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Urinary excretion ratio of xanthurenic acid/kynurenic acid as a functional biomarker of niacin nutritional status

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The present study was conducted to survey functional biomarkers for evaluation of niacin nutritional status. Over 500 enzymes require niacin as a coenzyme. Of these, we chose the tryptophan degradation pathway. To create niacin-deficient animals, quino- linic acid phosphoribosyltransferase-knock out mice were used in the present study because wild type mice can synthesize nicotinamide from tryptophan. When the mice were made niacin-deficient, the urinary excretion of xanthurenic acid (XA) was extremely low compared with control mice; however, it increased according to the recovery of niacin nutritional status. The urinary excretion of kynurenic acid (KA) was the reverse of XA. Kynurenine 3-monooxygenase, which needs NADPH, was thought to be suppressed by niacin deficiency. Thus, we calculated the urinary excretion ratio of XA:KA as a functional biomarker of niacin nutrition. The ratio increased according to recovering niacin nutritional status. Low values equate with low niacin nutritional status.

Key words: niacin-deficiency; QPRT; tryptophan catabolism; mice; vitamin

We reported that the urinary excretion of the sum of nicotinamide (Nam) and its catabolites reflects niacin nutritional status.1) However, it was pointed out that the urinary levels only reflect the intakes of preformed niacin and tryptophan, but it does not reflect physiological function of the niacin coenzyme pyridine nucleotide coenzymes (PNC). This was a very important caveat.

Niacin plays many biological roles of about 500 enzymes such as PNC. However, studies of niacin deficiency are scarce. One of the most important things is that mammals including humans can biosynthesize Nam from tryptophan. In studies focusing on niacin deficiency, animals have been fed niacin-free and tryptophan-imbalanced diets.2–7) However, such niacin deficiency is not true niacin deficiency, because tryptophan deficiency also occurred. Thus, inhibiting the tryptophan → Nam conversion pathway was necessary in studies of niacin nutritional status regardless of tryptophan nutritional status. We generated truly niacin-deficient mice that have no quinolinic acid phosphoribosyltransferase (QPRT) gene.8) In the present experiment, we surveyed a functional biomarker of niacin nutritional status using QPRT-KO mice.

Materials and methods

Chemicals. Vitamin-free milk casein, 1-methionine, and sucrose were purchased from Wako Pure Chemical Industries (Osaka, Japan). Corn oil was obtained from Ajinomoto (Tokyo, Japan). A mineral mixture (AIN-93-G-MX)9) and vitamin mixture (AIN-93-VX)9) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Thiamin hydrochloride (C₁₂H₁₇ClN₄OS·HCl, molecular weight [MW] = 337.27), riboflavin (C₁₇H₂₂NO₄·H₂O, MW = 376.37), pyridoxine hydrochloride (C₆H₁₁NO₇·H₂O·HCl, MW = 205.63), cyanocobalamin (C₆₃H₈₈CoN₁₄O₁₄P, MW = 1355.40), and Nam (C₆H₆N₂O, MW = 183.16) were made by ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.) and obtained through Wako Pure Chemical Industries. 4-Pyridoxic acid (4-PIC) (C₈H₉NO₄, MW = 183.16) was made by ICN Pharmaceuticals and Shibata et al.11) respectively. L-Trp, anthranilic acid (AnA), 3-hydroxykynurenine (3-HK), quinolinic acid (QA), and Nam were purchased from Wako Pure Chemical Industries. N₁-Methylnicotinamide (MNA) chloride (C₇H₈N₂O·HCl, MW = 159.61) was purchased from Tokyo Chemical Industry (Tokyo, Japan). N₂-Methyl-2-pyridone-5-carboxamide (2-Py) (C₆H₈N₂O₂, MW = 152.15) and N₇-methyl-4-pyridone-3-carboxamide (4-Py) (C₆H₈N₂O₂, MW = 152.15) were synthesized by the methods of Pullman and Colowick10) and Shibata et al.11) respectively. 1-Trp, anthranilic acid (AnA), 3-hydroxykynurenine (3-HK), quinolinic acid (QA), and Nam were purchased from Wako Pure Chemical Industries. Kynurenine sulfate, MNA chloride, xanthurenic acid (XA), kynurenic acid (KA) and 3-hydroxyanthranilic acid (3-HA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Corn oil was obtained from Ajinomoto (Tokyo, Japan). A mineral mixture (AIN-93-G-MX)9) and vitamin mixture (AIN-93-VX)9) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). Thiamin hydrochloride (C₁₂H₁₇ClN₄OS·HCl, molecular weight [MW] = 337.27), riboflavin (C₁₇H₂₂NO₄·H₂O, MW = 376.37), pyridoxine hydrochloride (C₆H₁₁NO₇·H₂O·HCl, MW = 205.63), cyanocobalamin (C₆₃H₈₈CoN₁₄O₁₄P, MW = 1355.40), and Nam (C₆H₆N₂O, MW = 183.16) were made by ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.) and obtained through Wako Pure Chemical Industries. 4-Pyridoxic acid (4-PIC) (C₈H₉NO₄, MW = 183.16) was made by ICN Pharmaceuticals and Shibata et al.11) respectively. L-Trp, anthranilic acid (AnA), 3-hydroxykynurenine (3-HK), quinolinic acid (QA), and Nam were purchased from Wako Pure Chemical Industries. Kynurenine sulfate, MNA chloride, xanthurenic acid (XA), kynurenic acid (KA) and 3-hydroxyanthranilic acid (3-HA) were purchased from Tokyo Chemical Industry (Tokyo, Japan). All other chemicals were of the highest purity available from commercial sources.

