Effects of Chronic Betaine Ingestion on Methionine-Loading Induced Plasma Homocysteine Elevation in Rats

Masako YAGISAWA1, Norihiro SHIGEMATSU3 and Rieko NAKATA2*

1Central Research Laboratory, Fancl Co., Totsuka-ku, Yokohama 244–0806, Japan
2Department of Food Science and Nutrition, Nara Women’s University, Kitauoya-Nishimachi, Nara 630–8506, Japan

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Summary The effects of chronic betaine ingestion were investigated in rats. Rats were fed an experimental diet containing 5% betaine for 4 wk and methionine was intravenously administered. The elevations of plasma homocysteine were assessed by comparing the increments to the initial measured value and the positive incremental area under the plasma homocysteine concentration curve over the 240-min post-methionine-loading period (ΔAUC0–240). In the betaine-ingesting rats, ΔAUC0–240 was significantly lower than in the control group (48% of the control), and the increments of plasma homocysteine were also lower compared with the control, especially 15–30 min after methionine loading. Choline, a precursor of betaine, did not alter the plasma homocysteine elevation. In a definite period immediately after methionine loading, carnitine, a methyl-group-rich amino acid, induced a significant increase of plasma homocysteine, compared to the control. Moreover, plasma homocysteine concentration was significantly decreased by 4 wk of betaine ingestion. Betaine enhanced liver BHMT activity whereas choline and carnitine did not show any effects on BHMT activity. These results suggest that betaine contributes to both the decrease in the plasma homocysteine concentration and the suppression of plasma homocysteine elevation through the activation of liver BHMT.

Key Words betaine, chronic ingestion, homocysteine, methionine loading, rats

Betaine (trimethylglycine) is widely found in most living organisms and is a significant component of many foods (1, 2). In the living body, it is an endogenous catabolite of choline. It has three methyl groups in its structure and serves as a methyl donor in the metabolic reaction to remethylate homocysteine into methionine, catalyzed by the enzyme betaine-homocysteine methyltransferase (BHMT; EC 2.1.1.5) (3). Homocysteine, a sulfur-containing amino acid, is a metabolite of methionine which can be either remethylated to methionine or metabolized to cysteine. Folic acid and cyanocobalamin, and pyridoxine regulate the remethylation pathway and the conversion, respectively (4).

Congenital defects in homocysteine metabolism and several lifestyle-related factors cause elevated plasma homocysteine concentrations (4–7). Elevated plasma homocysteine concentrations (hyperhomocysteinemia) have been demonstrated as an independent risk factor for arterial sclerosis and cardiovascular diseases, with no clear-cut threshold (8–12). Consequently, many studies have been conducted on how to reduce elevated homocysteine levels. Nutritional approaches to homocysteine are extremely important, since no medical product is known to act as a homocysteine regulator. Previous studies have shown that folic acid, cyanocobalamin, and pyridoxine lower the plasma homocysteine level (13, 14). In addition to these nutrients, we have paid close attention to betaine as a homocysteine regulator. As betaine plays a role in homocysteine remethylation independently of folic acid, it is strongly believed to promote homocysteine metabolism. Indeed, betaine supplementation reduces plasma homocysteine concentrations (3, 15, 16).

As mentioned above, attempts to reduce plasma homocysteine levels have achieved some progress; however, ways to avoid plasma homocysteine elevation have received little attention. It is conceivable that the prevention of elevated homocysteine levels is a crucial factor in lowering the risks of arterial sclerosis and cardiovascular diseases, while the reduction of elevated homocysteine levels is also effective. We have previously reported that a methionine loading-induced plasma homocysteine elevation was suppressed by intravenously administered betaine (17). This previous result indicates a close relationship between plasma betaine and plasma homocysteine, and the possibility that orally ingested betaine suppresses plasma homocysteine elevation when it is absorbed into the blood.

