Homocysteine levels in A/J and C57BL/6J mice: genetic, diet, gender, and parental effects

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Homocysteine levels in A/J and C57BL/6J mice: genetic, diet, gender, and parental effects. Physiol Genomics 21: 404–410, 2005. First published March 1, 2005; doi:10.1152/physiolgenomics.00199.2004.—Increased levels of homocysteine in the blood have been associated with various birth defects and adult diseases. However, the extent to which genetic factors control homocysteine levels in healthy individuals is unclear. Laboratory mice are valuable models for dissecting the genetic and environmental controls of total homocysteine (tHcy) levels. We assessed the inheritance of tHcy levels in two inbred strains, A/J and C57BL/6J (B6), under controlled physiological conditions and assessed the relative importance of genetic, diet, gender, and parental effects. Diet affected mean tHcy levels, whereas gender affected both the mean and variance of tHcy levels. Moreover, gender of the parents influenced mean tHcy levels in reciprocal F1 hybrids, suggesting maternal effects. Finally, gene-diet interactions affected heritability of mean tHcy levels. These studies showed that each of these factors contributes to tHcy levels and provided important clues to understanding homocysteine homeostasis in humans.

HYPERHOMOCYSTEINEMIA, or elevated levels of total homocysteine (tHcy) in the blood, is an independent risk factor for neural tube defects (NTDs) (27a, 37), vascular disease (4, 43, 46), and Alzheimer’s disease (36) and is often associated with diseases such as colon cancer (20), osteoporosis (25, 41), and Down syndrome (3, 16). Although it is unclear whether elevated tHcy levels are a cause or consequence of disease, at least one meta-analysis suggests that hyperhomocysteinemia causes cardiovascular disease (43).

Normal tHcy blood levels range from 5 to 12 μmol/l (31), and hyperhomocysteinemia is classified into three categories: mild (12–30 μmol/l), moderate (30–100 μmol/l), and severe (>100 μmol/l) (17, 31). Several environmental and genetic factors affect tHcy levels in the blood. Environmental factors include age (tHcy increases with age), gender (women tend to have lower levels than men), and diet (intake of vitamin cofactors such as folate and vitamin B6 and B12 usually reduces tHcy levels) (26). Deficiencies in genes involved in homocysteine and folate metabolism result in hyperhomocysteinemia: these genes include cystathionine β-synthase (CBS; Refs. 2, 28), methionine synthase (MTR; Ref. 45), methionine synthase reductase (MTRR; Ref. 45), and methylenetetrahydrofolate reductase (MTHFR; Ref. 9, 11, 19, 21).

Homocysteine and folate pathways are tightly linked, and their regulation is complex. tHcy and folate levels are usually inversely related (34). Homocysteine, a sulfur-containing amino acid, is remethylated through the trans-methylation pathway or irreversibly catabolized through the trans-sulfuration pathway (29) and contributes to methylation of DNA, proteins, and lipids and indirectly to glucose metabolism and citric acid cycle. Folate metabolism manages the major pool of single carbon units and participates in purine and pyrimidine biosynthesis and amino acid metabolism (34).

Because the levels of homocysteine in the blood are a complex trait that is affected by several genetic and environmental factors, its genetic control in humans is difficult to dissect. Genetic factors contribute to mild, moderate (43), and severe (29) hyperhomocysteinemia, whereas their role in the control of normal blood levels of tHcy is unclear (1, 6, 32). The laboratory mouse is an important and useful model to study the genetic control of normal tHcy levels, independent of disease. We investigated homocysteine metabolism in two inbred strains of mice, A/J and C57BL/6J (B6), on two diets with different amounts of folate. These two inbred strains are analogous to two humans with distinct genotypes, in contrast to average effects in families and populations (15). A unique aspect of our study was the analysis of inheritance, not only of mean tHcy levels but also of variance itself. Gender and diet affected the mean and variance of homocysteine in different extents in A/J and B6 mice. In addition, gender of the parents affected tHcy mean and variance differently in reciprocal F1 hybrids, suggesting a possible role for maternal effects in homocysteinemia. Physiological factors affect heritability, with genetic contributions being greatest in males on the “higher folate” diet and lowest in both females and males on the “lower folate” diet. These studies revealed important clues about the inheritance of normal tHcy levels and provided the first evidence of genetic control of variance in tHcy levels.