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Abbreviations: AnA, anthranilic acid; 3-HA, 3-hydroxyanthranilic acid; 3-HK 3-hydroxykynurenine; KA, kynurenic acid; MNA, N₁-methylnicotinamide; 2-Py, N₂-methyl-2-pyridone-5-carboxamide; 4-Py, N₇-methyl-4-pyridone-3-carboxamide; Nam, nicotinamide; 4-PIC, 4-pyridoxic acid; PNC, pyridine nucleotide coenzymes; QA, quinolinic acid; QPRT-KO, quinolinic acid phosphoribosyltransferase-knock out; XA, xanthurenic acid.
Animals and diets. The care and treatment of the experimental animals conformed to the University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals. The room temperature was maintained at around 22 °C and about 60% humidity with a 12:12 light:dark cycle (06:00–18:00 light/18:00–06:00 dark).

Male 9-week-old C57BL/6J mice and male 9- to 10-week-old Qprt−/− mice, which were backcrossed for over 10 generations onto a C57BL/6 genetic background, were used. Each mouse was housed in a metabolic cage (CL-0355, CLEA Japan, Tokyo). All mice had free access to a chemically defined 20% casein diet without nicotinic acid (Table 1) for 28 days (from Day 1 at 09:00 to Day 29 at 09:00 of the experiment) and then were fed a chemically defined 20% casein diet with nicotinic acid (Table 1) for another 12 days (from Day 29 at 09:00 to Day 41 at 09:00). Body weights and food intakes were measured daily at around 09:00, and food and water were renewed daily. Twenty-four-hour urine samples were collected seven times in amber colored bottles containing 1 mL of 1 mol/L HCl, and were stored at −25 °C until analysis: Day 28 urine, Day 29 at 09:00 to Day 29 at 09:00, the last day mice were fed the nicotinic acid-free diet; Day 30 urine, Day 30 at 09:00 to Day 31 at 09:00, the 2nd day fed the nicotinic acid-containing diet; Day 32 urine, Day 32 at 09:00 to Day 33 at 09:00, the 4th day fed the nicotinic acid-containing diet; Day 34 urine, Day 34 at 09:00 to Day 35 at 09:00, the 6th day fed the nicotinic acid-containing diet; Day 36 urine, Day 36 at 09:00 to Day 37 at 09:00, the 8th day fed the nicotinic acid-containing diet; Day 38 urine, Day 38 at 09:00 to Day 39 at 09:00, the 10th day fed the nicotinic acid-containing diet; and Day 40 urine, Day 40 at 09:00 to Day 41 at 09:00, the 12th day fed the nicotinic acid-containing diet.

Measurements of B-group vitamins in urine. The concentrations in urine of members of B-group vitamins and the catabolites such as thiamin, riboflavin, NAD, MNA, Py2, 4-Py, and Nam N-oxide were measured using high-performance liquid chromatography. The concentrations of L-Trp catabolites such as AnA, KA, 3-HK, XA, 3-HA, and QA were measured using high-performance liquid chromatography.

