Effect of Vitamin B<sub>6</sub> Deficiency on S-Adenosylhomocysteine Hydrolase Activity as a Target Point for Methionine Metabolic Regulation

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Summary The objective of this study was to clarify the relationship between the accumulation of S-adenosylhomocysteine (SAH) and the change in the SAH hydrolase activity in vitamin B<sub>6</sub> (B<sub>6</sub>)-deficient rats. Male Wistar rats were fed a control diet (control and pair-fed groups) or B<sub>6</sub>-free diet (B<sub>6</sub>-deficient group) for 5 wk. Although the SAH-synthetic activity of SAH hydrolase significantly increased in the B<sub>6</sub>-deficient group, SAH-hydrolytic activity of SAH hydrolase showed no significant difference in the liver among the three groups. On the other hand, SAH hydrolase mRNA in the liver did not show any significant change. Thus, the accumulation of SAH would be due to the increased SAH-synthetic activity of SAH hydrolase. The disturbed methionine metabolism by B<sub>6</sub>-deficiency, such as a significant increase of plasma homocysteine, might induce the activation of SAH hydrolase in the direction of SAH synthesis.

Key Words Vitamin B<sub>6</sub>, S-adenosylhomocysteine hydrolase, S-adenosylhomocysteine, homocysteine, methionine metabolism

Vitamin B<sub>6</sub> (B<sub>6</sub>) is known as a coenzyme related to amino acid metabolism, including methionine. Methionine is metabolized to S-adenosylmethionine (SAM) by l-methionine S-adenosyltransferase (EC 2.5.1.6) (1, 2). SAM is a methyl donor for transmethylation in vivo, involving protein, histones, DNA and RNA as cellular methyl acceptors (3). SAM is converted to S-adenosylhomocysteine (SAH) by some transmethylation reactions, and then SAH is hydrolyzed to homocysteine (Hcy) by SAH hydrolase (EC 3.3.1.1). Hcy was reported to be an independent risk factor for cardiovascular diseases and arterial sclerosis (4), so it is important to remove Hcy from the metabolic pathway. Hcy is catabolized by cystathionine β-synthase (EC 4.2.1.22; CBS) and γ-cystathionase (EC 4.4.1.1), both of which require PLP as a coenzyme. This route is called the transsulfuration pathway. On the other hand, Hcy is remethylated to methionine and this route is called the remethylation pathway. This pathway requires betaine, folic acid and vitamin B<sub>12</sub>. Under normal physiological conditions, the remethylation pathway predominates rather than the transsulfuration pathway (5). Additionally, it was reported that appropriate intake of vitamin B<sub>12</sub>, folic acid and B<sub>6</sub> is important to avoid Hcy accumulation (6, 7).

SAH hydrolase plays an important role in the metabolism of SAH. SAH is reversibly hydrolyzed to adenosine and Hcy by SAH hydrolase. Thermodynamically, an equilibrium of SAH hydrolase reaction favors the direction of SAH synthesis. However, under physiological conditions, SAH hydrolase directs toward the hydrolysis of SAH because adenosine and Hcy are rapidly catabolized.

Our previous report showed the accumulation of SAH and an increase of the SAH-synthetic activity of SAH hydrolase in the liver and thymus of B<sub>6</sub>-deficient rats (8). This indicated the possibility that SAH accumulated in the liver and thymus due to a dominant increase in the SAH-synthetic activity of SAH hydrolase. Since SAH hydrolase is a reversible enzyme, it is needed to examine whether the SAH-hydrolytic activity of SAH hydrolase was also activated or SAH hydrolase, per se, increased during B<sub>6</sub> deficiency. Therefore, we measured the activity of SAH hydrolase in both directions and examined the expression of SAH hydrolase mRNA to clarify the mechanism of SAH accumulation during B<sub>6</sub> deficiency.

MATERIALS AND METHODS

Reagents. Somnopentyl® was purchased from Schering-Plough Co. (NJ, USA). SAH, SAM, adenosine and l-homocysteine thiolactone were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). SBD-F (7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate) and 1-heptanesulfonic acid sodium salt were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methanol and acetonitrile were purchased from Kanto Kagaku Co. (Tokyo, Japan). Other reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise indicated.

Animal and diets. Male Wistar rats, 4-wk old and weighing 80–100 g, obtained from Japan SLC, Inc. (Hamamatsu, Japan) were acclimatized on AIN-76 diet

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for 3 d. The animals were housed individually in a stainless-steel cage in a room maintained at constant temperature (23 ± 1°C) with alternating 12-h cycles of light (light: 6:00 a.m. to 6:00 p.m.) and dark. They were randomly divided into three groups of six rats each. The B<sub>6</sub>-deficient (B<sub>6</sub>-def.) group and the control group were given free access to the respective diets (Table 1) for 5 wk, and the pair-fed control (PF) group was pair-fed to the B<sub>6</sub>-def. group. Food intake and body weight were measured daily.

