Urinary Branched-Chain 2-Oxo Acids as a Biomarker for Function of B-Group Vitamins in Humans

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Summary To find a functional biomarker of B-group vitamins, we collected 24-h urine samples from young Japanese women who lived in the community (n=29) to measure branched-chain 2-oxo acids such as 2-oxo-3-methylbutanoic acid, 2-oxo-3-methylpentanoic acid, and 2-oxo-4-methylpentanoic acid because B-group vitamins are involved in the catabolism of branched-chain amino acids. The relationships between each pair of the three urinary 2-oxo acids were very high (2-oxo-3-methylbutanoic acid and 2-oxo-3-methylpentanoic acid, \( p<0.001 \); 2-oxo-3-methylbutanoic acid and 2-oxo-4-methylpentanoic acid, \( p<0.001 \); 2-oxo-3-methylpentanoic acid and 2-oxo-4-methylpentanoic acid, \( p<0.001 \)). The participants were divided into three groups using the upper (n=10), middle (n=9), and lower tertiles (n=10) based on the urinary excretion amounts of the sum of the three branched-chain 2-oxo acids. The administration of capsules containing the daily necessary amounts of B-group vitamins led to a decrease in the urinary excretion of the sum of the three types of branched-chain 2-oxo acids in participants belonging to the upper tertile. A similar phenomenon was observed in the middle tertile, but not in the lower tertile. Intakes of B-group vitamins and the urinary excretion amounts of B-group vitamins were not observed to be significantly different among the upper, middle, and lower tertiles. These results indicate that some young Japanese women need much higher levels of B-group vitamins than the Dietary Reference Intakes for Japanese. Thus, urinary branched-chain 2-oxo acids are useful functional biomarkers for B-group vitamins in humans.

Key Words 2-oxo acids, branched-chain 2-oxo acids, urine, functional biomarker, human

To assess the nutritional status of healthy free-living humans, a weighted record method has been used widely to record dietary intake. However, this method has several problems. These occur because the description of food items and the amounts have to be recorded by participants themselves. Therefore, accuracy and precision may be very low. Calculation of the intakes of nutrients is done by using “Tables of Food Composition” (1). The contents of the essential-organic-micronutrients, such as vitamins, are variable within the same food item and these vitamins are generally unstable and may be destroyed during preservation, food processing, and cooking. Therefore, the accuracy and precision of the vitamin intakes are not reliable. Thus, we developed a more reliable method to predict the intakes of vitamins: the urinary excretory amounts of water-soluble vitamins closely reflect the surplus amount of water-soluble vitamins in the bodies of rats and humans (2, 3). Nutritional assessment using biomarkers is very persuasive and leads readily to the transformation of habitual dietary intakes. Thus, we tried to set tentative guidance of urinary excretion amounts of water-soluble vitamins for evaluation of individual vitamin nutrition (2, 3). However, it was pointed out that the urinary levels of vitamins just reflect the intakes of available free type forms of vitamins, but it does not reflect physiological functions of B-group vitamins such as the levels of coenzymes and enzyme activities needing coenzymes. This was a very important caveat.

Methods for evaluating the physiological functions of B-group vitamins of individuals are desired. B-group vitamins are involved in the catabolism of amino acids such as valine, isoleucine, and leucine (Fig. 1). Specifically, these vitamins are involved in the formation and breakdown of 2-oxo acids (Fig. 1). We developed high-performance liquid chromatographic methods for detecting 2-oxo acids in urine (4, 5) and reported that the urinary excretion amounts of some 2-oxo acids were increased by vitamin B₁, vitamin B₆, or pantothenic acid deficiencies (5). In the present paper, we investigated whether urinary levels of 2-oxo acids are useful functional biomarkers for B-group vitamins in humans.

MATERIALS AND METHODS

The study was conducted in April 2015 and the protocol was approved by the Ethics Committee of the University of Shiga Prefecture (Shiga, Japan). The study was conducted according to the guidelines set out in the Declaration of Helsinki. All participants provided written informed consent to participate in the study after being informed of the study protocol and purpose.

