Comparison of Metabolic Fates of Nicotinamide, NAD⁺ and NADH Administered Orally and Intraperitoneally; Characterization of Oral NADH

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Summary Since NADH has been implicated in medication for some symptoms and as a possible supplement for health, we characterized the metabolic fate of NADH orally given to mice by comparing with those of nicotinamide (Nam), NAD⁺ and NADH intraperitoneally or orally administered. Mice were individually housed in metabolic cages, and divided into two sets of four groups. Within each set, one group was intraperitoneally or orally administered saline and the other three groups received intraperitoneal or oral administration of a pharmacological dose of Nam, NAD⁺ or NADH (5 μ mol/mouse). Twenty-four hour urine samples for the day before and days 1 to 4 after administration were collected and analyzed for Nam and its metabolites. When mice were administered saline alone, urinary excretion of Nam and its metabolites, such as nicotinamide N-oxide (Nam N-oxide), N¹-methylnicotinamide (MNA), N¹-methyl-2-pyridone-5-carboxamide (2-Py), and N¹-methyl-4-pyridone-3-carboxamide (4-Py), was unchanged from day 0 to day 4. Intraperitoneal injection of Nam, NAD⁺ and NADH produced significant increases in urinary excretion of Nam and its metabolites. Similar results were obtained when Nam and NAD⁺ were given orally. On the other hand, oral administration of NADH did not bring about an increase in urinary excretion of Nam and its metabolites, suggesting that NADH in digestive organs has been decomposed to a compound(s) that cannot yield Nam. In fact, incubation of NADH at acidic pH to mimic the stomach resulted in rapid conversion of NADH to an unknown compound. Better understanding of the fate of oral NADH is needed for its therapeutic and supplemental use.

Key Words NADH, NAD⁺, oral administration, intraperitoneal administration, mouse

Reduced nicotinamide adenine dinucleotide (NADH) and its oxidized form (NAD⁺) in cells are synthesized mainly from dietary nicotinamide (Nam) (Fig. 1). Nam is actively absorbed into intestinal cells and distributed into various tissues, where it is used for biosynthesis of pyridine nucleotide coenzymes (1, 2). These coenzymes are also synthesized partly from tryptophan and nicotinic acid. Excess Nam is converted into nicotinamide N-oxide (Nam N-oxide), N¹-methylnicotinamide (MNA), N¹-methyl-2-pyridone-5-carboxamide (2-Py), and N¹-methyl-4-pyridone-3-carboxamide (4-Py) and these catabolites are excreted into urine in mice (3). It has been shown that orally taken NAD⁺ also supplies Nam, since NAD⁺ is metabolized to Nam in the small intestinal tract (1).

In contrast, the metabolic fate of oral NADH is unclear. It appears that instability of NADH in an acidic condition (gastric juice) (4) has made it difficult to pursue its fate. Nevertheless, NADH has been used as a novel medication for Parkinson's disease (PD) patients (5–7). Although support for this trial includes findings that NADH stimulates dopamine production through

activation of tyrosine hydroxylase (8), which is the rate-limiting step of dopamine biosynthesis (9), and the intravenous or oral NADH administration improves PD rating scale, it is still controversial for several reasons whether NADH is recommendable as a therapeutic agent of PD (5–7). Furthermore, it has been reported that NADH appears to act against jet lag (10) and malaise (11, 12).

Such a high incidence of NADH ingestion has prompted us to explore in vitro and in vivo changes of NADH. In the present study, we show that exposure of NADH to an acidic condition yields unknown products and that the metabolic fate of NADH orally given to mice markedly differs from that of intraperitoneal NADH. Metabolism of Nam, NAD⁺ and NADH administered either orally or intraperitoneally has also been compared by measuring urinary Nam and its metabolites.

MATERIALS AND METHODS

Chemicals. Vitamin-free milk casein, sucrose, L-methionine and Nam were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). N¹-Methylnicotinamide (MNA) chloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). NAD⁺ and NADH were

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Fig. 1. Proposed metabolic fate of the intraperitoneally injected NADH. Nam, nicotinamide; MNA, *N*¹-methylnicotinamide; Nam *N*-oxide, nicotinamide *N*-oxide; 2-Py, *N*¹-methyl-2-pyridone-5-carboxamide; 4-Py, *N*¹-methyl-4-pyridone-3-carboxamide; NMN, nicotinamide mononucleotide; PRPP, 5-phosphoribosyl 1-pyrophosphate.

purchased from Sigma-Aldrich Fine Chemicals (St. Louis, MO, USA). Nam *N*-oxide was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI, USA). *N*¹-Methyl-2-pyridone-5-carboxamide (2-Py) and *N*¹-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized by the methods of Pullman and Colowick (13) and Shibata et al. (14), respectively. Corn oil was purchased from Ajinomoto (Tokyo, Japan). The mineral and vitamin mixtures and the gelatinized cornstarch were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). All other chemicals used were of the highest purity available from commercial sources.

