Biochemical and Biophysical Research Communications 744 (2025) 151136

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

NAD^+ deficiency plays essential roles in the hyperuricemia of stroke-prone spontaneously hypertensive rat via xanthine dehydrogenase to xanthine oxidase conversion

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ARTICLE INFO

Keywords:
SHRSP
Uric acid
Xanthine oxidase
Xanthine dehydrogenase
ß-nicotinamide mononucleotide

ABSTRACT

Inhibition of xanthine oxidoreductase (XOR) was shown to ameliorate the stroke susceptibility in the stroke-prone spontaneously hypertensive rat (SHRSP), suggesting hyperuricemia had a pathological role in this rat model. In this study, we thus aimed to explore mechanisms inducing hyperuricemia in SHRSP. XOR is known to have two forms, xanthine dehydrogenase (XDH) as the prototype and xanthine oxidase (XO) as the converted form through cleavage of a peptide bond or through formation of disulfide bonds in the enzyme. XO was shown to have a greater activity to produce UA and oxidative stress. We thus hypothesized that the excess conversion to XO caused the higher UA level in SHRSP. Male SHRSP at 10 weeks of age showed a higher serum level of UA and a higher activity of XO than those in Wistar-Kyoto rat. As the protein level of the total XOR did not differ between the two strains, the conversion to XO seemed responsible for the high UA level in SHRSP. Meanwhile, NAD^+ level in SHRSP was lower than that in WKY, suggesting that low NAD^+ promoted the conversion to XO in this strain. ß-nicotinamide mononucleotide (NMN) supplementation for 2 weeks increased NAD^+ level and reduced the serum UA level as well as the XO activity in SHRSP. These observations supported that a low NAD^+ accelerated the conversion of XDH to XO in SHRSP, which resulted in high UA. The current study suggested the potential significance of NMN supplementation in the treatment of hyperuricemia in humans.

1. Introduction

Significance of oxidative stress in the pathophysiology of cardiovascular diseases has been pointed out repeatedly [1]. Several reports suggested that increased reactive oxygen species (ROS) production played a critical role in vascular dysfunction, induction of inflammatory responses and cardiovascular fibrosis [2], which eventually resulted in severe complications such as stroke [3].

The stroke-prone spontaneously hypertensive rat (SHRSP) is an excellent model to study the pathophysiology of hypertension and cardiovascular diseases [4]. Significant role of oxidative stress in the pathophysiology of hypertension as well as stroke in this rat model was indicated in many studies [5–7]. As NADPH oxidases (NOXs) and xanthine oxidoreductase (XOR) are considered to play a major role in ROS production [5–8], in a previous study, we evaluated effects of NOX and XOR activity on stroke in SHRSP using a gene depleted SHRSP and a pharmacological inhibitors. The results showed that inhibition of XOR by febuxostat (Febx) improved the stroke latency significantly, which suggested a major role of XOR in the stroke susceptibility in SHRSP possibly through generation of ROS [8].

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https://doi.org/10.1016/j.bbrc.2024.151136
Received 20 November 2024; Received in revised form 2 December 2024; Accepted 4 December 2024
Available online 12 December 2024

XOR is involved in the final stage of the purine metabolism yielding uric acid (UA) [9]. Epidemiological studies indicated that an elevated level of serum UA was associated with cardiovascular diseases including hypertension, stroke and heart failure [9]. It is enigmatic, however, how a high level of UA imposes deleterious effects on the cardiovascular system when considering antioxidant property of UA itself [10].

There are two interconvertible forms of XOR; (1) xanthine dehydrogenase (XDH), which uses NAD^+ as an electron acceptor, yielding NADH as a byproduct, and (2) xanthine oxidase (XO), which uses oxygen as an electron acceptor to give ROS along with UA [9]. The XDH form is the prototype in all organisms including mammals. In mammals, however, XDH can be converted to XO through two processes: (1) the reversible conversion in which sulfhydryl groups of the cysteine residues form disulfide bonds through oxidization, creating a configurational change. This type of conversion was reported to occur mainly in hypoxic/ischemic conditions [11]. The other process is (2) the irreversible proteolytic conversion in which a peptide bond is cleaved irreversibly to release a subunit of enzyme (130 kD in size) with XO activity [12]. In both cases, the enzyme in the XO form loses ability to interact with NAD^+. As a result, XO uses oxygen instead of NAD^+ generating ROS while the XDH form does not release ROS [13]. This observation suggests that the XO/XDH ratio is an important factor determining ROS production in the metabolic pathway of purine to UA [13]. Based on the discussion above, we hypothesized that excess of the XO conversion would have a key role in the pathophysiology in SHRSP.

