Choline deficiency in mice and humans is associated with increased plasma homocysteine concentration after a methionine load\textsuperscript{1–4}

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ABSTRACT

Background: Elevated concentrations of homocysteine in blood may be an independent risk factor for the development of atherosclerosis. Elevated homocysteine concentrations can be caused by decreased methylation of homocysteine to form methionine, as occurs in folate deficiency. A parallel pathway exists for methylation of homocysteine, in which choline, by way of betaine, is the methyl donor.

Objective: Our goal was to determine whether choline deficiency results in a decreased capacity to methylate homocysteine.

Design: C57BL/6J mice were fed diets containing 0, 10, or 35 mmol choline/kg diet for 3 wk. We then administered an oral methionine load to the animals and measured plasma homocysteine concentrations. Also, in a pilot study, we examined 8 men who were fed a diet providing 550 mg choline/d per 70 kg body weight for 10 d, followed by a diet providing almost no choline, until the subjects were clinically judged to be choline deficient or for \( \pm 42 \) d. A methionine load was administered at the end of each dietary phase.

Results: Two hours after the methionine load, choline-deficient mice had plasma homocysteine concentrations twice those of choline-fed mice. Four hours after the methionine load, clinically choline-depleted men had plasma homocysteine concentrations that were 35\% greater than those in men not choline depleted.

Conclusion: These results suggest that choline, like folate, plays an important role in the metabolism of homocysteine in humans and that response to a methionine load may be useful when assessing choline nutriture. \textit{Am J Clin Nutr} 2005;81:440–4.

KEY WORDS Choline deficiency, methionine load, homocysteine, mice, humans

INTRODUCTION

Epidemiologic studies have shown that an elevated total plasma homocysteine concentration is correlated with an increased risk of atherosclerotic vascular disease (1, 2). Homocysteine is metabolized by conversion to sulfur amino acids, and mutations in cystathionine \( \beta \)-synthase, the catabolic enzyme that requires vitamin B-6, result in hyperhomocysteinemia (3). In addition, homocysteine can be methylated to form methionine (3) by 2 parallel pathways, both of which lower homocysteine concentrations. In the first pathway, vitamin B-12 and folic acid are involved in a reaction catalyzed by methionine synthase (4). Deficiency of these nutrients (5, 6), or single nucleotide polymorphisms in the genes for the enzymes involved in this pathway (4, 6, 7), can result in elevated plasma homocysteine concentrations. The alternative pathway for the methylation of homocysteine to form methionine is catalyzed by betaine homocysteine methyltransferase (8), an enzyme whose activity was reported to increase in rats during methionine excess (9). Betaine, derived from dietary choline, is the methyl group donor in this reaction, and supplemental oral betaine can lower plasma homocysteine concentrations (10, 11). Choline is an essential nutrient (12), and humans can become depleted of it (13, 14). In this study we examined whether dietary choline deficiency was associated with a decreased capacity to methylate homocysteine and whether it results in elevated plasma homocysteine concentrations.

MATERIALS AND METHODS

Materials

\( L \)-Homocysteine, cysteamine, reduced glutathionine, and SBD-F (7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid, ammonium salt) were purchased from Sigma Chemical Co (St Louis). \( L \)-Cysteine came from Aldrich (Milwaukee), and TCEP-HCl [tris (2-carboxyethyl) phosphine hydrochloride] was obtained from Pierce (Rockford, IL). The other reagents were obtained from Fisher Scientific (Fair Lawn, NJ).

Animals and diets

All animal procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. Male C57BL/6J mice (Jackson Labs, Bar Harbor, ME), 25 g body weight, were housed individually in tubs in a climate-controlled room (24 °C) exposed to light from 0600 to 1800 daily. They

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were given free access to water and pelleted diets (Dyets, Bethlehem, PA) for 3 wk. Three AIN-93G diets were used, containing 35 mmol choline/kg diet (choline supplemented, CS); 10 mmol choline/kg diet (control), or 0 mmol choline/kg diet (choline deficient, CD). These AIN-93G diets contained 5.2 g methionine/kg, 2 g folate/kg, 7 mg vitamin B-6/kg, and 0.025 mg vitamin B-12/kg, which meets the rodent requirement for these nutrients (15).

