Effect of Age and Sex on Plasma Total Homocysteine in Taiwanese Subjects

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Abstract

Plasma total homocysteine (tHcy) is now established as a clinical risk factor for coronary artery disease, as well as for other arterial and venous occlusive diseases. Therefore, we measured the plasma tHcy concentrations in 385 healthy Chinese subjects in Taiwan and in 40 patients with occluded coronary artery disease or maintenance hemodialysis. The plasma tHcy levels in Taiwanese male and female volunteers were found to increase gradually with age (age group: 20-29, 30-39, 40-49, 50-59, and >60; mean ± SD 8.22 ± 2.00, 8.51 ± 2.67, 8.87 ± 2.22, 11.41 ± 2.50 and 13.28 ± 2.31 μM for male volunteers and 6.49 ± 1.75, 7.15 ± 1.20, 7.40 ± 1.30, 9.57 ± 3.01 and 10.95 ± 2.11 μM for female volunteers). At the same age, male volunteers were shown to have higher tHcy levels than female volunteers. In addition, the mean concentrations of plasma tHcy in occluded coronary artery disease (13.62 ± 5.43 μM) or in maintenance hemodialysis (21.28 ± 4.32 μM) were statistically higher than in age-matched normal subjects (11.02 ± 2.85 μM). This study emphasizes the significance of age and sex-associated difference in the plasma tHcy levels, and underlines the importance of the range for plasma homocysteine in normal Taiwanese subjects.

Key Words: homocysteine, age, sex, occluded coronary artery disease, hemodialysis

Introduction

Homocysteine (Hcy) is a sulfur amino acid formed during the metabolism of methionine. Once synthesized, homocysteiene may undergo remethylation to methionine or enter the transsulfuration pathway to cysteine synthesis. It exists in several forms in plasma. The major form of plasma Hcy is protein-bound (70%), and the remainder is mostly spontaneously oxidized to homocystine or forms the homocysteine-cysteine disulfide, with only 2-3 % remaining as free Hcy (37). The concentration of total homocysteine (tHcy) in human plasma is defined as the sum of all the homocysteine species in plasma, including free, bound to proteins and oxidized forms. The results of many recent studies have indicated that

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an elevated plasma level of tHcy is an independent risk factor for myocardial infarction (32), stroke (11) and peripheral vascular atherosclerotic disease. (10, 26, 34).

The normal values for tHcy are usually 5~15 μmole/L in healthy subjects (36), and plasma homocysteine levels are determined by both genetic (35) and nutritional factors (31). Moderate hyperhomocysteinemia (15~30 μmole/L) is now recognized as an independent risk factor for coronary artery disease (CAD) (7). Thus determination of the plasma tHcy concentration can be useful in the clinical diagnosis and be a potentially important prediction for premature cardiac and other occlusive disease. Although several studies have documented the plasma tHcy concentration in healthy subjects (15, 23, 29, 36), no literature on the tHcy concentration of healthy Chinese population in Taiwan is presently available. Thus, the objectives of this study were to measure plasma tHcy concentrations in healthy Taiwanese men and women selected for health according to strict criteria, in order to determine relations between tHcy and age separately by sex. From this we plan to provide plasma tHcy values from the normal Chinese subjects in Taiwan for others to reference on. The effect of age and sex on plasma tHcy concentrations was also studied. In addition, the results were compared with values established for patients with occluded coronary artery disease or undergoing hemodialysis.

Materials and Methods

Chemicals

Dithiothreitol (DTT), tetrabutylammonium hydroxide (TBA) and D, L-homocysteine were purchased from Sigma (St. Louis, MO, USA). Monobromobimane (mBBr) was purchased from Calbiochem (LaJolla, CA, USA). HPLC-grade methanol was purchased from Mallinckrodt (Kentucky, USA).

Human Subjects

The 385 healthy Chinese subjects (199 men and 186 women) enrolled in this study were volunteers undergoing physical examinations at the Kuang Tien General Hospital in Taichung without medical history of vascular disease, diabetes mellitus, renal disease or hepatic disease. The volunteers were divided into five different age groups (20-29, 30-39, 40-49, 50-59 and >60 years of age). Thirty-six patients over 50 years of age (mean 66; range 51-85) with occluded coronary artery disease who had been hospitalized for cardiac catheterization in Taichung Veterans General Hospital, and forty uremia patients with chronic hemodialysis (mean 65; range 50-83) in Kuang Tien General Hospital were also entered in this study.

