**Note**

**Increased Plasma Homocysteine Concentration in Rats from a Low Casein Diet**

Hiroshi O KAWA, Tatsuya MORITA, and Kimio Sugiyama†

Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422-8529, Japan

Received July 3, 2006; Accepted August 14, 2006; Online Publication, December 7, 2006

Rats were fed on a 10% casein (10C) diet, 30% casein (30C) diet, 10C + 0.5% methionine diet, or 30C + 0.5% methionine diet for 14 d to investigate the relationship between the dietary protein level and plasma homocysteine concentration. The plasma homocysteine concentration was significantly higher in the rats fed on the 10C diet than in the rats fed on the 30C diet, and this phenomenon persisted even under the condition of methionine supplementation. The activity of hepatic cystathionine β-synthase (CBS) was significantly lower in the rats fed on the 10% casein diets than in the rats fed on the 30% casein diets, irrespective of methionine supplementation. This is the first demonstration of a low-protein diet increasing the plasma homocysteine concentration in experimental animals. It is suggested that the decreased CBS activity might be associated, at least in part, with the hyperhomocysteinemia caused by the low-casein diet.

Key words: homocysteine; dietary protein level; low casein diet; methionine; rat

Homocysteine is the usual intermediate for methionine metabolism. It is widely recognized that an elevated plasma homocysteine concentration is an independent risk factor for cardiovascular disease.1–3) The normal plasma homocysteine concentration is in the range of 5 to 15 μM, and a 5-μM increase in this amino acid concentration is associated with an increased risk of 60–80% for coronary heart disease.21) It has been reported that the plasma homocysteine concentration was affected by various factors such as nutritional, pharmacological, hormonal, disease, lifestyle, and genetic factors.3,4) In recent years, attention has been paid to the effect of dietary protein on the plasma homocysteine concentration, since an intake of methionine, the sole precursor of homocysteine, is usually associated with the amount and type of dietary protein ingested. For instance, it has been shown that a single dose of a high-protein diet increased the postprandial plasma homocysteine concentration in humans.5,6) In contrast, a sustained intake of a high-protein diet did not alter5–9) or rather decreased the plasma homocysteine concentration in humans.10) In rats, the plasma homocysteine concentration was significantly higher when fed on a high-casein diet than when fed on a standard casein diet.11) Thus, the results of previous studies are not necessarily consistent.

We investigated in the present study the effect of dietary protein level on the plasma homocysteine concentration by using rats fed on a low (10%) casein diet and a relatively high (30%) casein diet. Since methionine loading has often been used to assess the capacity for homocysteine metabolism,12,13) the effect of dietary supplementation with methionine was also investigated.

Male six-week-old rats (120–140 g) of the Wistar strain were obtained from Japan SLC (Hamamatsu, Japan). They were individually housed in hanging stainless-steel wire cages kept in an isolated room at 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). Four experimental diets were used in the present study: a 10% casein (10C) diet, 30% casein (30C) diet, 10C + 0.5% L-methionine (10CM) diet, and 30C + 0.5% L-methionine (30CM) diet. The composition of the 10C and 30C diets was as follows (g/100 g): casein, 10 or 30; corn starch, 58.25 or 38.25; sucrose, 20; corn oil, 5; AIN-93G mineral mixture, 3.5; AIN-93G vitamin mixture, 1; choline bitartrate, 0.25; cellulose, 2. Methionine was added to the diet at the expense of starch. After rats had been given free access to water and the experimental diets for 14 d, they were killed by decapitation between 10.00 and 10.30 h to obtain the blood and liver. The experimental plan was approved by the Laboratory Animal Care Committee of the Faculty of Agriculture at Shizuoka University.

