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ORIGINAL ARTICLE

Intracerebroventricular Delivery of Recombinant NAMPT Deters Inflammation and Protects Against Cerebral Ischemia

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Abstract

Our previous study indicated that nicotinamide phosphoribosyltransferase (NAMPT) is released from cells and might be an important extracellular neuroprotective factor in brain ischemia. Here, we tested whether NAMPT protects against ischemic brain injury when administered directly into the intracerebroventricular (ICV) compartment of the cranium. Recombinant NAMPT protein (2 μg) was delivered ICV in mice subjected to 45-min middle cerebral artery occlusion (MCAO), and the effects on infarct volume, sensorimotor function, microglia/macrophage polarization, neutrophil infiltration, and BBB integrity were analyzed. The results indicate that ICV administration of NAMPT significantly reduced infarct volume, retained its beneficial properties even when ICV administration was delayed by 6 h after MCAO, and improved neurological outcomes. NAMPT treatment inhibited pro-inflammatory microglia/macrophages, promoted microglia/macrophage polarization toward the anti-inflammatory phenotype, and reduced the infiltration of neutrophils into the perilesional area after brain ischemia. In vitro studies indicated that multiple pro-inflammatory microglial markers/cytokines were downregulated while multiple anti-inflammatory microglial markers/cytokines were induced in primary microglial cultures treated with NAMPT protein. NAMPT treatment also fortified the blood–brain barrier (BBB), as shown by reduced extravascular leakage of the small-molecule tracer Alexa Fluor 555 Cadaverine and larger-sized endogenous IgGs into brain parenchyma. Thus, NAMPT may protect against ischemic brain injury partly through a novel anti-inflammatory mechanism, which in turn maintains BBB integrity and reduces the infiltration of peripheral inflammatory cells. Taken together, these results provide validation of recombinant NAMPT delivery into the extracellular space as a potential neuroprotective strategy for stroke.

Keywords Brain ischemia · Microglia/macrophages polarization · Inflammation · Neuroprotection · BBB integrity · NAMPT

Fenghua Chen and Zhongfang Weng contributed equally to this work.

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Introduction

Stroke is the second most common cause of death worldwide and the leading cause of long-term adult disability [1]. Tissue plasminogen activator (tPA) and mechanical thrombectomy are two effective therapies for ischemic stroke but are limited to a fraction of stroke patients [2, 3]. Furthermore, many patients receiving either tPA or thrombectomy treatment still suffer brain damage and/or functional deficits. Thus, neuroprotective intervention remains a promising and attractive approach for the treatment of ischemic stroke, especially the use of endogenous protective molecules or as combined treatments with tPA or with thrombectomy.

Previous work by us and other groups using transgenic overexpression and viral delivery methods indicate that protective effects of NAMPT (also known as pre-B cell colony-enhancing factor 1 or visfatin) can be achieved across multiple systems, including against brain injury [4–6]. NAMPT is a pleiotropic gene coding for an enzyme that exists in both the intracellular and extracellular compartments. The intracellular form of NAMPT converts nicotinamide into nicotinamide mononucleotide as the rate-limiting enzyme for mammalian NAD biosynthesis [7, 8], a critical mechanism for providing energy supplies under conditions with ATP depletion, such as hypoxia, stroke, and stress. However, the function of the extracellular form of NAMPT remains unknown.

Our previous study indicated that NAMPT is released into the extracellular space following cerebral ischemia and that the secreted form of NAMPT protects the surrounding neurons against brain ischemia [4]. Thus, in the present study, we tested the hypothesis that exogenous NAMPT offers protection against ischemic brain injury when delivered into the ICV space, and we examined the underlying mechanism.

Methods

Generation of Recombinant NAMPT Protein

Recombinant NAMPT protein was purified as we previously described [9, 10]. In brief, human NAMPT cDNA tagged with His6 at the C-terminus was inserted into pET-30a (Novagen, Madison, WI) and transformed into *E. coli* BL21. Recombinant protein was induced with isopropyl β-D-1-thiogalactopyranoside (0.1 mM) at 25 °C for 6 h. Recombinant protein was purified using the Ni-NTA Fast Start Kit (Qiagen, Germantown, MD) and dialyzed sequentially in 300 mM NaCl/10 mM imidazole and 300 mM NaCl/10 mM Tris-HCl (pH 8.0). To remove endotoxins, purified protein was passed through Detoxi-Gel Endotoxin Removing Columns as per the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). A NAD enzyme-inactive NAMPT with the H247A point mutation was generated with the same procedures as above. The purified protein was filtered through a 0.2-μm filter, aliquoted, and stored at − 70 °C. Purified proteins were verified by Coomassie blue staining and Western blot analyses, and protein concentrations were determined with the Pierce BCA Protein Assay (Thermo Fisher Scientific).

