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Plasma aminothiol oxidation in chronic hemodialysis patients

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Plasma aminothiol oxidation in chronic hemodialysis patients.

Background. Plasma aminothiols, including homocysteine, cysteine, and glutathione, function as an important extracellular redox system. We examined the plasma aminothiol concentration and redox status in ten chronic hemodialysis patients compared to ten age-matched healthy subjects.

Methods. Plasma levels of reduced, free oxidized, and protein-bound homocysteine, cysteine, cysteinylglycine, and glutathione were determined using high-pressure liquid chromatography (HPLC).

Results. Total plasma homocysteine, cysteine, and cysteinylglycine levels were significantly elevated in hemodialysis patients before dialysis compared to healthy subjects. Total plasma concentration of cysteine and homocysteine significantly decreased after hemodialysis. The ratio of free oxidized to free reduced homocysteine, cysteine, cysteinylglycine, and glutathione were each significantly elevated before dialysis compared to healthy subjects, and decreased significantly by the end of dialysis. The free oxidized to reduced ratio of cysteine and homocysteine were also significantly correlated with total plasma concentrations.

Conclusions. Plasma aminothiols are excessively oxidized in uremia, while the hemodialysis procedure is restorative of redox status. Oxidized aminothiols are candidate uremic toxins.

Increased oxidant stress has been implicated in the development of atherogenesis and cardiovascular complications for patients receiving chronic hemodialysis therapy [1–14]. An important manifestation of oxidative stress in uremic patients is an increase in the oxidation of thiol groups in plasma proteins, a process that can be modified by dialysis therapy [15]. In addition to plasma protein-associated thiol groups, there are also low-molecular-weight thiol-containing compounds in the plasma (known as aminothiols) that are largely derived from sulfur-containing amino acids (Fig. 1). Aminothiols have a critical function as intracellular redox buffers and are also increasingly recognized as constituting an important extracellular redox system [16, 17].

Key words: uremia, end-stage renal disease, oxidant stress, homocysteine, cysteine, glutathione, extracellular redox system, uremic toxin.

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Several important aminothiols, including homocysteine and cysteine, are retained as solutes in higher quantities in uremic patients than in healthy subjects and are candidates for uremic toxins [18–27]. Both homocysteine and cysteine can promote atherogenesis by virtue of their effect on endothelial function, vascular smooth muscle cell activation, and hemostatic activation [28–34]. Alterations in plasma homocysteine and cysteine concentrations have been associated in numerous clinical studies with the development of vascular disease [35–38]. In contrast, the aminothiol glutathione and its metabolite, cysteinylglycine, have important antioxidative and likely anti-atherogenic properties [39–41].

To date, few studies have specifically examined both the plasma concentration and the redox status of these aminothiols in uremic patients. Furthermore, the effects of hemodialysis on the redox status (for example, the ratio of oxidized to reduced molecular species) of plasma aminothiols have not been investigated. In order to examine whether increased plasma thiol oxidation in uremic patients is restricted to protein-associated thiol groups [15] or is a more generalized oxidative phenomena, we investigated the redox status of the major plasma aminothiols in patients receiving chronic hemodialysis therapy compared to healthy subjects. We further examined the effects of the hemodialysis procedure on both the concentration and the redox status of plasma aminothiols.

METHODS

Patient characteristics

Ten patients receiving chronic maintenance hemodialysis were compared to ten age-matched healthy controls. The mean age of patients and healthy controls was 43 years (range 32 to 53 years) and 46 years (range 33 to 56 years), respectively. The etiology of end-stage renal disease (ESRD) was diabetic nephropathy in two patients, polycystic kidney disease in three patients, complications of pregnancy in one patient, membranous nephropathy in one patient, and proliferative glomerulonephritis in one patient The etiology was unknown in two patients. Blood was drawn from patients just prior to dialysis after access cannulation and from efferent

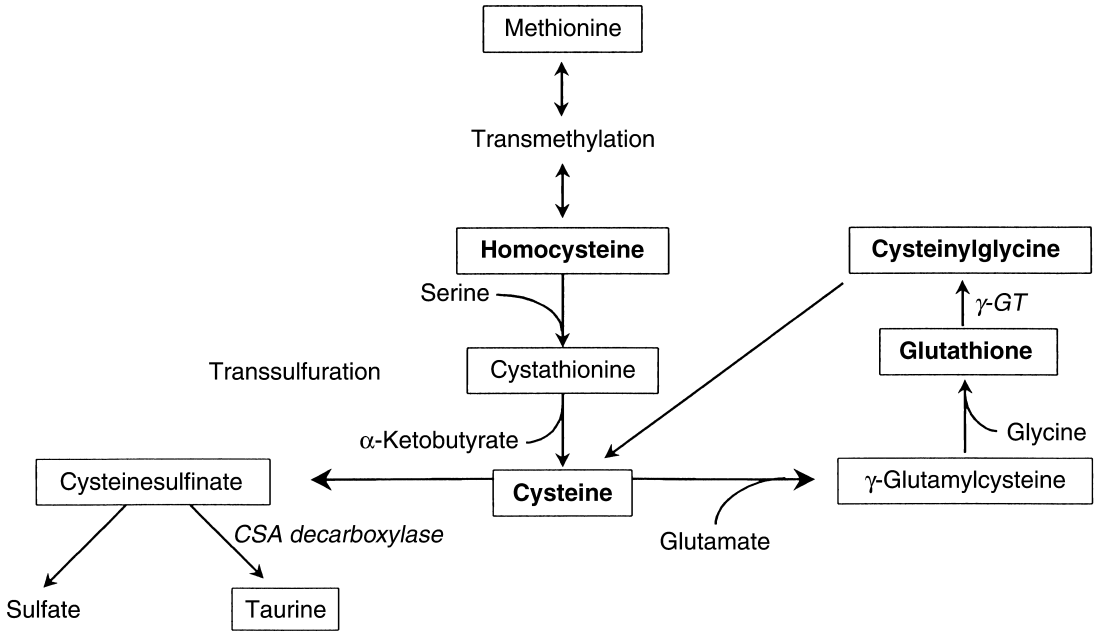

Fig. 1. Aminothiol synthesis and degradation pathways

dialyzer lines at the end of treatment. The dialysis prescription for all patients consisted of a treatment time of 240 minutes with the use of a high-flux polysulfone dialyzer (F80; Fresenius Medical Care, Lexington, MA, USA). Prescribed blood flow was 400 mL/min with a dialysate flow of 800 mL/min. The mean ± SEM urea reduction ratio for patients in this study was 72 ± 2. In all cases, heparin was used as a standard anticoagulant. All subjects gave informed consent and the Institutional Review Board at the Maine Medical Center approved all study procedures.

