Factors Contributing to the Resistivity of a Higher Casein Diet against Choline Deficiency-Induced Hyperhomocysteinemia in Rats

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(Received August 3, 2011)

Summary The mechanism by which feeding a higher casein diet results in resistance to choline deprivation-induced hyperhomocysteinemia was investigated in rats. Plasma homocysteine concentration was significantly lower in rats fed a 30% casein diet (30C) than in rats fed a 10% casein diet (10C). Choline deprivation did not enhance plasma homocysteine concentration in rats fed 30C, while it significantly enhanced plasma homocysteine concentration in rats fed 10C. The choline deprivation-induced enhancement of plasma homocysteine concentration in rats fed 10C was significantly suppressed by methionine supplementation in a dose-dependent manner in the range of 0.1 to 0.3%, but the suppressive effect of methionine became smaller with an increase in supplementation level in the range of 0.3 to 0.5%. At a 0.5% supplementation level, methionine did not exhibit any suppressive effect on choline deprivation-induced hyperhomocysteinemia. The higher plasma homocysteine concentration in rats fed choline-deprived 10C+0.5% methionine was significantly decreased by concurrent supplementation with 0.32% glycine+0.94% serine to the level of rats fed 10C. Raising dietary total amino acid level by adding 3.61% branched-chain amino acids (BCAA)+4.5% acidic amino acids (AAA) to choline-deprived 10C+0.5% methionine+0.32% glycine+0.94% serine resulted in a further decrease in plasma homocysteine concentration to a level lower than the level in rats fed 10C. Choline deprivation-induced increases in hepatic S-adenosylhomocysteine and homocysteine concentrations were significantly suppressed by supplementation with glycine+serine and further suppressed by BCAA+AAA. Hepatic cystathionine β-synthase activity and its gene expression were significantly increased by BCAA+AAA. Hepatic triglyceride concentration changed in a manner similar to that of plasma homocysteine concentration. The results indicate that there are at least three factors contributing to the resistivity of rats fed a higher casein diet (30C) to choline deprivation-induced hyperhomocysteinemia, i.e., higher intake of methionine, higher intake of glycine and serine, and higher intake of other amino acids such as BCAA and AAA.

Key Words choline deficiency, plasma homocysteine, methionine, glycine and serine, dietary amino acid level

Homocysteine is a usual intermediate metabolite in the metabolism of methionine (Fig. 1), but an elevated plasma homocysteine concentration is an independent risk factor for cardiovascular disease (1–4). Plasma homocysteine concentration is influenced by various factors such as genetic, physiological, lifestyle, nutritional and clinical factors. Of these factors, nutritional and genetic factors are thought to have a greater influence on the plasma homocysteine concentration (5). For instance, deficiencies of folate, vitamin B-12, and vitamin B-6 cause hyperhomocysteinemia, since folate and vitamin B-12 participate in the metabolism of homocysteine by methionine synthase and vitamin B-6 participates in the metabolism of homocysteine by cystathionine β-synthase (CBS). In addition to these vitamins, deficiency of choline also elevates plasma homocysteine concentration. Varela-Moreiras et al. (6) first demonstrated that choline deprivation in the diet, which contained methionine at a level of 0.2%, increased serum homocysteine concentration. We also showed that choline deprivation gave rise to hyperhomocysteinemia in rats fed a 10% casein diet (10C) or 25% soybean protein diet, whereas it did not elevate plasma homocysteine concentration in rats fed a 25% casein diet (7). It is apparent that choline-deprivation-induced hyperhomocysteinemia is associated with cho-
that there might exist some factors other than methionine (unpublished data). These results suggest the possibility of completely suppressing the hyperhomocysteinemia induced by hyperhomocysteinemia, whereas supplementation with choline-deprived 10C with methionine at a level of 0.35% of methionine might have stimulated the BHMT reaction. Previously, we have shown that decreases in hepatic phosphatidylcholine (PC) and choline were observed in this model of rats (8). PC is synthesized by two pathways, the CDP-choline pathway and phosphatidylethanolamine (PE) N-methylation pathway (9, 10). Dietary methionine level or methionine intake affects hepatic S-adenosylmethionine (SAM) concentration and thereby influences PC synthesis via the PE N-methylation pathway (8–10), indicating that choline status within the body is influenced not only by choline intake but also by methionine intake. The methionine contents of 10C and a 25% soybean protein diet are lower than that of a 25% casein diet. The elevation of plasma homocysteine in rats fed a choline-deprived 25% soybean protein diet was effectively suppressed by supplementation with a small amount (0.35%) of methionine (7). These results suggest that choline deprivation leads to hyperhomocysteinemia only when dietary methionine levels are relatively low and that methionine in dietary proteins acts as an anti-hyperhomocysteinemic amino acid under the condition of restricted choline intake. Since choline is the sole precursor of betaine, hepatic betaine concentration reflects choline status within the body. Hence, one of the mechanisms by which choline deficiency induces hyperhomocysteinemia is thought to be a decrease in homocysteine remethylation by betaine-homocysteine S-methyltransferase (BHMT) due to a decrease in hepatic concentration of betaine, a methyl-group donor for the BHMT reaction. Previously, we found an interesting phenomenon that supplementation of choline-deprived 10C with methionine at a level of 0.5% did not suppress choline deprivation-induced hyperhomocysteinemia, whereas supplementation with 0.5% methionine in combination with 2.5% serine completely suppressed the hyperhomocysteinemia (unpublished data). These results suggest the possibility that there might exist some factors other than methionine to prevent choline deficiency-induced hyperhomocysteinemia.