Murine Model of Transient Focal Ischemia

All animal procedures used in this study were conducted in strict compliance with The National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the University of Pittsburgh Institutional Animal Care and Use Committee. Focal cerebral ischemia was produced by intraluminal occlusion of the left middle cerebral artery (MCA) with a nylon monofilament suture as we previously described [9]. In brief, 10-week-old male C57BL/6 mice (22–25 g, The Jackson Laboratory, Bar Harbor, ME) were anesthetized with 1.5% isoflurane in a 30% O_2/70% N_2O mixture under spontaneous breathing. The left MCA was blocked with a silicone rubber-coated monofilament (602256PK10Re, Doccol, Sharon, MA) for 45 min followed by reperfusion for 72 h for infarct volume measurements and immunohistochemical staining, or for 2 weeks for behavioral tests. During all surgical procedures, rectal temperature was controlled at 37.0 ± 0.5 °C via a temperature-regulated heating pad (Harvard Apparatus, Holliston, MA). All mice underwent 1-h recovery under a heating lamp after the incision was closed and then returned to their cages with ad libitum access to food and water. Mice received 1 mL daily subcutaneous saline fluids throughout the recovery period. Detailed information regarding animal number/group, animal exclusions, and mortality for the entire project are listed in Supplemental Table 1.

ICV Administration of NAMPT

The mice were anesthetized with isoflurane and placed in a stereotaxic apparatus. A midline incision was made from behind the eyes 1 cm toward the posterior of the cranium, and the skull was exposed. A small burr hole on the left hemisphere was opened using a dental drill. A plastic cannula was implanted into the lateral ventricle using the following coordinates: 0.2 mm caudal to bregma, 1.0 mm lateral to the sagittal suture, and 2.5 mm ventral to the skull. Our previous study indicated that 5 μg/mL NAMPT significantly reduced neuronal death induced by oxygen glucose deprivation in vitro [4]. Based on this concentration and the brain weight (~ 400 mg for 10-week-old C57 mice), 2 μg of NAMPT or an equivalent volume of PBS (4 μL) was administered into the left lateral ventricle. The injection was performed with a UMP3 microsyringe pump equipped with a Micro4 microsyringe controller (World Precision Instruments Inc., Sarasota, FL) using a Hamilton syringe connected to a cannula at a rate of 0.5 μL/min at the following timepoints: at the onset of reperfusion, and 4, 6, or 8 h after MCAO. In a separate study, 2 μg of NAMPT H247A protein was injected 6 h after MCAO as an intracellular NAMPT control, and its role on infarct volume was compared with that of NAMPT at the same timepoints. The mice were placed on an isothermal pad at 37 °C and continuously observed following surgery until recovery.

Quantification of Cerebral Infarction

At 72 h after MCA occlusion, brains were removed and sliced into eight coronal sections (1 mm thick) with a rodent brain matrix (Ted Pella, Inc., Redding, CA). The sections were stained for 20 min with 2% 2,3,5-triphenyltetrazolium chloride monohydrate (TTC, Sigma, St Louis, MO) at 37 °C and then fixed in 4% paraformaldehyde. Sections were scanned, and the infarction area in each section was measured by a blinded observer using ImageJ analysis software (National Institutes of Health, Bethesda, MD). The hemispheric infarct area in each section was calculated by subtracting the area of the normal, TTC-staining brain in the ipsilateral ischemic hemisphere from the contralateral nonischemic area. Infarct volume was then calculated by summing the infarct areas over all sections and multiplying by the slice thickness. Animals were randomly divided into three groups: sham, MCAO with PBS, and MCAO with NAMPT. Animals displaying massive hematomas in the brain were omitted from histological and behavioral analyses.

Neurobehavioral Tests

Two different neurobehavioral tests were performed in sham and MCAO mice treated with PBS and NAMPT by an observer blinded to group assignments. The rotarod test was performed [11] by placing mice on a rotating drum (model 47650; Ugo Basile, Gemonio, Italy) with a speed accelerating from 5 to 40 rpm within 5 min. The time at which the animal fell off the drum (latency to fall) was recorded and expressed as the mean duration of time on the rotarod. Preoperative training was performed for three trials a day for 3 days, and the mean duration on the last day served as preoperative baseline. Postoperative testing was performed for three trials daily on days 3, 5, 7, 10, and 14 post-injury. The adhesive tape removal test was performed to assess sensorimotor deficits on days 3, 5, 7, 10, and 14 post-injury. Briefly, an adhesive tape (3 × 4 mm) was attached to the distal–radial region on the wrist of each forelimb. The time to touch and remove the tape from the forelimbs was recorded during five trials per day for each forepaw, with a minimal interval of 5 min between consecutive trials. Mice were trained twice daily for 3 days before surgery, and the mean times on the last day served as preoperative baseline.

Immunofluorescence Staining

After blocking with 5% bovine serum albumin (BSA) in PBST for 1 h, coronal sections (25 μm) were incubated with primary antibodies at 4 °C overnight followed by the appropriate secondary antibodies for 1 h at room temperature. The primary antibodies used in this study include rat anti-CD16 (1:200, BD Biosciences, Franklin Lakes, NJ), goat anti-CD206 (1:200, R&D Systems, Minneapolis, MN), rabbit anti-Iba1 (1:1000, Wako Diagnostics, Mountain View, CA), and rat anti-neutrophil (1:100, Abcam, Cambridge, MA).