For the study in mice, on the morning of the last day of the diet period, half of the mice in each group (n = 7/group in CD; n = 5/group in control and CS mice) were anesthetized (200 mg ketamine/kg and 16 mg xylazine/kg), and blood was collected by cardiac puncture. Heparin was added to the sample, and the plasma was separated by centrifugation (1000 × g for 10 min at 4 °C). The livers from the exsanguinated mice were then collected by freeze-clamping them with tongs precooled in liquid nitrogen, and the animals died. All the samples were then stored at −80 °C until further analysis. Methionine was administered to the remaining animals by oral gavage at a dose of 100 mg/kg body weight. Two hours later, these mice were anesthetized and exsanguinated by cardiac puncture, plasma and livers were collected and stored as stated above, and the mice died. Homocysteine was measured 2 h after the methionine load in the mice because previous work in rats indicated a maximal response by that time (16).

**Human study**

Eight healthy male volunteers aged 20–46 y (5 whites and 3 African Americans) with body mass indexes (in kg/m²) ranging from 20 to 33, were recruited for a protocol approved by the Institutional Review Board at the University of North Carolina at Chapel Hill. Subjects were admitted to the General Clinical Research Center and administered various research diets. A description of these diets has been published elsewhere (17, 18). Specifically, subjects were fed for 10 d a baseline diet of normal foods containing 550 mg choline/d per 70 kg body weight, which is approximately the current presumed adequate intake (12); the choline content of the diet was assayed by our laboratory (19). On the morning of day 10, a blood sample was collected from the subjects. Then the subjects were given l-methionine (100 mg/kg body weight) orally, and 4 h later blood was collected for the measurement of homocysteine concentrations. Subjects were then switched to a choline depletion diet containing <50 mg choline/d per 70 kg body weight for ≤42 d or until they were judged to be choline deficient [developed hepatic steatosis, as measured by magnetic resonance imaging (MRI)]. On the morning after subjects were determined to be choline deficient, or after 42 d on the experimental diet, a blood sample was collected from subjects. Then they were given l-methionine (100 mg/kg body weight) orally, and 4 h later blood was collected for the measurement of homocysteine concentrations. After these measurements, subjects not deemed to be clinically choline deficient by MRI were fed the 550-mg choline diet for 3 d and then discharged. Subjects deemed to be clinically choline deficient were fed diets containing graded amounts of choline (sequentially in 10-d periods; containing 138, 275, 413, or 550 mg choline/d per 70 kg body weight) until hepatic steatosis resolved by MRI and they were judged not to be clinically choline deficient. These subjects, on the last day of the repletion diet, were given l-methionine orally (100 mg/kg body weight), and 4 h later blood was collected and homocysteine concentrations were measured.

The diets were composed of 0.8 g high-biologic-value protein/kg body weight, with 30% kcal coming from fat and the remaining kcal from carbohydrate. The diets met or exceeded the estimated average requirement for methionine plus cysteine and the daily reference intake for vitamin B-6, vitamin B-12, and folate (400 dietary folate equivalents/d).

**Analytic methods**

We used an HPLC method to measure total plasma homocysteine concentrations (20). We used a Dynamax HPLC system (model SD-200; Rainin Instruments, Woburn, MA) with a Microsorb-MV C18 column (5 μmol, 100 A, 25 cm; Varian Inc, Walnut Creek, CA) and fluorescence spectrophotometric detector (Varian Prostar model 360) at EX 385 nm, EM 515 nm wavelength. Cysteamine was the internal standard used to correct for recovery. Blood samples from the human study also were analyzed by an independent laboratory at the University of Colorado Health Sciences Center (Denver) with use of gas chromatography–mass spectrometry methods to confirm plasma homocysteine concentrations (21).

