Regenerative Neurogenesis After Ischemic Stroke Promoted by Nicotinamide Phosphoribosyltransferase–Nicotinamide Adenine Dinucleotide Cascade

Yan Zhao, MD, PhD*; Yun-Feng Guan, BSc*; Xiao-Ming Zhou, MD*; Guo-Qiang Li, MD; Zhi-Yong Li, MD, PhD; Can-Can Zhou, BSc; Pei Wang, MD, PhD; Chao-Yu Miao, MD, PhD

Background and Purpose—Nicotinamide adenine dinucleotide (NAD) is a ubiquitous fundamental metabolite. Nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme for mammalian NAD salvage synthesis and has been shown to protect against acute ischemic stroke. In this study, we investigated the role of Nampt–NAD cascade in brain regeneration after ischemic stroke.

Methods—Nampt transgenic (Nampt-Tg) mice and H247A mutant enzymatic-dead Nampt transgenic (ΔNampt-Tg) mice were subjected with experimental cerebral ischemia by middle cerebral artery occlusion. Activation of neural stem cells, neurogenesis, and neurological function recovery were measured. Besides, nicotinamide mononucleotide and NAD, two chemical enzymatic product of Nampt, were administrated in vivo and in vitro.

Results—Compared with wild-type mice, Nampt-Tg mice showed enhanced number of neural stem cells, improved neural functional recovery, increased survival rate, and accelerated body weight gain after middle cerebral artery occlusion, which were not observed in ΔNampt-Tg mice. A delayed nicotinamide mononucleotide administration for 7 days with the first dose at 12 hours post middle cerebral artery occlusion did not protect acute brain infarction and neuronal deficit; however, it still improved posts ischemic regenerative neurogenesis. Nicotinamide mononucleotide and NAD+ promoted proliferation and differentiation of neural stem cells in vitro. Knockdown of NAD-dependent deacetylase sirtuin 1 (SIRT1) and SIRT2 inhibited the progrowth action of Nampt–NAD axis, whereas knockdown of SIRT1, SIRT2, and SIRT6 compromised the prodifferentiation effect of Nampt–NAD axis.

Conclusions—Our data demonstrate that the Nampt–NAD cascade may act as a centralizing switch in posts ischemic regeneration through controlling different sirtuins and therefore represent a promising therapeutic target for long-term recovery of ischemic stroke. (Stroke. 2015;46:1966-1974. DOI: 10.1161/STROKEAHA.115.009216.)

Key Words: ischemic stroke ■ NAD ■ neurogenesis ■ neural stem cells ■ sirtuin

Nicotinamide adenine dinucleotide (NAD) is a coenzyme found in all living cells, and nicotinamide phosphoribosyltransferase (Nampt) is the rate-limiting enzyme for NAD biosynthesis in mammal. Nampt catalyzes the reaction between nicotinamide and 5-phosphoribosyl-1-pyrophosphate to yield nicotinamide mononucleotide (NMN), an intermediate in the biosynthesis of NAD. Classically, NAD acts as a coenzyme and metabolite playing a fundamental role in electron transfer chain and energy generation (ATP production) through oxidative phosphorylation. Recent work has revealed an entirely different role of NAD as a critical signaling regulator. Moreover, with the identification of a group of NAD-consum ing proteins, such as the sirtuins, poly(ADP-ribose) polymerases, and cyclic ADP-ribose synthases, Nampt-regulated NAD pool has drawn much attention as a substrate and a regulator in cell signaling and cellular homeostasis, such as metabolism, circadian rhythms, immunity, and cell death.

Nampt–NAD cascade plays critical roles in cerebral ischemic injury. Cerebral ischemia caused brain NAD depletion followed by brain cell death. Replenishment of NAD conferred a marked neuroprotection against ischemic cell death. We and other groups have shown that Nampt is mainly localized on neurons rather than glial cells, and both intercellular and extracellular Nampt display potent neuroprotection in ischemic stroke models. We also showed that induction of autophagy

Received April 20, 2015; final revision received May 9, 2015; accepted May 14, 2015.

From the Department of Pharmacology, Second Military Medical University, Shanghai, China (Y.Z., Y.-F.G., G.-Q.L., Z.-Y.L., C.-C.Z., P.W., C.-Y.M.); Department of Science and Education, Shandong Provincial Hospital affiliated to Shandong University, Jinan, China (X.-M.Z.); and Center of Stroke, Beijing Institute for Brain Disorders, Beijing, China (C.-Y.M.).

*Y. Zhao, Y.-F. Guan, and X.-M. Zhou contributed equally.

The online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.115.009216/-/DC1.