Reagents

N-ethylmalemide (NEM), *N*-ethylmorpholine, dithioerythritol (DTE), glutathione (GSH), homocysteine (Hcy), cysteinylglycine (Cys-Gly), cysteine (Cys), 5-sulfosalicylic acid (SSA), and octanol were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Monobromobimane (mBrB) was purchased from Calbiochem (La Jolla, CA, USA) as Thiolyte® Reagent. Dimethyl sulfoxide (DMSO), hydrogen bromide (HBr), sodium borohydride ($NaBH_4$), and tetrabutylammonium dihydrogenphosphate were obtained from Aldrich (Milwaukee, WI, USA). Perchloric acid and acetic acid were obtained from JT Baker (Phillipsburg, NJ, USA). Acetonitrile (ACN) and high-pressure liquid chromatography (HPLC)–grade water were obtained from Fisher Scientific (Pittsburgh, PA, USA).

Solution B was 65% DMSO and 35% water (vol/vol) containing 51 mmol/L NaCl and 140 mmol/L HBr.

Sample collection

For each sample, blood was drawn into three 5 mL Vacutainer® tubes (Becton-Dickinson, Franklin Lakes, NJ, USA) containing heparin as an anticoagulant and either mBrB, NEM, or no additive. All tubes were immediately centrifuged at 10,000 × *g* for one minute at room temperature to remove cells and platelets. From the different plasma preparations, aliquots were removed and treated to determine the various thiol components. Prepared samples were stored in the dark at 4°C and analyzed within six hours.

Sample preparation for determination of the various thiol components

Total, reduced, free oxidized, and protein-bound forms of the plasma aminothiols were prepared and measured according to the method of Mansoor, Svardal, and Ueland [42]. Selective determination of each redox species was accomplished by varying the order of disulfide reduction with $NaBH_4$, protein precipitation, and derivatization. In each protocol, the resulting free sulfhydryl groups were derivatized with mBrB, forming a fluorescent thiol-bimane adduct. These compounds were separated and quantitated by reversed-phase HPLC. Octanol (2 μL) was added to the samples prior to reduction to reduce foaming.

Total plasma thiol component determination

Octanol (2 μL) and 30 μL of 2 mmol/L $NaBH_4$ in 0.05 mol/L NaOH were added to 30 μL of untreated plasma. Plasma proteins were then precipitated by adding 60 μL

of 20% sulfosalicylic acid (SSA) containing 100 μmol/L DTE. After allowing for the release of gas, precipitated protein was removed by centrifugation. Supernatant (60 μL) was derivatized by adding 30 μL of 1.4 mol/L $NaBH_4$ in 0.05 mol/L NaOH, 130 μL of solution B, 50 μL 1 mol/L *N*-ethylmorpholine (pH 9), and 10 μL of 20 mmol/L mBrB in 100% acetonitrile. After a ten-minute incubation at room temperature in the dark, 20 μL of 1.06 mol/L perchloric acid was added.

Free oxidized plasma thiol component determination

A combination of 55 μL 50% SSA with 500 μmol/L DTE was added to 500 μL NEM-treated plasma. Precipitated protein was removed by centrifugation. Supernatant (30 μL) was derivatized by adding 2 μL octanol, 30 μL 1.4 mol/L $NaBH_4$ in 0.05 mmol/L NaOH, 160 μL solution B, 50 μL 1 mol/L *N*-ethylmorpholine (pH 9), and 10 μL 20 mmol/L mBrB in 100% acetonitrile. After a ten-minute incubation at room temperature in the dark, 20 μL of 1.06 mol/L perchloric acid was added.

Protein-bound plasma thiol component determination

A combination of 150 μL 5% SSA with 50 μmol/L DTE was added to 30 μL untreated plasma. Following protein precipitation and centrifugation, the supernatant was discarded. To the pellet, 30 μL of 2 mol/L $NaBH_4$ in 0.05 mol/L sodium hydroxide was added. The dissolved pellet was derivatized by adding 30 μL 40% SSA with 100 μmol/L DTE, 30 μL 1.4 $NaBH_4$ in 0.05 mol/L NaOH, 130 μL solution B, 50 μL in 1 mol/L *N*-ethylmorpholine (pH 9), and 10 μL 20 mmol/L mBrB in 100% acetonitrile. After a 10-minute incubation at room temperature in the dark, 20 μL of 1.06 mol/L perchloric acid was added.

Reduced plasma thiol component determination

A combination of 55 μL 50% 5-sulfosalicylic acid with 500 μmol/L DTE was added to 500 μL mBrB-treated plasma. Precipitated protein was removed by centrifugation. Supernatant (30 μL) was treated with 2 μL octanol, 30 μL 5% SSA with 5 μmol/L DTE, 160 μL distilled water, 50 μL of 1 mol/L *N*-ethylmorpholine (pH 9), and 10 μL ACN to mimic the derivatization procedure. After a 10-minute incubation at room temperature in the dark, 20 μL of 1.06 mol/L perchloric acid was added.

Derivatized samples were stored in the dark at 4°C and analyzed within six hours of preparation.

Standards and calibration curves

Standard solutions of 800 μmol/L cysteine, 100 μmol/L cysteinylglycine, 40 μmol/L homocysteine, and 40 μmol/L glutathione were prepared in 5% SSA containing 50 μmol/L DTE. Equal volumes of each standard were mixed, giving a final standard containing 200 μmol/L cysteine, 25 μmol/L cysteinylglycine, 10 μmol/L homocysteine, and 10 μmol/L glutathione. Calibration curves for quantitation were prepared by adding known amounts of cysteine, cysteinylglycine, homocysteine, or glutathione to 5% SSA containing 50 μmol/L DTE. All standard samples were processed using the protocol for total thiol components.

Chromatography

Prior to injection, all samples were filtered using .2 μm polyvinyl idene difluoride (PVDF) Micro-Spin centrifuge filters (Alltech Associates, Inc., Deerfield, IL, USA). Aliquots (25 μL) were manually injected onto a Hypersil ODS (C_{18}) analytical column (3 μm; 4.6 × 150 mm; Waters, Milford, MA, USA). The temperature was 25°C and the flow rate 1.5 mL/min. Elution solvent A was 10 mmol tetrabutylammonium phosphate and 2.5 mL acetic acid, diluted to 1 L with HPLC-grade water, pH adjusted to 3.4 with 2 mol/L NaOH. Solvent B was 200 mL ACN, 10 mmol tetrabutylammonium phosphate and 2.5 mL acetic acid, diluted to 1 L with HPLC grade water, pH adjusted to 3.4 with 2 mol/L NaOH. Solvent C was 75% ACN. The elution profile was as follows: 0 to 13 minutes, 6 to 22% solvent B; 13.1 to 25 minutes, 22 to 40% solvent B; 25.1 to 30 minutes, 40 to 75% solvent B. After each run, the column was washed for five minutes with solvent C to remove late-eluting fluorescent material. (An increased amount of this late-eluting fluorescent material was found in all protein-bound and some free oxidized samples of hemodialysis patients. This material made it difficult to clearly resolve the glutathione peak. Therefore, data on protein bound glutathione was omitted from final analysis.) As the acetonitrile concentration in the column solvent was increased, the bimane derivatives of the aminothiols were eluted and detected by an in-line fluorescence detector (excitation λ400 nm, emission λ475 nm). The retention times for the bimane derivatives of cysteine, cysteinylglycine, homocysteine, and glutathione were 7.0, 9.0, 12.5, and 32.2 minutes, respectively (Fig. 2). Compounds were identified by their retention time and co-elution with derivatized aminothiol standards.

