Hyperhomocysteinemia Due to Short-Term Folate Deprivation Is Related to Electron Microscopic Changes in the Rat Brain

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ABSTRACT We investigated the effects of folate deprivation on plasma folate and homocysteine and its effects on cerebral microvasculature using electron microscopy. Two levels of folic acid (0 mg and 4 mg/kg diet) were fed to 6-mo-old male rats for 8 wk. Dietary folate deprivation decreased plasma folate from 353.0 +/− 29.7 nmol/L to 44.2 +/− 7.2 nmol/L with a concomitant increase in plasma homocysteine from 6.15 +/− 0.9 μmol/L to 19.5 +/− 2.7 μmol/L. Plasma folate was negatively correlated with plasma homocysteine at wk 8 (r = −0.876, P = 0.004). Electron microscopic studies of the brains of folate-deprived rats revealed cytoplasmic swelling and mitochondrial degeneration in the endothelium, perivascular amorphous fibrosis and pericytic degenerative appearance in the cerebrocortical microvascular wall. These morphologic changes might be helpful for elucidating the mechanisms underlying the cerebrovascular and neuropathology with folate deficiency. J. Nutr. 132: 3418–3421, 2002.

KEY WORDS: folate deprivation • hyperhomocysteinemia • cerebral microvascular wall • neurotoxicity • rats

Homocysteine is a sulfur-containing amino acid regulated by the nutritional status of the B-vitamins such as folate and vitamins B-6 and B-12 (1), of which folate is the most important (2). Folate deficiency causes plasma homocysteine to increase and the resulting hyperhomocysteinemia has been associated with cerebrovascular disease (3), stroke (4) and several neural and psychiatric disorders including depression, Alzheimer’s disease (5) and Parkinson’s disease (6).

Elevated plasma homocysteine may be an independent risk factor for cerebrovascular and neurodegenerative disorders, but the underlying mechanisms are not clear. Hyperhomocysteinemia may promote the development of dementia in several ways, such as through the development of cerebral microangiopathy (7), endothelial dysfunction (8,9), and oxidative stress (10), as well as the enhancement of β-amyloid peptide-dependent neurotoxicity (10) and neuronal apoptosis (11). Homocysteic acid, a metabolite of homocysteine, can also cause neuronal excitotoxicity by stimulating N-methyl-D-aspartate receptors (12). In addition, the effects of homocysteine on atherothrombosis in the cerebral vasculature can promote central nervous system ischemia, neuronal hypoxia and injury.

The strong association of homocysteine with cerebrovascular disease has been attributed to homocysteine-induced vascular injury leading to stroke and participate in the ensuing neurotoxic response in the brain (13,14). The growth-promoting effect of homocysteine on vascular smooth muscle cells (15), together with its inhibitory effect on endothelial cell growth (9), may explain homocysteine-induced atherosclerosis. Cerebral microvascularity in hyperhomocysteinemia should be investigated to elucidate the mechanism of homocysteine-induced neurotoxicity and the development of neurodegenerative changes.

To investigate the effects of folate deprivation on plasma homocysteine, and its cerebrovascular and neurotoxic effects, we induced hyperhomocysteinemia in rats by folate deprivation and examined the morphological alterations in cerebral microvasculature by electron microscopy.

MATERIALS AND METHODS

The experiment was conducted using an animal protocol approved by the Ewha Womans University College of Pharmacy Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (6-mo-old) (Samtaco, Osan, Korea) were housed individually in stainless steel cages under controlled conditions (25°C, 12-h light: dark cycle). Rats were divided into two groups: those fed a folate-deficient (folate-D)3,4 (3) diet and those fed a folate-supplemented (folate-S) diet for 8 wk.

Baseline samples were collected from four rats fed nonpurified diet (Diet #31; Samtaco) for 1 wk before experimentation. Experimental rats were then allowed free access to food and water and samples were collected at over 4 wk intervals.

After 8 wk, rats were anesthetized intraperitoneally with pentobarbital sodium. Blood samples were drawn by heart puncture and promptly centrifuged at 1,750 × g for 15 min at 4°C. The plasma was stored frozen at −70°C. Plasma homocysteine was analyzed by the HPLC (Waters 474, Millford, MA) fluorescence detection method developed by Araki and Sako (17). Plasma vitamin B-12 and folate were measured with the 57Co vitamin B-12 and 125I folic acid Dualcount SPNB (solid phase no boil) Radiodassay (Diagnostic Products, Los Angeles, CA).

