Role of aminothiols as a component of the plasma antioxidant system and relevance to homocysteine-mediated vascular disease

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ABSTRACT

Hyperhomocysteinaemia is considered to be an independent risk factor for vascular disease. Elevated plasma homocysteine may pose an oxidative stress, leading to the development of vascular damage. A component of this effect may be a disturbance of the extracellular aminothiol redox state. The relative contributions of plasma total homocysteine (tHcy) and plasma total cysteine (tCys) to the total antioxidant capacity (TAOC) of plasma was established in subjects with normal and elevated plasma tHcy. A total of 10 subjects with severe hyperhomocysteinaemia (due to inherited metabolic defects), 13 of their heterozygous parents and 72 normal healthy subjects were recruited to the study. The mean plasma tHcy in the patients was 91.8 \( \mu \text{mol/l} \), compared with 13.2 \( \mu \text{mol/l} \) in the parents and 14.7 \( \mu \text{mol/l} \) in healthy control subjects. Plasma tCys and plasma TAOC were significantly lower in the subjects with severe hyperhomocysteinaemia compared with the parents and healthy control subjects (P < 0.05). In blood samples from subjects with a normal tHcy, a positive correlation was observed between tCys and tHcy (P < 0.0001). In contrast, in blood samples with tHcy \(< 20 \, \mu\text{mol/l}\), plasma tCys was negatively correlated with tHcy (P = 0.0001). In samples with tHcy \(\geq 20 \, \mu\text{mol/l}\), tHcy was inversely correlated with TAOC (P = 0.0001), whereas tCys was positively associated with TAOC (P = 0.0001). Multiple regression analysis revealed that tCys was the most important independent determinant of TAOC in the patient and control groups when the effects of tHcy and several factors known to influence TAOC, such as urate, were taken into account. Thus hyperhomocysteinaemia may pose an oxidative stress not only through the direct cytotoxicity of homocysteine, but also from an associated fall in plasma cysteine.

INTRODUCTION

Homocysteine is a thiol-containing amino acid, and the total plasma concentration of this amino acid is markedly elevated (100 \( \mu \text{mol/l} \); normal range < 20 \( \mu \text{mol/l} \)) in subjects with deficiencies of the enzyme cystathionine \( \beta \)-synthase (severe hyperhomocysteinaemia). These patients are at risk of premature vascular disease and thrombotic complications in adolescence and in infancy [1]. More recently, epidemiological data have demonstrated that a mild elevation of plasma total homocysteine (tHcy) to \(> 20 \, \mu\text{mol/l}\) (hyperhomocysteinaemia) is an independent and graded risk factor for premature vascular disease in the general population [2]. Homocysteine is derived from the metabolism of the essential amino acid methionine. The homocysteine formed is either metabolized to cysteine via transulphuration, catalysed by cystathionine \( \beta \)-synthase (a vitamin \( \text{B}_{12} \)-dependent

Key words: antioxidants, cardiovascular disease, cysteine, hyperhomocysteinaemia, oxidant stress.

Abbreviations: ABTS, 2,2’-azino-di-(3-ethylbenzthiazoline sulphonate); TAOC, total antioxidant capacity; tCys, total cysteine; tHcy, total homocysteine.

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enzyme), or remethylated back to methionine, which involves folate and vitamin $B_{12}$-dependent enzymes [1].

Several plausible explanations have been proposed for homocysteine-mediated vascular disease, although there is growing evidence that endothelial dysfunction is an initiating event [3]. Numerous studies have been published demonstrating endothelial dysfunction associated with elevated plasma tHcy. Such studies have been performed in patients with severe hyperhomocysteinaemia [4], in subjects with moderately elevated plasma tHcy following an oral methionine load [5], and in animal models in which hyperhomocysteinaemia was induced by dietary means [6].

The autoxidation of homocysteine is associated with the generation of reactive oxygen species, including hydrogen peroxide, superoxide and hydroxyl radicals [7–9], which may be an important factor in the development of endothelial dysfunction [10,11]. Antioxidants have been shown to reverse or ameliorate homocysteine-related endothelial damage in vitro and in vivo, further supporting a role for reactive oxygen species in homocysteine-mediated vascular disease [12–14].