This study was conducted to clarify the mechanisms by which rats fed a higher casein diet resist choline deprivation-induced hyperhomocysteinemia. At first, we compared the effects of choline deprivation on plasma homocysteine concentration and related variables in rats fed 10C with the effects of choline deprivation in rats fed a 30% casein diet (30C) to confirm that diets containing higher levels of casein, e.g., 30C, do not cause choline deprivation-induced hyperhomocysteinemia. Secondly, we investigated the dose-response effects of methionine supplementation (0.1–0.5%) on plasma homocysteine concentration and related variables in rats fed choline-deprived 10C. Thirdly, we investigated the effects of supplementation of choline-deprived 10C+0.5% methionine with glycine plus serine, which are known to stimulate homocysteine metabolism (11–14), and the effects of branched-chain amino acids (valine, leucine and isoleucine) plus acidic amino acids (aspartate and glutamate), which comprise a considerable part (47.1% of casein) in choline-deprived diets, to determine whether choline deprivation-induced hyperhomocysteinemia can be suppressed when methionine is supplemented in combination with these amino acids.

### MATERIALS AND METHODS

#### Chemicals

Amino acids were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were purchased from Wako or Sigma-Aldrich (St. Louis, MO) and were of analytical grade. Vitamin-free casein, a mineral mixture (AIN-93G), a vitamin mixture (AIN-93) and cellulose powder were purchased from Oriental Yeast Co., Ltd. (Tokyo). The other ingredients of the diet were purchased from Wako.