Chemicals. Thiamin hydrochloride (C₁₂H₁₇ClN₄OS-
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HCl, molecular weight [MW]=337.27), riboflavin (C_{17}H_{20}N_{4}O_{6}, MW=376.37), pyridoxine hydrochloride (C_{8}H_{11}NO_{3}-HCl, MW=205.63), cyanocobalamin (C_{63}H_{88}CoN_{14}O_{14}P, MW=1,355.40), nicotinamide (C_{6}H_{6}N_{2}O, MW=122.13), calcium pantothenate (C_{18}H_{32}N_{2}O_{10}-Ca, MW=476.54), pteroylmonoglutamic acid (C_{19}H_{19}N_{7}O_{6}, MW=441.4), and biotin (C_{10}H_{16}N_{2}O_{3}S, MW=244.3) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 4-Pyridoxic acid (4-PIC) (C_{8}H_{9}NO_{4}, MW=183.16) was made by ICN Pharmaceuticals (Costa Mesa, CA) and obtained through Wako Pure Chemical Industries. N^{1}-Methylnicotinamide (MNA) chloride (C_{7}H_{9}N_{2}O-HCl, MW=159.61) was purchased from Tokyo Chemical Industry (Tokyo, Japan). N^{1}-Methyl-2-pyridone-5-carboxamide (2-Py) (C_{7}H_{8}N_{2}O_{2}, MW=152.15) and N^{1}-methyl-4-pyridone-3-carboxamide (4-Py) (C_{7}H_{8}N_{2}O_{2}, MW=152.15) were synthesized by the methods of Pullman and Colowick (6) and Shibata et al., (7) respectively. 2-Oxo-3-methylbutanoic acid (C_{5}H_{8}O_{3}, MW=116.1), 2-mercaptoethanol, and sodium hydrosulfit e were purchased from Wako Pure Chemical Industries. 2-Oxo-3-methylpentanoic acid (C_{6}H_{10}O_{3}, MW=130.1) and 2-oxo-4-methylpentanoic acid (C_{6}H_{10}O_{3}, MW=130.1) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO). 1,2-Diamino-4,5-methyleneedioxybenzene-dihydrochloride (DMB) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other chemicals were of the highest purity available from commercial sources.

Participants. Female Japanese students were recruited from the University of Shiga Prefecture. Participants diagnosed with a cold or influenza and those who had taken multivitamin supplements at least once during the previous month were excluded. All participants were non-smokers and passed a standard medical examination at the university. Of the 29 apparently healthy female Japanese students who participated in this study, all participants (age, 20–21 y) completed the study.

Study design. The experimental period was 3 wk (Fig. 2). Each experiment was done from Monday to Thursday. All food consumed during the 4-d period in each week was recorded using a weighed food record method (8). All of the participants lived and consumed food freely during the experiment. A capsule containing the daily necessary amounts of B-group vitamins (Table 1) was made based on the Dietary Reference Intakes for Japanese (2015) (9). A digital cooking scale (1 g unit; Tanita Inc., Tokyo, Japan), a set of dietary record forms and a dietary record manual were distributed to the participants in advance. Participants consumed only food for the 1st week, food and one vitamin capsule for the 2nd week, and food and three vitamin capsules for the 3rd week (Fig. 2). That is, the participants were requested to consume one vitamin capsule during the 2nd week and three vitamin capsules during the 3rd week at breakfast daily Monday morning to Thursday morning.

Twenty-four-hour urine samples from the 2nd urine on Thursday to the 1st urine on Friday of each week were collected in amber bottles by the participants to measure urinary levels of B-group vitamins and the branched-chain 2-oxo acids. After the urine samples were collected, the volumes of the samples were measured on Friday morning in our laboratory. Aliquots of the urine were stabilized to avoid destruction of B-group vitamins and of 2-oxo acids. For analyses of urine thiamin, riboflavin, 4-PIC, nicotinamide, MNA, 2-Py, 4-Py, and branched-chain 2-oxo acids, 1 mL of 1 mol/L HCl was added to 9 mL urine. The treated urine samples were then stored at −80˚C until analysis.