At weekly intervals, the 24-h urine of each rat was collected for measurement of xanthurenic acid excretion. It was determined colorimetrically in the presence of Fe<sup>2+</sup> at 610 nm (9).

The experiments were performed in accordance with the guidelines of governmental legislation in Japan on the proper use of laboratory animals (1980), and our experiments were approved by the ethical committee of the Faculty of Applied Biological Sciences in Gifu University.

**Sample preparation.** Under Somnopentyl® anesthesia, blood samples were drawn from the abdominal aorta with a heparinized syringe, and the liver was then immediately excised and weighed. Plasma was obtained by centrifugation at 2,000 × g (4°C) for 20 min. Plasma and liver were stored at −20°C until analyzed.

**Assay of plasma PLP.** The contents of PLP in the plasma were determined using the method described by Tsuge (10).

**Assay of plasma Hcy.** Total Hcy in the plasma was measured by the method of Yamaguchi et al. (11).

**Assay of SAM and SAH.** SAM and SAH were measured by the method of She et al. (12).

**Assay of hepatic SAH hydrolase.** The SAH-synthetic activity of SAH hydrolase was measured using a modified method of She et al. (12), and the SAH-hydrolytic activity of SAH hydrolase was determined using the method of Aksamit et al. (13). Livers were homogenized with five volumes of 0.25 M sucrose/3.3 mM MgCl<sub>2</sub>/2 mM glutathione (reduced form)/50 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 100,000 × g (4°C) for 1 h. The supernatant was used for assaying SAH hydrolase.

For the assay of the SAH-synthetic activity of SAH hydrolase, the incubation mixture consisted of 100 mM potassium phosphate buffer (pH 7.3), 2 mM dithiothreitol (DTT), 5 mM l-homocysteine thiolactone, 100 μM adenosine and 50 μL homogenate (1 mg protein/mL) in a total volume of 0.3 mL. For the assay of the SAH-hydrolytic activity of SAH hydrolase, the incubation mixture consisted of 50 mM potassium phosphate buffer (pH 6.9), 0.2 mM SAH, 2 mM DTT, 1.7 units of adenosine deaminase (purified from calf intestine following the method of Coddington (14)) and 50 μL homogenate (1 mg protein/mL) in a total volume of 0.3 mL. After incubation at 37°C for 10 min, the reaction was stopped by adding 50 μL of 3 N perchloric acid (PCA). The content was mixed and then centrifuged at 2,000 × g for 10 min. The supernatant was filtered through a Millipore membrane (0.45 μm) and applied to HPLC for the analysis of SAH. SAH-synthetic activity was estimated by the increase in SAH, and SAH-hydrolytic activity was estimated by the decrease in SAH.

**Extraction of total RNA.** Total RNA in each homogenate was isolated by the acid guanidium-phenol-chloroform method, using TRIZol (Invitrogen Corporation, Carlsbad, CA, USA). RNA concentration was determined by absorption at 260 nm using a Hitachi U-2001 spectrophotometer (Tokyo, Japan).

**Assay for expression of SAH hydrolase mRNA.** Expression of SAH hydrolase mRNA was determined by RT-PCR using a TaKaRa One Step RNA PCR Kit (TaKaRa Bio Inc., Otsu, Japan). The RT-PCR process partly followed the method of Ohmori et al. (15). The primer for SAH hydrolase was as follows: the upstream primer sequence was 5¢-AAGCTGCCATGGAGGGCTACGA-3¢, and the downstream primer sequence was 5¢-GATGGC-TGCTGGAAGGTTAAGG-3¢. For the primer of β-actin (used as an invariant control), the β-actin RT-PCR Primer set (Toyobo., Ltd. Osaka, Japan) was used.

The samples were amplified by 30 PCR cycles, where each consisted of denaturation at 94°C for 60 s, annealing at 58°C for 60 s and extension at 72°C for 75 s. Each PCR product was resolved by electrophoresis on 1% agarose gel stained with ethidium bromide, and photographed under UV light. Band intensity was evaluated by a NIH image program, which was developed by the U.S. National Institute of Health.

**Statistical analysis.** The statistical difference among means was estimated at p<0.05 according to ANOVA and Duncan’s multiple-range test (16).

