Mice Deficient in Methylenetetrahydrofolate Reductase Exhibit Tissue-Specific Distribution of Folates

Haifa Ghandour, Zhoutao Chen,* Jacob Selhub, and Rima Rozen*2

Vitamin Metabolism and Aging, Jean Mayer U.S. Department of Agriculture, Human Nutrition Research Center on Aging, Tufts University, Boston, MA and *Departments of Human Genetics, Pediatrics and Biology, McGill University-Montreal Children’s Hospital Research Institute, Montréal, QC, Canada

ABSTRACT Methylenetetrahydrofolate reductase (MTHFR) catalyzes the synthesis of 5-methyltetrahydrofolate (5-methylTHF), which is used for homocysteine remethylation to methionine, the precursor of S-adenosylmethionine (SAM). Impairment of MTHFR will increase homocysteine levels and compromise SAM-dependent methylation reactions. Mild MTHFR deficiency is common in many populations due to a polymorphism at bp 677. To assess how impaired MTHFR activity affects folate metabolism in various tissues in vivo, we used affinity/HPLC with electrochemical detection to analyze the distribution of folates in plasma, liver, and brain of Mthfr-deficient mice. The most pronounced difference in total folate was observed in plasma. In Mthfr−/− mice, plasma total folate levels were ~25% of those in wild-type (Mthfr+/+) mice. Only 40% of plasma folate in Mthfr−/− mice was comprised of 5-methylTHF, compared with at least 80% in the other 2 genotype groups. In liver and brain, there were no differences in total folate. However, the proportion of 5-methylTHF in both tissues was again markedly reduced in mice with the Mthfr−/− genotype. In this genotype group, 5-methylTHF is likely derived from the diet. Our study demonstrated reduced total circulating folate and altered distribution of folate derivatives in liver and brain in Mthfr deficiency. Decreased methylfolates and increased nonmethylfolates would affect the flux of one-carbon units between methylation reactions and nucleotide synthesis. This altered flux has implications for several common disorders, including cancer and vascular disease. J. Nutr. 134: 2975–2978, 2004.

KEY WORDS: • folate distribution • methylenetetrahydrofolate • formylated-folates • MTHFR deficiency

The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR,1 EC 1.5.1.20) catalyzes the irreversible reduction of 5,10-methylenetetrahydrofolate (5,10-methyleneTHF) to 5-methyltetrahydrofolate (5-methylTHF). This reduction is crucial because it serves several biological reactions as follows: 1) 5-methylTHF provides the methyl group for the remethylation of homocysteine to methionine. Methionine is a precursor of S-adenosylmethionine (SAM), the universal methyl donor for many methylation reactions including DNA, proteins, lipids, hormones and others (1–3). 2) The substrate 5,10-methyleneTHF is also used in thymidylate synthesis or converted to 10-formylTHF for purine synthesis (4). Because the MTHFR reaction is irreversible, it diverts folate coenzymes to 5-methylTHF, and potentially to the trapping of folate coenzymes (5). 3) It provides 5-methylTHF (monoglutamates), which serves in the transport and trafficking of folate in and out of peripheral tissues (6).

Severe deficiency of MTHFR results in the inborn error of metabolism, homocystinuria, a relatively rare disorder for which >25 different mutations in MTHFR were identified (7). A mild deficiency of MTHFR, a more common condition, is present in 10–15% of many North American and European populations, due to homozygosity for a polymorphism (677C→T) that encodes a thermolabile enzyme with reduced activity (8). We generated Mthfr knockout mice, providing a model with which to study the consequences of a disturbance in folate metabolism (9). The homozygous knockout mice (Mthfr−/−) have a 10-fold elevation in plasma homocysteine (~30 vs. ~3 μmol/L in wild-type mice), altered SAM and SAH levels, and decreased DNA methylation in several tissues. The heterozygous knockout mice (Mthfr+/−) have moderate changes in the above parameters; their plasma homocysteine values are ~5 μmol/L. Due to the above features, the Mthfr−/− and Mthfr+/− mice are considered good animal models for severe and mild MTHFR deficiency in humans, respectively.

Because MTHFR deficiency should result in the absence of newly synthesized 5-methylTHF, the present study was undertaken to determine how this deficiency affects plasma folate, which is normally made exclusively of 5-methylTHF (monoglutamate). Moreover, because plasma 5-methylTHF is con-

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2 To whom correspondence should be addressed.

E-mail: rima.rozen@mcgill.ca.