Stability of NAD⁺ and NADH in acidic solution. NAD⁺ and NADH were each dissolved in 0.1 mol/L HCl at 0.1 mg/mL and kept at 25°C. Each solution was directly

injected to an HPLC system. NADH was dissolved in H₂O at 0.1 mg/mL and immediately injected into the HPLC. NADH was dissolved in 0.1 mol/L HCl at 0.1 mg/mL at 25°C, and then injected into the HPLC at 1 min after the dissolution, 40 min after the dissolution, and 3 h after the dissolution. The chromatographic conditions were constant: column, Chemcosorb 7-ODS-L (4.6, i.d., ×250 mm); mobile phase, 10 mmol/L KH₂PO₄ (pH 3.0 adjusted by H₃PO₄):acetonitrile=96:4; column temperature, 30°C; detection, UV (260 nm); flow-rate, 1.0 mL/min; sample volume, 20 μL.

Animals and diets. This experimental design was approved by the Animal Experiment Committee of The University of Shiga Prefecture and the mice were handled according to Guidelines for Care and Use of Labora-

tory Animals.

Experiment 1: Male mice of the ICR strain (11 wk old) were obtained from CLEA Japan, Inc. (Tokyo, Japan). The mice were individually housed in metabolic cages (CM-10S; CLEA Japan, Inc.) and fed a complete

Table 1. Composition of the diets.

	Control diet (NiA-free, 20% casein diet)
	(g/kg of diet)
Milk casein (Vitamin-free)	200
L-Methionine	2
Gelatinized-cornstarch	459
Sucrose	229
Corn oil	50
Mineral mixture ¹	50
Vitamin mixture (NiA-free) ²	10

¹ Provided the following (g/kg of diet): CaCO₃, 14.645; CaHPO₄·2H₂O, 0.215; KH₂PO₄, 17.155; NaCl, 12.53; MgSO₄·7H₂O, 4.99; Fe(C₆H₅O₇)·6H₂O, 0.31115; CuSO₄·5H₂O, 0.078; MnSO₄·H₂O, 0.0605; ZnCl₂, 0.01; KI, 0.00025; and (NH₄)₆Mo₇O₂₄·4H₂O, 0.00125. Obtained from Oriental Yeast Co., Ltd., Tokyo, Japan.

² Provided the following (mg/kg of diet, except as indicated): retinyl acetate, 5,000 IU; cholecalciferol, 1,000 IU; tocopherol acetate, 50; menadione, 52; thiamine-HCl, 12; riboflavin, 40; pyridoxine-HCl, 8; cyanocobalamin, 0.005; ascorbic acid, 300; D-biotin, 0.2; folate, 2; calcium pantothenate, 50; *para*-aminobenzoic acid, 50; nicotinic acid, 60; inositol, 60; choline chloride, 2,000; and made up to 10 g with cellulose powder. Obtained from Oriental Yeast Co., Ltd.

20% casein diet (Table 1) and allowed free access to water throughout the experimental period. Body weight and food intake were measured daily at 09:00. The environmental conditions were constant: 12-h light/dark cycle, room temperature of 22±2°C, humidity of about 60%.

After 1 wk, they were divided into four groups of five each. One group was intraperitoneally injected with a sterile physiological saline solution (0.1 mL), while the other three groups were intraperitoneally injected with an appropriate dose of Nam, NAD⁺ or NADH (5 µmol/mouse) dissolved in sterile saline (0.1 mL) at 09:00. In the preliminary experiment of feeding a NiA-free 20% casein diet, the sum of Nam and its metabolites in the 24 h urine was about 1 µmol per mouse. For this reason, we decided dosage at 5 µmol as the amount of the lowest addition at which the increase of the excretion to the urine would be obviously confirmed. Twenty-four hour (09:00–09:00) urine samples from the day before (day 0) and days 1 to 4 after the injection were collected into bottles containing 1 mL of 1 mol/L HCl and stored at –25°C until analysis for Nam and its metabolites.

