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Effect of Diet and 677 C→T 5, 10-Methylenetetrahydrofolate Reductase Genotypes on Plasma Homocyst(e)ine Concentrations in Slovak Adolescent Population

K. RAŠLOVÁ, A. BEDEROVÁ[1], J. GAŠPAROVIČ, P. BLAŽÍČEK[2], B. SMOLKOVÁ

Department of Lipid and Glucose Metabolism, Institute of Preventive and Clinical Medicine, [1]Research Institute of Nutrition and [2]Hospital of Ministry of Defense, Bratislava, Slovak Republic

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Summary

The objective of this study was to evaluate the effect of diet and 677 C→T mutation of the methylenetetrahydrofolate reductase (MTHFR) gene on plasma homocyst(e)ine concentrations in an adolescent population (113 males, age: 14.2±2.4 years; 202 females, age: 14.9±2.1 years) from a region characterized by high cardiovascular mortality. Homocyst(e)ine levels did not differ between males and females (9.4±3.5 and 8.9±3.1 μmol/l, respectively). The homozygosity for the 677 C→T MTHFR mutation was found in 4.6 % of subjects. No differences in homocyst(e)ine levels were found between MTHFR genotypes. Analysis of the diet composition which was performed on a 24-hour daily recall basis and a food frequency questionnaire showed a low daily intake of vitamin B_6 (males: 1.13 mg/66 % RDA; females: 0.92 mg/61 % RDA). Daily folic acid intake was 0.21 g/105 % RDA in males and 0.23 g/115 % RDA in females. The results of our study show that the high homocyst(e)ine levels in the adolescent population were not affected by the 677 C→T MTHFR mutation. We conclude that an insufficient dietary intake of vitamin B_6 and folic acid is responsible for this finding. This is in accord with the recommendation that the dietary allowances for folate should be reset to the originally prescribed levels of 0.4 g/day which should be sufficient to control the homocysteine levels.

Key words

Homocysteine • 677 C→T methylenetetrahydrofolate reductase genotypes • Diet • Vitamin B_6 • Folic acid

Introduction

Many experimental, clinical and epidemiological studies (McCully 1993, Duell and Malinow 1997, Nygard *et al.* 1997) have suggested that increased plasma total homocyst(e)ine (Hcy) levels play a role in the development of atherosclerosis. Recent meta-analysis (Boushey *et al.* 1995) indicates that up to 10 % of the population's coronary artery disease risk in Western Europe and North America may be attributable to moderately increased Hcy concentrations (15-30 μmol/l). Plasma homocyst(e)ine levels are influenced by a number of environmental factors as well as by genetically based

Fax+4202 24920590
E-mail: physres@biomed.cas.cz
http://www.lf2.cuni.cz/physiolres/physiolres.htm

alterations of homocyst(e)ine transsulphurylation or remethylation (Selhub *et al.* 1993, Mudd *et al.* 1995, Rosenblatt 1995, Nygard *et al.* 1995, Toborek and Henneig 1996). The decreased activity of the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is one of the most frequent genetic causes of moderately elevated plasma levels of Hcy. A thermolabile variant of the enzyme MTHFR was described with a specific activity of less than 50 % of normal control values (Kang *et al.* 1988, 1993, Ueland *et al.* 1993). A common mutation (nucleotide 677 C→T) appears to be responsible for this functional defect which is inherited as an autosomal recessive trait (Kang *et al.* 1991, Frosst *et al.* 1995). The frequency of this mutation may vary among different ethnic groups (Van der Put *et al.* 1995, de Franchis *et al.* 1996, Motulsky 1996, Schmitz *et al.* 1996). Folate, vitamin B_6 and B_{12} are important cofactors for the metabolism of plasma Hcy. Clinical and prospective studies have shown that the intake of folate and vitamin B_6 above current recommended dietary allowances may be important in reducing Hcy levels as well as in the primary prevention of coronary heart disease (Ubbink *et al.* 1993, Rimm *et al.* 1998).