Correspondence to Chao-Yu Miao, MD, PhD or Pei Wang, MD, PhD, Department of Pharmacology, Second Military Medical University, Shanghai, China. E-mail cymiao@smmu.edu.cn or pwang@smmu.edu.cn

© 2015 American Heart Association, Inc.

Stroke is available at http://stroke.ahajournals.org

DOI: 10.1161/STROKEAHA.115.009216

1966
at the early stage of ischemia contributes to the neuroprotection of Nampt. Stein et al demonstrated that forebrain excitatory neurons mainly use intracellular Nampt-mediated NAD biosynthesis to maintain their survival and function. These observations support the view that Nampt–NAD cascade may be a critical therapeutic target in brain disorders. However, the role of Nampt–NAD signaling in brain regeneration after cerebral ischemia has not been examined. Given the crucial role of NAD signaling in cellular functions and biological consequences, we applied genetic and pharmacological approaches to study the effects of Nampt–NAD axis in postischemic brain regeneration.

We found that the number of neural stem cells (NSCs) in Nampt transgenic (Nampt-Tg) mouse brain was higher than that in H247A-enzymatic–dead Nampt (ΔNampt) transgenic (ΔNampt-Tg) mouse brain after ischemic stroke. Neural functional recovery was also better improved in Nampt-Tg mice than ΔNampt-Tg mice. A delayed administration of Nampt enzymatic product (NMN) did not protect brain infarction but successfully improved neurogenesis in vivo. NMN and NAD treatment also enhanced proliferation and differentiation of cultured NSCs in vitro. Knockdown of sirtuin deacetylases (SIRT1, SIRT2, or SIRT6) significantly reduced the proneurogenesis effect of Nampt enzymatic product. Thus, we provide first evidence that Nampt–NAD cascade is not only crucial against acute brain ischemic injury but also important for postischemic regeneration.

Methods

Mice

Ten-week-old male C57BL/6J mice were purchased from Sino-British SIPPR/BK Lab Animal Ltd (Shanghai, China). Nampt-Tg mice overexpressing normal mouse Nampt and ΔNampt-Tg mice overexpressing H247A-ΔNampt were generated as described previously. All experiments were performed according to the National Institutes of Health guidelines on the use of laboratory animal.

Generation of Transgenic Mice

Nampt-Tg mice and H247A mutant ΔNampt transgenic mice (ΔNampt-Tg mice) were described in our previous report. Linearized pIRES2-EGFP vector encoding full-length mouse Nampt or ΔNampt under control of the ubiquitin promoter was injected into fertilized 1-cell eggs. Injected fertilized eggs were transplanted into the oviducts of pseudopregnant F1 hybrids of C57BL/6J mice. Founder mice were hybridized with wild-type (WT) C57BL/6J mice to produce mice used in all the experiments.

Cerebral Ischemia Model

Cerebral ischemia/reperfusion model was induced by middle cerebral artery occlusion (MCAO) in mice as previously described.

Figure 1. Nicotinamide phosphoribosyltransferase (Nampt) overexpression promotes neurogenesis after ischemic stroke. A, Scheme showing the generation of Nampt transgenic (Nampt-Tg) and ΔNampt transgenic (ΔNampt-Tg) mice and the experiment design for BrdU injection and immunostaining. Fragments containing His-tagged mouse normal-Nampt or ΔNampt cDNA were subcloned into pIRES2-EGFP vector under control of the ubiquitin promoter. B, Representative images and quantitative analysis of neurogenesis (BrdU+/NeuN+) in dentate gyrus. C, Representative images and quantitative analysis of migrated neural progenitor cells (BrdU+/DCX+) in dentate gyrus and subventricular zone (SVZ). D, Representative images and quantitative analysis of proliferated neural stem cells (BrdU+/Nestin+, top) and radical glial stem cells (GFAP+/Nestin+, bottom) on day 7 after MCAO. n=8; *P<0.01 vs wild-type (WT), #P<0.01 vs Nampt-Tg.
Briefly, adult male mice weighing from 20 to 25 g were anesthetized with an intraperitoneal injection of chloral hydrate (400 mg/kg). Rectal temperature was monitored and maintained at 37°C during the surgical procedure and recovery period until the animals regained full consciousness. The right middle cerebral artery was occluded by silicone rubber–coated nylon monofilament with round tip and advanced 1.1 cm into the internal carotid artery to obstruct the right middle cerebral artery.

