Nutritional Factors That Affect the Formation of 5-Aminolevulinic Acid, a Key Intermediate of Heme Biosynthesis

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Summary 5-Aminolevulinic acid (ALA) is a key intermediate of heme biosynthesis, which is an essential component of the respiratory chain. Therefore, nutrients that affect ALA biosynthesis eventually affect ATP production, which is the basis of mitochondrial function. Although the effects of various non-nutrient components that affect ALA after biosynthesis have been reported, there are few reports on the effects of dietary amino acids/protein on ALA formation and the effects of dietary vitamins that are involved in amino acid metabolism. In mitochondria, ALA is synthesized from succinyl-CoA and glycine by the pyridoxal phosphate-dependent enzyme ALA synthase [EC 2.3.1.37]. In this study, the effects of dietary amino acids/protein and vitamins on the amount of ALA synthesized were investigated using mice, rats, and cultured cells. Amounts of ALA in plasma and urine, and porphyrins in plasma increased with increasing protein intake. Vitamin B1 insufficiency did not affect ALA synthesis. Vitamin B6 insufficiency increased the amount of ALA synthesized, while niacin deficiency markedly reduced ALA synthesis. Thus, for heme synthesis, an essential biological substance for life, the amounts of amino acids, as well as the pathways metabolizing amino acids to glycine and succinyl-CoA are very important. Specifically, it is important that niacin is associated with the formation of glycine and succinyl-CoA from amino acids.

Key Words 5-aminolevulinic acid, vitamins, protein, porphyrin, heme

The most important mitochondrial function is the production of ATP. Heme is a pivotal component of ATP production. Hemoglobin, myoglobin, cytochromes, catalase, nitric oxide synthases, and some oxygenases contain heme as a prosthetic group. Heme is a complex of protoporphyrin IX and Fe2+. Protoporphyrin IX is biosynthesized from glycine and succinyl-CoA (Figs. 1 and 2). 5-Aminolevulinic acid (ALA) is a rate-limiting compound in the protoporphyrin IX synthetic pathway (1) and, in mitochondria, is synthesized from succinyl-CoA and glycine (2) by the pyridoxal phosphate (PLP)-dependent enzyme ALA synthase (ALAS, [EC 2.3.1.37]) (3). Succinyl-CoA is mainly supplied from acetyl-CoA and oxaloacetic acid via citric acid, but the formed succinyl-CoA is used for reproduction of oxaloacetic acid, and not for ALA biosynthesis. For ALA biosynthesis, extra succinyl-CoA must be supplied from other pathways. Amino acids, such as valine, isoleucine, and methionine supply succinyl-CoA via propionyl-CoA (Fig. 1). Another possible supplier of succinyl-CoA is glutamic acid via 2-oxoglutaric acid (Fig. 1). In addition, the B-group vitamins are involved in metabolism of these amino acids (Fig. 1). Although recent studies have reported the effect of various non-nutritive components on heme biosynthesis from ALA (4), little data are available on the effects of dietary amino acids/protein and vitamins on ALA formation. In the present study, the influence of amino acids/protein intake, vitamin B1 insufficiency, vitamin B6 insufficiency, or niacin deficiency on the synthesis of ALA itself was investigated.

MATERIALS AND METHODS

Chemicals. Casein from vitamin-free milk, L-methionine, sucrose, thiamin chloride hydrochloride (C12H17ClN4OS-HCl, molecular weight [MW] = 337.27), riboflavin (C17H20N4O6, MW = 376.37), nicotinamide (C6H6N2O, MW = 122.13), and nicotinamide N-oxide (C6H6N2O2, MW = 138.12) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Corn oil was obtained from Ajinomoto (Tokyo, Japan). A mineral mixture (AIN-93-G-MX) (5) and a vitamin mixture (AIN-93-VX) (5) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). 4-Pyridoxic acid (4-PIC) (C6H7NO4, MW = 183.16), manufactured by ICN Pharmaceuticals (Costa Mesa, CA, USA), was obtained from FUJIFILM Wako Pure Chemical Corporation. ALA hydrochloride (C5H5NO3-HCl, MW = 167.6) and protoporphyrin IX (C34H34N4O4, MW = 562.66) were purchased from Sigma Chemicals (St. Louis, MO, USA), and 1-methylnicotinamide (MNA) chloride (C7H9N2O-HCl, MW = 159.61) was obtained from Tokyo Chemical In-
N\textsuperscript{1}-Methyl-2-pyridone-5-carboxamide (2-Py) (C\textsubscript{7}H\textsubscript{8}N\textsubscript{2}O\textsubscript{2}, MW 152.15) (6) and N\textsuperscript{1}-methyl-4-pyridone-3-carboxamide (4-Py) (C\textsubscript{7}H\textsubscript{8}N\textsubscript{2}O\textsubscript{2}, MW 152.15) (7) were synthesized as described. All other chemicals were of the highest purity available from commercial sources.