Measurements of B-group vitamins in urine

Thiamin: The acidified urine samples (1 mL) were thawed and centrifuged at 10,000 ×g for 10 min at 4˚C. The supernatant was retained and used to measure thiamin. The thiamin in the supernatant (500 μL) reacted with 1% cyanogen bromide (100 μL) in a strong alkali medium (5% NaOH, 100 μL) at room temperature. After the mixture had been kept for 10 min, 1.5 mol/L HCl (80 μL) was added. The mixture was then centrifuged at 10,000 ×g for 10 min at 4˚C. The resulting
Table 1. The contents of B-group vitamins per capsule.

| Vitamins                                                                 | Content/capsule | Vitamin content/capsule |
|--------------------------------------------------------------------------|-----------------|-------------------------|
| Thiamin-Cl HCl                                                           | 1.0 mg          | 1.0 mg as vitamin B₁    |
| Riboflavin                                                               | 1.0 mg          | 1.0 mg as vitamin B₂    |
| Pyridoxine HCl                                                           | 1.2 mg          | 0.96 mg as vitamin B₆   |
| Cyanocobalamin                                                           | 0.002 mg        | 0.002 mg as vitamin B₁₂ |
| Nicotinamide                                                             | 10 mg           | 10 mg as niacin         |
| Ca-pantothenate                                                          | 6.0 mg          | 5.5 mg as pantothenic acid |
| Biotin                                                                   | 0.045 mg        | 0.045 mg as biotin      |
| Pteroylmonoglutamic acid                                                 | 0.2 mg          | 0.2 mg as folacin       |
| Sucrose                                                                  | 980.55 mg       | 0 mg                    |

1 Major urinary compound of vitamin B₁.
2 Major urinary compound of vitamin B₂.
3 SUM = MNA + 2-Py + 4-Py. MNA: N¹-methylnicotinamide, 2-Py: N¹-methyl-2-pyridone-5-carboxamide, 4-Py: N¹-methyl-4-pyridone-3-carboxamide.
4 Major urinary compound of niacin catabolite.
5 Major urinary compound of vitamin B₆.
6 4-PIC: 4-pyridoxic acid.
7 Major urinary compound of pantothenic acid (PaA).
8 Values are means ± SD, n=29. The nonparametric Friedman test for repeated measures followed by Dunn’s post hoc test was used to analyze statistical differences among all treatments. A p-value of <0.05 was considered to be statistically significant. Labeled means in a row without a common letter differ with p<0.05.

Table 2. Various parameters of the participants (female Japanese students).

| Parameters                      | No vitamin capsule period | 1 vitamin capsule period | 3 vitamin capsules period |
|--------------------------------|---------------------------|--------------------------|---------------------------|
| Physical parameters            |                           |                          |                           |
| Age (y)                        | 20.0±0.3                  | 20.0±0.3                 | 20.0±0.3                  |
| Height (cm)                    | 159.3±6.6                 | 159.3±6.6                | 159.3±6.6                |
| Body weight (kg)               | 52.8±7.5                  | 52.8±7.5                 | 52.8±7.5                 |
| BMI (kg/m²)                    | 20.7±2.0                  | 20.7±2.0                 | 20.7±2.0                 |
| Energy and major nutrient intakes |                           |                          |                           |
| Energy (kcal/d)                | 1,722±360                 | 1,677±317                | 1,663±494                |
| Protein (g/d)                  | 63.3±14.0                 | 59.3±11.0                | 56.9±14.0                |
| Fat (g/d)                      | 54.3±17.1                 | 52.3±11.0                | 51.7±19.9                |
| Carbohydrate (g/d)             | 239±53                    | 237±47                   | 237±69                   |
| B-Group vitamin intakes        |                           |                          |                           |
| Vitamin B₁ (mg/d)              | 0.86±0.28                 | 1.83±0.24                | 3.75±0.28                |
| Vitamin B₂ (mg/d)              | 1.12±0.31                 | 2.15±0.27                | 4.27±0.30                |
| Niacin (mgNE/d)                | 23.9±6.3                  | 33.6±5.8                 | 53.9±6.7                 |
| Vitamin B₆ (mg/d)              | 0.002 mg                  | 0.002 mg                 | 0.002 mg                 |
| Vitamin B₁₂ (μg/d)             | 3.45±1.25                 | 5.49±1.57                | 9.29±1.48                |
| Folacin (μg/d)                 | 267±85οβ                 | 565±474οβ                | 849±99οβ                 |
| PaA (mg/d)                     | 5.83±1.70                 | 10.9±1.33                | 21.6±1.59                |
| Biotin (μg/d)                  | 32.0±11.3                 | 74.4±8.25                | 161±8.4                 |