For the electron microscopic study, rats were perfused transcardially with 1.55% calcium-free heparinized saline followed by 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and brain immediately removed and postfixed in the same buffer for 24 h. The tissue was then washed in 0.1 M cacodylate buffer (pH 7.4) and immersed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 2 h. Finally, the tissue was dehydrated in alcohol and embedded in epoxy resin (Epon 812). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and examined using a Hitachi H800 transmission electron microscope. The hippocampus, cerebral cortex and lateral ventricle were selected, and electron microscopic studies were performed from three rats in each group.

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3 Abbreviations used: BBB, blood-brain barrier; folate-D, folate-deficient; folate-S, folate-supplemented.

4 The folate-D diet contained comstarch (466.8 g/kg diet; Daesang, Seoul, Korea), casein (140 g/kg diet; Murray Goulburn Cooperative, Australia), dextrinized comstarch (155 g/kg diet, Daesang), sucrose (100 g/kg diet; Chee Jeol, Inchon, Korea), soybean oil (46 g/kg diet; Sindromeang Inchon, Korea), fiber (50 g/kg diet; Sigma-Aldrich, St. Louis, MO), mineral mixture (35 g/kg diet, AIN-93M), ω-methionine (1.8 g/kg diet; Sigma-Aldrich), choline chloride (1.4 g/kg diet; Acros Organics, Geel, Belgium), tert-butylhydroquinone (0.008 g/kg diet; Sigma-Aldrich) and vitamin mixture (15 g/kg diet, AIN-93M) without added folate. The folate-S diet was made by adding 0.004 g folate/kg diet as folic acid.
ally with glutaraldehyde (25 g/L) and paraformaldehyde (20 g/L) in 0.1 mol/L phosphate buffer, at pH 7.4 and decapitated. The brain was removed and the tissue blocks were immersed in 0.1 mol/L phosphate buffer, at pH 7.4 and decapitated. The brain was

microscopy was unaware of the dietary treatments of the brain specimens.

The analysis was performed with transmission electron microscope (Hitachi H-600, Tokyo, Japan). The person performing the electron microscopy was unaware of the dietary treatments of the brain specimens.

Initial body weights, weight gain, and food intake were analyzed by Student’s t test. Plasma homocysteine and folate concentrations were analyzed by two-way ANOVA. After a significant ANOVA ($P < 0.05$), differences were tested by Duncan’s multiple range test. Pearson’s correlation analysis was used to evaluate the correlation between plasma homocysteine and folate levels. Results are presented as mean ± SEM.

**RESULTS**

Initial body weights, weight gain, and food intake of folate-D rats were not different from those of folate-S rats (Table 1). Dietary folate deprivation caused a decrease in the plasma folate from 353.0 ± 29.7 nmol/L to 44.2 ± 7.2 nmol/L with a concomitant increase in the plasma homocysteine from 6.15 ± 0.9 nmol/L to 19.5 ± 2.7 nmol/L by 8 wk (Fig. 1). Plasma folate was negatively correlated with plasma homocysteine at 8 wk ($r = -0.876; P = 0.004$). Electron microscopic data for cerebrocortical microvascular wall in brain cross sections from folate-S groups (panel A) and folate-D groups (panels B–D) are shown in Figure 2. Panel A shows a normal cerebral capillary wall with a relatively smooth luminal surface and a regular thin layer of basement membrane around the endothelium, surrounding a pericyte. The endothelial cell formed a thin, regular sheet on the inner side of the basement membrane. Panels B–D show a degenerative appearance of the cerebrocortical microvascular wall in rats fed the folate-D diet for 8 wk. Lesser degenerative changes were also observed after 4 wk of feeding (not shown). High amplitude mitochondrial swelling (m) with disintegration of mitochondrial cristae and dissolution of cytoplasmic organelles in the endothelial cell cytoplasm are exhibited (panel B). Abnormal electron-lucent structures indicating perivascular amorphous fibrosis are observed within the basement membrane and in the perivascular area (panel C). Locally and irregularly thickened basement membrane is also observed in cerebral microvessels of folate-deficient rats. Frequent perivascular detachment was observed in relatively large cerebral microvessels. Characteristic degenerative pericytes including mitochondrial and cytoplasmic swollen profiles are also shown (panel D).