3 Abbreviations used: DHF, dihydrofolate; FA, folic acid; 10-formylTHF, 10-formyltetrahydrofolate; 5,10-methyleneTHF, 5,10-methylenetetrahydrofolate; 5,10-methylTHF, 5-methyltetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; SAM, S-adenosylmethionine; THF, tetrahydrofolate.
sidered to be the transport species across the cell membrane, this study also investigated folate distribution in peripheral tissues. Because our earlier report of these mice demonstrated that peripheral tissues from Mthfr-deficient mice did contain tissues. Because our earlier report of these mice demonstrated this study also investigated folate distribution in peripheral tissues. Because our earlier report of these mice demonstrated that peripheral tissues from Mthfr-deficient mice did contain tissues.

**MATERIALS AND METHODS**

**Mthfr-deficient mice.** Mice with severe (Mthfr −/−) and mild (Mthfr +/−) deficiency were generated and housed as described previously (9). Mice were progeny from matings of Mthfr +/− mice that had been backcrossed for at least 10 generations from 129/Sv-BALB/c F1 heterozygotes (9) to BALB/cAnNCrBR (Charles River Canada). Animal experimentation was approved by the Animal Care Committee of the Montreal Children’s Hospital and complied with the guidelines of the Canadian Council for Animal Care. The mice were fed standard mouse food (Harlan Teklad LM-485 Mouse, code 7012). After overnight food deprivation, blood was collected from the tail vein in vacutainer tubes containing EDTA. Plasma was obtained after centrifugation at 2000 × g for 5 min and stored at −70°C until analysis. Mice were killed and liver and brain tissues were immediately removed and also stored at −70°C until analysis. Mthfr genotypes were determined by a PCR-based method as previously described (9).

**Analysis of folate distribution.** Folate distribution was determined in liver, brain, and plasma using the affinity/HPLC method with electrospray (coulometric) detection (10) with minor modifications. Briefly, plasma was diluted 10-fold with extraction buffer (0.05 mol/L potassium tetraborate, 1% sodium ascorbate, pH 9.2), heat extracted (100°C for 15 min) and centrifuged for 15 min at 36,000 × g. Liver and brain samples were homogenized with extraction buffer MES (0.05 mol/L MES, 0.1 mol/L DET, 1% sodium ascorbate, pH 6.0), heat extracted (100°C for 15 min), and centrifuged for 15 min at 36,000 × g. Conjugase from folate-free rat plasma was added to the supernatant and incubated at 37°C for 1.5 h. The samples were then heated at 100°C for 5 min, and centrifuged for 20 min at 36,000 × g. The supernatant fraction (2 mL) was injected into the affinity column (10 × 4.6 mm), which contained purified milk folate binding protein covalently bound to AffiPrep 10 support (Bio-Rad). After washing the affinity column sequentially with 0.05 mol/L potassium phosphate (pH 7) and water, folate in the affinity column was eluted onto the analytical column (Betalis Phenyl, 250 × 4.6 mm, Keystone Scientific) with an acid mobile phase (0.028 mol/L potassium phosphate (pH 7) and water, folate in the affinity column 10-formylTHF and 5-formylTHF, and referred to as formyl residues. Folate forms elute in the following order: tetrahydrofolate (THF), 5-methylTHF, 5-10-formylTHF, 5,10-methenylTHF, dihydrofolate (DHF), and folic acid (pteroylglutamate). Folate activity was determined using an ESA Four Channel Coularray Detector with channels 1 through 4 set at 0, 300, 500, and 600 mV, respectively. Quantification and identification of individual folates were achieved by comparison with external folate standards of known concentration. To simplify the comparison of various folate forms, the values of 5,10-methenylTHF were added to those of the corresponding 10-formylTHF and 5-formylTHF, and referred to as formylated THF. 5-MethylTHF and total folate in the mouse diet were determined using the combined affinity and ion-pair chromatography detection as described previously (11,12).

**Statistical analysis.** All values are reported as means ± SEM. Differences among groups were evaluated by ANOVA. When the ANOVA was significant (P < 0.05), individual post-hoc tests were performed among groups using Tukey’s Studentized range statistic for pairwise comparisons. All stated differences are significant (P < 0.05), unless indicated otherwise. All statistical analyses were performed using SYSTAT software (version 10.0, SPSS).

**RESULTS**

Folate in plasma from the Mthfr +/+ and Mthfr +/+ mice consisted primarily (82–94%) of 5-methylTHF (Table 1). In mice with the Mthfr −/− genotype, however, plasma folate consisted of more nonmethylated (60%) and less methylated (40%) folate. The percentage of 5-methylTHF in plasma of Mthfr +/− mice tended to be lower (82.1%) than that in Mthfr +/+ mice (93.6%; P = 0.06). Another important difference between the genotypes was the significant decrease, ~75% lower, in total plasma folate in mice with the Mthfr −/− genotype, compared with total plasma folate in mice with the other 2 Mthfr genotypes (Table 2).