Primary Microglial Cultures and Real-Time PCR

Primary microglial cultures were prepared as described previously [12]. In brief, neural cell suspensions from the whole brains of 1-day-old pups were harvested and seeded on flasks coated with poly-D-lysine. At 12–14 days after seeding, microglia were shaken off, collected, and seeded on 6-well plates at 1×10^6 cells/well. Six hours after seeding, microglial cultures were treated with NAMPT (1 μg/mL) or PBS and collected 48 h later. Total RNA was isolated using the RNeasy Mini Kit (Qiagen), and cDNA was synthesized using the RT^2 Easy First Strand Kit (Qiagen) according to the manufacturer's instructions. Quantitative polymerase chain reaction (PCR) was performed with the Opticon-2 Real-Time PCR System (Bio-Rad, Hercules, USA) using the RT^2 SYBR green qPCR MasterMixes (Qiagen). PCR was performed at 95 °C for 2 min and 35 cycles of 30 s at 94 °C, 20 s at 65 °C, and 20 s at 72 °C with two sets of primers from pro- and anti-inflammatory microglial markers/cytokines listed in Supplemental Table 2. The cycle time values of the genes of interest were first normalized to levels of glyceraldehyde-3-phosphate dehydrogenase, and gene expression levels were then expressed as fold changes versus PBS controls.

Assessment of BBB Permeability after MCAO

Twenty-four hours after MCAO, the fluorescent tracer Alexa Fluor 555 Cadaverine (Thermo Fisher Scientific) was injected through the femoral vein at a dose of 200 μg per mouse. Mice were sacrificed 60 min after tracer injections, and 25-μm-thick coronal brain sections were collected. The leakage of tracers into the brain parenchyma was directly viewed on an EVOS FL Auto microscope (Thermo Fisher Scientific). To measure the extravasation of endogenous IgGs, sections were blocked with 5% (wt/vol) BSA for 1 h, then incubated with biotinylated anti-mouse IgG antibodies (1:500, Vector Laboratories, Burlingame, CA) at 4 °C overnight. Sections were then incubated with Alexa 488 Streptavidin (1:1000, Jackson ImmunoResearch Laboratories, West Grove, PA). Images were acquired using the EVOS GFP light tube. To measure the brain volume with tracer/IgG staining, brain slices spaced 500 μm apart (i.e., every 20th slice) were stained and imaged. The brain area with tracer/IgG leakage and the mean fluorescent intensity of each slice were measured with ImageJ by a blinded observer. These areas were summed and multiplied by the distance between sections (0.5 mm) to yield leakage volume. The total intensity of tracer or IgG leakage was calculated with the following formula: brain volume with tracer or IgG leakage × mean fluorescent intensity.

Statistical Analyses

Statistical analysis was performed using GraphPad Prism software (version 7.03). Data with normal distributions were expressed as the mean ± SEM, whereas data with non-normal distributions were expressed as the median and interquartile ranges. Statistically significant differences between two groups with non-normal distribution were analyzed by the Mann–Whitney *U* test. Differences among multiple groups were analyzed using one- or two-way ANOVA followed by a Bonferroni/Dunn post hoc correction. A *P* value smaller than 0.05 was considered statistically significant.

Results

ICV Administration of Recombinant NAMPT Reduces Infarct Volume After Focal Brain Ischemia

The neuroprotective effects of NAMPT against focal ischemia were assessed in the MCAO model after ICV delivery. NAMPT protein did not affect physiological parameters (data not shown) or survival rates after MCAO (Supplemental Table 1). Quantitative analyses of TTC staining (Fig. 1a, b) revealed that 45-min MCAO caused infarcts that occupied, on average, 36% (or ~ 72 mm^3) of the ipsilateral brain volume. ICV administration of 2 μg NAMPT at the onset of reperfusion offered potent cerebral protection agaist ischemia, as the infarct volume was reduced to 5.5% ($P < 0.001$). To further test whether NAMPT could protect brain tissue when delivered after injury, NAMPT was administered ICV at 4, 6, or 8 h after MCAO. NAMPT administration at both 4 and 6 h after MCAO significantly reduced infarct volumes (7.5% and 15.6%, $P < 0.01$ and $P < 0.05$, respectively). However, the protective effects of NAMPT faded when administered 8 h after MCAO. ICV administration of enzyme-inactive NAMPT H247A at 6 h after MCAO also significantly reduced infarct volumes when compared to PBS controls ($P < 0.05$) with efficacy similar to that of as wild- type NAMPT (21.3% versus 15.6%), suggesting that ICV-delivered NAMPT exerted neuroprotection mainly by extracellular mechanisms.