Animals and diets. In “Experiment 1,” the care and treatment of experimental animals conformed to Konan Women’s University guidelines for the ethical treatment of laboratory animals (Approval number, No. 3201907) and in “Experiments 2, 3, 4, and 5,” to the University of Shiga Prefecture guidelines (Approval number, No. 26-2). Room temperature was maintained at approximately 22˚C and humidity at approximately 60% with a 12/12-h light/dark cycle (06:00–18:00/18:00–06:00). The diets used in these experiments did not contain preformed niacin because mice and rats fed diets containing 20% casein biosynthesize sufficient nicotinamide (8, 9). Body weight and food intake were measured daily at approximately 09:00, and food and water were renewed daily.

Dietary protein levels in rats (Experiment 1). Five-week-old male Wistar rats were purchased from CLEA Japan, Inc. (Tokyo, Japan) and placed individually in metabolic cages (CL-0353; CLEA Japan, Inc.). Rats were divided into three groups and fed a 20%, 40%, or 70% casein diet (Table 1) ad libitum for 14 d. Twenty-four-hour urine samples were collected from 18:00 on day 13 to 18:00 on day 14 into amber colored bottles containing 1 mL 1 mol/L HCl and stored at 220˚C until use. At 09:00 on day 15, all rats were euthanized under anesthesia of pentobarbital and isoflurane. Blood samples were collected into a tube containing heparin and centrifuged at 3,000 \times g for 10 min at room temperature to obtain plasma. Plasma samples were stored at −20˚C. The liver of each rat was harvested and stored at −80˚C.

Vitamin B\textsubscript{1}-insufficiency in mice (Experiment 3). Five-
week-old female ICR mice were purchased from Charles River Laboratories and housed individually in metabolic cages. Mice were divided into two groups, and fed a control diet and a vitamin B1-free diet ad libitum for 7 d (Table 1). Twenty-four-hour urine samples were collected from 09:00 on day 7 to 09:00 on day 8 in amber colored bottles containing 1 mL 1 mol/L HCl and stored at \(-20^\circ\)C until use.

**Vitamin B6-Insufficiency in mice (Experiment 4).** Nine-week-old male C57BL6/J mice were purchased from Charles River Laboratories and housed individually in metabolic cages. Mice were divided into two groups, and fed a control diet and a vitamin B6-free diet ad libitum for 23 d. Twenty-four-hour urine samples were collected from 09:00 on day 23 to 09:00 on day 24 into amber colored bottles containing 1 mL 1 mol/L HCl and stored at \(-20^\circ\)C until use.

**Niacin-deficiency in mice (Experiment 5).** Nine-week-old male C57BL6/J mice (wild-type; Charles River Japan) and 9- to 10-wk-old male quinolinic acid phosphoribosyltransferase knock-out (QPRT-KO) mice, unable to synthesize nicotinamide from the dietary tryptophan contained in casein (8), were housed individually in metabolic cages. All mice had free access to a chemically defined 20% casein diet (Table 1) for 27 d. Twenty-four-hour urine samples were collected from 09:00 on day 27 to 09:00 on day 28 into amber colored bottles containing 1 mL 1 mol/L HCl and stored at \(-20^\circ\)C until use.

**Cell culture (Experiment 6).** The human hepatocellular carcinoma (HepG2) cell line was obtained from the Japanese Collection of Research Bioresources Cell Bank (JCRB1054) (Osaka, Japan) and cultured in low-glucose Dulbecco’s modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO2. Cells were harvested after culture in amino acid-free DMEM (FUJIFILM Wako Pure Chemical Corporation) supplemented with 1× or 2× amino acid solution (Sigma Aldrich, St. Louis, MO, USA) for 24 h and were immediately used.

**Measurements of B-group vitamins in urine.** Acidified frozen urine samples were thawed and centrifuged at 10,000 \(\times g\) for 10 min at 4°C. Each supernatant sample was used for analysis of the B-group vitamins.

Thiamin (10, 11), riboflavin (12), 4-PIC (13), Nam
and its catabolites (Nam N-oxide, 2-Py, and 4-Py) (14), and MNA (15) were measured as described.

Measurement of ALA in plasma and urine. Frozen plasma samples were thawed and an equivalent amount of 25% trichloroacetic acid solution was added and centrifuged at 10,000 × g for 10 min at 4˚C. Frozen acidified urine samples were thawed and centrifuged at 10,000 × g for 10 min at 4˚C. Both supernatants were withdrawn and used for ALA analysis using the method of Ota et al. (16). Briefly, 0.02 mL of the supernatant samples was mixed with 0.23 mL water, 0.25 mL 200 mmol/L CH₃COOH-CH₃COONa buffer (pH 3.8), 1.25 mL acetylacetone reagent (acetylacetone : ethanol : 68.4 mmol/L NaCl, 3 : 2 : 15), and 0.25 mL 3.3% formaldehyde, and the mixture was immersed in boiling water for 15 min. After cooling in ice water, the reaction mixture was centrifuged at 10,000 × g for 5 min at 4˚C. A total of 20 μL of the resulting supernatant was passed through a 0.45-μm microfilter and applied to a high-performance liquid chromatography system (LC-2040C-Plus, Shimadzu Co., Kyoto, Japan), consisting of a Tosoh TSK-GEL ODS-80Ts (average particle size, 5 μm, 250 mm×4.6 mm inner diameter) column maintained at 40˚C, an elution phase of 0.63 mmol/L acetic acid containing 45% methanol (flow rate, 0.6 mL/min), and a fluorescence detector (excitation wavelength 363 nm, emission wavelength 473 nm).