In liver, the chromatogram peak for 5-methylTHF was barely discernible in mice with the Mthfr −/− genotype; it comprised only 0.5% of total folate, compared with 7.7–9.1% in extracts from mice with the +/+ and +/+ genotypes (Table 1). In spite of these differences in the relative proportion of 5-methylTHF, total folate in liver extracts did not differ among the 3 genotypes (Table 2).

As in plasma and liver, the peak in brain corresponding to 5-methylTHF was attenuated in mice with the Mthfr −/− genotype and was 13% of the total folate, compared with other genotypes in which the proportion of 5-methylTHF was 30–35% of the total (Table 1). Extracts from brains contained significantly higher percentages of 5-methylTHF compared with liver, irrespective of the genotype, and a lower percentage of THF and formylTHF (P < 0.05). As with the liver, however, MTHFR deficiency did not affect brain total folate (Table 2).

Mthfr +/+ mice had lower 5-methylTHF in plasma, compared with the Mthfr +/+ group; the decrease in 5-methylTHF levels also tended to occur in liver and brain of this genotype (P < 0.08).

Because of the presence of 5-methylTHF in extracts of mice that lack MTHFR, we conducted an HPLC analysis of the folate in their standard mouse food. This analysis revealed that

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**Table 1**

<p>| Table 1 Percentage of individual folate forms in total folate from plasma, liver and brain of mice stratified by Mthfr genotype |</p>
<table>
<thead>
<tr>
<th>+/+</th>
<th>−/+</th>
<th>−/−</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma [n]</td>
<td>[7]</td>
<td>[7]</td>
<td>[7]</td>
</tr>
<tr>
<td>THF</td>
<td>3.0 ± 1.5</td>
<td>6.7 ± 2.5</td>
<td>49.4 ± 7.8</td>
</tr>
<tr>
<td>5-methylTHF</td>
<td>93.6 ± 2.5</td>
<td>82.1 ± 4.8</td>
<td>39.7 ± 6.8</td>
</tr>
<tr>
<td>FormylTHF</td>
<td>1.5 ± 1.5</td>
<td>6.4 ± 4.3</td>
<td>10.9 ± 10.9</td>
</tr>
<tr>
<td>DHF</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FA</td>
<td>2.0 ± 2.0</td>
<td>4.8 ± 2.4</td>
<td>0</td>
</tr>
<tr>
<td>Liver [n]</td>
<td>[7]</td>
<td>[7]</td>
<td>[7]</td>
</tr>
<tr>
<td>THF</td>
<td>75.6 ± 4.9</td>
<td>72.9 ± 5.1</td>
<td>86.0 ± 5.4</td>
</tr>
<tr>
<td>5-methylTHF</td>
<td>9.1 ± 1.3</td>
<td>7.7 ± 1.7</td>
<td>6.5 ± 0.3</td>
</tr>
<tr>
<td>FormylTHF</td>
<td>10.5 ± 2.7</td>
<td>15.1 ± 3.8</td>
<td>10.1 ± 3.2</td>
</tr>
<tr>
<td>DHF</td>
<td>4.7 ± 2.1</td>
<td>4.4 ± 1.4</td>
<td>3.4 ± 2.3</td>
</tr>
<tr>
<td>FA</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Brain [n]</td>
<td>[7]</td>
<td>[7]</td>
<td>[7]</td>
</tr>
<tr>
<td>THF</td>
<td>54.2 ± 3.0</td>
<td>61.7 ± 3.0</td>
<td>56.4 ± 11.1</td>
</tr>
<tr>
<td>5-methylTHF</td>
<td>35.5 ± 3.9</td>
<td>29.5 ± 2.7</td>
<td>31.3 ± 9.9</td>
</tr>
<tr>
<td>FormylTHF</td>
<td>2.4 ± 1.8</td>
<td>1.8 ± 0.3</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>DHF</td>
<td>3.1 ± 2.0</td>
<td>1.1 ± 1.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>FA</td>
<td>4.8 ± 2.3</td>
<td>5.9 ± 1.7</td>
<td>10.8 ± 6.8</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM. Symbols indicate a difference from the Mthfr +/+ and +/+ genotypes, *P < 0.001, **P < 0.05.
the food contained 231 μg 5-methylTHF and 6.7 mg folic acid/kg diet (data not shown).

