The Effects of *Morinda citrifolia* (Noni) Fruit Juice on the Prevention of Stroke by Promoting Production of Nitric Oxide through the Brain of the Spontaneously Hypertensive Stroke Prone (SHRSP) Rats

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Abstract: *Morinda citrifolia* (Noni) is a traditional folk medicinal plant and has a long history of use as a food and medicine. In order to reveal the effects of Noni fruit juice (NFJ) on stroke prevention, we performed experiments using spontaneously hypertensive stroke prone (SHRSP) rats. NFJ did not change rat body weight, food intake, and water intake. However, both systolic blood pressure (SBP) and diastolic blood pressure (DBP) were significantly decreased after NFJ treatment in SHRSP rats. Furthermore, NFJ significantly increased the survival rate, urinary nitric oxide (NO) concentration was significantly higher in the NFJ group, and endothelial NO synthase (eNOS) phosphorylation levels increased in the brain after NFJ treatment. Two pathways regulate eNOS phosphorylation: the insulin-dependent pathway and the insulin-independent pathway. For the insulin-dependent pathway, phosphorylation of insulin receptor substrate 1 (IRS1) and protein kinase B (Akt) did not change in the NFJ group. For the insulin-independent pathway, expression of adenosine monophosphate-activated protein kinase (AMPK) phosphorylation, liver kinase B 1 (LKB1), and silent information regulator 1 (Sirt1) significantly increased in the brain of SHRSP rats after NFJ treatment. These data suggested that NFJ prevented stroke by improved blood circulation, increased NO production, and elevated eNOS phosphorylation by stimulating the insulin-independent pathway (Sirt1-LKB1-AMPK-eNOS).

Keywords: *Morinda citrifolaia*, stroke, spontaneously hypertensive stroke prone rats, nitric oxide, endothelial nitric oxide synthase.

INTRODUCTION

Stroke is a lifestyle disease, and the stroke mortality rate decreases by advanced medical treatment. However, there are still many stroke patients in Japan, which places a large healthcare burden on society. One of the main causes of stroke is hypertension [1]. If patients have hypertension for a long time, functional modifications in the cerebrovascular system can occur [2]. Therefore, it is important to avoid and treat hypertension. However, long-term drug therapy may cause side effects, so it is necessary to research and develop novel treatments with few side effects.

Nitric oxide (NO) is important for vascular tone [3]. In animal models of hypertension and patients with hypertension, NO production is abnormal, which leads to hypertensive vascular lesion formation [4]. NO is generated by endothelial NO synthase (eNOS) in blood vessels, and eNOS is protective against pathological vascular remodeling [5], hypertension [6], atherosclerosis [7], and complications associated with diabetes [8, 9]. There are two pathways that regulate eNOS phosphorylation: the insulin-dependent pathway (IRS1-Akt) and the insulin-independent pathway (Sirt1-LKB1-AMPK) (Figure **1**).

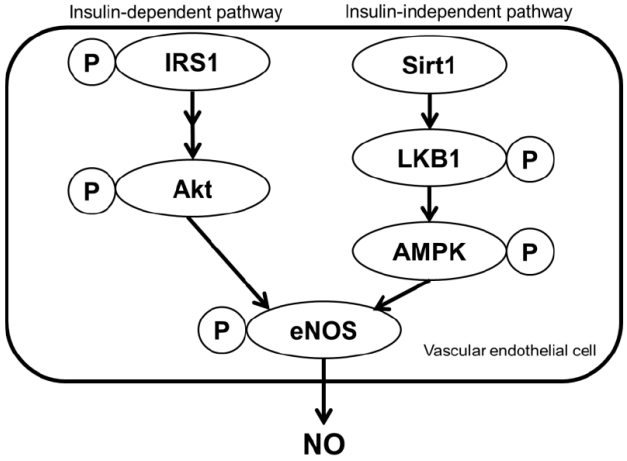

Figure 1: NO secretion signaling in the vascular endothelial cell.

Spontaneously hypertensive stroke prone (SHRSP), a substrain of SHR created by selective breeding, is a model for basic research on hypertension-associated

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cerebrovascular injury because the SHRSP model presents with marked elevation of blood pressure and almost all of these animals develop stroke. Numerous nutritional and pathological studies using SHRSP have revealed that various foods are protective against hypertension and stroke [1, 10, 11].

Morinda citrifolia (Noni) is a traditional folk medicinal plant [12] and has a long history of use as a food in tropical regions [13]. Noni has been reported to have a broad range of therapeutic and nutritional effects [14]. There is some evidence that Noni fruit juice (NFJ) has been used for the successful treatment of the cold, influenza, diabetes, hypertension, cancer, and other illnesses [15, 16]. However, there are no reports related to stroke protection. Therefore, we investigated whether NFJ has preventive effects against hypertension and stroke and clarified the mechanisms of these effects in the brain of SHRSP rats.

MATERIALS AND METHODS

Study 1: Investigation of the Effects of NFJ on Protection against Stroke and Survival.

Experimental Animals

Six-week-old male SHRSP rats were obtained from Japan SLC (Shizuoka, Japan). All rats were maintained at 22–24°C and 40–60% humidity under artificial lighting with 12 h light and dark cycles. Rats were randomly assigned to two groups (n = 10 for each group): a control group (not treated) and a NFJ group (administered water containing 10% NFJ, which was supplied by M&K Laboratories Co., Ltd, Nagano, Japan, Lot number: OR2919D1). All groups consumed an SP diet (Funakoshi Co., Tokyo, Japan) (Table **1**) and had free access to rat chow and water throughout the experiment. We administered NFJ to SHRSP rats by free intake and measured food intake, water intake, body weight, blood pressure, survival rates, and days of survival. Rats were identified for potential termination if they showed clinical signs caused from severe stroke such as paralysis, tremor, and spasms. After rats died, we confirmed that rats developed stroke by necropsy. The skulls of SHRSP rats were carefully opened and stroke was diagnosed if intracranial hemorrhagic was present in the form of meningeal or intraventricular bleeding or blood clots. All procedures were performed in accordance with the guiding principles for the care and use of animals in the field of physiological sciences established by the Physiological Society of Japan and guidelines for the care and use of animals set by Mukogawa Women's University.

