Effects of Acute Hyperhomocysteinemia on the Neurovascular Coupling Mechanism in Healthy Young Adults

B. Rosengarten, MD; S. Osthaus; D. Auch, MD; M. Kaps, MD

Background and Purpose—Hyperhomocysteinemia is a vascular risk factor that interferes with the nitric oxide signaling pathway of endothelial vasoregulation. Most investigations in young healthy humans on the peripheral vasculature using a standardized methionine challenge demonstrated altered vascular reactivity. In contrast, the cerebral autoregulation mechanism was shown to be unaffected by the same methionine load. To obtain additional insight into the compensatory range of the cerebral vasculature during a methionine challenge, we tested the neurovascular coupling mechanism that adjusts cerebral blood flow in accordance with cortical activity.

Methods—Fifteen healthy young adults (age, 24.7±2.3 years; 7 men) were tested with a functional transcranial Doppler test before and 3, 8, and 24 hours after administration of placebo, 20 mg folic acid, 20 mg folic acid and 0.1 g/kg body weight L-methionine, or L-methionine alone. Evoked blood flow response was evaluated according to a control system approach. Plasma concentrations of homocysteine, resting blood flow velocities, and control system parameters of flow velocity change were compared for each time point using a multiple analysis of variance test.

Results—Homocysteine levels increased significantly compared with baseline (before, 7.6±1.9 μmol/L; 3 hours, 22.2±6.0 μmol/L [P<0.0001]; 8 hours, 27.9±8.6 μmol/L [P<0.0001]; 24 hours, 12.6±7.8 μmol/L [P=NS]). Resting flow velocities and control system parameters remained statistically nonsignificant.

Conclusions—Compared with the peripheral vasculature, the regulatory mechanisms controlling adequate cerebral blood flow appear to have a wider compensatory range. This is concluded from statistically nonsignificant results comparing the vascular reactivity in young adults undergoing a standardized methionine challenge. Our data confirm indirectly the reports of high concentrations of homocysteine needed to affect the cerebral vasculature in animal experiments. (Stroke. 2003;34:446-451.)

Key Words: endothelium ■ homocyst(e)ine ■ ultrasonography, Doppler ■ vasodilation

The neurovascular coupling (NC) mechanism adjusts local cerebral blood flow in accordance with the underlying cortical activity.1-4 Whereas the detailed mechanisms remain to be resolved, there is good evidence that the biological elements of the NC mechanism functionally resemble a complex and cascaded control system.1,2,4-8 The vascular endothelium and its nitric oxide system play a crucial role in the NC-related vasoregulation.4,9-13

Hyperhomocysteinemia is an emerging vascular risk factor leading to endothelial dysfunction, atherosclerosis, and consequently parenchymal ischemia.10-13 The term “endothelial dysfunction” is assumed to describe in general a functional alteration of the vasculature, depending on some vasoregulatory mechanism.14,15 Under acute hyperhomocysteinemia, it is hypothesized to be caused by a decrease in availability of nitric oxide.16-19

Several studies in healthy young volunteers undergoing an oral methionine loading of 0.1 g/kg body weight demonstrated an impaired peripheral vascular reactivity.20-24 However, some reports found neither an altered peripheral vascular reactivity25,26 nor a change in arterial rigidity under the same homocysteine challenge.27 Similarly, for the cerebral autoregulation (CA) mechanism that maintains constant cerebral perfusion despite changes in arterial blood pressure, it was demonstrated that vascular reactivity was affected only in aged but not in young healthy subjects.28 An attenuation of the endothelium-dependent cerebrovascular reactivity was found consistently only in animal experiments using high concentrations of homocysteine (1 mmol/L).16-19

Because it is known that the cerebral vasculature differs in many functional and morphological aspects from the peripheral vasculature, the suggestion was made that an unchanged CA mechanism might be caused by a higher compensatory range of the cerebral vasculature.28 To obtain additional information, we performed a visual stimulation test and measured the resultant flow velocity response in the posterior cerebral artery (PCA) with transcranial Doppler (TCD). Relying on the NC mechanism, the method allows measure-
ment of the vascular reactivity of cerebral vessels.\textsuperscript{1-3,5,6} The Doppler approach is a noninvasive, painless, and nearly physiological test for investigating evoked flow velocity changes in basal cerebral vessels with high accuracy and time resolution.\textsuperscript{1,2} With the use of standardized stimulation paradigms, the NC was demonstrated to be highly reliable and fine tuned.\textsuperscript{3,6}