#### Animals and diets

Six-week-old male rats (120–140 g) of the Wistar strain were obtained from Japan SLC, Inc. (Hamamatsu, Japan). They were individually housed in hanging stainless-steel wire cages in an isolated room kept in a controlled temperature (23–25 °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 acclimated to the facility for 5 d and given free access to water and a 25% casein diet. In this study, three separate animal experiments were conducted. In experiment 1, rats were randomly assigned to the following four diet groups to investigate the relationship between dietary casein level and effect of choline deprivation: (1) 10% casein diet (10C), (2) choline-deprived 10C (10CCD), (3) 30% casein diet (30C), and (4) choline-deprived 30C (30CCD). In experiment 2, rats were randomly assigned to the following seven diet groups to investigate the dose-dependent effects of methionine supplementation of 10CCD: (1) 10C, (2) 10CCD, (3) 10CCD+0.1% L-Met, (4) 10CCD+0.2% L-Met, (5) 10CCD+0.3% L-Met, (6) 10CCD+0.4% L-Met, and (7) 10CCD+0.5% L-Met. In experiment 3, rats were randomly assigned to the following five diet groups to investigate the effects of dietary amino acid level: (1) 10C, (2) 10CCD, (3) 10CCD+0.5% L-Met (10CCDM), (4) 10CCDM+1.26%
GS (glycine and L-serine) (10CCDMGS), and (5) 10CCDMGS+3.61% BCAA (branched-chain amino acids) + 4.50% AAA (acidic amino acids) (10CCDMGSSBA). The 1.26% GS consisted of 0.32% glycine and 0.94% L-serine, 3.61% BCAA consisted of 1.11% L-valine, 1.51% L-leucine and 0.99% L-isoleucine, and 4.50% AAA consisted of 0.97% L-aspartic acid and 3.53% L-glutamic acid. Amino acids were added to the diet to make their amino acid levels comparable to those of 30C, based on the literature (15). The 10C consisted of the following ingredients (g/kg): vitamin-free casein, 100; α-cornstarch, 582.5; sucrose, 200; corn oil, 50; mineral mixture (AIN-93G), 35; vitamin mixture (AIN-93), 10; choline bitartrate, 2.5 and cellulose powder, 20. In 10CCD, choline bitartrate was omitted with an increase in cornstarch content. Amino acids were added to 10CCD at the expense of cornstarch. Rats were given free access to the experimental diets and water for 10 d and killed by decapitation between 10:00 and 11:00 h without prior food deprivation. This study was approved by the Animal Use Committee of Shizuoka University, and the animals were maintained in accordance with the “Guidelines for the Care and Use of Laboratory Animals” of Shizuoka University.

**Tissue collection and fractionation.** 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 blood, the whole liver was quickly removed, rinsed in ice-cold saline, blotted on filter paper, cut into three 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 0.3 M trichloroacetic acid solution 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, betaine and serine. Another portion of the liver was homogenized in 4 volumes (vol/wt) of a 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M KCl, and the resulting homogenate was centrifuged at 14,000 × g for 10 min at 4˚C. The post-mitochondrial supernatant was subjected to enzyme assays. For the assay of hepatic triglyceride concentration, an aliquot of the liver homogenate was lyophilized, and total lipids were extracted by the method of Folch et al. (16). The third portion of the liver was subjected to analysis of mRNA, and total mRNA was isolated using a kit, ISOGEN (Nippon Gene, Tokyo), according to manufacturer's instructions.

**Biochemical analysis.** The concentrations of homocysteine and cysteine in the plasma and liver were measured by HPLC using the method of Durand et al. (17). The concentrations of SAM and S-adenosylhomocysteine (SAH) in the liver were measured by HPLC according to Cook et al. (18). The concentration of betaine in the liver was measured by HPLC according to Laryea et al. (19) and the concentration of serine in the liver was measured by an amino acid autoanalyzer (Model L-8500; Hitachi). The activity of BHMT in the liver was measured according to Finkelstein and Mudd (20), but HPLC was used in the assay of the reaction product, N,N-dimethylglycine (DMG), according to Laryea et al. (19). The activity of CBS in the liver was measured according to Mudd et al. (21), but HPLC was used in the assay of the reaction product, cystathionine, according to Einarsson et al. (22). The amounts of mRNA for BHMT and CBS relative to β-actin in the liver were measured by quantitative real-time PCR analysis as described previously (14). The hepatic triglyceride concentration was measured enzymatically using a commercial kit (Triglyceride E-Test Wako, Wako). The protein concentration was measured according to Lowry et al. (23) using bovine serum albumin as a standard.

**Statistical analysis.** Each value is expressed as the mean ± SE. Data were analyzed by a two-way ANOVA (experiment 1) or one-way ANOVA (experiments 2 and 3), 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 software (version 1.5; Esumi, Tokyo).