EXPERIMENTAL PROCEDURES

Mice

A/J and C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained under specific pathogen-free conditions. All mice shared the same animal room with controlled temperature, humidity, and 12:12-h light-dark cycle. Mice were provided food and water ad libitum. The use of mice in these studies was approved by the Case Western Reserve University Institutional Animal Care and Use Committee.
Diets

Inbred strain survey. Female mice were maintained on the 5010 LabDiet (Richmond, IN) for 3 wk upon arrival from the Jackson Laboratory.

Inheritance of homocysteine levels in A/J and B6 strains. Mice were maintained either on the 5010 LabDiet (6.0 mg folic acid/kg chow, laboratory autoclavable rodent diet 5010; Richmond, IN) or 7013 Harlan Teklad (1.83 mg folic acid/kg chow; NIH-31 modified mouse/rat sterilizable diet; Madison, WI). Progeny were raised from conception on either diet. In many cases, mice used in these studies had been maintained on the same diet for several generations.

Mouse Breeding

Parental strains. Separate mating pairs of A/J and B6 mice were bred on each diet to produce progeny that were tested for gender, diet, and strain effects.

F1 progeny. Crosses between A/J females and B6 males produced ABF1 mice, and crosses between B6 females and A/J males produced reciprocal BAF1 mice.

F2 progeny. Crosses between ABF1 mice produced ABF2 mice, and crosses between BAF1 mice produced reciprocal BAF2 progeny.

Blood Samples

Blood samples were obtained from the retroorbital sinus of male and virgin female mice that were 6–8 wk old and were collected in nonheparinized tubes. After centrifugation, serum samples were stored at −80°C. Virgin females were used to control for possible effects of pregnancy on tHcy levels (18, 38).

Homocysteine Measurements

tHcy levels were measured by HPLC and tandem mass spectrometry (TMS). Both methods consist of reducing protein-homocysteine disulfide bonds before analysis.

HPLC. The HPLC method of Ubbink et al. (39) was used.

TMS. The TMS method of McCann et al. (24) was used.

TMS vs. HPLC: normalization of tHcy levels. Preliminary data showed a consistent bias in which samples measured by HPLC gave higher tHcy levels than those measured by TMS. To pool data from each technique, the following method was used: tHcy levels from 400 samples that were obtained with both HPLC and TMS and from 4 different strains were used. Of the 490 samples, 282 were measured by HPLC and the remainder by TMS. The mean tHcy levels were calculated for each group, and the correction factor was applied as follows:

$$
\mu_{\text{HPLC}} - \mu_{\text{TMS}} = \text{mean tHcy levels for all samples measured by HPLC} - \text{mean tHcy levels for all samples measured by TMS}
$$

$$
\mu_{\text{HPLC}}(5.849 \mu\text{mol/l}) - \mu_{\text{TMS}}(4.238 \mu\text{mol/l}) = 1.611 \mu\text{mol/l}
$$

tHcy levels differed by 1.611 μmol/l with a correlation factor (r) of 0.92 between the two methods after correction. Each sample that was measured with HPLC had its tHcy level reduced by 1.611 μmol/l, and then the results for the two methods were combined into a single data set for analysis.

Data Analysis

Statistical analysis. To assess differences in mean tHcy levels, Student’s t-test was used and significance levels were subjected to the appropriate Bonferroni correction factor to account for multiple testing. To assess differences in variance, Levene’s test was used (23). When equality of variance was not met, we normalized the data, but in most cases the inequality of variance persisted, raising the possibility that variance itself is a genetically controlled trait. Therefore, we analyzed two measures of tHcy levels: mean and variance. To test for differences in mean, the Student’s t-test was used. Levene’s test was used to test for differences in variance. In both cases, significance levels were subjected to the appropriate Bonferroni correction factor to account for multiple testing.

Heritability estimates. Heritability is the proportion of genetic variance that affects a trait and uses data from the two parental strains, F1 hybrids, and F2 population for its estimation (8, 48).

