# Hyperhomocysteinemia does not Perturb Protein C and S Activity in Patients with Cerebral Ischemia

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**Abstract-** Homocysteine is an independent risk factor for vascular thrombosis involving the coagulation cascades and vascular wall. An increase of homocysteine is known to perturb the protein C anticoagulant system by generating activated protein C resistance or suppressing thrombomodulin. Its effect to protein C and S activity is rarely mentioned in stroke patients. In this study, we examined the relation between plasma homocysteine level and protein C or S activity in cerebral ischemia patients. There were 27.7% patients to have plasma homocysteine levels over 15 μM/L. The plasma homocysteine levels were significantly higher in hypertensive ($p = 0.004$) and male ($p = 0.024$) patients respectively. Hyperhomocysteinemia is not associated with the change of protein C or S activity. There was no correlation between plasma homocysteine levels and protein C or S activity. These findings did not change in relating to the size of infarct. Therefore, it is possible that hyperhomocysteinemia does not perturb protein C or S activity in cerebral ischemia subjects.

**Key Words:** Homocysteine, Protein C, Protein S, Cerebral ischemia

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## INTRODUCTION

Homocysteine is a thiol-containing amino acid that is formed by demethylation of methionine. It is metabolized by two major pathways: remethylation and trans-sulfuration. Homocysteine is converted back to methionine in remethylation cycle while metabolized to cysteine in trans-sulfuration pathway. A perturbation of either pathway would result in an accumulation of homocysteine. The folate and cyanocobalamin are essential cofactors for the remethylation cycle and the pyridoxal phosphate trans-sulfuration pathway respectively[1].

The link between vascular diseases and elevated homocysteine levels has already been known for more than 30 years. It is until recent years that their relation becomes more apparent. Homocysteine is currently recognized to perturb both coagulation and vascular system, and is relevant to thrombosis and atherosclerosis[1,2]. In regard to the effect of hyperhomocysteinemia (Hcy) to protein C anticoagulant system, most data come from

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in vitro or ex vivo study. In human, homocysteine mainly reduces thrombomodulin synthesis and thrombin binding, induces activated PC resistance[1-4], or associates with a decrease of protein C antigen. However, credible information is lacking for cerebral ischemia thoroughly despite a tight relation has been established with hyperhomocysteinemia. In this study, we examined the relation between plasma homocysteine level (PHL) and protein C and S activities in cerebral ischemia.

## MATERIALS AND METHODS

### Population sampling

In this study, patients with noncardiac cerebral ischemia (NCCI) were enrolled. The clinical diagnosis of NCCI had been well described in our previous studies[5,6]. In brief, NCCI is diagnosed in patients with acute focal neurological deficits examined by two neurologists, corresponding infarct in cranial computed tomogram or magnetic resonance image reviewed by neuroradiologist, and exclusion of caridac diseases based on cardiac echogram and electrocardiogram. Patients were excluded if they had any of the followings: systemic lupus erythematosus, collagen diseases, recent craniofacial trauma or alcoholism (> 30 gm/day of alcohol) in recent 6 months; consumption of herb remedy, anticoagulant, estrogen, androgen, or corticosteroids; abnormal renal function (serum creatinine > 1.4 mg/dl), liver function (alanine aminotransferase or aspartate aminotransferase > 40 IU/L, or cirrhosis), thyroid or adrenal function; a positive antinuclear antibody (> 1:40), or transient ischemic attack. The diagnosis of SLE was made according to the American College of Rheumatology with an updated revision[7,8].