Instrumentation

A Waters 626 system coupled to a Waters 474 fluorescence detector was used. Plotting and peak integration were performed using Waters Millennium32 software.

Total plasma protein-free sulfhydryl group determination

Free sulfhydryl groups were assayed as previously described [15]. Blood was drawn into a Vacutainer tube containing ethylenediaminetetraacetic acid (EDTA), centrifuged at 1700 × *g* for 15 minutes to obtain plasma.

A buffer (1 mL) containing 0.1 mol/L Tris, 10 mmol/L EDTA (pH 8.2) and 50 μL plasma were added to cuvettes, followed by 50 μL 10 mmol/L 5′5′dithio-bis (2-

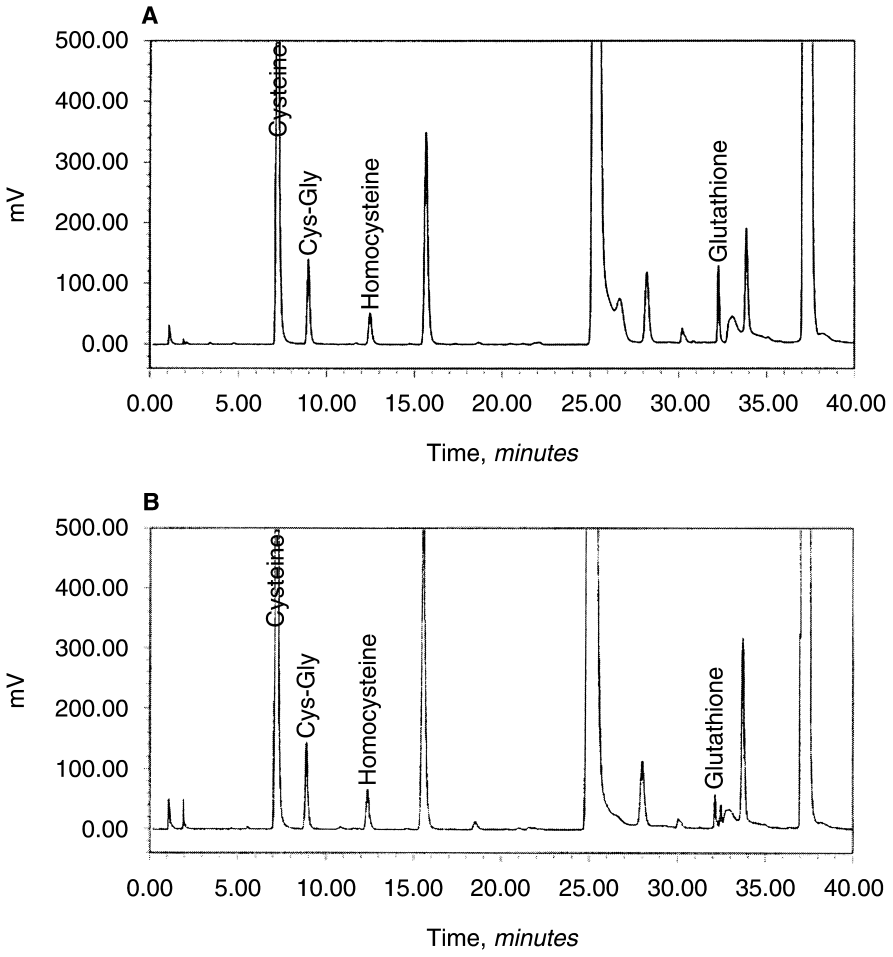

Fig. 2. Representative high-performance liquid chromatograms. (*A*) Authentic standards. (*B*) Sample from healthy subject

nitro benzoic acid) (DTNB) in methanol. Blanks were run for each sample, prepared as above, with the exception that there was no DTNB in the methanol. Following incubation for 15 minutes at room temperature, sample absorbance was read at 412 nm on a lambda-2 spectrophotometer (Perkin Elmer, Norwalk, CT, USA). Sample and reagent blanks were also subtracted. The concentration of sulfhydryl groups was determined using the TNB molar extinction coefficient of 14,100 $mol/L^{-1} \cdot cm^{-1}$, and results were reported as micromoles per liter. The coefficient of variation for this assay was 2.7%.

Data analysis and statistics

Results are expressed as mean ± SEM. Total values represent the sum of reduced, free oxidized and protein-bound values. For expressions of aminothiol ratios, values were calculated prior to statistical analyses. Unpaired Student *t* tests (assuming equal or unequal variance, depending on the results of an *f* test) evaluated the significance of differences between healthy subjects and chronic hemodialysis patients. A two-tailed paired *t* test was performed on data comparing pre- and post-dialysis samples. For linear regression analyses, Pearson correlation coefficients are given with accompanying *P* values.

RESULTS

Total plasma aminothiols in hemodialysis patients and healthy subjects

Total plasma concentrations of the aminothiols cysteine, homocysteine, cysteinylglycine, and glutathione in patients before and after hemodialysis compared to age-matched healthy subjects are depicted in Figure 3. There was a significant increase in total plasma cysteine (478 ± 16 μmol/L vs. 372 ± 13 μmol/L, $P < 0.0001$) in patients before dialysis compared to healthy subjects. Similarly, there was an increase in total plasma homocysteine (41 ± 5 μmol/L vs. 14 ± 1 μmol/L, $P = 0.0002$) and cysteinylglycine (77 ± 6 μmol/L vs. 56 ± 6 μmol/L, $P = 0.02$) in patients before dialysis versus healthy subjects. In contrast, there were no significant differences in total plasma glutathione concentration between patients before dialysis and healthy subjects. At the end of the dialysis session, the total plasma concentrations of homo-

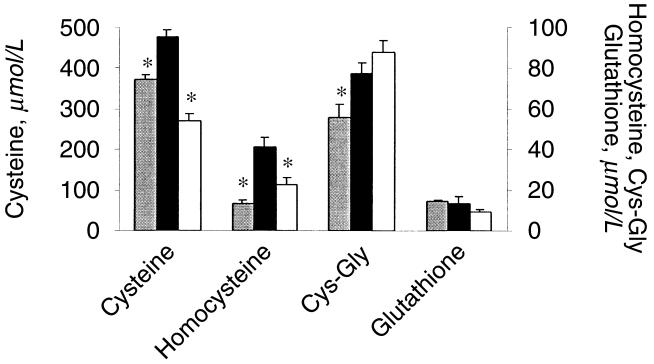

Fig. 3. Total plasma aminothiol concentration in hemodialysis patients before (■) and (□) after dialysis, and age-matched healthy subjects (▩). $N = 10$ hemodialysis patients and healthy subjects for cysteine, homocysteine, and cysteinylglycine (Cys-Gly). $N = 5$ for glutathione in hemodialysis patients. *$P < 0.02$ vs. before hemodialysis

cysteine and cysteine were significantly lower than before dialysis, while there was no significant change in glutathione or cysteinylglycine concentrations.

