Abstract

Despite the growing evidence that plasma homocysteine is a cardiovascular risk factor, the mechanism behind the vascular injuries is still unknown. Studies are difficult as a result of the fact that little is known about the formation of different homocysteine species in vivo. Since extracellular glutathione and cysteine may influence the formation of different homocysteine species, we have in the present study investigated the different fractions of homocysteine and their relation to the different fractions of glutathione and cysteine in stroke patients and control subjects. We found a ratio of about 32–33% between reduced and total plasma glutathione concentrations and 2.6–3.0% between reduced and total plasma cysteine concentrations both in patients and in healthy control subjects. We noted an elevated concentration of total plasma homocysteine in stroke patients, but no difference in the ratio between reduced and total plasma homocysteine concentrations in patients and control subjects (mean value 1.20 and 1.10%, respectively). However, in a subgroup of patients with higher concentrations of total plasma homocysteine, we observed a significantly lower ratio of reduced to total plasma homocysteine compared to a subgroup of patients with lower concentration of total plasma homocysteine. A low reduced/total ratio of plasma homocysteine in combination with elevated plasma homocysteine concentration might reflect an increased pro-oxidant activity in plasma from these patients. Thus, increased pro-oxidant activity in plasma might be one factor, besides genetic and nutritional factors, that could explain hyperhomocysteinemia. Since substantial evidence indicates that progression of atherosclerosis is related to enhanced pro-oxidant activity, the premature vascular disease associated with increased plasma homocysteine concentration might be as a result of increased pro-oxidant activity and the elevated plasma homocysteine concentration may only reflect the increased oxidative stress. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Markedly elevated plasma levels of homocysteine, a sulfhydryl amino acid, are found in subjects with homocystinuria [1]. These patients exhibit early manifestations of arteriosclerotic disease as well as arterial and venous thrombosis. Numerous studies have indicated that a milder degree of hyperhomocysteinemia ( > 15 μmol/l) is also associated with an increased risk of developing occlusive vascular diseases [2–7]. Genetic factors, such as heterozygosity for homocystinuria, and the thermolabile variant of methylenetetrahydrofolate reductase, or nutritional factors such as deficiency of folate and/or cobalamin partly seem to explain the etiology of the hyperhomocysteinemia.

Adenosyl-methionine is the immediate precursor of homocysteine and is the principal methyl donor in mammals [1]. After a methyl transfer reaction (transmethylation), adenosyl-homocysteine is hydrolyzed to homocysteine and adenosine. Homocysteine may either be catabolized in the transulfuration pathway via cystathionine to cysteine or remethylated back to methionine, mainly by the folate and cobalamin-dependent enzyme methionine synthase. A third way of disposal of intracellular homocysteine is an export to the extracellular environment, which is suggested to occur when the intracellular production of
homocysteine exceeds its utilization. The facile extracellular oxidation of thiols results in a variety of disulfides in plasma [8–10]. These include low-molecular-weight disulfides and mixed disulfides with proteins, in plasma mainly albumin. Almost all homocysteine is therefore found as disulfide products with other thiol-containing molecules. The reduced homocysteine is reactive, and may produce peroxides during its oxidation, and it is generally supposed to be the form of homocysteine which is related to the atherogenic process. Despite the growing evidence that elevated plasma homocysteine is a cardiovascular risk factor, the mechanism behind the vascular injuries is still unknown. Studies of the possible pathogenic mechanisms of increased plasma homocysteine concentrations are difficult as a result of the fact that little is known about the mechanism for formation of different homocysteine species in vivo.

One of the major supplies of reduced thiol equivalents to the plasma is glutathione production. The major source for plasma glutathione is the liver, and the primary organ for clearance of circulating glutathione is the kidney [11–13]. The liver efflux contributes more than 90% of the total glutathione influx to circulation. The half-life of glutathione in plasma is short, about 15 min [13,14]. Reduced cysteine represents the most abundant low-molecular-weight thiol in plasma [10], and reduced cysteine might also be a component of extracellular antioxidant defense system [10,15]. The relation between reduced glutathione/cysteine and different homocysteine fractions in plasma is unknown.

We have recently developed a procedure for determination of reduced and total thiols in human plasma. Using this method we found that the reduced plasma homocysteine was fairly normal in spite of raised total plasma homocysteine concentration in patients with renal insufficiency [16] and in patients with stroke [9]. In a recent study [17] in patients with renal failure we observed a decreased ratio between reduced and total plasma thiol concentrations (homocysteine, cysteine and glutathione), which probably reflected an increased oxidative activity in plasma from these patients. In the present study, we have further studied the relation between reduced and total fractions of homocysteine and glutathione/cysteine in patients with stroke.

