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ORIGINAL RESEARCH

Effects of Plasma Homocysteine Levels on Serum HTase/PON Activity in Patients with Type 2 Diabetes

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ABSTRACT

***Introduction*:** Homocysteine is a predictor of vascular disease and may have an important role in diabetes. In this study, we examined the effects of folic acid and methylcobalamin supplementation on changes in homocysteine (Hcy) levels and homocysteine thiolactonase/paraoxonase (HTase/PON) activity in a short-term trial.

***Methods*:** Ninety patients with type 2 diabetes were randomly divided into three groups: Group I received no vitamin supplementation, group II received 5 mg/day folic acid (orally), group III received folic acid (5 mg/day) in combination with methylcobalamin (500 μg/day; intramuscularly, on prescription). All patients were treated for 2 weeks. Plasma Hcy, HTase/PON activity, vitamin B_{12}, and folic acid were measured before and after supplementation in each group. In addition, forty healthy (nondiabetic) controls were enrolled.

***Results*:** Serum HTase/PON activity was significantly higher in diabetics compared with controls, plasma Hcy levels were significantly lower

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(P<0.05). After vitamin supplementation there was a significant reduction in plasma Hcy levels. The mean percentage reduction in Hcy was 2.75% in group I, 14% in group II and 37.3% in group III. There was a significant inverse correlation between the changes in HTase/PON activity and Hcy levels (r=–0.29, P=0.004). A 2.72% increase in HTase/PON activity was seen in group I, an 8.03% increase was detected when folic acid was given in group II (P<0.001), and a 17.59% increase in HTase/PON activity was seen in group III (P<0.001).

***Conclusion*:** Short-term oral folic acid (5 mg/day) supplementation with or without methylcobalamin appeared to be an effective approach to decrease Hcy levels and increase HTase/PON activity in patients with type 2 diabetes. A decrease in plasma Hcy levels may partly account for the elevation of serum HTase/PON activity. This could be a novel mechanism to protect against vascular diabetic complications.

Keywords: diabetes mellitus; folic acid; homocysteine; homocysteine thiolactonase; methylcobalamin

INTRODUCTION

Homocysteine (Hcy) is a sulfhydryl amino acid, which has been widely accepted as a novel risk factor in vascular diseases.[1] It has been shown that diabetes is associated with an acutely increased risk of cardiovascular disease and mortality.[2] However, research has shown that the conventional risk factors, such as hypertension, dyslipidemia, and obesity, do not fully explain the excessive cardiovascular disease risk in diabetic patients.[3] Studies have demonstrated that hyperhomocysteinemia is more frequent in diabetic patients and there is a close relationship between plasma Hcy and macro- or microangiopathy.[3] Human serum paraoxonase (PON)—an important antioxidant enzyme in high-density lipoproteins—is a key enzyme in Hcy metabolism, and hydrolyzes homocysteine thiolactone (HcyT) to Hcy, thereby minimizing protein N-homocysteinylation.[4] A lowered PON activity has been reported to be associated with atherosclerotic diseases.[5] Recently, studies have shown that low PON activity is related to the increased risk of diabetic macro- or microvascular diseases.[6] Research has also shown that vitamin supplementation may have an impact on Hcy levels.[7] The present study was undertaken to evaluate the effects of short-term folic acid and methylcobalamin supplementation on serum homocysteine thiolactonase/paraoxonase (HTase/PON) activity and Hcy levels in patients with type 2 diabetes mellitus, and to investigate the relationship between HTase/PON activity and Hcy levels.