## RESULTS

**Effect of B<sub>6</sub> deficiency on growth parameters and liver weight**

The final body weight, body weight gain and total food intake are shown in Table 2. The parameters of the B<sub>6</sub>-def. group were the lowest of the three groups. Liver

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**Table 1. Composition of the experimental diets (g/kg).**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control and PF.</th>
<th>B&lt;sub&gt;6&lt;/sub&gt;-def.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (vitamin-free)</td>
<td>700</td>
<td>700</td>
</tr>
<tr>
<td>Sucrose</td>
<td>173</td>
<td>173</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Vitamin mix. (AIN-76)</td>
<td>10</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin B&lt;sub&gt;6&lt;/sub&gt;-free vitamin mix.</td>
<td>—</td>
<td>10</td>
</tr>
<tr>
<td>Mineral mix. (AIN-76)</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

1 Pair-fed group: Control diet was pair-fed to the B<sub>6</sub>-deficient group.
2 Vitamin B<sub>6</sub>-deficient group: Vitamin B<sub>6</sub>-deficient diet was fed ad libitum.
3 Composition of vitamins except vitamin B<sub>6</sub> basically followed the AIN-76 vitamin mixture.

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weights (g/100 g body weight) in the B<sub>6</sub>-def. group were higher than those of the P.F. group (Table 2). Urinary xanthurenic acid excretion as an index of B<sub>6</sub> deficiency (<sup>17</sup>) was significantly high in the B<sub>6</sub>-def. group, indicating B<sub>6</sub> deficiency in this group (data not shown).

Concentrations of plasma PLP and Hcy

In general, it is known that the plasma PLP concentration is the most suitable index for internal B<sub>6</sub> nutrition (<sup>18</sup>). Concentrations of plasma PLP after the feeding period are shown in Fig. 1. The plasma concentrations of PLP in the B<sub>6</sub>-def. group were lower than those of the other groups. This indicates that the B<sub>6</sub>-def. group did not hold enough B<sub>6</sub> in the body.

Concentrations of plasma Hcy are shown in Fig. 2. It was significantly higher in the B<sub>6</sub>-def. group than in the other groups. Hcy concentration was about two times higher in the B<sub>6</sub>-def. group as compared to those in the other groups. This would be caused by impaired CBS and H<sub>9253</sub>-cystathionase activities in the liver due to B<sub>6</sub> deficiency, as reported previously (<sup>19</sup>).

Contents of SAM and SAH in liver

The content of SAM in the livers of the B<sub>6</sub>-def. group was significantly lower than that of the control group; however, it was almost the same as in the P.F. group (Fig. 3). On the other hand, the content of hepatic SAH was significantly higher in the B<sub>6</sub>-def. group than in that of the other groups (Fig. 3).

Expression of SAH hydrolase mRNA

The expression of SAH hydrolase mRNA was evaluated by RT-PCR. There were no significant differences in mRNA expression in the livers among the three groups (data not shown).

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### Table 2. Growth parameters and tissue weights of the experimental rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>P.F.</th>
<th>B&lt;sub&gt;6&lt;/sub&gt;-def.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Final body weight (g)</td>
<td>303±11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>152±2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>146±9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>194±10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>43±4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>37±10&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total food intake (g)</td>
<td>515±17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>268±3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>254±15&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Liver weight (g/100 g B.W.)</td>
<td>2.82±0.41&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.12±0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.92±0.18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are means±SE for six rats.  
1,2 See the legend for Table 1.  
Values without a common superscript letter are significantly different at p<0.05.
DISCUSSION

Nguyen et al. reported abnormal methionine metabolism using B6-deficient rats (8), in which the accumulation of SAH and increase of SAH-synthetic activity in both the liver and thymus were observed. There were two explanations for the mechanism of SAH accumulation. One explanation is the inhibition of SAH hydrolysis by adenosine, because SAH hydrolase is an adenosine binding protein and bound adenosine was reported to inhibit the activity (20). Another explanation is due to enhancement of the SAH-synthetic rate from adenosine and Hcy. According to previous results (8), it was presumed that SAH accumulation was caused by the increased SAH-synthetic activity of SAH hydrolase. However, we could not ascribe SAH accumulation to the increased activity of SAH synthesis, because this enzyme is a reversible one. Namely, we could not explain the accumulation of SAH until we measured both directions of SAH hydrolase activity.

In this study, we observed a significant decrease of PLP in the plasma (Fig. 1) and accumulation of SAH in the liver (Fig. 3) of the B6-def. group, and these results were similar to a previous report (8). On the other hand, the content of SAM in the liver was significantly lower in the B6-def. and PF groups than in the control group, which would reflect the amount of methionine supplied from the diet.

