Methylation status in healthy subjects with normal and high serum folate concentration

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Abstract

Objective: We assessed the impact of high serum folate concentration on erythrocyte S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) concentrations, SAM/SAH ratio, CpG methylation levels across the promoter region of the extracellular superoxide dismutase (ec-SOD) gene, and ec-SOD activity in healthy men.

Methods: Serum folate levels were measured in 111 subjects who were categorized in quintiles according to their folate status. Subjects located at the lowest, middle, and upper quintiles were selected for assessment of SAM and SAH by high-performance liquid chromatography, C677T genotype of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, ec-SOD methylation of CpG sites in lymphocytes genomic DNA by bisulfate treatment, and ec-SOD activity by a chemical assay.

Results: Sixteen subjects were in the lowest serum folate quintile (<23.6 nmol/L), 17 in the middle (>34–<42 nmol/L), and 14 in the highest (>45nmol/L). SAM concentration was higher in the upper than in the middle and lowest quintiles (5.57 ± 1.58, 2.52 ± 0.97, 2.29 ± 1.2 μmol/L; $P < 0.0001$). SAH concentration was higher in the upper compared with the lowest quintile (0.76 ± 0.24 versus 0.52 ± 0.23 μmol/L, $P < 0.001$). There were no differences in the SAM/SAH ratio, ec-SOD activity, methylation status of CpG sites of the ec-SOD gene, and TMTHFR C677T genotype between groups.

Conclusion: Serum folate concentrations in the highest quintile among healthy humans are associated with increased erythrocyte SAM and SAH concentrations, but not with SAM/SAH ratio or with methylation levels of CpG sites across the promoter region of the ec-SOD gene. Further research is required to determine if these findings are beneficial or harmful.

Keywords: Folate; S-adenosylmethionine; S-adenosylhomocysteine; Methylation; Extracellular superoxide dismutase gene

Introduction

Folate acts as a methyl donor in the remethylation of homocysteine to methionine, which is converted to S-adenosylmethionine (SAM), a direct methyl donor involved in numerous methyltransferase reactions, related to DNA, RNA, neurotransmitters, phospholipids, and proteins. The end product and inhibitor of these methylation reactions is S-adenosylhomocysteine (SAH); therefore, the SAM/SAH ratio has been termed the methylation index [1].

It has been suggested that the association between hyperhomocystinemia and cardiovascular disease may be explained by a low SAM or a high SAH concentration or a low SAM/SAH ratio. Loehrer et al. [2] detected lower whole

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blood SAM concentrations in patients with coronary artery disease as compared with healthy controls. In healthy subjects, plasma SAH paralleled the increases in total homocysteine (tHcy) and lymphocyte global DNA hypomethylation after homocysteine and methionine loading [1]. In an elderly population, SAM and 5-methylenetetrahydrofolate (MTHF; the active form of folate) were positively associated with endothelium-dependent flow-mediated vasodilatation [3]. Carotid intima–media thickness in non-diabetic individuals also was inversely associated with high SAM levels [4].

Animals fed on diets deficient in methyl donors have shown global and gene-specific hypomethylation levels that return to normal after 1- or 2-wk administration of an adequate diet [5]. Folate deficiency is associated with DNA hypomethylation in animals and humans, which could potentially result in changes in gene expression. In postmenopausal women, moderate folate depletion increased plasma homocysteine, as expected, and decreased lymphocyte DNA methylation, which was reversed within 3 wk by folate repletion [6]. Castro et al. [7] observed that patients with vascular disease had significantly higher plasma SAH concentrations, decreased plasma SAM/SAH ratios, and lower global DNA methylation status as compared with controls. In healthy young women, plasma SAH levels were positively correlated with serum homocysteine levels and lymphocyte global DNA hypomethylation [1]. In endothelial cell cultures, an inverse association between intracellular SAH and global DNA methylation has been shown [8].

In a previous study, we observed that moderate folate depletion in adult Wistar rats induces a significant reduction in SAM and the SAM/SAH ratio in the liver, which was not related to alterations in endothelial function when measured in aortic rings in vitro (unpublished observations) [9].

DNA methylation, which occurs primarily in the C5 position of the cytosine ring in 5′- to 3′-oriented CG dinucleotides (CpG), affects gene expression in many biological processes such as differentiation, genomic imprinting, and DNA mutation and repair [10,11]. DNA hypermethylation, usually occurring at promoter CpG islands, is a major epigenetic mechanism for silencing the expression of genes [12] and is tightly regulated by three different DNA methylases involved in de novo and maintenance methylation during replication [9,13]. Other DNA methyl group transfer-related enzymes are demethylases, which act by demethylating DNA during differentiation [14].

