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Note

β-Nicotinamide Mononucleotide, an Anti-Aging Candidate Compound, Is Retained in the Body for Longer than Nicotinamide in Rats

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Summary The turnover of the oxidized form of nicotinamide adenine dinucleotide (NAD^+) has attracted interest in regard to longevity. Thus, compounds that can rapidly increase the cellular NAD^+ concentration have been surveyed by many researchers. Of those, β-nicotinamide mononucleotide (β-NMN) has been focused on. Studies on the biosynthesis of NAD^+ from β-NMN have been reported at the cellular level, but not at the whole animal level. In the present study, we investigated whether β-NMN is superior to nicotinamide (Nam) as a precursor of NAD^+ in whole animal experiments. To this end we compared the NAD^+ concentration in the blood and the urinary excretion amounts of NAD^+ catabolites. Rats were intraperitoneally injected with β-NMN or Nam. After the injection, blood samples and urine samples were collected at 3-h intervals. The concentration of blood total NAD (NAD^++NADH) in each sample showed no significant differences between the two groups. The urinary excretion amounts of NAD^+ catabolites in the urine samples collected at 3–6 h after the injection were lower in the β-NMN group than in the Nam group. These results suggest that β-NMN is retained in the body for longer than Nam.

Key Words β-nicotinamide mononucleotide, nicotinamide phosphoribosyltransferase, NAD^+, urine, rat

More than 2,200 kinds of enzymes are known, and about 500 of them need the oxidized form of nicotinamide adenine dinucleotide NAD^+, the oxidized form of nicotinamide adenine dinucleotide phosphate ($NADP^+$), and their reduced forms NADH and NADPH as coenzymes. Recently, it has been shown that the deacetylation reaction of the aging-related molecule, sirtuin, needs NAD^+ as a substrate (*1*). It has attracted attention as a link between metabolism and aging. The NAD^+ biosynthesis pathway has been classified into a de novo pathway from tryptophan (Trp) and a salvage pathway from nicotinamide (Nam). In particular, the salvage pathway couples with the sirtuin deacetylation reaction, and reuses Nam from deacetylation, converting it into β-nicotinamide mononucleotide (β-NMN) by nicotinamide phosphoribosyltransferase (Nampt). Moreover, nicotinamide mononucleotide adenylyltransferase (Nmnat) synthesizes NAD^+ from β-NMN and ATP. Nampt is the rate-limiting NAD^+ biosynthesis enzyme, and it plays a critical role in biological aspects (e.g., metabolism, inflammation and aging) by adjusting the NAD^+ level in response to nutrition and the environment (*2*). In addition, it has been reported that β-NMN improves age-associated metabolic diseases, such as diabetes and Alzheimer, because NAD^+ metabolites such as β-NMN increase the NAD^+ level (*3*–*5*). It has also been reported that after β-NMN treatment, the NAD^+ level in a 22-mo-old mouse is similar to that in a 6-mo-old mouse (*6*). Hence, it is thought that β-NMN has anti-aging effects.

It has been reported that the liver NAD^+ level in rat was significantly increased by injection of a large amount of Nam, which is an NAD^+ precursor (*7*). However, Nam is not converted into NAD^+ by the Nam→β-NMN→NAD^+ pathway. NAD^+ derived from Nam is made by the Nam→nicotinic acid (NiA)→nicotinic acid mononucleotide (NaMN)→nicotinic acid adenine dinucleotide (NaAD)→NAD^+ pathway, because Nampt is inhibited by NAD (*8*, *9*). On the other hand, NAD^+ synthesis from β-NMN is not regulated by the cellular NAD^+ level. In light of metabolic control mechanisms and many reports on β-NMN, β-NMN is likely to be more effective as an NAD^+ precursor than Nam. Studies on NAD^+ biosynthesis from β-NMN have been conducted at the cellular levels, but not at the whole animal level.