Since 1960, marked differences of the mortality from coronary artery disease between Western and Eastern European countries have been shown by WHO and MONICA statistical data (Food and Health Indicators in Europe 1995, Ginter 1995a, 1998). However, the analysis of the prevalence of the "old" risk factors of atherosclerosis did not explain this situation. Therefore, it was suggested that factors which increase the level of oxidative stress may be responsible for the high cardiovascular mortality in Central and Eastern Europe (Ginter 1995a,b, Ziedén *et al.* 1999). Hcy represents a risk factor which is supposed to be connected with increased atherosclerosis risk by its role in oxidative stress and thrombogenesis (Duell and Malinow 1997).

The objective of this study was to evaluate the Hcy concentrations in a representative sample of the adolescent population from a South-Eastern region of Slovakia. This region is characterized by high cardiovascular mortality and by a population that traditionally consumes high amounts of animal fat and small amounts of fruit and vegetables. We therefore supposed that the dietary habits may be reflected in Hcy concentrations. We also looked into the extent to which the 677 C→T MTHFR mutation may affect Hcy concentrations.

Methods

Subjects

One hundred and fourty-nine boys (age: 14.2±2.4 range 11-18 years) and two hundred and thirty-seven girls (age: 14.9±2.1 range 11-18 years) were examined as a part of a prospective study of the Research Institute of Nutrition of School Population in Bratislava in different regions of Slovakia (Béderová *et al.* 1995).

The personal and family history and data on the presence of exogenous risk factors for atherosclerosis were gathered by a questionnaire. A positive family history of atherosclerosis was defined as the diagnosis of myocardial infarction or stroke in parents by the age of 55 years.

The body mass index (BMI) was calculated as the body weight divided by the height squared. Overweight was defined as BMI > 23.5. Percentage of body fat was measured by a Harpenden caliper as thickness of four skinfolds. Computation was performed using the Durnin tables (Durnin and Womerslez 1974). Blood pressure was measured with a digital sphygmomanometer.

Analysis of diet composition was done using a 24-hour daily recall and a detailed food frequency questionnaire (FFQ). The FFQ was completed at home, usually with the help of a parent. At a visit, a day after, the dietician checked the daily recall and made adjustments according to the child. Computation of daily intakes of nutrients and foods was carried out using a food database and software system developed at the Research Food Institute, Bratislava (Strmiska *et al.* 1992). Nutrients, fiber, micronutrients and vitamin intake were calculated and related to the percentage of recommended dietary allowances (% RDA).

Informed consent was obtained from all the parents of examined children and the study was approved by the Institute's Ethics Committee.

Laboratory measurements

Blood samples were obtained in EDTA vacutainers after a 12-14 h overnight fast. Blood counts, erythrocyte rate and chemical urine analysis were then carried out.

Lipid and apolipoprotein measurements

Plasma total cholesterol and triglycerides were determined enzymatically, high density lipoprotein cholesterol (HDL-C) was measured by precipitation, followed by enzymatic determination of cholesterol (Warnick *et al.* 1982). Low density lipoprotein cholesterol (LDL-C) was calculated by the Friedewald formula (triglyceride levels in all patients were less than 4.6 mmol/l) (Friedewald *et al.* 1972). The risk index (RI) was calculated as a proportion of LDL-C to HDL-C.

Mutation analysis

Leukocyte DNA was extracted from 10 ml of EDTA-anticoagulated whole venous blood by phenol extraction. 677 C→T MTHFR mutation was analyzed by PCR using forward primer 5′TGAAGGAGAAGGTGT CTGCGGGA and reverse primer 5′-AGGACGGTGCGG TGAGGAGGTG. Amplified DNA was digested by Hinf I enzyme at 37 °C for 1 h. Restriction fragments were separated by electrophoresis in Visigel (Stratagene) and visualized under ultraviolet light (Frosst *et al.* 1995).