In this report, we therefore evaluated the XO/XDH conversion in SHRSP and the effect of β-nicotinamide mono nucleotide (NMN) supplementation on XO activity since NMN supplementation was reported to increase NAD^+, which promoted the reversible conversion from XO to XDH conversion *in vivo* [14,15].

2. Materials and Methods

2.1. Animal procedures

For measurements of basic UA level in serum, and for western blotting analysis and measurements of XO and XOR activity in liver, ten weeks old male WKY/Izm, SHR/Izm and SHRSP/Izm fed with normal chaw were used. Rats were sacrificed via euthanizing by isoflurane (3 %) at 5mL/rat and blood samples were withdrawn from the abdominal aorta. Blood samples were centrifuged at 3000 rpm for 5 min at 4 °C to separate serum. Liver tissue was collected in two Eppendorf tubes. One tube was snap-frozen in liquid nitrogen and the other tube containing fresh liver tissue was used measuring XO and XOR activity. For further analyses, serum and frozen liver samples were stored at −20 °C and at −80 °C, respectively.

For evaluation of effects of NMN and Febx, 18 male SHRSP at 8 weeks old were employed. After blood pressure (BP) and body weight (BW) were measured, they were divided into 3 groups and fed with; (1) plain water [control (Cont) group], (2) Febx at 30 mg/L in drinking water [Febx group] or (3) NMN at 3 mg/mL in drinking water [NMN group] for two weeks [8,16]. Water and food consumption were monitored every day. At the end of the experimental period (at 10 weeks old), BP and BW were measured again, and all the rats were sacrificed for collecting serum and liver tissue as described above. NMN was generously provided by Abe Yoando Pharma, CO. Ltd. (Tokyo, Japan).

WKY/Izm, SHR/Izm and SHRSP/Izm were provided by the Disease Model Cooperative Research Association (Kyoto, Japan). All the animal procedures were conducted according to the approval of the Local Committee of Animal Research in Shimane University (#IZ2-22, IZ5-80).

2.2. Biochemical analyses

Liver lysates were prepared by homogenizing the tissues (60 mg/mL RIPA buffer) 20 times with loosely fitted homogenizer first and then 6 times with the tightly fitted homogenizer on ice. The component of RIPA Lysis buffer was 1x TBS, 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.15 SDS, 0.004 % sodium azide, a protease inhibitor cocktail and (100 mM) sodium orthovanadate (sc-24948, Santa Cruz Biotechnology, Dallas, Texas). Liver lysates were centrifuges at 15000 rpm for 10 min at 4 °C and the supernatant was collected and stored at −20 °C. Protein concentration was determined by the method of Bradford with bovine serum albumin as a standard.

Serum UA was measured using Spotchem (SPOTCHEM™ EZ SP-4430, ARKRAY, Inc., Kyoto, Japan) according to the instruction. The XO activity measured by hydrogen peroxide production was conducted using the Xanthine Oxidase Assay Kit (EXOX-100, BioAssay Systems, Hayward, CA). The total XOR activity and XO activity measured by UA production was performed according to the previous study [17]; UA production with and without addition of 500 μM NAD^+ was assumed to represent the total XOR (i.e., XDH + XO) activity and the XO activity alone, respectively. The NAD^+ level was measured using NAD^+/NADH Assay kit (E2ND-100, BioAssay Systems, Hayward, CA). Enzymatic activity assay of the liver uricase was conducted using Uricase Assay Kit (Fluorometric, ab234042, Abcam, Cambridge, UK).