Superoxide dismutase (SOD) is a major component of antioxidant defenses in blood vessels and has powerful cardioprotective properties. In plasma, extracellular SOD (ec-SOD) is by far the dominant SOD isoenzyme in most mammalian species examined [15]. High levels of ec-SOD expression in the arterial wall maintain low extracellular $O_2^{\cdot -}$ concentrations, which prevents oxidation of cellular proteins and low-density lipoprotein and promotes inactivation of endothelium-derived relaxing factor (nitric oxide) [16]. In atherosclerotic aortas of rabbits, Laukkanen et al. [17] found hypomethylation of the ec-SOD gene. Although a causal relation between the methylation level and ec-SOD expression has yet not been established, those results suggest that hypomethylation may be associated to atherosclerotic lesions [17].

The aim of this study was to assess the impact of high serum folate concentrations on erythrocyte SAM, SAH concentrations, SAM/SAH ratio, CpG methylation levels across the promoter region of the ec-SOD gene, and ec-SOD activity in healthy men.

Materials and methods

Subjects and blood collection

One hundred eleven healthy male volunteers 24 to 68 y of age were studied. Exclusion criteria for entry to the study were intake of vitamin supplements, personal history of hypothyroidism or hyperthyroidism, cardiovascular or renal disease, hypertension, diabetes, dyslipidemia, or other chronic diseases.

All subjects signed an informed and written consent. The study was approved by the local ethics committee.

After an overnight fast, a 40-mL venous blood sample was obtained from each subject, kept in disposable vacuum tubes with the adequate preservers, and placed immediately on ice. After centrifugation, plasma, serum, erythrocytes, and white blood cells were separated and frozen at −70°C for subsequent analyses within 2 mo. Routine clinical chemistry of serum folate, vitamin B12, and homocysteine levels, ec-SOD activity, SAM and SAH in erythrocytes, 5,10-MTHF reductase (MTHFR) C677T genotype, and ec-SOD methylation of promoter CpG sites of genomic DNA in lymphocytes were measured.

The SAM, SAH, and ec-SOD activity and methylation levels across ec-SOD promoter CpG sites were measured only in volunteers in the upper, middle, and lower quintiles of folate levels.

Analyses

Total proteins, creatinine, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triacylglycerol, homocysteine, and liver function tests (routine chemistry) were measured by routine laboratory automated methods using Abbott kits (Abbott Laboratories, Abbott Park, IL, USA).

Serum homocysteine was measured using an Abbott kit (Abbott IMx homocysteine, Abbott Laboratories). This procedure is based on the fluorescence polarization immunoassay technology.

Folic acid and vitamin B12 were measured by the DPC BioMediq Immulite 2000 analyzer using a chemiluminescent enzyme immunoassay (DPC, Los Angeles, CA, USA).

The C677T polymorphism on the MTHFR gene was determined by polymerase chain reaction (PCR) and the analysis of the digestion pattern of *Hinf*I restriction enzyme as described by Stegmann et al. [18].

Determination of SAM and SAH in erythrocytes.

The SAM and SAH concentrations in erythrocytes were quantified by high-performance liquid chromatography as described by Fu et al. [19], with some modifications by using an Agilent-1100 DAD detector (Hewlett-Packard, Palo Alto, CA, USA) operating at 260 nm. Frozen erythrocytes (400 μL) were treated with 0.5 M $HClO_4$ (600 μL) and incubated at 4°C for 30 min. The precipitated proteins were removed by centrifugation at 20 000 $\times$ *g* for 15 min at 4°C and supernatants containing SAM and SAH were filtered through a 0.22-μm Millipore filter. Samples (100 μL) were directly injected into the high-performance liquid chromatograph with a C18 MCM column (4.6 mm $\times$ 150 mm, ESA, Chelmsford, MA, USA) operating at 35°C. The mobile phase (operated at 20°C) consisted of 50 mM NaH_2PO_4, 6.7 mM 1-heptanesulfonic acid, and 5% (v/v) methanol at a pH of 4.4 adjusted with HPO_4. High-performance liquid chromatographic analyses were conducted at a flow rate of 1 mL/min. Calibration curves were performed using pure SAM and SAH (Sigma, St. Louis, MO, USA), and linear responses were obtained between 0–2 and 0–10 μmol/L for SAH and SAM, respectively, with a correlation coefficient higher than 0.999 for each curve. Under these conditions, retention times for SAH and SAM were 13 and 18 min, respectively. The concentrations of SAM and SAH were linearly related to the areas under the high-performance liquid chromatogram. Results were expressed as micromoles per liter.