It has been previously shown that urinary excretion of water-soluble vitamins correlates with their intake (*10*). In addition, urine collection is easy and noninvasive (*10*). That is, the measurement of metabolite excretion in the urine is very useful.

In the present study, we investigated whether β-NMN is superior to Nam as a precursor of NAD^+ in whole animal experiments.

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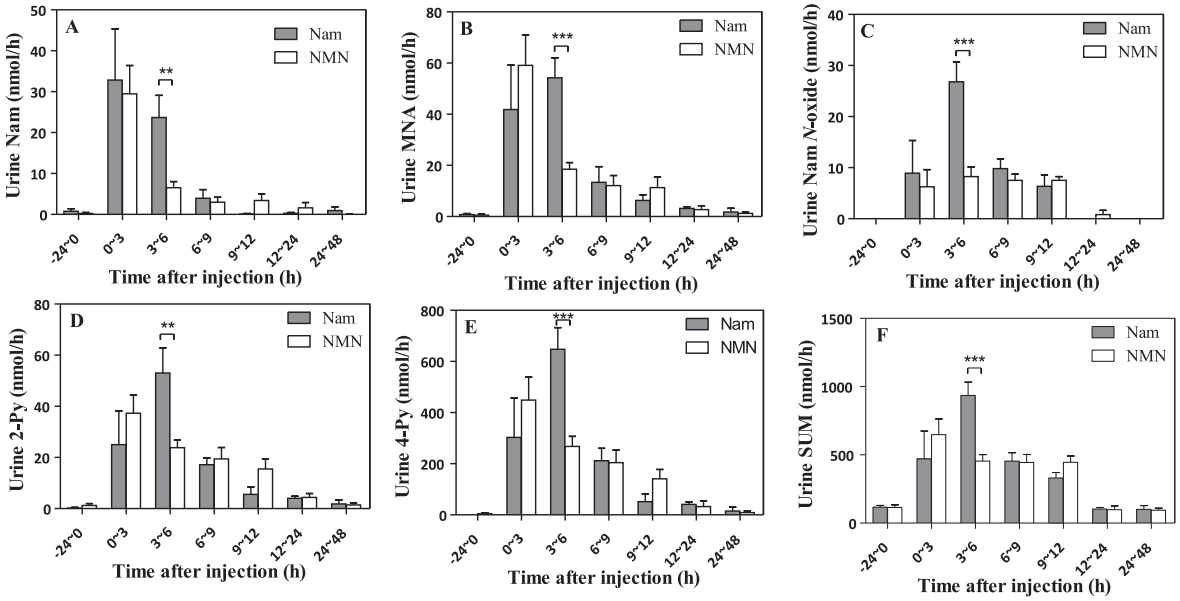

Fig. 1. The changes in NAD^+ metabolites excreted to the urine at −24–0, 0–3, 3–6, 6–9, 9–12, 12–24 and 24–48 h after Nam or β-NMN was administered to rats. Forty-five micromoles of Nam or β-NMN per kg body weight were intraperitoneally injected into rats at 0 h. Values are mean±SE, $n=5$–6. The values in a row without a common superscript letter differ, $p<0.05$, as determined by one-way ANOVA followed by Tukey's multiple comparison tests. (A) Urine Nam. (B) Urine MNA. (C) Urine Nam *N*-oxide. (D) Urine 2-Py. (E) Urine 4-Py. (F) Urine SUM.

Materials and Methods

Reagents. Vitamin-free milk casein, sucrose and L-methionine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was purchased from Ajinomoto (Tokyo, Japan). Gelatinized cornstarch, a mineral mixture (AIN-93G mineral mixture) (*11*), a vitamin mixture, NiA-free AIN-93 vitamin mixture containing 25% choline bitartrate (*11*), and β-NMN were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Nam ($C_6H_6N_2O$, MW=122.13) was purchased from Wako Pure Chemical Industries. NAD^+ was purchased from Sigma (St. Louis, MO). N^1-Methylnicotinamide (MNA) chloride ($C_7H_9N_2O$-HCl, MW=159.61) was purchased from Tokyo Chemical Industry (Tokyo, Japan). N^1-Methyl-2-pyridone-5-carboxamide (2-Py) ($C_7H_8N_2O_2$, MW=152.15) and N^1-methyl-4-pyridone-3-carboxamide (4-Py) ($C_7H_8N_2O_2$, MW=152.15) were synthesized by the methods of Pullman and Colowick (*12*), and Shibata et al. (*13*), respectively. All other chemicals used were of the highest purity available from commercial sources.

