Folate Deficiency Increases Postischemic Brain Injury

Matthias Endres, MD; Michael Ahmadi, MD; Inna Kruman, PhD; Detlev Biniszewicz, PhD; Andreas Meisel, MD; Karen Gertz, MD

Background and Purpose—Folate deficiency and resultant hyperhomocysteinemia impair vascular function and increase stroke risk. We tested the hypothesis that folate deficiency and high homocysteine levels promote DNA damage and increase brain injury after cerebral ischemia/reperfusion.

Methods—129/Sv mice, uracil-DNA glycosylase–deficient (Ung−/−) mice, and Ung+/- littermate mice were exposed to a folate-deficient diet for 3 months and then subjected to 30-minute middle cerebral artery (MCA) occlusion and reperfusion. Plasma homocysteine levels and physiological parameters were measured in selected animals. Outcome measures were neurological sensorimotor deficits, infarct size measured by computer-assisted volumetry, and oxidative DNA damage measured by a colorimetric assay.

Results—Exposure to a folate-deficient diet for 3 months conferred ~6- to 10-fold higher plasma homocysteine levels than those associated with a normal diet. Cerebral lesion volumes and neurological deficits after MCA occlusion and 72-hour reperfusion were significantly 2.1-fold increased in folate-deficient 129/SV wild-type mice compared with those associated with a normal diet, which could not be explained by obvious differences in physiological parameters. Abasic sites, hallmarks of oxidative DNA damage, were significantly increased in DNA from the ischemic brain of folate-deficient animals at early time points after MCA occlusion. Folate deficiency further increased brain lesion size in animals lacking uracil-DNA glycosylase compared with wild-type littermate mice.

Conclusions—Folate deficiency and resultant hyperhomocysteinemia are not only associated with increased stroke risk but increase oxidative DNA damage and ischemic lesion size after MCA occlusion/reperfusion. (Stroke. 2005;36:321-325.)

Key Words: animal models ■ cerebral ischemia ■ folic acid ■ homocysteine

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Folate is a cofactor in 1-carbon metabolism, during which it promotes the remethylation of homocysteine. Homocysteine is a metabolite of methionine, an amino acid critical for the generation of S-adenosylmethionine, which is the principal biological methyl donor on all cellular transmethylation reactions. Dietary folate has a major impact on all cellular transmethylation reactions. Dietary folate has a major impact on homocysteine levels, with an inverse relationship between plasma folate and homocysteine levels. In 1969, McCully was the first to implicate homocysteine in the pathogenesis of atherosclerosis. A number of epidemiological studies have linked folate deficiency and resultant elevated plasma total homocysteine levels with an increased risk of vascular disease and ischemic stroke. However, it has remained unclear whether this association is causal, and the recent Vitamin Intervention for Stroke Prevention (VISP) randomized controlled trial to lower homocysteine in patients with ischemic stroke was negative.

In addition to augmenting the risk for vascular events, low folate/high homocysteine may directly increase the susceptibility of neurons to brain injury. Folate deficiency causes uracil misincorporation into DNA and chromosomal breakage, which has implications for neuronal damage. In fact, folic acid deficiency and high homocysteine levels can be directly toxic to cultured neurons by mechanisms that involve uracil misincorporation, impaired DNA repair, and DNA damage. When maintained on a folate-deficient diet, amyloid precursor protein mutant transgenic mice, but not wild-type mice, exhibited increased cellular DNA damage and hippocampal neurodegeneration. Moreover, epidemiological studies have linked folate deficiency and hyperhomocysteinemia to neurodegenerative and neuropsychiatric disease, including Alzheimer and Parkinson disease, depression, and schizophrenia.

