reduced cysteinylglycine was not altered. Plots of protein-bound cysteine and cysteinylglycine vs total homocysteine formed hysteretic loops, showing a time-dependent relation between these analytes. After the initial decrease, protein-bound cysteine and cysteinylglycine showed a slight, transient increase. From 12 to 24 h after loading, protein-bound cysteine approached preloading concentrations in two subjects and declined further in four subjects. The response pattern was similar for cysteine and cysteinylglycine in each subject. Simple displacement could not account for these effects, which suggests that plasma homocysteine may affect the disposition of other thiols through complex mechanisms. The presence of reduced homocysteine and the dynamic relation that exists between homocysteine, cysteine, and related compounds in plasma should be taken into account when evaluating plasma homocysteine as an indicator or causative agent of human disease.

Homocysteine is a sulfur-containing amino acid formed from methionine during transmethylation. Once formed, homocysteine is either salvaged to methionine by remethylation or is condensed with serine to form cystathionine, which is further catabolized to cysteine. Remethylation of homocysteine to methionine is catalyzed either by 5-methyltetrahydrofolate–homocysteine methyltransferase (methionine synthase, EC 2.1.1.13) or betaine-homocysteine methyltransferase (EC 2.1.1.5). The former enzyme is widely distributed and requires 5-methyltetrahydrofolate as methyl donor and

methylcobalamin as a cofactor. Betaine–homocysteine methyltransferase is confined to the liver, with only minor activity occasionally found in the kidneys and adrenal glands. The catabolic pathway is catalyzed by the sequential action of two vitamin B₆-dependent enzymes, cystathionine β-synthase (EC 4.2.1.22) and cystathionine γ-lyase (EC 4.4.1.1) (1).

Intracellular homocysteine probably occurs in the reduced form and is kept at a low concentration. Substantial amounts of homocysteine also exist in extracellular fluids, e.g., plasma and urine. Under conditions of increased homocysteine production or inhibition of homocysteine metabolism, homocysteine is exported into the extracellular compartment, so that the extracellular concentrations increase markedly. In the extracellular fluids, the major portion is analyzed as oxidized homocysteine. In human plasma, ~30% is determined as free, acid-soluble cysteine–homocysteine mixed disulfide, whereas the major portion (70%) forms a protein-bound mixed disulfide with albumin. Rapid protein binding and oxidation of homocysteine in blood in vitro may obscure assessment of the species prevailing in vivo (2).

Patients with inborn errors of remethylation or catabolism of homocysteine, collectively termed homocystinuria, have a marked increase in concentrations of plasma and urinary homocysteine. Patients with cystathionine β-synthase deficiency, the most common form of homocystinuria, have vascular disease in childhood and early adolescence (3).

Increases in plasma homocysteine have also been demonstrated in acquired conditions, e.g., deficiencies of such cofactors as folate, cobalamin, or vitamin B₆. Also, individuals who are heterozygous for cystathionine β-synthase deficiency have plasma homocysteine concentrations above normal; other heterozygous states involving homocysteine metabolism may predispose to hyperhomocysteinemia (2). This is particularly important because moderate hyperhomocysteinemia seems to be an independent risk factor for premature vascular disease and may therefore by of major concern to the public health (4).

The methionine loading test was first described by Brenton et al. (5) as a diagnostic procedure to detect homocystinuria due to cystathionine β-synthase deficiency; it has since been found useful for identifying heterozygotes among patients with premature vascular disease (6–13). This test involves the oral administration of methionine, which induces a transient increase in both free and protein-bound homocysteine in plasma, peaking between 4 and 8 h after loading in normal subjects (14, 15).

The rapidly changing concentrations of homocysteine

Table 1. Fasting Concentrations of Thiol Components and Methionine in Plasma (μmol/L)

Subject	Total homocysteine	Total cysteine	Total cysteinylglycine	Total glutathione	Methionine
1	12.4	244.6	27.9	5.4	23.3
2	12.0	252.5	30.8	9.0	28.5
3	10.4	237.1	40.1	8.1	51.4
4	9.9	222.7	26.2	4.4	17.7
5	11.4	257.9	27.0	6.2	31.0
6	7.35	221.0	32.3	5.5	41.3

Table 2. Methionine in Plasma (μmol/L) after Methionine Loading

Subject	Time after loading, h						
	0	2	4	6	8	12	24
1	23.3	512.1	466.3	444.7	426.5	388.7	155.8
2	28.5	499.1	445.1	405.2	357.0	231.1	31.9
3	51.4	441.6	363.8	259.7	178.2	85.5	43.0
4	17.7	618.4	472.8	374.3	309.7	219.6	41.6
5	31.0	662.5	553.7	520.3	475.8	400.0	127.3
6	41.3	769.3	658.5	578.2	476.5	395.6	97.6

in plasma after methionine loading may allow investigation of the possible relations between various forms of homocysteine and other thiols in plasma from healthy subjects in vivo. This can be accomplished by a procedure we developed (16) for determining reduced, oxidized, and protein-bound homocysteine, cysteine, cysteinylglycine, and glutathione (GSH), in which whole blood is collected directly into tubes containing thiol-specific reagents.