MATERIALS AND METHODS

Ninety patients with type 2 diabetes who visited the Chinese PLA General Hospital (Beijing, China) were randomly divided into three groups: group I received no vitamin supplementation; group II received only folic acid (5 mg/day, orally); group III received methylcobalamin (500 μg/day; intramuscularly, on prescription; Methycobal, Eisai Co., Ltd, Tokyo) in combination with folic acid (5 mg/day, orally). All patients were treated for 2 weeks. Forty healthy (nondiabetic) controls were also recruited from the same hospital, none of which were given any treatment. Enrolled patients were type 2 diabetic patients aged from 30 to 75 years, who visited the Chinese PLA General Hospital (Beijing, China) in 2007. Type 2 diabetes was defined based on the World Health Organization criteria of 1999.[8]

Patients were excluded if they had a history of either diabetic ketoacidosis, other systemic diseases, severe hepatic or renal diseases (serum creatinine >133 μmol/L), fever, congestive heart failure, or acute cardiovascular and cerebrovascular events. Patients were also excluded if they had taken vitamin B complex or folic acid during the previous 6 months. The study was approved by the ethics committee affiliated to Chinese PLA General Hospital and written informed consent was obtained from all participants.

Plasma Hcy, vitamin B_{12}, folic acid, and HTase/PON activity were measured before and after supplementation in all subjects; blood samples (11 mL) were drawn from subjects after overnight fasting.

Plasma Hcy levels were measured by a polarization immunoassay; the intra- and inter-assay coefficients of variation were 2.0%-4.6% and 2.2%-5.1%, respectively. Serum HTase/PON activity was measured based on its capacity to hydrolyze phenylacetate to phenol as has been previously described.[4] The absorbance of the sample was monitored at 270 nm using spectrophotometery (UV/VIS-731 Spectrophotometer, China). The intra- and inter-assay coefficients of variation were 2.8%-6.5% and 3.2%-7.4%, respectively.

Vitamin B_{12} and folic acid in plasma were determined using a competitive protein binding assay. Glycosylated hemoglobin (HbA_{1c}) was measured with chromatography (BIO-RAD Variant, USA). Other biochemical parameters (eg, cholesterol, triglycerides, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, creatinine, fasting blood glucose levels) were determined using an autoanalyzer (HITACH7600, Japan). Urinary albumin/creatinine (Alb/Cr) was assayed with a radioimmunoassay method (DCA2000, USA). Adverse events were recorded at the end of the study protocol.

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS, version 11.0 for Microsoft Windows, Chicago, USA). Normally distributed clinical data are expressed as the mean±SD; nonnormally distributed results are shown as the median with 25th and 75th quartiles. Kruskal-Wallis test/analysis of variance (ANOVA) were used to assess the dif-

ferences between the groups. Wilcoxon signed ranks/paired-samples *t* tests were used to evaluate intragroup differences. Correlations analysis was performed using Spearman's correlation analysis. $P<0.05$ was considered to be significant.

RESULTS

Serum HTase/PON activity ($n=90$; 97.8±21.9 KU/L) and folic acid concentrations (7.0 [4.7-10.2] ng/mL) in the diabetic groups overall were significantly lower

Table 1. Baseline charachteristics of type 2 diabetic patients and normal (nondiabetic) controls.

	Controls ($n=40$)	Group I ($n=30$)	Group II ($n=30$)	Group III ($n=30$)
Gender, female/male	23/17	14/16	13/17	12/18
Age, years, mean±SD	58.9±10.1	57.9±10.4	59.4±10.4	58.0±9.8
Duration of diabetes, years, median (25th and 75th quartiles)	–	11.0 (3.0-14.0)	9.0 (4.5-12.5)	11.0 (8.0-15.0)
BMI, kg/m^2, mean±SD	24.2±2.7	25.0±2.8	25.1±3.2	25.1±3.8
SBP, mmHg, mean±SD	123.0±9.6*	135.3±18.2	135.3±20.4	138.2±19.5
DBP, mmHg, mean±SD	81.0±6.6	79.2±8.5	78.9±10.7	81.3±11.6
Ch, mmol/L, mean±SD	4.8±0.7	5.0±1.0	4.6±1.0	4.9±1.4
TG, mmol/L, mean±SD	1.3±0.4*	2.2±0.8	2.0±0.4	2.7±0.6
LDL-C, mmol/L, mean±SD	2.8±0.5	3.2±0.8	2.8±1.0	3.0±1.1
HDL-C, mmol/L, mean±SD	1.5±0.3*	1.3±0.4	1.3±0.3	1.3±0.3
HbA_{1C}, %, mean±SD	5.4±0.9*	8.1±2.1	7.8±1.5	7.9±2.0
Cr, μmol/L, mean±SD	54.3±9.3*	61.7±23.3	63.7±21.5	67.6±28.1
Alb/Cr, mg/g, mean (range)	(not measured)	13.0 (8.2-43.7)	22.5 (8.5-103.5)	22.0 (7.7-272.0)
Hcy, μmol/L, median (25th and 75th quartiles)	8.1 (5.9-9.7)*	9.3 (7.7-12.6)	9.4 (8.2-12.4)	9.6 (7.3-12.9)
HTase/PON, KU/L, mean±SD	147.8±38.5*	101.6±21.3	98.3±26.9	93.1±17.1
Folic acid, ng/mL, median (25th and 75th quartiles)	13.6 (12.7-15.1)*	8.3 (5.7-9.6)	7.2 (4.3-8.1)	6.8 (3.7-11.2)
$VitB_{12}$, pg/mL, median (25th and 75th quartiles)	495.0 (434.8-560.8)	504.0 (395.3-626.3)	455.5 (284.3-555.8)	452.0 (358.0-620.0)