In this study we tested the hypothesis that impaired 1-carbon metabolism resulting from folate deficiency and resultant high homocysteine levels increases oxidative DNA damage and aggravates tissue injury after cerebral ischemia/reperfusion in wild-type mice. Moreover, since uracil misincorporation and uracil base excision repair (BER) have been implicated in neuronal damage caused by folate deficiency, we tested whether ischemic brain injury is further aggravated in animals lacking uracil-DNA glycosylase, an enzyme that excises aberrant uracil residues from DNA during BER.
Materials and Methods

Mice, Diets, and Measurements of Homocysteine Levels

All experimental procedures conformed to institutional guidelines and were approved by an official committee. 129/Sv wild-type mice (Bundesinstitut für Risikobewertung, Berlin, Germany), uracil-DNA glycosylase–deficient (Ung<sup>−/−</sup>) mice, and Ung<sup>+/−</sup> littermate mice (from a 129/SvJae/balb/c background)<sup>21</sup> aged 6 to 8 weeks were exposed to a control versus experimental diet for 3 months. The control diet was a standard mouse diet, which contained defined choline and folate (Altromin control diet C1000), whereas the experimental diet lacked folic acid (<0.5 mg/kg; Altromin, special diet C1027). Both chows were supplemented with 1% succinylsulfathiazole (Sigma-Aldrich) for selective intestinal decontamination. Mice (aged 6 to 8 weeks) were fed with the respective diet ad libitum for 3 months. After 3 months on the diets, blood was taken for analysis of homocysteine levels. Homocysteine levels in serum samples were quantified with the use of an IMx immunoassay analyzer (Abbott Laboratories) according to the protocol provided by the manufacturer.

Model of Cerebral Ischemia

Mice (18 to 20 g) were first anesthetized by isoflurane anesthesia (1.2% for induction and 0.8% for maintenance) in 70% N<sub>2</sub>O and 30% O<sub>2</sub> with a face mask. Focal cerebral ischemia was produced as described.<sup>21,22</sup> Briefly, the left middle cerebral artery (MCA) was occluded with an 8-0 silicone-coated monofilament, and the filament was withdrawn after 30 minutes. Rectal temperature was controlled and kept constant at 36.5 ± 0.5°C by means of a feedback temperature-control unit. Regional cerebral blood flow was measured by means of a flexible probe and laser-Doppler monitoring (Perimed).<sup>21,22</sup> There was an equivalent drop in regional cerebral blood flow to <20% of baseline at filament insertion and an equivalent rise in regional cerebral blood flow at filament withdrawal. In randomly assigned animals, the left femoral artery was cannulated for arterial blood pressure monitoring and blood withdrawal. Arterial blood samples were analyzed for pH, arterial oxygen pressures, and partial pressure of carbon dioxide as described.<sup>22</sup>

Determination of Neurological Deficits

Mice were tested for neurological sensorimotor deficits and scored from 0 to 3 as described by Bederson et al.<sup>23</sup> Animals were rated in a blinded fashion by an observer naive to the experimental groups.

Determination of Infarct Size

After 72-hour reperfusion, animals were killed by a pentobarbital overdose, and brains were quickly removed from the skull and snap-frozen in isopentane on dry ice for cryostat sectioning. Infarct areas were quantified with an image analysis system (Sigma Scan Pro 4.0; Jandel Scientific) on 20-μm hematoxylin-eosin (H&E)–stained cryostat sections (2-mm distance) as described.<sup>21,22</sup>

Assessment of Oxidative DNA Damage

Apurinic/apyrimidinic abasic sites (AP sites) were quantitatively measured in nuclear DNA extracted from the ischemic striatum or corresponding tissue from the contralateral hemisphere at various time points after MCA occlusion/reperfusion (10 minutes, 3 hours, and 24 hours). Measurements were performed with the use of a commercially available kit for AP site counting (DNA Damage Quantification Kit, Dojindo Molecular Technologies) with minor modifications as described in detail elsewhere.<sup>24-26</sup> The colorimetric assay uses a biotin-labeled reagent specific for the aldehyde group in the ring-open form of AP site, termed aldehyde reactive probe (ARP), for the detection of AP sites.<sup>24-26</sup> All ARP assays were performed in triplicate, and the means were calculated. Data were calculated on the basis of a linear calibration curve with ARP-DNA standard solution (Dojindo Molecular Technologies) and expressed as number of AP sites per 10<sup>7</sup> nucleotides.

Statistical Analysis

Experiments were performed in a blinded fashion. Values are presented as mean ± SEM. Statistical comparisons were performed by unpaired Student t test, Mann-Whitney rank sum test, and 1-way or 2-way ANOVA followed by Tukey test. Probability values <0.05 were considered statistically significant.