Animal treatment and diets.

Ethical approval of the study protocol: The care and treatment of experimental animals conformed to the guidelines set by the University of Shiga Prefecture (Shiga, Japan) for the ethical treatment of laboratory animals. The room was maintained at ~22°C with ~60% humidity. A 12-h light–dark cycle (06:00–18:00/18:00–06:00) was used.

Kinetics of the urinary excretion of NAD^+ catabolites after Nam or β-NMN administration. To acclimatize rats to their new environment, male Wistar rats (7 wk old; CLEA Japan, Inc., Tokyo) were kept in individual rat metabolic cages (CT-10; CLEA Japan). Rats were fed ad libitum a 20% casein diet consisting of 20% vitamin-free milk casein, 0.2% L-methionine, 46.9% gelatinized-cornstarch, 23.4% sucrose, 5% corn oil, 3.5% AIN-93G mineral mixture and 1% NiA-free AIN-93 vitamin mixture containing 25% choline bitartrate for 5 d. The rats were then divided into three groups: on the 6th day at 09:00 h, 45 μmol/kg Nam dissolved in 0.5 mL of sterilized saline was intraperitoneally injected into the rats of the first group; 45 μmol/kg β-NMN dissolved in 0.5 mL of sterilized saline was injected into rats of the second group; and 0.5 mL of sterilized saline was injected into the rats of the third group (control). Urine samples were collected in amber bottles containing 1 mL of 1 mol/L HCl at −24–0 h, 0–3 h, 3–6 h, 6–9 h, 9–12 h, 12–24 h and 24–48 h after the injection and stored at −25°C until use.

Kinetics of blood NAD^+ concentrations after Nam or NMN administration. To acclimatize rats to their new environment, male Wistar rats (7 wk old; CLEA Japan) were kept in cages and fed ad libitum the 20% casein diet described above for 5 d. The rats were then divided into three groups: at 09:30 h on the 6th day, 45 μmol/kg body weight (BW) Nam dissolved in 0.5 mL of sterilized saline was intraperitoneally injected into the rats of the first group; 45 μmol/kg BW β-NMN dissolved in 0.5 mL of sterilized saline was injected into the rats of the second group; and 0.5 mL of sterilized saline was injected into the rats of the third group (control). Blood samples were collected from the tail vein every 30 min for 6 h after the injection.

Comparison of the liver NAD concentrations, 3 h after

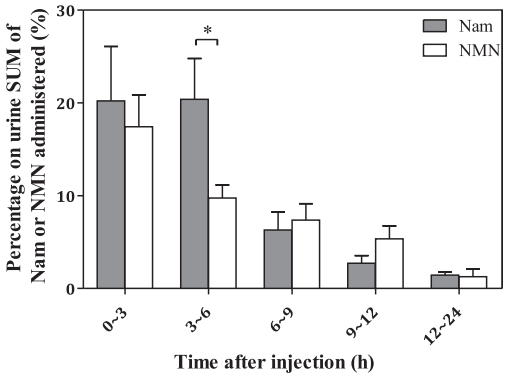

Fig. 2. Percentage of the urinary SUM of Nam or β-NMN administered. Forty-five micromoles of Nam or β-NMN per kg body weight were intraperitoneally injected into rats at 0 h. Values are mean±SE, n=5–6. * Statistical difference from the Nam group, determined by Student's t-test.