Homocyst(e)ine assay

Blood was collected in EDTA tubes and centrifuged within one hour for 15 min at 4 °C. The plasma was stored at –70 °C until analysis. Hcy plasma levels were quantified by HPLC using a reverse phase, C-18 column and fluorescence detection. The samples and an internal standard (2.5 mM acetylcysteine, Sigma) were pretreated with tri-n-butylphosphine to allow the reduction of thiols and mixed sulphides. After precipitation of proteins, the supernatant was derivatized using 0.1 % SBD-F and then injected onto the column. The peak height ratio in the unknown sample of the homocysteine and internal standard was compared to the ratio in the standard (Homocyste(i)ne standard Sigma). The ratios were directly proportional to the concentration of Hcy present. The interassay coefficient of variation of the method was 5 %.

Statistical analyses

The normal distribution of examined parameters was tested by the Kolmogorov-Smirnoff test (Statgraphics statistical software). Differences between the examined quantitative parameters were compared by Student′s t-test or by the U-test for parameters which showed a skewed distribution (triglycerides and Hcy). Allele frequencies were calculated using Hardy-Weinberg equilibrium (Strachan and Read 1996). The relationship between genotypes and Hcy were assessed using analysis of variance. Correlation between age, vitamin B_6 and Hcy was tested using the Spearman correlation test.

Statistical analyses were made using the Statgraphics software. Results are expressed as mean ± S.D. All statistical tests were provided at the alpha level = 0.05.

Table 1. Risk factors for atherosclerosis in adolescents.

	Males n=149	**Females n=237**
BMI	19.4 ± 3.0	19.0±2.9
Systolic blood pressure (mmHg)	113.1±15.9	107.5±14.8
Diastolic blood pressure (mmHg)	68.3±13.6	68.0±10.7
Cholesterol (mmol/l)	4.02±0.72	4.28±0.70
Triglycerides (mmol/l	1.06±0.42*)*	1.08±0.35
LDL-cholesterol (mmol/l)	2.34±0.68	2.51±0.69
HDL-cholesterol (mmol/l)	1.21±0.21	1.28±0.28
LDL-cholesterol: HDL-cholesterol	2.0±0.7	2.0±0.65
% of body fat	17.7±7.8	25.7±5.7
BMI>23.5 (%)	6.8	9.1

Table 2. Plasma homocyst(e)ine concentrations in adolescents.

Homocyst(e)ine μmol/l	Males n=113	Females n=203
Mean ± S.D.	9.4±3.5	8.9±3.1
Minimum	2.0	4.0
Maximum	19.0	31.0

Results

Table 1 summarizes the major risk factors (RF) for CAD in adolescents showing normal levels of plasma lipids and blood pressure and the relatively high incidence of moderate degree of obesity. A positive family history of premature myocardial infarction and/or stroke was found in 16 subjects (4.1 %).

Hcy concentrations were measured in 113 boys and 202 girls (Table 2) who did not exhibit any sex differences. Statistically significant correlations of Hcy levels with age were found in boys and girls (r: 0.49, p = 0.001, r: 0.35, p = 0.001, respectively).

The 677 C→T MTHFR mutation was analyzed in 323 adolescents. Table 3 shows the distribution of alleles and genotypes and corresponding values of Hcy. No statistically significant differences of Hcy levels were found between T/T , C/T and C/C MTHFR genotypes.

The assessment of daily energy, nutrient, fiber and vitamin intake at 24 hour-daily recalls showed a high consumption of proteins, lipids and cholesterol, and a low consumption of carbohydrates and B_6 and C vitamins (Tables 4 and 5). According to the RDA criteria the consumption of vitamin B_{12} and folates was not found to be low. The evaluation of the relationship between RDA of vitamin B_6 and Hcy concentrations did not exhibit statistically significant correlation.

Table 3. Distribution of 677 C→T MTHFR allele, genotypes and corresponding homocyst(e)ine concentrations.