After collecting the blood, the whole liver was quickly removed, rinsed in ice-cold saline, blotted on filter paper, cut into two portions, weighed, quickly frozen in liquid nitrogen and stored at −80°C until needed for analyses. One portion of the liver was

† To whom correspondence should be addressed. Fax: +81-54-238-4877; E-mail: aksugi@agr.shizuoka.ac.jp
homogenized in 4 volumes (vol/wt) of ice-cold 25% perchloric acid and then centrifuged at 10,000 \( \times g \) for 10 min at 4°C. The supernatant of the deproteinized liver homogenate was subjected to assays of methionine metabolites. The other portion of the liver was homogenized in 4 volumes (vol/wt) of a 10 mM Tris–HCl buffer (pH 7.4) containing 150 mM KCl, and the resulting homogenate was centrifuged at 14,000 \( \times g \) for 10 min at 4°C. The supernatant was subjected to an enzyme assay. The concentrations of total homocysteine and cysteine in the plasma were measured by HPLC according to the method of Durand et al.\(^{14}\). The concentrations of \( S \)-adenosylmethionine (SAM) and \( S \)-adenosylhomocysteine (SAH) in the liver were measured by HPLC essentially according to the method of Cook et al.\(^{15}\). The activity of cystathionine \( \beta \)-synthase (E.C. 4.2.1.22) in the liver was measured according to the method of Mudd et al.\(^{16}\) but HPLC was used for the assay of the reaction product, cystathionine, according to the method of Einarsson et al.\(^{17}\). Each data value is expressed as the mean ± SEM. Data were analyzed by a two-way analysis of variance, and the differences among experimental groups were analyzed by the Tukey test when the \( F \) value was significant. The statistical analysis was performed with Mac Tokei-Kaiseki ver. 1.5 software (Esumi, Tokyo, Japan).

The body weight gain was significantly lower in the rats fed on the 10C diet than in the rats fed on the 30C diet (Table 1). Methionine supplementation promoted the growth of the rats fed on the 10% casein diet to the level of the rats fed on the 30C diet, but it did not affect the growth of the rats fed on the 30% casein diet. The food intake was significantly higher in the rats fed on the 10C diet than in the rats fed on the other diets. Figure 1 summarizes the effects of the dietary casein level and methionine supplementation on the plasma concentrations of homocysteine and cysteine, and on the hepatic concentrations of methionine metabolites and cystathionine \( \beta \)-synthase activity in the liver. The plasma homocysteine concentration was significantly higher by about 50% in the rats fed on the 10C diet than in the rats fed on the 30C diet. Although methionine supplementation enhanced the plasma homocysteine concentration, the plasma homocysteine concentration was still significantly higher in the rats fed on the 10CM diet than in those fed on the 30CM diet. The plasma cysteine concentration was unaffected either by the dietary casein level or methionine supplementation. The hepatic concentrations of SAM, SAH, and homocysteine were significantly lower or tended to be lower in the rats fed on the 10C diet than in those fed on the 30C diet. These profiles were not essentially altered by methionine supplementation. The hepatic CBS activity was significantly lower in the rats fed on the 10% casein diets than

<table>
<thead>
<tr>
<th>Diet</th>
<th>Body wt. gain (g/14 d)</th>
<th>Food intake (% of body wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% Casein (10C)</td>
<td>38 ± 3(^{b})</td>
<td>210 ± 7(^{a})</td>
</tr>
<tr>
<td>30% Casein (30C)</td>
<td>61 ± 2(^{a})</td>
<td>192 ± 5(^{a})</td>
</tr>
<tr>
<td>10C + 0.5% l-Met</td>
<td>57 ± 3(^{b})</td>
<td>197 ± 3(^{a})</td>
</tr>
<tr>
<td>30C + 0.5% l-Met</td>
<td>62 ± 4(^{a})</td>
<td>183 ± 7(^{b})</td>
</tr>
</tbody>
</table>

\(^{b}\)Each value is the mean ± SEM for six rats; values with different superscripts are significantly different at \( p < 0.05 \).