### Laboratory examinations

The blood protein C, protein S, homocysteine, prothrombin time, activated partial thromboplastin time, albumin, alanine aminotransferase and aspartate aminotransferase were examined in each patient. Venous blood was collected from antecubital vein using a sterile vacutainer system after an overnight fasting for ten hours. The blood samples were anticoagulated by 3.8% trisodium citrate (1:9 citrate:blood) in all tests except tripotassium EDTA for homocysteine. For activated partial thromboplastin time, prothrombin time, protein C, protein S and homocysteine, the blood samples were centrifuged within one hour after collection for 10 minutes at 3000 g under 4°C to yield platelet-poor plasma. The activated partial thromboplastin time and prothrombin time were done within 2 hours after collection. The platelet-poor plasma was aliquoted and stored at -20°C for batch measurement within 14 days. For albumin, alanine aminotransferase and aspartate aminotransferase, serum was examined within 2 hours after collection (Hitachi 7450 autoanalyzer, Tokyo, Japan).

The activated partial thromboplastin time and prothrombin time assays wre performed on the Sysmex CA-6000 coagulometer (Australian Diagnostics Corporation, Sydney, Australia). Thromboplastins were used with APTT-LS (Fisher Diagnostics Middletown, VA, USA) for the activated partial thromboplastin time and Innovin (Dade Behring Marburg GmbH, Marburg, Germany) for the prothrombin time. The protein C and protein S were measured using the chromogenic assay (STA-STACHROM®-Protein C, Diagnostica Stago, France), and homocysteine was measured using the fluorescence polarization immunoassay (AxSYM Homocysteine kit, Abbott laboratories, Oslo, Norway). The protein C and protein S were expressed as activity in percentage and homocysteine in μgM/L. In protein C, protein S and homocysteine assay, the coefficient variance of intravariation was 1.8%, 1.8% and 2.0%; and intervariation was 3.6%, 3.6% and 4.6%, respectively.

### Type of infarct

The type of infacrt was classified into lacunar and nonlacunar according to the clinical symptom, infarct size and location of infarct measured by conventional diffuse-weighted MRI (Delta = 36.6 ms, TR/TE = 10,000/99 ms, diffusion sensitivity b = 1000 s/mm) with a 1.5-T MRI scanner modified with hardware for echoplanar imaging (GE Signa Horizon Echospeed). Lacunar infarct was defined as a presentation of lacunar syndrome[9] and volume of solitary infarct < $1.8 \times 10$ $mm^3$[10]. Nonlacunar infarct was the lesion involving the hemisperic parenchyma or brainstem with a volume > $1.8 \times 10$ $mm^3$ but less than one-third territory irrigated by anteri-

or, middle or posterior cerebral artery, or half territory of hemibrainstem.

**Statistical analysis**

Descriptive statistics for continuous variables were reported as mean and standard deviation. For categorical variables, number and percentage of patients was given. Differences between means were assessed with unpaired Student's t-test and least significant difference (LSD) test for independent samples for variables with a normal distribution. Categorical variables (hypertension, diabetes mellitus, PHL) were evaluated with Chi-Square test. Simple regression tests wre calculated between all variables in each subset of data (protein C, protein S, and homocysteine) in order to elucidate the correlation of each variable of a subset. Multiple variables linear regression test was calculated for the effect of PHL influenced by sex, age, protein C, protein S, hypertension and diabetes mellitus. A probability less than 0.05 was considered significant.

## RESULTS

A total of ninety-four patients were enrolled in this study. There were 66 men and 28 women. The age ranged from 28 to 89 years; average being 61.22 years. Patients were divided into three groups according to their PHL, that were, Group I: PHL ≤ 10.00 μML, Group II: 10.01 - 14.99 U/ml and Group III: ≥ 15.00 U/ml. Accordingly, 23 patients belonged to Group I, 43 patients Group II and 26 patients Group III, respectively. An increase of PHL over 10.00 U/ml as in Group II and III patients was considered abnormal.