Results

Folate Deficiency Renders Wild-Type Mice Susceptible to Cerebral Ischemia/Reperfusion

Wild-type male 129/Sv mice were placed on either a normal diet or a diet deficient in folate. After 3 months on the diets, serum levels of homocysteine were determined and were found to be 7-fold increased in animals subjected to the folate-deficient diet (Figure 1a).

To determine whether folate deficiency increases the susceptibility to ischemic brain injury, animals were subjected to 30-minute left filamentous MCA occlusion followed by
TABLE 1. Neurological Sensorimotor Deficit Score

<table>
<thead>
<tr>
<th>Group</th>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Median</th>
<th>25%</th>
<th>75%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=11)</td>
<td></td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Folate-deficient diet (n=10)</td>
<td></td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>0</td>
<td>1.5</td>
<td>1.0</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Wild-type Sv/129 mice were subjected to a diet deficient in folate or regular diet for 3 months. Neurological scoring was determined by the use of a previously published method performed 72 hours after 30-minute MCA occlusion/reperfusion.23 The numerical scores are as follows: 0, normal motor function; 1, flexion of contralateral torso and forelimb on lifting the whole animal by the tail; 2, circling to the contralateral side; 3, loss of walking or righting reflex.

reperfusion for 72 hours. Cerebral lesion size was measured on H&E-stained cryostat sections and compared between groups. Folate-deficient mice had 2.2-fold larger lesion volumes than control mice (Figure 1b). Significantly larger lesion areas were evident in 3 of the 5 standardized coronal brain sections (ie, sections 2, 3, and 4; Figure 1c). All animals exhibited a neurological deficit score $\geq$2 (ie, moderate or severe deficit) after onset of cerebral ischemia. By 72 hours, the deficits were improved in either group but were significantly higher in animals exposed to the folate-deficient diet (P<0.05, Mann–Whitney rank sum test; Table 1).

It is well known that changes in physiological parameters may modify outcome after cerebral ischemia. Therefore, we carefully monitored these parameters in randomly selected animals before, during, and after brain ischemia. As indicated in Table 2 we did not observe any differences in arterial blood pressure or blood gases (ie, PO2, PaCO2). pH levels were lower in the control group; however, it is unlikely that physiological parameters may have contributed to the differences in ischemia outcome.

TABLE 2. Physiological Parameters in 129/Sv Mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Diet</th>
<th>Folate-Deficient Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABP, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>111±3</td>
<td>110±2 (P=NS)</td>
</tr>
<tr>
<td>During</td>
<td>122±5</td>
<td>122±2 (P=NS)</td>
</tr>
<tr>
<td>After</td>
<td>119±5</td>
<td>116±5 (P=NS)</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>7.25±0.01</td>
<td>7.31±0.01 (P&lt;0.05)</td>
</tr>
<tr>
<td>PaCO2, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>48±2</td>
<td>45±2 (P=NS)</td>
</tr>
<tr>
<td>PaO2, mm Hg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>97±5</td>
<td>104±3 (P=NS)</td>
</tr>
<tr>
<td>Core temperature, °C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>During</td>
<td>36.9±0.1</td>
<td>36.8±0.2 (P=NS)</td>
</tr>
<tr>
<td>Weight, g</td>
<td>24.5±0.7</td>
<td>24.4±1.0 (P=NS)</td>
</tr>
</tbody>
</table>

Animals were subjected to 30-minute filamentous MCA occlusion followed by reperfusion. Mean arterial blood pressure (MABP) was measured before ischemia (baseline), during ischemia, and 30 minutes after reperfusion.21,22 Fifty microliters of blood was withdrawn during cerebral ischemia for blood gas determination (pH, PaO2, PaCO2). Animals were weighed before onset of the experiment. Rectal temperature was controlled and kept constant by means of a feedback temperature-control unit (n=6 animals per group; ANOVA and Tukey post hoc test).

Figure 2. 129/Sv wild-type mice were exposed to a normal (ND) or folate-deficient (FD) diet for 3 months and then exposed to 30-minute left filamentous MCA occlusion and 72-hour reperfusion. The number of AP sites was determined in DNA extracted from ischemic brain tissue and corresponding tissue from the contralateral hemisphere with the use of a DNA damage quantification kit at various times points after MCA occlusion. In control brains, AP sites are typically $<1\times10^6$ nucleotides.24 Values are mean±SEM; n=3 animals per group. *P<0.05 vs control, ANOVA and Tukey post hoc test.