Plasma ec-SOD activity.

Plasma ec-SOD activity was measured by using a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine (Cayman Assay kit, Ann Arbor, MI, USA). The detection limit of the assay was 0.05 U/mL. Inter- and intra-assay coefficients of variation were 3% and 5.5%, respectively [20].

Isolation of nucleic acids from human lymphocytes.

DNA from human lymphocytes was isolated using the proteinase K method described by Zuccotti and Monk [21], with slight modifications: lymphocytes obtained from 3 mL of blood were homogenized in 1.5-mL microtubes using a DNAse-free pestle with 180 μL of a solution (lysis buffer) containing 280 μg/mL of proteinase K, 1% sodium dodecylsulfate, and 2 μg/mL of tRNA from *Escherichia coli* (Sigma). Samples were incubated at 55°C for 90 min. Then, 60 μL of Promega protein precipitation solution (Madison, WI, USA) was added and centrifuged for 10 min at 4000 rpm and 4°C. The supernatant was transferred to new tubes, 240 μL of isopropanol was added for precipitation of the DNA, and then tubes were centrifuged for 6 min at 13 000 rpm. The supernatant was discarded and DNA was washed with 80 μL of 80% EtOH. Samples were centrifuged again at 13 000 rpm for 6 min. The supernatant was then discarded and precipitated DNA samples were air-dried for 20 min. Samples were resuspended in 80 μL of lysis buffer. All DNA samples were stored at −20°C until analysis of DNA methylation through bisulfite sequencing.

Bisulfite treatment of DNA.

The DNA bisulfite treatment was essentially carried out as previously described by Clark et al. [22,23], with modifications introduced by Warnecke and Clark [24]. DNA samples were treated with sodium metabisulfite for 16 h. The bisulfite reaction was desalted using a DNA clean-up column (Promega), as instructed by the manufacturer. Bisulfite-treated DNA was eluted in 30 μL of H_2O.

PCR conditions

Each PCR reaction for bisulfite-treated DNA was carried out in a total volume of 20 μL containing 10 μL of 1$\times$ Promega master mix, 7 μL of DNAse-free H_2O, 1 μL of forward and reverse primers, and 1 μL of the template. PCR conditions used for all genes studied were described elsewhere [21], with the exception of annealing temperatures, which are described below. For the human ec-SOD gene, we designed the following semi-nested primers for PCR amplification of a fragment in the promoter region of which nine CpG sites were analyzed: forward 5′-GGATTTAGAGATTTAGATTTTTTATAAGTTT-3′, reverse internal 5′-CTCAATCATAACAACCCTTTCCAAA-3′, reverse external 5′-CACTCATAAATAATCATCATTATCATTATCA-3′. The annealing temperature for ec-SOD was 57°C for the first and second rounds. This set of primers amplified a part of the promoter region (−459 to −112) that contains several elements of regulation of gene expression [25].

Sequencing and CpG site methylation analysis.

Samples were sequenced at Macrogen (Seoul, Korea) with the reverse primers in all cases. The level of methylation per CpG site was quantified using the program Bioedit (North Carolina State University, Raleigh, NC, USA), which allows quantifying the ratio of methylated versus non-methylated peaks on raw-data electrophenograms.

Statistical analyses were done using STATA 7.0 for Windows (STATA Corp., College Station, TX, USA). Descriptive data are expressed as mean ± standard deviation. Comparisons between groups were performed using one-way analysis of variance. Post hoc comparisons between groups when analysis of variance was significant were done using Bonferroni's test. Correlations between variables were analyzed by Pearson's and multiple regression analyses.