There were 32 patients sustained diabetes mellitus. The mean value of PHL did not differ between diabetic (12.58 ± 4.55 μM/L) and nondiabetic patients (13.43 ± 4.43 μM/L) (t = -0.869, p > 0.1). The frequency of Hcy did not increase in diabetic patients (Chi-Square test, $X^2$ = 0.162). There were 65 patients to have hypertension. The mean value of PHL was higher in hypertensive (13.66 ± 4.69 μM/L) than normotensive patients (11.98 ± 3.73 μM/L) but the statistic was insignificant (t = -1.856, p = 0.068). However, Hcy was more frequent in hypertensive patients (Chi-Square test, $X^2$ = 15.51, p < 0.01; LSD, Group II and III, p = 0.012). In the Chi-Square test, the expected value in all cells was over 5.

The alanine aminotransferase, aspartate aminotransferase and albumin were within reference ranges in all patients. The protein C was 100.35 ± 20.81%, 97.49 ± 22.48 % and 92.53 ± 23.79 % in Group I, II and III patients, respectively. There was no significant difference among them (LSD test, F = 0.775, p = 0.464) (Fig. 1A). The protein S was 81.00 ± 22.09 %, 80.47 ± 20.97 % and 77.77 ± 17.94 % in Group I, II and III patients, respectively. There was no significant difference among them (LSD test, F = 0.190, p = 0.827) (Fig. 1B). The PHL correlated neither to protein C ($r^2$ = 0.0261) nor to protein S ($r^2$ = 0.0007) (Fig. 2). In multiple variables regression test, the PHL is correlated to gender (p = 0.004) and hypertension (p = 0.024) (Table 1).

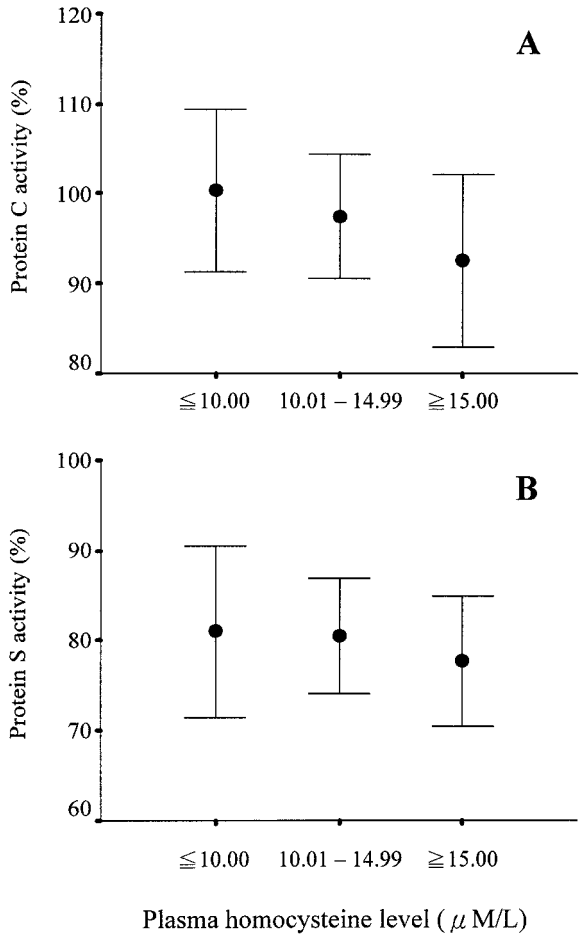


**Figure 1.** The protein C (A) and S activities (B) in different groups of cerebral ischemia patients classified according to their plasma homocysteine level.