2.3. Western blotting analysis

SDS-PAGE was performed using a polyacrylamide gradient gel (Mini Protein Any KD, Bio-Rad Lab Inc, Hercules, CA). After electrophoresis for 40 min at 200 V, the gels were electrically blotted onto PVDF or nitrocellulose membrane. The membranes were then blocked with 5 % (w/v) skim milk dissolved in TBS-T (0.05 M Tris-HCl at pH 7.6, 0.15 M NaCl, 0.5 % Tween 20) for 1 h at room temperature in a mild shaker. A rabbit monoclonal anti-XO antibody (EPR4605, Abcam, Cambridge, UK) was diluted by 1:2500 in 1 % skim milk. The membranes were then incubated overnight with mild shaking at 4 °C. Then the membranes were incubated with a secondary antibody (HRP-linked anti-rabbit IgG antibody, Cell Signaling Technology, Danvers, MA) at room temperature for 1 h. The ECL Prime reagent (Pierce ECL western blotting substrate, Thermo Fisher scientific # 32106, Waltham, MA) was used to visualize the protein bands by a gel imaging system-Image quant-800 (Amersham™ ImageQuant™ 800 Systems, Washington DC).

2.4. Sequence analysis

Whole genome sequencing (WGS) was performed for SHRSP/Izm, SHR/Izm, and WKY/Izm. DNA libraries were prepared using a TruSeq DNA PCR-free kit (Illumina, San Diego, CA), and 459–482 million 150-bp paired-end reads were sequenced on a HiSeq Sequencer (Illimina, San Diego, CA) with an average read depth of 42-44x. We used the BWA program (version 0.7.15) for mapping the reads on the mRatBN7.2 reference genome, the GATK program (version 3.8) for variant discovery, and Ensembl Variant Effect Predictor for annotation. DNA variants (SNPs, insertions, deletions, and nucleotide repeats) that might predispose to the inter-strain differences of the XO activity were searched using the WGS data in and around the *Xdh* gene of the three strains.

2.5. Statistical analyses

In this study, all values were expressed as mean ± SEM. The biochemical data were compared using ANOVA, Student's *t*-test or Dunnett's test. The analyses were conducted with Prism (v.8, GraphPad Prism Software Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

3. Results

BP and BW of SHRSP and WKY at 10 weeks of age are summarized in the Supplementary Table 1. Fig. 1 indicates that, at 10 weeks of age, serum UA was significantly higher in SHRSP than in WKY (Fig. 1A). In