We therefore tested the effect of chronic betaine ingestion on the post-methionine-loading elevation of plasma homocysteine in rats. We also tested the effects of choline, a precursor of betaine, and carnitine, similar in its methyl group-containing structure to betaine, and...
compared the effects to those of betaine. In addition, the activities of liver BHMT, which catalyzes the homocysteine metabolism with betaine, were investigated simultaneously.

**MATERIALS AND METHODS**

*Chemicals.* All chemicals were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) unless otherwise noted.

*Animals and experimental diets.* All experimental protocols were approved by the Animal Care Committee of Nara Women’s University, according to the guidelines from the Prime Minister’s Office in Japan (No. 6 of March 27, 1980).

Weaning, male Wistar rats weighing 130 to 150 g (Japan SLC Ltd., Hamamatsu, Shizuoka, Japan) were used throughout this study. They were housed individually in stainless steel cages (W150×L210×H170 mm) at 22±2°C with a 12-h light/dark cycle (light: 6:00 to 18:00). Rats were acclimated for 3 d, during which time they were fed a commercial laboratory diet (certified Diet MF, Oriental Yeast Co. Ltd., Tokyo, Japan). The body weights and food intakes of the animals were recorded daily.

*Methionine loading and sample preparation.* Methionine loading was performed using a slight modification of the method described by Yagisawa et al. (17).

After 4-wk feeding and 18-h fasting periods, rats were intravenously injected with 50 mg/kg of L-methionine. L-Methionine was dissolved in saline (Otsuka Pharmaceutical Co., Tokyo, Japan). Approximately 250 μL of blood was taken from the jugular vein under diethyl ether anesthesia immediately before and 5, 15, 30, 60, 120 and 240 min after methionine loading. Blood samples were promptly collected into polypropylene tubes containing heparin-Na and centrifuged at 5,000×g for 10 min. The supernatants were separated as plasma samples and kept at −80°C until analysis. The liver tissue was weighed, minced with scissors and homogenized in 5 volumes of phosphate buffered saline with a Teflon homogenizer. The homogenates were stored at −80°C until the assay for liver BHMT activity (liver homogenate).

*Analytical methods.* The plasma homocysteine concentration was determined according to previous methods (17).

The plasma homocysteine concentration increments (ΔHcy) were calculated according to the following equation: ΔHcy = post-loading concentration – pre-loading concentration.

The area under the ΔHcy time curve from 0 to 240 min (ΔAUC_0–240) was calculated using the linear trapezoidal rule (20, 21). Individual preloading plasma homocysteine concentrations were used as the baseline for the calculation of ΔAUC_0–240.

**Assay for liver BHMT activity.** Liver BHMT activity was measured as follows. The volume of the reaction mixture was 500 μL, comprising 50 μL of the liver homogenate, 50 mM K-phosphate buffer (pH 7.3), 5 mM dl-homocysteine, and 10 mM betaine (reagent grade, Wako Pure Chemical Industries, Ltd.). The reaction mixture was incubated at 37°C for 30 min and chilled on ice, and the reaction was terminated by the addition of 1 mL of 0.15 N HCl. After centrifugation at 12,000×g for 10 min, the supernatant was filtered using a 0.45-μm filter (Chromato Disc, Kurabo Industries Ltd., Osaka, Japan). The amount of synthesized methionine in the supernatant was measured using an amino acid analyzer (L8500, Hitachi Ltd., Tokyo, Japan). Total protein in the liver homogenate was determined using the procedure of Lowry et al. (22), using bovine serum albumin as a standard. A unit of BHMT activity is defined as the amount of synthesized methionine per milligram protein per minute.

**Statistical analysis.** The statistical significance among means was estimated at p<0.05 according to standard ANOVA and Bonferroni/Dunn’s multiple-range test using Stat View J. 5.0 for Windows (SAS Institute Inc., NC, USA).