**RESULTS**

**Dietary casein level and effects of choline deprivation (experiment 1)**

Food intake during the 10-d experimental period was significantly lower or tended to be lower in rats fed 30C and 30CCD than in rats fed 10C and 10CCD, whereas the intake of methionine was reversed (Table 1). Body weight gain and relative liver weight were significantly higher in rats fed 30C and 30CCD than in rats fed 10C and 10CCD. Choline deprivation did not affect these variables. Plasma homocysteine concentration was significantly lower in rats fed 30C than in rats fed 10C. Choline deprivation significantly increased plasma homocysteine concentration in rats fed 10C, but it did not increase plasma homocysteine concentration in rats fed 30C. Plasma cysteine concentration, which was measured for comparison with homocysteine, was significantly higher in rats fed 30C than in rats fed 10C and unaffected by choline deprivation. Hepatic SAM concentration was significantly higher in rats fed 30C than in rats fed 10C, and choline deprivation significantly decreased SAM concentration in both rats fed 10C and those fed 30C. Hepatic SAH concentration was slightly higher in rats fed 30C than in rats fed 10C and choline deprivation significantly increased SAH concentration in rats fed 10C but not in rats fed 30C. Likewise, the SAM : SAH ratio was significantly higher in rats fed 30C than in rats fed 10C, and choline deprivation significantly increased SAH concentration in rats fed 10C but not in rats fed 30C. Choline deprivation significantly increased hepatic homocysteine concentration in rats fed 10C, but it did not enhance homocysteine concentration in rats fed 30C but not in rats fed 30C. Choline deprivation significantly increased hepatic homocysteine concentration in rats fed 10C, but it did not enhance homocysteine concentration in rats fed 30C. Hepatic BHMT activity was significantly higher in rats fed 30C than in rats fed 10C, and choline deprivation significantly decreased the enzyme activity in rats fed 10C but not in rats fed 30C. The relative level of mRNA for BHMT was significantly higher in rats fed 30C than in rats fed 10C, and choline deprivation significantly decreased the...
mRNA level in rats fed 30C but not in rats fed 10C. Choline deprivation markedly decreased hepatic concentration of betaine, a substrate of BHMT, in rats fed 10C, whereas the effect of choline deprivation in rats fed 30C was relatively small. Hepatic CBS activity was significantly higher in rats fed 30C than in rats fed 10C, and choline deprivation slightly decreased the enzyme activity in both rats fed 10C and those fed 30C. The relative levels of mRNA for CBS significantly higher in rats fed 30C than in rats fed 10C, and choline deprivation did not affect the mRNA level. Hepatic concentration of serine, a substrate of CBS, was markedly lower in rats fed 30C than in rats fed 10C, and choline deprivation did not affect the amino acid concentration. Choline deprivation markedly increased hepatic triglyceride concentration in rats fed 10C, but it did not affect the lipid concentration in rats fed 30C.

Effects of methionine supplementation at graded levels (experiment 2)

Body weight gain and relative liver weight were significantly increased by methionine supplementation at levels of 0.3% or more, whereas food intake did not differ among the groups (Table 2). The choline deprivation-induced enhancement of plasma homocysteine concentration was significantly suppressed by methionine supplementation in a dose-dependent manner in the range of 0.1 to 0.3%, but the suppressive effect of methionine became smaller with an increase in supplementation level in the range of 0.3 to 0.5% (Fig. 2A). Choline deprivation or methionine supplementation had little effect on plasma cysteine concentration (Fig. 2B). Plasma triglyceride concentration was significantly decreased by choline deprivation and this decrease was restored by methionine supplementation in the range of 0.1 to 0.5% (Fig. 2C). The choline deprivation-induced increase in hepatic triglyceride concentration was significantly suppressed by methionine supplementation in a dose-dependent manner in the range of 0.1 to 0.3%, but the suppressive effect of methionine became smaller with an increase in supplementation level in the range of 0.3 to 0.5% (Fig. 2D). The profile of hepatic triglyceride concentration was similar to that of plasma homocysteine concentration.

The choline deprivation-induced decrease in hepatic SAM concentration was suppressed by methionine supplementation in a dose-dependent manner (Fig. 3A). The increase in hepatic SAH concentration was significantly suppressed by methionine supplementation in the