Materials and Methods

Materials and Subjects

N-Ethylmaleimide (NEM), N-ethylmorpholine, dithioerythritol, GSH, glutathione disulfide (GSSG), homocysteine, and cysteine were obtained from Sigma Chemical Co. (St. Louis, MO); oxidized cysteinylglycine was from Serva Chemicals (Heidelberg, FRG). NaBH₄ was from Fluka Chemie AG (Buchs, Switzerland). Dimethyl sulfoxide, hydrogen bromide, 5-sulfosalicylic acid (dihydrate), perchloric acid, acetic acid, phosphoric acid, and methanol (for chromatography) were purchased from Merck AG (Darmstadt, FRG); monobromobimane (mBrB) was from Molecular Probes, Inc. (Eugene, OR). Tetrabutylammonium hydroxide was obtained from Aldrich Chemie (Steinheim, FRG). ODS Hypersil (3-μm particles) chromatographic packing was obtained from Shandon Southern Ltd. (Cheshire, UK). Columns for reversed-phase liquid chromatography (3-μm Hypersil; 150 × 4.6 mm) were slurry packed at 62.1 MPa with a Shandon column packer.

Six healthy male volunteers participated in the study; their mean age was 31.5 years (range 24–35 years). All had plasma homocysteine and plasma cysteine concentrations (Table 1) within the normal range, i.e., within 2 SD of the mean of a healthy population (5.5–15 μmol/L for total homocysteine and 200–300 μmol/L for total cysteine). All had a normal methionine profile, which approached preloading concentrations within 24 h after loading (Table 2).

Procedures

Methionine loading, blood sampling, and processing. The methionine-loading test was performed by oral administration of methionine (100 mg/kg of body wt.) in ~200 mL of orange juice after an overnight fasting. Blood samples were collected immediately before the methionine intake and 2, 4, 6, 8, 12, and 24 h afterwards.

Blood was routinely collected into three evacuated tubes containing either mBrB or NEM as thiol-derivatizing reagent or no additions. The blood was centrifuged without delay at 10 000 × g for 1 min at room temperature to remove the formed elements of blood.

Determination of reduced, oxidized, and protein-bound thiol components in plasma. Thiols in blood react with mBrB to form fluorescent adducts. The blood cells are removed by centrifugation, the plasma proteins by acid precipitation. Chromatographic analysis of the acid-soluble supernatant gives results for the free, reduced forms of homocysteine, cysteine, cysteinylglycine, and GSH.

In blood collected into a solution containing NEM, the reduced sulfhydryl groups are rapidly trapped as their NEM adducts. The plasma fraction is then deproteinized with acid, and the disulfides are reduced in the presence of NaBH₄ to their corresponding thiols, which are then derivatized with mBrB. This is the procedure for determining the oxidized, free forms of homocysteine, cysteine, cysteinylglycine, and GSH.

Proteins in a sample from untreated plasma were precipitated with sulfosalicylic acid and dissolved in a solution containing NaBH₄ and NaOH. The free thiols formed from reduction of protein-bound mixed disulfides by NaBH₄ were derivatized with mBrB for determination of the protein-bound species.