The values of urinary excretion amounts of vitamins were shown in terms of mol per g food intake because the food intakes were difference between WT mice and QPRT-KO mice.

Results

Effects of recovery from niacin deficiency on food intakes and body weights. The QPRT-KO mice cannot biosynthesize niacin from tryptophan. Therefore, they absolutely need preformed niacin such as dietary niacin acid or Nam. When the QPRT-KO mice were fed a 20% casein diet without preformed niacin acid, daily food intakes became lower compared with those of WT mice (Fig. 1(A)). The food intakes of the QPRT-KO mice became significantly lower at Day 5 of the experiment. The food intakes of the QPRT-KO mice decreased over time, dropping to the lowest value at Day 12. This bottom value remained constant. After the diet was changed to the 20% casein diet with niacin acid from the 20% casein diet without niacin acid, food intakes of the QPRT-KO mice increased dramatically (Fig. 1(A)). Food intakes of the WT mice remained constant during the experiment regardless of the type of diet, the 20% casein diet with or without niacin acid.

The body weights of the QPRT-KO mice increased within the first 7 days; however, weights decreased after this time period (Fig. 1(B)). The lowest value was attained at around Day 22 of the experiment. Body weight was regained by changing the diet from the 20% casein diet without niacin acid to the diet with niacin acid (Fig. 1(B)). In WT mice, body weight was gained regardless of the type of diet, the 20% casein diet with or without niacin acid.

Table 1. Food compositions.

<table>
<thead>
<tr>
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<th>20% Casein diet with nicotinic acid</th>
<th>20% Casein diet without nicotinic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/kg of diet</td>
<td></td>
<td></td>
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<tr>
<td>Vitamin-free milk casein</td>
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<td>200</td>
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<td>Gelatinized cornstarch</td>
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<td>334</td>
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<tr>
<td>Sucrose</td>
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<td>167</td>
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<td>Corn oil</td>
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<td>50</td>
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<td>35</td>
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<tr>
<td>Vitamin mixture (AIN-93)</td>
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<td>10</td>
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<tr>
<td>NIA-free vitamin mixture (AIN-93)</td>
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<td>0</td>
</tr>
<tr>
<td>L-Methionine</td>
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<td>4</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>1000</td>
</tr>
</tbody>
</table>

1Ref. 9.

Effects of recovery from niacin deficiency on urinary excretion amounts of Nam and its catabolites

NAD+ and Nam are synthesized from niacin acid as follows: niacin acid → niacin acid mononucleotide → niacin acid adenine dinucleotide → NAD+. NAD+ is then hydrolyzed to Nam. These subsequent reactions occur only in the liver. The synthesized Nam from niacin acid in the liver is distributed to non-hepatic tissues. Surplus Nam in non-hepatic tissues returns to the liver where it is catabolized to Nam.
N-oxide, MNA, 2-Py, and 4-Py, which are eliminated in the urine.

The urinary excretion amounts of Nam and its catabolites such as Nam N-oxide, MNA, 2-Py, and 4-Py were nearly zero when the QPRT-KO mice were fed the 20% casein diet without nicotinic acid (Fig. 2, −NiA, left side part). These results are because the QPRT-KO mice cannot biosynthesize Nam from tryptophan. The Nam and its catabolites were clearly detected in urine when the diet was changed from the nicotinic acid-free diet to the nicotinic acid-containing diet as shown in Fig. 2 (+NiA, right side part).

The urinary excretion amounts of Nam and its catabolites from the QPRT-KO mice fed the nicotinic acid-containing diet was lower compared with that in the WT-mice. This is because the QPRT-KO mice cannot biosynthesize Nam from tryptophan. Namely, the difference equates to the biosynthesized amounts from tryptophan.

Effects of recovery from niacin deficiency on urinary excretion of B-group vitamins

The urinary excretion of each B-group vitamin was not significantly different between the QPRT-KO and WT mice when fed a nicotinic acid-free diet as shown in Fig. 3 (−NiA, left side part). Specifically, the urinary excretion of each B-group vitamin was not affected by niacin-deficiency. Therefore, when mice were fed a nicotinic acid-containing diet, the urinary excretion of each B-group vitamin was not observed to be different between the QPRT-KO and WT mice as shown in Fig. 3 (+NiA, right side part).