Urinary excretion amounts of vitamins

1 Major urinary compound of vitamin B₁.
2 Major urinary compound of vitamin B₂.
3 SUM = MNA + 2-Py + 4-Py. MNA: N¹-methylnicotinamide, 2-Py: N¹-methyl-2-pyridone-5-carboxamide, 4-Py: N¹-methyl-4-pyridone-3-carboxamide.
4 Major urinary compound of niacin catabolite.
5 Major urinary compound of vitamin B₆.
6 4-PIC: 4-pyridoxic acid.
7 Major urinary compound of pantothenic acid (PaA).
8 Values are means ± SD, n=29. The nonparametric Friedman test for repeated measures followed by Dunn’s post hoc test was used to analyze statistical differences among all treatments. A p-value of <0.05 was considered to be statistically significant. Labeled means in a row without a common letter differ with p<0.05.
supernatant was passed through a 0.45-μm microfilter equipped with Hydrophilic Durapore™ (PVDF) (Millipore, Bedford, MA). The filtrate (20 μL) was injected directly into a high-performance liquid chromatography (HPLC) system to measure thiochrome (10, 11).

Riboflavin: The acidified urine samples (100 μL) were thawed and centrifuged at 10,000 × g for 10 min at 4˚C. The supernatant was retained and used to measure riboflavin. The resulting supernatant was passed through the 0.45-μm microfilter. The filtrate (20 μL) was injected directly into an HPLC system (12).

4-PIC: The acidified urine samples (100 μL) were thawed and centrifuged at 10,000 × g for 10 min at 4˚C. The supernatants were retained and used to measure 4-PIC, a catabolite of vitamin B₆. The resulting supernatant was passed through the 0.45-μm microfilter. The filtrate (20 μL) was injected directly into an HPLC system (13).

Nicotinamide and its catabolites: The acidified urine samples (1.5 mL) were thawed and centrifuged at 10,000 × g for 10 min at 4˚C. The supernatant (1.0 mL) was withdrawn and added to 10 μL of 1.0 g/L isonicotinamide (used as an internal standard), and diethyl ether (5.0 mL) was then added in the presence of K₂CO₃ (1.2 g) to extract nicotinamide, 2-Py, and 4-Py from the water layer into the organic solvent layer. This extraction procedure was repeated twice. The combined diethyl ether layer was dried at 40˚C, and the dried materials were dissolved with 0.5 mL of water. The water solution was passed through a 0.45-μm microfilter. The filtrate (20 μL) was injected directly into an HPLC system to measure nicotinamide, 2-Py, and 4-Py simultaneously as previously reported (7).

MNA, another catabolite in the urine samples (800 μL), reacted with 0.1 mol/L acetonophenone in ethanol (500 μL) in a strong alkali medium (1.0 mL of 6 mol/L NaOH) at 0˚C in the presence of a large amount of isonicotinamide (200 μL of 1 mol/L isonicotinamide added for an interference of deamination of MNA under the strong alkali medium). After the mixture had been kept for 10 min, formic acid was added and the mixture was kept for another 15 min at 0˚C. The mixture was then heated to >93˚C for 5 min. The reaction product is 1-methyl-7-phenyl-1,5-dihydro-5-oxo-1,6-naphthyridine. After cooling in ice water, the mixture was passed through a 0.45-μm microfilter. The filtrate (20 μL) was injected directly into an HPLC system as previously described (14).

Pantothenic acid: The acidified urine samples (100 μL) were thawed and centrifuged at 10,000 × g for 10 min at 4˚C. The supernatant was retained and used to measure pantothenic acid by the microbioassay method using Lactobacillus plantarum (ATCC 8014) (15).

Measurement of branched-chain 2-oxo acids in urine. The DMB solution was prepared by mixing the following in sequence: 8.7 mL of H₂O, 0.049 g of sodium hydroxide, 0.7 mL of 2-mercaptoethanol, 0.58 mL of concentrated HCl, and 0.016 g of DMB. The DMB solution was stable for 1 mo when stored at 4˚C.

The acidified urine samples (100 μL) were thawed and centrifuged at 10,000 × g for 10 min at 4˚C. The supernatant was retained and used to measure branched-chain 2-oxo acids. A total of 0.1 mL of the DMB solution was added to 0.1 mL of a urine sample diluted to a suitable concentration in a microtube with a sealed cap. The reaction was conducted by immersing the microtube in a bath of boiling water for 45 min, after which the microtube was cooled in ice water for at least 5 min. The resulting reaction mixture was filtered through the 0.45-μm filter, and the filtrate (5 μL) was then separated using a TSKgel ODS-80Ts column (average particle diameter: 5 μm, 4.6 i.d.×250 mm) (TOSOH, Tokyo, Japan) with 30 mmol/L of KH₂PO₄ (pH 3.0) : acetoni- trile (7 : 3) at a flow rate of 1.0 mL/min (5).