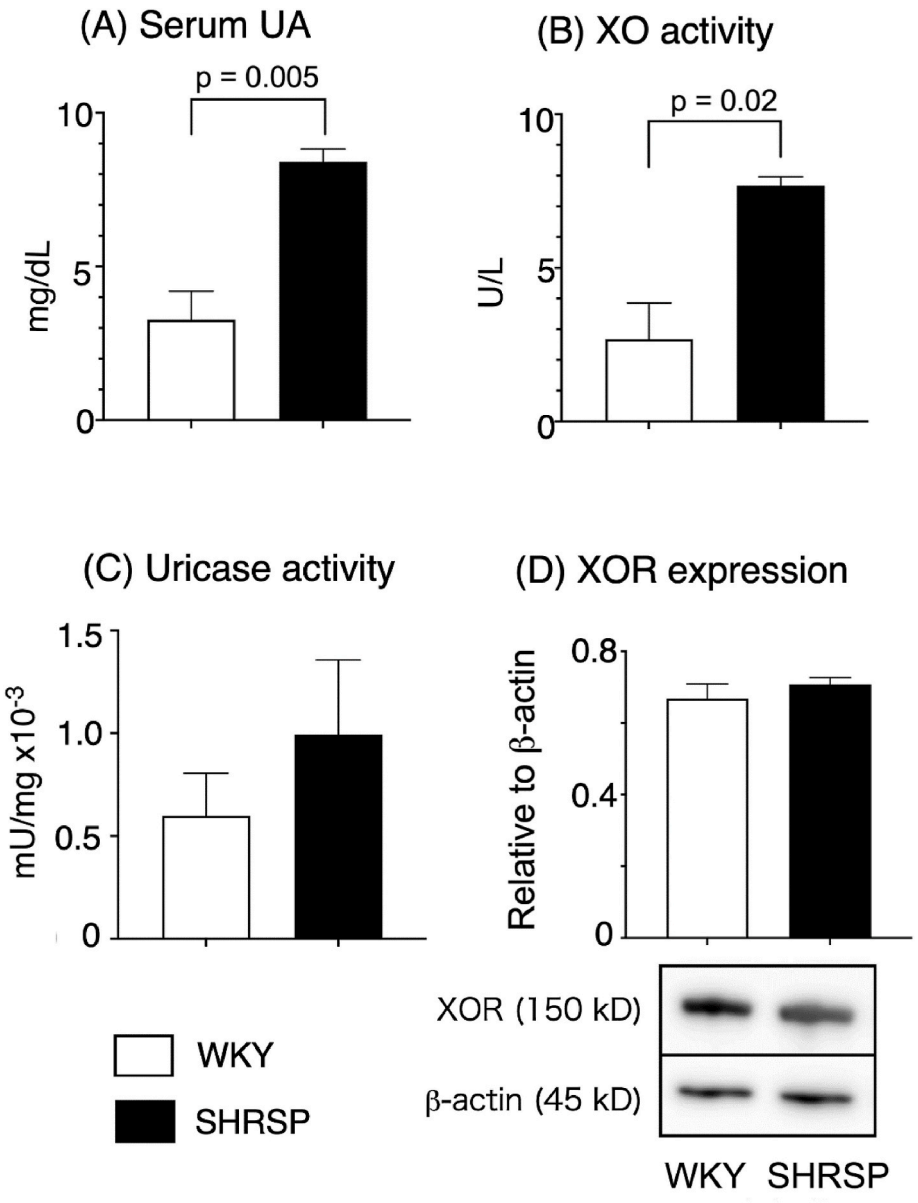

Fig. 1. Comparison of serum UA, XO activity, uricase activity and XOR protein expression between WKY and SHRSP.
Serum UA level (A), liver XO activity (B), uricase activity (C) and XOR protein expression (D) were measured in WKY and SHRSP (n = 6 for each strain) as described in Materials and Methods. Each column and error bar shows mean and SEM, respectively. Student's test was performed for a statistical evaluation.

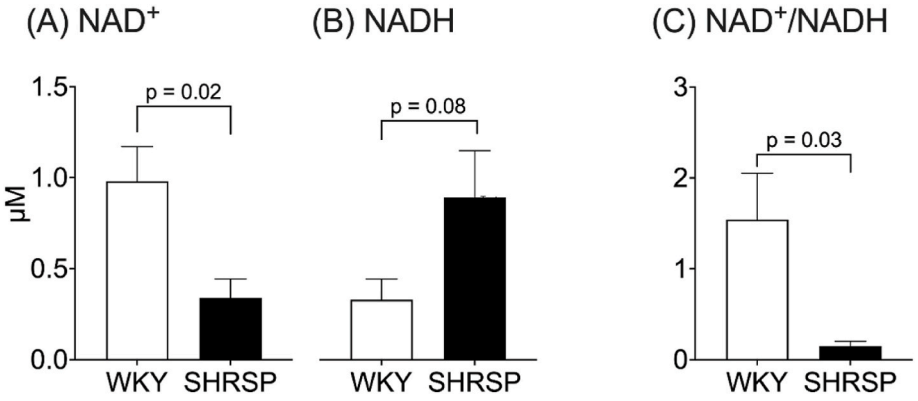

Fig. 2. NAD^+ and NADH levels in WKY and SHRSP.
NAD^+(A) and NADH (B) were measured in the liver lysates of WKY and SHRSP (n = 6 for each strain) as described in Materials and Methods. NAD^+/NADH ratio was calculated accordingly. Each column and error bar shows mean and SEM, respectively. Student's test was performed.

accordance with it, XO activity in the liver was significantly greater in SHRSP than in WKY (Fig. 1B). On the other hand, uricase activity did not differ significantly between the two strains (Fig. 1C). To confirm the results of XO activity measurement above, we additionally examined the total XOR (i.e., XDH + XO) activity and the XO activity alone by measuring UA production. The results indicated that the total XOR activity under the presence of NAD^+ and the XO activity under the absence of NAD^+ were significantly ($P = 0.046$) and marginally ($P = 0.071$) different, respectively, between WKY and SHRSP (see Supplementary Fig. 1). Western blotting analysis indicated no significant difference in the expression level of XOR protein between SHRSP and WKY (Fig. 1D). Fig. 1D further indicates that, neither in WKY nor in SHRSP, any additional bands other than the band for the full XOR protein at 150 kD were observed, implying no proteolytic irreversible conversion to XO occurred in the two strains.