The relationships between total plasma concentrations of aminothiols in both healthy subjects and uremic patients are illustrated in Figure 4. Figure 4A demonstrates a significant positive correlation between total plasma cysteine and homocysteine concentrations, particularly in hemodialysis patients before and after dialysis ($R = 0.81$; $P = 1 \times 10^{-5}$). In contrast, there were no significant positive correlations between plasma total homocysteine and cysteine concentrations and plasma cysteinylglycine concentrations in hemodialysis patients or healthy subjects (Fig. 4 B–F). These data indicate that there is a positive relationship between the extent of hyperhomocysteinemia and hypercysteinemia, but not between other aminothiols in patients with uremia.

Increased oxidation of plasma aminothiols in hemodialysis patients compared to healthy subjects

The plasma aminothiols constitute a complex of free reduced compounds, free oxidized compounds, and oxidized protein-bound compounds. To compare the redox status of plasma aminothiols in hemodialysis patients and healthy subjects, we first compared the ratio of free oxidized and free reduced cysteine, homocysteine, cysteinylglycine, and glutathione in healthy subjects and hemodialysis patients before and after dialysis (Fig. 5). Of note, all four free plasma aminothiols were more oxidized in hemodialysis patients compared to healthy subjects. For all plasma aminothiols, the hemodialysis procedure resulted in a significant change in the free oxidized to free reduced ratio toward a ratio similar to those seen in healthy subjects. Thus, there was a generalized alteration in the redox status of amino acid thiol groups in uremic patients and the hemodialysis procedure had a restorative effect on redox balance.

To determine the importance of total plasma concentrations of plasma aminothiols on redox status, total plasma levels and the ratio of free oxidized to free reduced homocysteine, cysteine, cysteinylglycine, and glutathione were compared (Fig. 6). Figure 6 A and B demonstrate that for homocysteine and cysteine, there was a positive relationship between total plasma concentration and the redox ratio of free aminothiols ($R = 0.60$ for homocysteine, $P = 0.005$; $R = 0.68$ for cysteine, $P < 0.001$). In contrast, there was no clear-cut relationship between total plasma concentration and the redox ratio of free glutathione (Fig. 6D). To further clarify the relationship between total plasma aminothiol concentration and redox status, the concentration of free oxidized and total plasma homocysteine, cysteine, cysteinylglycine, and glutathione were compared (Fig. 7). Figure 7 A and B demonstrate a striking positive relationship between total plasma cysteine, homocysteine, and cysteinylglycine concentration and their respective concentrations of the free oxidized aminothiol.

Protein binding of aminothiols in patients with uremia

In addition to oxidation of free plasma aminothiols, the extent to which oxidized aminothiols are protein-bound was examined in both hemodialysis patients and healthy subjects (Fig. 8). The ratio of free to protein-bound cysteine and homocysteine but not cysteinylglycine was higher in patients with uremia before dialysis compared to healthy subjects. The extent of protein binding of glutathione could not be determined due to inability to resolve this peak in protein-containing fractions, a phenomenon that has been observed by other investigators [43]. Of note, the hemodialysis procedure resulted in a significant decrease in the free to protein-bound ratio of homocysteine, cysteine, and cysteinylglycine. These results suggest that oxidized aminothiols in hemodialysis patients are less likely to bind to plasma protein than in healthy subjects, perhaps due to an increased saturation of plasma protein ligands for aminothiols. The hemodialysis procedure also proportionately cleared more free oxidized aminothiols compared to protein-bound aminothiols, as would be expected for any solute given the pore size of conventional high-flux hemodialysis membranes.

To further examine the hypothesis that the ratio of free to protein-bound plasma aminothiols may be altered due to relative saturation of available protein binding groups, we compared the slopes of protein-bound to total plasma homocysteine, cysteine and cysteinylglycine, respectively (Fig. 9). Figure 9A demonstrates that the slope (0.31) was different than the slopes for homocysteine (0.74) and cysteinylglycine (0.74). Thus, at high plasma total cysteine concentrations, protein binding of