Folate Deficiency Increases Oxidative DNA Damage at Early Time Points After MCA Occlusion/Reperfusion

AP sites, hallmarks of oxidative damage, are the most commonly produced DNA lesion and occur in neurons as early as minutes after transient ischemia.24 To determine whether folate deficiency increased oxidative DNA damage as an important contributory factor for ischemic cell death, we quantified AP sites in the ischemic versus contralateral striatum after 3 months on a folate-deficient versus normal diet. AP sites were significantly increased in DNA from ischemic tissue of folate-deficient animals compared with animals fed with a normal diet as early as 10 minutes after MCA occlusion/reperfusion. This increase in AP sites in folate-deficient mice was sustained until 24 hours (Figure 2).

Folate Deficiency Confers Further Increases in Infarct Size in Animals Lacking Uracil-DNA Glycosylase

We have recently demonstrated that Ung-deficient animals have increased posts ischemic brain injury, which could not be explained by obvious alterations in vascular anatomy.21 Since uracil misincorporation and uracil BER have been implicated in neuronal cell death induced by folate deficiency and hyperhomocysteinemia,15,16,17,20 we exposed Ung−/− and Ung+/+ littermates to a folate-deficient versus control diet for 3 months. Similar to 129/Sv mice, folate deficiency conferred a significant increase in serum homocysteine levels in either genotype compared with normal diet at 3 months (Figure 3a). Animals were subjected to 30-minute MCA occlusion and 72-hour reperfusion. There were major increases in lesion...
increases in ischemic lesion size in Ung−/− normal-diet on 5 anterior-posterior serial coronal H&E-stained cryostat sections (20 μm). Values are mean±SEM. *P<0.05 vs normal diet, ANOVA plus Tukey test; n=5 animals per group. Brain lesion volume (b) and lesion areas (c) were determined by worse neurological scores, suggesting that the increases in tissue damage corresponded to functional outcome. Lesion volumes and deficits after 30-minute MCA occlusion in mice were exposed to MCA occlusion/reperfusion, folate-deficient mice compared with animals that were fed a normal diet; we did not measure AP sites in Ung−/− mice, however. These differences between groups were already evident at 10 minutes and were sustained until 24 hours. Chen and coworkers also reported increased AP sites at a frequency similar to that in our study after ischemia/reperfusion. Indeed, although they present evidence that these DNA lesions may be efficiently repaired in the cortex, the levels of AP sites remained increased in the caudate putamen up to 72 hours. In the present study we demonstrate that significant increases in these oxidative nuclear DNA lesions herald subsequent increases in ischemic lesion size in folate-deficient animals; however, we did not causally link homocysteine levels with increased oxidative DNA damage.

Dietary deficiency of folate increases dUMP incorporation into DNA, via inhibition of thymidylate synthase and depletion of the TTP pool, and this may result in enzymatic fragmentation of the newly synthesized DNA. In mitotic cells such DNA damage can lead to cancer, while in postmitotic cells such as neurons it promotes cell death. In fact, neurons are more vulnerable to folate deficiency than are mitotic astrocytes. Although little is currently known about the repair capabilities of neurons, we have recently reported that animals lacking uracil-DNA glycosylase have increased postischemic brain injury, implying uracil BER in ischemic neuronal cell death. In the present study we demonstrate that exposing Ung−/− animals to folate deficiency further increases ischemic brain injury after MCA occlusion. Brain lesion volumes in folate-deficient Ung−/− mice were 1.7-fold larger than in folate-deficient wild-type mice, 2.2-fold larger than in normal-diet Ung−/− mice, and 4.4-fold larger than in normal-diet wild-type mice (Figure 3). It should be noted that we did not determine uracil residues in DNA; in addition, the

Discussion
This study provides evidence that a folate-deficient diet resulting in hyperhomocysteinemia renders wild-type mice susceptible to cerebral ischemia/reperfusion. It is known that bacteria in the intestinal flora are capable of synthesizing folate; therefore, both control and experimental diets were supplemented with antibiotics for intestinal decontamination. Diets were administered for 3 months, a time interval that is known to induce relevant carcinogenesis. Indeed, 129/Sv animals behaved normally and had no macroscopic evidence of tumor formation after 3 months. However, when animals were exposed to MCA occlusion/reperfusion, folate-deficient mice had major increases in brain lesion volumes compared with normal-diet animals. Moreover, this was accompanied by worse neurological scores, suggesting that the increases in tissue damage corresponded to functional outcome. Lesion volumes and deficits after 30-minute MCA occlusion in mice fed with a normal diet are in agreement with published data. No alterations in systemic physiological parameters were demonstrated; it seems therefore unlikely that the observed differences in ischemia susceptibility can be explained by obvious effects of folate deficiency on these parameters.

