Hyperhomocysteinemia Induced by Guanidinoacetic Acid Is Effectively Suppressed by Choline and Betaine in Rats

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Rats were fed 25% casein (25C) diets differing in choline levels (0–0.5%) with and without 0.5% guanidinoacetic acid (GAA) or 0.75% L-methionine for 7 d to determine the effects of dietary choline level on experimental hyperhomocysteinemia. The effects of dietary choline (0.30%) and betaine (0.34%) on GAA- and methionine-induced hyperhomocysteinemia were also compared. Dietary choline suppressed hyperhomocysteinemia induced by GAA, but not by methionine, in a dose-dependent manner. GAA-induced enhancement of the plasma homocysteine concentration was suppressed by choline and betaine to the same degree, but the effects of these compounds were relatively small on methionine-induced hyperhomocysteinemia. Dietary supplementation with choline and betaine significantly increased the hepatic betaine concentration in rats fed a GAA diet, but not in rats fed a methionine diet. These results indicate that choline and betaine are effective at relatively low levels in reducing plasma homocysteine, especially under the condition of betaine deficiency without a loading of homocysteine precursor.

Key words: homocysteine; choline; betaine; guanidinoacetic acid; methionine

Homocysteine is an intermediate metabolite in the metabolism of methionine (Fig. 1), but it is recognized that an elevated plasma homocysteine concentration is an independent risk factor for cardiovascular disease.1–3) The plasma homocysteine concentration is known to be influenced by various factors, e.g., genetic, physiological, lifestyle, nutritional, and clinical factors. For instance, deficiencies of vitamins such as folate, vitamin B8, and vitamin B12 result in hyperhomocysteinemia, since these vitamins are involved in the metabolism of homocysteine. It is thought that plasma homocysteine derives mainly from the liver, since it is the central organ of methionine metabolism.4) In the liver, the homocysteine concentration is affected by the rates of the following processes: (i) the production of homocysteine from S-adenosylhomocysteine (SAH) or its precursor S-adenosylmethionine (SAM), (ii) remethylation of homocysteine to methionine using the methyl-group of either 5-methyltetrahydrofolate or betaine, (iii) cystathionine formation from homocysteine and serine, and (iv) the export of homocysteine into the blood plasma. The plasma homocysteine concentration can be manipulated by modulating any of the above processes.

An effective means of lowering the plasma homocysteine concentration is to increase homocysteine remethylation. Hence, many attempts have been made to lower the plasma homocysteine concentration by administering folate or betaine.5–7) Since choline is the precursor of betaine, it would be interesting to determine whether choline has a significant effect on the plasma

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Fig. 1. Methionine and Homocysteine Metabolism.

C1, one-carbon unit; DMG, N,N-dimethylglycine; GAA, guanidinoacetic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; THF, tetrahydrofolate. Enzymes: (1) guanidinoacetic acid N-methyltransferase [EC 2.1.1.2], (2) phosphatidylethanolamine N-methyltransferase [EC 2.1.1.17], (3) cystathionine β-synthase [EC 4.2.1.22], (4) methionine synthase [EC 2.1.1.13], (5) betaine-homocysteine S-methyltransferase [EC 2.1.1.5].

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16 homocysteine concentration. With regard to this, recent reports have indicated that choline and phosphatidylcholine (PC) are effective in decreasing the plasma homocysteine concentration in humans.8–10 Choline might elicit its effect through the supply of the remethylation substrate betaine, enhancement of betaine-homocysteine S-methyltransferase (BHMT) activity, or both, but the mechanism underlying the effect of choline has not yet been fully elucidated.

Various hyperhomocysteinemic animal models have been reported in investigating the regulation of the plasma homocysteine concentration. These fall into several groups: (i) hyperhomocysteinemia induced by loading of a homocysteine precursor such as methionine11,12 or homocystine,13 (ii) hyperhomocysteinemia induced by loading of a methyl-group acceptor such as GAA14,15 or the precursor of a methyl-group acceptor such as nicotinic acid,16 and (iii) hyperhomocysteinemia induced by feeding diets deficient in certain vitamins that participate in the metabolism of homocysteine, such as folate, vitamin B12, and vitamin B6.12,17 It appears that the GAA-induced hyperhomocysteinemia model has physiological relevance, since creatine synthesis from GAA is estimated to consume a major part (about 75%) of the methyl-group of SAM in humans.18,19

In this study, we investigated the effects of dietary choline level on hyperhomocysteinemia induced by GAA or methionine in rats. The effects of choline and betaine were also compared using two hyperhomocysteinemic rat models.