As shown in Fig. 2, the NAD^+ and NADH levels were significantly lower and higher in SHRSP, respectively, when compared with those in WKY (Fig. 2A and B). As a result, NAD^+/NADH ratio was significantly greater in SHRSP than in WKY (Fig. 2C). The observations in Figs. 1 and 2 suggested that the reversible conversion to XO was accelerated in SHRSP due to the lower NAD^+ level [12].

To obtain further evidence supporting this hypothesis, we next examined effects of NMN supplementation on XO activity and serum UA level in SHRSP. As shown in Supplementary Fig. 2, oral supplementation of NMN, as well as of Febx, did not affect food or water consumption in rats. Under this condition, BW increase after two weeks was significantly less in NMN group than in Cont group while BW increase in Febx group did not differ from that in Cont group (Fig. 3A). systolic BP (SBP) tended to be lower in Febx and NMN group than in Cont group though difference was not significant (Fig. 3B).

As shown in Fig. 4, NMN supplementation significantly increased serum NAD^+ level (Fig. 4A), which resulted in a greater NAD^+/NADH ratio (Fig. 4C). Under this condition, serum UA and liver XO activity were both significantly decreased in NMN group (Fig. 5A and B). As expected, Febx reduced serum UA and liver XO activity significantly (Fig. 5A and B). No changes in XOR protein expression were observed after Febx or NMN treatment (Fig. 5C).