MTHFR	T-allele %	C-allele %	C/C n/%	C/T n/%	T/T n/%
Frequency	27.5	72.5	160/49.5	148/45.8	15/4.6
Homocyst(e)ine (μmol/l)			9.3±3.6	8.9±2.7	9.6±3.5

n – number of subjects carrying the MTHFR genotypes; homocyst(e)ine levels expressed as mean ± SD.

Table 4. Daily energy and nutrient intake in adolescents.

	Energy kcal/%RDA	Proteins g/% RDA	Carbohydrates g/% RDA	Fat g/% RDA	Cholesterol mg/day
Males	2918/98	99/165	392/79	114/134	633
Females	2081/89	68/135	299/79	78/120	353

% RDA – percentage of recommended dietary allowances.

Discussion

Plasma homocyst(e)ine concentrations are influenced by several genetic (MTHFR, cystatione β-synthase, methionine synthase) and exogenous factors (diet, smoking, blood pressure, concomitant chronic disorders) among which diet composition and 677 C→T mutation of the MTHFR gene are accepted as the major regulators of Hcy levels (Duell and Malinow 1997). This study was conducted in the adolescent population of the South-Eastern district of Slovakia which is characterized by high cardiovascular mortality and a high

unemployment rate. Therefore, it was supposed that this might be reflected in the level of risk factors of atherosclerosis, especially those which are related to dietary habits. The examination of the adolescent population also provides an opportunity to select subjects whose Hcy levels are not affected by smoking, age, blood pressure or lipid variables but mostly by genetic factors and/or the diet.

Table 5. Daily fiber and vitamin intake in adolescents.

	Fiber g/% RDA	Vitamin C mg/% RDA	Vitamin B_6 mg/% RDA	Vitamin B_{12} mg/% RDA	Folic acid g/% RDA
Males	20/90	55/55	1.13/66	0.005/250	0.21 / 105
Females	18/100	54/60	0.92/61	0.006/270	0.23 / 115

The analysis of traditional risk factors showed that adolescents of both sexes had normal levels of lipids, lipoproteins and blood pressure. Although the evaluation of dietary habits indicated a very high intake of animal fat and cholesterol, this was not reflected in plasma lipid levels.

A growing number of reports have appeared dealing with the role of Hcy as a risk factor for atherosclerosis in adult individuals, either healthy or affected by atherosclerotic disorders (Duell and Malinow 1997). However, few reports have studied children and/or adolescents. In our cross-sectional population study, plasma Hcy concentrations in male and female adolescents were much higher when compared with Norwegian pediatric data in which the mean levels of Hcy ranged from 5.6 to 6.6 μmol/l (Tonstad *et al.* 1996, 1997). Similarly to Norway, Hcy levels also showed no sex differences and a positive correlation with age in Slovaks. In a Spanish study, Hcy levels in adolescent males and females were also lower than in our population (expressed as medians: 7.8 and 7.4, 8.7 and 8.6 μmol/l, respectively) (Vilaseca *et al.* 1997), although the differences were not as marked as in the Norwegian report. One would expect that the Hcy concentrations which are strongly influenced by dietary habits might be lower in the Mediterranean region compared to Scandinavia, however, the contrary seems to be true. The significant effects of the socio-economic status on plasma Hcy levels which were shown in Norwegian children (Tonstad *et al.* 1996) seem to be at least partially responsible for the differences between children and adolescents from different countries.

The association of plasma Hcy levels with nucleotide 677 C→T MTHFR mutation has been frequently found in the Caucasian populations (Kang *et al.* 1991, Frosst *et al.* 1995, Kluijtmans *et al.* 1996). Studies dealing with the frequency of nucleotide 677 C→T MTHFR mutation in apparently healthy populations and in patients with coronary heart disease (CHD) show ethnic differences (Brulhart *et al.* 1997, Stevenson *et al.* 1997), but also differences according to the characterization of the nucleotide 677 C→T MTHFR mutation as a direct or an indirect risk factor of CHD (Kluijtmans *et al.* 1996, Wilcken *et al.* 1996, van Bockxmeer *et al.* 1997). In our study, the frequency of homozygote 677 T/T MTHFR genotype was 4.6 % which was comparable with that found in the Dutch healthy population (Kluijtmans *et al.* 1996) and in our case-control study (Rašlová *et al.* in press).