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**Table 1. Effects of choline deprivation on plasma homocysteine concentration and other variables in rats fed 10% and 30% casein diets (experiment 1).**

| Metric                                | 10C         | 10CCD      | 30C         | 30CCD      | ANOVA^2 |
|---------------------------------------|-------------|------------|-------------|------------|---------|
| Met level in diet, g/kg               | 2.5         | 2.5        | 7.5         | 7.5        |         |
| Food intake, g/10 d                   | 171±8ab,1   | 175±6b     | 148±5b      | 154±4b     | P       |
| Met intake, g/10 d                    | 0.43±0.02b  | 0.44±0.02b | 1.11±0.04a  | 1.16±0.03a | P       |
| Body wt gain, g/10 d                  | 26±2b,1     | 30±2b      | 44±2e       | 47±1e      |         |
| Liver wt, g/100 g body wt             | 4.08±0.04b  | 4.03±0.07b | 4.67±0.10a  | 4.73±0.08a | P       |
| Plasma homocysteine, μmol/L           | 18.0±0.2b   | 32.5±0.5a  | 12.3±0.3c   | 12.2±0.4c  | C, P, CP|
| Cysteine, μmol/L                      | 104±3b      | 107±3b     | 122±4a      | 124±3a     | P       |
| Liver                                 |             |            |             |            |         |
| SAM, nmol/g                           | 66.1±1.3c   | 41.8±0.6d  | 103.4±1.2a  | 80.3±1.0b  | C, P    |
| SAH, nmol/g                           | 13.6±0.3c   | 19.3±0.2a  | 15.8±0.3b   | 12.8±0.4c  | C, P CP |
| SAM : SAH ratio                       | 4.88±0.18b  | 2.17±0.03c | 6.58±0.17a  | 6.32±0.25a | C, P CP |
| Homocysteine, nmol/g                  | 2.59±0.04b  | 3.66±0.06a | 2.71±0.04b  | 2.75±0.03b | C, P CP |
| BHMT activity^1                       | 0.99±0.01b  | 0.46±0.01c | 1.95±0.07a  | 1.82±0.08a | C, P CP |
| BHMT mRNA^4                           | 1.00±0.02c  | 0.75±0.04c | 1.71±0.12a  | 1.37±0.10b | C, P    |
| CBS activity^1                        | 4.86±0.12c  | 4.12±0.09d | 8.50±0.11a  | 7.93±0.18b | C, P    |
| CBS mRNA^4                            | 1.00±0.17b  | 0.91±0.04b | 2.11±0.11a  | 1.79±0.14a | P       |
| Betaine, μmol/g                       | 2.69±0.09a  | 3.33±0.01d | 1.22±0.05b  | 0.94±0.04c | C, P CP |
| Serine, μmol/g                        | 2.52±0.27a  | 2.60±0.13a | 0.28±0.03b  | 0.31±0.02b | P       |
| Triglyceride, μmol/g                  | 25.7±0.4b   | 79.4±1.2a  | 24.3±0.4b   | 25.5±0.4b  | C, P CP |

10C, 10% casein diet; 30C, 30% casein diet; 10CCD, choline-deprived 10C; 30CCD, choline-deprived 30C; BHMT, betaine-homocysteine S-methyltransferase; CBS, cystathionine β-synthase; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine.

^1 Each value is the mean±SE, n=8. Values without a common letter differ, p<0.05.

^2 Two-way ANOVA: C, affected by choline deprivation, p<0.05; P, affected by protein level, p<0.05; CP, interactively affected by choline deprivation and protein level, p<0.05.

^3 Expressed as nmol/(min·mg protein).

^4 Values represent BHMT mRNA/β-actin or CBS mRNA/β-actin and are expressed as relative values to the value in the 10C group.
Table 2. Body weight gain, food intake and liver weight of rats fed the experimental diets (experiments 2 and 3).

| Diet                  | Body wt gain (g/10 d) | Food intake (g/10 d) | Liver wt (g/100 g body wt) |
|-----------------------|------------------------|----------------------|----------------------------|
| **Experiment 2**      |                        |                      |                            |
| 10C                   |                        |                      |                            |
| 10CCD                 |                        |                      |                            |
| 10CCD+0.1% Met        | 27±2bc,1               | 152±3                | 4.00±0.05b                 |
| 10CCD+0.2% Met        | 35±1ac                 | 152±3                | 4.11±0.08b                 |
| 10CCD+0.3% Met        | 41±2a                  | 151±4                | 4.48±0.04a                 |
| 10CCD+0.4% Met        | 39±1a                  | 143±4                | 4.51±0.07a                 |
| 10CCD+0.5% Met        | 41±2a                  | 145±5                | 4.55±0.09a                 |
| **Experiment 3**      |                        |                      |                            |
| 10C                   |                        |                      |                            |
| 10CCD                 |                        |                      |                            |
| 10CCD+0.5% Met (10CCDM) | 42±2a               | 163±6                | 4.71±0.12a                 |
| 10CCDM+0.32% Gly +0.94% Ser (10CCDMGS) | 43±2a | 153±5 | 4.69±0.09a |
| 10CCDMGS+3.6%, BCAA +4.5% AAA (10CCDMGSBA) | 35±2abc | 157±3 | 4.79±0.11a |