**Materials and Methods**

**Subjects and Study Design**

Fifteen healthy students (age, 24.7±2.3 years; 7 men, 8 women) without family or personal history of migraine, premature vascular disease, hypertension, diabetes mellitus, or hyperlipidemia were included in the double-blind crossover study. All volunteers were clinically healthy nonsmokers who did not take regular medication. The body mass index was 21.9±1.8 kg/m\(^2\). A normal vascular status examined by an extracranial and transcranial Duplex scan was an inclusion criterion. The study was approved by the institutional review committee, and each volunteer gave written, informed consent. The tests were performed in a quiet room at about 25°C while the subjects were sitting comfortably. All volunteers had abstained from caffeine overnight (10 to 14 hours) before the study. Venous blood samples were drawn from all volunteers to measure the concentrations of basal (fasting) homocysteine \((7.9±2.2 \mu\text{mol/L}),\) total cholesterol \((169±16 \text{mg/dL}),\) high-density lipoprotein (HDL) cholesterol \((68±16 \text{mg/dL}),\) triglycerides \((85±21 \text{mg/dL}),\) glucose \((84±8.2 \text{mg/dL}),\) HbA1c \((5.1±0.4\%),\) serum folate \((8.5±3.9 \text{ng/mL}),\) vitamin B6 \((1.74±0.17 \text{nmol/L}),\) and vitamin B12 \((315±134 \text{pg/mL}).\) Arterial blood pressure (ABP) was measured noninvasively \((\text{systolic ABP, } 118±7 \text{ mm Hg}; \text{diastolic ABP, } 74±4 \text{ mm Hg}),\) and a functional TCD (fTCD) test was performed as described below for all subjects after they sat for 10 minutes. In addition to the baseline recordings, ABP, evoked blood flow velocity, and plasma levels of total homocysteine were measured repeatedly at 3, 8, and 24 hours after administration of oral methionine \((1\text{-methionine, } 0.1 \text{ g/kg body weight}; \text{Acimethin, Gry-pharma}),\) methionine \((0.1 \text{ g/kg body weight})\) with 20 mg folic acid \((\text{Folsan, Solvay Arzneimittel}),\) 20 mg folic acid alone, or placebo. All agents were administered in a mixture with orange juice. The sequence of the 4 experiments was alternated by chance with a time interval between tests of >1 week.

**Vascular Studies**

Two 2-MHz probes were mounted on an individually fitted headband. In all cases, the P2 segment of the left PCA and the right middle cerebral artery (MCA) in its M1 segment were sonicated. The MCA was recorded to determine nonspecific effects in the test situation. The procedure of finding and verifying the vessels followed the descriptions of published protocols.\textsuperscript{29}

Peak systolic and end-diastolic blood flow velocities were recorded with a Multidop T2 Doppler device (DWL). The reason for the separated evaluation of systolic and end-diastolic blood flow velocities was that the indexes show different time courses in dynamic blood flow regulation.\textsuperscript{30}

As a stimulation paradigm, we used a news magazine that the volunteers could read freely. This “reading” test was validated against a checkerboard stimulation paradigm.\textsuperscript{6} The stimulation protocol consisted of 10 cycles each with a resting phase of 20 seconds and a stimulating phase of 40 seconds for each cycle. During resting periods, volunteers were instructed to close their eyes; during stimulation phases, they read silently. Changes between phases were signaled acoustically with a tone.