The damaging effects of elevated plasma tHcy may not be entirely attributable to homocysteine autoxidation and the associated generation of reactive oxygen species. The plasma aminothiols (cysteine, homocysteine, cysteinylglycine and glutathione) exist in reduced, oxidized and protein-bound forms, and may be important components of the extracellular antioxidant defence system [15]. In subjects with hyperhomocysteinaemia, elevated plasma tHcy is associated with a shift in the redox state of cysteine towards the oxidized form, cystine [16,17]. In contrast with the pro-oxidative nature of homocysteine in vitro, cysteine has been shown to have antioxidative properties [18].

The aim of the present study was to establish the relative contributions of plasma tHcy and plasma total cysteine (tCys) to the total antioxidant capacity (TAOC) of the plasma in subjects with normal and elevated plasma tHcy.

**METHODS**

**Subjects**

The patient study group consisted of 10 subjects (six male and four female), mean age 26 years (range 6–66 years), with inherited defects of homocysteine metabolism (nine of these subjects had cystathionine $\beta$-synthase deficiency; the remaining patient had a functional methionine synthase defect). These patients were recruited after obtaining written consent from the patients or their parents. A total of 41 blood samples were collected over a 6-month period, following an overnight fast, as part of their routine monitoring. Subjects with homocystinuria were treated with pyridoxine (50–300 mg/day), folic acid (5–20 mg/day) and betaine (150–250 mg·kg$^{-1}$·day$^{-1}$). The subject with the functional methionine synthase defect received folic acid, betaine and hydroxycoabalamin (2 mg, intramuscular). Single fasting blood samples were also collected from 13 of the obligate heterozygous parents (only 13 of the total parent group gave informed consent). The protocol for this study was approved by the South Sheffield Research Ethics Committee.

Blood samples were also used that had been obtained from 72 apparently healthy subjects (32 male and 40 female), mean age 42 years (range 18–65 years), recruited to an existing study of homocysteine and vascular disease. Exclusion criteria included: previous history of cardiovascular disease, diabetes, hypertension or smoking, and the use of vitamin supplements. Ethical committee approval was granted from the University of Wales College of Medicine (Bro Taf Health Authority), from where the study was co-ordinated.

Fasting venous blood samples were drawn from an antecubital vein into lithium heparin tubes, placed on ice and centrifuged at 3000 g for 10 min at 4 °C within 30 min of venepuncture. Plasma samples for analysis of vitamin C were treated with 9 vol. of 5% metaphosphoric acid to produce a deproteinized extract. Deproteinized plasma extracts and plasma were divided into aliquots and stored at −70 °C until required for analysis.

**Biochemical analyses**

The accurate determination of the concentrations of reduced and oxidized forms of thiols in plasma is difficult due to redox cycling following venepuncture. We therefore measured tHcy and tCys in plasma (protein-bound and free forms). Plasma tHcy and plasma tCys were measured as described by Spaapen et al. [19]. Plasma samples were treated with tri-n-butyl phosphate to reduce disulphide bonds between homocysteine and plasma protein and between mixed disulphides. Plasma proteins were then removed by deproteinization with trichloroacetic acid, and the liberated thiols were derivatized using 7-fluoro-2,1,3-benzoxadiazole-4-sulphonic acid. Cysteine and homocysteine were separated by HPLC, using a Waters 600 pumping and controller system, a Waters 717 autosampler and a Waters 474 fluorescence detector (Waters Company Millipore, Milford, MA, U.S.A.).

Plasma TAOC was measured using a Cobas Bioanalyser (Roche Diagnostica) using a commercial kit (NX2332; Randox Laboratories). This assay is based on that of Miller et al. [20]. In this method, the relatively long-lived blue–green radical cation ABTS$^+$ is generated following the addition of hydrogen peroxide to the chromagen, which contains ABTS$^+$ (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) and a peroxidase (metmyoglobin). The radical generated absorbs light at 600 nm. Antioxidants added to the reaction system will reduce the radical, with a concentration-dependent de-
crease in absorbance. The measurement was standardized by the use of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble α-tocopherol analogue.