**DISCUSSION**

In this study, the hyperhomocysteinemia induced by folate deprivation was accompanied by ultrastructural degenerative changes in the cerebral microvasculature, including endothelial and pericytic degeneration, mitochondrial destruction and cytoplasmic dissolution. These alterations were similar to previously reported microvascular degenerative features typically found in cerebral diseases such as Alzheimer’s disease, Parkinson’s disease and aging processes (18–20). Perivascular fibrosis and pericytic changes were frequently observed in cerebral vessels of aged animals and senescence-accelerated mice in the studies by other investigators (18,20). The electron-lucent image structures that we observed in the perivascular area reveal features that are consistent with features observed in aged animal brain (20) and findings in Alzheimer’s disease (18).

It is possible that the observed amorphous deposition in the basement membrane and the perivascular areas indicating cerebrovascular fibrosis may have been part of a compensatory response to the dysfunctional endothelium. The cerebral capillary endothelium is the anatomical basis of the blood-brain barrier (BBB) and provides the energy necessary for the active transport of a variety of essential nutrients into the neurons. We contend that the pathological alterations in the endothelial structure we observed suggest a BBB failure in which the perivascular deposits could have compromised an optimum energy-dependent nutrient transport, resulting in impaired neuronal functioning. The increased number of pericytic cytoplasmic inclusions and the enlarged mitochondria, in contrast, may represent an activated, phagocytic state of these cells because it has been suggested that the pericytes may function as immune cells in the cerebral microvasculature (21). Such an increased activity suggests action against potential rupture and leakage in the BBB.

Homocysteine is a key functional metabolite in methionine metabolism. It has two major metabolic fates: transmethylation and transsulfuration. The importance of the homocysteine-dependent transsulfuration pathway lies in the maintenance of the intracellular glutathione pool, and the regulation of this pathway is strongly influenced by oxidative stress. The mechanism whereby homocysteine leads to endothelial cell damage has been found to be via its auto-oxidation to homocystine and H$_2$O$_2$ (22). In vitro studies on the role of homo-

**TABLE 1**

<table>
<thead>
<tr>
<th>Wk</th>
<th>Group</th>
<th>Initial body weight</th>
<th>Weight gain</th>
<th>Food intake</th>
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<tr>
<td></td>
<td></td>
<td>g</td>
<td>g/week</td>
<td>g/day</td>
</tr>
<tr>
<td>0</td>
<td>Baseline</td>
<td>589.6 ± 17.0</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>Folate-D</td>
<td>602.1 ± 11.9</td>
<td>7.7 ± 2.1</td>
<td>26.6 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Folate-S</td>
<td>596.3 ± 15.7</td>
<td>8.2 ± 2.8</td>
<td>26.2 ± 1.3</td>
</tr>
<tr>
<td>8</td>
<td>Folate-D</td>
<td>583.5 ± 17.0</td>
<td>9.6 ± 1.5</td>
<td>28.4 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>Folate-S</td>
<td>582.1 ± 16.6</td>
<td>8.3 ± 1.1</td>
<td>29.8 ± 1.3</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, n = 4.
cysteine in aging have previously shown a positive link between the amount of exposure to homocysteine and the rate of senescence (23). It also has been reported that the peroxidase scavenger catalase attenuates the ability of homocysteine to accelerate the rate of senescence (23).

We have previously confirmed that dietary folate deprivation does not affect the plasma levels of vitamin B-12 (24). Also, unlike plasma folate, plasma vitamin B-12 levels were not correlated with plasma homocysteine in these rats. This indicates that the ultrastructural changes in the brain are most likely due only to the folate deficiency and its influence on homocysteine metabolism, not to the vitamin B-12 deficiency.

We have presented for the first time electron microscopic images of ultrastructural cerebro-microvascular damage in folate deficiency-induced hyperhomocysteinemia in rat brains, similar to the cerebrovascular degeneration typically found in cerebral diseases such as Alzheimer’s, and Parkinson’s and with age. We have confirmed that these ultrastructural changes are due only to the folate deficiency and its influence on homocysteine metabolism, not to vitamin B-12 deficiency.

We are currently investigating the effect of folate administration on cerebral microvasculature that has been damaged by hyperhomocysteinemia due to folate deficiency. We are also conducting light microscopic and ultrastructural investigations on the vascular permeability to examine whether hyperhomocysteinemia induces BBB damage with subsequent ultrastructural changes in the cerebrovascular structures that we observed in this study.

LITERATURE CITED


