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

Sandra Hirsch, M.D., M.Sc. a,*, Ana María Ronco, Ph.D. b, Carlos Guerrero-Bosagna, Ph.D. b, María Pía de la Maza, M.D., M.Sc. a, Laura Leiva, M.Sc. a, Gladys Barrera, R.N. a, Miguel Llanos, Ph.D. b, M. Angelica Alliende, M.Sc. c, Francisca Silva, M.D. a, and Daniel Bunout, M.D. a

a Aging and Chronic Diseases Laboratory, INTA, University of Chile, Santiago, Chile
b Hormone and Receptors Laboratory, INTA, University of Chile, Santiago, Chile
c Genetic and Metabolic Disease Laboratory, INTA, University of Chile, Santiago, Chile

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 (34.3–<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. © 2008 Elsevier Inc. All rights reserved.

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 hyperhomocysteinemia 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...
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-methyltetrahydrofolate (MTHF; the active form of folate) were positively associated with endothelium-dependent flow-mediated vasodilation [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 sites, 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 isozyme in most mammalian species examined [15]. High levels of ec-SOD expression in the arterial wall maintain low extracellular O₂⁻ 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 Hinf1 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 HClO₄ (600 μL) and incubated at 4°C for 30 min. The precipitated proteins were removed by centrifugation at 20 000 × 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 × 150 mm, ESA, Chelmsford, MA, USA) operating at 35°C. The mobile phase (operated at 20°C) consisted of 50 mM NaH₂PO₄, 6.7 mM 1-heptanesulfonic acid, and 5% (v/v) methanol at a pH of 4.4 adjusted with HPO₄. 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 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 H₂O.

**PCR conditions**

Each PCR reaction for bisulfite-treated DNA was carried out in a total volume of 20 μL containing 10 μL of 1× Promega master mix, 7 μL of DNase-free H₂O, 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'-GGATTTAGAGATTATTATATAAGTTT-3', reverse internal 5'-CTCAATCATACAACCCCTTTCAAA-3', reverse external 5'-CACTTAAATATCATATATTATATC-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)

<table>
<thead>
<tr>
<th>Feature</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>53.0 ± 7.4</td>
<td>24–68</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.3 ± 3.7</td>
<td>17.4–37.5</td>
</tr>
<tr>
<td>Serum albumin (g/L)</td>
<td>45 ± 3.3</td>
<td>34–51</td>
</tr>
<tr>
<td>Serum creatinine (μmol/L)</td>
<td>74.3 ± 15.9</td>
<td>38.0–138.8</td>
</tr>
<tr>
<td>Serum total cholesterol (mmol/L)</td>
<td>4.98 ± 0.93</td>
<td>2.78–7.05</td>
</tr>
<tr>
<td>Serum LDL cholesterol (mmol/L)</td>
<td>2.65 ± 0.78</td>
<td>1.23–4.62</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.48 ± 0.59</td>
<td>0.68–3.91</td>
</tr>
<tr>
<td>Serum triacylglycerols (mmol/L)</td>
<td>1.90 ± 1.14</td>
<td>0.57–5.95</td>
</tr>
<tr>
<td>Serum vitamin B12 (pmol/L)</td>
<td>393.7 ± 247.2</td>
<td>80.6–1945.0</td>
</tr>
<tr>
<td>Serum folate (nmol/L)</td>
<td>34.6 ± 12.8</td>
<td>12.9–76.3</td>
</tr>
<tr>
<td>Serum total homocysteine (μmol/L)</td>
<td>11.78 ± 5.3</td>
<td>6.02–36.7</td>
</tr>
</tbody>
</table>