10C, 10% casein diet; 10CCD, choline-deprived 10C; AAA, acidic amino acids (0.97% Asp + 3.53% Glu); BCAA, branched-chain amino acids (1.11% Val + 1.51% Leu + 0.99% Ile).

1 Each value is the mean±SE, n=8. Values without a common letter differ, p<0.05.
2 In experiment 3, several amino acids were added to the diet to make it comparable to the 30% casein diet.

Fig. 2. Plasma concentrations of homocysteine (A), cysteine (B), and triglyceride (C) and hepatic triglyceride concentration (D) in rats fed the experimental diets (experiment 2). Each value is the mean±SE, n=8. Means in a panel without a common letter differ, p<0.05. 10C, 10% casein diet; 10CCD, choline-deprived 10C; Cys, cysteine; Hcy, homocysteine.

Fig. 3. Hepatic concentrations of S-adenosylmethionine (A), S-adenosylhomocysteine (B), their ratio (C), and homocysteine (D) in rats fed the experimental diets (experiment 2). Each value is the mean±SE, n=8. Means in a panel without a common letter differ, p<0.05. SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine. See the legend of Fig. 2 for other abbreviations.
range of 0.2 to 0.4%, but supplementation with methionine at a level of 0.5% significantly increased SAH concentration to a level higher than the level in the 10CCD group (Fig. 3B). The decrease in the SAM:SAH ratio was suppressed by methionine supplementation in a dose-dependent manner (Fig. 3C). The increase in hepatic homocysteine concentration was significantly suppressed by methionine supplementation at a level of 0.3%, but methionine supplementation at a level of 0.5% increased homocysteine concentration to a level higher than the level in the 10CCD group (Fig. 3D). The profile of hepatic homocysteine concentrations was similar to that of hepatic homocysteine concentrations. The choline deprivation-induced decrease in hepatic BHMT and CBS activities were suppressed by methionine supplementation in a dose-dependent manner (Fig. 4A and B). The decrease in hepatic betaine concentration was partially restored by methionine supplementation in a dose-dependent manner (Fig. 4C). In contrast, hepatic serine concentration was decreased by methionine supplementation in a dose-dependent manner (Fig. 4D).

Effects of supplementation with glycine+serine and dietary amino acid level (experiment 3)

Body weight gain and relative liver weight were significantly higher or tended to be higher in rats fed diets supplemented with methionine irrespective of other supplements, whereas food intake did not differ among the groups (Table 2). The results of experiment 2 showing that choline deprivation-induced increase in plasma homocysteine concentration cannot be suppressed by supplementation with methionine alone at a level of 0.5% were reproduced (Fig. 5A). In contrast, choline deprivation-induced increase in plasma homocysteine concentration was completely suppressed by supplementation with methionine+GS. Plasma homocysteine concentration was further decreased by supplementation with BCAA+AAA in addition to methionine+GS. Plasma cysteine concentration was slightly higher in rats fed diets supplemented with GS or GS+BCAA+AAA than in rats fed other diets (Fig. 5B). The decrease in plasma triglyceride concentration was restored by supplementation with methionine irrespective of other supplements (Fig. 5C). The increase in hepatic triglyceride concentration was partly suppressed by supplementation with methionine alone or methionine+GS and...
was completely suppressed by supplementation with methionine+GS+BCAA+AAA (Fig. 5D).