We additionally evaluated serum UA, XO activity, expression of XOR

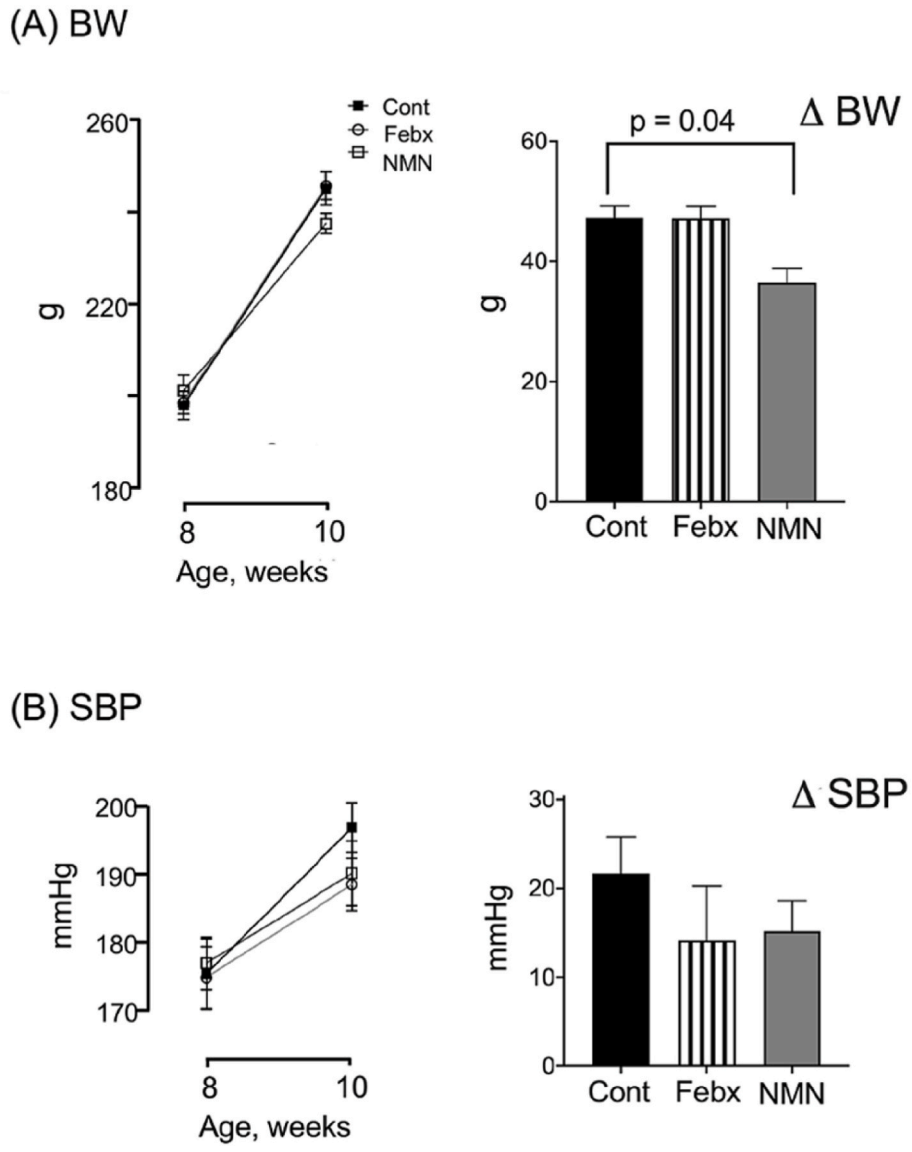

Fig. 3. Effects of Febx and NMN supplementation on the BW and SBP in SHRSP.
Febx or NMN in drinking water was given to SHRSP for 2 weeks from 8 to 10 weeks of age as described in Materials and Methods (n = 6 for each group). Changes in BW(A) and SBP (B) were shown. Pannels on the right indicate increase of BW and SBP during the experimental period. Each column and error bar shows mean and SEM, respectively. Dunnett's test was performed for a statistical analysis.

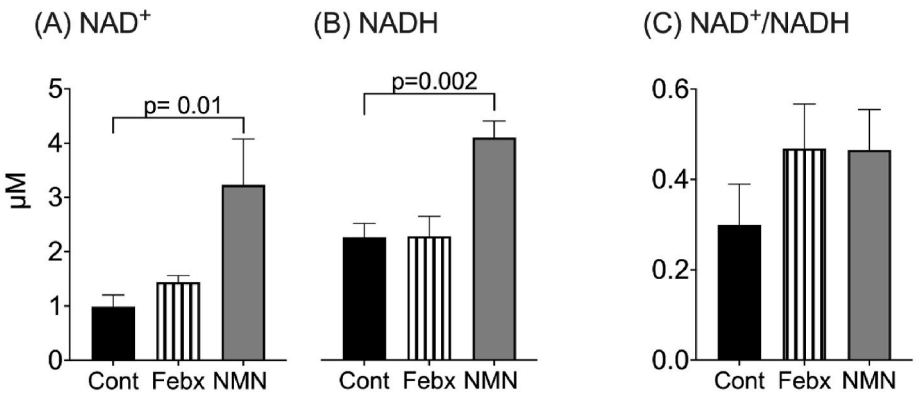

Fig. 4. NAD^+ and NADH level in SHRSP treated with Febx or NMN.
NAD^+ (A), NADH (B) and NAD^+/NADH ratio (C) were evaluated in liver lysates of SHRSP after 2 weeks administration of Febx and NMN. Each column and error bar shows mean and SEM, respectively. Dunnett's test was performed (n = 6 for each group).

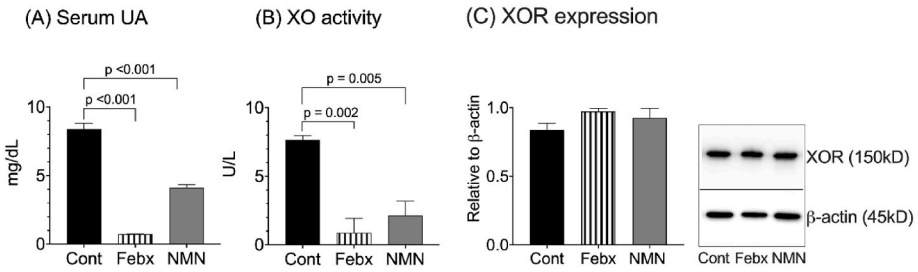

Fig. 5. Effects of Febx and NMN supplementation on serum UA, liver XO activity, liver XOR protein expression in SHRSP.
Serum UA (A), liver XO activity (B) and XOR protein expression (C) were measured in SHRSP treated with Febx or NMN (n = 6 for each group) as described in Materials and Methods. Each column and error bar shows mean and SEM, respectively. Dunnett's test was performed.