Stratification by urine total branched-chain 2-oxo acids excretion. The number of participants (n=29) was divided into tertiles in response to urinary levels of total branched-chain 2-oxo acids, that is the lower ter-
Table 3. Comparison of various parameters among the upper, middle, and lower tertiles of urinary branched-chain 2-oxo acids of female Japanese students without supplementation.

| Parameters | Upper tertile | Middle tertile | Lower tertile |
|------------|--------------|---------------|--------------|
| | 11.25–29.47 μmol/d (high total branched-chain 2-oxo acids group, n=10) | 7.91–11.00 μmol/d (middle total branched-chain 2-oxo acids groups, n=9) | 5.19–7.18 μmol/d (low total branched-chain 2-oxo acids groups, n=10) |
| Urinary excretion amounts of branched-chain 2-oxo acids | | | |
| Total branched-chain | 17.13±7.16a | 9.33±1.11b | 6.15±0.74c |
| 2-oxo acids (μmol/d) | 11.67±7.16a | 6.15±1.11b | 3.84±0.74c |
| 2-Oxo-3-methylbutanoic acid (μmol/d) | 3.48±1.46a | 1.99±0.40b | 1.34±0.21c |
| 2-Oxo-4-methylpentanoic acid (μmol/d) | 2.98±1.44a | 1.97±0.28b | 1.47±0.22c |
| 2-Oxo-3-methylpentanoic acid (μmol/d) | 10.67±4.76a | 5.38±0.79b | 3.33±0.73c |
| Physical parameters | | | |
| Age (y) | 20.1±0.3 | 20.0±0.0 | 20.1±0.3 |
| Height (cm) | 161.1±2.8 | 158.0±8.1 | 159.0±6.7 |
| Body weight (kg) | 54.4±6.2 | 52.3±8.3 | 52.0±7.6 |
| BMI (kg/m²) | 21.0±1.9 | 20.2±1.8 | 21.0±2.3 |
| Energy and major nutrient intakes | | | |
| Energy (kcal/d) | 1,702±374 | 1,680±336 | 1,786±398 |
| Protein (g/d) | 63.4±14.4 | 62.4±14.5 | 64.2±14.5 |
| Fat (g/d) | 54.6±17.5 | 48.9±14.9 | 59.4±18.8 |
| Carbohydrate (g/d) | 229±56 | 243±43 | 244±62 |
| B-Group vitamin intakes | | | |
| Vitamin B₁ (mg/d) | 0.81±0.24 | 0.88±0.32 | 0.87±0.39 |
| Vitamin B₂ (mg/d) | 1.03±0.15 | 1.20±0.43 | 1.14±0.30 |
| Niacin (mgNE/d) | 25.3±6.7 | 22.2±4.4 | 24.2±7.7 |
| Vitamin B₆ (mg/d) | 1.04±0.48 | 1.14±0.47 | 1.02±0.42 |
| Vitamin B₁₂ (μg/d) | 3.78±1.49 | 3.20±1.14 | 3.37±1.16 |
| Folacin (μg/d) | 250±49 | 276±95 | 274±107 |
| Pantothenic acid (μg/d) | 5.54±1.03 | 6.01±1.96 | 5.95±2.06 |
| Biotin (μg/d) | 29.8±6.7 | 35.5±16.2 | 30.6±9.2 |
| Urine excretion amounts of B-group vitamins | | | |
| Thiamin¹ (nmol/d) | 962±571 | 1,050±842 | 927±488 |
| Riboflavin² (nmol/d) | 400±271 | 677±502 | 486±257 |
| SUM³ (μmol/d) | 96.8±27.9 | 74.2±33.2 | 71.1±31.1 |
| MNA⁴ (μmol/d) | 37.5±17.8 | 30.0±14.8 | 25.4±10.5 |
| 2-Py⁵ (μmol/d) | 52.3±13.9 | 39.5±19.4 | 40.6±20.2 |
| 4-Py⁵ (μmol/d) | 6.97±2.80 | 4.71±2.64 | 5.10±1.98 |
| 4-PIC⁵ (μmol/d) | 14.5±21.6 | 11.5±5.9 | 10.9±5.3 |
| Pantothenic acid (μmol/d) | 12.5±3.6 | 13.8±4.1 | 13.9±3.4 |