Blood was withdrawn from overnight fasting subjects and plasma was collected immediately followed by centrifuging blood samples 3000 × g for 10 min under 4°C and removal of blood cells. An equal volume of 50mM sodium phosphate buffer (pH 7.4) containing dithiothreitol (final concentration 500 μM) was added to the plasma. The mixture was heated to 50°C for 10 min, with subsequent cooling and addition of an equal volume of 2 mM methanolic mBBr. The derivatized material was incubated for 20 min prior to HPLC analysis. The concentrations of total homocysteine in human plasma were calculated from calibration graphs established by injection of standard Hcy solutions of various concentrations repeatedly. For each sample serie, a new calibration graph was used. Standard Hcy solutions were prepared by dissolving Hcy in 50 mM phosphate buffer containing 50 μM dithiothreitol.

High Performance Liquid Chromatography Analysis

The high performance liquid chromatography (HPLC) system consisted of an 1100 Series quaternary pump, an 1100 Series on-line degasser, an 1100 Series autosampler and a Model 1462A HPLC fluorescence detector with two holographic diffraction monochromators, all obtained from Hewlett-Packard (Waldbronn, Germany). Optimum responses of homocysteine were observed when the excitation and emission wavelengths were set at 270 and 474 nm, respectively. Peak areas and concentrations were determined using a Hewlett-Packard Chem Station Chromatographic Management System.

Separations were achieved using an Econosphere C_{18} cartridge column (150 × 4.6 mm I.D., particle size 5 μm) obtained from Merck. Prior to the preparation of mobile phase containing TBA, a commercially available 40% aqueous solution of tetrabutylammonium hydroxide was neutralized to pH 6.4 with phosphoric acid. All mobile phases containing TBA were 30 mM in TBA. After addition of TBA, the pH of the mobile phase was adjusted to 3.2-3.4 (apparent) as measured with a glass electrode. In all cases, the mobile phases were filtered through filters (pore size 0.2 μm) and degassed. Binary gradient elution at a flow rate of 1.1 ml/min was used. Mobile phase A consisted of 30 mM TBA in 20% methanol, while mobile phase B consisted of 30 mM TBA in 100% methanol. The elution profile was 0-7 min 90% mobile phase A and 10% mobile phase B, followed by a column wash with mobile phase B for 5 min. Then the column was regenerated with 90% mobile phase A for 5 min before the next injection.

Table 1. The Level of Plasma tHcy in Normal Taiwanese Subjects

	Male (199)		Female (186)	
Age	Mean±SD	95% CI	Mean±SD	95% CI
20-29*	8.22±2.00 (60)[a]	7.70-8.7	6.49±1.75 (24)[a]	5.75-7.23
30-39*	8.51±2.67 (31)[a]	7.53-9.49	7.15±1.20 (26)[a]	6.67-7.63
40-49*	8.87±2.22 (41)[a]	8.17-9.57	7.40±1.30 (46)[a]	7.01-7.78
50-59*	11.41±2.50 (32)[b]	10.30-12.52	9.57±3.01 (43)[b]	8.64-10.49
60+*	13.28±2.31 (35)[c]	12.21-14.37	10.95±2.11 (47)[c]	10.33-11.57

Data are presented as mean ± standard deviation. CI indicates confidence interval. Letters (a-c) denote significant difference in one column ($p<0.05$). * Significant difference between male and female at the same age ($p<0.05$).

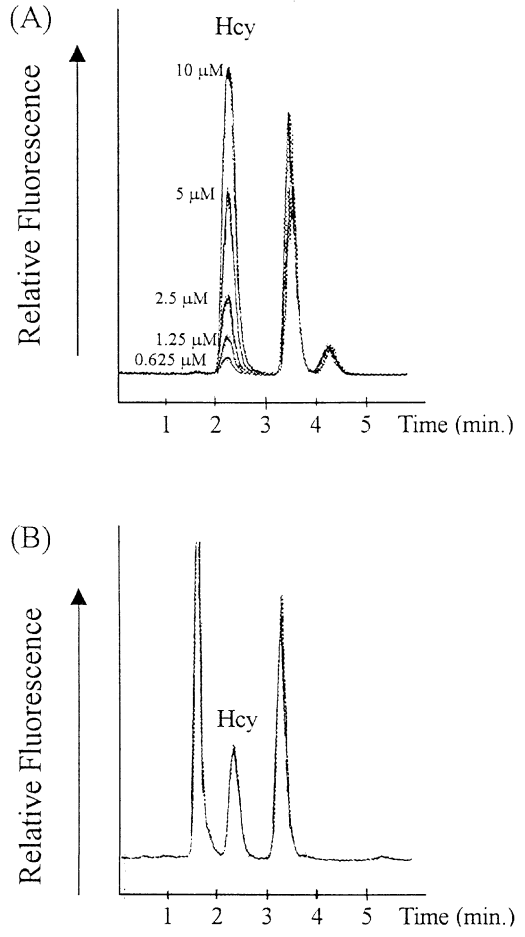

Fig. 1. Typical chromagrams of homocysteine in standard solutions (A: 0.625 ~ 10 μM) and in plasma sample (B: 4.2 μM)

Statistical Methods

Results had a normal distribution, and thus are expressed as means ± standard deviation. Simple correlations were calculated using Pearson's correlation coefficient. Analysis of variance was performed by ANOVA procedures and $p < 0.05$ was considered to be statistically significant.