**RESULTS**

*Body weight and daily food consumption.* Rats were fed an experimental diet as shown in Table 1. Compared with the control group, neither the betaine nor carnitine group showed any difference in the accumulated weight gain or the total food intake for 4 wk (Table 2). The accumulated weight gain of the choline group was significantly lower than that of the control group (Table 2); however, the rats in the choline group appeared to be healthy. Since the physical states of the choline group were not adversely affected, these rats were used for subsequent studies.

*Plasma homocysteine concentration immediately before methionine loading.* In the betaine group, the plasma homocysteine concentration immediately before methionine loading was significantly lower compared with the control group (48% of the control) (Fig. 1). No significant differences in plasma homocysteine were observed among the control, choline and carnitine groups.

*Elevation of plasma homocysteine after methionine loading.* The plasma homocysteine concentration increments after methionine loading and the positive incremental area under the plasma homocysteine concentration curve over the 240-min post-methionine-loading period (ΔAUC_0–240) are shown in Table 3 and Table 4.
In the betaine group, the time course of plasma homocysteine incrementation was low compared with that of the control group. The increments were significantly lower than those of the control group at 15 and 30 min after methionine loading. In the choline group, although the peak time of plasma homocysteine elevation was shifted to 15 min later than that in the control group, there was no significant difference in plasma homocysteine concentration incrementation at any time point. In the carnitine group, the plasma homocysteine concentration increments were significantly 1.3–1.4-fold higher at 15–30 min compared with the control group. However, the time course of plasma homocysteine incrementation of the carnitine group was similar to that of the control group after 60 min. Only in the betaine group was AUC\(_{0-240}\) significantly lower compared with the control group (83% of the control). No significant differences in AUC\(_{0-240}\) were observed between the control and choline groups, or the control and carnitine groups.

Table 1. Composition of the experimental diets.

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control (n=14)</th>
<th>Betaine (n=10)</th>
<th>Choline (n=8)</th>
<th>Carnitine (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino acid mixture(^1)</td>
<td>178</td>
<td>178</td>
<td>178</td>
<td>178</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Corn oil</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Dextrin</td>
<td>404</td>
<td>404</td>
<td>404</td>
<td>404</td>
</tr>
<tr>
<td>Sucrose</td>
<td>211</td>
<td>161</td>
<td>161</td>
<td>161</td>
</tr>
<tr>
<td>Vitamin mixture(^2)</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mineral mixture(^2)</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Choline chloride</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Succinylsulfathiazole</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Betaine</td>
<td>—</td>
<td>50</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Choline hydrogen tartrate</td>
<td>—</td>
<td>—</td>
<td>50</td>
<td>—</td>
</tr>
<tr>
<td>Carnitine tartrate</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>50</td>
</tr>
</tbody>
</table>

\(^1\) Amino acid mixture provided the following amounts per kilogram diet: DL-alanine 7.12 g, L-arginine 11.4 g, L-asparagine 6.10 g, L-cystine 3.56 g, L-glutamic acid 35.6 g, L-glycine 23.7 g, L-histidine 3.36 g, L-isoleucine 8.34 g, L-leucine 11.3 g, L-lysine 14.6 g, L-methionine 8.34 g, L-phenylalanine 11.8 g, L-proline 3.56 g, L-serine 3.56 g, L-threonine 8.34 g, L-tryptophan 1.77 g, L-tyrosine 3.56 g, L-valine 8.34 g.

\(^2\) The vitamin mixture and the mineral mixture based on AIN-76 were used (19).

Table 2. Growth of rats fed the experimental diets for 4 wk.

<table>
<thead>
<tr>
<th>Growth parameter</th>
<th>Control (n=14)</th>
<th>Betaine (n=10)</th>
<th>Choline (n=8)</th>
<th>Carnitine (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial body weight (g)</td>
<td>152.3±5.92</td>
<td>150.3±7.70</td>
<td>154.6±6.85</td>
<td>150.5±7.13</td>
</tr>
<tr>
<td>Accumulated weight gain (g)</td>
<td>143.7±12.1</td>
<td>138.6±6.94</td>
<td>70.3±14.4*</td>
<td>119.4±6.98</td>
</tr>
<tr>
<td>Total food intake (g)</td>
<td>422.0±54.3</td>
<td>415.2±37.5</td>
<td>264.8±22.8</td>
<td>335.5±12.9</td>
</tr>
<tr>
<td>Feed efficiency (g gain/g feed)</td>
<td>34.6±5.30</td>
<td>33.6±2.87</td>
<td>26.4±3.32*</td>
<td>33.8±0.75</td>
</tr>
</tbody>
</table>

Values are means±SD. *Significantly different from control, Bonferroni/Dunn’s multiple range test (p<0.05).