Experiment 2: The methods were the same as in Experiment 1 except for the route of administration. After 1 wk, they were divided into four groups of five. One group was orally administered a sterile physiological saline solution (0.1 mL), while to the other three groups was orally administered Nam, NAD⁺ or NADH (5 µmol/mouse) dissolved in sterile saline (0.1 mL) at 09:00.

Analyses. The quantities of Nam, 2-Py and 4-Py in the urine were measured simultaneously by the HPLC method of Shibata et al. (14). The urinary content of

Fig. 2. Time-dependent changes in the HPLC chromatogram of NADH dissolved in 0.1 mol/L HCl. A: NADH was dissolved in H₂O at 0.1 mg/mL and immediately injected into the HPLC. B–D: NADH was dissolved in 0.1 M HCl at 0.1 mg/mL at 25°C, and then injected into the HPLC at (B) 1 min after the dissolution, (C) 40 min after the dissolution, and (D) 3 h after the dissolution.

Fig. 3. Effects of intraperitoneal injection of Nam, NAD⁺ or NADH on the urinary excretion of nicotinamide and its metabolites in mice. On day 1 at 09:00, 5 μ mol/mouse of Nam (●), NAD⁺ (□) or NADH (△) dissolved in 0.1 mL of sterile saline was intraperitoneally injected into mice. As a control (○), 0.1 mL of sterile saline was injected. Twenty-four hour (09:00–09:00) urine samples were collected for 1 d before the administration (day 0) and days 1 to 4 after the injection. Sum=Nam+Nam N-oxide+MNA+2-Py+4-Py. Each point is the mean $\pm$ SE for 5 mice. Values with different superscript letters in the same figure are statistically significantly different at $p<0.05$ vs. the control group on the same day. *Significant at $p<0.05$ compared with the value of the respective day 0.

MNA or Nam N-oxide was measured by HPLC as previously described (15, 16).

Statistics. All data are presented as means $\pm$ SE, $n=5$. Statistical analysis was carried out by two-way ANOVA followed by Dunnett's multiple comparison test; the mice injected or administered with a sterile physiological saline solution were defined as the control groups (Stat View 5.0, SAS Institute Inc.; Cary, NC, USA).

RESULTS

Changes of NADH and NAD⁺ in acidic solution

It is widely accepted that NADH is unstable under acidic conditions but it is stable under alkaline conditions, while NAD⁺ shows the opposite properties (4). We first examined the breakdown of NADH dissolved in 0.1 mol/L HCl (Fig. 2). Figure 2A is a HPLC chromatogram of NADH dissolved in water; a single peak of

NADH was observed. Figure 2B, C, and D are HPLC chromatograms of the acidified NADH solution after incubation at 25°C for 1 min, 40 min and 3 h, respectively. Significant breakdown of NADH was seen after exposure for only 1 min (Fig. 2B) and incubation for 40 min resulted in a major breakdown product eluted at around 3 min (Fig. 2C). After incubation for 3 h, most NADH had been converted to this major product whose structure remains yet unknown (Fig. 2D). This product does not correspond to Nam, because Nam is eluted at around 6 min under the HPLC conditions used. As expected, NAD⁺ in acidic solution was unchanged after 3 h incubation at 25°C (data not shown).

Body weight and food intake

The daily changes in body weight and food intake among all the groups in Experiments 1 and 2 were almost constant (data not shown). Therefore, there was

Fig. 4. Effects of oral administration of Nam, NAD⁺ or NADH on the urinary excretion of nicotinamide and its metabolites in mice. On day 1 at 09:00, 5 μ mol/mouse of Nam (●), NAD⁺ (□) or NADH (△) dissolved in 0.1 mL of sterile saline was orally administered to mice. As a control (○), 0.1 mL of sterile saline was orally administered. The others are the same as in the legend for Fig. 3.

no influence on body weight or food intake by the route of administering NADH.