Environmental variance (VE) = (V_{A/J} + V_{B6} + V_{F1})/3

Total variance (VT) = V_{F2}

Genetic variance (VG) = V_{F1} - V_{F2}

Heritability = V_{G}/V_{T}

where V is variance, F1 is F1 population, and F2 is F2 population.

Estimating the number of independent segregating genes. Wright’s formula (48) was used to estimate the number of genes contributing to variation of mean tHcy levels

$$
N = (\mu_{F1} - \mu_{P})/8 \times V_{G}
$$

where $\mu_{F1}$ is mean tHcy level, $\mu_{P}$ is parental strains (A/J or B6), and $V_{G}$ is genetic variance.

This equation assumes that 1) the phenotype follows a normal distribution; 2) variance of the parents and F1 progeny is equal; and 3) all genes involved are unlinked, acting in an additive manner with an equivalent contribution to the phenotype (8, 48).

RESULTS

Inbred Strain Survey

Little is known about normal tHcy levels in mice. The levels of tHcy in serum were measured in thirteen different inbred strains. The survey showed that tHcy levels ranged from 3.2 to 7.0 μmol/l in females fed the LabDiet 5010 diet (Fig. 1). Two main groups of inbred strains were found. One group included EL/SuZ, NOD/LJ, DYY/Jel, SWR/J, and NOR/LJ females with an overall tHcy mean of $3.6 \pm 0.1 \mu\text{mol/l}$ (range 2.2–4.8 μmol/l), whereas the other group included CBA/CaJ, SWXL-4, BALB/cByJ, CBA/J, DBA/2J, C3H/HeJ, and ABP/Lc females with an overall tHcy mean of $6.2 \pm 0.1 \mu\text{mol/l}$ (range 4.6–8.4).

Inheritance of tHcy Levels in A/J and C57BL/6J Mice

The levels of tHcy in serum were measured in A/J and B6 inbred strains as well as in their reciprocal F1 hybrids and F2 progeny. Homocysteine levels were analyzed in males and females on two different diets, lower folate (Harlan Teklad 7013) and higher folate (Lab Diet 5010). For simplicity, folate content was used to distinguish the two diets, although they differed in the amounts of many other ingredients (Supplementary Table S1; available at the Physiological Genomics web site).

We analyzed patterns of variation in both mean and variance of tHcy levels. Subsequent studies should examine the effects of specific constituents in each diet.

Variation in Mean tHcy Levels

Gender effects. tHcy levels differed significantly between females and males, in both A/J and B6 strains, that were raised on either diet (Table 1). However, contrary to the general trend

1The Supplemental Material for this article (Supplemental Table S1) is available online at http://physiolgenomics.physiology.org/cgi/content/full/00199.2004/DC1.
in humans (30), females had significantly higher levels than males in both strains (Table 1).

Diet effects. Only in B6 female and male mice were tHcy levels significantly different between the two diets. tHcy levels were elevated on the lower vs. higher folate diets in both genders (Table 1).

Strain effects. tHcy levels were compared between AJ and B6 mice to study the effects of genetic background. AJ females and males both had significantly lower tHcy levels than B6 mice of the respective gender. These differences were found regardless of the diet on which the mice were raised (Figs. 2 and 3).

Parental effects. To test the effects of the gender of the parents, tHcy levels were compared between reciprocal F1 hybrid mice, i.e., ABF1 (where A refers to the AJ mother) vs. BAF1 (where B refers to the B6 mother). Gender of the parents did not affect tHcy levels in F1 females raised on either diet, but, tHcy levels in ABF1 females were lower than in BAF1 females (Figs. 2, A and B). However, tHcy levels differed in reciprocal hybrid males, i.e., tHcy levels in ABF1 < BAF1 (Figs. 2B and 3B).

Dominance effects. tHcy levels for the parental strains and F1 hybrids were compared to determine dominance of homocysteinemia; comparisons are presented according to gender and diet effects.

Females on the lower folate diet. Because ABF1 and BAF1 females had similar tHcy levels (see above), their data were pooled. F1 hybrids had significantly higher tHcy levels than AJ mice and significantly lower levels than B6 females, demonstrating that tHcy levels were inherited as a semidominant trait (Fig. 2A).