Plasma vitamin C was measured spectrofluorimetrically using a Cobas Bio-autoanalyser, by the method of Vuilleumier and Keck [21]. The assay is based on the oxidation of ascorbic acid to dehydroascorbic acid during incubation of the plasma extract with ascorbic acid oxidase. The dehydroascorbic acid produced then reacts with 1,2-diphenylenediamine to produce a compound which fluoresces at an excitation wavelength of 350 nm. The fluorescence readings of the test samples were compared with a standard curve of L-ascorbic acid. A Vitros Clinical Chemistry dry film analysser (Johnson & Johnson, Clinical Diagnostics, Amersham, Bucks., U.K.) was used for the measurement of plasma urate, albumin and bilirubin.

Statistical analysis
Data were analysed using SPSS for Windows. Comparisons of mean plasma concentrations of thiols and antioxidant variables between subject groups were made using a one-way ANOVA, followed by a Scheffe’s post hoc analysis when significant differences were observed. Linear regression analysis was performed to explore associations between variables. Significance was taken at the 95% confidence interval ($P < 0.05$).

RESULTS
The inter-batch coefficient of variation was determined for all analytes. For tHcy and tCys this was $< 5\%$, and for vitamin C, TAOC, bilirubin, uric acid and albumin it was $< 3\%$.

Plasma tHcy, tCys, tHcy/tCys ratio and TAOC
The mean ($±$ S.E.M.) and range of concentrations for plasma tHcy, tCys and TAOC in patients with severe hyperhomocysteinaemia, their heterozygous parents and healthy normal controls are shown in Table 1. A single mean value for each patient was included in this analysis. The mean plasma tHcy concentration was significantly greater in subjects with severe hyperhomocysteinaemia when compared with their parents and with the healthy control group ($P < 0.0001$). Mean plasma tCys concentrations, tCys/tHcy ratios and TAOC were significantly lower in the subjects with severe hyperhomocysteinaemia compared with the parents and the healthy control group ($P < 0.0001$). Mean values for all plasma variables measured were not statistically different between the parent and healthy control groups.

Relationship between plasma tHcy and plasma tCys
For further data analysis, samples from the patients with severe hyperhomocysteinaemia, their heterozygous parents and normal healthy controls were classified into those in which the plasma tHcy concentration was $< 20\; \mu$mol/l or $\geq 20\; \mu$mol/l. This cut-off was derived from the mean ± S.D. of the tHcy data derived from the normal healthy population group ($n = 72$) used in the present study.

For blood samples with tHcy $\geq 20\; \mu$mol/l, tCys showed a strong negative correlation with tHcy (Figure 1) ($r = -0.869, n = 45, P = 0.0001$). In contrast, a positive

| Table 1 Plasma tHcy, plasma tCys, tCys/tHcy ratio and plasma TAOC in patients with severe hyperhomocysteinaemia, their heterozygous parents and apparently healthy control subjects |
|---|---|---|---|
| Analyte | Patients ($n = 10$) | Parents ($n = 13$) | Apparently healthy controls ($n = 72$) |
| tHcy ($\mu$mol/l) | 91.8 ± 22.3 (10.1–196.0)* | 13.2 ± 1.27 (5.0–21.0) | 14.7 ± 0.72 (7.4–37.8) |
| tCys ($\mu$mol/l) | 175.5 ± 20.7 (55.0–272.5)* | 264.7 ± 12.7 (183.0–332.0) | 256.9 ± 3.4 (180.8–351.7) |
| tCys/tHcy | 6.61 ± 2.44 (0.28–18.9)* | 22.5 ± 2.31 (9.67–36.89) | 19.35 ± 0.67 (6.68–39.4) |
| TAOC (mmol/l) | 1.39 ± 0.02 (1.26–1.48)* | 1.49 ± 0.02 (1.38–1.61) | 1.49 ± 0.01 (1.29–1.66) |

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correlation was observed between plasma tCys and tHcy in blood samples with tHcy \( < 20 \mu mol/l \) (results not shown).