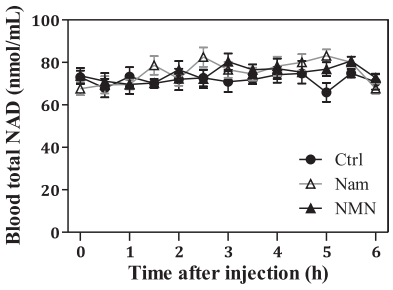

Fig. 3. The changes in blood total NAD (NAD^++NADH) concentration at 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 h after Nam or β-NMN administration into rats. Forty-five micromoles of Nam or β-NMN per kg body weight were intraperitoneally injected into rats at 0 h. Values are mean±SE, n=6. A significant difference was not observed, as determined by one-way ANOVA followed by Tukey's multiple comparison tests.

Nam or β-NMN administration. To acclimatize rats to their new environment, male Wistar rats (12 wk old; CLEA Japan) were kept in cages and fed ad libitum the 20% casein diet described above for 5 d. The rats were then divided into three groups: at 09:30 h on the 6th day, 45 μmol/kg BW Nam dissolved in 0.5 mL of sterilized saline was intraperitoneally injected into the rats of the first group; 45 μmol/kg BW β-NMN dissolved in 0.5 mL of sterilized saline was injected into the rats of the second group; and 0.5 mL of sterilized saline was injected into the rats of the third group (control). The rats were killed by decapitation at 3 h after the administration, the liver of each animal was dissected, and a portion (~0.25 g) was immediately treated as described in the literature (*14*).

Measurement of NAD^+ catabolites. NAD^+ catabolites, such as Nam (*13*, *15*), Nam *N*-oxide (*15*, *16*), MNA (*17*), 2-Py (*13*, *15*) and 4-Py (*13*, *15*), in the urine were measured as described previously. The SUM was defined as the total amount of Nam+Nam *N*-oxide+MNA+2-Py+4-Py.

Calculation method for the percentage of the urinary SUM of Nam or β-NMN administered. We assumed that the urinary SUM originating from food was constant during the experiment. Each percentage of the urinary SUM of Nam or β-NMN administered (%)={(urinary SUM of the Nam or β-NMN-administered group, mol/urine collected hours)−(urinary SUM of the control group, mol/urine collected hours)}÷(Nam or β-NMN administered, mol)×100. The urine collection hours were 0–3 h, 3–6 h, 6–9 h, 9–12 h, and 12–24 h.

Measurement of total NAD in the blood and liver. Total NAD (NAD^++NADH) in whole blood and liver was measured using the methods of Shibata and Murata (*14*).

Statistical analysis. Values are reported as mean±SE. Differences between the Nam group and the β-NMN group were analyzed by Student's t-test. $p<0.05$ was considered statistically significant. Prism version 5.0 was used for all analyses.

Results

Kinetics of the urinary excretion of NAD^+ catabolites after Nam or β-NMN administration

Figure 1 shows the changes in urinary excretion amounts of NAD^+ catabolites, such as Nam, Nam *N*-oxide, MNA, 2-Py and 4-Py, and their SUM (=Nam+Nam *N*-oxide+MNA+2-Py+4-Py) after Nam or β-NMN administration. Interestingly, the urinary excretion of all the tested catabolites was lower in the β-NMN group than in the Nam group at 3–6 h. Moreover, the percentage of the urinary SUM was lower in the β-NMN-administered group than in the Nam group at 3–6 h (Fig. 2). On the other hand, the percentages at 6–9 and 9–12 h tended to be higher in the β-NMN group than in the Nam group.

Kinetics of the blood total NAD (NAD^++NADH) concentration after Nam or β-NMN administration

The diets used in the present study did not contain niacin. Therefore, all total NAD was derived from dietary Trp and injected Nam or β-NMN. As shown in Fig. 3, the concentration of blood total NAD at each time point showed no significant differences among the three groups.