Inclusion and Exclusion Criteria

Animals with an adequacy of MCAO as evidenced by >75% drop in the cerebral blood flow determined by Laser-Doppler flowmetry (VMSTM-LDF1; Moor Instruments, Axminster, United Kingdom) were included in the study. Animals were excluded from analysis when the following occurred: inadequacy of MCAO as evidenced by incomplete occlusion (<75%), subarachnoid hemorrhage on postmortem analysis, death because of anesthesia or surgery, and death that occurred within 24 hours post MCAO.

NMN Treatment in mice

Intraperitoneal injection of NMN (500 mg/kg) was given at different times (30 minutes and 12 hours) post MCAO to explore the potential neuroprotective effect of NMN on acute ischemia cerebral injury. For delayed NMN treatment, mice were administrated with intraperitoneal injection of NMN (500 mg/kg per d)6,11 for 7 days with the first dose at 12 hours post MCAO. After NMN administration, the mice were maintained for another week and examined with water maze test for 3 days. Then, the mice were anesthetized and executed to obtain brain tissue for immunohistochemistry.

Measurement of NAD in Brain Tissue

NAD concentration was determined using NMN quantification kit (BioVision) as described previously.11,20 Brain tissue (2 g) was cut and washed with cold PBS and homogenized with 400 μL of extraction buffer supplied by the kit in a microcentrifuge tube. Sample was centrifuged at 12,000g for 5 minutes. The extracted supernatant was transferred into a new tube. Then, the NAD levels were determined by the kit according to the instructions of manufacturer.

Morris Water Maze Test

Morris water maze tests were performed as described previously.21 Experiments were performed on days 21, 23, 25, and 27 post MCAO comprising 20 trials (5 trials per day for every mouse). The data were collectively analyzed with the HVS Image 2020 Plus Tracking System (US HVS Image, San Diego, CA). On day 28 post MCAO, 24 hours after the previous training, probe trials were started in which the escape platform was removed and the mice were allowed to swim freely for 2 minutes, and the times of crossing the platform (passing times) were calculated.

Immunohistochemistry Staining

BrdU (Sigma-Aldrich, St. Louis, MO) was consecutively injected (50 mg/kg per d IP, dissolved in saline) as indicated in Figure 1. For immunostaining, the mice were anesthetized and then fixed by transcardiac perfusion with 4% paraformaldehyde (pH 7.4). After dissection, the brains were immersed in 4% paraformaldehyde at 4°C for 24 hours, cryoprotected in 15% to 30% (wt/vol) sucrose/paraformaldehyde, and cut into slices (20 μmol/L). For BrdU immunostaining, the brain sections were incubated in 1 N HCl at 45°C for 30 minutes and then neutralized in 0.1 mol/L sodium borate buffer (pH 8.0). The sections or cells were sequentially incubated with 10% goat serum for 2 hours and incubated in specific primary antibodies as follows: anti-BrdU antibody (1:200; number ab1893, Abcam), NeuN (1:500; number 427917, Chemicon), GFAP (1:500; number 3670, Cell Signaling Technology), Nampt (1:400; number H-300, Stanta Cruz Biotechnology), Nestin (1:400; number H-85, Stanta Cruz Biotechnology), CNP (1:400; number BA1754, Boster China), and Tuj-1 (1:400; number 5568s, Cell Signaling Technology). After being washed by 3× with PBS, the sections and cells were incubated with corresponding secondary antibodies (Alexa 488-conjugated and Cy3-conjugated). The fluorescence-labeled sections were examined with FLUOVIEW FV1000 Confocal.
laser scanning microscope (Olympus, Japan). Immunohistochemical-positive cells were counted in the dentate gyrus and subventricular zone in at least 10 sections per animal.

**Neurosphere Culture, Differentiation, and Proliferation**

Culture of neurospheres was based on the previously published methods with minor modifications. Dissected telencephalons of embryonic day 13 mice were digested with Accutase solution (Life Technologies, Gaithersburg, MD) for 5 minutes at 37°C. The single and suspended cells were plated at a density of 1×10⁴ cells per milliliter on 6-well plate. Growth medium was composed of DMEM-F-12 medium (Life Technologies), 20 ng/mL of epidermal growth factor (Life Technologies), and 20 ng/mL basic fibroblast growth factor (Life Technologies). At 7 days, the neurospheres were plated on polylysine- (Sigma) coated glass coverslips. Differentiation was induced by withdrawal of epidermal growth factor and basic fibroblast growth factor. At day 7, the differentiation was evaluated. Cell viability was evaluated by a nonradioactive cell counting kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) as described previously.