Materials and Methods

Choline bitartrate, betaine, and guanidinoacetic acid were purchased from Sigma-Aldrich (St. Louis, MO). L-Methionine and other chemicals were from Wako Pure Chemical (Osaka, Japan). Mineral and vitamin mixtures (AIN-93G) were from Oriental Yeast (Tokyo). Male six-week-old rats of the Wistar strain were obtained from Japan SLC (Hamamatsu, Japan). They were individually housed in hanging stainless-steel wire cages in an isolated room kept at controlled temperature (24–26°C) and humidity (40–60%). Lighting was maintained on a 12-h cycle (lights on from 07:00 to 19:00 h). Before starting the experiments, all rats were acclimatized to the facility for 5 d and were given free access to water and a 25% casein (25C) diet.

In this study, two experiments were conducted. In experiment 1-A, rats were fed 25C + 0.5% GAA (25CG) diets differing in choline levels (0, 0.1, 0.25, and 0.5%). In experiment 1-B, rats were fed 25C + 0.75% L-methionine (25CM) diets differing in choline levels (0, 0.1, 0.25, and 0.5%). As a normal group, rats were fed the 25C diet containing choline at a level of 0.1%. In experiment 2-A, the rats were fed a 25CG without choline diet (control), a 25CG with 0.3% choline diet, or a 25CG with 0.34% betaine diet. In experiment 2-B, rats were fed a 25CM without choline diet (control), a 25CM with 0.3% choline diet, or a 25CM with 0.34% betaine diet. As a normal group, rats were fed the 25C diet containing choline at a level of 0.3%. The composition of the 25C diet containing choline at a level of 0.1% was as follows (g/100 g): casein, 25; α-corn starch, 43.25; sucrose, 20; corn oil, 5; AIN-93G mineral mixture, 3.5; AIN-93G vitamin mixture, 1; choline bitartrate, 0.25; cellulose, 2. The addition of GAA or methionine and alteration of the choline level were adjusted by changing the starch content. The rats were fed the experimental diets for 7 d, and then killed by decapitation between 10:00 and 11:00 h without prior starvation. The experimental plan was approved by the Laboratory Animal Care Committee of the Faculty of Agriculture at Shizuoka University.

Blood plasma was separated from heparinized whole blood by centrifugation at 2,000 × g for 15 min at 4°C, and was stored at −30°C until needed for analysis. After collection of the blood, the whole liver was quickly removed, rinsed in ice-cold saline, blotted on filter paper, cut into several portions, weighed, quickly frozen in liquid nitrogen, and stored at −80°C until needed for analysis. One portion of the liver was homogenized in 4 volumes (vol/wt) of ice-cold 5% perchloric acid, and then centrifuged at 10,000 × g for 10 min at 4°C. The supernatant of the deproteinized liver homogenate was subjected to assays for methionine metabolites. For determination of betaine, the liver was likewise homogenized in 5% trichloroacetic acid solution. The other portion of the liver was homogenized in 4 volumes (vol/wt) of a 10 mM sodium phosphate buffer (pH 7.4) containing 150 mM KCl, and the resulting homogenate was centrifuged at 14,000 × g for 10 min at 4°C. The supernatant was subjected to enzyme assays.

The concentrations of total homocysteine and cysteine in the plasma were measured by HPLC by the method of Durand et al.20 The concentrations of SAM and SAH in the liver were measured by HPLC, essentially according to Cook et al.21 The concentration of betaine in the liver was measured by HPLC according to Laryea et al.22 The activity of cystathionine β-synthase (E.C. 4.2.1.22) (CBS) in the liver was measured according to Mudd et al.,23 but HPLC was used in the assay of the reaction product, cystathionine, according to Einarsson et al.24 The activity of betaine-homocysteine S-methyltransferase (EC 2.1.1.5) (BHMT) in the liver was measured according to Finkelstein et al.,25 but HPLC was used in the assay of the reaction product, N,N-dimethylglycine, according to Laryea et al.22 The activities of CBS and BHMT were expressed as nmol/min/mg of protein. The protein concentration was measured according to Lowry et al.,26 using bovine serum albumin as the standard.