**Table 1.** Body Weight Gain, Food Intake and Liver Weight of Rats Fed on the Experimental Diets

**Fig. 1.** Effects of Dietary Casein Level with or without Methionine Supplementation on the Plasma Concentrations of Homocysteine (A) and Cysteine (B), the Hepatic Concentrations of \( S \)-Adenosylmethionine (C), \( S \)-Adenosylhomocysteine (D) and Homocysteine (E), and on the Activity of Hepatic Cystathionine \( \beta \)-Synthase (F) in Rats.

Each value is the mean ± SEM for six rats. Values with different letters are significantly different at \( p < 0.05 \). CBS, cystathionine \( \beta \)-synthase; Cys, cysteine; Hcy, homocysteine; SAH, \( S \)-adenosylhomocysteine; SAM, \( S \)-adenosylmethionine.
in those fed on the 30% casein diets, irrespective of methionine supplementation.

The results of the present study clearly demonstrate that a low (10%) casein diet, as compared with a higher (30%) casein diet, led to an enhanced plasma homocysteine concentration in the rats. These results were unexpected, since it was anticipated that low-protein diet might decrease the plasma homocysteine concentration because of the reduced intake of methionine. Indeed, Stead et al.\textsuperscript{11} have demonstrated that a high (60%) casein diet increased the plasma homocysteine concentration when compared with a standard (20%) casein diet in rats, suggesting that higher protein intake would increase the plasma homocysteine concentration. As for the effect of the low-protein diet, Smolin and Benevenga\textsuperscript{18} have shown in an earlier report that there was no difference in the plasma homocysteine concentration between rats fed on a 10% casein diet and rats fed on a 60% casein diet. There is no other information, to our knowledge, about the effect of dietary protein level on the plasma homocysteine concentration in experimental animals. Therefore, the present study is the first demonstration that a low-protein diet did not decrease but rather increased the plasma homocysteine concentration in experimental animals. Our results appear to be in conflict with the finding by Stead et al.\textsuperscript{11} but the reason for the discrepancy is currently unclear. On the other hand, there are some human epidemiological studies to suggest that a low-protein diet might increase the plasma homocysteine concentration. For instance, Stolzenberg-Solomon et al.\textsuperscript{10} have reported that there was no inverse dose-response relationship between the dietary protein and plasma homocysteine concentration in older human populations in USA. Furthermore, Ingenbleek et al.\textsuperscript{19} have reported that subclinical protein malnutrition might be a determinant of hyperhomocysteinemia observed in goiter patients in Africa.

The liver is the central organ of methionine metabolism, and one of the key enzymes in the metabolism of homocysteine is CBS that catalyzes the formation of cystathionine. Our results clearly show that the CBS activity was significantly lower in rats fed on the 10C diet than in rats fed on the 30C diet, suggesting that the low activity of CBS might be associated, at least in part, with the hyperhomocysteinemia in the rats fed on the 10C diet. As a modification of the methionine-loading test, we added methionine to the diet and fed rats for 14 d instead of a one-shot administration. The results indicate that the homocysteine-metabolizing capacity was lower in the rats fed on the 10CM diet than in those fed on the 30CM diet, judging from the plasma homocysteine concentration, supporting the notion that the hyperhomocysteinemia caused by the 10C diet might be attributable to the decreased capacity for homocysteine metabolism. It is known that the activity of CBS is regulated in two ways, allosteric activation by SAM\textsuperscript{20} and induction by dietary protein with an increase in mRNA for the enzyme.\textsuperscript{21} The findings that methionine supplementation did not enhance the CBS activity (Fig. 1F), while it significantly increased the hepatic SAM level (Fig. 1C), suggest that the CBS activity might have been influenced mainly by the dietary protein level rather than by the dietary methionine level under the experimental conditions used. Although homocysteine can be remethylated to methionine, the proportion of remethylation relative to trans-sulfuration is thought to be increased in rats fed on a low-methionine diet\textsuperscript{22} and probably also in rats fed on a low-protein diet. Further studies are needed to clarify whether the low-protein diet would generally increase the plasma homocysteine concentration regardless of the dietary protein source and to elucidate the detailed mechanism(s) by which the dietary protein level affects the plasma homocysteine concentration.

References