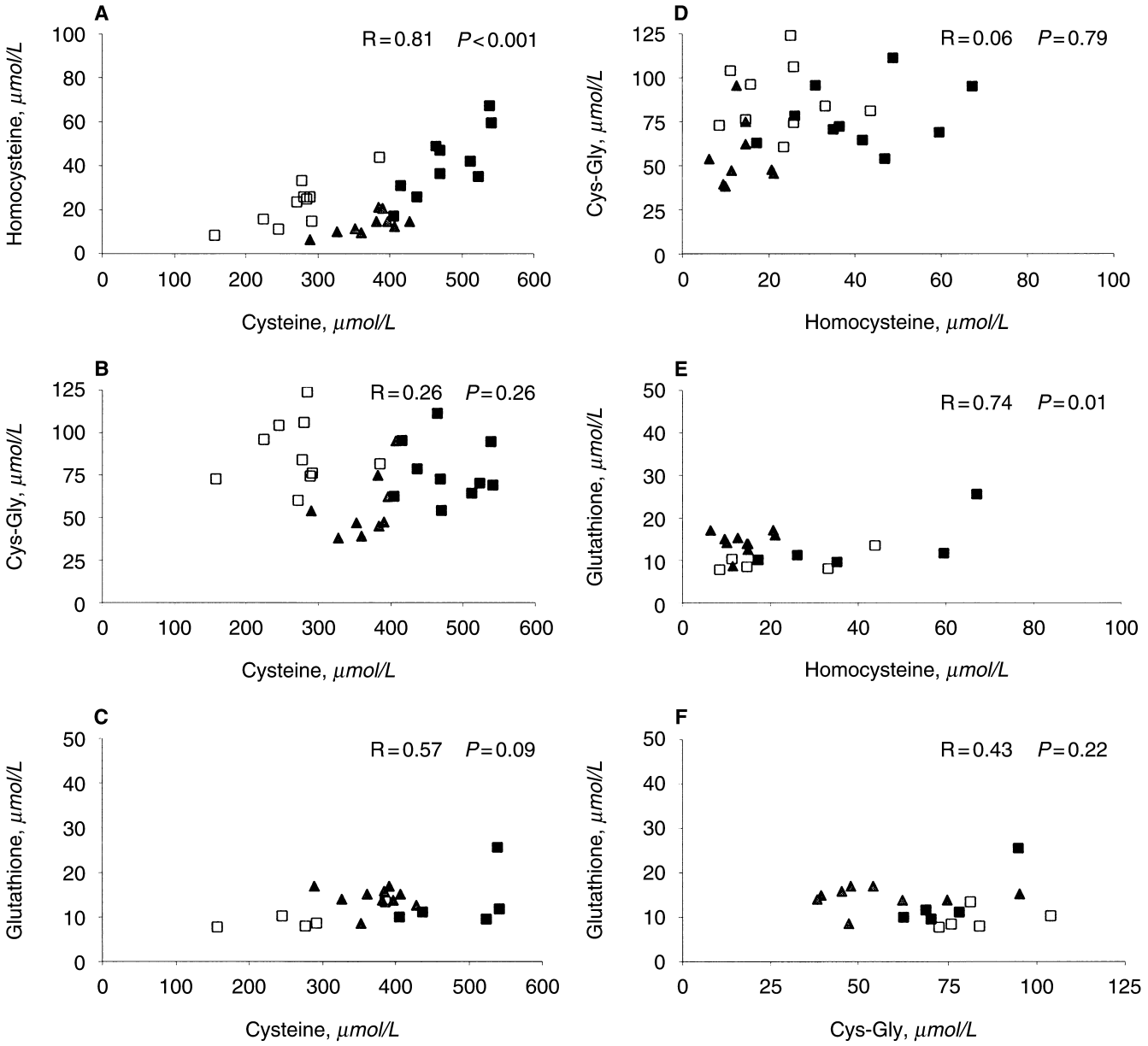

Fig. 4. Relationships between total plasma concentrations of aminothiols in healthy subjects (▲) and hemodialysis patients pre- (■) and post- (□) dialysis. $N = 10$ hemodialysis patients and healthy subjects for cysteine, homocysteine, and cysteinylglycine (Cys-Gly). $N = 5$ for glutathione in hemodialysis patients. R and P values were calculated for all hemodialysis patient samples.

oxidized cysteine may be relatively inhibited due to saturation.

Plasma protein thiol redox status in patients with uremia and healthy subjects

Since protein thiols quantitatively constitute the major source of antioxidant potential in the plasma [15, 44], the extent to which protein-associated thiols are oxidatively conjugated to aminothiols was investigated (Fig. 10). Hemodialyzed uremic subjects had a significantly higher ratio of protein-bound aminothiols to available plasma thiol compared to healthy subjects (1.01 ± 0.07 vs. 0.61 ± 0.05; $P < 0.001$). In comparison, the ratio of protein-bound aminothiols to available plasma protein free thiol groups at the end of the dialysis procedure was significantly different than before hemodialysis (0.63 ± 0.06 vs. 1.01 ± 0.07, $P = 0.002$) and similar to the ratio seen in healthy subjects. These results clearly demonstrate a similar pattern of altered redox status of the plasma protein thiol pool (similar to the free aminothiol pool) in uremic subjects before hemodialysis compared to

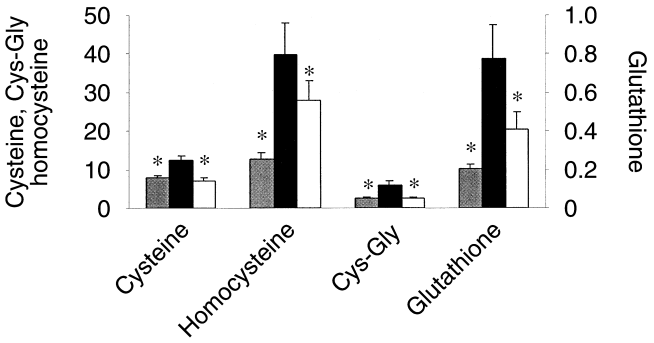

Fig. 5. Ratio of free oxidized to reduced aminothiols in healthy subjects (■) and hemodialysis patients before (■) and after (□) dialysis. N = 10 hemodialysis patients and healthy subjects for cysteine, homocysteine, and cysteinylglycine (Cys-Gly). $N = 5$ for glutathione in hemodialysis patients. *$P < 0.05$ vs. before hemodialysis

healthy subjects, which was restored to levels similar to healthy subjects at the end of the dialysis procedure.

DISCUSSION

Patients with ESRD on hemodialysis therapy experience accelerated atherogenesis as evidenced by excessively high cardiovascular mortality. Recent studies demonstrate that even after adjustment for age, race, gender, and the presence of diabetes, cardiovascular mortality is 10- to 20-fold higher than in the general population [45]. Furthermore, risk factors such as hypertension and hypercholesterolemia, which are predictive of cardiovascular morbidity and mortality in the general population, are not as predictive of cardiovascular complications in patients with chronic kidney disease, suggesting that processes related to uremia may contribute in unique ways to cardiovascular complications in this patient population [9, 46]. A major focus of current efforts to lower cardiovascular complications in uremic patients is a better understanding of the contributions of solute retained in uremia to cardiovascular toxicity.

Patients with chronic kidney disease, particularly those requiring chronic dialysis therapy, have been reported to have numerous disturbances in the metabolism of plasma sulfur amino acids [23, 25]. In recent years, much attention has been focused on elevated total plasma homocysteine levels in uremic patients as a potential risk factor for early-onset cardiovascular disease [18–27]. Hyperhomocysteinemia is extremely prevalent in this patient population and is not entirely normalized by supplementation with vitamin B_6 and folic acid. Homocysteine, once formed, is either salvaged to methionine by remethylation, or is condensed with serine to form cystathionine, which is further catabolized to cysteine in the transsulfuration pathway (Fig. 1) [47]. Abnormalities in both remethylation and transulfuration pathways of homocysteine metabolism have been described in uremic patients. While intracellular homocysteine is kept at a low concentration and likely occurs in the reduced form, in extracellular fluids such as plasma, homocysteine is predominantly oxidized, either as a mixed disulfide or as a protein-bound disulfide with albumin.