ICV Administration of Recombinant NAMPT Improves Long-Term Neurological Outcomes After Brain Ischemia

To test whether the tissue protection was associated with functional improvements, NAMPT (2 μg) was injected ICV at the onset of reperfusion, and sensorimotor function was assessed by the rotarod test (Fig. 2a) and adhesive tape removal test (Fig. 2b, c). For the rotarod test, mice subjected to MCAO and treated with PBS displayed a shorter latency to fall off the rod compared with sham mice ($P < 0.05$), suggesting that MCAO causes severe motor deficits. However, mice treated with NAMPT stayed longer on the rod than MCAO mice treated with PBS ($P < 0.05$), and this difference existed for at least 10 days after MCAO. As shown in Fig. 2b, mice subjected to MCAO and treated with PBS were slower to touch the tape than sham mice ($P < 0.05$), verifying that MCAO causes sensorimotor deficits. However, mice treated with NAMPT touched the tape significantly faster ($P < 0.05$), and this difference existed for at least 10 days after MCAO. Similarly, mice treated with NAMPT removed the tape significantly faster

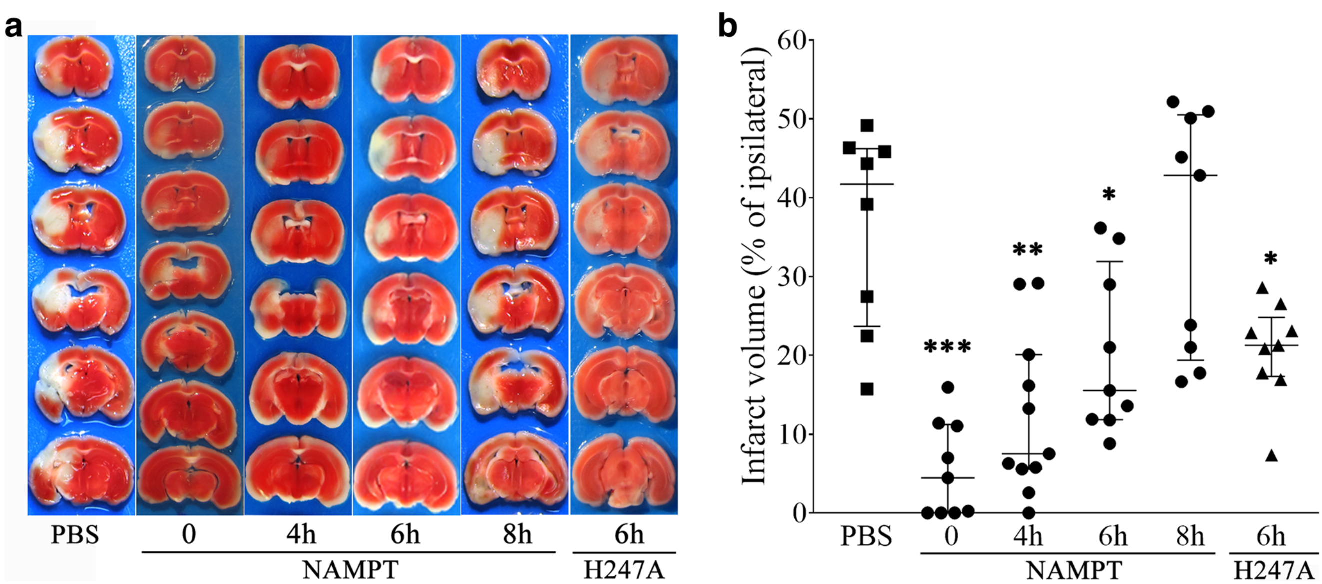


Fig. 1 ICV administration of recombinant NAMPT reduces infarct volume in a murine model of focal cerebral ischemia. MCAO was induced for 45 min, and 2 μg of NAMPT protein were injected by the ICV route at the onset of reperfusion, or 4, 6, and 8 h after ischemia. NAMPT H247A (2 μg) was injected 6 h after MCAO as an inactive control. An equivalent volume of PBS was administered as the vehicle control. Infarction volume was determined by TTC staining at 72 h after MCAO. **a** Representative TTC images of brain sections. **b** Quantitative analysis of infarct volume. The infarction ratio was calculated as the volume of infarcted tissue divided by the volume of the contralateral nonischemic area. Data are expressed as median with interquartile range, $n = 8$, 9, 11, 9, 9, and 9 for PBS, onset, 4, 6, and 8 h after MCAO and NAMPT H247A, respectively. Data were analyzed by ANOVA followed by the Bonferroni/Dunn post hoc correction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus PBS