The total amounts of homocysteine, cysteine, cysteinylglycine, and GSH in plasma were determined according to a modification (16) of a procedure described
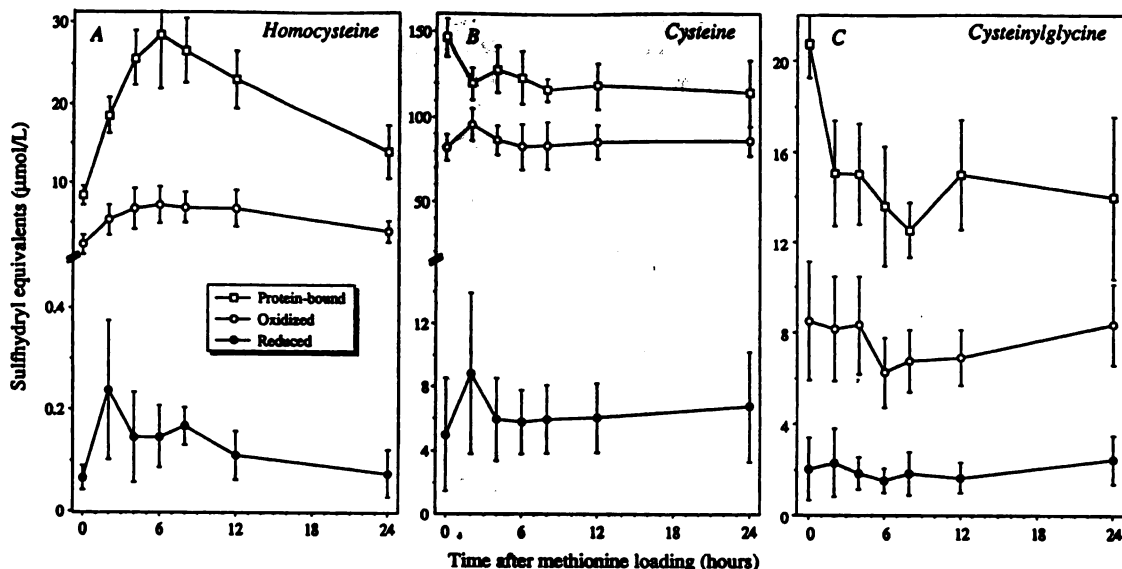
Fig. 1. Various forms of homocysteine, cysteine, and cysteinylglycine in six healthy men after methionine loading
Results are given as mean (SD)

previously (17), which involved reduction of disulfides in whole plasma with $NaBH_4$ and derivatization of the free thiols with mBrB. The thiol–mBrB adducts are separated by ion-paired liquid chromatography on an ODS-Hypersil column. Details on the set up and performance of these assays are described elsewhere (16).

Methionine in plasma. Methionine was determined in deproteinized plasma by an assay based on derivatization with o-phthaldialdehyde and fluorescence detection (18).

Statistical analysis. Data obtained for a particular analyte over a 24-h period were analyzed by the Friedman test (nonparametric analysis of variance). In cases where significant ($P < 0.05$) changes were obtained, the values before loading were compared with the values at 2 h after loading, by use of the Wilcoxon matched-pair signed-range test. All P-values are given as two tailed.

Results

Various forms of homocysteine in plasma during methionine loading. The homocysteine response after methionine loading is shown in Figure 1A. Free oxidized and protein-bound homocysteine in plasma increase, showing a peak 6 h after methionine intake, as has been previously demonstrated (14, 19, 20).

The mean concentration of reduced homocysteine in these individuals before loading was 0.07 (SD 0.02) μmol/L. There was a significant change ($P < 0.001$, Friedman test) and a significant ($P < 0.05$) increase to 0.24 (SD 0.14) μmol/L within 2 h. At that time, large variations in reduced homocysteine were observed (range 0.1 to 0.46 μmol/L; Table 3), after which the concentration of reduced homocysteine declined (Figure 1A).

Various forms of cysteine, cysteinylglycine, and glutathione. Protein-bound cysteine (Figure 1B) and cysteinylglycine (Figure 1C) showed significant ($P < 0.025$ and <0.001, respectively) changes after loading; concentrations of both analytes decreased ($P < 0.05$) within 2 h

Table 3. Plasma Concentrations (μmol/L) of Reduced Homocysteine and Cysteine before and 2 H after Methionine Loading

Subjects	Before loading (fasting)		2 h after loading	
	Homocysteine	Cysteine	Homocysteine	Cysteine
1	0.05	2.9	0.10	4.1
2	0.05	3.2	0.46	10.9
3	0.08	12.2	0.17	17.5
4	0.03	4.1	0.21	6.4
5	0.09	3.7	0.34	9.8
6	0.09	3.9	0.14	4.4
Mean (SD)	0.07 (0.02)	5.0 (3.6)	0.24 (0.14)	8.8 (5.0)

of methionine intake. The rate and extent of normalization of protein-bound cysteine varied from individual to individual, but approached preloading concentrations in two (subjects 2 and 6) of six subjects 24 h after methionine administration (Figure 2).

Free oxidized cysteine showed no significant change after loading. Changes in the concentration of free oxidized cysteinylglycine could be demonstrated ($P < 0.001$, Friedman test), but there was no significant increase after 2 h (Figure 1).