Males on the lower folate diet. Dominance of tHcy levels in hybrid males was parent dependent. ABF1 hybrid males had similar levels to AJ males and significantly lower levels than B6 males, demonstrating that the low tHcy trait, which was characteristic of the AJ parent, was inherited in a dominant manner. In contrast, BAF1 hybrid males had significantly higher and lower levels than AJ and B6 males, respectively, showing that tHcy levels in these mice were inherited in a semidominant manner (Fig. 2B).

Females on higher folate diet. Because ABF1 and BAF1 females had similar levels, data were pooled. Interestingly, F1 hybrid females had lower levels than both B6 and AJ males, indicating that the homocysteine trait was underdominant (Fig. 3A). In this context, “underdominant” means that the trait value in hybrids is less than the value in either parent strain.)

Males on higher folate diet. Dominance of tHcy levels in hybrid males was parent dependent. ABF1 hybrid males had lower levels than both B6 and AJ males, indicating that the homocysteine trait was underdominant. By contrast, BAF1 hybrids had similar levels to B6 males and significantly higher levels than AJ males, indicating that the high tHcy trait in BAF1 males was inherited in a dominant manner from the B6 parent (Fig. 3B).

Heritability. Heritability is the proportion of genetic variance that affects a trait and uses data from the two parental strains, F1 hybrids, and F2 population for its estimation. When parental gender influenced tHcy levels, which was the case for males on both diets (Figs. 2A and 3A), heritability was estimated separately. Genetic factors contributed significantly to the tHcy levels in males on the higher folate diet (0.58 for the ABF1-ABF2 population; 0.61 for the BAF1-BAF2 population) but more modestly in both females (0.40) and males (0.49 for the ABF1-ABF2 population; 0.10 for the BAF1-BAF2 population) raised on the lower folate diet. Heritability estimates are

Table 1. Gender and diet effects on mean tHcy levels

<table>
<thead>
<tr>
<th>Gender Effects (F vs. M)</th>
<th>Lower Folate Diet</th>
<th>Higher Folate Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>F (3.6 ± 0.2) &gt; M (2.5 ± 0.1)</td>
<td>F (3.9 ± 0.2) &gt; M (2.4 ± 0.1)</td>
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<tr>
<td></td>
<td>P = 5E-10 (44 F; 41 M)</td>
<td>P = 2E-21 (43 F; 45 M)</td>
</tr>
<tr>
<td>B6</td>
<td>F (5.9 ± 0.4) &gt; M (4.0 ± 0.2)</td>
<td>F (4.4 ± 0.3) &gt; M (2.8 ± 0.2)</td>
</tr>
<tr>
<td></td>
<td>P = 1E-14 (60 F; 55 M)</td>
<td>P = 7E-13 (46 F; 49 M)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diet Effects (L vs. H folate)</th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td>L (3.6 ± 0.2) &gt; H (3.9 ± 0.2)</td>
<td>L (2.5 ± 0.1) = H (2.4 ± 0.1)</td>
</tr>
<tr>
<td></td>
<td>NS (44 L; 43 H)</td>
<td>NS (41 L; 45 H)</td>
</tr>
<tr>
<td>B6</td>
<td>L (5.9 ± 0.4) &gt; H (4.4 ± 0.3)</td>
<td>L (4.0 ± 0.2) &gt; H (2.8 ± 0.2)</td>
</tr>
<tr>
<td></td>
<td>P = 1E-08 (60 L; 46 H)</td>
<td>P = 1E-12 (55 L; 49 H)</td>
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</tbody>
</table>

Total homocysteine (tHcy) levels are expressed as means ± 1.96 SE (μmol/l); values are indicated in parentheses. Diet effects: tHcy levels were compared between females (F) and males (M). Diet effects: tHcy levels were compared between mice raised on the lower folate diet (L) and those raised on the higher folate diet (H). NS, not significant.
not available for females that were raised on the higher folate diet because the F2 population was not studied.