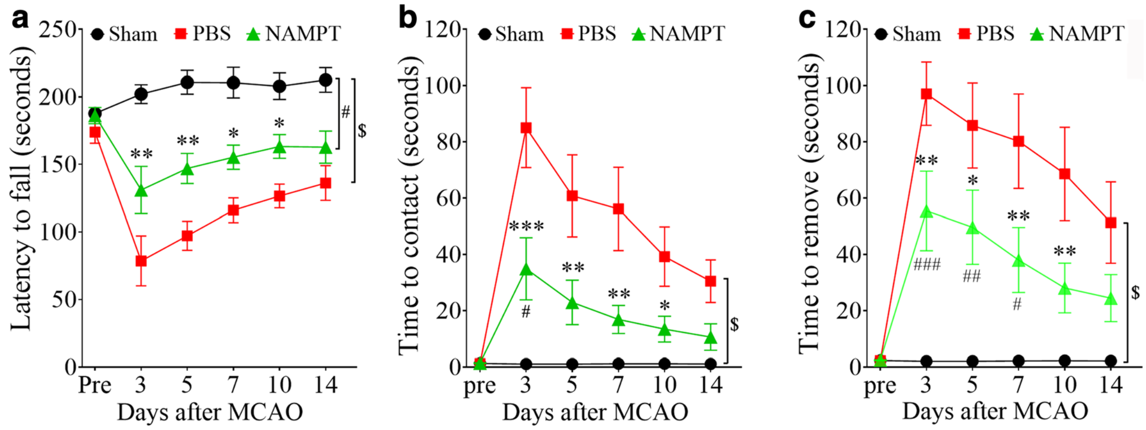


Fig. 2 ICV administration of recombinant NAMPT improves neurological outcomes after brain ischemia. NAMPT (2 μg) was injected by the ICV route at the onset of reperfusion, and sensorimotor functions were assessed by the rotarod test (**a**) and adhesive tape removal test (**b**, **c**) on days 3, 5, 7, 10, and 14 post-injury. Preoperative training was performed, and the final preoperative trial served as the baseline. PBS was administered as the vehicle control. $n = 12$, 10, and 11 for sham, PBS-, and NAMPT-treated mice. Data were expressed as mean ± SEM and were analyzed by ANOVA followed by the Bonferroni/Dunn post hoc correction. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ versus PBS; $\#P < 0.05$, $\#\#P < 0.01$, $\#\#\#P < 0.001$ versus sham; $\$P < 0.05$ versus sham

than mice treated with PBS ($P < 0.05$), and this difference also existed for at least 7 days after MCAO. Together, these data suggest that ICV administration of NAMPT enhances sensorimotor outcomes after ischemic brain injury.

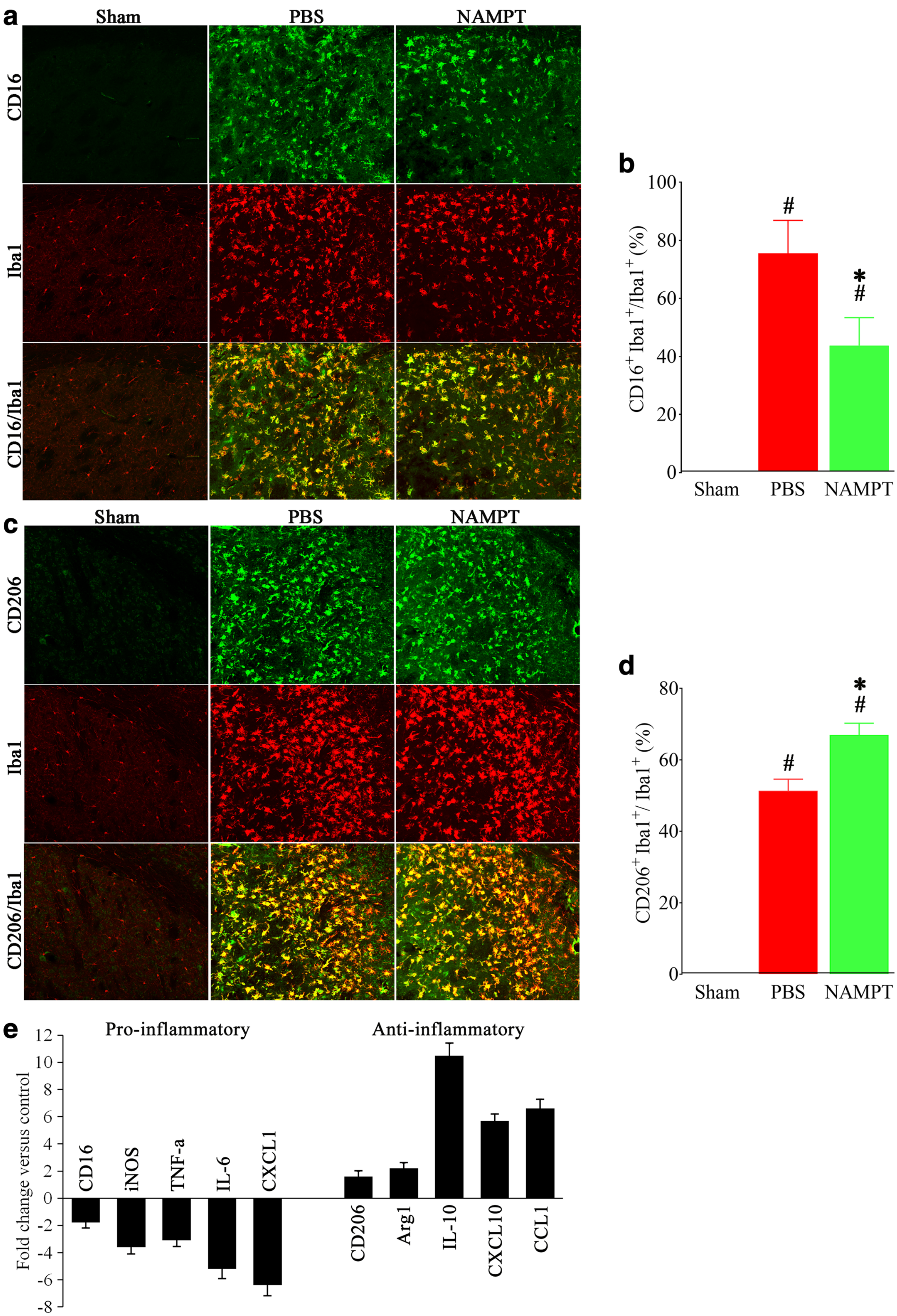
NAMPT Promotes Microglia/Macrophage Polarization Toward Anti-Inflammatory Phenotypes After Brain Ischemia