Choline deprivation-induced decrease in hepatic SAM concentration was completely restored and significantly increased to levels higher than the level in rats fed 10C by supplementation with methionine irrespectively of other supplements (Fig. 6A). The increase in hepatic SAH concentration was further increased by supplementation with methionine alone, but it was unaffected by supplementation with methionine+GS and was significantly decreased by supplementation with methionine+GS+BCAA+AAA (Fig. 6B). The decrease in the SAM:SAH ratio was completely restored by supplementation with methionine+GS or methionine+GS+BCAA+AAA (Fig. 6C). The increase in hepatic homocysteine concentration was further increased by supplementation with methionine alone or methionine+GS, but it was unaffected by supplementation with methionine+GS+BCAA+AAA (Fig. 6D). Choline deprivation-induced decrease in hepatic BHMT activity was completely restored by supplementation with methionine alone and further increased to levels higher than the level in rats fed 10C by supplementation with methionine+GS or methionine+GS+BCAA+AAA (Fig. 7A). The decrease in hepatic CBS activity was restored by supplementation with methionine alone or methionine+GS and further increased to a level higher than the level in rats fed 10C by supplementation with methionine+GS+BCAA+AAA (Fig. 7E). Hepatic serine concentration was markedly decreased by supplementation with methionine alone and this decrease was significantly suppressed or tended to be suppressed by concurrent supplementation with...
GS or GS+BCAA+AAA (Fig. 7F).

**DISCUSSION**

Our previous study demonstrated that choline deprivation significantly enhanced plasma homocysteine concentration when rats were fed a low casein diet, whereas it did not enhance plasma homocysteine concentration when rats were fed a higher casein diet (7). This was confirmed in experiment 1 of the present study. It appears that the different responses of rats fed 10C and rats fed 30C to choline deprivation were associated with different choline status within the body, because fatty liver, which is one of the indices of deficiency of PC or choline (8), developed only in rats fed choline-deprived 10C. The results also support the assumption that one of the mechanisms underlying choline deprivation-induced hyperhomocysteinemia is due to deficiency of betaine, since hepatic betaine concentration was markedly decreased by choline deprivation in rats fed 10C, in contrast to only a limited decrease in betaine concentration in rats fed 30C. Betaine status within the body is closely linked to choline status. It is known that choline status within the body is determined by intake of both choline and methionine (8). Therefore, it is reasonable to assume that in rats fed 30C, which contained a higher level of methionine, choline deprivation did not cause deficiencies of choline-containing compounds such as PC, choline, and betaine and, therefore, did not result in hyperhomocysteinemia.

One of the important findings obtained in experiment 2 is that methionine had two opposing effects on plasma homocysteine concentration, which were dependent on the supplementation level of methionine, i.e., a plasma homocysteine-lowering effect of methionine in the supplementation range of 0.1 to 0.3% and a plasma homocysteine-elevating effect in the supplementation range of 0.3 to 0.5%, as shown in Fig. 2. It appears that the former effect of methionine is mainly ascribed to stimulation of PC synthesis via the PE N-methylation pathway and resulting increase in betaine supply. On the other hand, the latter effect might be mainly ascribed to the augmented production of homocysteine, since methionine is the sole precursor of homocysteine. In support of this, dietary supplementation with methionine increased plasma homocysteine concentration in a dose-dependent manner in rats fed a 25% casein diet, although the diet contained choline at a level of 0.1% (13). The results showing that methionine supplementation at around 0.3% led to the lowest plasma homocysteine concentration suggest that the plasma homocysteine-lowering effect of methionine at this supplementation level was greater than the plasma homocysteine-elevating effect of methionine in rats fed choline-deprived 10C. The fact that methionine supplementation at a level of 0.5% did not have any suppressive effect on choline deprivation-induced hyperhomocysteinemia indicates that 10C+0.5% methionine is definitively distinct from 30C despite the methionine contents of the two diets being comparable. Interest-ingly, hepatic triglyceride concentration was influenced by methionine supplementation in a manner similar to that of plasma homocysteine concentration despite hepatic betaine concentration being increased with increase in methionine supplementation level.