Measurement of ALAS1 activity. ALAS1 activity in the rat liver and HepG2 cells was measured using the method of Yasuda et al. (17). ALA production amount was determined similar to that of the urine samples (16).

Measurement of total porphyrins. Porphyrin concentration in plasma and liver were measured according to the method of Westerlund et al. (18). Protoporphyrin IX was used as a standard.

Biochemical analysis. The concentrations of glucose, triglyceride, cholesterol, and creatinine in plasma were measured using glucose, triglyceride, cholesterol, and creatinine tests (FUJIFILM Wako Pure Chemical Corporation). Plasma insulin concentration was measured using an ultra-sensitive rat insulin ELISA kit (Morinaga, Kanagawa, Japan).

Real-time polymerase chain reaction (PCR) analysis. Total RNA was isolated from the frozen rat liver and cultured cells using RNAiso Plus (Takara Bio, Shiga, Japan). Subsequently, cDNA was synthesized from the RNA template using Moloney murine leukemia virus, according to the standard method. SYBR® green-based quantitative real-time PCR was performed using the synthesized cDNA and an appropriate primer set for each target gene with a StepOne™ real-time PCR system (Applied Biosystems, Foster City, CA, USA).

Western blotting. Tissue was lysed in cold radioimmunoprecipitation assay buffer supplemented with a protease inhibitor cocktail. The extracted protein (20 μg) was separated on a 10% polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 h at room temperature and treated with each antibody: ALAS1 (Novus Biologicals, Centennial, CO, USA) or β-actin (Cell Signaling Technology, Beverly, MA, USA) overnight at 4˚C. After washing with Tris-buffered saline containing 0.05% Tween® 20, the membrane was incubated with an appropriate secondary antibody for 1 h at room temperature and visualized using a luminescent image analyzer (ImageQuant LAS-500, GE Healthcare, Tokyo).

Table 1. Diet compositions.

| Diet Composition                        | NiA-free | NiA-free | NiA-free | NiA-free | NiA-free |
|-----------------------------------------|----------|----------|----------|----------|----------|
| 20% casein diet (Control diet)          | 200      | 400      | 700      | 200      | 200      |
| 40% casein diet                         | 463      | 336      | 136      | 463      | 463      |
| 70% casein diet                         | 240      | 167      | 67       | 240      | 240      |
| 20% casein vitamin B₃-free diet         | 50       | 50       | 50       | 50       | 50       |
| NiA-free vitamin mixture (AIN-93)³     | 35       | 35       | 35       | 35       | 35       |
| NiA and vitamin B₁-free vitamin mixture (AIN-93)³ | 0 | 0 | 0 | 10 | 0 |
| NiA and vitamin B₆-free vitamin mixture (AIN-93)³ | 0 | 0 | 0 | 0 | 10 |
| t-Methionine                            | 2        | 2        | 2        | 2        | 2        |
| Total                                   | 1,000    |          |          |          |          |

¹ Ref. 5).
NiA: nicotinic acid.
Animals do not need preformed niacin for optimum growth when fed a 20% casein diet (8, 9).
The band was quantified using ImageJ software version 1.52.

Statistical analysis. Values are expressed as the mean ± standard error (SE). Statistical significance was determined using one-way analysis of variance, followed by Tukey’s multiple comparison test (Tables 2–5, Figs. 3–6) and by Student’s t-test (Tables 6–8, Fig. 7). Pearson correlation coefficients were calculated for the data shown in Figs. 4 and 6. A p value < 0.05 was considered significant. All analyses were performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

Effects of dietary protein levels on basal nutritional variables in rats (Experiment 1)

In the rat experiment, as shown in Table 2, final body weight, body weight gains, and food intake are slightly or significantly lower in the 70% casein diet group compared to those of the 20% and 40% casein diet groups. However, the food efficiency ratio (weight gain/food intake) did not differ among the groups. Mass of the liver, epididymal fat, and gastrocnemius muscle were not affected by amount of dietary protein intake (Table 2). A high protein diet did not affect plasma glucose, insulin, triglyceride, and cholesterol concentrations (Table 3).

Effect of dietary protein levels on the ALA content in rats (Experiment 1)

In rats, urinary ALA excretion significantly increased according to dietary protein content (Fig. 3A). Plasma ALA concentrations were significantly increased in the 70% casein diet group compared to that in the 20% and 40% casein diet groups (Fig. 3B). Hepatic ALA concentration was not increased according to the dietary protein content (Fig. 3C).

Effect of dietary protein levels on urinary vitamin excretion in rats (Experiment 1)

As described in Fig. 3A, the urinary excretion of ALA was increased according to protein intakes, we analyzed the relationship between urinary excretion of ALA and urinary excretion of each of the B-group vitamins in rats, instead of the relationship between protein intakes. In Fig. 4, the left hand closed circle is the data for the
Fig. 3. Effects of dietary protein content on 5-aminolevulinic acid (ALA) content in rats (Experiment 1). Rats were fed a 20%, 40%, or 70% casein diet. Urine samples (24 h urine) were collected on the last day of the experiment. Samples of plasma and liver were collected after urine samples were collected. (A) Urine, (B) Plasma, (C) Liver. Each value is the mean ± SE for five animals; a different superscript letter in the same figure indicates a significant difference; p < 0.05.