When comparing the Hcy concentrations between the three 677 MTHFR genotypes (CC, CT, and TT), we did not find any differences. Such differences were confirmed in the study of children with familial hypercholesterolemia (Wilcken *et al.* 1996), but also in some studies conducted on adult populations (Frosst *et al.* 1995, Kluijtmans *et al.* 1996). However, in the US Physicians Study, the authors observed significantly higher Hcy levels only in the low-folate group of MI-survivors with the homozygote T/T MTHFR genotype (Ma *et al.* 1996).

The Hordaland study (Nygard *et al.* 1995) demonstrated that many lifestyle factors influence Hcy concentrations. The reduction of Hcy concentrations has been achieved by supplementation of the diet with folate and/or vitamins B_6 and B_{12} (Boushey *et al.* 1995, Jacque *et al.* 1996). Boushey *et al.* (1995) suggested that, if the population were to eat two or three more servings of fruit and vegetables daily, dietary folate ingestion would be expected to cause an average decrease in Hcy of about 2 μmol/l. When evaluating the daily intake of vitamins

which play the role of cofactors in remethylation or transsulphurylation of plasma homocyst(e)ine, we found that the adolescents consumed only 60 % of RDA for vitamin B_6 that plays the role as a cofactor in both metabolic processes. The examination of the relationship between RDA for vitamin B_6 and Hcy levels did not reveal any significant correlation. However, this finding is not surprising because different methods were used for assessments of the two variables (HPLC measurement for Hcy and the questionnaire for vitamin B_6). Nevertheless, the daily intake of folate in adolescent boys and girls seems to be in accordance with current RDA values (Food and Nutrition Board 1989). However, there is increasing evidence that this level is not sufficient to minimize the risk of neural tube defects, and possibly CHD. In the Nurses' Health Study (Rimm *et al.* 1998), the evaluation of the dose-response relationship for folate in women with higher folate intake than the RDA of 180 μg/day, the authors found a significantly decreased risk for CHD for each 200 μg increase in folate. In this study, the multiple vitamin supplements were by far the largest contributors to total folate and vitamin B_6 intake. Each 100 μg/day increase in folate was associated with 5.8 % lower risk of CHD. Therefore, it was suggested that current RDA for folate of 180 μg/d should be reset to the earlier levels of 400 μg/day to be sufficient to minimize the risk of CHD (Oakley 1997). Despite the epidemiological findings of a positive effect of vitamin supplements (Malinow *et al.* 1997), clinical data show that supplementation with B vitamins can normalize increased Hcy levels only in 25-50 % of subjects with hyperhomocysteinemia and a suboptimal status of vitamin B_6, B_{12} and folate (Ubbink *et al.* 1993). In accordance with the supplementation data, neither genetic nor dietary factors could explain more than 47 % of elevated Hcy concentrations in the studied individuals (Tsai *et al.* 1999).

The etiopathogenesis of moderate hyperhomocysteinemia is multifactorial, and the data from different populations help to improve the understanding of this problem. The results of our study show that the high levels of Hcy in the adolescent population from a region with socio-economic problems are affected by the diet. It seems that insufficient dietary intake of two vitamins, B_6 and folic acid, but not mutation in MTHFR gene, are responsible for this finding.

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Reprint requests

K. Rašlová, Department of Lipid and Glucose Metabolism, Institute of Preventive and Clinical Medicine, Limbová 14, 833 01 Bratislava, Slovak Republic. Tel/Fax: +421 7 59369161, e-mail: raslova@upkm.sk