Table 1
Demographic and laboratory features of studied subjects ($n = 111$)

	Mean ± SD	Range
Age (y)	53.0 ± 7.4	24–68
Body mass index (kg/m^2)	25.3 ± 3.7	17.4–37.5
Serum albumin (g/L)	45 ± 3.3	34–51
Serum creatinine (μmol/L)	74.3 ± 15.9	38.0–138.8
Serum total cholesterol (mmol/L)	4.98 ± 0.93	2.78–7.05
Serum LDL cholesterol (mmol/L)	2.65 ± 0.78	1.23–4.62
HDL cholesterol (mmol/L)	1.48 ± 0.59	0.68–3.91
Serum triacylglycerols (mmol/L)	1.90 ± 1.14	0.57–5.95
Serum vitamin B12 (pmol/L)	393.7 ± 247.2	80.6–1945.0
Serum folate (nmol/L)	34.6 ± 12.8	12.9–76.3
Serum total homocysteine (μmol/L)	11.78 ± 5.3	6.02–36.7

HDL, high-density lipoprotein; LDL, low-density lipoprotein

Table 3
DNA methylation levels at specific CpG sites across the promoter region of the extracellular superoxide dismutase gene*

	Quintile 1 ($n = 16$)	Quintile 3 ($n = 17$)	Quintile 5 ($n = 14$)
CpG1	37.4 ± 14.0	31.9 ± 12.0	36.7 ± 11.6
CpG2	51.7 ± 16.8	49.2 ± 11.0	54.4 ± 10.6
CpG3	67.1 ± 8.2	59.3 ± 10.0	57.6 ± 21.6
CpG4	62.0 ± 10.3	56.3 ± 10.4	55.2 ± 9.1
CpG5	63.0 ± 10.8	58.1 ± 11.7	62.3 ± 14.1
CpG6	59.6 ± 17.0	52.2 ± 12.9	54.4 ± 17.1
CpG7	60.1 ± 15.4	49.9 ± 14.5	44.1 ± 20.4
CpG8	60.2 ± 21.2	52.2 ± 15.0	67.7 ± 16.3
CpG9	49.1 ± 24.5	45.8 ± 21.1	53.8 ± 22.3

* Values are means ± SDs for percentage of methylation.

Results

The characteristics of the overall sample are presented in Table 1. Folate deficiency (normal value 7–39 nmol/L; No Boil Dualcount kit, Los Angeles, CA, USA) was not observed, 5.4% of subjects had vitamin B12 deficiency (normal value 150–700 pmol/L; No Boil Dualcount kit), and 10% had hyperhomocysteinemia (5–15 μmol/L; Abbott IMx homocysteine kit). No subject had abnormal laboratory values indicating chronic disease.

The SAM, SAH, and ec-SOD activity and methylation levels of ec-SOD CpG sites were measured in 47 subjects. According to their serum folate levels, 16 subjects were in the lowest quintile (<23.6 nmol/L), 17 in the middle quintile (>34 and <42 nmol/L), and 14 in the upper quintile (>45 nmol/L). Serum vitamin B12 levels were higher in the upper quintile ($P < 0.05$), but this difference disappeared when two outliers were excluded from the calculations. Erythrocyte SAM concentrations were higher in the upper than in the middle and lowest quintiles (5.57 ± 1.58, 2.52 ± 0.97, and 2.29 ± 1.2 μmol/L, respectively, $P < 0.0001$). Erythrocyte SAH concentrations were higher only in subjects in the highest quintile compared with the lowest quintile (0.76 ± 0.24 versus 0.52 ± 0.23 μmol/L, $P < 0.03$). The SAM/SAH ratio was similar in the three groups (Table 2). There were no differences between groups in ec-SOD activity (Table 2) and promoter CpG methylation of ec-SOD (Table 3). The TMTHFR C677T genotype was similar in the three groups.

Subjects in the upper quintile were younger and had a higher body mass index (BMI) than subjects in the middle and lowest quintiles. Serum albumin, creatinine, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triacylglycerol levels were comparable in all serum folate groups. No subject had abnormal creatinine or albumin values.

Erythrocyte SAM levels correlated with erythrocyte SAH levels ($R = 0.41$, $P < 0.01$), serum folate levels ($R = 0.70$, $P < 0.001$), and vitamin B12 ($R = 0.31$, $P = 0.02$), BMI ($R = 0.43$, $P = 0.03$) and negatively with age ($R = -0.37$, $P < 0.02$). Serum folate levels correlated with vitamin B12 levels ($R = 0.47$, $P < 0.01$), erythrocyte SAH levels ($R = 0.42$, $P < 0.01$), Hcy concentration ($R = -0.38$, $P < 0.01$), and BMI ($R = 0.43$, $P = 0.03$) and negatively with age ($R = -0.35$, $P < 0.02$). Erythrocyte SAH levels did not correlate with serum homocysteine levels, age, or BMI. In a multiple regression model using SAM as the dependent variable and folate, vitamin B12,