Fig. 4. Relationship between urinary excretion of 5-aminolevulinic acid (ALA) and each of the B-group vitamins in rats (Experiment 1). The left hand closed circle is the data for the group of 20% casein diet, the middle circle is 40% casein diet, and the right hand circle is 70% casein diet in each graph. (A) Thiamin, (B) Riboflavin, (C) 4-Pyridoxic acid (4-PIC), (D) SUM. Each value is the mean ± SE for five rats; a different superscript letter in the same figure indicates a significant difference: p < 0.05. SUM = nicotinamide + N^1-methylnicotinamide + N^1-methyl-2-pyridone-5-carboxamide + N^1-methyl-4-pyridone-5-carboxamide.
345

5-Aminolevulinic Acid and Vitamins in Urine

In each graph.

Thiamin excretion was low only in the 70% casein diet group and did not have a correlation with urinary ALA levels (Fig. 4A). Riboflavin excretion was not changed among the three groups and did not have a correlation with urinary ALA levels (Fig. 4B). The correlation between urine 4-PIC and ALA is shown in Fig. 4C. The correlation between urine SUM (sum of nicotinamide and its metabolites) and ALA is shown in Fig. 4D.

When analyses were performed with an individual rat instead of among of groups as shown in Fig. 4, much more significant relationship was observed between ALA and 4-PIC ($p=0.0009$), and between ALA and SUM ($p=0.016$).

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**Table 4.** Water intake, urine volume, and plasma creatinine levels in rats fed 20%, 40%, or 70% casein diets (Experiment 1).

|            | 20% casein diet group | 40% casein diet group | 70% casein diet group |
|------------|-----------------------|-----------------------|-----------------------|
| Water intake (g/d) | 21±2.5$^a$            | 34±5.3                | 57±9.5$^b$            |
| Urine volume (mL/d) | 4.7±0.6$^a$            | 13.1±0.8$^b$         | 23.5±1.4$^c$         |
| Plasma creatinine (mg/dL) | 0.35±0.03            | 0.36±0.05             | 0.32±0.03             |

Each value is the mean±SE for five mice; a different superscript letter in the same row indicates a significant difference; $p<0.05$.

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**Effect of dietary protein levels on ALAS1 in rat liver (Experiment 1)**

Mammals have two ALAS isozymes, one is the ubiquitous housekeeping ALAS1 and the other is erythroid-specific, ALAS2 (19, 20). ALAS1 mRNA expression levels were not affected by dietary protein levels (Fig. 5A). The ALAS1 protein concentration (Fig. 5B) and ALAS1 activity (Fig. 5C) increased according to dietary protein levels.

**Effect of dietary protein levels on porphyrin content in rat liver and plasma (Experiment 1)**

Total porphyrin levels in the livers did not change among the three groups (Table 3). The 70% casein diet group showed high total porphyrin concentrations in plasma (Table 3).

**Effect of high protein diet on kidney function in rats (Experiment 1)**

Unzu et al. (21) have indicated that renal insufficiency influences hepatic ALAS1. In the present study,
rats fed a high protein diet for 14 d enhanced water intake and urine volume, but did not affect plasma creatinine concentration (Table 4).

Effects of dietary protein levels on basal nutritional variables and urinary ALA excretion in ICR mice (Experiment 2)

Mice fed 20%, 40%, or 70% casein diets for 15 d had little effect on food intake and body weight (Table 5). Same as rats, urinary ALA levels were also increased by dietary protein intake (Fig. 6A) and had positive correlation with urinary SUM levels (Fig. 6B) in mice.

Table 5. Basal nutritional parameters in ICR mice fed 20%, 40%, or 70% casein diets (Experiment 2).

|                        | 20% casein diet group | 40% casein diet group | 70% casein diet group |
|------------------------|-----------------------|-----------------------|-----------------------|
| Initial body weight (g) | 20.60±0.52            | 20.40±0.27            | 20.80±0.50            |
| Final body weight (g)   | 24.3±0.65             | 23.4±0.35             | 23.3±0.64             |
| Final body weight change (g/15 d) | 3.80±0.38± | 3.10±0.17± | 2.60±0.33± |
| Total food intake during the experiment (g/15 d) | 65.1±2.5     | 71.5±5.3             | 69.6±2.8             |

Each value is the mean±SE for five mice; a different superscript letter in the same row indicates a significant difference; p<0.05.