Table 1: Composition of SP Diet

Component	Content (%)
Moisture	7.0
Total protein	20.5
Total fat	5.0
Total fiber	3.2
Ash	5.0
Non-nitrogen soluble compound	59.3
Minerals	
Calcium	0.74
Magnesium	0.21
Potasium	0.75
Sodium	0.40

Blood Pressure and Heart Rate Measurements

Systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate were measured by using a sphygmomanometer (UR-1000, Ueda Co., Chiba) using the tail-cuff method.

Statistical Analysis

Data are expressed as mean ± standard deviation of the mean (S.D). Cumulative survival was analyzed for differences according to Kaplan Meier followed by the Wilcoxon test. Statistical analyses of the data were performed by Student's t-test. A *P* value of less than 0.05 was considered significant.

Study 2: Analysis of the Mechanism of Stroke Improvement after NFJ Treatment

Experimental Animals

Male SHRSP rats at 6 weeks of age were housed as in Study 1. All rats at 6 weeks of age were divided into two groups: a control group and a NFJ groups administered water containing 10% NFJ (n = 10 for each group). We measured food and water intake daily. Body weight and blood pressure were measured every two weeks. At the conclusion of the 4-week treatment period, 24 h urine samples were collected by placing the rats in metabolic cages. Food was withdrawn but water was provided. All rats were anesthetized with pentobarbital (65 mg/kg body weight). The tissues were immediately dissected, cleaned to measure organ weight, and the organs were promptly frozen in liquid nitrogen and stored at -80°C for experiments in the future. All procedures were carried out in accordance with the guiding principles for the care and use of

animals in the field of physiological sciences established by the Physiological Society of Japan and guidelines for the care and use of animals set by Mukogawa Women's University.

Blood Pressure Measurement

Systolic blood pressure (SBP) was measured using a sphygmomanometer (UR-1000, Ueda Co., Chiba) using the tail-cuff method.

Urinary NO_2/NO_3 Concentration Measurements

Urine samples were centrifuged at 3000 rpm for 5 min and supernatants were analyzed. NO_2/NO_3 concentration in the urine was measured using a NO_2/NO_3 Assay Kit-FX (Fluorometric) (Dojin laboratories Co., Ltd., Japan) following the manufacturer's protocol.

Primary and Secondary Antibodies

Immunoblotting was performed with the following commercially available antibodies: anti-rabbit eNOS, anti-rabbit phospho-eNOS (Ser1177 and Thr495), anti-rabbit AMPK, anti-rabbit phospho-AMPK, anti-rabbit phospho-LKB1, anti-rabbit Sirt1, anti-rabbit Akt, anti-rabbit phospho-Akt (Ser473), anti-rabbit phospho-IRS1 (Ser1101, 318, and 612), anti-rabbit IgG and anti-mouse IgG from Cell Signaling Technology (Beverly, MA); anti-rabbit phospho-IRS1 (Tyr989) and anti-rabbit IRS1 from Santa Cruz Biotechnology; and anti-mouse β-actin from Sigma (St. Louis, Mo, US).

Protein Extraction Experiments

Brain tissue was homogenized in ice-cold homogenization buffer containing 50 mM Tris-HCl (pH7.4), 100 mM NaCl, 1% Nonidet P-40, 0.25% Na deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM NaF, 2 mM Na_3VO_4, 30 mM Na pyrophosphate, 2 mM PMSF, 1 mM benzamidine, 0.02 g/mL trypsin inhibitor, 0.02 g/mL leupeptin, and 0.02 g/mL aprotinin. After incubation for 30 min on ice, lysates were centrifuged at 12,000 rpm for 10 min and supernatants were isolated. Proteins were extracted by boiling the tissues in 0.5 mM Tris-HCl (pH 6.8), glycerol, 10% SDS, 0.1% bromophenol blue, and 2-mercaptoethanol.

Western Blot Analysis

Proteins (20 μg/lane) were electrophoresed using a 10–12.5% SDS-PAGE gel at 100 V for 90 min. After electrophoresis, proteins were transferred onto a PVDF membrane (Amersham Life Science Inc. Buckinghamshire) at 100 mA for 1 h. The membrane was blocked with Blocking One or Blocking One-P (Nacalai Tesque, Japan) for 30 min. After blocking, the membrane was incubated overnight with primary antibody in antibody solution 1 (Toyobo, Japan). Blots were washed with TTBS containing 1 M Tris-HCl (pH 7.5), NaCl, and 20% Tween 20 and incubated for 1 h with a 1:10000 dilution of anti-rabbit, goat, or mouse IgG-horseradish peroxidase. Detection was achieved using a Chemi-Lumi One Super (Nacalai Tesque, Japan). β-actin was used as an internal control. Densities of the bands were measured using Image J software.

Statistical Analysis

Statistical analysis was performed the same as in Study 1.

RESULTS

Study 1: Investigation of the Effect of NFJ on Survival

Effects of NFJ on Body Weight, Food Intake, and Water Intake

First, we examined the effects of NFJ on body weight, food intake, and water intake in SHRSP rats. When rats were treated daily with NFJ or control for 4 weeks, there were no significant differences in body weight, food intake, and water intake between the two groups (Figure **2A**, **B**, **C**).

Effects of NFJ on Blood Pressure, Heart Rate, and Survival Rate in SHRSP Rats

Next, we measured two types of blood pressure: systolic blood pressure (SBP) and diastolic blood pressure (DBP) using the tail-cuff method. The elevation of both SBP and DBP is significantly inhibited after 2 weeks of NFJ treatment (Figure **3A**). However, heart rate did not change between the two groups (Figure **3C**). Kaplan-Meier survival curves were significantly higher and the average number of survival days also expanded in the NFJ group (Figure **3D**, **E**). When all rats died, we sacrificed the rats immediately and confirmed that the cause of death was a developing stroke.