**siRNA-Mediated Knockdown of SIRT1-7 by Nucleofection**

For plasmids encoding siRNAs of SIRT1-7, we used pRNAi-U6.2/Lenti vector (Genescript, Piscataway, NJ) as described previously. Sequences of effective siRNAs targeting SIRT1-7 are listed in Table I in the online-only Data Supplement. Nucleofection of embryonic mouse NSCs were performed by electroporation as reported previously. We used 5×10⁴ neurosphere-derived NSCs per sample and resuspended them in 20 μL of P3 primary cell solution containing 5 μg of plasmid DNA. Cells were transferred into the nucleofector cuvette (20 μL) according to the manufacturer’s protocol (Lonza, Walkersville, MD). After the pulse application, 180 μL of prewarmed neurosphere medium was added to the electroporated NSCs in the cuvette. NSCs were gently resuspended in the cuvette and transferred into a 1.5-mL sterile tube. After centrifugation with 80g for 5 minutes at room temperature, the supernatant was discarded and the cell pellet was resuspended in 500 μL of neurosphere medium containing epidermal growth factor (20 ng/mL) and basic fibroblast growth factor (20 ng/mL).

**Statistical Analysis**

Data are presented as mean±SEM. ANOVA was used for comparisons. Neuroscore was analyzed by Mann–Whitney test. Survival curve was analyzed by Log-rank test. Statistical significance was set at P<0.05.

**Results**

**Nampt-Mediated NAD Promotes NSC Activation and Neurogenesis After Ischemic Stroke**

In H247A mutant-Nampt (ΔNampt), the histidine at site 247 was mutated into alanine to inactivate its NAD-biosynthetic activity (Figure 1A and IB in the online-only Data Supplement). We generated 2 strains of transgenic mice: normal Nampt transgenic (ΔNampt-Tg) mice and ΔNampt-Tg mice (Figure 1A). DNA assay, immunoblotting, and immunohistochemistry confirmed the overexpression of normal-Nampt and ΔNampt in mice (Figures IC, ID, and II in the online-only Data Supplement). Transgene of normal-Nampt increased the NAD concentration in brain tissue, whereas transgene of ΔNampt decreased the NAD concentration in brain tissue (Figure III in the online-only Data Supplement). MCAO was performed in (WT), Nampt-Tg, and ΔNampt-Tg mice to study the activation of NSCs (Figure 1B). At days 7, 14, and 28 post MCAO, the number of BrdU+/NeuN+ cells in dentate gyrus of ΔNampt-Tg mice was higher than that in WT mice, which was not observed in ΔNampt-Tg mice (Figure 1B). At day 9 post MCAO, the number of BrdU+/DCX+ cells in dentate gyrus and subventricular zone of Nampt-Tg mice, but not ΔNampt-Tg mice, was higher than that in WT mice (Figure 1C). The numbers of BrdU+/Nestin+ cells in dentate gyrus and GFAP+/Nestin+ cells in subventricular zone were significantly higher in Nampt-Tg mice (Figure 1D). These results indicate that Nampt overexpression elevates brain NAD levels and promotes NSC activation and neurogenesis after ischemic stroke via its enzymatic activity.
Transgene of Nampt Accelerates Postischemic Neurological Function Recovery and Body Weight Gain

We used Morris water maze to examine brain learning and memory function after ischemic stroke. In visible platform tests, Nampt-Tg mice exhibited a decreased latency to escape onto the visible platform and a shorter swimming distance before escaping onto the visible platform (Figure 2A). Nampt-Tg mice also had more platform-passing times to escape onto the hidden platform (Figure 2A). These improvements in Nampt-Tg mice were not found in ΔNampt-Tg mice (Figure 2A). Accordingly, Nampt-Tg mice, but not ΔNampt-Tg mice, displayed a higher survival rate (Figure 2B) and a better recovery of body weight (Figure 2C) compared with WT mice. Moreover, the cortical cavitation caused by ischemic injury was attenuated in Nampt-Tg mice but not in ΔNampt-Tg mice (Figure 2D).

Delayed Administration of NMN Improves Postischemic Regenerative Neurogenesis

Intraperitoneal injection of NMN, the enzymatic product of Nampt (Figure 1A in the online-only Data Supplement) increased cerebral NAD/nicotinamide adenine dinucleotide phosphate ratio (Figure 1B in the online-only Data Supplement). Early NMN administration with the first dose at 30 minutes post MCAO for 7 days decreased brain infarction and neurological deficit (Figure 3A), enhanced animal survival, and accelerated body weight recovery (Figure 5 in the online-only Data Supplement). However, because the early NMN administration with the first dose at 30 minutes post MCAO is able to protect acute cerebral ischemic injury, the possibility that the difference of neuronal damage is likely to lead to inconsistent levels of survived NSCs and thereby affects post ischemic neurogenesis cannot be ruled out.