As the key enzyme for NAD biosynthesis, NAMPT plays an important neuroprotective role by synthesizing NAD for extra energy reserves [4–6]. We previously showed that mutant NAMPT without NAD biosynthetic activity retains its neuroprotective influence against ischemic brain injury, suggesting that other functions of NAMPT also contribute to its effects [4]. Anti-inflammatory microglia/macrophages play a beneficial role in neuroprotection, and extracellular NAMPT has been shown to promote anti-inflammatory macrophage polarization in chronic lymphocytic leukemia [13]. Therefore, we tested whether the neuroprotective effects of NAMPT are associated with anti-inflammatory microglia/macrophage polarization following ischemic brain injury. Microglia/macrophage polarization was analyzed by the pro-inflammatory marker CD16 and the anti-inflammatory marker CD206, and counterstained for the activated microglia/macrophage marker Iba1 at 72 h after MCAO, the peak time for microglia/macrophage infiltration/activation after ischemic brain injury [14–16]. As shown in Fig. 3a, b, few activated microglia/macrophages ($Iba1^+$ cells) and pro-inflammatory ($CD16^+/Iba1^+$) were found in sham brains, and these markers were significantly induced after MCAO. In the activated microglia/macrophage ($Iba1^+$) population, ~75% were immunopositive for CD16. However, NAMPT treatment significantly reduced the $CD16^+$ cell percentages to 43% ($P < 0.05$ versus PBS). Similarly, few activated anti-inflammatory microglia/macrophages ($CD206^+/Iba1^+$) were detected in sham conditions, and this marker was also induced after MCAO (51%, $P < 0.05$ versus sham). NAMPT treatment significantly increased the $CD206^+$ cells to 67% ($P < 0.05$ versus PBS), as shown in Fig. 3c, d. Taken together, these results suggest that NAMPT may exert neuroprotection by reducing pro-inflammatory microglia/macrophages and promoting microglia/macrophage polarization toward anti-inflammatory phenotypes after brain ischemia.

Next, we verified the effects of NAMPT on microglial polarization in primary microglial cultures. Primary cultured microglia were treated with NAMPT at 1 μg/ml for 48 h, and two sets of pro- and anti-inflammatory microglia markers/cytokines were quantitatively analyzed with real-time PCR. NAMPT treatment significantly decreased the expression of CD16 and iNOS, two representative markers of pro-inflammatory microglia, and the expression of TNF-α, IL6, and CXCL1, three representative cytokines produced by pro-inflammatory microglia. However, the expression of anti-inflammatory markers CD206 and Arg1 and the cytokines IL-10, CXCL10, and CCL1 was significantly induced (Fig. 3e). These collective observations demonstrate that NAMPT may modulate the phenotypic polarization of microglia.

NAMPT Reduces Neutrophil Infiltration After Brain Ischemia

We then tested whether NAMPT affects the infiltration of neutrophils, the predominant inflammatory cells entering the brain after MCAO [17]. Brains were collected 72 h after MCAO and stained with anti-neutrophil, a neutrophil marker that recognizes the Ly-6B.2 antigen. As shown in Fig. 4a, b, there were no $neutrophil^+$ cells in sham mouse brains, but the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◀ **Fig. 3** NAMPT treatment primes microglia/macrophage polarization after brain ischemia and in primary microglia cultures. NAMPT (2 μg) was injected by the ICV route at the onset of reperfusion. Brains were collected 72 h after MCAO and stained with pro-inflammatory (**a**, **b**) and anti-inflammatory (**c**, **d**) microglia/macrophage markers. PBS was administered as the vehicle control. **a** Representative images of CD16/Iba1 staining. **b** Percentage of $CD16^+/Iba1^+$ cells among Iba1+ cells. **c** Representative images of CD206/Iba1 staining. **d** Percentage of $CD206^+/Iba1^+$ cells among Iba1+ cells. Data were expressed as mean ± SEM. *$P < 0.05$ versus PBS; #$P < 0.05$ versus sham; \$$P < 0.05$ versus sham. **e** Pro- and anti-inflammatory microglial markers/cytokines in primary microglial cultures treated with NAMPT or PBS. Microglia were treated with either NAMPT or PBS for 48 h, and the expression of microglial markers/cytokines were analyzed with real-time PCR. $N = 3$ for each treatment. Data were expressed as fold changes versus control

number of $neutrophil^+$ cells rose to 150 cells/mm^2 ($P < 0.05$ versus sham) in MCAO mice treated with PBS. NAMPT treatment decreased $neutrophil^+$ cell densities to 76 cells/mm^2 ($P < 0.05$ versus PBS), suggesting that NAMPT inhibits neutrophil infiltration into the brain following brain ischemia.