Beat-to-beat intervals of cerebral blood flow velocity data were interpolated linearly with a “virtual” time resolution of 50 ms for averaging procedures. To ensure independence from the sononation angle and to allow comparisons between volunteers, absolute data were transformed into relative changes of cerebral blood flow velocity in relation to baseline. Baseline was calculated from the blood flow velocity averaged for a time span 5 to 0 seconds before the beginning of the stimulation phase. The method and algorithm for analyzing the data sets in terms of a control system are described in detail in an earlier work.\textsuperscript{6,7} Evaluation was performed separately for the systolic and end-diastolic blood flow velocity data for each test condition. The following parameters were specified: K represents the gain; T\(_c\), the rate time; \(\omega\), the undamped natural angular frequency (natural frequency); and \&xi;\(\text{, the attenuation parameter of the system. Additionally, the time delay, } T\), was calculated. The parameters describe different dynamic features of the assumed regulative principle of the NC. Because the parameters are all derived from a mathematical approximation, they are at first glance theoretical and do not have a direct correlation to physiological processes.

**Laboratory Assays**

Venous blood samples were collected in tubes containing EDTA. Samples were centrifuged within 10 minutes at 4000 rpm for 10 minutes. The plasma was then separated and stored at −70°C until analysis.

Total plasma concentrations of homocysteine were measured by fluorescence polarization immunoassay (Axis Biochemicals ASA). Plasma total cholesterol, HDL cholesterol, low-density lipoprotein (LDL) cholesterol, and triglyceride levels were analyzed by enzymatic methods (Roche Diagnostics). HbA1c was measured by a latex immunoagglutination inhibition method (Bayer Diagnostics). The reference range for HbA1c was 4.3% to 5.7%. Plasma folic acid and vitamin B12 were obtained by a competitive immunoassay (Bayer Diagnostics). Plasma vitamin B6 was estimated by enzymatic photometry.

**Statistical Analysis**

Continuous data were expressed as mean±SD. Statistical comparisons between chemical measurements, resting blood flow velocities, and each of the independent control system parameters were performed with a 2-way analysis of variance (ANOVA) for repeated measurements (study arms and flow measurements were repeated in each subject). The 4 test experiments and the 4 different time points of each test were assumed to be fixed treatments according to ANOVA model 1. Statistical significance was inferred at \(P<0.05.\) When statistical significance occurred, Scheffe’s post hoc test was performed. Tests for normal distribution were performed, and the homogeneity of the variances was checked by an \(F\) test.

**Results**

None of the volunteers had to be excluded because of results from the duplex scan. Data from all volunteers were used for evaluation. Comparison of ABP and blood flow velocities in the MCA during rest and stimulation revealed no significant differences (data not shown).

Placebo (before, 7.9±2.0 \(\mu\text{mol/L}); 3 \text{ hours, } 7.9±1.9 \mu\text{mol/L}; 8 \text{ hours, } 8.1±2.1 \mu\text{mol/L}; 24 \text{ hours, } 7.8±1.8 \mu\text{mol/L}; \text{P=NS)} and folic acid (before, 7.7±2.0 \mu\text{mol/L); 3 \text{ hours, } 7.6±1.8 \mu\text{mol/L}; 8 \text{ hours, } 7.7±1.8 \mu\text{mol/L}; 24 \text{ hours, } 7.3±1.8 \mu\text{mol/L}; \text{P=NS)} did not affect plasma levels of homocysteine, whereas the administration of methionine (before, 7.6±1.9 \mu\text{mol/L); 3 \text{ hours, } 22.2±6.0 \mu\text{mol/L}; 8 \text{ hours, } 27.9±8.6 \mu\text{mol/L}; 24 \text{ hours, } 12.6±7.8 \mu\text{mol/L}; \text{P<0.0001 for 3 and 8 hours}) and methionine with folic acid (before, 8.0±2.6 \mu\text{mol/L); 3 \text{ hours, } 23.7±7.8 \mu\text{mol/L}; 8 \text{ hours, } 28.54±10.2 \mu\text{mol/L}; 24 \text{ hours, } 12.3±5.1 \mu\text{mol/L}; \text{P<0.0001 for 3 and 8 hours}) increased homocysteine levels significantly. As an example, the averaged flow velocity time courses for each test condition of the 8-hour fTCD tests are shown in Figure 1 for the peak systolic and in Figure 2 for the end-diastolic data. Results of the 8-hour test were chosen for
Discussion