To further tease out the specific effect of Nampt–NAD axis on neurogenesis, we applied a delayed administration of NMN for 7 days with the first dose at 12 hours post MCAO. Delayed NMN administration was unable to decrease brain infarction and neurological deficit (Figure 3A), suggesting that delayed NMN administration does not have neuroprotection. Delayed NMN administration also did not affect the body weight (Figure 3B). However, delayed NMN treatment reduced MCAO-induced death (Figure 3C) during the first weeks post MCAO. Furthermore, delayed NMN treatment not only improved the neuronal recovery (Figure 3C) but also increased neurogenesis (BrdU+/NeuN+ cells) in subventricular zone and dentate gyrus of MCAO mice (Figure 3D). Taken together, these results indicate that Nampt–NAD+ axis is able to improve postischemic neurogenesis.

NMN and NAD Induce NSC Neurosphere Proliferation and Differentiation

We further investigated the potential effect of Nampt-mediated NAD pathway (Figure 3A) on neurogenesis in cultured NSC neurospheres. Nampt was expressed abundantly in the online-only Data Supplement. However, because the early NMN administration with the first dose at 30 minutes post MCAO is able to protect acute cerebral ischemic injury, the possibility that the difference of neuronal damage is likely to lead to inconsistent levels of survived NSCs and thereby affects post ischemic neurogenesis cannot be ruled out.

To further tease out the specific effect of Nampt–NAD axis on neurogenesis, we applied a delayed administration of NMN for 7 days with the first dose at 12 hours post MCAO. Delayed NMN administration was unable to decrease brain infarction and neurological deficit (Figure 3A), suggesting that delayed NMN administration does not have neuroprotection. Delayed NMN administration also did not affect the body weight (Figure 3B). However, delayed NMN treatment reduced MCAO-induced death (Figure 3C) during the first weeks post MCAO. Furthermore, delayed NMN treatment not only improved the neuronal recovery (Figure 3C) but also increased neurogenesis (BrdU+/NeuN+ cells) in subventricular zone and dentate gyrus of MCAO mice (Figure 3D). Taken together, these results indicate that Nampt–NAD+ axis is able to improve postischemic neurogenesis.

NMN and NAD Induce NSC Neurosphere Proliferation and Differentiation

We further investigated the potential effect of Nampt-mediated NAD pathway (Figure 3A) on neurogenesis in cultured NSC neurospheres. Nampt was expressed abundantly in the online-only Data Supplement. However, because the early NMN administration with the first dose at 30 minutes post MCAO is able to protect acute cerebral ischemic injury, the possibility that the difference of neuronal damage is likely to lead to inconsistent levels of survived NSCs and thereby affects post ischemic neurogenesis cannot be ruled out.

To further tease out the specific effect of Nampt–NAD axis on neurogenesis, we applied a delayed administration of NMN for 7 days with the first dose at 12 hours post MCAO. Delayed NMN administration was unable to decrease brain infarction and neurological deficit (Figure 3A), suggesting that delayed NMN administration does not have neuroprotection. Delayed NMN administration also did not affect the body weight (Figure 3B). However, delayed NMN treatment reduced MCAO-induced death (Figure 3C) during the first weeks post MCAO. Furthermore, delayed NMN treatment not only improved the neuronal recovery (Figure 3C) but also increased neurogenesis (BrdU+/NeuN+ cells) in subventricular zone and dentate gyrus of MCAO mice (Figure 3D). Taken together, these results indicate that Nampt–NAD+ axis is able to improve postischemic neurogenesis.

NMN and NAD Induce NSC Neurosphere Proliferation and Differentiation

We further investigated the potential effect of Nampt-mediated NAD pathway (Figure 3A) on neurogenesis in cultured NSC neurospheres. Nampt was expressed abundantly in the online-only Data Supplement. However, because the early NMN administration with the first dose at 30 minutes post MCAO is able to protect acute cerebral ischemic injury, the possibility that the difference of neuronal damage is likely to lead to inconsistent levels of survived NSCs and thereby affects post ischemic neurogenesis cannot be ruled out.