Fig. 1. Plasma homocysteine concentration immediately before methionine loading. Values are means±SD, n=14 for control, n=10 for betaine, n=8 for choline and n=5 for carnitine. *Significantly different from control (Bonferroni/Dunn’s multiple range test) (p<0.05).
Effects of Betaine on Plasma Homocysteine

Table 3. Effects of betaine, choline and carnitine on the increments of plasma homocysteine.

<table>
<thead>
<tr>
<th>Time after methionine loading (min)</th>
<th>Control (n=14)</th>
<th>Betaine (n=10)</th>
<th>Choline (n=8)</th>
<th>Carnitine (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1.49±0.43</td>
<td>1.50±0.41</td>
<td>1.27±0.83</td>
<td>2.04±1.11</td>
</tr>
<tr>
<td>15</td>
<td>2.04±0.87</td>
<td>1.65±0.38’</td>
<td>1.75±0.44</td>
<td>2.74±0.53’</td>
</tr>
<tr>
<td>30</td>
<td>1.87±0.48</td>
<td>1.50±0.23’</td>
<td>2.04±0.67</td>
<td>2.65±0.36’</td>
</tr>
<tr>
<td>60</td>
<td>0.99±0.52</td>
<td>0.79±0.19</td>
<td>1.22±0.50</td>
<td>1.30±0.18</td>
</tr>
<tr>
<td>120</td>
<td>0.02±0.06</td>
<td>0.07±0.13</td>
<td>0.51±0.29</td>
<td>0.12±0.21</td>
</tr>
<tr>
<td>240</td>
<td>0.02±0.07</td>
<td>0.01±0.05</td>
<td>0.14±0.24</td>
<td>0.01±0.02</td>
</tr>
</tbody>
</table>

Methionine was intravenously administered to rats at a dose of 50 mg/kg. Concentrations of plasma homocysteine were measured at various time points and post-methionine-loading increments of plasma homocysteine were calculated. Values are means±SD. *Significantly different from control at the particular time point (Bonferroni/Dunn’s multiple range test) (p<0.05).

Table 4. Comparison of ΔAUC0–240 and liver BHMT activity.

<table>
<thead>
<tr>
<th>Group</th>
<th>ΔAUC0–240 (μmol·min/L)</th>
<th>% Control</th>
<th>BHMT (nmol methionine synthesized/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=14)</td>
<td>130.0±30.3</td>
<td>—</td>
<td>0.91±0.10</td>
</tr>
<tr>
<td>Betaine (n=10)</td>
<td>108.2±20.2*</td>
<td>83.2</td>
<td>1.08±0.11*</td>
</tr>
<tr>
<td>Choline (n=8)</td>
<td>138.2±4.68</td>
<td>106.3</td>
<td>1.02±0.23</td>
</tr>
<tr>
<td>Carnitine (n=5)</td>
<td>179.5±7.73*</td>
<td>138.0</td>
<td>0.84±0.19</td>
</tr>
</tbody>
</table>

Values are means±SD. *Significantly different from control (Bonferroni/Dunn’s multiple range test) (p<0.05).

Liver BHMT activity

The liver tissue was removed 240 min after methionine loading and the BHMT activities were measured (Table 4). Compared with the control group, the betaine group showed a significantly higher activity (1.2-fold of the control), whereas there were no significant differences between the control, choline and carnitine groups.