Fig. 6. Effects of dietary protein content on relationship between urinary excretion of 5-aminolevulinic acid (ALA) and SUM in mice (Experiment 2). (A) Relationship between dietary protein level and urinary excretion of ALA. (B) Relationship between urinary excretion of ALA and urinary excretion of SUM. Mice were fed a 20%, 40%, or 70% casein diet. Urine samples (24 h urine) were collected on the last day of the experiment. Each value is the mean±SE for five mice; a different superscript letter in the same figure indicates a significant difference; p<0.05. SUM=nicotinamide+nicotinamide N-oxide+N\textsuperscript{1}-methylnicotinamide+N\textsuperscript{1}-methyl-2-pyridone-5-carboxamide+N\textsuperscript{1}-methyl-4-pyridone-5-carboxamide.

Fig. 7. 5-Aminolevulinic acid synthase (ALAS1) activity and mRNA expression in HepG2 cells treated with various amounts of amino acids (Experiment 6). (A) ALAS1 activity. (B) ALAS1 mRNA expression. Each value is the mean±SE (n=4). *p<0.05 by Student’s two-tailed t-test.

Effect of amino acid mixture addition on ALAS activity and mRNA expression in HepG2 cells (Experiment 6)

In HepG2 cells supplemented with amino acid solution, ALAS1 activity was also significantly increased (Fig. 7A), although ALAS1 mRNA expression was not changed (Fig. 7B).

Effects of a vitamin B\textsubscript{1}-free diet on urinary ALA excretion in ICR mice (Experiment 3)

Mice fed a 20% casein diet without vitamin B\textsubscript{1} for 7 d did not experience reductions in food intake and body weight (Table 6), but showed significant reductions in urinary vitamin B\textsubscript{1} excretion (Table 6). Vitamin B\textsubscript{1} insufficiency, however, did not alter urinary ALA excre-
5-Aminolevulinic Acid and Vitamins in Urine

Effects of a vitamin B6-free diet on urinary ALA excretion in C57BL6/J mice (Experiment 4)

Mice fed a 20% casein diet with or without vitamin B6 for 23 d showed no significant between-group differences in body weight gain and food intake (Table 7). In contrast, the urinary vitamin B6 excretion was significantly lower, while the urinary ALA excretion was higher in mice fed the vitamin B6-free diet (Table 7).

Table 6. Body weight changes, food intake, and urinary excretion amounts of thiamin and ALA in ICR mice fed a 20% casein diets with or without vitamin B1 (Experiment 3).

|                      | Control diet group       | Vitamin B1-free diet group   |
|----------------------|--------------------------|-----------------------------|
| Initial body weight (g) | 24.44±0.84               | 23.76±0.59                  |
| Final body weight (g)  | 26.22±1.09               | 24.80±0.59                  |
| Final body weight change (g/7 d) | 1.78±0.32               | 1.20±0.33                  |
| Total food intake during the experiment (g/7 d) | 29.2±0.8                | 25.7±0.9                   |
| Urine thiamin (nmol/d) | 41.6±7.6                 | 1.39±0.24*                  |
| Urine ALA (nmol/d)    | 36.4±10.0                | 41.1±10.7                  |

ALA: 5-aminolevulinic acid.
Each value is the mean±SE for five mice.
*p<0.05 by Student’s two-tailed t-test.

Table 7. Body weight changes, food intake and urinary excretion of 4-PIC and ALA in C57BL6/J mice fed a 20% casein diet with or without vitamin B6 (Experiment 4).

|                      | Control diet group       | Vitamin B6-free diet group   |
|----------------------|--------------------------|-----------------------------|
| Initial body weight (g) | 17.8±1.57                | 19.7±0.55                   |
| Final body weight (g)  | 22.0±2.10                | 23.6±0.91                   |
| Final body weight change (g/23 d) | 4.12±1.20               | 3.90±0.30                   |
| Total food intake during the experiment (g/23 d) | 83.3±6.88               | 90.7±3.92                   |
| Urine 4-PIC (nmol/d)  | 67.9±11.4                | 1.2±0.7*                    |
| Urine ALA (nmol/d)    | 31.2±3.4                 | 48.5±6.3*                   |

4-PIC: 4-pyridoxic acid.
Each value is the mean±SE for five mice.
*p<0.05 by Student’s two-tailed t-test.

Table 8. Body weight changes, food intake, and urinary excretion of SUM and ALA in wild-type and QPRT-KO C57BL6/J (niacin-deficient) mice fed a 20% casein diet without nicotinic acid (Experiment 5).

|                         | WT mice                     | QPRT-KO mice               |
|-------------------------|----------------------------|---------------------------|
| Initial body weight (g) | 19.88±3.61                 | 19.32±0.77                |
| Final body weight (g)   | 22.40±3.63                 | 17.59±0.77*               |
| Final body weight change (g/27 d) | 2.53±0.75               | −1.71±0.50*               |
| Total food intake during the experiment (g/27 d) | 94.2±2.8                | 67.3±1.6*                 |
| SUM (nmol/d)            | 639±142                    | 1.24±0.48*                |
| ALA (nmol/d)            | 32.2±5.0                   | 10.9±1.6*                 |

SUM=nicotinamide+nicotinamide N-oxide+N1-methylnicotinamide (MNA)+N1-methyl-2-pyridone-5-carboxamide (2-Py)+N1-methyl-4-pyridone-5-carboxamide (4-Py).
Each value is the mean±SE for five mice.
*p<0.05 by Student’s two-tailed t-test.