protein, uricase activity, and NAD^+ and NADH level in SHR. As shown in Supplementary Fig. 3, those parameters in SHR did not differ significantly from those in SHRSP. Further, analysis of a high-quality WGS of WKY, SHR and SHRSP of Izm colony identified no sequence variations in the coding sequence of the *Xdh* gene (data not shown). The coding sequences of *Xdh* of the three strains of Izm colony were the same as WKY, SHR and SHRSP of other colonies, which were available in the Rat Genome Database (https://rgd.mcw.edu).

4. Discussion

The present study suggested that high XO activity in SHRSP, which was due to a greater XO/XDH ratio caused by the reversible conversion from XDH to XO (see Fig. 1). As no differences between the two strains were found either in the expression level of XOR protein nor in the coding sequence of the *Xdh* gene, the high XO activity in SHRSP was not due to increase of the total amount of XOR enzyme nor due to functional sequence variations in the *Xdh* gene. XO was reported to have a greater ability to produce UA when compared with XDH [18]. Accordingly, a high XO/XDH ratio would give greater production of UA as well as of ROS in SHRSP [19]. Different from humans, rodents have uricase, an enzyme metabolizing UA to allantoin [20–22]. We showed no difference of the uricase activity between WKY and SHRSP, indicating that uricase did not contribute to the different level of UA between the two strains (see Fig. 1).

It was of interest how SHRSP acquired the excess conversion to XO. The fact that SHRSP had a low NAD^+ and that restoration of NAD^+ level by NMN supplementation reduced XO activity suggested that a low NAD^+ level in this rat strain would play an important role in the conversion of XDH to XO [12]. As NAD^+ is the electron acceptor for XDH, NAD^+ deficiency may promote the XO conversion [12,13]. Recently, CD38 was found to be over-expressed in SHRSP [23]. As CD38 catabolizes NAD^+, over-expression of CD38 may reduce NAD^+ level [24]. In addition, a recent study indicated that the tryptophan-kynurenine pathway, the pathway for *de novo* synthesis of NAD^+ from tryptophan, was suppressed in SHRSP due to alteration in the microbiota [25]. Some genes in this pathway were found mutated in SHR as well [26]. These observations may emphasize the role of the tryptophan-kynurenine pathway on the NAD^+ level in SHRSP. On the other hand, previous studies reported that multiple ROS-producing and erasing systems were causally related to the high level of oxidative stress in SHRSP [7]. As oxidative stress is known to accelerate the reversible conversion from XDH to XO [27], exacerbation of the XO conversion may be exacerbated through such a secondary process after the high oxidative stress in SHRSP. Further investigation would be warranted in future.

In the present study, we did not focus on the association of the high XO activity with cardiovascular phenotypes observed in SHRSP. However, previous studies indicated that reduction of XOR activity by Febx ameliorated both stroke latency and BP increase in SHRSP [8], and that NMN administration significantly suppressed stroke occurrence suggesting that an increase in NAD^+ level was important to prevent cerebral stroke in this rat model [24]. Although, in the latter study, it was suggested that activation of intracellular autophagy was the key mechanism of the beneficial effect of NMN administration [24,28], reduction of XO activity might have some contribution to it if the observations of the present study was considered.

Despite of firm epidemiological evidence showing that hyperuricemia was a risk factor of various cardiovascular diseases, the etiology is still controversial [29]. As UA itself is an antioxidant, it is attractive to assume that not UA *per se* but oxidative stress generated by XO is responsible for the deleterious effects [19]. On the other hand, recent studies suggested that UA activated inflammasome in cells, which induced sterile inflammation [30]. Therefore, it is possible that

increased UA *per se* induced hypertension as well as other cardiovascular complications through activation of inflammatory reactions [31]. In this context, it is of note that studies of uricase-knockout mice showed varying effects of the loss of uricase activity on BP; although most of the knockout mice suffered from nephropathy due to hyperuricemia, hypertension was observed in only a few studies [32]. As depletion of uricase activity is expected to increase UA level without activating XO, the observations on the uricase-knockout mice above may support that high UA has only a modest effect on BP if any. Recently, two mice genetically modified to have either XDH activity alone or XO activity alone were developed [17]. It would be interesting to make genetically-modified SHRSP to have XDH activity alone to examine phenotypic effects of XO in SHRSP in a future study.