2. Material and methods

2.1. Study population

The control subjects were six women and eight men (71 ± 8 years, mean age ± S.D.); all were apparently healthy and without current or previous stroke. The patients, 13 women and 20 men (69 ± 10 years) had all had a stroke 1.5–3 years before the sampling was performed. The patients were all examined by computer tomography of the brain. A total of 29 patients had cerebral infarct and four patients intracerebral hemorrhage. Ten patients had heart disease (defined as current or previously received medical or surgical treatment for heart disease), eight patients were current smokers, six patients were receiving medical treatment for hypertension and five patients had diabetes mellitus. Two patients had elevated serum cholesterol and two patients had elevated serum triglycerides. The study design was approved by the Ethics Committee of the Medical Faculty, University of Lund, Sweden, and informed consent was obtained from all subjects.

2.2. Sample collection

Blood was drawn in pre-chilled (ice-water) EDTA-containing Vacutainer tubes (Becton Dickinson, Rutherford, NJ), placed within 10 s in ice-water and chilled for 5 min before centrifugation (10 000 × g, 4°C, 4 min). Protein-free plasma was immediately prepared, by transferring 1.2 ml of plasma to tubes, which contained 0.3 ml 150 g/l sulfosalicylic acid. After mixing, the precipitate was kept at 4°C for 30 min before centrifugation (10 000 × g, 4°C, 4 min). The supernatant was frozen at −70°C until analysis within 1 week. A total of 80 μl was used for the determination of reduced thiols.

Of the remaining chilled plasma (400 μl) in the Vacutainer tubes was transferred, as soon as possible after sampling, to a separate tube and mixed with 100 μl 0.1 mol/l dithiothreitol and incubated at 37°C for 15 min followed by protein precipitation with 100 μl 150 g/l sulfosalicylic acid. The precipitate was kept at 4°C for 30 min before centrifugation (10 000 × g, 4°C, 4 min). The supernatant was stored at −70°C until analysis within 1 week when 30 μl were used for the determination of total thiols in plasma. The assays for reduced and total plasma thiols have been described previously [9,18]. In short, the analyses were performed with a high-performance liquid chromatographic method, which utilized isocratic reversed-phase ion-pair liquid chromatography at pH 2.4 and postcolumn derivatization with 4,4'-dithiopyridine and colorimetric detection at 327 nm.

2.3. Statistics

The results are presented as means and S.D. All statistic tests were two-tailed and the 5% level of significance was used to evaluate the results. The following non-parametric methods were used: Mann–Whitney U-test in the case of two independent samples and Spearman’s rank correlation coefficients were calculated to test for monovariate relationship between different variables.
3. Results

Table 1 shows that total plasma homocysteine concentration was increased in patients but there were no other significant differences between thiol concentrations in plasma from healthy subjects and patients with stroke. Likewise, there was no difference between healthy subjects and the patients concerning the ratios between reduced and total thiols concentrations (Table 2).

The subgroup of patients (Table 3) with total plasma homocysteine concentration above 15 μmol/l exhibited lowered ratio between reduced and total plasma homocysteine concentrations compared to the subgroup of patients with plasma homocysteine concentration below 15 μmol/l. The ratios for reduced to total plasma cysteine or glutathione showed no differences in these subgroups of patients.

Correlation tests were performed between the reduced and total plasma thiol concentrations in both patients and healthy control subjects. Both in patients ($\rho = 0.57$, $P < 0.01$, $n = 33$) and in control subjects ($\rho = 0.59$, $P < 0.05$, $n = 14$) the correlation between reduced and total plasma glutathione concentrations was significant. Reduced plasma homocysteine concentration correlated to reduced plasma cysteine concentration both in controls ($\rho = 0.73$, $P < 0.01$) and patients ($\rho = 0.61$, $P < 0.001$). Likewise, total plasma homocysteine concentration correlated to total plasma cysteine concentration both in controls ($0.56$, $P < 0.05$) and in patients ($\rho = 0.41$, $P < 0.05$). There were no other significant relations between the different plasma thiol fractions.