Effects of a nicotinic acid-free diet on urinary ALA excretion in QPRT-KO mice (Experiment 5)

QPRT is the enzyme that connects the complete tryptophan degradation pathway and the nicotinamide adenine dinucleotide (NAD) cycle and converts quinolinic acid, formed during the complete degradation of tryptophan, to nicotinic acid mononucleotide in the presence of 5-phosphoribosyl-1-pyrophosphate. Therefore, QPRT-KO mice become niacin deficient when fed a 20% casein diet without niacin. QPRT-KO mice fed a niacin-free, 20% casein diet for 27 d showed reduced food intake
and body weight compared to that of wild-type mice (Table 8). The urinary concentration of nicotinamide and its catabolites was almost zero in the QPRT-KO group. Under such nutritional conditions, urinary ALA excretion was lower in QPRT-KO mice than that in wild-type mice (Table 8).

**DISCUSSION**

ALA is a key intermediate in heme biosynthesis; there are many reports on the ALAS enzyme (19, 20). However, few reports are available from a nutritional standpoint; for example, effects of the amount of proteins and vitamins on ALA biosynthesis. The present study showed that increasing dietary protein intake enhanced urinary and plasma ALA and plasma porphyrin amounts without an influence on basal metabolic markers. In addition, vitamins, such as niacin and vitamin B6, were found to be crucial for the conversion of amino acids to direct precursors of ALA, such as glycine and succinyl-CoA. Therefore, the proper intake of protein and vitamins is essential for maintaining a high degree of mitochondrial function.

Increased urinary and plasma ALA excretion generally suggests surplus ALA formation compared to the required amount of heme in the body. ALAS1 activity in the liver increased with increasing protein intake in rats. Recent study has reported that urinary ALA excretion and hepatic ALAS1 mRNA expression are upregulated by renal dysfunction in mice (21). Another study shows that glucose or insulin administration downregulates ALAS1 mRNA expression in hepatocyte (22). In the present study, high protein diet did not affect plasma creatinine, glucose, and insulin levels. Moreover, hepatic ALAS1 mRNA expression levels were not changed by high protein intake in rats, although ALAS1 protein expression was increased. Therefore, this increased enzyme activity was due to upregulation of the ALAS1 protein rather than increased expression of ALAS1 mRNA. The results of HepG2 cells supplemented with amino acid solution suggest that ALAS1 activity is enhanced by increasing of amino acid or its metabolites. However, the detailed mechanism explaining why surplus ALA formation occurred in rats fed a high protein diet remains unclear.

Hauschildt (23) reports a curious finding on the relationship between vitamin B1 deficiency and heme synthesis, that vitamin B1 deficiency inhibits porphyrin synthesis but increases heme-containing enzymes levels. Vitamin B1 is a coenzyme of 2-oxoglutarate: glyoxylate carboligase [EC 2.2.1.5], which converts 2-oxoglutaric acid to 2-hydroxy-3-oxoadipic acid in the presence of glyoxylic acid (24) (Fig. 1). This enzyme is the same enzyme as 2-oxoglutarate: lipoate oxidoreductase [EC 1.2.4.2], which converts 2-hydroxy-3-oxoadipic acid to

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**Fig. 8. Heme catabolism.** (1) Heme oxygenase [EC 1.14.99.3], (2) biliverdin reductase [EC 1.3.1.24], (3) uridine 5′-diphospho (UDP)-glucuronosyltransferase [EC 2.4.1.17], (4) β-glucuronidase [EC 3.2.1.31].
5-hydroxy-4-pentanoic acid (25) (Fig. 1). 5-Hydroxy-4-pentanoic acid is oxidized to form 4,5-dioxypentanoic acid, and 4,5-dioxypentanoic acid is converted to ALA (26, 27).

In the present study, vitamin B1 insufficiency had no effect on ALA synthesis. A similar result has been reported by Stewart et al. (28), however, there is a report that vitamin B1 deficiency leads to significantly lower 2-oxoglutarate-glyoxylate carboligase activity (29).

In animals, ALA is synthesized by ALAS which is a PLP-dependent enzyme (30). Therefore, vitamin B6-free diet should reduce ALA production. However, the present study showed that urinary ALA was higher in vitamin B6-insufficient mice than that in control mice. ALA is reversibly metabolized to 4,5-dioxypentanoic acid and alanine in the presence of pyruvic acid by the PLP-dependent enzyme ALA transaminase [EC 2.6.1.43] (Fig. 1) (31). Elevated production of 4,5-dioxypentanoic acid would therefore decrease ALA levels. On the other hand, ALA is metabolized to porphobilinogen by the enzyme ALA dehydratase (ALAD, [EC 4.2.1.24]) which requires Zn^{2+} as a cofactor (32). ALAD deficiency causes ALAD-deficiency porphyria (33). Accordingly, the concentration of ALA is controlled by three enzymes. The current finding that vitamin B6 insufficiency increased ALA formation might be due to inhibition of the PLP-dependent ALA transaminase. In contrast, vitamin B6 insufficiency would have little effect on the activity of the PLP-dependent ALAS in the mitochondrial matrix. Further study is needed to verify this hypothesis.