Gene number. Wright’s (48) formula was used to estimate the number of genes that control the mean in tHcy levels. The results were sometimes less than one, which is problematic, both because the trait is genetic and because multiple genes are involved. Instead, it is likely that assumptions underlying the Wright’s formula were violated (8, 48): normality (this assumption was not violated), equality of variance in parents and F1 hybrids (this assumption was violated in females raised on the lower folate diet), and additivity among loci (this assumption was violated; unpublished results from a survey of chromosome substitution strains).

Variation in tHcy Variance

We then studied variance in tHcy levels as a measure of the ability of distinct genetic systems to modulate environmental

Fig. 2. tHcy levels (μmol/l) in parental strains, F1, and F2 mice on the lower folate diet. Top nos. represent sample size (n), and bottom nos. represent means ± 1.96 *SE. A: tHcy levels in females. B: tHcy levels in males. P values are indicated.

Fig. 3. tHcy levels (μmol/l) in parental strains, F1 mice, and F2 mice on the higher folate diet. Top nos. represent sample size (n), and bottom nos. represent means ± 1.96; *SE. A: tHcy levels in females. B: tHcy levels in males. P values are indicated.
and stochastic perturbations of homocysteine metabolism (12, 35).

**Gender effects.** tHcy variance differed significantly between females and males only in mice that were raised on the lower folate diet (A/J, P = 4E-04; B6, P = 2E-04). In general, females had significantly more variable tHcy levels than males (Fig. 2).

**Diet effects.** Diet had no effect on tHcy variance in either A/J or B6 mice (Figs. 2 and 3).

**Strain effects.** To study the effects of genetic background, tHcy variance was compared between A/J and B6 mice. When raised on the lower folate diet, A/J females had less variable tHcy levels than B6 females (Fig. 2A and Table 2). Genetic background did not affect tHcy variance in males raised on the lower folate diet (Fig. 2B) or in both females and males raised on the higher folate diet (Fig. 3).

**Parental effects.** To test the effects of parental gender on variance in tHcy levels in hybrid mice, reciprocal ABF1 vs. BAF1 mice were compared. In F1 females on the lower folate diet, parental effects affected variance, with tHcy levels being more variable in ABF1 than in BAF1 females (Fig. 2A and Table 2). Not surprisingly, gender of the parents did not affect tHcy variance in males raised on the lower folate diet or in females and males on the higher folate diet (Figs. 2B and 3).

**Dominance effects.** To measure dominance effects on variance, tHcy variances for the parental strains and F1 hybrids were compared. Dominance of tHcy variance was parent dependent for F1 females raised on the lower folate diet. In ABF1 females, variance was underdominant, because hybrids had significantly less variable levels than B6 and A/J females (Fig. 2A and Table 2). In BAF1 females, the low variance trait was inherited in a dominant manner from the A/J parent, with hybrids having similar variance to A/J females and significantly less variable levels than B6 females (Fig. 2A and Table 2).

### DISCUSSION

Both genetic and environmental factors control blood levels of homocysteine. However, little is known about the relative impact of these factors on normal tHcy levels among individuals who do not have hyperhomocysteinemia. Therefore, we studied the inheritance of homocysteinemia in mouse models to gain insight into the genetic and environmental controls of variation in normal tHcy levels. Using A/J and B6 inbred mouse strains, we conducted systematic studies to characterize influences, genetic, diet, gender, and parental effects, on patterns of tHcy variation. In our mouse models, these factors affected both the mean and variance in tHcy levels. These results provide crucial information that will guide the design of future studies in mice and humans.

### Variation in Mean tHcy Levels

Female mice tended to have higher tHcy levels than males. In humans, hormonal effects account for gender differences, where higher estrogen levels are often associated with decreased tHcy levels (13, 27, 49). In A/J and B6 mice, however, tHcy levels were usually higher in females than males (Table 1). This trend was also found in other inbred strains that were analyzed in our laboratory (unpublished data). Therefore, estrogen may have contrasting effects on mean tHcy levels in humans and mice, perhaps of sequence differences in estrogen-response elements in key genes.

Diet affected tHcy levels. In humans, dietary supplementation with folic acid usually lowers tHcy levels (15a, 40), but some individuals do not respond to vitamin supplementation (40). We also observed differences in responses to folate in our mouse models (Table 1). The higher folate diet reduced tHcy levels to a greater extent in female and male B6 mice, whereas it did not affect tHcy levels in A/J females and males (Table 1). The A/J mouse might correspond to humans whose tHcy levels are resistant to the beneficial effects of folate supplementation, whereas the B6 mouse might correspond to humans whose tHcy levels are folate responsive.