4. Discussion

In healthy subjects, cysteine is the most abundant low-molecular-weight plasma thiol and about 3% of the total amount is in reduced form, whereas 1–2% of total plasma homocysteine is in reduced form [8–10]. Glutathione differs from these thiols in that a larger part exists in reduced form [8,17,18]. In the present study we have analyzed the reduced and total thiols in plasma from stroke patients and control subjects. The reduced proportion of cysteine and homocysteine in both patients and controls was as described previously [8–10]. Likewise, glutathione differed from the homocysteine and cysteine in that a large part exists in the reduced form in vivo. In the present study we found a ratio of about 30% in both patients and healthy control subjects, which is in agreement with our recent study [17]. We also found a positive correlation between reduced and total glutathione in both patients and control subjects. The reason a larger part of glutathione exists in reduced form is probably its continuous secretion in the reduced form from the liver and its rapid elimination by the kidneys [11–13]. Thus, the constant supply of reduced glutathione to the blood is sufficient to maintain a high level of this substance in vivo. The high production of reduced glutathione from the liver and the elimination by the kidney leads to a rapid turnover of plasma glutathione [14].

In the present study we observed significant correlations between reduced plasma homocysteine concentration and plasma cysteine concentration. It has previously been reported [10] that alteration of redox
status of homocysteine rapidly affects and is related to the redox status of cysteine. These observations may be as a result of a continuous disulfide exchange reaction in plasma. Since the production of homocysteine is small compared to the high and continuous efflux of reduced glutathione, it is not likely that the production of homocysteine has any profound influence on the redox status of plasma glutathione. This hypothesis is supported by the lack of correlations between concentrations of plasma homocysteine fractions and glutathione fractions and the finding of similar ratios between the concentrations of reduced and total glutathione in both control subjects and stroke patients. Likewise, there were no relations between the concentrations of plasma cysteine fractions and concentrations of plasma glutathione fractions. Thus, neither changes in the concentrations of plasma homocysteine fractions nor the concentrations of plasma cysteine fractions seem to be influenced by the concentrations of plasma glutathione fractions, which is in agreement with our recent study [17].

Mansoor et al. [19] reported that total plasma cysteine and both reduced and total plasma homocysteine concentrations were increased in patients with early-onset peripheral vascular disease, but the reduced plasma cysteine concentration was not changed. Our findings in stroke patients are somewhat different from those of Mansoor et al. [19]. However, the concentrations of reduced and total plasma homocysteine fractions in stroke patients and control subjects observed in the present study are similar to our previous findings [9]. As in the previous study [9], we noted an elevated concentration of total plasma homocysteine but no change in the concentration of reduced plasma homocysteine. In both the present and the previous study [9] the ratios between reduced and total plasma homocysteine concentrations did not differ between stroke patients and control subjects. The control subjects in the present study were few, which may account for the fact that only few differences were observed. However, in the present study we observed that a subgroup of patients with higher concentrations of total plasma homocysteine (cut-off level 15 μmol/l) had a significantly lower ratio between reduced and total plasma homocysteine concentrations compared to a subgroup of patients with lower concentrations of plasma homocysteine. We also used the cut-off level 16 μmol/l of plasma homocysteine and obtained similar findings (not shown). A low reduced/total ratio of plasma homocysteine and increased concentration of total plasma homocysteine in subgroups of stroke patients might reflect an increased overall pro-oxidant activity in those patients. It is likely that even healthy subjects with elevated concentration of total plasma homocysteine exhibit a lowered reduced/total ratio of plasma homocysteine, possibly reflecting an increased pro-oxidant activity, because there was a significant inverse correlation between concentration of total plasma homocysteine and reduced/total ratio of plasma homocysteine in 29 healthy subjects [17]. The reduced/total ratios for concentrations of plasma cysteine and glutathione showed, in the present study, no differences in the subgroups of patients. This finding might indicate that the ratio between reduced and total plasma homocysteine concentrations is a more sensitive marker for increased oxidative activity than the ratio between reduced and total plasma cysteine or glutathione concentrations.

Our hypothesis is that increased pro-oxidant activity in plasma from some patients with vascular disease leads to a rapid oxidation of reduced homocysteine to disulfides. Homocysteine engaged in disulfide bonds with plasma proteins is not as metabolically available as homocysteine in low-molecular-weight disulfides or in its reduced form, and therefore accumulates in the circulation [20]. Therefore increased pro-oxidant activity in plasma might lead to an increase of total plasma homocysteine. The lowered elimination of plasma homocysteine observed in patients with renal failure [21] could also be explained by increased disulfide formation of homocysteine with plasma proteins and consequently a lower metabolic availability. Thus, increased pro-oxidant activity in plasma might be a further factor, besides the genetic and nutritional factors mentioned in the introduction, that could explain hyperhomocysteinemia. Since substantial evidence indicates that progression of atherosclerosis is related to enhanced pro-oxidant activity [22], the premature vascular disease associated with increased plasma homocysteine concentration might be as a result of increased pro-oxidant activity and not to increased plasma homocysteine concentration.

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References