To further tease out the specific effect of Nampt–NAD axis on neurogenesis, we applied a delayed administration of NMN for 7 days with the first dose at 12 hours post MCAO. Delayed NMN administration was unable to decrease brain infarction and neurological deficit (Figure 3A), suggesting that delayed NMN administration does not have neuroprotection. Delayed NMN administration also did not affect the body weight (Figure 3B). However, delayed NMN treatment reduced MCAO-induced death (Figure 3C) during the first weeks post MCAO. Furthermore, delayed NMN treatment not only improved the neuronal recovery (Figure 3C) but also increased neurogenesis (BrdU+/NeuN+ cells) in subventricular zone and dentate gyrus of MCAO mice (Figure 3D). Taken together, these results indicate that Nampt–NAD+ axis is able to improve postischemic neurogenesis.
in NSC neurospheres (Figure 4A), and NSCs were able to secrete Nampt (Figure 4A). Flow cytometer assay showed that the number of NSCs was increased by NMN (Figure VI in the online-only Data Supplement). Double immunostaining (BrdU+/Nestin+, Figure VII in the online-only Data Supplement) assay also showed that NMN and NAD promoted NSC proliferation, whereas Nampt inhibitor FK866 (10 nmol/L, also known as daporinad) induced converse effect (Figure 4B). NSCs cultured with differentiation medium exhibited phenotypes of neurons, astrocytes, and oligodendrocytes (Figure VIII in the online-only Data Supplement). NMN and NAD increased the Tuj-1/DAPI ratio and GFAP/DAPI ratio, suggesting a prodifferentiation effect of NMN and NAD (Figure 4C).

**Sirtuin Deacetylases Mediate the Proneurogenesis Effect of Nampt–NAD Cascade**

Sirtuins, a family of NAD-dependent deacetylases, regulate neurogenesis. Most of biological functions of Nampt–NAD cascade were mediated by sirtuins, including SIRT1 for neuroprotection against acute cerebral ischemia, SIRT3/4 for protecting against cell death, and SIRT6 for synthesis of tumor necrosis factor. SIRT1 to 7 were knocked down with specific siRNA by nucleofection in NSCs (Figure IX in the online-only Data Supplement). Knockdown of SIRT1 or SIRT2 partly blocked the increase of cell viability induced by either NMN or NAD (Figure 5A and 5B). BrdU+/Nestin+ immunostaining showed that knockdown of SIRT1 or SIRT2 blocked the increase of NSC proliferation by either NMN or NAD (Figure 5C). Meanwhile, Tuj-1 immunostaining showed that knockdown of SIRT1, SIRT2, or SIRT6 postponed the prodifferentiation (Tuj-1/DAPI ratio) effect of either NMN or NAD (Figure 6A and 6B).

**Discussion**

Here, we contribute new insights into the function of Nampt–NAD axis in regeneration after ischemic stroke. Previously, we and others demonstrate that Nampt–NAD axis is a potent defense system in acute ischemic brain injury. Nampt confers neuroprotection against ischemic

![Figure 5. Sirtuins 1 (SIRT1) and SIRT2 are required for the proliferative effect of nicotinamide mononucleotide (NMN) and nicotinamide adenine dinucleotide (NAD). A and B, Effects of NAD (300 μmol/L, A) and NMN (300 μmol/L, B) on cell viability in siRNA1-7–transfected neural stem cells (NSCs) for 7 days. C, Effects of NAD (300 μmol/L) and NMN (300 μmol/L) on BrdU+/Nestin+ cell number in siRNA1-7–transfected NSCs for 7 days. n=8; *P<0.05 vs siRNA-scramble.](https://stroke.ahajournals.org/doi/fig/10.1161/STROKEAHA.117.019111)
potentiates autophagy salvage mechanism at early stage of cerebral ischemia, and ultimately promotes neuronal survival. In this study, we focused on neurogenesis post ischemic stroke. Nampt-Tg mice, but not H247A-ΔNampt transgenic mice, displayed enhanced NSC activation and improved neurological functional recovery after ischemic stroke. Furthermore, delayed NMN treatment, which cannot confer neuroprotection, still improved postischemic neurogenesis. Either NMN or NAD promoted NSC proliferation/differentiation via a sirtuin deacetylase–dependent manner, whereas Nampt chemical inhibitor FK866 induced converse effect. In addition, Nampt has a proangiogenic capacity. All these results suggest that Nampt–NAD+ cascade may have both neuroprotective and proneurogenesis effects ensuring that adequate NAD content in the brain may have advantages on facilitating both angiogenesis and neurogenesis after cerebral ischemia.