As noted above, SAH hydrolase is a reversible enzyme in the methionine metabolism and catalyzes the conversion between SAH and Hcy. Although the equilibrium dynamics of the SAH hydrolase reaction strongly favor SAH formation rather than SAH hydrolysis, the reaction under the physiological conditions was supposed to proceed in the hydrolytic direction of SAH because adenosine was removed rapidly through adenosine deaminase or adenosine kinase (21–23), and Hcy was consumed via transsulfuration and remethylation so as to be removed from this system (5). In this experiment, we measured the activity of SAH hydrolase in both directions. SAH-synthetic activity of SAH hydrolase was significantly higher in the livers of the B6-def. group; however, the SAH-hydrolytic activity of SAH hydrolase in this group did not show a significant increase (Table 3). Although the same enzyme catalyzes the hydrolysis and synthesis of SAH, the changes in both activities were not parallel. These results indicate that SAH-synthetic activity increased, in particular. Furthermore, we examined hepatic SAH hydrolase mRNA expression. There was no significant difference in SAH hydrolase mRNA expression in the livers among the three groups. It was confirmed that B6 deficiency did not affect SAH hydrolase at the mRNA level. Therefore the accumulation of SAH in B6 deficiency would be caused by an increase in the SAH-synthetic activity of SAH hydrolase because the SAH-hydrolytic activity of SAH hydrolase was not increased and there was no significant difference in the expression of SAH hydrolase mRNA among the three groups.

On the other hand, we observed an increase of plasma Hcy in the B6-def. group (Fig. 2). Smolin and Benevenga (24) reported the accumulation of plasma Hcy under B6 deficiency using the model for CBS-deficient rats. She et al. (19) also reported that CBS and γ-cystathionase were greatly diminished in the livers of the B6-def. group, and suggested that the accumulation of Hcy was derived from B6 deficiency. It was reported that excess methionine impaired the Hcy metabolism in B6-deficient rats (25). In our study, we added methionine at 1% into the diet and excess methionine might be metabolized through an unusual fashion because the enzyme activities involved in the transsulfuration pathway were significantly decreased by B6 deficiency (19). Under such conditions, there was an increase in the Hcy concentration, and the resulting increase needed to be metabolized by either remethylation or a SAH-synthetic reaction. The remethylation pathway was reported to be suppressed by excess methionine intake (25). Therefore, under B6 deficiency, accumulated Hcy might be re-converted to SAH again due to limitations of the remethylation pathway together with a diminished transsulfuration pathway. This would be the circumstances under which the SAH-synthetic activity of SAH hydrolase needs to increase.

In in vitro experiments, some factors were reported to be involved in the increase of SAH hydrolase in SAH-synthetic direction. Rat liver SAH hydrolase is a homotetrametric enzyme which contains NAD+ as a co-factor, and its activity is regulated by the oxidation-reduction cycle of the enzyme-bound NAD+ (26). Kloor et al. (27) reported that the SAH-synthetic activity of SAH hydrolase increased in the presence of phosphate at physiological concentrations in bovine kidney. They suggested that the phosphate would facilitate adenosine-mediated conversion of enzyme-bound NAD+ to NADH. Furthermore, Finkelstein and Harris (28)

Table 3. Activity of SAH hydrolase in the livers of the experimental rats.

<table>
<thead>
<tr>
<th>Activity (nmol/mg protein/min)</th>
<th>Control</th>
<th>PF 1</th>
<th>B6-def. 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAH hydrolysis</td>
<td>6.1±1.3</td>
<td>6.2±1.4</td>
<td>7.3±1.0</td>
</tr>
<tr>
<td>SAH synthesis</td>
<td>22.3±2.6</td>
<td>26.8±0.8</td>
<td>37.7±1.3</td>
</tr>
</tbody>
</table>

Values are means±SE for six rats.
1,2 See the legend for Table 1.
Values without a common superscript letter are significantly different at p<0.05.
SAH-synthetic activity was estimated by increased SAH, and SAH-hydrolytic activity was estimated by decreased SAH. For the details about the assay, see “Materials and Methods.”
reported that hormone treatment caused a marked change in the SAH-synthetic activity of SAH hydrolase. It is also reported that B6 binds to steroid hormone receptors and controls steroid hormone functions. These factors might be involved in the specific increase of SAH-synthetic activity of SAH hydrolase observed in this study.

In summary, we measured methionine metabolites and SAH hydrolase activity to determine the mechanism of SAH accumulation in B6-deficient rats. We observed SAH accumulation and a significant increase of SAH-synthetic activity of SAH hydrolase in the liver although SAH-hydrolytic activity showed no significant increase. Furthermore, there was no significant difference in the hepatic mRNA level of SAH hydrolase. Thus, SAH hydrolase mRNA was not involved in the changes in SAH hydrolase. We assume that the observed non-parallel increase in the SAH-synthetic activity of SAH hydrolase might be a cause of SAH accumulation. Further research is needed to clarify the mechanism why the SAH concentration and SAH-synthetic activity of SAH hydrolase alone were significantly enhanced in the liver under our experimental conditions.

REFERENCES