*Statistically significant difference ($P<0.05$) when compared with the diabetic groups as whole. Group I received no vitamin supplementation, group II received 5 mg/day folic acid (orally), group III received folic acid (5 mg/day) in combination with methylcobalamin (500 μg/day; intramuscularly, on prescription). Alb/Cr=albumin/creatinine; BMI=body mass index; Ch=cholesterol; Cr=creatinine; DBP=diastolic blood pressure; HbA_{1C}=glycosylated hemoglobin; Hcy=homocysteine; HDL-C=high-density lipoprotein cholesterol; HTase/PON=homocysteine thiolactonase/paraoxonase; LDL-C=low-density lipoprotein cholesterol; SBP=systolic blood pressure; TG=triglycerides; $VitB_{12}$=vitamin B_{12}.

than those of the normal controls (n=40; 147.8±38.5 KU/L, and 13.6 [12.7-15.1] ng/mL) (P<0.05, P<0.01, respectively). Median plasma Hcy in the diabetic groups overall was 9.4 (7.6-11.9) μmol/L, being significantly higher than that of the normal controls (8.1 [5.9-9.7] μmol/L [P<0.05]), while vitamin B_{12} was not significantly different from the normal controls.

The demographic and clinical characteristics of each group are shown in Table 1. There were no significant differences in baseline values of plasma Hcy, folic acid, vitamin B_{12} levels, or serum HTase/PON

Table 2. Post-treatment levels of biochemical parameters in three groups of diabetic patients.

	Group I (n=30)	Group II (n=30)	Group III (n=30)	F	P
Ch, mmol/L, mean±SD	3.47±1.51	3.65±0.71	3.92±0.75	1.731	0.164
TG, mmol/L, mean±SD	1.75 ±0.83	1.50±0.58	1.58±0.86	1.267	0.289
LDL-C, mmol/L, mean±SD	2.50±0.67	2.23±0.51	2.20±0.56	1.788	0.153
HDL-C, mmol/L, mean±SD	1.15±0.40	0.98±0.22	1.09±0.30	1.483	0.223
Cr, μmol/L, mean±SD	64.6±21.0	69.5±18.9	67.3±23.4	0.477	0.699
FBG, mmol/L, mean±SD	7.11±1.67	6.75±1.79	6.91±2.10	1.359	0.259
$VitB_{12}$, pg/mL, median, (25th and 75th quartiles)	467 (358-593)	383 (297-593)	15360 (11720-17600)	92.47	<0.001
Folic acid, ng/mL, median, (25th and 75th quartiles)	8.1 (6.3-9.7)	35.1 (30.5-38.6)	35.2 (28.6-38.4)	92.62	<0.001
Hcy, μmol/L median, (25th and 75th quartiles)	9.2 (7.4-12.5)	9.0 (6.3-11.5)	6.0 (4.9-7.8)	17.49	0.001
HTase/PON, KU/L, mean±SD	103.2±17.5	108.2±27.8	112.7±16.4	2.456	0.049

Group I received no vitamin supplementation; group II received 5 mg/day folic acid (orally); group III received folic acid (5 mg/day) in combination with methylcobalamin (500 μg/day; intramuscularly, on prescription).