Study 2: Analysis of the Mechanism of Stroke Improvement after NFJ Treatment

Effects of NFJ on Body Weight and SBP in SHRSP Rats

In Study 1, we observed rats after death to determine the cause of death. Next, to clarify the mechanism of the developing stroke, we examined the

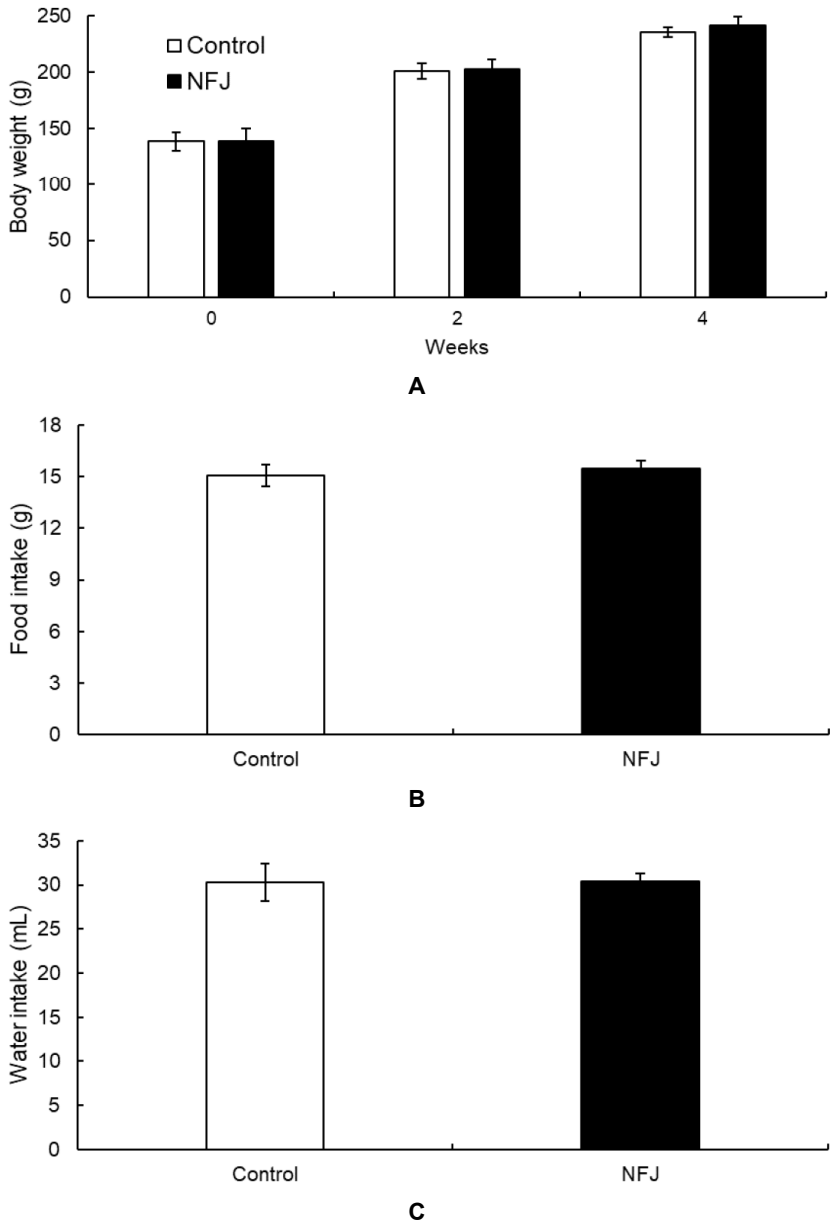
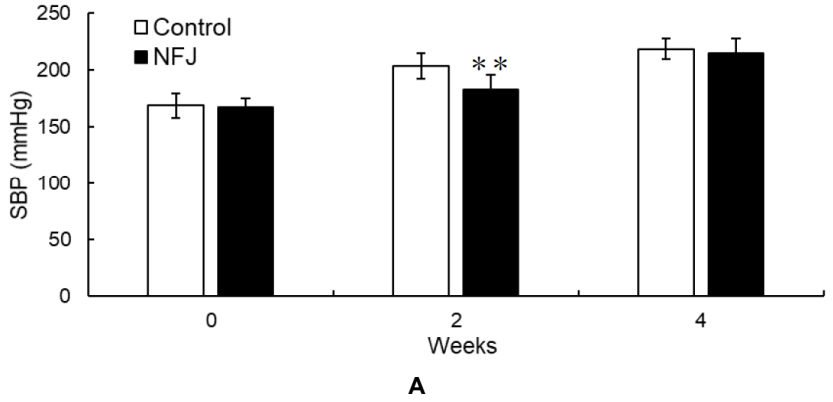


Figure 2: Effects of NFJ on body weight, food intake, and water intake in SHRSP rats.

Six-week-old male SHRSP rats were treated daily with 10% NFJ or vehicle for 4 weeks. Body weight development (**A**), food intake (**B**), and water intake (**C**) are shown. Data are expressed as mean ± S.D.; n = 10 for each group.



(Figure 3). Continued.