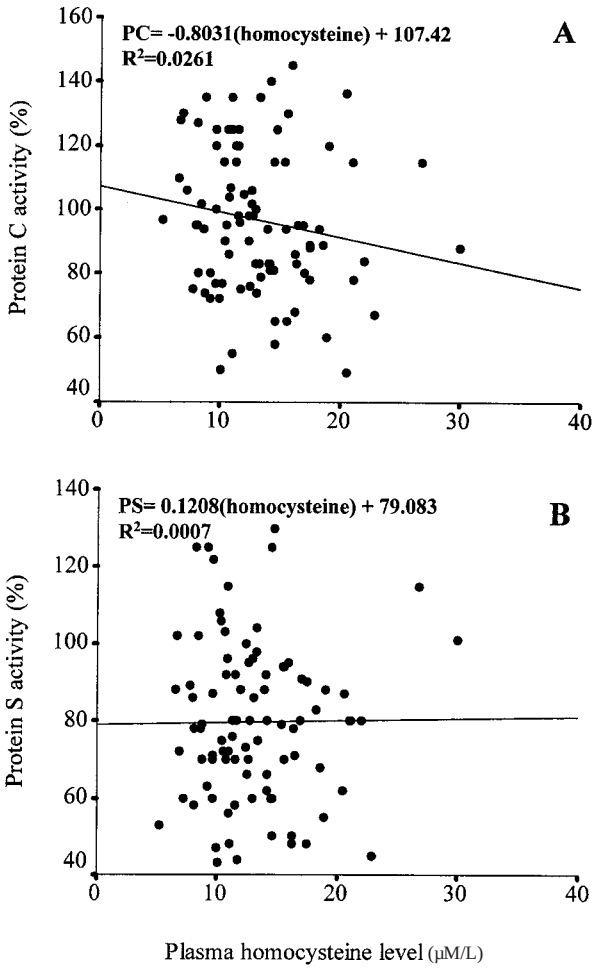


**Figure 2.** The correlation between plasma homocysteine levels and protein C (A) or S activities (B).

***Table 1.*** *The multiple variables linear regression analysis of the plasma homocysteine level in 94 patients*

| Parameters | E coefficient | Standard error | t-value | p-value |
|---|---|---|---|---|
| Sex | -2.968 | 0.991 | -2.994 | 0.004 |
| Hypertension | 2.264 | 0.985 | 2.299 | 0.024 |
| Age | 0.05228 | 0.046 | 1.128 | 0.263 |
| Protein C | -0.02200 | 0.022 | -0.997 | 0.322 |
| Diabetes mellitus | -0.702 | 0.955 | -0.736 | 0.464 |
| Protein S | 0.00670 | 0.024 | 0.282 | 0.779 |

There were 22 lacunar and 72 nonlacunar patients. We did not find any difference of the frequency of hypertension or diabetes mellitus, age gender, protein C, protein S or PHL between the two types of infarct (Table 2). The PHL was still positively correlated to gender (lacunar patients, p = 0.003; nonlacunar patients, p = 0.025) and hypertension (lacunar patients, p = 0.002; nonlacunar patients, p = 0.003).

***Table 2.*** *The parameters in lacunar and nonlacunar patients*

| Parameters | Lacunar (n=22) | Nonlacunar (n=72) |
|---|---|---|
| Protein C (%) | 94.50 ± 19.35 | 97.60 ± 23.04 |
| Protein S (%) | 79.36 ± 23.27 | 81.07 ± 19.42 |
| Plasma homocysteine level (U/ml) | 12.16 ± 4.14 | 13.44 ± 4.55 |
| ≤10.00 μM/L | 9 (40.9%) | 14 (19.4%) |
| 10.01 - 14.99 U/ml | 10 (45.5%) | 35 (48.6%) |
| ≥15.00 U/ml | 3 (13.6%) | 23 (31.9%) |
| Age (year, mean±SD) | 59.64 ± 12.00 | 61.71 ± 9.12 |
| Gender, male (%) | 18 (81.8%) | 48 (66.7%) |
| Hypertension, case (%) | 12 (54.6%) | 53 (73.6%) |
| Diabetes mellitus, case (%) | 5 (22.7%) | 27 (37.5%) |

No significant difference (unpaired Student's t-test or Chi-Square test, p > 0.05) in any Parameters between the two subtypes.