**Choline and metabolites, folate, and triacylglycerols**

Choline and its metabolites were extracted from liver tissue and human plasma by the method of Bligh and Dyer (22). Aqueous and organic compounds were separated, analyzed, and quantified directly by HPLC-mass spectrometry (liquid chromatography and electrospray ionization–isotope dilution mass spectrometry) after the addition of internal standards labeled with stable isotopes that were used to correct for recovery (23). Plasma folate concentrations were measured with the use of a microbiological assay (24). Lipids were extracted from mouse liver tissue by the method of Bligh and Dyer (22), dried, and resuspended in 100 μL isopropanol, 1% Triton X-100 for ≥1 h. Triacylglycerols were quantified against a standard with use of the Stanbio enzymatic colorimetric triacylglycerol assay (Boerne, TX).

**Statistical analysis**

In the mouse study, significant differences between fasting and methionine load homocysteine concentrations in plasma were determined by Student’s t test. Data were analyzed by two-factor analysis of variance (ANOVA) with interaction to determine whether differences in methionine-load status varied by diet. Differences among the 6 groups defined by combinations of methionine-load status and diet were determined by using 1-factor ANOVA and the Tukey-Kramer critical difference test. In the human study, for each metabolite, a two-sample t test based on the differences between the metabolite concentrations for the 2 diets was used to compare the clinically depleted and the not depleted groups. Significant differences between the diets were determined by paired t test, and 95% CIs were calculated for changes in homocysteine concentration by depletion status. Statistics were performed with the use of JMP software, version 3.2 (SAS Institute Inc, Cary, NC).

**RESULTS**

The mean (±SE) homocysteine concentration in mouse plasma at the beginning of our study was 4.4 ± 0.6 μmol/L (n = 4), which was similar to published values (25). After 3 wk of the...
various diets, no significant difference was observed in plasma homocysteine concentrations between the CS (4.4 ± 0.4 μmol/L; n = 5), control (4.8 ± 0.2 μmol/L; n = 5), and CD (4.2 ± 0.2 μmol/L; n = 7) groups (Figure 1, hatched bars). There was, however, a significant interaction between methionine-load status and diet (P < 0.001). In choline-fed mice (control + CS groups), an oral dose of methionine increased plasma homocysteine concentrations at least 2-fold 2 h after the dose compared with mice not given a methionine load (4.6 ± 0.7 compared with 11.4 ± 3.9 μmol/L; P < 0.01, Student’s t test; Figure 1). After a methionine load, CD mice had plasma homocysteine concentrations 5 times (25.0 ± 3.7 μmol/L; P < 0.01, Student’s t test; Figure 1, solid bars) those of CD mice not given a methionine load and twice those in the CS (12.6 ± 2.3 μmol/L) and control (10.2 ± 1.1 μmol/L; P < 0.01, ANOVA; Figure 1 solid bars) mice given a methionine load.

After 3 wk on the experimental diets, livers from the CD mice had 68% less phosphocholine (Table 1; P < 0.001, Student’s t test), 38% less choline (P < 0.05), and 14% less phosphatidylcholine (P < 0.01) than livers from the mice fed 10 mmol choline/kg diet. Concentrations of other metabolites of choline such as betaine, glycerophosphocholine, and sphingomyelin did not change with choline deficiency (Table 1), nor did hepatic triacylglycerols (Table 1; P = 0.142, Student’s t test). Plasma folate concentrations were within the normal range and also did not differ between the groups (Table 1; P = 0.867, Student’s t test). We previously reported that choline and triacylglycerol data separated, and homocysteine concentrations were measured by HPLC before and 2 h after (solid bars) the methionine load; *Significantly different from fasting (no methionine load), P < 0.01 (Student’s t test). *Significantly different from all other groups, P < 0.01 (ANOVA and Tukey-Kramer test).