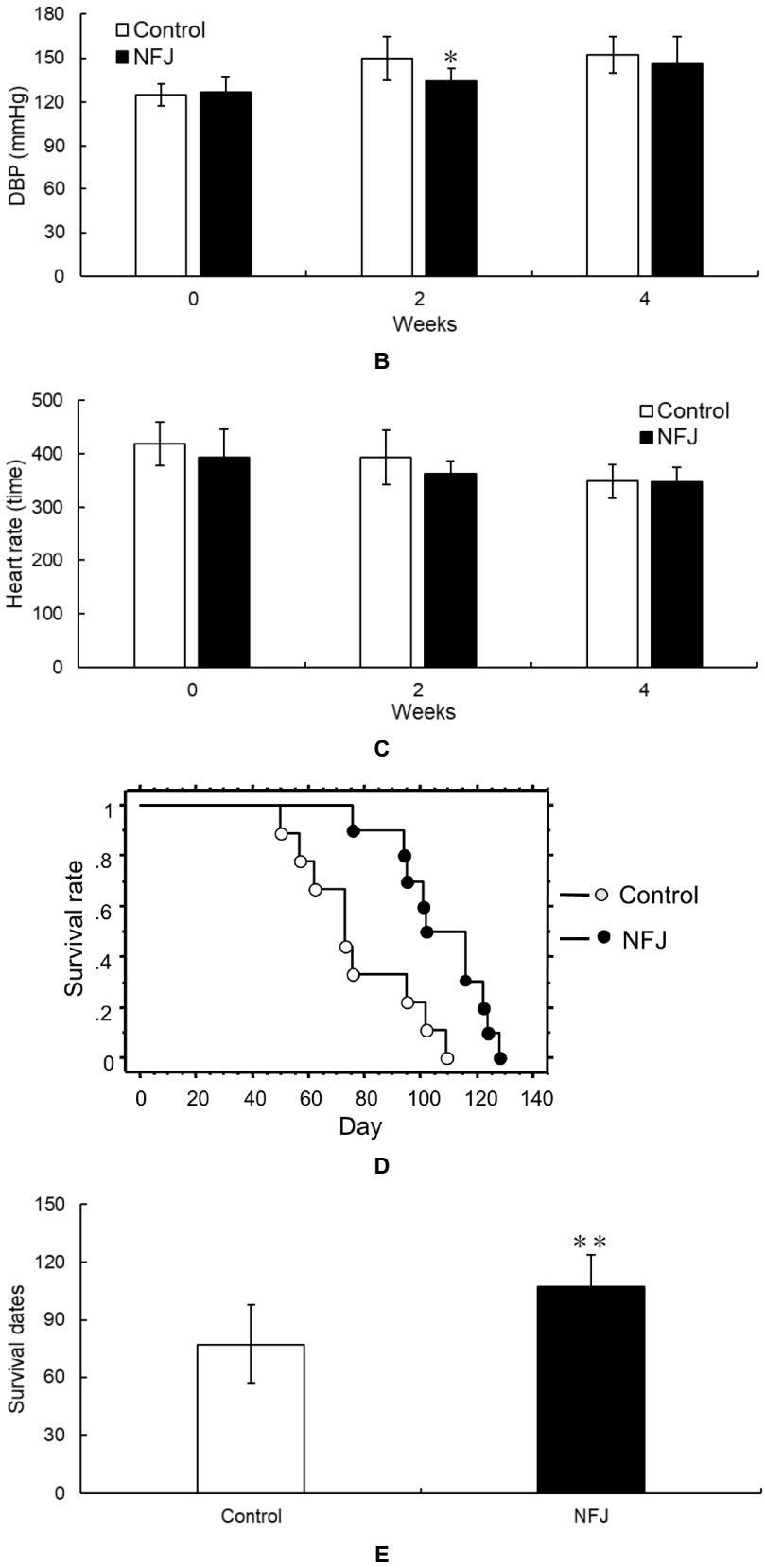


Figure 3: Effects of NFJ on systolic blood pressure (SBP), diastolic blood pressure (DBP), heart rate, survival rate, and days of survival in SHRSP rats.

Measurement of SBP (**A**) and DBP (**B**) performed by tail-cuff method and heart rate (**C**); survival rate (**D**) and days of survival (**E**) are shown. Data are expressed as the mean ± S.D.; n = 10 for each group. *$P < 0.05$, **$P < 0.01$ vs. control group.

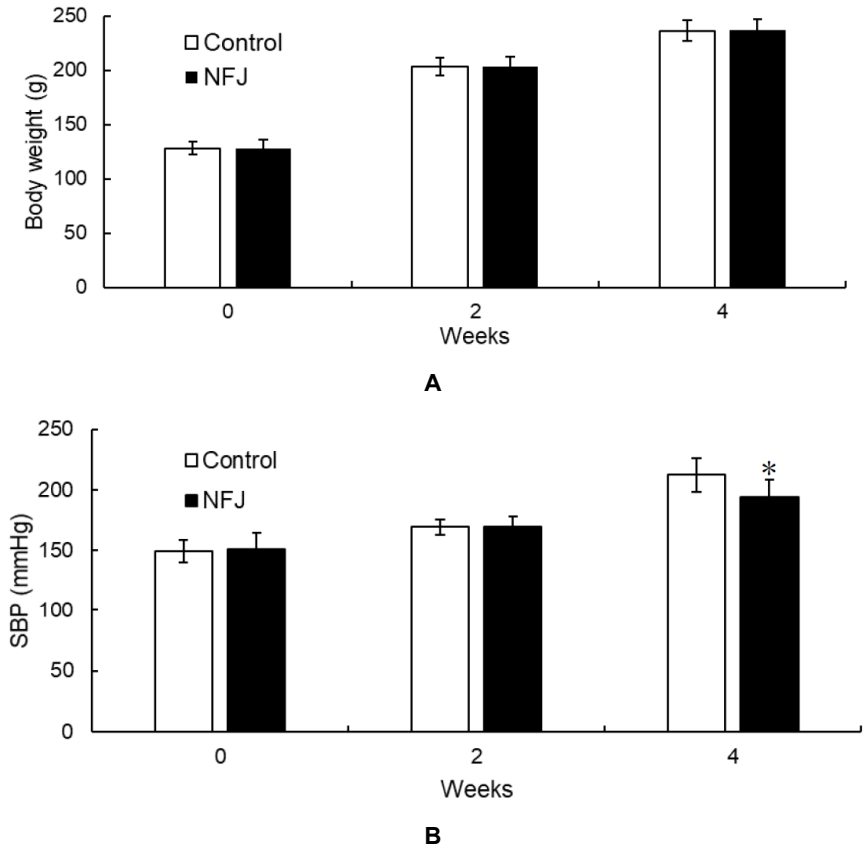


Figure 4: Effects of NFJ on body weight and SBP in SHRSP rats (Study 2).

Six-week-old male SHRSP rats were treated daily with 10% NFJ or vehicle for 4 weeks. Body weight development (**A**) and SBP (**B**) are shown. Data are expressed as the mean ± S.D.; n = 10 for each group. *P < 0.05 vs. control group.

effects of NFJ on body weight and SBP in SHRSP rats. When rats were treated daily with NFJ or a control for 4 weeks, there were no significant differences in body weight (Figure **4A**). However, SBP was significantly decreased in the NFJ group compared with that in the control group (Figure **4B**) similar to the results of the first experiment.

Effects of NFJ on NO Concentration ([NO^{2-}] + [NO^{3-}]) in Urine of SHRSP Rats

Urinary NO concentration ([NO^{2-}] + [NO^{3-}]) in the NFJ group was 2–3 fold higher than that in the control group (Figure **5**).

Effects of NFJ on Phosphorylated eNOS Protein Expression in SHRSP Rats

To investigate the regulation of NO synthases, we dissected the cerebral cortex from SHRSP rats and examined factors involved in NO production. eNOS Ser1177 significantly increased in the brain with NFJ treatment (Figure **6A**). In addition, eNOS Thr495 was reduced significantly in the NFJ group (Figure **6B**), which suggested that NFJ increased NO production by promoting eNOS phosphorylation.