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

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

<table>
<thead>
<tr>
<th>Feature</th>
<th>Quintile 1 (n = 16)</th>
<th>Quintile 2 (n = 17)</th>
<th>Quintile 3 (n = 17)</th>
<th>Quintile 4 (n = 15)</th>
<th>Quintile 5 (n = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>55.7 ± 4.2b</td>
<td>56.2 ± 4.5b</td>
<td>56.3 ± 4.5</td>
<td>55.7 ± 4.7</td>
<td>54.1 ± 4.9</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>23.7 ± 2.1b</td>
<td>23.8 ± 2.2</td>
<td>22.8 ± 3.2</td>
<td>23.3 ± 2.5</td>
<td>23.5 ± 3.0</td>
</tr>
<tr>
<td>Serum folate (nmol/L)</td>
<td>20.5 ± 3.0b</td>
<td>20.7 ± 3.0</td>
<td>20.4 ± 2.9</td>
<td>20.6 ± 2.7</td>
<td>21.0 ± 3.1</td>
</tr>
<tr>
<td>Serum total homocysteine (μmol/L)</td>
<td>15.3 ± 19.3</td>
<td>15.2 ± 15.4</td>
<td>15.1 ± 20.2</td>
<td>15.4 ± 19.7</td>
<td>15.6 ± 19.8</td>
</tr>
<tr>
<td>Serum vitamin B12 (pmol/L)</td>
<td>44.2 ± 249.5b</td>
<td>53.5 ± 166.0</td>
<td>53.1 ± 166.0</td>
<td>53.2 ± 166.0</td>
<td>53.3 ± 166.0</td>
</tr>
<tr>
<td>Erythrocyte SAM (μmol/L)</td>
<td>2.29 ± 1.2b</td>
<td>2.52 ± 0.97b</td>
<td>2.52 ± 0.97</td>
<td>2.53 ± 0.97</td>
<td>2.54 ± 0.97</td>
</tr>
<tr>
<td>Erythrocyte SAH (μmol/L)</td>
<td>0.52 ± 0.23b</td>
<td>0.55 ± 0.27</td>
<td>0.55 ± 0.27</td>
<td>0.56 ± 0.27</td>
<td>0.57 ± 0.24</td>
</tr>
<tr>
<td>SAM/SAH ratio</td>
<td>6.1 ± 5.9</td>
<td>9.4 ± 16.6</td>
<td>9.4 ± 16.5</td>
<td>9.4 ± 16.3</td>
<td>9.8 ± 3.1</td>
</tr>
<tr>
<td>Plasma ec-SOD activity (U/mL)</td>
<td>112.9 ± 51.9</td>
<td>126.5 ± 44.6</td>
<td>126.5 ± 44.4</td>
<td>126.5 ± 43.2</td>
<td>126.5 ± 43.2</td>
</tr>
</tbody>
</table>

* 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.

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 lowest quintile (1.58, 2.52 μmol/L, respectively, P < 0.0001). Erythrocyte SAM 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).

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

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

* Values are means ± SDs for percentage of methylation.

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,
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 $R^2 = .62$, $P < 0.01$; Table 4).

**Table 4**

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Explanatory variable</th>
<th>Proportion of explained variance (adjusted $R^2$)</th>
<th>Adjusted regression coefficient $\beta$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAM</td>
<td>Folate</td>
<td>0.55</td>
<td>0.45</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>SAH</td>
<td>0.22</td>
<td>0.073</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>−0.19</td>
<td>0.096</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>0.20</td>
<td>0.99</td>
<td>0.872</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12</td>
<td>0.08</td>
<td>0.52</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>SAM</td>
<td>0.44</td>
<td>0.002</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Vitamin B12</td>
<td>0.31</td>
<td>0.007</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Age</td>
<td>−0.006</td>
<td>0.872</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Hcy</td>
<td>−0.26</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>SAH</td>
<td>0.10</td>
<td>0.87</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>BMI</td>
<td>0.22</td>
<td>0.06</td>
<td>0.62</td>
</tr>
</tbody>
</table>

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

**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 folate 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-methyltetrahydrofolate 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.

Acknowledgments

The authors appreciate the excellent technical assistance of Mrs. Nancy Cruz and Mr. Fernando Garrido G.

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