¹ Major urinary compound of vitamin B₁.
² Major urinary compound of vitamin B₂.
³ SUM=MNA+2-Py+4-Py. MNA: N⁴-methylnicotinamide, 2-Py: N¹-methyl-2-pyridone-5-carboxamide, 4-Py: N¹-methyl-4-pyridone-3-carboxamide.
⁴ Major urinary compound of niacin catabolite.
⁵ Major urinary compound of vitamin B₆.
⁶ Major urinary compound of pantothenic acid (Pantothenic acid).

Values are means±SD, n=10. The significance of the differences in the means among groups was determined by one-way analysis of variance followed by Tukey’s post hoc analysis for comparisons among groups. Differences with p<0.05 were considered to be statistically significant. Labeled means in a row without a common letter differ with p<0.05.
tile (low branched-chain 2-oxo acids group, median 6.18 μmol/d; 5.19–7.18 μmol/d, n = 10), middle tertile (middle branched-chain 2-oxo acids group, median 9.44 μmol/d; 7.91–11.00 μmol/d, n = 9), and upper tertile (high branched-chain 2-oxo acids group, median 13.65 μmol/d; 11.25–29.47 μmol/d, n = 10).

Statistical analysis. Pearson coefficients were calculated to determine the correlation between concentrations of 2-oxo-3-methylbutanoic acid and 2-oxo-3-methylpentanoic acid, 2-oxo-3-methylbutanoic acid and 2-oxo-4-methylpentanoic acid, and 2-oxo-3-methylpentanoic acid and 2-oxo-4-methylpentanoic acid (Fig. 3). The nonparametric Friedman test for repeated measures followed by Dunn’s post hoc test was used to analyze statistical differences among all treatments (Fig. 4, Fig. 5, and Table 2). The significance of the differences in the means among groups was determined by one-way analysis of variance followed by Tukey’s post hoc analysis for comparisons among groups (Table 3). Differences with p < 0.05 were considered to be statistically significant. All statistical analyses were undertaken using Prism version 5.0 (GraphPad, San Diego, CA).

RESULTS

Various parameters in the participants (Japanese female students)

Table 2 shows basic physical parameters such as body height, body weight, and body mass index of the participants. Each value was as the same as the reference values described in the Dietary Reference Intakes for Japanese in 2015 (9). Therefore, the participants were considered typical university female students in Japan. During the 3-wk experimental period, the 1st week (no vitamin capsule period), the 2nd week (one vitamin capsule period), and 3rd week (three vitamin capsules period), all participants lived freely and the intakes of energy, major nutrients (protein, fat, and carbohydrate) (Table 2), and minerals among the three periods were not observed to be significantly different (data not shown). The intakes of B-group vitamins almost doubled in the 2nd week and increased about four fold in the 3rd week because the participants were requested to take one vitamin capsule daily during the 2nd week and three vitamin capsules daily during the 3rd week. Therefore, the urinary excretion amounts of vitamins were increased based on the intakes of vitamins (Table 2).

Relationship between each of the urinary excretion amounts of branched-chain 2-oxo acids

Figure 3 shows the relationship between each pair of the urinary excretion amounts of branched-chain 2-oxo acids. Very high relationships were observed between each pair of the branched-chain 2-oxo acids such as 2-oxo-3-methylbutanoic acid, 2-oxo-3-methylpentanoic acid, and 2-oxo-4-methylpentanoic acid.  

Tertile analysis of the urinary excretion amounts of branched-chain 2-oxo acids

The urinary excretion amounts of total branched-chain 2-oxo acids (sum of the three branched-chain 2-oxo acids) in the 1st week (no vitamin supplement period) were divided into three groups: the upper (n = 10), middle (n = 9), and lower (n = 10) tertiles. Data including physical parameters, intakes of energy and major nutrients, and B-group vitamin intakes of the upper, middle, and lower tertile groups are shown in Table 3. No significant difference in any of these parameters was observed among the upper, middle, or lower tertile (Table 3). In addition, the urinary excretion amounts of thiamin, riboflavin, the sum of nicotinamide and its metabolites, 4-pyridoxic acid, and pantothenic acid, which are involved with the formation and degradation of the 2-oxo acids (Fig. 1), were not
Effects of B-group vitamin supplementation on the urinary branched-chain 2-oxo acids (tertile analysis)