It has been suggested that folate deficiency and elevated homocysteine levels increase the vulnerability of cultured neurons partly via mechanisms related to uracil misincorporation, oxidative DNA damage, and impaired DNA repair. In this study we demonstrate that AP sites, hallmarks of oxidative DNA damage, are significantly elevated in DNA from ischemic brain tissue of folate-deficient mice compared with animals that were fed a normal diet; we did not measure AP sites in Ung−/− mice, however. These differences between groups were already evident at 10 minutes and were sustained until 24 hours. Chen and coworkers also reported increased AP sites at a frequency similar to that in our study after ischemia/reperfusion. Indeed, although they present evidence that these DNA lesions may be efficiently repaired in the cortex, the levels of AP sites remained increased in the caudate putamen up to 72 hours. In the present study we demonstrate that significant increases in these oxidative nuclear DNA lesions herald subsequent increases in ischemic lesion size in folate-deficient animals; however, we did not causally link homocysteine levels with increased oxidative DNA damage.

Figure 3. Ung−/− and Ung+/+ littermate mice were exposed to a folate-deficient (fd) or normal diet (nd) for 3 months and then exposed to 30-minute left MCA occlusion/reperfusion. a, Levels of homocysteine in serum samples. Values are mean±SEM. *P<0.05, **P<0.01 vs normal diet, ANOVA plus Tukey test; n=5 animals per group. Brain lesion volume (b) and lesion areas (c) were determined on 5 anterior-posterior serial coronal H&E-stained cryostat sections (20 μm). Values are mean±SEM. #P<0.05 vs normal-diet and wild-type controls, 2-way ANOVA plus Tukey test; n=5 animals per group.
lesion studies in knockout mice do not provide conclusive evidence that uracil misincorporation is mechanistically involved in neuronal cell death induced by folate deficiency. Nevertheless, our results clearly emphasize the importance of both 1-carbon metabolism and uracil BER in ischemic cell death.

In regard to the risk of cardiovascular disease and stroke in humans, the relationship between plasma homocysteine levels and vascular events is as follows: <7 μmol/L, low; 8 to 11 μmol, moderate; 12 to 16 μmol/L, high; >16 μmol/L, very high. Data from the Framingham Study suggest a similar relationship for neurodegenerative disorders. In our study the level of homocysteine in the blood of wild-type mice was in the range of 2 to 4 μmol/L, while longer trials in different populations demonstrated that folic acid supplementation enhances growth, only to reduce stroke risk but to protect the brain from cerebral ischemia/reperfusion. By inference, lowering homocysteine levels as potential causes of increased stroke risk cannot differentiate between the lack of folate and increased homocysteine levels potentially caused by increased stroke vulnerability in our study. The VISP randomized controlled trial recently investigated whether lowering homocysteine by high doses of folic acid, pyridoxine (vitamin B6), and cobalamin (vitamin B12) reduced the risk of recurrent strokes. Disappointingly, the treatment was not associated with a reduction of vascular events, which may relate to the relatively short follow-up of 2 years and the fact that homocysteine levels were only moderately reduced (by ~2 μmol/L) in the treatment group. While longer trials in different populations may be necessary to confirm the utility of homocysteine lowering to reduce stroke risk, animal studies recently demonstrated that folic acid supplementation enhances growth, repair, and recovery in the injured adult central nervous system.

In conclusion, we demonstrate that folate deficiency and resultant elevated homocysteine levels not only increase stroke incidence but directly augment brain damage after cerebral ischemia/reperfusion. By inference, lowering homocysteine levels by vitamin therapy may provide a therapy not only to reduce stroke risk but to protect the brain from neurodegeneration during cerebral ischemia.

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References

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