An interesting and unexpected phenomenon is that the proneurogenesis effect of Nampt–NAD axis requires several sirtuins rather than SIRT1 solely. We found that SIRT1 and SIRT2 contribute to NSC proliferation, whereas SIRT1, SIRT2, and SIRT6 contribute to NSC differentiation. Our study seemed to be the first work revealing the effects of SIRT2 and SIRT6 in posts ischemia neurogenesis. The transcription levels of sirtuin family in NSCs are selectively regulated by different histone acetylation in neural development process. Most of studies devoting to reveal the molecular mechanism underlying the biological functions of Nampt have highlighted the importance of SIRT1 for the roles of Nampt in neuron. Stein et al showed that Nampt is critical for oligodendrocytic lineage fate decisions in NSCs through a mechanism mediated by SIRT1 and SIRT2. SIRT6 was reported to be involved in proliferation/differentiation of bone marrow mesenchymal stem cells. We did not observe the influence of knockdown of SIRT3, 4, 5, and 7 on NSC proliferation and differentiation. This interesting and unexpected phenomenon may have to do with the distribution of sirtuins family proteins. SIRT1, SIRT6, and SIRT7 were mainly localized to nucleus; SIRT2 is a predominantly cytoplasmic deacetylase that colocalizes with microtubules; SIRT3, SIRT4, and SIRT5 were predominantly present in mitochondria. There were still no data on the effects of SIRT3, SIRT4, SIRT5, and SIRT7 in biological functions of NSCs. According to our data, SIRT1, SIRT4, SIRT5, and SIRT7 might not be involved in the regulatory roles of Namp-NAD+ axis in NSCs. Several specified sirtuins, including SIRT1, SIRT2, and SIRT6, may cooperate with each other to regulate NSC proliferation and differentiation.

Our data indicate a potentially clinical importance of Nampt–NAD axis. Obviously, modulation of Nampt genetically is far from clinical application. Supplementation of NMN and activation of Nampt are 2 potential ways to provide adequate NAD pool. NMN is a small-molecule chemical easy to be administrated to modulate intracellular NAD level. Our previous data demonstrate that NMN can pass through blood–brain barrier in the mouse. NMN has been reported to treat diabetes mellitus and aging in animal models. Our data provide evidence that brain NAD was increased by intraperitoneal injection of NMN, and a delayed supplementation of

Figure 6. Sirtuin 1 (SIRT1), SIRT2, and SIRT6 are required for the prodifferentiation effect of nicotinamide mononucleotide (NMN) and nicotinamide adenine dinucleotide (NAD). A, siRNA-transfected neural stem cells (NSCs) were treated with NMN (300 μmol/L) and NAD (300 μmol/L) for 7 days, and then the Tuj-1 staining was performed to evaluate the differentiation of NSCs. B. Quantitative analysis on the differentiation of NSCs treated by NMN and NAD. n=8; *P<0.05 vs siRNA-scramble.
NMN may be of benefit for regeneration post ischemic stroke. NMN may be safe used in stroke rehabilitation in long-term treatment because it is an endogenous substrate. Activation of Nampt may be another way to maintain intracellular NAD pool. Prolonged administration of P7C3 chemicals, a class of newly discovered small molecules screened from 1000 drug-like compounds, impeded neuron death and preserved cognitive capacity in aged rodent. The mechanism by which P7C3 chemicals initiate the neuroprotection has been proposed in a recent study; P7C3 chemicals may function via activating Nampt. However, it is yet unknown for the efficacy of P7C3 chemicals in stroke model.

In conclusion, we demonstrate that Nampt promotes regeneration after cerebral ischemia via its enzymatic activity. Our results provide new insights into the critical roles of Nampt–NAD axis in cerebral ischemia and would add weight to the notion that modulation of Nampt–NAD axis, such as supplementation of NMN, may be a promising treatment for ischemic stroke.

Acknowledgments
We thank Dr Cynthia Wolberger (Johns Hopkins University School of Medicine) for providing H247A mutant Nampt plasmid. C.-Y. Miao and P. Wang designed the study. Y. Zhao, Y.-F. Guan, and X.-M. Zhou performed most of the experiments. G.-Q. Li performed nicotinamide mononucleotide experiments in vivo. Z.-Y. Li screened effective siRNAs for SIRT1–7 knockdown. C.-C. Zhou performed some of animal experiments. P. Wang, Y. Zhao, and C.-Y. Miao wrote the article. All authors contributed to the analysis of experimental data.

Sources of Funding
This work was supported by grants from National Natural Science Foundation of China (81373414, 81130061, 81473208, and 8122049), National 863 Plan Young Scientist Program (2015AA020943), National Basic Research Program of China (2009CB521902), Shanghai Qimingxing project (14QA140700), and Shandong Province Natural Science Foundation (ZR2014HQ003).

Disclosures
C.-Y. Miao, P. Wang, and Y.-F. Guan are inventors of a Chinese patent (no. CN101601679A) named Application of nicotinamide mononucleotide, which is related to the content of this article.