**Aminothiols and plasma antioxidant status (TAOC)**

Linear regression analysis showed that plasma TAOC was significantly inversely associated with plasma tHcy in the blood samples with tHcy \( \geq 20 \mu mol/l \) (Figure 2) \( (r = -0.509, n = 45, P = 0.0001) \). In contrast, plasma TAOC was positively associated with plasma tCys in this same series of blood samples (Figure 3) \( (r = 0.673, n = 45, P = 0.0001) \). Measurements made in serial samples \( (n = 16) \) taken from a single patient with severe hyperhomocysteinaemia from a period of poor compliance with homocysteine-lowering therapy to a period of better control (adherence to homocysteine-lowering therapy) showed an equally strong negative correlation between plasma TAOC and tHcy (Figure 4) \( (r = -0.753, n = 16, P = 0.0008) \) and a positive correlation between plasma TAOC and tCys (Figure 5) \( (r = 0.781, P = 0.0004) \). A positive correlation between plasma TAOC and tCys was also observed in blood samples with tHcy \( < 20 \mu mol/l \) (Figure 6) \( (r = 0.337, n = 81, P = 0.002) \). In contrast, no association was found between plasma TAOC and tHcy in blood samples with tHcy \( < 20 \mu mol/l \) \( (r = -0.18, n = 81, P = 0.108) \) (results not shown).
Relative importance of plasma thiols and other plasma antioxidants for plasma antioxidant activity

This part of the study was carried out using blood samples from the patient and control groups only. The plasma TAOC assay evaluates the combined effects of antioxidants present in the plasma [22]. The concentration of plasma antioxidants was determined in samples in which plasma tHcy was < 20 μmol/l and compared with that in samples in which tHcy was ≥ 20 μmol/l (Table 2). The mean (± S.E.M.) plasma tHcy for these groups was 12.7 ± 0.91 μmol/l and 112.8 ± 11.02 μmol/l respectively. Values of tCys, tCys/tHcy, uric acid and TAOC were significantly lower in samples with plasma tHcy ≥ 20 μmol/l compared with samples with tHcy < 20 μmol/l. Vitamin C was significantly higher in subjects with tHcy ≥ 20 μmol/l. Neither albumin nor bilirubin concentrations differed between the two groups.

To ascertain the relative contributions of tHcy, tCys and individual antioxidants to plasma TAOC, linear regression analysis was performed on all samples collected from the patients and parents (n = 54). Table 3 shows the correlation coefficients between plasma TAOC and individual antioxidants. We found a significant positive correlation between TAOC and tCys (r = 0.691, P = 0.0001) and between TAOC and tCys/tHcy (r = 0.639, P = 0.0001). In contrast, we found a significant negative correlation between plasma TAOC and plasma tHcy (r = -0.656, P = 0.0001). A significant positive correlation was also observed between plasma TAOC and plasma urate (P = 0.014). No significant correlations were observed between bilirubin, albumin or vitamin C and plasma TAOC. A multiple regression analysis was carried out to determine the relative importance of tCys, tHcy and urate as independent predictors of TAOC. tCys was the only independent predictor of TAOC (P < 0.05).

DISCUSSION

The reported association between elevated plasma tHcy and cardiovascular risk does not prove cause and effect, although there are plausible mechanisms for damaging effects of homocysteine, including changes in the extracellular thiol redox state of plasma, leading to a decrease in plasma TAOC.

Cysteine is a product of homocysteine metabolism and a precursor of glutathione, which is the most abundant thiol in plasma. In normal healthy subjects cysteine is present in plasma at a mean concentration of ~250 μmol/l (~65% is protein bound, ~30% exists in the free disulphide form, and the remaining 5% exists in the reduced form). Homocysteine has a mean concentration in plasma of ~10 μmol/l, which is mostly bound to plasma proteins, with very little present in the reduced form. Glutathione is less abundant (~6 μmol/l) and is mostly present in the reduced form [17].