<table>
<thead>
<tr>
<th>Substance</th>
<th>Control mice</th>
<th>Choline-deficient mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Betaine (nmol/mg protein)</td>
<td>1.3 ± 0.3</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Choline (nmol/mg protein)</td>
<td>2.5 ± 0.4</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Glycerophosphocholine (nmol/mg protein)</td>
<td>2.1 ± 0.3</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Phosphocholine (nmol/mg protein)</td>
<td>1.5 ± 0.1</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>Phosphatidylcholine (nmol/mg protein)</td>
<td>123.9 ± 2.5</td>
<td>107.0 ± 5.2</td>
</tr>
<tr>
<td>Sphingomyelin (nmol/mg protein)</td>
<td>10.8 ± 0.6</td>
<td>10.1 ± 0.8</td>
</tr>
<tr>
<td>Triacylglycerols (nmol/mg protein)</td>
<td>277.2 ± 28.2</td>
<td>330.0 ± 13.5</td>
</tr>
<tr>
<td>Folate (nmol/mL plasma)</td>
<td>20.8 ± 2.2</td>
<td>20.1 ± 4.0</td>
</tr>
</tbody>
</table>

All values are ± SE; n = 6/group unless noted otherwise. Mice were fed a control (10 mmol choline/kg diet) or choline-deficient (0 mmol choline/kg diet) diet for 3 wk. Livers were collected, extracted, and analyzed by liquid chromatography and electrospray ionization–isotope dilution mass spectrometry for choline and its metabolites and by a colorimetric assay for triacylglycerols. Plasma folate was measured with a microbiological assay.

Significantly different from control mice, P < 0.05 (Student’s t test).

n = 4.

n = 7.

Develops in choline-deficient humans because choline moiety is needed to secrete VLDL from liver (13, 27); we selected this as a clinically important marker for functional changes associated with choline deficiency. During the choline-depletion phase, hepatic steatosis was measured by MRI at 21 and 42 d. Four of the subjects on the 50 mg choline depletion diet were judged to be clinically choline depleted (2 at day 21, 2 at day 42) because they developed hepatic steatosis, and 4 subjects were not judged to be clinically depleted by the end of the study (day 42). At the end of the depletion diet phase, fasting plasma homocysteine concentrations (Figure 2) increased by 1.3 μmol/L (95% CI: 0.8, 1.8; P = 0.004) in the subjects judged to be clinically choline depleted.

![Image of plasma homocysteine concentrations in mice fed AIN-93G diets containing 0 (choline deficient, CD), 10 (control, C), or 35 (choline supplemented, CS) mmol choline/kg diet for 3 wk followed by a methionine load (100 mg/kg body weight). Blood was collected, plasma was separated, and homocysteine concentrations were measured by HPLC before (cross-hatched bars) and 2 h after (solid bars) the methionine load; n = 5–7/group. There was a significant (P < 0.001) methionine-load status × diet interaction. *Significantly different from fasting (no methionine load), P < 0.01 (Student’s t test). *Significantly different from all other groups, P < 0.01 (ANOVA and Tukey-Kramer test).

![Image of liver concentrations of choline and its metabolites in control and choline-deficient mice.](image-url)
and increased by 0.8 \mu mol/L (95% CI: −1.3, 2.9; \( P = 0.309 \)) in the subjects not deemed to be clinically depleted. After a methionine load (at the end of the depletion diet phase), in the clinically choline-depleted group, plasma homocysteine concentrations rose 5.2 \mu mol/L (95% CI: 1.2, 9.1; \( P = 0.025 \); Figure 2) above that which was previously observed after a methionine load with the 550-mg choline diet. After a methionine load (at the end of the depletion diet phase), in the group that was not judged to be clinically choline depleted, plasma homocysteine concentrations did not increase above the amounts previously observed when subjects were on the 550-mg diet and received a methionine load (−0.3 \mu mol/L; 95% CI: −3.5, 2.87; \( P = 0.761 \)). By two-sample \( t \) test, there was a significant interaction between methionine load and clinical status (\( P = 0.014 \)). After choline-deficient subjects consumed the repletion diets and were found to no longer have hepatic steatosis, plasma homocysteine concentrations returned to baseline values (6.5 ± 0.4 \mu mol/L), and response to a methionine load was not different from that seen on the 550-mg diet (increasing to homocysteine of 15.2 ± 1.5 \mu mol/L after the load).