References


Regenerative Neurogenesis After Ischemic Stroke Promoted by Nicotinamide Phosphoribosyltransferase–Nicotinamide Adenine Dinucleotide Cascade

Yan Zhao, Yun-Feng Guan, Xiao-Ming Zhou, Guo-Qiang Li, Zhi-Yong Li, Can-Can Zhou, Pei Wang and Chao-Yu Miao

Stroke. 2015;46:1966-1974; originally published online June 9, 2015; doi: 10.1161/STROKEAHA.115.009216

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/46/7/1966

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2015/06/22/STROKEAHA.115.009216.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/
SUPPLEMENTAL MATERIAL

Regenerative neurogenesis after ischemic stroke promoted by Nampt-NAD cascade
Zhao Y, et al.

Supplementary Table I. Sequences of siRNA.

<table>
<thead>
<tr>
<th>siRNA-SIRT1 pool</th>
<th>TGAAGTGCCCTCAGATATTA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GCCATGTTTGATATGAGTAT</td>
</tr>
<tr>
<td>siRNA-SIRT2</td>
<td>GACTCCAAGAAGGCCTACA</td>
</tr>
<tr>
<td>siRNA-SIRT3 pool</td>
<td>GCCCAATGTCACTCACTACTT</td>
</tr>
<tr>
<td></td>
<td>GCGTTGTGAAACCCCGACAT</td>
</tr>
<tr>
<td>siRNA-SIRT4 pool</td>
<td>TGGAGAGTGCTGCTTTTAAT</td>
</tr>
<tr>
<td></td>
<td>TTGACTTTTGCAAGCGACAAAG</td>
</tr>
<tr>
<td>siRNA-SIRT5</td>
<td>GCGGATTCAATTTCCAGTTGT</td>
</tr>
<tr>
<td>siRNA-SIRT6</td>
<td>GGTGCAGTGCTGTTCGTAT</td>
</tr>
<tr>
<td>siRNA-SIRT7 pool</td>
<td>CCTCCCTCTTTCTACTCCTTA</td>
</tr>
<tr>
<td></td>
<td>TGCATCCCTAACAGAGATAT</td>
</tr>
</tbody>
</table>

Supplementary Figure I

Characterization of Nampt-transgenic mice (Tg mice) and H247A mutant Nampt-transgenic mice (ΔTg mice). (a) The schematic illustration of ΔNampt protein. The histidine [H] at the site 247 was mutated to alanine [A]. (b) NAD biosynthesis activities of recombinant Nampt and ΔNampt protein. (c) DNA assay for transgene of exogenous Nampt. (d) Confirmation of exogenous Nampt and H247A mutant Nampt (ΔNampt) overexpression in mice by immunoblot targeting His-tag (vector) and full-length Nampt. The antibody specific for full-length Nampt can not recognize the ΔNampt protein.
Supplementary Figure II

Double immunofluorescent staining of Nampt (green) and His-tag (red) in dentate gyrus and lateral ventricle of WT, Tg and ΔTg mice. DAPI was used to stain nuclei.

Supplementary Figure III

NAD concentrations in brain tissues from WT, Tg and ΔTg mice. *P < 0.05 vs WT, n = 6 per group.
**Supplementary Figure IV**

(A) Scheme showing Nampt-NAD cascade. Nicotinamide is converted to nicotinamide mononucleotide (NMN) by Nampt and then to NAD by nicotinamide mononucleotide adenylyltransferase (Nmnat). (B) NAD/NADH ratio in brain tissue 2 hours after intraperitoneal injection of NMN.

**Supplementary Figure V**

Percent survival curve (left panel) and body weight curve (right panel) in mice treated with saline (control) or early NMN treatment (500 mg/kg/day, i.p., first dose at 30 minutes post MCAO) for 7 days post MCAO. n=26 and 17 in control and early NMN treatment groups, respectively.
Supplementary Figure VI

(a) Representative flow cytometry images for cell number determination of NSCs in single well which was added into with different concentrations of NMN. (b) Quantitative analysis of cell number of NSCs. *P<0.05 vs without NMN. n = 8.

Supplementary Figure VII

Double immunochemistry of BrdU (green) and nestin (red) in primary NSCs-formed neurospheres.
Under pro-differentiative condition, NSCs exhibited phenotypes of neurons (Tuj1), astrocytes (GFAP), and oligodendrocytes (2',3'-cyclic-nucleotide 3'-phosphodiesterase, CNP).

Knockdown of SIRT1-7 with specific siRNA by nucleofection in NSCs. (a) NSCs in neurosphere cultures were nucleotransfected with plasmids encoding GFP-tagged siRNA targeting SIRT1-SIRT7. (b) Diagrammatic sketch of the plasmid structure. (c) Evaluation of the efficiency of siRNA-mediated knockdown using real-time PCR. *P < 0.05 vs. control. n = 8.