NAMPT Maintains BBB Integrity After Brain Ischemia

As ICV NAMPT may not directly contact neutrophils and macrophages, we then tested whether NAMPT inhibits neutrophil and microphage infiltration by maintaining BBB integrity. BBB integrity was assessed by measuring the extravascular leakage of the small-molecule tracer Alexa Fluor 555 Cadaverine (MW 950) and larger-molecule endogenous plasma IgG (MW 155 kD) leakage 24 h after MCAO. As shown in the top panel of Fig. 5a, the intact brain was impermeable to Alexa Fluor 555 Cadaverine. However, BBB integrity was significantly compromised after MCAO in PBS-treated mice, as shown by leakage of Alexa Fluor 555 Cadaverine into the infarct region. NAMPT administration not only reduced the infarct region but also reduced the fluorescence intensity of Alexa Fluor 555 Cadaverine. Similarly, MCAO elicited the leakage of plasma IgGs into the brain parenchyma (bottom panel), and NAMPT administration attenuated this effect. As the quantity of leaked tracer or IgGs is closely related to both the area of the leakage and the intensity of the leaked tracer or IgGs, we accounted for this by using the following formula: total intensity = brain leakagearea × mean fluorescent intensity. The total intensity of Alexa Fluor 555 Cadaverine in PBS-treated mice was 16,732 but decreased to 8992 in NAMPT-treated mice ($P < 0.05$ versus PBS). The total intensity of IgGs was 12,965 in PBS-treated mice and decreased to 4542 in NAMPT-treated mice ($P < 0.05$ versus PBS). These findings reveal that NAMPT protects the BBB and prevents the extravasation of both small- and large-sized molecules.

Discussion

The present findings demonstrate that NAMPT delivered into the ventricles reduces infarction in preclinical stroke, even when administered in a clinically translatable regimen of 6 h after the ischemic injury. Importantly, the tissue protection was associated with neurobehavioral improvements, suggesting that the histologically preserved neurons were indeed functional. These findings are consistent with our previous study showing that released extracellular NAMPT protects against ischemic brain injury [4] and other studies showing that NAMPT protein is neuroprotective against brain ischemia [18]. However, our results are inconsistent with Zhao and colleagues' study, in which ICV administration of NAMPT exacerbated infarct volume [19]. The Zhao et al. study also showed that NAMPT exacerbated oxygen–glucose–deprivation (OGD)-induced neuronal death only in neuron-glia mixed cultures, but not in neuron cultures, which contradicts other reports that NAMPT inhibits OGD-induced neuronal death in neuron culture [4, 18].

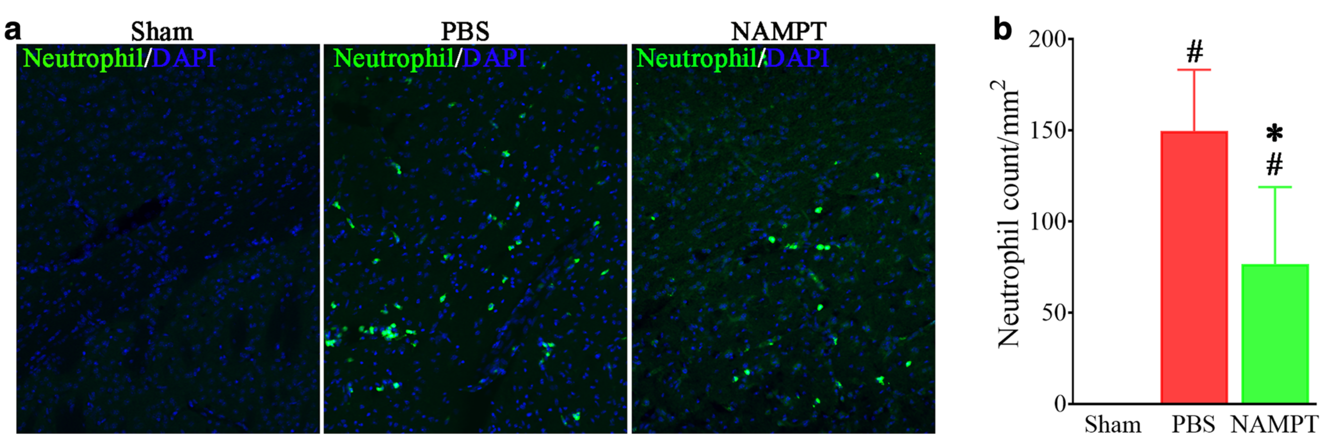

Fig. 4 NAMPT reduces neutrophil infiltration after brain ischemia. Brains were collected 72 h after MCAO and stained with an anti-neutrophil antibody. Nuclei were counterstained with DAPI. **a** Representative images of neutrophils/DAPI. **b** Quantitative analysis of neutrophils. Data were collected from three slices per brain out of six brains per group and expressed as mean ± SEM. Data were analyzed by ANOVA followed by the Bonferroni/Dunn post hoc correction. *$P < 0.05$ versus PBS; #$P < 0.05$ versus sham