Table 2
Erythrocyte SAM and SAH concentrations and ec-SOD activity in healthy subjects with different serum folate concentrations*

	Quintile 1 ($n = 16$)	Quintile 3 ($n = 17$)	Quintile 5 ($n = 14$)
Age (y)	55.7 ± 4.2^b	56.9 ± 4.5^b	48.1 ± 8.9^a
Body mass index (kg/m^2)	23.7 ± 2.1^b	22.8 ± 3.2^b	27.7 ± 2.2^a
Serum folate (nmol/L)	20.5 ± 3.0^c	38.4 ± 4.7^b	62.9 ± 10.1^a
Serum total homocysteine (μmol/L)	15.3 ± 19.3	10.3 ± 1.8	10.5 ± 1.6
Serum vitamin B12 (pmol/L)	444.2 ± 249.5^b	535.1 ± 166.0	703.0 ± 349.0^a
Erythrocyte SAM (μmol/L)	2.29 ± 1.2^b	2.52 ± 0.97^b	5.57 ± 1.58^a
Erythrocyte SAH (μmol/L)	0.52 ± 0.23^b	0.55 ± 0.27	0.76 ± 0.24^a
SAM/SAH ratio	6.1 ± 5.9	9.4 ± 16.6	8.8 ± 3.1
Plasma ec-SOD activity (U/mL)	112.9 ± 51.9	126.5 ± 44.6	126.3 ± 43.2

ec-SOD, extracellular superoxide dismutase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine

* Values are means ± SDs. One-way analysis of variance ($P < 0.03$). Values with superscript letters (a versus b, c versus a and b) are significantly different by Bonferroni's post hoc analysis.

Table 4
Multiple linear regression analysis using SAM and folate as dependent variables

Response variable	Explanatory variable	Proportion of explained variance (adjusted R^2)	Adjusted regression coefficient β	P
SAM	Folate	0.55	0.45	0.005
	SAH		0.22	0.073
	Age		−0.19	0.096
	BMI		0.20	0.99
	Vitamin B12		0.08	0.52
Folate	SAM	0.62	0.44	0.002
	Vitamin B12		0.31	0.007
	Age		−0.006	0.872
	Hcy		−0.26	0.06
	SAH		0.10	0.87
	BMI		0.22	0.06

BMI, body mass index; Hcy, homocysteine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine

SAH, BMI, and age as independent variables, only serum folate level was included in the model (adjusted $R^2 = .55$, $P < 0.01$; Table 4). Using folate as the dependent variable and SAM, SAH, vitamin B12, Hcy, BMI, and age as independent variables, SAM and vitamin B12 levels were included in the model (adjusted $R^2 = 0.62$, $P < 0.01$; Table 4).

Discussion

In this study we did not observe differences on the methylation status of CpG sites at the promoter region of ec-SOD among healthy subjects with different serum folate levels.

Folate deficiency has been associated with DNA hypomethylation. However, within the samples used in the present study, folate deficiency was not observed in any volunteer. On the contrary, serum levels of the upper quintile were above the normal range (7–39 nmol/L). This finding is not unexpected, since January 2000, the government of Chile initiated a program of wheat flour fortification with folic acid (220 μg of synthetic folic acid/100 g of wheat flour) to reduce women's risk of bearing a child with a neural tube defect. The distribution of serum folate levels was similar to those observed in the United States, Canada, and then United Kingdom after the fortification program with folate started and with our previous results [26–28]. The linearity of the curve between folate levels and SAM precludes the existence of a threshold folate level on this parameter.

A previous report showed a significant CpG methylation reduction in the ec-SOD gene in atherosclerotic aortas from rabbits [17]. Because folate depletion alters DNA methylation, it was feasible to hypothesize that variations in folate concentrations could affect the methylation status of ec-SOD. However, there are no reports on the effects of high folate concentrations on DNA methylation. We did not observe any effect of high serum folate levels on methylation patterns across the ec-SOD promoter gene.

The increased serum folate levels concomitant with increased SAM levels has been previously reported [1]. However, its biological significance is unclear. Basten et al. [29] reported that supplementation with 1.2 mg of folic acid in healthy subjects decreased misincorporation of uracil into cellular DNA, whereas DNA strand breakage, global DNA methylation status, and DNA repair capacity in response to oxidative DNA damage did not change. Stam et al. [30] demonstrated that folic acid supplementation increased whole-body remethylation and transmethylation flux in healthy subjects as measured by the Hcy remethylation rate and SAM/SAH ratio. However, Becker et al. [31], in an elderly population, did not demonstrate an association between plasma or erythrocyte concentrations of SAM or SAH and serum folate levels. It is important to mention that serum folate levels were lower in Becker's study, which may explain the differences between these studies.