Each data value is expressed as the mean ± SEM. Data were analyzed by a one-way analysis of variance, and differences among the experimental groups were analyzed by the Tukey test when the F value was
significant. Statistical analysis was performed with Mac Tokei-Kaiseki ver. 1.5 software (Esumi, Tokyo).

### Results

**Effect of dietary choline level (experiment 1)**

Body weight gain, food intake, and relative liver weight were not affected by supplementation with GAA or methionine or by dietary choline level (Table 1), except that the body weight gain was slightly lower in rats fed the 25CM diet containing 0.5% choline than in rats fed the other diets. Dietary GAA-induced increases in the plasma homocysteine concentration were suppressed by dietary choline in a dose-dependent manner; there was no significant difference in plasma homocysteine concentration between the normal group and the GAA group of rats fed diets containing choline at the 0.25% or 0.5% level (Fig. 2A). Dietary GAA-induced increases in hepatic SAH and homocysteine concentrations were suppressed by dietary choline in a dose-dependent manner (Fig. 2C). In contrast, dietary methionine-induced increases in the plasma homocysteine concentration were not suppressed by raising the dietary choline level, at least up to 0.5% (Fig. 3A). Likewise, dietary methionine-induced increases in hepatic SAH and homocysteine concentrations were not suppressed by dietary choline (Fig. 3C, D), whereas the hepatic SAM concentration was further increased by raising the dietary choline level (Fig. 3B).

**Comparison of the effects of choline and betaine (experiment 2)**

Since the effect of dietary choline was different between the GAA-induced hyperhomocysteinemia and methionine-induced hyperhomocysteinemia models in experiment 1, the effect of choline was re-examined and compared with the effect of betaine using the two models. In this experiment, the effects of dietary levels of 0.3% choline and 0.34% betaine (equivalent to 0.3% choline on a molar basis) were compared. The body weight gain was slightly higher in rats fed a 25CG diet containing 0% choline and a diet containing 0% choline plus 0.34% betaine than in rats fed the normal diet (experiment 2-A, Table 1). In experiment 2-B, there were no differences in body weight gain, food intake,
or relative liver weight among the four groups. Supplementation of the 25CG diet with choline and with betaine markedly deceased the plasma homocysteine concentration to the same degree, the degrees of decrease due to choline and betaine being 67.4% and 72.5% respectively (Fig. 4A). The plasma cysteine concentration was slightly lower in the rats fed the 25CG diet containing betaine than in the rats fed diets containing choline (Fig. 4B). Supplementation with choline and with betaine increased the hepatic SAM concentration and the SAM/SAH ratio to the same degree, whereas the hepatic SAH concentration was decreased due to choline and betaine (Fig. 4C–E). Supplementation with choline and with betaine increased or tended to increase hepatic CBS and BHMT activities (Fig. 4F, G). The hepatic betaine concentration was significantly increased by choline and betaine, although the effect of betaine was significantly stronger than the effect of choline (Fig. 4H).

Fig. 3. Effects of Dietary Choline Level on the Plasma Homocysteine Concentration (A) and Hepatic Concentrations of S-Adenosylmethionine (B), S-Adenosylhomocysteine (C), and Homocysteine (D) in Rats Fed a 25% Casein + 0.75% Methionine Diet (Experiment 1-B).

Each value is the mean ± SEM for six rats. Values with different letters are significantly different at p < 0.05. 25C, 25% casein; Hcy, homocysteine; Met, methionine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

Fig. 4. Comparison of the Effects of Dietary Choline (0.3%) and Betaine (0.34%) on the Plasma Homocysteine Concentration and Other Variables in Rats Fed a 25% Casein + 0.5% Guanidinoacetic Acid Diet (Experiment 2-A).