The present study demonstrated that in uremic subjects, total plasma concentrations of homocysteine and cysteine were elevated before dialysis compared to healthy subjects, an observation similar to several previous reports. In addition, the present study demonstrated a correlation between serum homocysteine and serum cysteine levels in hemodialysis patients. Since the metabolism of homocysteine via the transsulfuration pathway may account for up to 50% of plasma cysteine levels, this correlation is not surprising. Though not as well studied as homocysteine as a cardiovascular risk factor, the potential vascular toxicity of cysteine has recently been emphasized. In patients with cardiovascular disease, both free and protein-bound cysteine levels are higher in the plasma of patients compared to healthy subjects [48]. In vitro, auto-oxidation of cysteine occurs more readily than homocysteine, thereby generating reactive oxygen species that can, by oxidation, modify low-density lipoprotein [49]. Of note, a recent study demonstrated that auto-oxidation of homocysteine can be dramatically accelerated by the presence of either cysteine or cystine [50]. It also has been demonstrated that cysteine, and not homocysteine, leads to increased reactive oxygen species production by vascular smooth muscle cells [30, 51]. The potential synergistic toxicity of hyperhomocysteinemia and hypercysteinemia in patients with chronic kidney disease has not been well explored and likely will be a subject for further investigation.

In this study, we did not observe a significant difference in total plasma glutathione levels in uremic patients prior to dialysis compared to healthy subjects. A recent study, also using HPLC methodology, demonstrated similar results in a study of nine hemodialysis patients [52]. Previous studies of total plasma glutathione levels in hemodialysis patients have yielded mixed results, with some studies demonstrating elevated plasma levels and other studies demonstrating normal or decreased levels compared to healthy subjects [13, 24, 52–54]. These discrepancies are likely due to the fact that glutathione is primarily an intracellular antioxidant. Thus, even minimal degrees of hemolysis during plasma sampling will artifactually elevate plasma glutathione levels. As an example, Suliman et al recently compared plasma and erythrocyte glutathione levels in hemodialysis patients and demonstrated that erythrocyte levels are approximately 700-fold higher than plasma levels [24].

To elucidate the in vivo redox status of aminothiols, it is important to recognize that each of these plasma thiols can exist as free reduced compounds, mixed disulfides, and as mixed disulfides with plasma protein; thus,

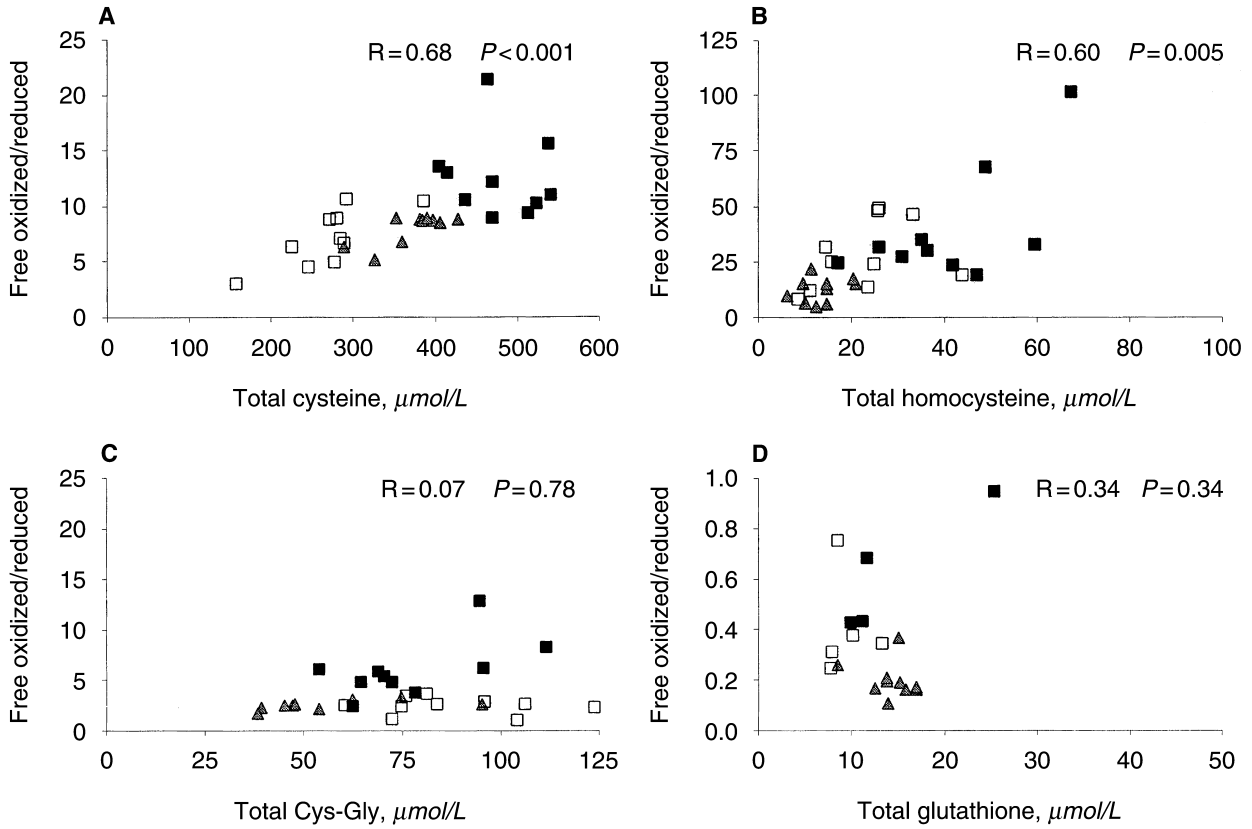

Fig. 6. Comparison of total plasma concentrations and the ratio of free oxidized and reduced aminothiols in healthy subjects (■) and uremic patients before (■) and after (□) dialysis. $N = 10$ hemodialysis patients and healthy subjects for cysteine (*A*), homocysteine (*B*), and cysteinylglycine (Cys-Gly; *C*). $N = 5$ for glutathione (*D*) in hemodialysis patients. R and *P* values were calculated for all hemodialysis patient samples.

their interrelated pathways are highly complex [43]. This complexity is increased by interrelations in the synthesis and metabolism of the individual aminothiols. Thus, while homocysteine can be metabolized to cysteine, cysteine is a precursor of glutathione. Catalysis of glutathione by gamma glutamyl transpeptidase (located on the external surface of cell membranes and found in the plasma) results in the formation of cysteinylglycine. In the past, several analytic approaches were used to measure aminothiol oxidation. Glutathione has been analyzed using a variety of spectrophotometric, chromatographic, and enzymatic techniques. The disulfides formed by low-molecular-weight thiols have been analyzed by chromatography, by enzymatic assays, and by HPLC. Recently, methodology employing gas chromatography-mass spectroscopy, has been employed to analyze reduced plasma homocysteine concentrations [55]. A common feature of many existing methods is their inability to simultaneously quantify the oxidized and reduced form of all the important aminothiols in biological systems.

