
Endothelial-cell–derived nitric oxide (NO) relaxes vascular smooth muscle cells, causes vasodilation, and inhibits platelet aggregation. It has been demonstrated that NO is the predominant and stable form of NO present in plasma and represents the bioavailability of NO (1, 2). NO generation is depressed in blood vessels affected by atherosclerosis, and a loss of NO activity is associated with impaired vasoreactivity, enhanced platelet aggregation, and increased endothelial cell–leukocyte interactions (3).

Recently, evidence has accumulated that suggests that increased concentrations of the amino acid homocysteine (Hcy) also increase the risk for cardiovascular disease (CVD) (4). Hcy concentrations can be lowered by supplementing with folate, vitamin B6, vitamin B12, and vitamin B12 (5, 6). In particular, folate may also contribute in the prevention of CVD because folate seems to restore impaired NO metabolism (7).

In the present investigation, we included apparently healthy persons. Individuals on all types of medications were excluded from this cross-sectional study. Blood samples were collected from participants who had been fasting for 12 h. The blood samples were centrifuged at 4 °C within 1 h and stored at −80 °C until analysis. Informed written consent was obtained from all participants. The study was approved by the Regional Ethics committee, University of Bergen, Norway.

Total homocysteine (tHcy) concentrations in plasma were measured by an HPLC method (8). Plasma NO3 concentrations were measured by capillary electrophoresis (9), and total serum cholesterol, HDL-cholesterol, and serum triglyceride concentrations were measured by a routine enzymatic colorimetric method (Roche/Hitachi).

The concentrations of erythrocyte folate, serum folate, and vitamin B12 were measured on an Access Immuno Assay System (Sanofi Pasteur Diagnostics).

The program “Power and Precision” was used for the power calculations. Power computations were performed for the comparison of smokers vs nonsmokers with serum folate as the primary endpoint, based on a 2-sample t-test for In-transformed data. All P values given are 2-tailed. The statistical program StatView for the Macintosh (Abacus Concepts) was used for all calculations.

The characteristics of the study participants are given in Table 1. In a simple regression analysis, we found a significant positive relationship between the concentra-

### Table 1. Characteristics and biochemical values in plasma/serum from the study participants.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Analyte</th>
<th>n</th>
<th>M/F</th>
<th>M</th>
<th>F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td></td>
<td>205</td>
<td></td>
<td>156</td>
<td>49</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td></td>
<td>24.9</td>
<td>(17.2–34.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking, years</td>
<td></td>
<td>22.5</td>
<td>(7.0–43.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma nitrate, µmol/L (n = 205)</td>
<td>25.4</td>
<td>(12.3–135.5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma folate, nmoL/L (n = 200)</td>
<td>11.0</td>
<td>(4.0–39.0)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocyte folate, nmoL/L (n = 148)</td>
<td>685</td>
<td>(230–1300)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hcy, µmol/L (n = 205)</td>
<td>10.8</td>
<td>(6.0–29.2)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B12, pmol/L (n = 204)</td>
<td>350</td>
<td>(146–924)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol, mg/L (n = 205)</td>
<td>5.8</td>
<td>(3.0–13.7)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDL, mg/L (n = 109)</td>
<td>1.4</td>
<td>(0.6–2.3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL, mg/L (n = 109)</td>
<td>3.8</td>
<td>(2.2–6.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides, mg/L (n = 203)</td>
<td>1.2</td>
<td>(0.5–6.9)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All data except for the numbers of males/females and the numbers of smokers/nonsmokers are presented as the median (range).

BMI, body mass index.

DOI: 10.1373/clinchem.2004.047274
tions of serum folate and plasma NO₃ \((R = 0.17; P = 0.017)\) and a significant inverse relationship between the concentrations of serum folate and tHcy \((R = 0.244; P = 0.0005)\). In a multiple regression analysis that included age, sex, body mass index, NO₃, tHcy, and vitamin B₁₂ as independent variables and serum folate as dependent variable, we found a significant positive relationship between the concentrations of serum folate and plasma NO₃ \((P = 0.0037)\), and a significant negative relationship between the concentrations of serum folate and tHcy \((P = 0.0005)\); Table 1B of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue7/). The present findings may indicate that folate is involved in the intra-cellular formation and release of NO into plasma. There are at least 2 mechanisms by which NO could be converted to NO₃. The first possibility is the direct and rapid oxidative conversion of NO to NO₃ in the presence of oxy-hemoproteins in the extracellular fluids. The second possibility is that the oxidation of NO to NO₃ takes place through the formation of an NO₂ intermediate, also in the presence of oxy-hemoproteins \((10)\).

5-Methyltetrahydrofolate (5-MTHF) is the principal form (perhaps as much as \(~95\%) of folate in serum; therefore, most studies have used serum folate as an appropriate marker for folate status in their aim to explore the relationship between serum folate and tHcy concentrations \((11)\). As suggested in Fig. 1, 5-MTHF is related to the formation of both NO and Hcy in the cell. In the presence of 5-MTHF, the enzyme dihydrobiopterin reductase can reduce quinoid dihydrobiopterin to tetrahydrobiopterin \((BH₄)\) \((12)\). 5-MTHF also stabilizes BH₄, enhances its binding to NO synthase \((NOS)\), increases the activity of NOS, and thus regulates the ratio of NO to superoxide \((O₂\^{-})\) \((13)\). Therefore, our findings may indicate that the intracellular concentrations of 5-MTHF may regulate the bioavailability of NO.