Comparison of the liver total NAD (NAD^++NADH) concentration 3 h after Nam or β-NMN administration

The concentration of liver total NAD at 3 h after Nam or β-NMN administration also showed no significant differences among the three groups (the values were around 800 nmol/g of liver).

Discussion

The cellular NAD^+ concentration is kept constant by the Nampt step, which catalyzes the reaction, Nam+5-phosphoribosyl-1-pyrophosphate (PRPP)→β-NMN+inorganic pyrophosphate. NAD^+ synthesis from Nam in the liver proceeds only when the NAD^+ level decreases below the physiological concentration, because Nampt is inhibited by NAD^+ (*18*). Consequently, even if Nam is injected, the NAD^+ level is hard to increase. However, the reaction that produces NAD^+

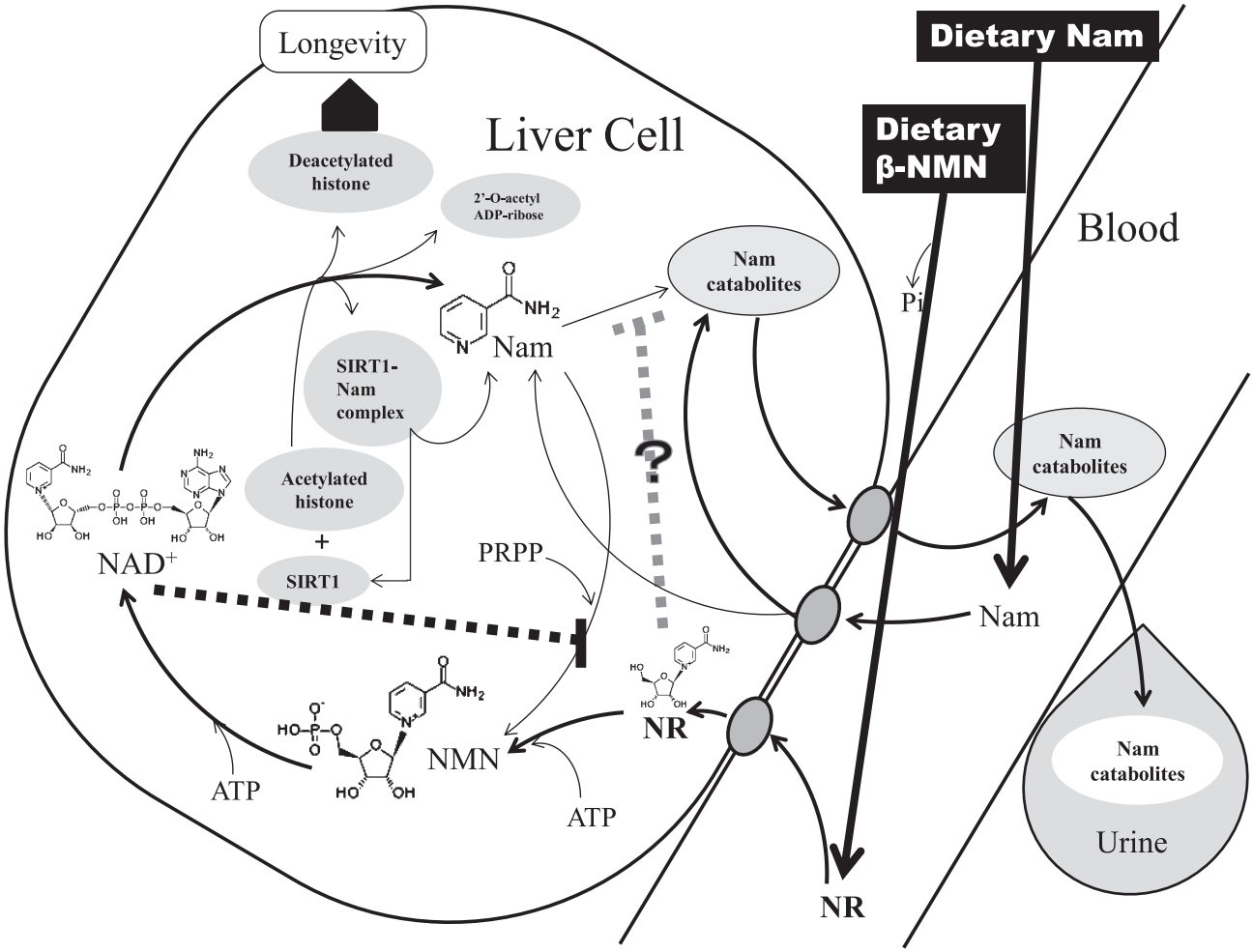

Fig. 4. Proposed NAD^+ biosynthesis pathways from Nam and β-NMN. Nam mainly catabolized to form inactive niacin compounds such as Nam *N*-oxide, MNA, 2-Py and 4-Py, which are eliminated into the urine. This is because the reaction, Nam + 5-phosphoribosyl-1-pyrophosphate (PRPP) → β-NMN + pyrophosphate, which is catalyzed by Nam phosphoribosyltransferase (Nampt), is inhibited by the physiological concentration of NAD^+. Therefore, administration of Nam does not contribute to the increased turnover of *salvage* biosynthesis of NAD^+ biosynthesis. If β-NMN is administered, it is dephosphorylated and is converted to nicotinamide riboside (NR) and it appears as NR in the bloodstream. NR is transported into liver cells and is re-phosphorylated to form β-NMN. This step (NR + ATP → β-NMN + ADP) should be the rate-limiting step of the conversion of NR to NAD^+. Thus, accumulation of NR should be observed in liver cells. The increased concentration of NR may inhibit the reaction of Nam → Nam catabolites. The resulting phenomenon accelerates the turnover of *salvage* biosynthesis of NAD^+, which activates the SIRT1 reaction, because SIRT1 (histone deacetylase) needs NAD^+. Deacetylated histone molecules induce DNA silencing, contributing to anti-aging and longevity.