Parental effects influenced tHcy levels only in hybrid male progeny. In humans, the tHcy phenotype of the mother is often implicated in homocysteinemia of the fetus; mothers with elevated tHcy levels are more likely to give birth to a child who also has elevated tHcy levels (22). In humans, however, it is often difficult to establish effects of parental gender on the phenotype of the progeny in humans. Our mouse models provide valuable information about parental effects on normal tHcy. Parental effects strongly influenced tHcy levels in F1 males but not in females (Figs. 2 and 3), suggesting that genomic imprinting, as well as many other maternal effects, may account for normal tHcy in the F1 hybrid males. imprinting is known to be sensitive to folate level (44, 47), which is the source of methyl groups for DNA methylation (34), but direct tests of imprinting on homocysteine levels in humans and mice remain to be done. This type of gender bias is not unusual and is also observed in neural tube defect-affected pregnancies; among human anencephalies and certain mouse exencephalies, such as Cd, females are more frequently affected than males (5).

### Heritability of tHcy

Twin studies have been used to estimate heritability of homocysteine in populations of healthy humans, but results are conflicting. Two studies found that normal tHcy levels are heritable (~0.72; Refs. 1, 32), and another study suggested that genetic factors do not contribute significantly to variation in tHcy levels (6). In our studies, gene-diet interaction
strongly influenced heritability estimates; heritability was greater for mice that were raised on the higher folate than on the lower folate diet. Interestingly, mice raised on the lower folate diet showed a greater environmental variance than those raised on the higher folate diet, suggesting that metabolic pathways that are stressed with low levels of essential factors, such as folate, are more vulnerable to stochastic influences. Similar factors, such as the diet, might complicate estimation of heritability in humans.

**Variation in tHcy Variance**

Perhaps our most striking observation was the evidence for genetic control of variance of tHcy levels in genetically identical inbred mice, which were expected to have comparable variances. Females had more variable tHcy levels than males (Fig. 2A and Table 2), and hormonal changes associated with the estrus cycle may explain these differences. In humans, estrogen affects mean tHcy levels (13, 27, 49); however, it is unknown whether it can also affect tHcy variance.

tHcy levels were more variable in B6 than in A/J females raised on the lower folate diet (Fig. 2A and Table 2). Perhaps B6 females had difficulty maintaining stable tHcy levels on the lower folate diet, and, consequently, they may be at greater risk for homocysteine-related disease than A/J females on the lower folate diet. This type of variability is not unusual in humans, and genetic factors, which are generally silent, may increase certain disease risks under specific environmental conditions (35). The same situation could apply to homocysteine.

Gender of the parents affected variance of F1 females raised on the lower folate but not the higher folate diet. The low tHcy variance trait was inherited in a dominant manner from the A/J parent in both reciprocal F1 female progeny, suggesting that greater variance under conditions of “low folate” and high tHcy levels may expose some fetuses to risk for birth defects because of stochastic factors.

These studies provide new clues about the variability of tHcy levels in two commonly used inbred strains of mice. We showed that, under defined husbandry and physiological conditions, the mean and variance of normal tHcy levels were independently modulated by gender, diet, and parental effects in A/J and B6 mice. Studies of complex traits are often initiated with an inadequate characterization of the underlying genetics and biology of the system. The results reported in this manuscript document the complex patterns of inheritance that characterize the levels of homocysteine in an important mouse model and will serve as guides to the design and interpretation of experimental studies of the multigenic basis for tHcy variability (quantitative trait loci analysis) and profiling expression and metabolite levels in normal and mutant mice (Refs. 10, 33, 42, and unpublished data). In particular, because of the limited ability to conduct sufficient replicates to gain statistical confidence in profiling studies, rigorous control of study design is needed to control experimental noise and improve the signal-to-noise ratio among transcripts and metabolites. The results reported here provide guidelines for designing more rigorous profiling studies.

**REFERENCES**