Effects of NFJ on the Insulin-Dependent Pathway in the Brain of SHRSP Rats

Next, we examined the effects of NFJ on the insulin-dependent pathway in the brain of SHRSP rats. The Akt (Ser473) ratio of phosphorylation was not significantly different between the two groups (Figure **7A**). In addition, phosphorylated IRS1 (Tyr989, Ser1101, 318, and 612) (Figure **7B**, **C**, **D**, **E**) was not significantly different, suggesting that insulin-dependent pathway (IRS1-Akt) was not stimulated by NFJ treatment.

Effects of NFJ on the Insulin-Independent Pathway in the Brain of SHRSP Rats

Because NFJ was not involved in the insulin-dependent pathway, we examined the effects of NFJ on the insulin-independent pathway, which regulates eNOS phosphorylation as well as the insulin-dependent pathway in SHRSP rats. We measured phosphorylated AMPK expression. AMPK phosphorylation significantly increased in the NFJ group compared with that of the control group (Figure **8A**). Moreover, the level of LKB1 and Sirt1, which are upstream factors of AMPK in the insulin-independent pathway, also increased in the NFJ

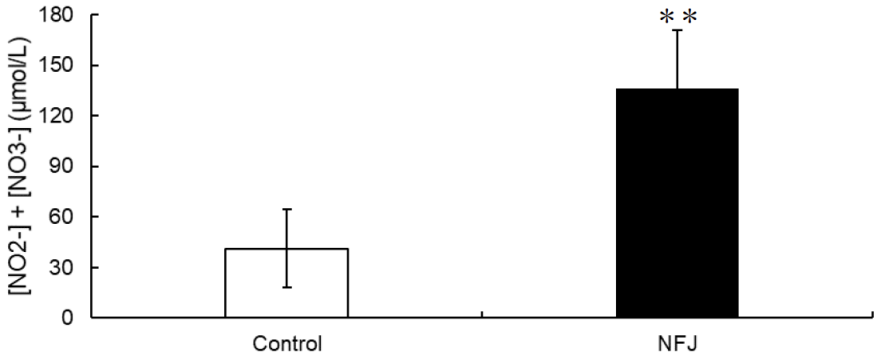


Figure 5: Effects of NFJ on NO concentration ($[NO^{2-}] + [NO^{3-}]$) in SHRSP rats.

NO concentration measurement was performed with urine from SHRSP rats treated with NFJ for 4 weeks. Data are expressed as the mean ± S.D.; n = 10 for each group. ***P* < 0.01 vs. control group.

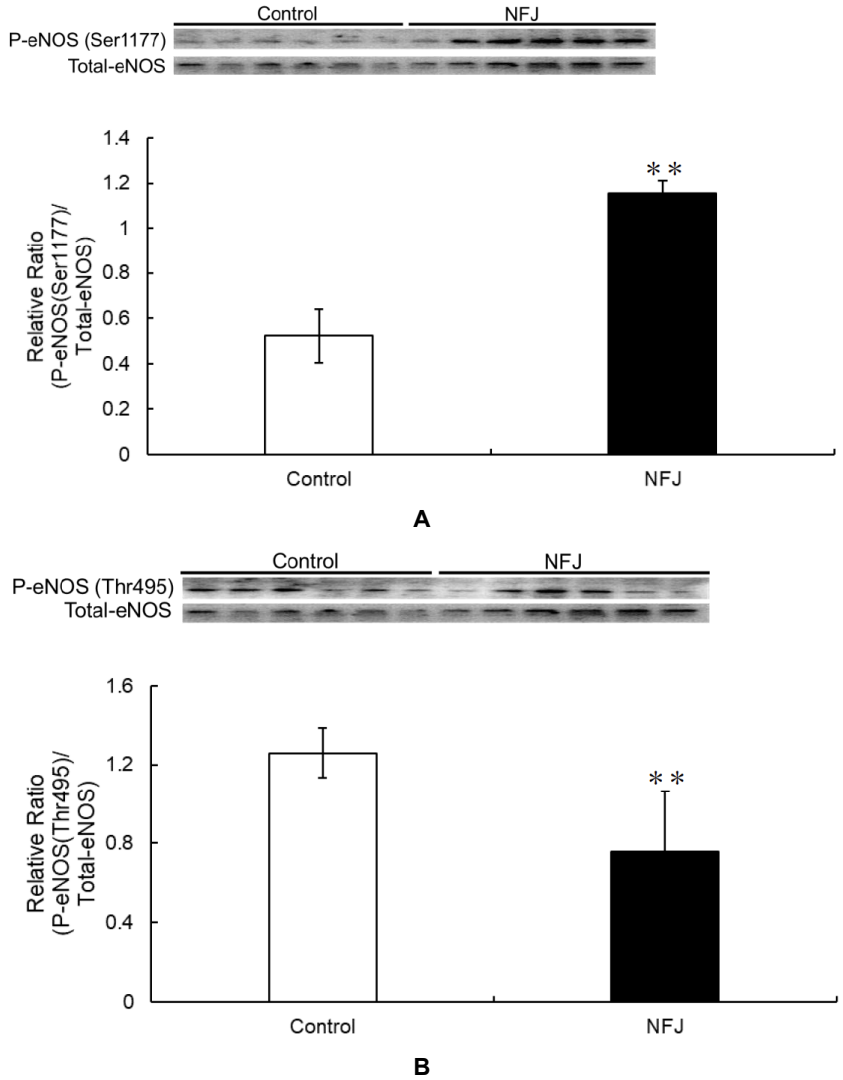
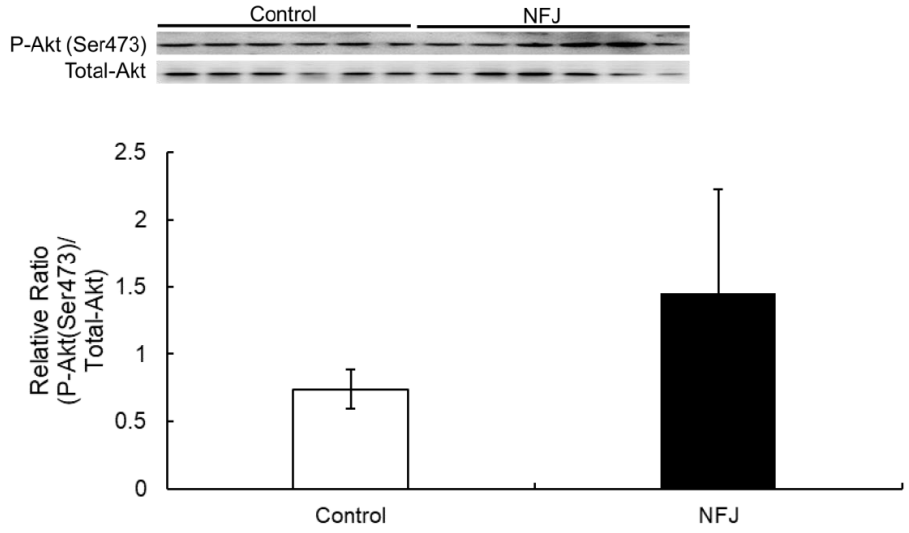
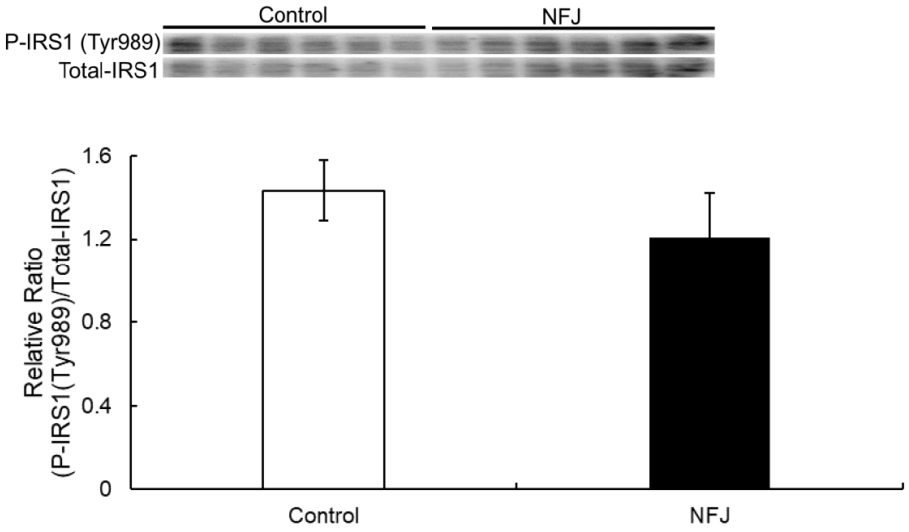