These results showed that betaine contributes to the suppression of plasma homocysteine elevation accompanied by activation of BHMT, and the effect of betaine was far superior to its precursor, choline and its analogue, carnitine.

DISCUSSION

Homocysteine is an independent risk factor for atherosclerosis and cardiovascular diseases, and the relationship between homocysteine and Alzheimer dementia, cancer and fracture have been recognized (23–26). Regulation of homocysteine is critically important for health maintenance since various diseases are related to homocysteine.

In the present study, we investigated the effect of chronic betaine ingestion on methionine-loading-induced plasma homocysteine elevation in rats, following both the time course of plasma homocysteine incrementation and ΔAUC0–240. We have formerly demonstrated the suppression of plasma homocysteine elevation by intravenous betaine (17). As Schwahn et al. reported that orally ingested betaine could be detected in the plasma (27); chronic betaine intake was expected to prevent the elevation of plasma homocysteine.

Our result that the chronic intake of betaine suppressed plasma homocysteine elevation substantiated our hypothesis. Furthermore, betaine-ingesting rats showed significantly lower pre-methionine-loading plasma homocysteine concentrations than control animals. This result corresponds to the data of Schwab et al. (13) and Steenge et al. (16). BHMT, which is predominantly located in the liver, catalyzes the remethylation of homocysteine (28), and chronic betaine intake enhanced liver BHMT activity in this study. These findings indicate that chronic betaine ingestion increases BHMT activity and enhances the remethylation of homocysteine, resulting in the suppression of plasma homocysteine elevation, and are in accord with the report of Kuhn et al. that betaine ingestion increased the level of BHMT in rats (29). It is not yet known whether betaine worked as a methyl donor for BHMT or activated BHMT activity. In this study, liver BHMT activities were measured 4 h after methionine loading in which the momentarily elevated plasma homocysteine reached in the same level as the pre-methionine-loading level. Drastically enhanced BHMT activity is observed if an investigation is performed when there is a significant difference in plasma homocysteine elevations between the betaine and control groups (15–30 min after methionine loading). Park and Garrow
reported that a combination of methionine and betaine induced BHMT mRNA expression (30). It is of interest how betaine ingestion itself effects BHMT gene expression.

We expected that choline might work as a methyl donor, analogue of betaine or precursor of betaine in the remethylation pathway of homocysteine. In choline-ingesting rats, the average amount of ΔAUC$_{0-240}$ was lower than the control. However, the difference in ΔAUC$_{0-240}$ between the choline-ingesting and control rats was not significant, whereas betaine ingestion led to a significant decrease in ΔAUC$_{0-240}$ compared to the control. Considering the above results and the constrained body weight gain in choline-ingested rats, choline is not a desirable homocysteine regulator.

Since three methyl groups are contained in its structure, betaine is presumed to work as a methyl donor. We selected carnitine for its methyl-group-rich component; however, it did not show any effects on either plasma homocysteine elevation or liver BHMT activity. Although carnitine seemed to enhance plasma homocysteine elevation induced by methionine loading, the enhancement was extremely temporary and further studies will be needed to clarify the effects of carnitine on homocysteine metabolism. This means that both BHMT and methyltetrahydrofolate-homocysteine methyltransferase (EC 2.1.1.13) catalyze the remethylation of homocysteine (4), and do not recognize carnitine as their methyl donor.

Effects of betaine on methionine-loading-induced plasma homocysteine elevation in humans were reported by Steenge et al. (16) and Holm et al. (31). In this study, we compared the effect of betaine to those of choline and carnitine. Moreover, we investigated liver BHMT activity along with the plasma homocysteine level in rats. Control of the homocysteine level using nutrients is really important since we have not yet found any medical treatment to reduce homocysteine level. Folic acid and cyanocobalamin, and pyridoxine are effective for controlling plasma homocysteine. A combination of betaine and these nutrients will be more effective for the avoidance of high-level plasma homocysteine concentrations and homocysteine-related diseases.

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REFERENCES