Effects of recovery from niacin deficiency on urinary excretion of tryptophan catabolites

From the findings described above (Figs. 1 and 2), the niacin-deficient mice (QPRT-KO mice fed a nicotinic acid-free diet) showed true niacin-deficiency. Under such conditions, we investigated how recovery from niacin-deficiency affects tryptophan catabolism to survey a functional biomarker of niacin nutritional status. Interesting phenomena were observed as shown in Fig. 4: The urinary excretion of KA began to decrease along with the recovery from the niacin deficiency (Fig. 4(B)) and the other hand, the urinary excretion of XA began to increase (Fig. 4(D)). Other urinary tryptophan catabolites such as AnA (Fig. 4(A)), 3-HK (Fig. 4(C)), and 3-HA (Fig. 4(E)) were not affected and kept constant regardless of the type of diet. Namely, the urinary excretion amounts of AnA, 3-HK, 3-HA were not changed by niacin nutritional status.

Higher urinary excretion of QA in QPR-KO mice compared with WT mice, regardless of the type of diet (Fig. 4(F)), indicates that QPRT-KO mice cannot metabolize QA. A specific event was the increase in QA formation along with the recovery from the niacin deficiency.

Effects of recovery from niacin deficiency on the urinary excretion ratio of XA:KA

A ratio of some two values is generally a superior indicator versus a single value. We calculated the ratio of urine XA to urine KA. The urinary excretion ratio of XA:KA increased according to recovery of niacin nutritional status (Fig. 5). A lower value equates to a lower niacin nutritional status.

Discussion

The members of the eight kinds of B-group vitamins bring to bear the full spectrum of function when the balance is well-maintained. Niacin deficiency could destroy the balance. Understanding the effects of niacin deficiency on levels of other members of B-group vitamins may shed light on the mechanism of niacin-deficiency syndrome. Many B-group vitamins are involved in the tryptophan degradation pathway. As described above, niacin-deficiency did not cause any

Fig. 1. Effects of recovery from niacin deficiency on food intake (A) and body weight change (B) in mice.
Notes: Each value is the mean ± SE for three mice. *Significant difference from WT mice in the same day.
Fig. 2. Effects of recovery from niacin deficiency on the urinary excretion amounts of nicotinamide (Nam) (A), and its catabolites nicotinamide N-oxide (Nam N-oxide) (B), 7-N-methylnicotinamide (MNA) (C), 7-N1-methyl-2-pyridone-5-carboxamide (2-Py) (D), 7-N1-methyl-4-pyridone-3-carboxamide (4-Py) (E), and SUM (Nam + Nam N-oxide + MNA + 2-Py + 4-Py) (F) in mice.
Notes: Each value is the mean ± SE for three mice. *Significant difference from WT mice in the same day.
Fig. 3. Effects of recovery from niacin deficiency on the urinary excretion of B-group vitamins. (A) Thiamin, (B) riboflavin, (C) 4-pyridoxic acid (4-PIC), (D) pantothenic acid (PaA), (E) folates, (F) biotin, and (G) vitamin B12 in mice.

Notes: Each value is the mean ± SE for three mice.
Fig. 4. Effects of recovery from niacin deficiency on the urinary excretion of tryptophan catabolites. (A) Anthranilic acid (AnA), (B) kynurenic acid (KA), (C) 3-hydroxykynurenine (3-HK), (D) xanthurenic acid (XA), (E) 3-hydroxyanthranilic acid (3-HA), and (F) quinolinic acid (QA) in mice.

Notes: Each value is the mean ± SE for three mice. *Significant difference from WT mice in the same day.
effects against levels of other B-group vitamins. Namely, niacin deficiency caused only low concentrations of niacin but did not affect the concentrations of other B-group vitamins.