Results

Effect of Age and Sex on Plasma Total Homocysteine in Healthy Chinese Subjects

In this study, plasma tHcy was determined by HPLC assay with fluorimetric detection. Fig. 1 illustrates a typical chromatogram of homocysteine in standard solutions and in plasma of a normal person, derivatized with mBBr. The homocysteine peak was well separated and the migration time was 2.68 min. As shown in Table 1, the male and female volunteers were divided into five different age groups and the plasma total homocysteine concentrations in the 385 normal Chinese subjects in Taiwan were presented. Plasma homocysteine levels in both male and female increase with age. Both normal females and males above the age of 50 years old exhibited statistically significant increases of plasma tHcy compared to subjects below this age. In addition, the concentrations of plasma tHcy were statistically significantly higher in male volunteers than in female volunteers of the same age.

We also investigated the relationship between age and plasma tHcy in normal Taiwanese subjects, with the results as shown in Fig. 2. A significant positive correlation was observed between plasma tHcy concentration and age in both males and females (Person correlation $r = 0.607$, $p<0.01$ in male; $r = 0.595$, $p<0.01$ in female). In view of the fact that plasma tHcy in Taiwanese male and female volunteers were found to increase with the increase of age.

Patients with Occluded Coronary Artery Disease or with Hemodialysis

Fig. 3 shows the plasma tHcy levels of thirty-six patients over 50 years old (mean 66, range 51-85) with occluded coronary artery disease and forty uremia

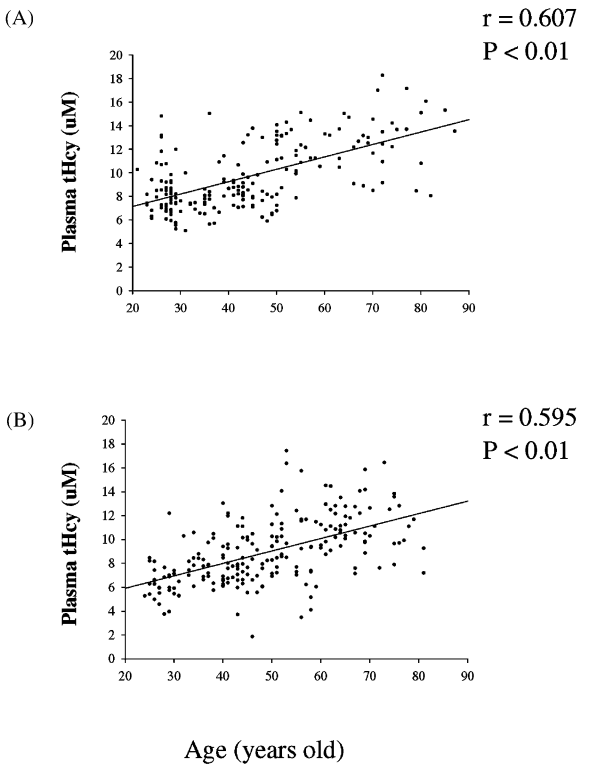

Fig. 2. Correlation of plasma tHcy concentration with age in normal Taiwanese (A) males (n=199) and (B) females (n=186).

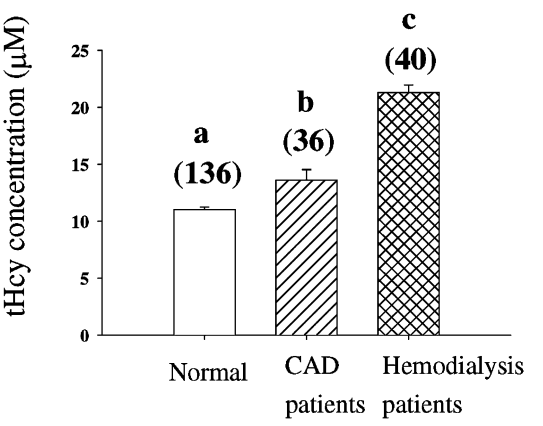

Fig. 3. Plasma tHcy levels in patients and in age-matched normal subjects. Data are presented as mean ± standard deviation. The numbers in the parentheses represent the number of subjects undergoing blood withdrawal. Letters (a-c) denote significant difference between groups ($p < 0.05$).