The results obtained in experiment 3 clearly demonstrate that choline deprivation-induced hyperhomocysteinemia was effectively suppressed by methionine when supplemented in combination with GS alone or with GS+BCAA+AAA. The effect of methionine+GS is essentially consistent with our previous finding that choline deprivation-induced hyperhomocysteinemia was completely suppressed by supplementation with 0.5% methionine+2.5% serine in rats fed 10C. It is assumed that GS stimulated cystathionine synthesis by supplying serine, a substrate of CBS, and thereby diminished the plasma homocysteine-elevating effect of methionine, since supplementation with GS did not increase hepatic CBS activity. The results showing that hepatic SAH and homocysteine concentrations were significantly lower in rats fed 10CCD supplemented with methionine+GS than in rats fed 10CCD supplemented with methionine alone support the idea of GS-stimulated homocysteine removal. An important finding in experiment 3 is that BCAA+AAA had a plasma homocysteine-lowering effect when supplemented in combination with methionine+GS. We added BCAA+AAA to the diet so as to raise the dietary amino acid level, since these amino acids comprise a major part of total amino acids of casein. The addition of BCAA+AAA was found to increase hepatic CBS and BHMT activities and their gene expression, which are favorable for the removal of homocysteine. It is thought that cystathionine formation is critical in homocysteine metabolism under the condition of relatively high levels of dietary methionine (24, 25). Therefore, the effect of BCAA+AAA on plasma homocysteine concentration appears to be explained mainly by increased CBS activity, although the increase in BHMT activity cannot be ignored. It has been shown that CBS activity increased in response to dietary casein level (14, 26), but it did not respond to dietary methionine level within the nutritional range (14). The latter phenomenon is unexpected, since CBS is a member of the family of sulfur-containing amino acid-metabolizing enzymes. The increased CBS activity and its gene expression may be mainly attributable to BCAA, since our previous study showed that hepatic CBS activity was significantly increased by dietary addition of BCAA alone at a high (12%) level (unpublished data). In any case, the results obtained in experiment 3 indicate that a higher casein diet results in resistance to choline deficiency-induced hyperhomocysteinemia by at least three mechanisms: (i) supply of methionine, which increases hepatic SAM concentration and thereby stimulates PC synthesis via the PE N-methylation pathway, (ii) supply of glycine and serine, which stimulate homocysteine metabolism as an indirect or direct substrate of CBS, and (iii) supply of a relatively large amount of amino acids such as BCAA and AAA, which induces CBS and thereby enhances...
homocysteine metabolism.

We previously demonstrated that plasma homocysteine concentration was lower in rats fed casein or soybean protein diets containing higher levels of protein than in rats fed casein or soybean protein diets containing lower levels of protein (14, 27–29). Since higher protein diets inevitably increase methionine intake, such a phenomenon seems to be paradoxical. One of the possible mechanisms by which higher protein diets decrease plasma homocysteine concentration is that higher protein diets increase CBS and BHMT activities and thereby effectively enhance homocysteine removal despite homocysteine production being augmented (29). We previously demonstrated that the plasma homocysteine-elevating effect of dietary supplementation with 0.5% methionine was smaller in rats fed 30C than in rats fed 10C (27). Dietetic addition of 0.5% guanidinoacetic acid did not increase plasma homocysteine concentration in rats fed a 40% casein diet, while it markedly enhanced plasma homocysteine in rats fed 10C (30). Furthermore, folate deficiency-induced enhancement of plasma homocysteine concentration was significantly smaller in rats fed a 20% casein diet than in rats fed 10C (unpublished data). These results indicate that higher casein diets generally cause resistance to various types of hyperhomocysteinemic treatment. Some of the factors contributing to the resistivity to choline deficiency, as presented here, may also be associated with the suppressive effects of higher casein diets on hyperhomocysteinaemia induced by methionine, guanidinoacetic acid, or folate deficiency. However, this remains to be clarified experimentally in further studies.

It is concluded that there are at least three factors contributing to the resistivity of rats fed 30C to choline deprivation-induced hyperhomocysteinaemia, i.e., higher intake of methionine, higher intake of glycine+serine, and higher intake of other amino acids such as BCAA and AAA. This is also the case for choline deprivation-induced development of fatty liver. The information provided here might be useful for studies on prevention of hyperhomocysteinaemia and fatty liver.

Acknowledgments
This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Culture, Sports and Technology of Japan.

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