Niacin deficiency markedly reduced ALA synthesis. Niacin is not involved in ALA synthesis and the subsequent heme synthesis, but niacin has a strong influence on the synthesis of 2-oxoglutaric acid, a precursor of succinyl-CoA. 2-Oxoglutaric acid is synthesized both from isocitric acid via the tricarboxylic acid (TCA) cycle and from glutamic acid. Glutamine, arginine, proline, and histidine are catabolized in the TCA cycle via the glutamate pathway and niacin functions as a coenzyme in many steps (Fig. 1). Succinyl-CoA is also synthesized from isoleucine, valine, and methionine via propionyl-CoA. This pathway is required for the B-group vitamins, including niacin and vitamin B12 (Fig. 1). Kawata et al. (34) have reported that vitamin B12 deficiency enhances urinary ALA excretion in rats. Considering this report (34), the major origin of succinyl-CoA for ALA synthesis is via the route of glutamic acid to 2-oxoglutaric acid, but not of propionyl-CoA.

The amount of urinary ALA excretion and the total excretion of nicotinamide and its catabolites had a very strong direct proportional relationship; niacin deficiency also caused lower ALA formation. Considering the present results and the highly direct proportional relationship between amounts of urinary ALA and nicotinamide catabolite excretion in a previous human observation study (35), and the NAD^{+}-dependent reactions of glutamic acid → 2-oxoglutaric acid → succinyl-CoA, there is a possibility that the ALA synthesis is strongly influenced by the metabolic fate of glutamic acid as the origin of succinyl-CoA.

The skin symptoms of pellagra dermatitis and porphyria are similar (36). Figure 8 shows the heme catalytic pathway. Reaction 1 (heme oxygenase [EC 1.14.99.3]) and reaction 2 (biliverdin reductase [EC 1.3.1.24]) require niacin as NADPH. Pellagra is caused by a niacin-deficiency. Thus, a niacin deficiency may cause accumulation of abnormal porphyrins, but this causal relationship will be explored in a future study.

In conclusion, these data highlight the pivotal role of amino acid/protein and vitamin intake, which is involved in the formation of glycine and succinyl-CoA required for heme synthesis, an essential biological substance for life. Specifically, niacin had a crucial role in the production of glycine and succinyl-CoA from amino acids.

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REFERENCES

1) Granick S, Urata G. 1963. Increase in activity of alphaminolevulinic acid synthetase in liver mitochondria induced by feeding of 3,5-dicarbethoxy-1,4-dihydrocollidine. J Biol Chem 238: 821–827.
2) Heinemann IU, Jahn M, Jhan D. 2008. The biochemistry of heme biosynthesis. Arch Biochem Biophys 474: 238–251.
3) Stojanovski BM, Ferreira GC. 2015. Asn-150 of murine erythroid 5-aminolevulinate synthase modulates the catalytic balance between the rates of the reversible reaction. J Biol Chem 290: 30750–30761.
4) Fujino M, Nishio Y, Ito H, Tanaka T, Li XK. 2016. 5-Aminolevulinic acid regulates the inflammatory response and alloimmune reaction. Int Immunopharmacol 37: 71–78.
5) Reeves PG. 1998. Components of the AIN-93 diets as improvements in AIN-76A diet. J Nutr 127: 8385–8418.
6) Pullman ME, Colowick SP. 1954. Preparation of 2- and
6-pyridones of N1-methylnicotinamide. J Biol Chem 206: 121–127.

7) Shibata K, Kawada T, Iwai K. 1998. Simultaneous micro-determination of nicotinamide and its major metabolites, N1-methyl-2-pyridone-5-carboxamide and N1-methyl-4-pyridone-3-carboxamide, by high-performance liquid chromatography. J Chromatogr 424: 23–28.

8) Terakata M, Fukuw66 atari T, Sano M, Nakao N, Sasaki R, Fukuoka S, Shibuta K. 2012. Establishment of true niacin deficiency in quinolinic acid phosphoribosyltransferase knockout mice. J Nutr 142: 2148–2153.

9) Shibata K, Mushiake M, Kondo T, Hayakawa T, Tsuge H. 1995. Effects of vitamin B6 deficiency on the conversion ratio of tryptophan to niacin. Biosci Biotechnol Biochem 59: 2060–2063.

10) Iswata H, Matsuda T, Tonomura H. 1988. Improved high-performance liquid chromatographic determination of thiamine and its phosphate esters in animal tissues. J Chromatogr 26: 317–321.

11) Shibata K, Hirose J, Fukuwatari T. 2014. Relationship between urinary concentrations of nine water-soluble vitamins and their vitamin intakes in Japanese adult males. Nutr Metab Insights 7: 61–75.

12) Ohkawa H, Ohishi N, Yagi K. 1983. New metabolites of riboflavin appear in human urine. J Biol Chem 258: 5623–5628.

13) Gregory JP 3rd, Kirk JR. 1979. Determination of urinary 4-pyridoxic acid using high performance liquid chromatography. Am J Clin Nutr 32: 879–883.