The SAM and SAH concentrations are substrate dependent. When 5-MTHF concentration exceeds the equilibrium, more conversion of Hcy to methionine and an increase in SAM levels might be expected. Under these circumstances, SAH also increases because it is generated from SAM and is an inhibitor of this reaction. However, if the SAM/SAH ratio remains constant, the generation of SAH from Hcy is inhibited by the reversal of the SAH hydrolase reaction. In contrast, folate deficiency increases Hcy and the SAH hydrolase reaction is then activated, thus increasing SAH concentration and inhibiting the SAM-dependent methyltransferase reaction, such as of 5-cytosine DNA methyltransferases, leading to lower SAM/SAH ratio and methylation capacity [1,32]. Apparently, DNA methylation is more dependent on the SAM/SAH ratio or SAH concentration than on absolute SAM levels. It has been suggested that the SAM/SAH ratio is predictive of reduced methylation capacity only when it is associated with an increase in SAH. This conclusion was reached in a combined genetic and dietary approach using wild-type or heterozygous cystathionine b-synthase (CBS 1/2) mice consuming a control or methyl-deficient diet for 24 wk. In that model, they showed that a decrease in the SAM/SAH ratio due to SAM depletion alone was not sufficient to affect DNA methylation [33].

In this study we did not observe differences in the SAM/SAH ratio between groups; therefore, the similar methylation levels observed on CpG sites across the promoter region of the ec-SOD gene is not surprising. However, it must be emphasized that DNA methylation occurs in intrinsic correlation with other epigenetic mechanisms such as RNA factors, histone methylation, and chromatin-remodeling enzymes, which appear to act together with DNA methyltransferase for the establishment and maintenance of methylation patterns and the generation of site-specific methylation and tissue-specific differences [34]. Moreover, the establishment of these methylation patterns is a process that

occurs at very specific times during development [35]. Therefore, it is more feasible that environmental stimuli (as nutritional folates) would have an effect when methylation patterns are established, i.e., early during early development, rather than when changes in methylation are already set in the adult, in which case the alteration would be due to a process of failure of maintenance [36].

Our results are consistent with those in rats, where supranormal supplementation with folic acid during 4 wk did not alter the erythrocyte SAM/SAH ratio, DNA methylation, enzymatic activities, or concentrations of vitamins involved in the nutritional regulation of the methionine cycle, except for folate [37]. However, most studies reported in the literature have measured total folate in serum or tissues and not the active form, 5-methyltetrahydrofolate. This could lead to an erroneous interpretation of the results, because an increase in SAM levels inhibits methyltetrahydrofolate reductase [38]. Thus, normal 5-methytetrahydrofolate concentrations in conditions of high folate concentrations could maintain the stability of the methylation process. Probably the capacity of adaptation to diverse folate concentrations involves the existence of sensitive regulatory mechanism of the methionine cycle.

The SOD activity was similar in all groups, in concordance with the lack of variation in the methylation status of ec-SOD CpG sites. Nevertheless, it is important to point out that the promoter region of the ec-SOD gene includes several elements of regulation of gene expression, such as a metal regulatory element, a cyclic adenosine monophosphate responsive element, and an Activator Protein-1 binding site [22]. Therefore, in addition to DNA methylation, ec-SOD expression may be regulated in many ways.

Vitamin B12 or cobalamin apparently does not affect SAM or SAH concentration, even in severe cobalamin deficiency and anemia. However, SAH concentration is influenced by renal status in elderly subjects with cobalamin deficiency [39]. Therefore, because our subjects had normal creatinine levels, we did not expect an association between SAM and SAH concentration with vitamin B12 concentration.

The slight but significantly differences between the age and BMI of the subjects in the upper quintile of serum folate concentration compared with those in the lowest and middle quintiles are a limitation of the study. However, both parameters had no independent influence on folate levels and SAM in the multiple regression analysis. We did not observe an association between SAH and age that has been previously described with SAH concentration in the cerebral cortex of rats [40].

In conclusion, this study demonstrated that serum folate concentrations in the highest quintile among healthy humans are associated with increased erythrocyte SAM and SAH concentrations, but not with the global methylation index (SAM/SAH ratio) or with methylation levels of CpG sites across the promoter region of the ec-SOD gene. Further research needs to elucidate if this finding is beneficial or harmful.