Reduced cysteine (mean, 5.0 μmol/L) and reduced cysteinylglycine (mean, 2.1 μmol/L) were also detected in plasma. Cysteine was significantly ($P < 0.05$) increased (to 8.8 μmol/L) 2 h after loading (Table 3), whereas the concentration of cysteinylglycine was unchanged (Figure 1).

The various species (reduced, oxidized, protein-bound, and total) of GSH remained stable after methionine loading. Total GSH varied within the range 4.4–9 μmol/L (data not shown).

Relation between homocysteine and protein-bound cysteine and cysteinylglycine. We plotted the sum of total homocysteine, cysteine, and cysteinylglycine (total thiol components, i.e., the reduced plus oxidized plus protein-
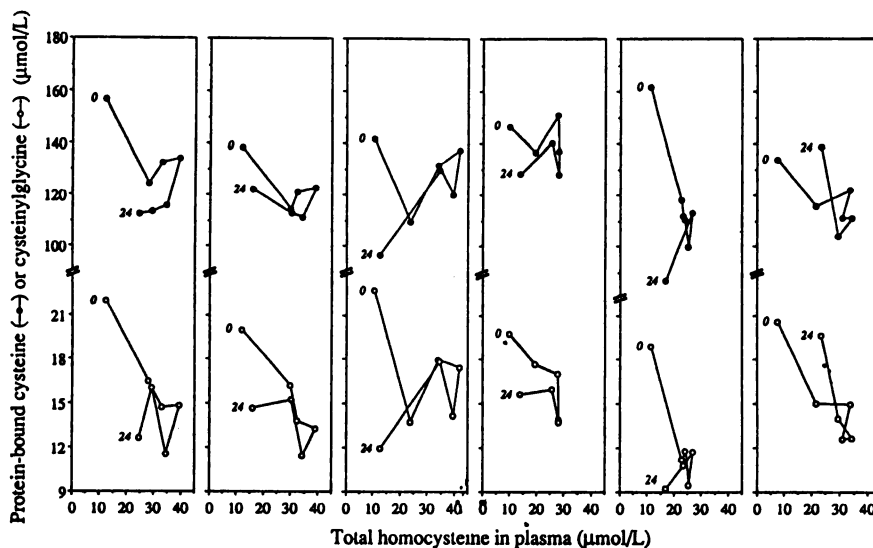
Fig. 2. Relation between the transient hyperhomocysteinemia induced by methionine loading and the protein binding of cysteine and cysteinylglycine in the individual subjects

The data points are connected by lines according to increasing time after loading. The point marked "0" indicates the value before loading and "24" indicates the value 24 h after methionine intake. From left to right, panels represent results for subjects 1–6, respectively.

bound thiol moieties) and the sum of protein-bound homocysteine, cysteine, and cysteinylglycine vs time after methionine loading (data not shown). There was a trend towards a decrease in the sum of total thiol components, but this decrease was not significant. In contrast, significant changes in the sum of protein-bound thiol compounds were observed after methionine loading ($P < 0.05$, Friedman test), and the values decreased ($P < 0.05$) 2 h after loading.

The changes in protein-bound cysteine and cysteinylglycine after methionine loading were plotted against total homocysteine. A time-dependent relation between protein-binding and total homocysteine in plasma was observed, and hysteretic loops or curves were obtained (Figure 2). The curves for cysteine and cysteinylglycine showed a similar pattern (Figure 2), consistent with a parallel alteration in protein-binding of these two compounds. All subjects showed a rapid decline in protein-bound cysteine within 2 h; after this time, a transient increase was observed (Figure 2). Next, the response was characterized by a clockwise or counterclockwise loop, followed by either a further decrease in protein binding (for subjects 1, 3, 4, and 5) or a partial increase to values approaching the preload binding (subjects 2 and 6).

Discussion

Evaluation of the Method

This study is based on recently developed procedures for the determination of reduced, oxidized, protein-bound, and total concentrations of homocysteine, cysteine, cysteinylglycine, and GSH in human plasma. The technique for determining the reduced and oxidized species is based on collecting whole blood directly into tubes containing rapidly reacting thiol-specific reagents (e.g., mBrB or NEM) (16). The oxidized forms are detected after the free thiol groups are blocked with NEM, followed by reduction of disulfides with $NaBH_4$,

and finally derivatization of free thiols with mBrB. The sequential combination of these reagents allows the separate determination of all these sulfur compounds in plasma. The assay is characterized by a CV <8% and the total amount of each compound assayed directly fits with the sum of the separate species (16).