Ch=cholesterol; Cr=creatinine; FBG=fasting blood glucose; Hcy=homocysteine; HDL-C=high-density lipoprotein cholesterol; HTase/PON=homocysteine thiolactonase/paraoxonase: LDL-C=low-density lipoprotein cholesterol; TG=triglycerides; $VitB_{12}$=vitamin B_{12}.

activity among the three treatment groups. There were also no significant differences with respect to age, sex, diabetes duration, body mass index (BMI), blood pressure, lipid profile, serum creatinine, HbA_{1c}, or Alb/Cr. There were no reported incidences of adverse events in any of the patients.

There were no significant differences in lipid profile, serum creatinine, or fasting blood glucose among the groups after the 2-week intervention (Table 2). Regarding the levels of vitamin B_{12}, Hcy, folic acid, and HTase/PON activity, there were significant differences among all of the groups (P<0.05).

Effects of Vitamin Supplementation on Reducing Plasma Hcy Levels

Post-treatment plasma Hcy concentrations in the vitamin supplementation groups were significantly lower in groups II and III compared with baseline values (P<0.05). However, in group I, the Hcy levels did not significantly change (P>0.05). Eleven patients from group III had normalized Hcy levels (<10 μmol/L) after treatment, but only three patients with supplementation of folic acid alone experienced normalization of fasting Hcy levels. None of the twelve patients with hyperhomocysteinemia from group I had normal Hcy levels after 2 weeks.

Effects of Changes in Plasma Hcy Levels on Serum HTase/PON Activity

Across the diabetic groups, a rise in serum HTase/PON activity was associated with a reduction in plasma Hcy levels

Table 3. Pre- and post-treatment values and percent variations of Hcy and HTase/PON in three groups of diabetic patients.

		Hcy, μmol/L, median (25th and 75th quartiles)				HTase, KU/L, mean±SD			
Group	***n***	**Pre-**	**Post-**	**Variation, %**	***P***	**Pre-**	**Post-**	**Variation, %**	***P***
Group I	30	9.31 (7.65-12.61)	9.24 (7.35-12.53)	–2.75	0.376	101.70±21.32	103.09±17.51	+2.72	0.499
Group II	30	9.43 (8.17-12.36)	8.98 (6.29-11.45)	–14.00	0.046	98.38±26.99	108.18±27.82	+8.03	<0.001
Group III	30	9.55 (7.25-12.92)	5.97 (4.92-7.82)	–37.30	<0.001	93.18±17.12	112.69±16.36	+17.59	<0.001

Group I received no vitamin supplementation; group II received 5 mg/day folic acid (orally); group III received folic acid (5 mg/day) in combination with methylcobalamin (500 μg/day; intramuscular, on prescription). Hcy values are shown as the mean (range); HTase values are shown as the mean±SD.
Hcy=homocysteine; HTase=homocysteine-thiolactonase.

(r=–0.29, P=0.004). In general, we found that the greater the decrease of plasma Hcy levels, the larger the elevation of serum HTase/PON activity. In the three groups, the greatest increase of HTase/PON activity (17.59%) was seen with the combined administration of methylcobalamin and folic acid group (P<0.001; Table 3); a 8.03% increase was detected in group II (P<0.001).

DISCUSSION

In this study we found that the groups given vitamin supplementation showed a significant reduction in plasma Hcy levels and a significant increase in HTase/PON activity. We also found a significant inverse correlation between the changes in HTase/PON activity and Hcy levels.