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References

[1] Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. J Biol Chem 2000;275:29318–23.

[2] Loehrer FM, Tschöpl M, Angst CP, Litynski P, Jäger K, Fowler B, Haefeli WE. Disturbed ratio of erythrocyte and plasma S-adenosylmethionine/S-adenosylhomocysteine in peripheral arterial occlusive disease. Atherosclerosis 2001;154:147–54.

[3] Spijkerman AM, Smulders YM, Kostense PJ, Henry RM, Becker A, Teerlink T, et al. S-adenosylmethionine and 5-methyltetrahydrofolate are associated with endothelial function after controlling for confounding by homocysteine: the Hoorn Study. Arterioscler Thromb Vasc Biol 2005;25:778–84.

[4] Becker A, Henry RM, Kostense PJ, Jakobs C, Teerlink T, Zweegman S, et al. Plasma homocysteine and S-adenosylmethionine in erythrocytes as determinants of carotid intima-media thickness: different effects in diabetic and non-diabetic individuals. The Hoorn Study. Atherosclerosis 2003;169:323–30.

[5] Niculescu MD, Zeisel SH. Diet, methyl donors and DNA methylation: interactions between dietary folate, methionine and choline. J Nutr 2002;132:2333S–5.

[6] Jacob RA, Gretz DM, Taylor PC, James SJ, Pogribny IP, Miller BJ, et al. Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women. J Nutr 1998;128:1204–12.

[7] Castro R, Rivera I, Struys EA, Jansen EE, Ravasco P, Camilo ME, et al. Increased homocysteine and S-adenosylhomocysteine concentrations and DNA hypomethylation in vascular disease. Clin Chem 2003;49:1292–6.

[8] Castro R, Rivera I, Martins C, Struys EA, Jansen EE, Clode N, et al. Intracellular S-adenosylhomocysteine increased levels are associated with DNA hypomethylation in HUVEC. J Mol Med 2005;83:831–6.

[9] Hirsch S, Ronco AM, Pinardi G, Montequin MJ, Leiva L, de la Maza MP, et al. Lack of effect of diet-induced hypomethylation on endothelium-dependent relaxation in rats (abstract OC7-482). Ann Nutr Metab 2007;51:271.

[10] Laird PW, Jaenisch R. The role of DNA methylation in cancer genetic and epigenetics. Annu Rev Genet 1996;30:441–64.

[11] Singal R, Ginder GD. DNA methylation. Blood 1999;93:4059–70.

[12] Johnson IT, Belshaw NJ. Environment, diet and CpG island methylation: epigenetic signals in gastrointestinal neoplasia. Food Chem Toxicol 2008;46:1346–59.

[13] Chen Z, Riggs A. Maintenance and regulation of DNA methylation patterns in mammals. Biochem Cell Biol 2005;83:438–48.

[14] Richardson B, Raymond Y. Role of DNA methylation in the regulation of cell function. J Lab Clin Med 1999;134:333–40.

[15] Karlsson K, Marklund SL. Extracellular superoxide dismutase in the vascular system of mammals. Biochem J 1988;255:223–8.

[16] Fattman CL, Schaefer LM, Oury TD. Extracellular superoxide dismutase in biology and medicine. Free Radic Biol Med 2003;35:236–256.

[17] Laukkanen MO, Mannermaa S, Hiltunen MO, Aittomäki S, Airenne K, Jänne J, Ylä-Herttuala S. Local hypomethylation in atherosclerosis found in rabbit ec-sod gene. Arterioscler Thromb Vasc Biol 1999;19:2171–8.

[18] Stegmann K, Ziegler A, Kohlschmidt N. Linkage disequilibrium of MTHFR genotypes 677C/T-1298A/C in the German population and

association studies in probands with neural tube defects (NTD). Am J Med Genet 1999;87:23–9.

[19] Fu W, Dudman NPB, Perry MA, Young K, Wang XL. Interrelations between plasma homocysteine and intracellular S-adenosylhomocysteine. Biochem Biophys Res Commun 2000;271:47–53.

[20] Di Massimo C, Scarpelli P, Di Lorenzo N, Caimi G, di Orio F, Tozzi M, Ciancarelli MG. Impaired plasma nitric oxide availability and extracellular superoxide dismutase activity in healthy humans with advancing age. Life Sci 2006;78:1163–7.