14) Maeta A, Sano M, Fukuwatari T, Shibata K. 2014. Simultaneous measurement of nicotinamide and its catabolites, nicotinamide N-oxide, N1-methyl-2-pyridone-5-carboxamide, and N1-methyl-4-pyridone-3-carboxamide, in mice urine. Biosci Biotechnol Biochem 78: 1306–1309.

15) Shibata K. 1987. Ultramicro-determination of N1-methylnicotinamide in urine by high-performance liquid chromatography. Vitamins 61: 599–604 (in Japanese).

16) Ota U, Fukuhara H, Ishizuka M, Abe F, Kawada C, Tamura K, Tanaka T, Inoue K, Osawa SI, Shuin T. 2015. Plasma protoporphyrin IX following administration of 5-aminolevulinic acid as a potential tumor marker. Mol Clin Oncol 3: 797–801.

17) Yasuda M, Gian L, Chen B, Kadirvel S, Yu C, Phillips JD, New MI, Liebow A, Fitzgerald K, Querbes W, Desnijck RJ. 2014. RNAi-mediated silencing of hepatic Alas1 effectively prevents and treats the induced acute attacks in acute intermittent porphyria mice. Proc Natl Acad Sci USA 111: 7777–7782.

18) Westerlund J, Pudek M, Schreiber WE. 1988. A rapid and accurate spectrophotometric method for quantification and screening of urinary porphyrins. Clin Chem 34: 345–351.

19) Hunter GA, Ferreira GC. 2009. 5-Aminolevulinate synthase: catalysis of the first step of heme biosynthesis. Cell Mol Biol 55: 102–110.

20) Stojanovski BM, Hunter GA, Na I, Uversky VN, Jiang RHY, Ferreira GC. 2019. 5-Aminolevulinate synthase catalysis: The catcher in heme biosynthesis. Mol Genet Metab 128: 178–189.

21) Unzu C, Sampedro A, Sardh E, Mauleón I, Salamanca RE, Prieto J, Salido E, Harper P, Fontanellas A. 2012. Renal failure affects the enzymatic activities of the three first steps in hepatic heme biosynthesis in the acute intermittent porphyria mouse. PLoS One 7: e32978.

22) Handschin C, Lin J, Rhee J, Peyer AK, Chin S, Wu PH, Meyer UA, Spiegelman BM. 2005. Nutritional regulation of hepatic heme biosynthesis and porphyrins through PGC-1α. Cell 122: 505–515.

23) Hauschildd S. 1975. The influence of thiamine deficiency on porphyrin synthesis and porphyrin proteins. J Nutr Sci Vitaminol 21: 403–410.

24) Stewart J, Quayle JR. 1967. The synergistic decarboxylation of glyoxylate and 2-oxoglutarate by an enzyme system from pig-liver mitochondria. Biochem J 102: 885–897.

25) Saito T, Tuboi S, Nishimura Y, Kikuchi G. 1971. On the nature of the enzyme which catalyzes a synergistic decarboxylation of alpha-ketoglutarate and glyoxylate. J Biol Chem 69: 265–273.

26) Kissel HJ, Heilmeyer L. 1969. Detection and determination of gamma, delta-dioxovaleric acid: reversible formation of gamma, delta-dioxovaleric acid and delta-aminolevulinic acid in rats. Biochim Biophys Acta 177: 78–87 (in German).

27) Porra RJ, Klein O. 1981. The determination of 4,5-dioxovaleric acid in plant preparations and a procedure for the assay of L-alanine:4,5-dioxovaleric acid aminotransferase (EC 2.6.1.43) activity. Anal Biochem 116: 511–518.

28) Stewart CB, Grammer J, Brosemer RW. 1981. Thiamine deficiency and glyoxyllic acid. Ann Nutr Metab 25: 289–298.

29) Sidhu H, Gupta R, Thind SK, Nath R. 1987. Oxalate metabolism in thiamine-deficient rats. Ann Nutr Metab 31: 354–361.

30) Kikuchi G, Kumar A, Talmage P, Shemin D. 1958. The enzymatic synthesis of 5-aminolevulinic acid. J Biol Chem 233: 1214–1219.

31) Gibson KD, Matthew M, Neuberger A, Tait GH. 1961. Biosynthesis of porphyrins and chlorophylls. Nature 192: 204–208.

32) Jaffe EK, Lawrence SH. 2012. Allosteric and the dynamic oligomerization of porphobilinogen synthase. Arch Biochem Biophys 519: 144–153.

33) Jaffe EK, Stith L. 2007. ALAD porphyria is a conformational disease. Am J Human Gen 80: 329–337.

34) Kawata T, Maekawa A, Suzuki T. 1982. Effect of vitamin B12 deprivation on the activities of 5-aminolevulinic acid synthase and 5-aminolevulinic acid dehydratase in rat liver. Vitamins 56: 409–412 (in Japanese).

35) Morita N, Shibata K. 2019. Relationship between urinary excretion amounts of 5-aminolevulinic acid and of urinary nicotinamide catabolites in humans. Bull Konan Women’s Univ II 13: 45–52.

36) Harber LC, Baer RL. 1972. Pathogenic mechanisms of drug-induced photosensitivity. J Invest Dermatol 58: 327–342.