Each value is the mean ± SEM for six rats. Values with different letters are significantly different at p < 0.05. 25C, 25% casein; 25CG, 25C + 0.5% guanidinoacetic acid; Bet, betaine; BHMT, betaine-homocysteine S-methyltransferase; CBS, cystathionine β-synthase; Cho, choline; Cys, cysteine; Hcy, homocysteine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.
of the 25CM diet with choline and with betaine significantly decreased the plasma homocysteine concentration, but the degrees of decrease due to choline and betaine were only 31.1% and 18.9% respectively (Fig. 5A). The plasma cysteine concentration was significantly lower in the rats fed methionine-supplemented diets than in those fed the normal diet (Fig. 5B). Supplementation with betaine, but not choline, significantly increased the hepatic SAM concentration and the SAM/SAH ratio (Fig. 5C, E), whereas the hepatic SAH concentration was unaffected by choline or betaine (Fig. 5D). Supplementation with choline and with betaine did not affect hepatic CBS activity (Fig. 5F), whereas hepatic BHMT activity was significantly increased by choline and betaine (Fig. 5G). The hepatic betaine concentration was not increased by choline or betaine (Fig. 5H).

**Discussion**

Several studies have suggested that dietary choline level might be a determinant of plasma homocysteine concentration.8–10,27) It is usually assumed that dietary choline affects homocysteine metabolism by stimulating homocysteine remethylation after being converted to betaine, but other mechanisms have also been considered. For instance, restoration of the hepatic SAM concentration can be associated with the effect of choline or betaine under some physiological conditions, since the SAM concentration affects the activity of CBS in an allosteric manner.28) In fact, a decreased SAM concentration was one of the features of hyperhomocysteinemia induced by folate deficiency17) and by GAA.15) It is also uncertain whether a change in BHMT activity is essential to the hypohomocysteinemic effects of choline and betaine, although a study by Finkelstein et al.29) showed that dietary supplementation with choline or betaine at a level of 0.2% increased hepatic BHMT activity in rats fed low-methionine diets without choline. The present study was conducted to clarify these points using experimental hyperhomocysteinemic rats. We used GAA- and methionine-induced hyperhomocysteinemia models to assess the effect of dietary choline and betaine. The results demonstrate that dietary supplementation with choline and with betaine was markedly effective in decreasing the plasma homocysteine concentration in GAA-induced hyperhomocysteinemic rats, whereas choline and betaine were apparently less effective in methionine-induced hyperhomocysteinemic rats, at least under the experimental conditions. The results also demonstrate that the plasma homocysteine-normalizing effect of choline was nearly comparable to the effect of betaine.

Stead et al.14) first demonstrated that dietary supplementation with 0.34% GAA increased the plasma homocysteine concentration in rats, and they postulated that GAA supplementation increases the plasma homocysteine concentration by accelerating the conversion of

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**Fig. 5.** Comparison of the Effects of Dietary Choline (0.3%) and Betaine (0.34%) on the Plasma Homocysteine Concentration and Other Variables in Rats Fed a 25% Casein + 0.75% Methionine Diet (Experiment 2-B). Each value is the mean ± SEM for six rats. Values with different letters are significantly different at p < 0.05. 25C, 25% casein; 25CM, 25C + 0.75% methionine; Bet, betaine; BHMT, betaine-homocysteine S-methyltransferase; CBS, cystathionine β-synthase; Cho, choline; Cys, cysteine; Hcy, homocysteine; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.
SAM to SAH and further to homocysteine. Consistently with this, we found that GAA supplementation led to a decrease in the SAM concentration and to increases in the SAH and homocysteine concentrations in a dose-dependent manner in the liver of rats.\textsuperscript{15} The finding that choline and betaine were quite effective in reducing plasma homocysteine in a GAA-induced hyperhomocysteinemia model suggests that GAA supplementation might cause betaine deficiency. In support of this, GAA supplementation significantly decreased the hepatic betaine concentration in the rats fed a diet containing 0.3\% choline (Fig. 4H). One possible explanation for the decrease in the hepatic betaine concentration due to GAA loading is that GAA enhanced the rate of the methionine cycle (the homocysteine remethylation pathway), and thereby increased the consumption of betaine. Although 5-methyltetrahydrofolate can donate its methyl-group to homocysteine, this pathway might not be as functional as the betaine pathway, for folate did not suppress GAA-induced hyperhomocysteinemia when added to the diet at up to 40 mg/kg (unpublished data).