SHRSP is a substrain derived from SHR. Although the two strains share some cardiovascular phenotypes such as hypertension, they have some phenotypes different from each other [4]. To obtain some clues about the association of high XO activity and cardiovascular phenotypes, we compared serum UA, XO activity, XOR expression, NAD^+ and NADH levels in SHR. As shown in Supplementary Fig. 2, the high XO activity (and the resulting high UA level) and the low NAD^+ level were shared by SHR and SHRSP. Contribution of low NAD^+ and high XO activity to cardiovascular complications should be carefully evaluated in these hypertensive models.

In the present study, the effect of Febx on BP was not statistically significant (see Fig. 3), which was inconsistent with the result of our previous study [8]. This may be due to difference in the experimental design, i.e., with and without salt loading. BP tended to decrease by 5–8 mmHg after NMN or Febx treatment in the present study, which was comparable with the data in other studies [32,33]. It may be necessary to increase the sample number and/or to take a longer experimental period to obtain a significant result.

In concluding remarks, our present study showed the significantly high XO activity in SHRSP. Although the mechanisms behind it were not thoroughly clarified yet, the key role of NAD^+ level on the reversible conversion to XO was suggested as the higher XO activity was ameliorated by NMN supplementation. These observations imply that inhibition of the conversion to XO by NMN supplementation would be a possible therapeutic strategy to reduce UA and oxidative stress to prevent cardiovascular complications in humans.

CRediT authorship contribution statement

Sara Amelia Ferdaus: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Hiroki Ohara:** Resources, Investigation, Data curation. **Hiroyuki Matsuo:** Resources, Methodology, Investigation, Data curation. **Kohei Kawakami:** Resources, Methodology, Investigation, Data curation. **Fumihiko Takeuchi:** Formal analysis. **Koichi Fujikawa:** Investigation, Data curation. **Emi Kawakita:** Resources, Methodology. **Norihiro Kato:** Formal analysis. **Toru Nabika:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Keizo Kanasaki:** Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:K. K. Lecture Fees: Boehringer Ingelheim Japan, Eli Lilly Japan, Astellas, Novo Nordisk Pharma, Mitsubishi Tanabe, Daiichi Sankyo, Sanofi, Sumitomo Pharma, Kowa, Kyowa Kirin, Taisho, AstraZeneca, Bayer, Novartis, OtsukaCollaboration: Boehringer Ingelheim Japan, Boehringer-Ingelheim (Germany), Kowa, Mitsubishi Tanabe, BayerResearch support: Boehringer Ingelheim Japan, Nipro, Life ScanAcknowledgement: The author would represent cordial thanks to Abe Yoando Pharma, CO. Ltd for generously providing NMN.

Acknowledgement

Sara Amelia Ferdaus was supported by MEXT scholarship from Japanese Government. Majorioty of this work was supported by the funds from Shimane University. Additionally, Emi Kawakita and Keizo Kanasaki were supported by grants from the Japan Society for the Promotion of Science to KK (22K08330, 23K14567) and the Uehara Memorial Foundation to KK (2019). KK is under a consultancy agreement with Boehringer Ingelheim. KK collaborated with Boehringer Ingelheim (Germany), Kowa, Bayer, Tanabe-Mitsubishi for projects not related to this manuscript. Shimane University was supported by funds from Boehringer Ingelheim, Mitsubishi Tanabe Pharma, Taisho Pharmaceutical, Ono Pharmaceutical, Kowa, Nipro, and Life Scan Japan. KK received lecture honoraria from Dainippon-Sumitomo Pharma, Astellas, Astra Zeneca, Ono, Otsuka, Taisho, Tanabe-Mitsubishi, Eli Lilly, Boehringer-Ingelheim, Novo Nordisk, Sanofi, Bayer, Novartis, Kyowa Kirin, Eisai, Medtronic, Abbott, and Kowa.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2024.151136.

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