Figure 6: Effects of NFJ on eNOS phosphorylation in the brain of SHRSP rats.

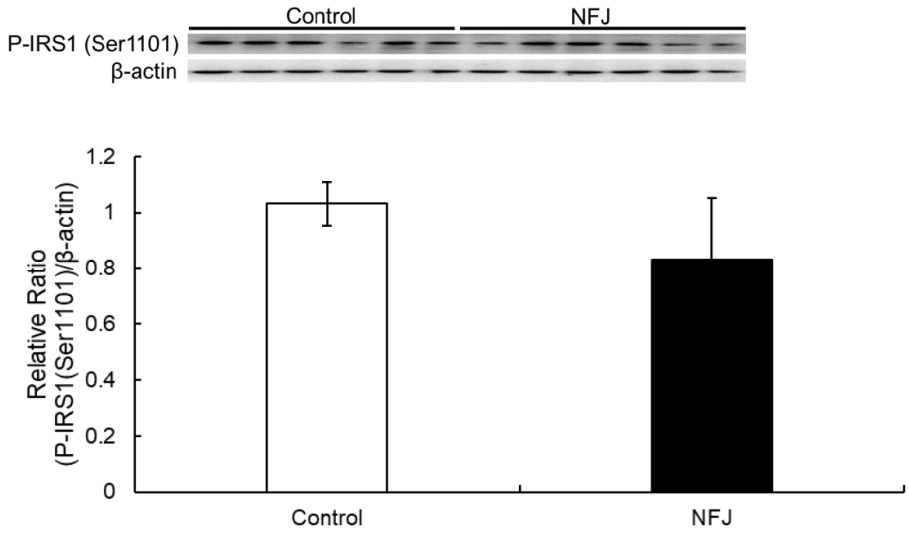
Western blotting was performed for eNOS Ser1177 (**A**) and Thr495 (**B**) in the brain of SHRSP rats. Data are expressed as the mean ± S.D.; n = 6 for each group. ***P* < 0.01 vs. control group.


Control
NFJ
P-Akt (Ser473)
Total-Akt
Relative Ratio
(P-Akt(Ser473)/
Total-Akt)
2.5
2
1.5
1
0.5
0
Control
NFJ

A


Control
NFJ
P-IRS1 (Tyr989)
Total-IRS1
Relative Ratio
(P-IRS1(Tyr989)/Total-IRS1)
1.6
1.2
0.8
0.4
0
Control
NFJ

B


Control
NFJ
P-IRS1 (Ser1101)
β-actin
Relative Ratio
(P-IRS1(Ser1101)/β-actin)
1.2
1
0.8
0.6
0.4
0.2
0
Control
NFJ

C

(Figure 7). Continued.