It is thought that choline status within the body is determined by both the dietary intake of choline and the \textit{de novo} synthesis of PC via the phosphatidylethanolamine (PE) N-methylation pathway. It has been confirmed that many transmethylation reactions using SAM as a methyl-group donor are dependent on the SAM concentration and hence the dietary methionine level rather than the enzyme mass of PE N-methyltransferase.\textsuperscript{30,31} Furthermore, different methyltransferases compete for SAM.\textsuperscript{32} One of the characteristic features of GAA loading is a decrease in the hepatic SAM concentration, as confirmed in the present study. Hence, it is reasonable to assume that GAA supplementation suppresses PC synthesis \textit{via} PE N-methylation through dual mechanisms: (i) a decrease in the hepatic SAM concentration, and (ii) competition between PE N-methyltransferase and GAA N-methyltransferase for SAM. PC or choline deficiency leads to betaine deficiency. Hence, we postulate that betaine deficiency also contributes to GAA-induced hyperhomocysteinemia, in addition to the acceleration of homocysteine formation. This might be a basis for the effectiveness of choline and betaine in GAA-induced hyperhomocysteinemia. Our results also suggest that choline and betaine exert their effects by increasing both the hepatic betaine concentration and BHMT activity, since supplementation with 0.3\% choline and with 0.34\% betaine significantly increased the hepatic betaine concentration and tended to increase BHMT activity (Fig. 4G, H).

Furthermore, an increase in hepatic CBS activity might also contribute to the plasma homocysteine-normalizing effects of choline and betaine (Fig. 4F). Previous studies have shown that betaine is effective in suppressing methionine-induced hyperhomocysteinemia in humans\textsuperscript{33,34} and rats.\textsuperscript{35,36} In rats, Yagisawa et al.\textsuperscript{35,36} found that hyperhomocysteinemia induced by intravenous injection with methionine was suppressed by concurrent injection with betaine or dietary supplementation with betaine. In contrast, we did not detect any strong effect of betaine or choline in a methionine-induced hyperhomocysteinemia model in the present study. Possible explanations of the discrepancy in the effects of dietary betaine between the results of Yagisawa et al.\textsuperscript{36} and ours are (i) the difference in the manner of methionine loading, one-shot methionine injection \textit{vs.} successive methionine feeding, and (ii) the difference in dietary betaine level, 5\% \textit{vs.} 0.34\%. It has been found that raising the dietary methionine level decreases the hepatic betaine concentration, and this phenomenon has been explained in terms of augmented betaine consumption following increased homocysteine production due to the supply of the homocysteine precursor methionine.\textsuperscript{37} In the present study, supplementation of a 25\% + 0.75\% methionine diet with 0.3\% choline and with 0.34\% betaine did not increase the hepatic betaine concentration, suggesting that the 0.75\% methionine-induced increase in betaine consumption might not be compensated by choline or betaine at these supplementation levels. Furthermore, in contrast to GAA loading, methionine loading increased the pool size of all of the metabolites of methionine in the liver, as shown in Fig. 3. Under this condition, homocysteine remethylation might not be an effective means of removing homocysteine. Indeed, Finkelstein et al.\textsuperscript{37,38} have found that homocysteine is metabolized mainly \textit{via} cystathionine formation rather than \textit{via} remethylation in rats fed high-methionine diets. Consistently with this, we have found that methionine-induced hyperhomocysteinemia can be effectively suppressed by glycine and serine in rats, probably through enhancement of cystathionine formation.\textsuperscript{15} In contrast, these amino acids were ineffective in GAA-induced hyperhomocysteinemia (unpublished data), confirming that remethylation is the limiting step of homocysteine metabolism in GAA-induced hyperhomocysteinemia. In any case, the results of the present study indicate that choline and betaine are less effective in reducing plasma homocysteine in a methionine (homocysteine precursor) loading-induced hyperhomocysteinemia model than in other types of hyperhomocysteinemia, such as GAA-induced hyperhomocysteinemia when choline and betaine are added to the diet at nutritional or physiological levels.

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