Homocysteine is an emerging vascular risk factor leading to a cascaded vascular damage that is assumed to begin with a state of endothelial dysfunction followed by progressive atherosclerosis that eventually, without treatment, can lead to increased cardiovascular or cerebrovascular ischemic risk.10–13 Endothelial dysfunction is hypothesized to be caused by the potency of homocysteine to interfere with the action of endothelial vasodilators such as nitric oxide.10–13,16,29 It is hypothesized that nitric oxide is scavenged by superoxide anion generated in the metabolic pathway of homocysteine.16,29,31,32

In animal experiments, it has been shown consistently that acute administration of high concentrations of homocysteine of 1 mmol/L results in several cerebrovascular effects.16–19 In healthy young humans, in which a standardized methionine challenge of 0.1 g/kg body weight was performed that resulted in only a 3- to 4-fold increase in plasma levels of homocysteine (from 7.4 to 20 to 30 μmol/L), discrepancies in results occurred. Whereas many reports described an altered peripheral vascular reactivity,20–24 other investigators did not find any differences in the functional integrity of peripheral vessels.25–27 In an investigation of the CA mechanism in young healthy volunteers, no differences were reported.28 In the present investigation, we found the NC mechanism to be unaffected by the same methionine challenge, as concluded from evaluation of Doppler data. The resting cerebral blood flow velocities in the MCA and PCA did not change under challenge, which is in agreement with results found by Chao et al28 before and 4 hours after a methionine load. Values of the control system parameters describing the main dynamic features of the flow adjustment also remained unchanged during the challenge. Our finding underlines further the special situation of the cerebral vasculature, which seems to have a wider compensatory range than peripheral vasculature in regulating adequate blood flow, because a standardized methionine challenge affected neither the CA nor the NC mechanism. The different compensatory capability of the central and peripheral vasculature can be seen even during normal aging. Several reports demonstrated the NC and CA mechanism to remain unchanged during normal aging, whereas many cardiovascular parameters change.1,4,33,34 However, the robustness of the cerebral vasculature was already ensured from the high homocysteine concentrations needed to obtain cerebrovascular effects in animal experiments.16

The nonefficacy of the methionine challenge in the present study, as in studies in which there is a lack of efficacy of a drug, demands careful consideration of the adequacy of dosage and access of the agent to the target tissue. These issues have been addressed in the present study in that a standardized methionine challenge was given. The given dosage of 0.1 g/kg body weight was demonstrated to increase reliably concentrations of homocysteine 3- to 4-fold.22,23 The plasma concentrations of homocysteine over the time points found in the present investigation are in good accordance with previous results.22,23 Therefore, it appears unlikely that the nonsignificant results are caused by an error in methionine application. However, from a statistical point of view, the nonsignificant results do not prove the statistical null hypothesis. Therefore, interpretations of results have to be treated with considerable care.

Vascular reactivity studies were performed mainly with the brachial artery ischemia test. Although the present investigation did not show significance, we assume the functional transcranial Doppler approach to be a feasible method for
investigating states of endothelial dysfunction in cerebral vasculature. Changes between states of rest and activation can be readily performed methodologically for the visual cortex and can be repeated many times.\textsuperscript{1-3} The Doppler method has a high temporal resolution that is needed for measuring the resultant flow velocity changes in the basal vessels. Because the basal cerebral vessels do not contribute to the NC mechanism, the vessel diameter remains constant, and thus flow velocity changes are closely related to blood flow changes.\textsuperscript{1,4} Although the spatial resolution is weak, the visual cortex is also the best candidate for sensory stimulation because it occupies a relatively large and well-defined area of the brain that is almost exclusively supplied by the PCA.\textsuperscript{1-3} Many fTCD investigations performed on the visual cortex have stated a reliable and close coupling between distinct features of the visual paradigm and blood flow change.\textsuperscript{1-3,6} Advantages compared with the brachial artery ischemia test include the fact that the test paradigm is nearly physiological and does not cause additional changes such as pain, ischemia, or vegetative stimulation, which might result in additional hemodynamic changes. In the present study, the occurrence of possible nonspecific changes in the systemic circulation was monitored by flow velocity recordings in the MCA. Comparison of flow velocities during rest and stimulation in the MCA yielded stable blood flow conditions. The use of a control system approach for evaluating the regulative hemodynamic response of the NC mechanism resulted in a higher sensitivity of the evaluation method for 2 reasons. First, compared with the conventionally used “overshoot” method, which specifies only the point of maximal velocity increase,\textsuperscript{1} the control system approach allows a much more refined description of the entire flow velocity time course of the NC mechanism that can be seen in the close matching of measured and modeled curves (Figures 1 and 2). The time course of the blood flow response is not evaluated by specifying only 1 point. The main hemodynamic features are sufficiently described by a control system model of low order. Second, because the overshoot value is statistically dependent on several control system parameters, the overshoot value consequently has a higher SD, which weakens statistical statements. Therefore, if differences in the flow response would have occurred in the present methionine loading, they more likely should have been detected with the new method.\textsuperscript{6,35} A further advantage of the NC test is that its methodology does not rely on test-inherent modulations of the systemic (CA test) or local (brachial artery ischemia test) blood flow as a result of the cuff deflation technique. Consequently, the relevant functional measurements are not