The HPLC methodology utilized in this study was developed by Mansoor, Svardal, and Ueland, and colleagues [42, 56–60] The assays are based on trapping the reduced species in plasma by collecting blood directly into tubes containing sulfhydryl-reactive agents such as monobromobimane [50] or *N*-ethylmaleimide and then immediately removing blood cells. This methodology previously has been used to examine changes in the redox status of aminothiols in healthy subjects during methionine or homocysteine loading, and in patients with hyperhomocysteinemia due to cobalamin deficiency and homocysteinuria [57–60]. Recently, an alternative strategy to measure the redox status of aminothiols in human plasma has been developed by Andersson et al [52]. In this system, the reduced sulfhydryl species in the plasma fraction were protected against in vitro oxidation by adding sulfosalicylic acid, thereby lowering pH. Under optimal sample processing, both methodologies obtain similar values, although they have not been simultaneously employed in the same study.

A major finding in our present study is that all of the important free plasma aminothiols (homocysteine, cysteine, cysteinylglycine, glutathione) are more oxidized in uremic subjects before dialysis compared to healthy subjects. Furthermore, the dialysis procedure has a beneficial effect in improving the redox status of each of the free plasma aminothiols, approaching levels by

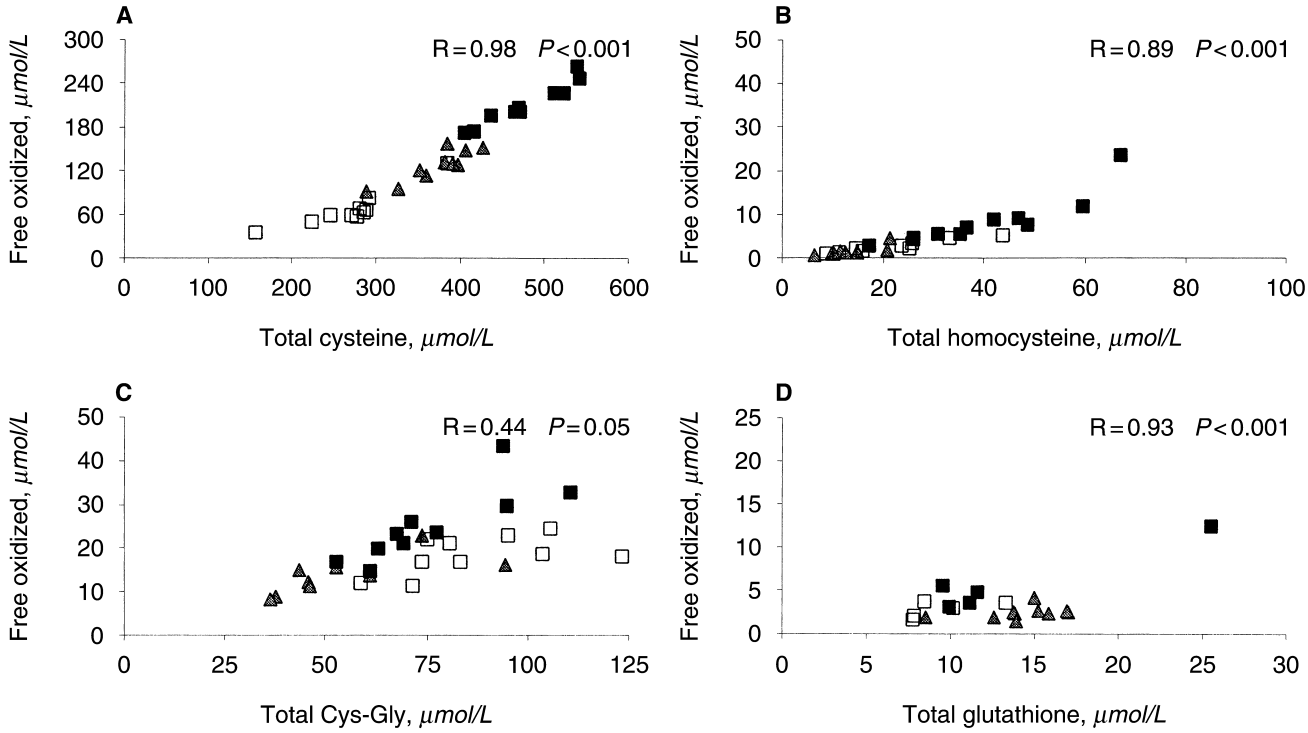

Fig. 7. Comparison of total plasma aminothiol concentrations and their respective free oxidized concentration in healthy subjects (■) and uremic patients before (■) and after (□) dialysis. $N = 10$ hemodialysis patients and healthy subjects for cysteine (*A*), homocysteine (*B*), cysteinylglycine (Cys-Gly; *C*). $N = 5$ for glutathione (*D*) in hemodialysis patients. R and *P* values calculated for all hemodialysis patient samples.

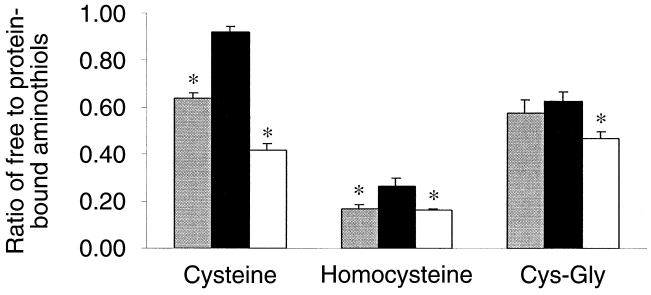

Fig. 8. Ratio of free to protein bound aminothiols in healthy subjects (■) and hemodialysis patients before (■) and after (□) dialysis. $N = 10$ patients in each group. $^*P < 0.05$ vs. before hemodialysis.

the end of the dialysis procedure observed in healthy subjects. There is concordance between the results observed in this study and our previous work examining plasma protein–associated thiol oxidation [15]. This suggests that a generalized derangement in thiol redox status exists in uremia that tends to be ameliorated by dialysis. This uremia-associated "thiol stress" is quantitatively the major demonstrable alteration in plasma redox chemistry induced by the uremic state. Furthermore, the extent of homocysteine, cysteine, and cysteinylglycine oxidation is related to total plasma concentrations. The concentration-dependent "thiol stress" observed in these studies is conceptually similar to the "substrate stress" hypothesis advanced to explain the development of vascular disease in diabetes mellitus [61]. These findings suggest that correcting the metabolic derangements leading to thiol oxidation in uremia may be an important approach to reducing cardiovascular complications in this patient population.