from β-NMN is not controlled by the cellular NAD^+ level; therefore when β-NMN is injected, the NAD^+ levels increase easily.

In the present study, we compared the blood total NAD (NAD^+ + NADH) and urinary excretion amounts of NAD^+ catabolites in β-NMN- and Nam-administered rats. The concentration of blood total NAD and liver total NAD showed no significant differences among the three groups. However, when we examined the kinetics of the urinary excretion, the urinary excretion of the SUM was lower in the β-NMN group than in the Nam group at 3–6 h after the administration. Moreover, the percentage on the urinary SUM was much lower in the β-NMN group than in the Nam group at 3–6 h. This result suggests that β-NMN is retained in the body for longer than Nam is. In addition, this result means that β-NMN has a higher turnover of *salvage* biosynthesis of NAD^+ than Nam does. The resulting phenomenon accelerates the turnover of *salvage* biosynthesis of NAD^+, which activates the SIRT1 reaction, because SIRT1 (histone deacetylase) needs NAD^+. Deacetylated histone molecules induce DNA silencing, contributing to anti-aging and longevity.

The proposed biosynthesis pathway from Nam and β-NMN in liver cells is shown in Fig. 4. When β-NMN is intraperitoneally injected, β-NMN is dephosphorylated in the blood to form Nam riboside (NR), which is then transported into the cells. NR is re-phosphorylated to form β-NMN, which is then synthesized to NAD^+ in the nucleus. In contrast to Nam, which is controlled at the Nam → β-NMN reaction, the β-NMN biosynthesis pathway is not regulated. To silence DNA, activation

of SIRT1 (histone deacetylase) is necessary (*1*). Thus, administration of β-NMN might be a good method for suppressing aging.

Acknowledgments

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Authors' contributions

TK, NM and KS designed the study. TK and KS drafted the manuscript. NM critically read the draft paper and gave valuable comments. TK and NM performed the experiments. All authors read and approved the final manuscript.

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