Reduced Homocysteine and Other Thiols in Plasma

There is one previous report on the presence of trace amounts of reduced homocysteine in human plasma (21), but the technique used did not quantify the reduced form because of the low reactivity (22) to thiols of the derivatization reagent used. By collecting blood directly into tubes containing mBrB, we could demonstrate reduced homocysteine at a concentration of 0.06 $\mu mol/L$ in plasma from healthy fasting men (Table 3); this accounts for ~1% of total homocysteine. The amount is drastically increased after methionine intake. Furthermore, the response is rapid, reaching maximal concentration after 2 h, and precedes the increase in oxidized and protein-bound species, which peak after 6 h (Figure 1A). Finally, the increase in reduced homocysteine in plasma shows marked interindividual differences (range 0.1 to 0.46 $\mu mol/L$ after 2 h; Table 3).

These observations show that the increase of reduced homocysteine is a component of the metabolic response to methionine intake, which does not merely mirror the transient increase of the disulfide forms. Therefore, the concentration of reduced homocysteine and the changed concentration after methionine loading should be investigated in patients with diseases characterized by increased concentrations of fasting or postload homocysteine, including homocystinuria, folate and cobalamin deficiencies, and premature vascular disease. In fact, modification of low-density lipoproteins by reduced homocysteine and the potential role of this modification in atherogenesis are suggested by experimental findings (23, 24) and may motivate such investigations.

We detected and measured reduced cysteine, cysteinylglycine (Figure 1, B and C), and GSH in human plasma. The presence of reduced cysteine in plasma was demonstrated by Brigham et al. (25) 30 years ago, and our finding of reduced GSH confirms recent observations from several laboratories (17). Reduced GSH represents a significant fraction of total glutathione (~60%), whereas the reduced form is only a minor fraction of total cysteine (~5%) or cysteinylglycine (~10%) (16). Whether this reflects different rates of formation or of removal (oxidation) of the reduced species remains to be determined.

The increase in reduced cysteine shortly after methionine intake (Figure 1B and Table 3) is another original observation of potential interest, especially because this thiol also oxidizes low-density lipoproteins, at least in vitro (23, 24).

Reduced cysteinylglycine was not significantly increased after methionine loading (Figure 1C). The selective increase in reduced cysteine may be explained by the fact that the metabolic relation between methionine and homocysteine vs cysteinylglycine is remote, whereas homocysteine is an immediate precursor of cysteine via the transsulfuration pathway (1). Therefore, methionine loading may enhance cysteinylglycine turnover to a lesser degree than it does the turnover of cysteine. The increased amount of reduced cysteine in plasma may reflect high turnover of this thiol during methionine loading.

Protein Binding

More than 50% of homocysteine, cysteine, and cysteinylglycine in the plasma of the healthy subjects investigated was protein-bound (Figure 1) (16). This agrees with values for protein binding previously reported for homocysteine (2, 26) and cysteine (26–28).

Both experimental (29, 30) and clinical studies (26) demonstrate the presence in plasma of binding sites for aminothiols, which preferentially interact with homocysteine. Binding of homocysteine seems to be saturable, the maximal binding capacity being ~140 μmol/L (26).

Methionine loading induces a transient hyperhomocysteinemia, which in all subjects is associated with a transient reduction in protein-bound cysteine and cysteinylglycine (Figure 2). This is not merely the replacement of these species with equivalent amounts of protein-bound homocysteine, because the sum of protein-bound cysteine, cysteinylglycine, and homocysteine is decreased (data not shown). The complex nature of this phenomenon is further indicated by the fact that a maximal decrease in protein-bound cysteine and cysteinylglycine in some subjects (1, 3, 4, 5) occurs at a time when the concentration of plasma homocysteine is totally or partially normalized (Figure 2).

Conceivably, several processes contribute to the hysteretic response depicted in Figure 2. One may speculate whether the rapid decrease in bound cysteine to ~120 μmol/L within 2 h after loading (Figure 2) represents displacement from a homogeneous population of binding

sites that have high affinity for homocysteine. The transient increase in bound cysteine (and cysteinylglycine) that follows may represent displacement from acceptor(s) in extravasal compartments.

Our data suggest that the plasma concentration and the protein binding of aminothiols are mutually regulated. Such regulation may either provide optimal amounts of these compounds for vital cellular function or protect cells against high, toxic concentrations of thiols and disulfides (31). The interrelation between aminothiols may also have implications for the use of plasma homocysteine in laboratory diagnosis. In line with this idea, we found that the concentration of serum folate in patients with rheumatoid arthritis was more strongly correlated to the ratio between homocysteine and cysteine than to the concentration of plasma homocysteine (32).

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