The participants were divided into the three groups based on total urinary branched-chain 2-oxo acids during the no vitamin supplemental period (Table 3). Excretion amounts of the participants belonging to the upper tertile were significantly decreased by the administration of one B-group vitamin capsule (Fig. 4A, upper tertile). The same phenomenon was also observed in each branched-chain 2-oxo acid (Fig. 5A, D, G). The administration of three capsules did not further decrease the urinary excretion amounts of total branched-chain 2-oxo acids (Fig. 4A, middle tertile) or any branched-chain 2-oxo acid (Fig. 5B, E, H).

The total urinary branched-chain 2-oxo acids excretion amounts of the participants belonging to the lower tertile did not change with the administration of one capsule of B-group vitamins (Fig. 4C, lower tertile). The same phenomena were observed in the lower tertile in terms of each of the branched-chain 2-oxo acids (Fig. 5C, F, I). The administration of three capsules did not further decrease the urinary excretion amounts of total branched-chain 2-oxo acids (Fig. 4C, middle tertile) or any branched-chain 2-oxo acid (Fig. 5B, E, H).

The total urinary branched-chain 2-oxo acids excretion amounts of the participants belonging to the lower tertile did not change with the administration of one capsule of B-group vitamins (Fig. 4C, lower tertile). The same phenomena were observed in the lower tertile in terms of each of the branched-chain 2-oxo acids (Fig. 5C, F, I). The administration of three capsules did not further decrease the urinary excretion amounts of total branched-chain 2-oxo acids (Fig. 4C, middle tertile) or any branched-chain 2-oxo acid (Fig. 5B, E, H).

The total urinary branched-chain 2-oxo acids excretion amounts of the participants belonging to the lower tertile did not change with the administration of one capsule of B-group vitamins (Fig. 4C, lower tertile). The same phenomena were observed in the lower tertile in terms of each of the branched-chain 2-oxo acids (Fig. 5C, F, I). The administration of three capsules did not further decrease the urinary excretion amounts of total branched-chain 2-oxo acids (Fig. 4C, lower tertile) or any branched-chain 2-oxo acid (Fig. 5C, F, I).

DISCUSSION

We investigated nutritional biomarkers of B-group vitamins and found that the urinary excretion amounts of B-group vitamins reflect the intakes of available free types of vitamins (2, 3). However, these levels only indicate the intakes of B-group vitamins but do not reflect the function of these vitamins, for an example, coenzyme function.

Branched-chain amino acids such as valine, isoleucine, and leucine are mainly catabolized in muscle cells in humans (16, 17). Thus, the urinary excretion...
amounts of branched-chain 2-oxo acids such as 2-oxo-3-methylbutanoic acid, 2-oxo-3-methylpentanoic acid, and 2-oxo-4-methylpentanoic acid reflect the catabolic activity of branched-chain amino acids in muscle cells. Generally speaking, higher levels of urinary branched-chain 2-oxo acids equate with weaker catabolic ability of branched-chain amino acids. In the present experiment, we explored whether urinary levels of branched-chain 2-oxo acids can be useful as a functional biomarker of B-group vitamins because the branched-chain 2-oxo acid dehydrogenase complex (BCKDC) [EC 1.2.4.4] needs coenzymes. We collected 24-h urine samples from young Japanese women. Concentrations of branched-chain 2-oxo acids such as 2-oxo-3-methylbutanoic acid, 2-oxo-3-methylpentanoic acid, and 2-oxo-4-methylpentanoic acid in urine samples were measured. The relationships between each pair of the urinary levels of the three types of branched-chain 2-oxo acids were very high. The participants were divided into three groups, the upper tertile (n = 10), the middle tertile (n = 9), and the lower tertile (n = 10), based on the urinary excretion of total branched-chain 2-oxo acids. The high urinary excretion of branched-chain 2-oxo acids means lower activity of BCKDC, which needs four types of coenzymes, namely TDP (functional form of vitamin B12), FAD (functional form of vitamin B2), NAD+ (functional form of niacin), and CoA (functional form of pantothenic acid). If B-group vitamin supplementation makes the urinary excretion of branched-chain 2-oxo acids decrease, it means that subjects need higher intakes of B-group vitamins. In the present experiment, all of the participants were apparently healthy and their B-group vitamin nutritional statuses were good as determined by the urinary excretion of B-group vitamins. Nevertheless, B-group vitamin supplementation led to decreased urinary excretion of total branched-chain 2-oxo acids in participants belonging to the upper tertile. A similar phenomenon was observed in participants in the middle tertile, but not in the lower tertile. Intakes and urinary excretion amounts of B-group vitamins were not observed to be significantly different among the participants in the upper, middle, and lower tertiles. This phenomenon means that subjects belonging in the upper and middle tertiles need higher B-group vitamin intakes than the requirement set by the Dietary Reference Intakes for Japanese (9). The values set by the Dietary Reference Intakes for Japanese (9) are calculated to prevent a deficiency, but are not calculated to attain optimum health for individuals. Therefore, measuring urinary 2-oxo acids might be a good method for finding the optimum vitamin requirements for individuals.