## DISCUSSION

In our series, 69 out of 94 patients (73.4%) had an increase of PHL, that is, over 10 μM/L. Although diabetes mellitus is a common cause for Hcy, a high frequency of Hcy is found in hypertensive patients instead of diabetic ones in our series. Actually, Hcy has been mentioned to be more prevalent in hypertensive than normotensive subjects, and associates with insulin resistance[11]. The causal relationship between Hcy and hypertension is unknown, but a concomitant association between them could worsen the vasorelaxative ability in hypertensive subjects[12].

The protein C anticoagulation system is one of the major physiological importance in the regulation of hemostatic mechanism. Protein C is encoded by a single gene on chromosome 2q13-14 while PS in chromosome 3. They are vitamin K-dependent serine proteases synthesized by the liver and degraded in the liver and kidney. Protein S is also synthesized by endothelial cells, platelets, testis, ovary, brain, thymus, and spleen. The circulating inactive PC is converted to the active form, activated protein C, by thrombomodulin-thrombin complex on the endothelial surface. Activated protein C then inactivates the factor Va and VIIIa biphasically under the presence of cofactor protein S[13]. In general, physiologi-

cal subcoagulant thrombin continuously stimulates the production of activated protein C in circulation to maintain an anticoagulant state[14]. According to our results, homocysteine seems not to perturb the protein C or protein S activity in circulation.

Although Hcy is considered to decrease generation of activated protein C by thrombomodulin hypofunction and resistance to activated protein C caused by decreased inactivation of factor Va[1-4], a number of clinical reports have revealed a normal protein C activity in Hcy subjects. In patients with Behcet's disease[15] and deep vein thrombosis[16], Hcy commits with a higher risk of thrombosis. However, the protein C and S activities were normal in these subjects.

Activated protein C resistance is not an uncommon hypercoagulation disorder and is examined by measuring the activated partial thromboplastin time with and without exogenous activated protein C. A dilution of patient's plasma with factor V deficient plasma could eliminate the effect of factor deficiency or increment that alters the baseline activated partial thromboplastin time. Activated protein C resistance is considered if the activated partial thromboplastin time does not prolong, indicating that the factor Va resists for proteolysis by activated protein C. In this study, we measured the amidasic activity of activated protein C cleavaged from protein C by the Agkistrodon contortrix contortrix venom. The results in this study represented that the functional protein C activity was vulnerable for activation, such as thrombin, in circulation. Therefore, a normal protein C activity in our patients did not equalize to activated protein C resistance that was found in Hcy. It was also similar for protein S in our patients.

Protein C, protein S and sulfur-containing homocysteine are synthesed from the liver. The methionine adenosyltransferase catalyzes the formation of S-adenosylmethionine, the principal biological methyl and propylamino donor, and is essential to normal cell function[1,17]. S-adenosylmethionine is converted to 5-adenosylhomocysteine, wthich is hydrolyzed to homocysteine and adenosine. Homocysteine enhances the inhibitory effect of extracellular adenosine on hepatic protein synthesis[18] and may therefore suppress hepatic protein C or protein S synthesis. In our series, we did not observe any decrease of protein C, protein S or albumin in Hcy patients. It might be that Hcy usually resulted from an inability of remethylation or transsulfuration. Either change might lead to an accumulation of homocysteine that in turn, suppresses the transmethylation process in feedback.

Increased hepatic synthesis of homocysteine after liver injury represents a modulation of expression of various genes, DNA methylation and substrates for regeneration of hepatic cells[19]. On contrary, the hepatic synthesis of protein C and S may decrease in liver disease. Therefore, a decrease of protein C or S activity in Hcy patients may result from liver damages. Our patients were selected for an absence of known liver disease and abnormal liver function, and the results herein apparently supported that Hcy did not repress protein C or S activity in patients with normal liver function.

In our study, two limitations must be acknowledged. First, the number of patients was small to make a better conclusion. Second, only the functional protein C and protein S activity were measured. An examination of the antigenic level of these proteins may provide quantitative information. Nevertheless, our results rectify the effect of Hcy in different parts of the protein C anticoagulant system.

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