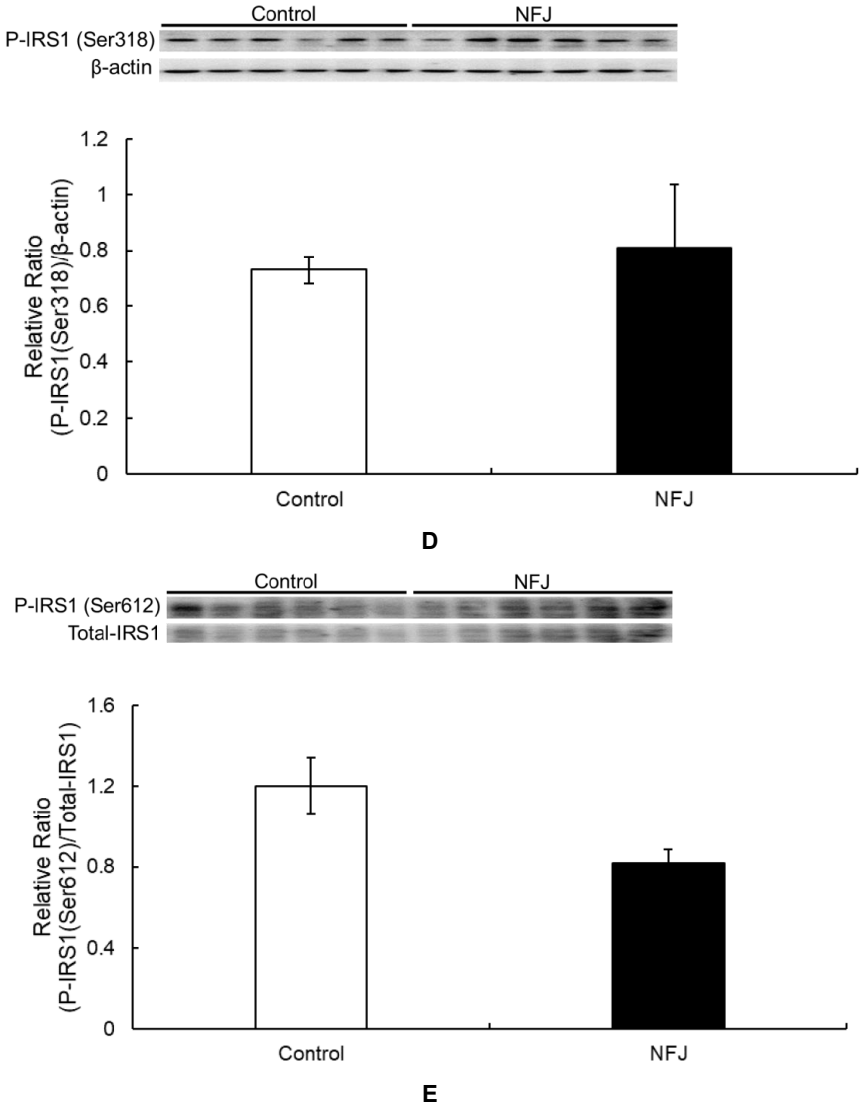
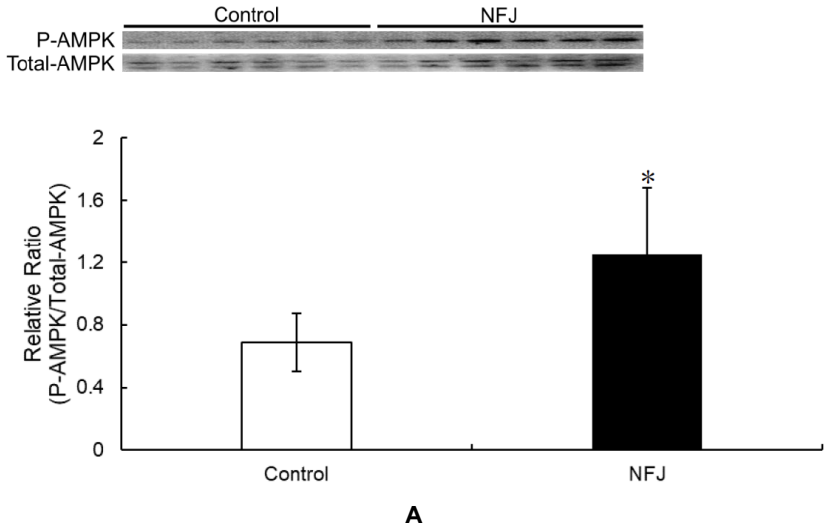


Figure 7: Effects of NFJ on the insulin-dependent pathway in the brain of SHRSP rats.

Analysis of the insulin-dependent pathway by western blotting in the brain in SHRSP rats. Expression of Akt (A) and IRS1 (Tyr989, Ser1101, 318, and 612) (B, C, D, and E, respectively). Data are expressed as the mean ± S.D.; n = 6 for each group.



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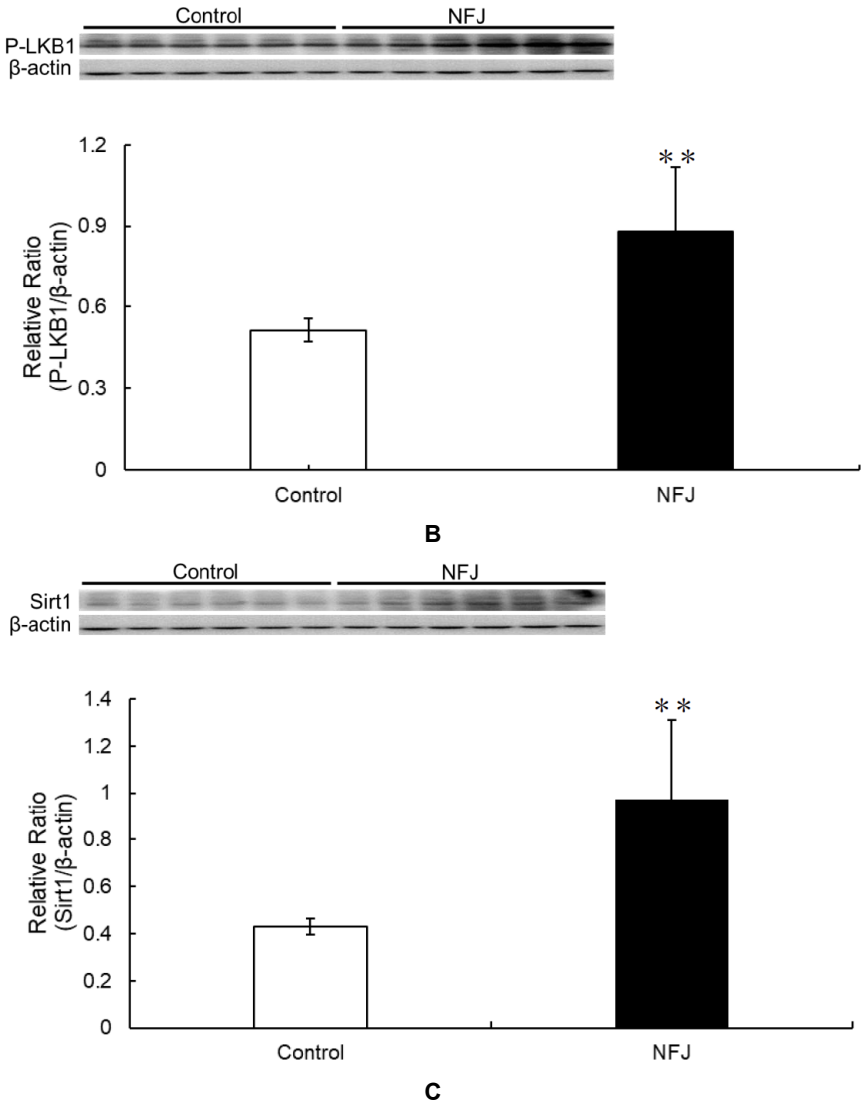

Figure 8: Effects of NFJ on the insulin-independent pathway in the brain of SHRSP rats.