As a general screening method for maple syrup urine disease (MSUD), urinary concentrations of branched-chain amino acids such as valine, leucine, and isoleucine are measured. MSUD is a defect of BCKDC that consists of E1α + β/E2/E3 subunits. Dihydrolipoamide dehydrogenase (E3) subunit is the same protein subunit of pyruvate dehydrogenase complex and 2-oxoglutarate dehydrogenase complex (18, 19). MSUD is often classified into five types by the pattern of signs and symptoms: the classic severe MSUD, intermediate MSUD, intermittent MSUD, dihydrolipoamide dehydrogenase (E3)-deficient MSUD with lactic acidosis, and thiamin-responsive MSUD (19). We have interest in thiamin-responsive MSUD because we have conducted studies on the optimum requirement of B-group vitamins for individuals. This variant of the disease is less severe than the classic form and has some enzyme activity. The first observed thiamin-responsive patient was reported by Scriver et al., in 1971 (20) and followed by Chuang et al., in 1982 (21). Chuang et al. (21) clarified that the primary defect in the thiamin-responsive MSUD patient is a reduced affinity of the mutant BCKDC for TDP that results in impaired oxidative decarboxylation of branched-chain 2-oxo acids. BCKDC needs four B-group vitamins, namely vitamin B1, vitamin B2, niacin, and pantothenic acid. The products of BCKDC are the CoA forms of branched-chain 2-oxo acids, namely isobutanol-CoA, 2-methylbutanol-CoA, and 3-methylbutanol-CoA (Fig. 1), which need other B-group vitamins such as vitamin B6, biotin, and vitamin B12 for complete degradation. Therefore, the administration of B-group vitamins is better for the improvement of branched-chain amino acid degradation than that of only vitamin B1. Recently, novel mutations of BCKDC and the related enzyme genes associated with MSUD have been reported (22, 23). The participants belonging to the upper tertile in the present experiment might have minor gene mutations of BCKDC and the related genes. The gene mutations of BCKDC would cause an increase in the requirement of the vitamins.

Another explanation might be possible because Shimomura et al. (24, 25) reported that exercise training modulates the gene expression of BCKDC and increases the BCKDC activity. Namely, it is that the difference of urinary excretion of branched-2-oxo acids is not dependent on the functional biomarker of B-group vitamins but it is dependent on the levels of physical activity. We did not precisely check their physical activities in the present experiment, but none of them did severe exercise during the experiment. In addition, the urinary excretion of total amount of branched-chain 2-oxo acids of the participants belonging to the upper tertile was significantly decreased by the administration of the B-vitamin capsule. Therefore, such a possibility is considered to be weak.

In summary, we found a high concentration of urinary branched-chain 2-oxo acids among the general population. These individuals had normal intakes and urinary excretion amounts of B-group vitamins. However, their urinary excretion amounts of branched-chain 2-oxo acids were significantly decreased by the administration of B-group vitamins. This result is a very important fact, nutritionally: some people need higher intakes of B-group vitamins than recommended by the Dietary Reference Intakes for Japanese (9). In future studies, we will clarify what types of B-group vitamins are most effective to decrease the urinary excretion amounts of branched-chain 2-oxo acids to the normal
range. Thus, urinary levels of branched-chain 2-oxo acids are useful as a functional biomarker for B-group vitamins in individuals.

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Author contributions
K.S. designed the study. M.S. conducted the experiments. K.S. drafted the manuscript. Both authors read and approved the final manuscript.

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