A decrease in the folate concentration leads to an accumulation of tHcy. Oxidation of Hcy in the presence of trace elements generates \(O₂\) and hydrogen peroxide \((H₂O₂)\) \((14)\); it has therefore been suggested that folate deficiency may contribute to an increase in the concentration of \(O₂\). Furthermore, increased concentrations of Hcy also reduce the availability of BH₄ in vitro and thus increase the formation of \(O₂\) \((15)\).

In a recent study it was suggested that folic acid supplementation improves endothelial function in coronary artery disease \((CAD)\) by a mechanism largely independent of Hcy \((16)\). It is reasonable to assume that the improvement in endothelial function was attributable to increased formation of NO during the folic acid intervention. A different study showed that folic acid restored endothelial dysfunction in patients with hypercholester-

---

Fig. 1. Proposed metabolic relationships between folate and NO₃ and folate and Hcy \((1, 5)\).

Enzymes: 1, methionine adenosyltransferase; 2, methyltransferase; 3, S-adenosylhomocysteine hydrolase; 4, betaine-homocysteine methyltransferase; 5, methionine synthase; 6, dihydrofolate reductase; 7, serine hydroxymethyltransferase; 8, 5,10-methylene-tetrahydrofolate reductase; 9, dihydropterin reductase; 10, NOS.

Abbreviations: SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; DHF, dihydrofolate; THF, tetrahydrofolate; qBH₂, quinoid 7,8-dihydrobiopterin; BH₂, 7,8-dihydrobiopterin; Hcy-S-NO, homocysteine-nitrosothiol; ONOO⁻, peroxynitrite; Hcy-SH, reduced Hcy; Hcy-S-S-P, protein-bound Hcy; Hcy-Tlc, Hcy thiolactone.
olemia who had tHcy concentrations within the reference interval (17).

Our observation that smokers have higher plasma concentrations of NO\(_3\) than do nonsmokers is in accordance with previously published data (Fig. 1B of the online Data Supplement) (18), but are contradictory to the findings of Node et al. (19). Oxidants, free radicals, and trace elements in cigarette smoke may activate macrophages and neutrophils in the circulation, which may generate increased amounts of NO and nitrite (20). It is also possible that cigarette smoke may prevent reaction of NO with thiols to form nitrosothiols, important reservoirs for NO in blood (21).

From our findings in the present investigation, we postulate the following possible clinical implications: Increased dietary consumption of fruits and green vegetables may increase the concentrations of folate in serum/cells and thus may increase the ratios NO/Hcy and NO/O\(_2\)\(_{3}\). Other possible benefits from increased concentrations of serum folate may be decreases in the concentrations of soluble circulating adhesion molecules. Increased concentrations of circulating adhesion molecules seem to be associated with the process of inflammation.

We thank Drs. Øyvind Hetland and Peter H. Evans for reading the manuscript and for useful comments.

References


DOI: 10.1373/clinchem.2004.046409

Liquid Chromatography–Tandem Mass Spectrometry Method for the Analysis of Asymmetric Dimethylarginine in Human Plasma, Edzard Schwedhelm,1 Ting Tan-Andresen, Renke Maas, Ulrich Riederer, Friedrich Schulze, and Rainer H. Böger1 (1 Institute of Experimental and Clinical Pharmacology, University Hospital Hamburg-Eppendorf, and 2 Institute of Pharmacy, University of Hamburg, Hamburg, Germany;* address correspondence to this author at: Institute of Experimental and Clinical Pharmacology, University Hospital Hamburg-Eppendorf, 20246 Hamburg, Germany; fax 49-40-42803975, e-mail schwedhelm@uke.uni-hamburg.de)

Nitric oxide (NO) is essential in numerous physiologic processes and may be involved in related pathologic processes. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of isoforms of NO synthase in humans (1). ADMA originates from protein arginine methylation by protein arginine methyltransferases after protein hydrolysis (2). Enzymatic hydrolysis by dimethylarginine dimethylaminohydrolases to dimethylamine and citrulline is the major pathway for elimination of ADMA (3). Circulating ADMA is altered in patients with cardiovascular and neurologic diseases, erectile dysfunction, and many other disorders (4–6), and increased circulating ADMA independently predicts future cardiovascular events and mortality (7, 8). Short-time infusion of ADMA affects hemodynamics and cardiac function in humans (3, 9).

Analytical methods for the measurement of ADMA include HPLC, capillary electrophoresis, ELISA, and mass spectrometry (MS). The commonly used HPLC methods with fluorescence detection measure \(\alpha\)-phthalaldehydederivatives of ADMA. These derivatives are not stable and must be analyzed on-line. Moreover, ADMA must be separated by chromatographic means from its biologically inactive isomer, symmetric dimethylamine.