**TABLE 1. Systolic Data**

<table>
<thead>
<tr>
<th>Challenge</th>
<th>Parameter</th>
<th>0 h</th>
<th>3 h</th>
<th>8 h</th>
<th>24 h</th>
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<tbody>
<tr>
<td>Homocysteine</td>
<td>Baseline, cm/s</td>
<td>62.8±8.5</td>
<td>62.8±8.8</td>
<td>66.12±7.3</td>
<td>58±6.6</td>
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<td>Gain, %</td>
<td>12.6±5.2</td>
<td>11.8±5.1</td>
<td>12.6±2.8</td>
<td>14.3±6.4</td>
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<tr>
<td></td>
<td>Nat freq, 1/s</td>
<td>0.23±0.04</td>
<td>0.22±0.03</td>
<td>0.22±0.05</td>
<td>0.22±0.03</td>
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<tr>
<td></td>
<td>Attenuation</td>
<td>0.41±0.14</td>
<td>0.42±0.1</td>
<td>0.39±0.18</td>
<td>0.41±0.12</td>
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<tr>
<td></td>
<td>Rate time, s</td>
<td>2.4±1.2</td>
<td>2.6±0.9</td>
<td>3.2±1</td>
<td>3.1±1.5</td>
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<td>Placebo</td>
<td>Baseline, cm/s</td>
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<td>59.6±8.9</td>
<td>58.2±7.5</td>
<td>57.6±6.8</td>
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<tr>
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<td>12.4±6</td>
<td>15.1±6.1</td>
<td>14±6</td>
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<tr>
<td></td>
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<td>0.21±0.04</td>
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<tr>
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<td>Attenuation</td>
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<td>0.42±0.14</td>
<td>0.44±0.1</td>
<td>0.44±0.14</td>
</tr>
<tr>
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<td>Rate time, s</td>
<td>3.5±1.6</td>
<td>2.8±1.4</td>
<td>3.8±1.5</td>
<td>3.1±1.4</td>
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<tr>
<td>Folic acid</td>
<td>Baseline, cm/s</td>
<td>61.3±8.6</td>
<td>63.5±9.2</td>
<td>61.16±8.5</td>
<td>59.2±7.3</td>
</tr>
<tr>
<td></td>
<td>Gain, %</td>
<td>14.5±6</td>
<td>12.5</td>
<td>14±7</td>
<td>14±6</td>
</tr>
<tr>
<td></td>
<td>Nat freq, 1/s</td>
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<td>0.22±0.05</td>
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<td>2.9±1.8</td>
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<td>Homocysteine + folic acid</td>
<td>Baseline, cm/s</td>
<td>60.6±7</td>
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<td>12.1±5.5</td>
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<td>Nat freq, 1/s</td>
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<td>2.9±1.7</td>
<td>3.4±0.66</td>
<td>3.1±1.4</td>
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Average data from all volunteers as mean±SD for the different metabolic challenges and time points of peak systolic blood flow velocity measurements. Absolute baseline flow velocities and control system parameters were obtained. Nat freq indicates natural frequency.
Major portions of this article were taken from the doctoral thesis of S. Osthaus.

References


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