Urinary excretion of Nam and its metabolites, Nam N-oxide, MNA, 2-Py and 4-Py

Experiment 1. Mice received intraperitoneal injection of saline, Nam, NAD⁺ or NADH, and 24-h urine samples were collected to investigate their metabolic fates. The results are shown in Fig. 3. Day 0 means the urine sample of the day before administration and day 1 indicates the day of administration. When saline only was administered to mice, daily excretions into the urine of Nam and its metabolites, such as Nam N-oxide, MNA, 2-Py, and 4-Py, were almost constant from day 0 to day 4 (Fig. 3A–E). Injection of Nam, NAD⁺ and NADH produced significant increases in urinary excretions of Nam (Fig. 3A), Nam N-oxide (Fig. 3B), MNA (Fig. 3C), 2-Py (Fig. 3D), and 4-Py (Fig. 3E). The increment was seen in the 24-h urine samples after injection, but their metabolite excreta into the urine samples from day 2 to 4 were similar to those in saline-injected mice. Likewise, the sums of Nam, Nam N-oxide, MNA,

2-Py and 4-Py excreted into the 24-h urine samples just after the injection of Nam, NAD⁺ or NADH were significantly higher than those in saline-injected mice (Fig. 3F). It is noted that NADH injected intraperitoneally is nearly equivalent to that of Nam or NAD⁺ with respect to increases in urinary excretion of Nam and its metabolites. These results suggest that the intraperitoneally injected NADH is efficiently converted to NAD⁺, which is then deglycosidated into Nam and ADP-ribose in cells. Then Nam would take its normal metabolic pathway including urinary excretion.

Experiment 2. Saline, Nam, NAD⁺ or NADH was orally administered to mice, and 24-h urine samples were collected to investigate their metabolic fates. The results are shown in Fig. 4. The administration of Nam and NAD⁺ produced significant increases in Nam (Fig. 4A), Nam N-oxide (Fig. 4B), MNA (Fig. 4C), 2-Py (Fig. 4D) and 4-Py (Fig. 4E). The sums of Nam, Nam N-oxide, MNA, 2-Py and 4-Py excreted into urine were significantly increased (Fig. 4F) by administration of Nam or NAD⁺ as compared with that of saline. These increases

were seen in the urine samples collected on day 1 but thereafter the urinary levels of metabolites returned to those of controls, which are similar to when Nam or NAD⁺ was given intraperitoneally. In contrast, oral administration of NADH did not produce any increases in Nam or its metabolites.

DISCUSSION

This study was undertaken to investigate metabolic fate of NADH, because this compound has been tested as a pharmacological agent to ameliorate some symptoms including Alzheimer's disease (17), chronic fatigue syndrome (11, 12), and jet lag (10). Primarily, in the patient with PD to which NADH was intravenously administered, a beneficial clinical effect was observed (18); therefore, it was investigated whether orally given NADH has a similar effect (19, 20). Afterwards, the safety of the stabilized orally absorbable form of NADH tablet was tested in the rat (21) and the dog (22). Rainer et al. (23) reported that they found no evidence for any cognitive effect by oral NADH in dementia. NADH might also be used as a dietary supplement, but it is important to understand the fate of orally given NADH.

NADH is unstable in acidic conditions, while NAD⁺ is stable (4). Gross and Henderson (1) revealed that NAD⁺ is efficiently digested in the small intestinal tract, producing Nam that is transported into the blood and distributed to various tissues. Nam in the circulation appears to take two metabolic pathways depending on the cellular conditions; Nam is reused for biosynthesis of NAD⁺ and NADH or it gives rise to some downstream metabolites whose physiological functions are not known. Excess Nam and its metabolites are also excreted into the urine. In agreement with this notion, NAD⁺ given orally increased urinary excretion of Nam (Fig. 4) and its metabolites in a manner similar to that found when NAD⁺ was intraperitoneally administered (Fig. 3). NAD⁺-induced elevation of the urinary excretion was also similar to that caused by oral (Fig. 4) or intraperitoneal (Fig. 3) administration of Nam.

NADH appears to be almost equivalent to Nam and NAD⁺ when it was given intraperitoneally (Fig. 3) but the fate of orally administered NADH (Fig. 4) is entirely different; oral administration of NADH showed little effect on the urinary excretion of Nam and its metabolites (Fig. 4). These results present three possibilities that orally administered NADH a) may not be oxidized to NAD⁺, b) may not be absorbed by the mouse gastrointestinal system, or c) may have been converted to a compound(s) before absorption that cannot yield Nam. Incubation of NADH under acidic conditions similar to gastric juice has made the third possibility very likely; treatment only for 1 min caused degradation of NADH and after 40 min most of the NADH was converted into an unknown product (Fig. 2). Although degradation of NADH in the gastric juice may be more complex, structural analyses of this product is important, because oral NADH-induced improvement of some symptoms might be attributable to this compound. Thus at the present

time the fate of NADH orally given is poorly understood and therefore recommendation of its use as a therapeutic or supplement appears to be premature.

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