Hcy is a sulphur-containing amino acid, which is formed from dietary-obtained methionine.[9] In humans, Hcy is detoxified by folic acid, vitamin B_{12}-dependent remethylation (to methionine), or vitamin B_6-dependent transsulfuration (to cysteine).[9] Defects in Hcy metabolism can lead to the elevation of plasma Hcy—this is the case with the inadequate intake of vitamins, or in the inherited enzyme deficiency of cystathione β-synthetase/5,10-methylenetetrahydrofolic acid reductase.[10] Folic acid, vitamin B_6, and vitamin B_{12} are the main cofactors for Hcy metabolism.[9] It has been estimated that approximately two-thirds of cases of elevated Hcy levels result from mild or moderate deficiency of these vitamins.[11] Research has shown that elevated Hcy levels could be reduced effectively by supplementation with folic acid alone or in combination with vitamin B_{12} or vitamin B_6.[12] The recommended folic acid dose is 0.5-5 mg/day, and higher doses appear to have no additional benefit.[13] One study showed that plasma Hcy reduced to more than 40% in patients with cardiovascular diseases, after they were administered folic acid (5 mg, orally) per day for 3 months.[7]

As a methyl group provider, methylcobalamin—one of the subtypes of vitamin B_{12}—plays a critical role in the remethylation of Hcy to methionine.[14] In a randomized, double-blind study, patients were given methylcobalamin (1500 μg/day, orally) for 8 weeks, and plasma Hcy levels were decreased by 10.9%.[15] In our short-term study, in order to observe the effects of changes in Hcy levels on serum HTase/PON activity, both folic acid and methylcobalamin were given in high doses. The data showed that a daily oral intake of 5 mg folic acid decreased Hcy levels by 14%; a 37.3% reduction in plasma Hcy level was also observed with combined administration of methylcobalamin and folic acid. Similarly to Koyama's study,[12] these data support the finding that combined supplementation of folic acid and methylcobalamin is most effective in reducing and normalizing Hcy in diabetic patients.

Human serum PON is an important antioxidant enzyme, which eliminates some free radicals in circulation.[16] It has been reported that PON could in fact be HTase, which hydrolyzes HcyT to Hcy.[17] HcyT is a reactive by-product of Hcy, metabolized by methionyl-tRNA synthetase

in all cell types.[18] The extent of thiolactone formation in human umbilical vein endothelial cells is positively correlated with concentrations of Hcy, and negatively correlated with methionine, folic acid, and HTase.[19] Jakubowski has demonstrated that HcyT might be involved in the molecular basis of Hcy-induced vascular damage.[19] Studies have revealed that HTase/PON activity is closely associated with diabetic macroangiopathy.[20] A lower HTase/PON activity was observed in diabetic patients in this study. Only a small number of studies have examined how reductions in Hcy levels affect human serum HTase/PON activity, as caused by supplementation with folic acid and methylcobalamin. This is the first study to reveal that administration of folic acid and methylcobalamin are associated with increased serum HTase/PON activity; we also found an inverse correlation between changes in HTase/PON activity and Hcy concentration. HTase/PON activity was elevated most markedly by the administration of both methylcobalamin and folic acid—the reduction of Hcy concentration was the greatest in this group.

In the murine model of hyperhomocysteinemia, the activity of PON is downregulated threefold.[21] Therefore, it is supposed that plasma Hcy levels could influence HTase/PON activity. Moreover, serum HTase/PON activity could be inactivated by lipid peroxidation.[22] Research has shown that oxidized low-density lipoprotein concentration is negatively related to serum HTase/PON activity.[22] Folic acid has been recently defined as an antioxidant, and a deficiency of this substance could cause an increase in the products of lipid peroxidation.[23] Henning et al. reported that reduced levels of glutathione and impairment in the activities of antioxidant enzymes were observed in rats deficient in methyl/folic acid.[24] Thus, the antioxidative property of folic acid may also account for the increase in HTase/PON activity shown in this study. Alternatively, plasma Hcy levels might affect HTase/PON activity through an unknown mechanism.

In conclusion, the present data show that oral folic acid (5 mg/day) supplementation could be an effective approach to decrease Hcy and elevate HTase/PON activity in patients with type 2 diabetes, while methylcobalamin plus folic acid therapy may be much more effective. However, there are some limitations in the present study. Firstly, the treatment lasted for only 2 weeks—in other similar short-term studies, a 3-week period (or longer) has generally been used. Secondly, the number of the subjects enrolled was relatively small. To confirm the effects of Hcy levels on serum HTase/PON activity and to elucidate the role of the intervention in the development of diabetic vascularopathy, large, long-term, prospective studies are needed.

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