**DISCUSSION**

In the present study, we demonstrated that the absence of MTHFR activity is associated with altered distribution of folate, i.e., a decrease in methylated folates and an increase in unsubstituted THF or formylated THF. Nevertheless, tissue from mice with the Mthfr −/− genotype did contain traces of 5-methylTHF that amounted to 40% in plasma, 0.5% in liver, and 13% in brain, compared with mice with the +/+ genotype (94% in plasma, 9% in liver and 35% in brain). We found that the mouse food used in this study contained a substantial amount of 5-methylTHF. Therefore the 5-methylTHF detected in tissues of Mthfr −/− mice must be derived from the diet (13) because Mthfr −/− mice were already shown to have undetectable Mthfr mRNA and enzymatic activity (9). There was also a trend toward lower 5-methylTHF in Mthfr +/+ mice in the 3 tissues examined (P = 0.08 for the overall trend). It is likely that these mice were also obtaining 5-methylTHF from dietary sources and therefore not exhibiting a more significant decrease as might be expected for a mutant allele. As suggested for humans with mild MTHFR deficiency, the Mthfr +/+ mice may require low-folate diets to manifest a disruption in folate and homocysteine metabolism.

In our original report on Mthfr-deficient mice, we examined the percentage of 5-methylTHF in liver and brain of mice with the 3 genotypes. We observed a similar trend i.e., a decreased percentage of 5-methylTHF in the Mthfr −/− genotype, although the absolute values were different (9). In that report, the mice were newly generated animals; consequently, they were of a mixed genetic background. The mice examined in this report were of a uniform BALB/c background. Consequently, our measurements across the 3 genotypes in this study reflect changes due to Mthfr deficiency alone, rather than to other unidentified strain-related factors.

Another feature of mice with the Mthfr −/− genotype is the lower concentration of plasma total folate, 75% lower, than that in mice with the +/+ genotype. Usually 5-methylTHF is the predominant circulatory form. With the decrease in plasma 5-methylTHF, unsubstituted THF becomes the predominant form in Mthfr −/− mice. Under normal circumstances, THF may be less efficiently released from body stores into the circulation or it is degraded in plasma due to its susceptibility to oxidative cleavage. However, despite the fact that the concentration of plasma folate in Mthfr −/− mice was substantially lower than that in the +/+ genotype, peripheral tissues were able to maintain sufficient levels of total folate. This would suggest either that the transport of nonmethylated folate is enhanced to maintain intracellular stores or that the folate concentration in plasma of the wild-type mouse is excessive, beyond that required for saturable tissue transport.

We found a higher proportion of formylated-THF in the liver compared with the brain, independently of Mthfr genotype. In contrast, the proportion of 5-methylTHF in the brain relative to that in the liver was significantly higher in mice with all 3 Mthfr genotypes [ratio (mean ± SEM): +/+ 3.89 ± 3; +/−, 3.84 ± 1.5 vs. −/−, 26.6 ± 13; P < 0.05]. This may indicate a special ability of the brain to preserve a higher proportion of 5-methylTHF at the cost of the liver. Because MTHFR activity in mouse brain is similar to that in mouse liver (9), the higher percentage of 5-methylTHF in brain may be due to increased uptake, rather than synthesis. Patients with homocystinuria due to severe MTHFR deficiency have a wide variety of neurological features (8), consistent with an important role for 5-methyltetrahydrofolate or preformed methyl donors for methylation reactions in normal brain development.

Bagley and Selhub (14) reported higher formylated-THF in RBC of individuals homozygous for the 677C→T mutation, and implied impaired conversion to methylated folates in immature RBC. As mentioned above, the tissue/cell type may determine the requirement of certain folate forms for a particular pathway such as cellular methylation. Recent studies by Stover and co-workers reported enzymatic competition between thymidylate synthase and MTHFR for one-carbon units (15,16). Scott and Weir (17) suggested that a shift in folate distribution may create a competition among folate metabolic pathways, which may play a regulatory role between the DNA synthesis and methionine synthesis pathways. These metabolic variations may contribute to disturbances in methylation (18) or homocysteine elimination (8), with an effect on certain pathological conditions such as cancer and vascular diseases, and can provide additional insights into the role of MTHFR deficiency in these and other common disorders (19,20).

**ACKNOWLEDGMENTS**

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**LITERATURE CITED**


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**TABLE 2**

**Total folate in plasma, liver and brain of mice stratified by Mthfr genotype**

<table>
<thead>
<tr>
<th>Mthfr genotype</th>
<th>Plasma, nmol/L</th>
<th>Liver, pmol/mg protein</th>
<th>Brain, pmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>−/−</td>
<td>52.8 ± 17.2 [4]*</td>
<td>346.2 ± 76.4 [3]</td>
<td>4.5 ± 0.45 [4]</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM [n]. * Different from the Mthfr +/+ and +/− genotypes, P < 0.001.