Previous studies that systematically examined aminothiol oxidation in uremic patients and the effects of the hemodialysis procedure have been sparse. Andersson and colleagues have used HPLC methodology with acidified plasma to examine the redox status of aminothiols in uremic patients [21, 52]. In their most recent publication, nine uremic patients on dialysis therapy were noted to have elevations in total plasma concentrations of cysteine, cysteinylglycine, and homocysteine (but not glutathione) compared with healthy subjects [52]. These findings are in accordance with the results of our present study. These investigators also noted a lower reduced to total plasma concentration of cysteine, glutathione, cysteinylglycine, and homocysteine in uremic patients compared to healthy subjects, findings that are a different measure but conceptually consistent with our present study. These authors did not directly measure the ratio of free reduced to free oxidized aminothiols, nor did they examine the effect of hemodialysis on aminothiol concentration or redox status. In a recent publication, Hoffer et al used a highly sensitive gas chromatography mass spectrometry

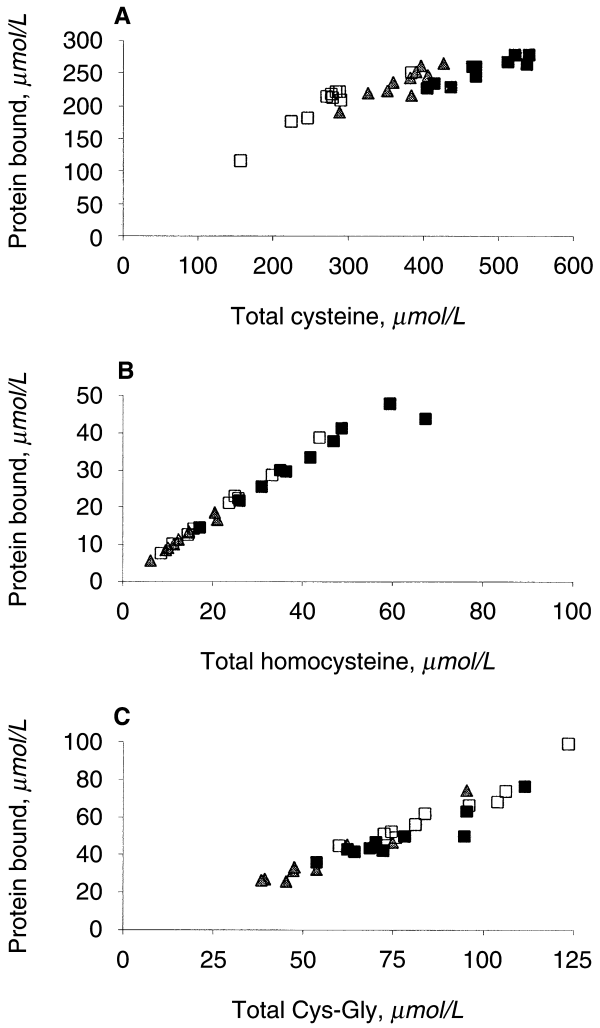

Fig. 9. Comparison of total plasma aminothiol concentrations and their respective protein-bound concentration in healthy subjects (▲) and uremic patients before (■) and after (□) dialysis. $N = 10$ in each group. Slopes calculated for all samples.

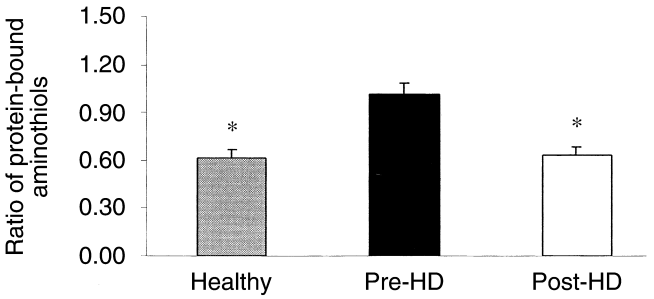

Fig. 10. Ratio of protein-bound aminothiols to available plasma thiols in healthy subjects (▩) and hemodialysis patients before (■) and after (□) dialysis. $N = 5$ for healthy subjects, $N = 6$ for hemodialysis patients
*$P \leq 0.002$ vs. before hemodialysis.

method for measuring plasma-reduced homocysteine concentrations in hemodialysis patients [55]. These authors demonstrated elevated plasma-reduced homocysteine levels in dialysis patients that were significantly related to plasma homocysteine concentration. Both reduced and total plasma homocysteine concentrations were lowered by the hemodialysis procedure.

A notable finding in the present study is that in uremic subjects, the redox state of the various plasma aminothiols is not in equilibrium. Specifically, the oxidized to reduced ratio of free homocysteine is greater than cysteine, which is greater than cysteinylglycine, which is greater than glutathione. That the redox state of the plasma aminothiols is not in equilibrium is not surprising given that they likely derive from different sources (Fig. 1). Of note, Jones et al recently performed a similar analysis in healthy subjects and also demonstrated that cysteine tended to be more oxidized than glutathione, with other plasma aminothiols being intermediate. As previously noted, glutathione is largely an intracellular antioxidant and plasma glutathione levels likely reflect cellular secretion of this antioxidant. Plasma cysteine is derived both from the metabolism of glutathione and from the transsulfuration of homocysteine, while cysteinylglycine is predominantly a metabolic product of the degradation of glutathione. Thus, while the redox status of plasma glutathione would be a response to both intracellular and extracellular oxidative processes, the redox status of cysteine and cysteinylglycine would be a more accurate reflection of extracellular oxidative status [43]. Furthermore, because the thiols of both cysteine and cysteinylglycine have lower pK values, they more readily undergo auto-oxidation at physiologic pH.

An interesting finding in the present study is that the ratio of free to protein-bound cysteine and homocysteine are higher before dialysis in uremic subjects compared to healthy subjects. A potential explanation for this finding is that higher levels of plasma cysteine and homocysteine seen in uremic subjects leads to a relative saturation of protein binding with a subsequent increase in free fractions. Support for this concept comes from previous work by Mansoor, Ueland, and Svardal, who demonstrated relative saturability of protein-bound homocysteine as plasma total homocysteine levels approach 140 μmol/L in patients with cobalamin deficiency [60]. In uremic patients, an additional potential explanation is that there may be a decrease in available plasma protein thiol binding sites as a function of oxidative stress [15]. Reactive carbonyl compounds, which have been demonstrated to accumulate in uremia, also may compete with plasma aminothiols for plasma protein-associated thiol binding. Also of interest, the hemodialysis procedure results in a marked decrease in the ratio of free to protein-bound cysteine, cysteinylglycine and homocysteine (compared to before dialysis levels) in uremic subjects. This finding is consistent with the relatively greater clearance of unbound compared to protein-bound solute re-

lated to pore size with high flux hemodialysis membranes.

Conclusion

This study demonstrates that both the plasma concentration and the redox status of aminothiols are altered in uremia and improved with the hemodialysis procedure. Plasma aminothiol oxidation in uremia is part of a more generalized "thiol stress," which is also manifested by plasma protein thiol oxidation [15]. Potential vascular toxicity of oxidant stress in general and aminothiol oxidation in particular in chronic hemodialysis patients suggests that amelioration of thiol stress in chronic hemodialysis patients may have beneficial cardiovascular effects. Further research is needed to examine the safety and efficacy of therapeutic approaches designed to improve plasma thiol oxidative stress status in chronic hemodialysis patients.

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