In all 8 human subjects, plasma choline and betaine concentrations fell 30% and 47%, respectively, at the end of the depletion phase (Table 2; \( P < 0.005 \), paired \( t \) test). Subjects who were judged to be clinically depleted had decreases in plasma choline and betaine concentrations that were not different from those observed in subjects not deemed to be clinically depleted (Table 2). Phosphatidylcholine concentrations in plasma did not change significantly with the diet (Table 2; \( P = 0.304 \), paired \( t \) test), nor did plasma folate concentrations (Table 2; \( P = 0.233 \), paired \( t \) test).

### DISCUSSION

The present study shows that, among its other functions, choline may be important for lowering plasma homocysteine concentrations even when dietary consumption of folate and other B vitamins is adequate. In our mouse study, for a better comparison with the human study, we used AIN 93G diets for the animals [instead of the more restrictive diets we used in the past that limited folate and methionine as well as choline (28)] and this depleted liver choline compounds but did not cause hepatic steatosis in mice. With this mild choline depletion, the plasma homocysteine concentrations in all the groups at 3 wk on experimental diets were not significantly different from each other. This latter observation was noted by others (29). This modest choline depletion could not be detected by only measuring plasma homocysteine concentrations in the absence of a methionine load. This lack of detection was probably because, when homocysteine flux is low, the folate-dependent methylation pathways are sufficient for homocysteine removal. We used a methionine load test to increase homocysteine synthesis and consequently to test the capacity of the homocysteine catabolic system under stress. Mice given a methionine load had almost 3-fold higher plasma homocysteine concentrations than mice not given a methionine load. The increase in homocysteine concentration after load was significantly higher in the CD mice than the other groups. Thus, when homocysteine flux was high, folate-dependent methylation was limiting and choline-betaine dependent methylation became critical.

In the human study, the diets similarly contained the recommended daily amounts of folate and B vitamins. Half of the subjects fed a choline-deficient diet eventually became clinically symptomatic of choline depletion (developing hepatic steatosis). The fasting plasma homocysteine concentrations in these depleted humans were increased more than in individuals who were judged not to be clinically depleted. This difference was even greater after a methionine load. Thus, in both mice and humans we showed that, when homocysteine flux was high, folate-dependent methylation was limiting and choline-betaine dependent methylation became critical.

We do not understand why half of the human subjects did not develop hepatic steatosis while on a choline-deficient diet. We hypothesized that their endogenous synthesis of choline by way of phosphatidylethanolamine N-methyltransferase (30) may have been greater than that in the subjects who did develop steatosis. It is important to note that measurement of plasma choline or betaine concentrations was not sufficient to predict which subjects would develop organ dysfunction, because all depleted subjects had low plasma choline and betaine concentrations. We were able to identify subjects who were sufficiently depleted of choline so as to develop hepatic steatosis by using a methionine-loading test. Also, this test would yield yet another important indicator, betaine after a methionine load, which was found in a recent report (9) to have a strong inverse association with the increase in homocysteine after methionine load. We suggest that clinicians use this test as a complement to plasma choline concentrations to identify patients who are clinically...
choline depleted. For example, the methionine-load test would be useful for evaluating patients fed total parenteral nutrition who become choline depleted and can develop steatosis (14, 31–33). If it is known that the patient has normal plasma or erythrocyte folate concentrations, a plasma choline determination and a methionine-loading test could predict which patients are likely to be at risk of organ dysfunction associated with choline deficiency. We suggest that it would be this group that would be most responsive to treatment with a supplemental source of choline (14).

Our study showed that when homocysteine flux is high, the folate-dependent pathway for methylation of homocysteine is limiting and the choline-betaine–dependent pathway becomes folate-dependent pathway for methylation of homocysteine is most responsive to treatment with a supplemental source of folate concentrations, a plasma choline determination and a methionine-load test is useful for detecting choline deficiency in humans.

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KAD-C participated in the design of the studies, planned and executed the animal experiments, supervised blood collection and processing, participated in the statistical analyses and interpretation, and was responsible for writing the manuscript. CEG validated the homocysteine assay, analyzed the mouse samples, and provided significant input in the writing of the manuscript. LMF participated in the design and supervision of the human study and the recruitment of patients. SHZ was responsible for the design of the studies, planned and executed the animal experiments, supervised blood collection and processing, participated in the statistical analyses.

REFERENCES