patients (mean 65, range 50-83) with chronic hemodialysis. The results were also compared with the 136 age-matched (mean 63, range 50-87) normal subjects. Patients with either occluded coronary artery disease (13.62 ± 5.43 μmole/L) or hemodialysis (21.28 ± 4.32 μmole/L) had significantly higher ($p < 0.05$) tHcy level than controls (11.02 ± 2.85 μmole/L). These results indicate that the plasma tHcy in patients with occluded coronary artery disease or with uremia hemodialysis were about 1.00 ~ 2.34 times and 1.8 ~ 3.13 times greater than the controls, respectively. We also evaluated the prevalence of moderate hyperhomocysteinemia in a group of patients with occluded coronary artery disease (CAD) and in a group of patients with uremia hemodialysis. Hyperhomocysteinemia was diagnosed when fasting plasma tHcy levels exceeded the upper limit of the normal range (mean ± 2 SD of values obtained in the control group). Total homocysteine levels exceeded the upper limit of the normal range in five patients (14%) with occluded CAD and thirty-four patients (85%) with uremia hemodialysis.

Discussion

High-performance liquid chromatographic (HPLC) methods employing fluorescence detection have been successfully applied to the measurement of tHcy in plasma (1, 14). The analytical procedures using a derivatization reagent, monobromobimane, are of interest because of the selectivity for thiols, leading to an easy interpretation of the chromatograms. In this study, we used the reliable and sensitive technique (12) that gives values (4.8 ~ 15.6 μmole/L) in normal subjects, comparing well with those (5 ~ 15 μmole/L) obtained by other analytical procedures (36).

Like others (2, 8, 28), we observed age-related increases in plasma tHcy concentration in both males and females. Several causes of increased plasma tHcy levels with age are known. Decreases in cofactor levels such as folate and vitamin B12 in elderly people (19, 22), or coexisting age-related deterioration of renal function (28) may be responsible. Furthermore, age-dependent reductions in cystathionine β-synthase activity may also be implicated (27). The enzyme cystathionine β-synthase with pyridoxine as a vitamin cofactor catalyzes the reaction of homocysteine with serine to form cystathionine, which is then split to form cysteine (33). A deficiency of this enzyme results in abnormal accumulation of homocysteine. In this study, we also observed that there were significantly differences in tHcy levels among Taiwanese male and female volunteers of simlar age, the tHcy concentrations in men being about 16.1 ~ 21.1% higher than the concentrations in women. This is similarly to other studies (3, 10, 32, 34), which also noted an important difference between males and females. The gender difference for tHcy has been ascribed to various factors, including rates of Hcy formation, the presence of larger muscles and greater creatine phosphate

synthesis in men, as well as a reducing effect from estrogen in women (10, 25).

Moderate hyperhomocysteinemia is a possible risk factor for vascular disease and its determination in clinical investigation of vascular disease is now indicated. In this study, we found that the plasma tHcy concentrations were significantly higher in patients with occluded artery disease, as is usually observed (5, 21, 32). Similarly to other studies (21), the mean plasma tHcy level was 2 to 3 μmole/L (20 to 30%) higher in coronary patients than in healthy control subjects. The way that homocysteine elicits pathological effect is not complete understood. Homocysteine is thought to cause endothelial damage, possibly through formation of free radicals and homocysteine thiolactone, a known cellular toxin (23). Furthermore, there is evidence that homocysteine has prothrombotic effect (30).

Consistent with previous reports (17, 20), the tHcy levels in patients with chronic renal failure may be two to four times normal. The cause of increased tHcy level in chronic renal failure has not been definitely clarified. One hypothesis links hyperhomocysteinemia in chronic renal failure with decreased plasma serine, since both degradation pathways of homocysteine, by either remethylation or cystathionine formation, consume serine (13). Furthermore, the significant reduction of homocysteine clearance (16) and reduced renal glomerular filtration rate (4) in renal patients may also be implicated. Indeed, hyperhomocysteinemia contributes to premature atherosclerosis in dialysis patients (6, 9). However, daily supplementation with folic acid has been shown to have a beneficial effect on the cardiovascular risk profile in dialysis (18).

To our knowledge, this is the first reported the range for plasma total homocysteine in Taiwanese subjects. However, a larger representative sample study should be needed to establish the reference value for plasma tHcy in normal Taiwanese subjects. In conclusion, we observed that males have markedly higher levels of plasma tHcy than females, and the plasma tHcy levels in both genders increase with increasing age. The mean concentrations of plasma tHcy in occluded artery disease or in maintenance hemodialysis patients were statistically higher than in age-matched normal Taiwanese subjects. The reported levels of plasma tHcy in Taiwanese subjects should be useful for clinical diagnosis and applications.

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