Under these conditions, we investigated how recovery from niacin deficiency affects tryptophan catabolism to survey a functional biomarker of niacin nutritional status. We have already reported that the urinary excretion amount of SUM of Nam and its catabolites reflect niacin status and the urinary excretion amounts of vitamins are useful biomarker. In the present experiment, the urinary excretion of SUM almost recovered within four days after changing to niacin-containing diet from niacin-free diet. As mentioned in “Introduction”, the urinary excretion of SUM just reflect the storage amounts of free form of Nam in the body, but does not reflect physiological functions of PNC. This point is very important caveat. Of the tryptophan metabolites, significant changes during recovery from niacin deficiency were observed in KA and XA. KA is formed from kynurenine and XA from 3-HK. Of the upper stream of tryptophan catabolism, one step needs NADPH as a coenzyme, which is kynurenine 3-monooxygenase [EC 1.14.13.9]. Although we did not measure the activity of kynurenine 3-monooxygenase, its activity would be suppressed by lower concentration of NADPH. The suppression of kynurenine 3-monooxygenase makes the accumulation of kynurenine, which leads the increased formation of KA. At the same time, the inhibition of kynurenine 3-monooxygenase makes the decrease of 3-HK, which leads the decreased formation of XA. So, we choose a ratio of XA/KA, a putative functional biomarker of niacin nutritional status. The ratio was slowly recovered compared to the urinary excretion of SUM. Namely, feeding a niacin-containing diet for about 10 days were needed to recover from niacin deficiency if a urine ratio of XA/KA was used as a functional biomarker.

Considering the tryptophan catabolism, we have to think organ correlation. Upstream metabolites such as KA and XA in the urine were originated from non-hepatic tissues, and not from the liver (Fig. 6).

The characteristic phenomenon observed in the present study was that the urinary excretion amount of KA decreased and that of XA gradually increased with recovery of niacin deficiency. However, the urinary excretion amounts of 3-HK and 3-HA did not change.

Fig. 5. Effects of recovery from niacin deficiency on the urinary excretion ratio of XA:KA in mice.
Notes: Each value is the mean ± SE for three mice. *Significant difference from WT mice in the same day.

Fig. 6. Organ correlation with tryptophan catabolism.
Notes: ACMS, 2-amino-3-carboxymuconate-6-semialdehyde; 3-HA, 3-hydroxyanthranilic acid; 3-HADO, 3-hydroxyanthranilic acid 3,4-dioxogenase; KA, kynurenic acid; Kyn, kynurenine; MNA, N1-methylnicotinamide; Nam, nicotinamide; Nam N-oxide, nicotinamide N-oxide; NiA, nicotinic acid; NaAD. Nicotinic acid adenine dinucleotide; NaMN, nicotinic acid mononucleotide; NMN, nicotinamide mononucleotide; 2-Py, N1-methyl-2-pyridone-5-carboxamide; 4-Py, N1-methyl-4-pyridone-3-carboxamide; QA, quinolinic acid; XA, xanthurenic acid.
with recovery of niacin deficiency. These findings mean that the urine KA and XA mainly originate in non-hepatic tissues and that urine 3-HK and 3-HA originate from liver. Niacin deficiency might be more severe in non-hepatic tissues than in liver because liver can make Nam from tryptophan, but non-hepatic tissues cannot. As a result, the concentration of NADPH decreased much more in non-hepatic tissues and the reaction of kynurenine → 3-HK decreased and the formation of QA from the accumulated kynurenine occurred in non-hepatic tissues. On the other hand, during the recovery of niacin nutritional status, the concentration of NADPH in non-hepatic tissues recovered and as a result, the reaction of kynurenine → 3-HK was normalized and the formation of kynurenine → KA decreased. On the contrary, the increased 3-HK accelerated the formation of XA.

Another characteristic phenomenon was the increase in QA with recovering from niacin deficiency. This phenomenon is thought to be a specific event in QPRT-KO animals. The increase in QA means that the concentration of NADPH in liver was recovered to normal in KO animals. The increase in QA means that the phenomenon is thought to be a specific event in recovering from niacin deficiency. This finding is thought to be a specific event in QPRT-KO animals. The increase in QA means that the concentration of NADPH in liver was recovered to normal in KO animals. The increase in QA means that the phenomenon is thought to be a specific event in recovering from niacin deficiency.

According to the recovery from niacin deficiency, the urinary excretion of KA decreased and the urinary excretion of XA increased. Therefore, the urinary excretion ratio of XA:KA is a suitable functional biomarker of niacin nutritional status. Low values equate to low nutritional status.

In future studies, we intend to measure levels of XA and KA in human urine to survey whether niacin administration can decrease the ratio among subjects who have a lower excretion ratio of XA:KA.

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

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Disclosure statement
No potential conflict of interest was reported by the authors.

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