In samples from the parent and healthy control groups, we report plasma tHcy and tCys concentrations that are consistent with those in other studies [17, 23]. In blood samples with tHcy < 20 μmol/l, a positive correlation was demonstrated between tHcy and tCys. This is consistent with the fact that, under normal physiological conditions, cysteine is a product of homocysteine in the transsulphuration pathway. In contrast, a negative correlation was observed between tHcy and tCys in samples with tHcy ≥ 20 μmol/l (of which 36 out of the 45 samples were collected from the patient group), which may be explained by the reduced activity of cystathionine β-synthase, resulting in a concurrent decrease in plasma tCys [24]. Elevated plasma homocysteine may also displace cysteine from plasma protein binding sites [24, 25], which could further explain the associated lowering of tCys, through urinary excretion. A similar change in the relationship between tHcy and tCys has been reported in a large healthy control group (the
Hordaland study), in which tCys concentrations fell as tHcy concentrations increased above 20 μmol/l [26].

The present study demonstrates the role that plasma aminothiols play in determining a measure of plasma TAOC. The decrease in the free radical scavenging activity of the plasma associated with elevated tHcy may occur through a shift in the balance of other aminothiols and their redox states. A lowered concentration of plasma cysteine in association with elevated homocysteine may have specific adverse effects on the vasculature through impaired uptake of cysteine into endothelial cells, to prevent intracellular depletion of the important antioxidant glutathione [27]. In addition, a decreased plasma concentration of cysteine, which is a substrate for nitric oxide production, may decrease the availability of endothelium-derived relaxing factor and the function of S-nitrosocysteine, a more potent vasodilator than endothelium-derived relaxing factor [28]. Endothelial dysfunction following an oral methionine load has been ascribed to the transient increase in plasma tHcy [5,14], but this effect may be due partly to the accompanying transient decrease in plasma tCys [17,29].

Our interpretation of cysteine as a potential antioxidant in plasma is in contrast with that of Jacob et al. [30], who have suggested that plasma tCys might be a cardiovascular risk factor in patients with hyperlipidaemia. This suggestion was based on increased plasma tCys in patients with cardiovascular disease, compared with asymptomatic subjects. In their study tHcy was also elevated in patients relative to controls, but adjusted odds ratios for cardiovascular disease for the highest tertile were elevated in patients relative to controls, but adjusted odds ratios for cardiovascular disease were higher for the highest tertile compared with the lowest was greater for tCys than for tHcy.

Decreased plasma antioxidant activity has been demonstrated in patients with vascular disease [31] and in a hyperhomocysteinaemia rabbit model [32]. Plasma antioxidant capacity can be measured in a number of ways, but functional methods examine the ability of plasma to inhibit some oxidation process [33]. Plasma TAOC is a measure of the ability of plasma constituents acting in synergy to scavenge a specific radical generated in vitro [20]. A particular strength of this method is that it can be automated and runs with a high degree of precision (< 3% in our hands). Decreased concentrations of individual antioxidants were observed in samples with tHcy ≥ 20 μmol/l compared with samples with tHcy < 20 μmol/l. Although linear regression analysis suggested a predictive effect of tCys, tHcy and uric acid on TAOC, a multiple regression analysis revealed that only tCys had an effect independent of the other variables. These data further suggest that cysteine has an antioxidant role. Other measures of plasma antioxidant activity have also indicated that plasma protein thiols make an important contribution to the antioxidant activity of plasma and provide the first line of defence during peroxyl radical attack [34].

In conclusion, we have demonstrated for the first time that plasma tCys is an independent predictor of a measure of plasma TAOC in normal healthy subjects and in subjects with elevated plasma tHcy. The increased vascular risk associated with elevated plasma tHcy may be partly explained by an associated fall in plasma tCys.

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Aminothiols and extracellular antioxidant status


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