Western blotting was performed for AMPK (**A**), LKB1 (**B**), and Sirt1 (**C**) in SHRSP rats treated with NFJ for 4 weeks. Data are expressed as the mean ± S.D.; n = 10 for each group. **P* < 0.05, ***P* < 0.01 vs. control group.

group (Figure **8B**, **C**), which suggested that NFJ enhanced eNOS phosphorylation by stimulating the insulin-independent pathway (Sirt1-LKB1-AMPK) in SHRSP rats.

DISCUSSION

The main results of this study were as follows: (1) administration of NFJ to SHRSP rats prevented stroke and inhibited the elevation of blood pressure and (2) the underlying mechanism was identified as an increase in NO production by enhancement phosphorylation of eNOS (Ser1177) via the insulin-independent pathway (Sirt1-LKB1-AMPK) in the brain of SHRSP rats.

The SHRSP is a model of hypertension, and blood pressure in SHRSP is significantly higher than that in WKY rats, which is a normal blood pressure model. The data in our survival studies showed that NFJ protected against stroke death. Furthermore, administration of NFJ inhibited the elevation of blood pressure, which suggests that NFJ may improve vascular function including that in blood vessels in the brain.

NO regulates hypertension, and the patients with hypertension have abnormal NO production. NO in the brain inhibits sympathetic nerve activity [17, 18], may directly affect vasodilatation, and reduces blood pressure. NFJ induced a higher level of NO_2/NO_3 in urine compared with that in the control group. NO is

regulated by post-translational modifications and protein-protein interactions. In addition, NO is synthesized by eNOS present in vessel endothelial cells, which is known to regulate vascular tone. NO generation from eNOS may initially limit stroke [19] and brain injury by inducing vasodilation. eNOS is activated by phosphorylation at two sites – Ser1177 and Thr495. Thr495 phosphorylation inhibits NO production, but Ser1177 phosphorylation activity increases NO production levels and enhances blood flow. In our study, phosphorylation of eNOS (Ser1177) was elevated and increased NO production in SHRSP rats after NFJ treatment.

There are two pathways regulating eNOS (Ser1177) phosphorylation: the insulin-dependent pathway and the insulin-independent pathway. The insulin-dependent pathway begins with the binding of insulin to the insulin receptor (IR). Akt, an upstream enzyme of eNOS [20], is then phosphorylated by IRS1, and Akt activation has been implicated in various endothelial functions including cell survival, cell migration, and activation of nitric oxide synthase (NOS). Finally, NO production is promoted and blood pressure elevation is inhibited.

On the other hand, the insulin-independent pathway is regulated by AMPK phosphorylation. AMPK has a role in cellular energy homeostasis and is a well conserved heterotrimer protein that consists of the α, β, and γ subunits encoded by 2 or 3 genes each [21]. An increased AMP/ATP ratio activates AMPK through LKB1, which phosphorylates a number of protein kinases at distinct sites [22]. AMP/LKB1 signaling is activated by Sirt1, which plays an important role in the maintenance of vascular endothelial cell homeostasis [23, 24, 25]. Therefore, the stimulation of NO production from eNOS involves both the insulin-dependent pathway and the insulin-independent pathway.

In this study, we showed that NFJ did not phosphorylate Akt in the brain of SHRSP rats. In addition, IRS1, which is upstream factor of Akt, did not change between the two groups. These results suggested that changes in eNOS phosphorylation after NFJ treatment did not depend on insulin-dependent signaling.

In addition, AMPK phosphorylation significantly increased in the NFJ group. Moreover, phosphorylation of LKB1 and Sirt1, which are upstream of AMPK, significantly increased by NFJ treatment. These data suggested that NFJ promoted eNOS phosphorylation by enhancing the insulin-independent pathway in the SHRSP brain.

In conclusion, Noni fruit juice may delay stroke through the improvement of blood circulation by promoting NO production via enhancement of the insulin-independent pathway (Sirt1-LKB1-AMPK-eNOS) in the brain of SHRSP rats (Figure **9**).

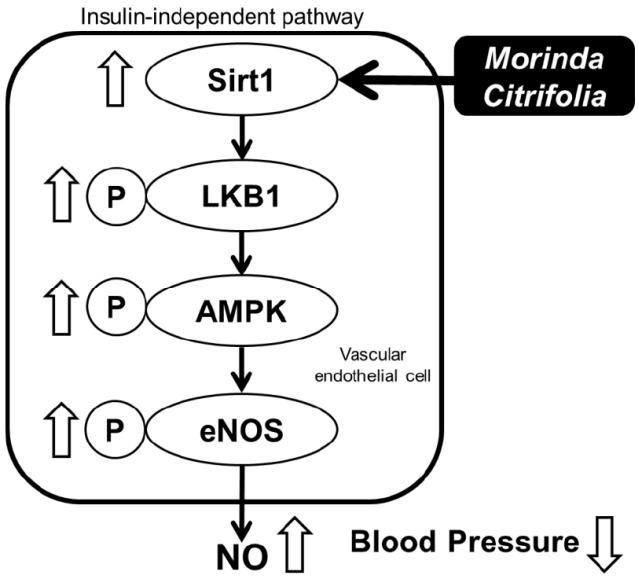

Figure 9: Enhancement of NO secretion signaling in vascular endothelial cells of *Morinda citrifolia*-treated SHRSP rats.

This suggests that the fruit and juice of *Morinda citrifolia* may be useful to help prevent stroke and in clinical studies in the future.

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