[21] Zuccotti M, Monk M. Methylation of the mouse Xist gene in sperm and eggs correlates with imprinted Xist expression and paternal X-inactivation. Nat Genet 1995;9:316–20.

[22] Clark SJ, Harrison J, Paul CL, Frommer M. High sensitivity mapping of methylated cytosines. Nucleic Acids Res 1994;22:2990–7.

[23] Clark SJ, Frommer M. In: Taylor GR, editor. Laboratory methods for the detection of mutations and polymorphisms in DNA. Boca Raton, FL: CRC Press; 1997, p. 151–62.

[24] Warnecke P, Clark S. DNA methylation profile of the mouse skeletal alpha-actin promoter during development and differentiation. Mol Cell Biol 1999;19:164–72.

[25] Folz RJ, Crapo JD. Extracellular superoxide dismutase (SOD3): tissue-specific expression, genomic characterization, and computer-assisted sequence analysis of the human EC SOD gene. Genomics 1994;22:162–71.

[26] Dietrich M, Brown CJ, Block G. The effect of folate fortification of cereal-grain products on blood folate status, dietary folate intake, and dietary folate sources among adult non-supplement users in the United States. J Am Coll Nutr 2005;24:266–74.

[27] Clarke R, Sherliker P, Hin H, Molloy AM, Nexo E, Ueland PM, et al. Folate and vitamin B12 status in relation to cognitive impairment and anaemia in the setting of voluntary fortification in the UK. Br J Nutr 2008;17:1–6.

[28] Hirsch S, de la Maza P, Barrera G, Gattas V, Petermann M, Bunout D. The Chilean flour folic acid fortification program reduces serum homocysteine levels and masks vitamin B-12 deficiency in elderly people. J Nutr 2002;132:289–91.

[29] Basten GP, Duthie SJ, Pirie L, Vaughan N, Hill MH, Powers HJ. Sensitivity of markers of DNA stability and DNA repair activity to folate supplementation in healthy volunteers. Br J Cancer 2006;94:1942–7.

[30] Stam F, Smulders YM, van Guldener C, Jakobs C, Stehouwer CD, de Meer K. Folic acid treatment increases homocysteine remethylation and methionine transmethylation in healthy subjects. Clin Sci (Lond) 2005;108:449–56.

[31] Becker A, Smulders YM, Teerlink T, Struys EA, de Meer K, Kostense PJ, et al. S-adenosylhomocysteine and the ratio of S-adenosylmethionine to S-adenosylhomocysteine are not related to folate, cobalamin and vitamin B6 concentrations. Eur J Clin Invest 2003;33:17–25.

[32] Finkelstein JD: Metabolic regulatory properties of S-adenosylmethionine and S-adenosylhomocysteine. Clin Chem Lab Med 2007;45:1694–9.

[33] Caudill MA, Wang JC, Melnyk S, Pogribny IP, Jernigan S, Collins MD, et al. Intracellular S-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine b-synthase heterozygous mice. J Nutr 2001;131:2811–18.

[34] Chen ZX, Riggs AD. Maintenance and regulation of DNA methylation patterns in mammals. Biochem Cell Biol 2005;83:438–48.

[35] Mac Phee D. Epigenetics and epimutagens: some new perspectives on cancer, germ line effects and endocrine disrupters. Mutat Res 1998; 400:369–79.

[36] Guerrero-Bosagna C, Sabat P, Valladares L. Environmental signaling and evolutionary change: can exposure of pregnant mammals to environmental estrogens lead to epigenetically induced evolutionary changes in embryos. Evol Dev 2005;7:341–50.

[37] Achón M, Alonso-Aperte E, Ubeda N, Varela-Moreiras G. Supranormal dietary folic acid supplementation: effects on methionine metabolism in weanling rats. Br J Nutr 2007;98:490–6.

[38] Kutzbach C, Stokstad EL. Feedback inhibition of methylenetetrahydrofolate reductase in rat liver by S-adenosylmethionine. Biochim Biophys Acta 1967;139:217–20.

[39] Stabler SP, Allen RH, Dolce ET, Johnson MA. Elevated serum S-adenosylhomocysteine in cobalamin-deficient elderly and response to treatment. Am J Clin Nutr 2006;84:1422–9.

[40] Trolin CG, Lofberg C, Trolin G, Oreland L. Brain ATP:L-methionine S-adenosyltransferase (MAT), S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH): regional distribution and age-related changes. Eur Neuropsychopharmacol 1994;4:469–77.