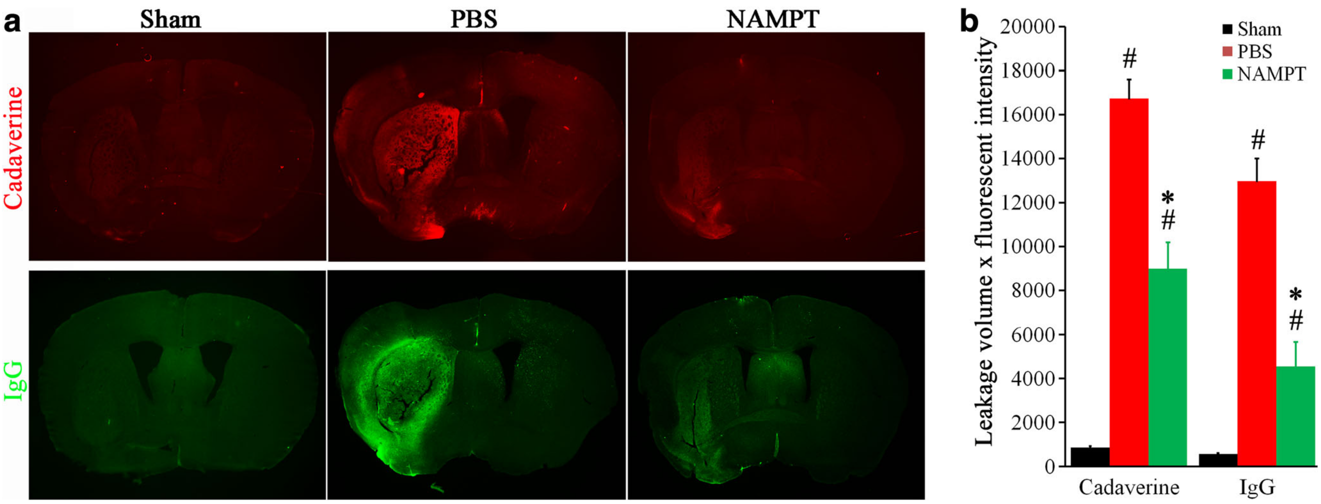

Fig. 5 NAMPT maintains BBB integrity after brain ischemia. Twenty-four hours after MCAO, Alexa Fluor 555 Cadaverine was injected through the femoral vein, and brains were collected 1 h later in sham, PBS, and NAMPT-treated mice. **a** Representative images show the leakage of Alexa Fluor 555 Cadaverine (red, top panel) or endogenous plasma IgGs (green, bottom panel) into the brain parenchyma in sham, PBS, and NAMPT-treated mice. **b** Quantitative measurements of total fluorescence intensity for Alexa Fluor 555 Cadaverine and IgG staining. Data are presented as leakage volume × mean fluorescent intensity. $N = 8$ for all three groups. $^*P < 0.05$ versus PBS; $\#P < 0.05$ versus sham

One potential reason for the contradictory results may be the lipopolysaccharide (LPS) content in the protein sample. LPS is the major component of the outer membrane of *E. coli* BL21, the most commonly used bacterial stain for recombinant protein production, and proteins obtained from these bacteria contain LPS at an approximate concentration of 108 EU/mg protein, based on our ELISA assay (data not shown). In our study, LPS was removed using Detoxi-Gel Endotoxin Removing Columns, and LPS was reduced to minimal levels (< 0.5 EU/mg). LPS triggers the inflammatory response, exacerbates infarct size, and results in worsened neurobehavioral outcomes in ischemic brain injury [20]. Thus, it seems likely that LPS contamination in NAMPT protein samples may negate the neuroprotective effects of NAMPT. To investigate this possibility, we compared the infarct volumes of mice subjected to MCAO and treated with NAMPT protein with or without LPS removal (Supplemental Fig. 1) and found that NAMPT protein without LPS removal failed to reduce infarct volume, as expected.

Our results indicate that NAMPT can reduce pro-inflammatory microglia/macrophages and activate anti-inflammatory microglia/macrophages, suggesting that NAMPT promotes microglia/macrophage polarization toward anti-inflammatory phenotypes after brain ischemia. Our in vitro data further demonstrated that NAMPT treatment decreased expression of pro-inflammatory microglial markers/cytokines, but induced expression of anti-inflammatory markers/cytokines, further verifying that NAMPT promotes microglial polarization. Consistent with these findings, a recent study by Audrito and colleagues reported that NAMPT protein treatment can increase the differentiation of resting monocytes, polarizing them into anti-inflammatory macrophages, and that this effect is independent of its NAD bioactivity [13]. However, these findings contradict Zhao and colleagues' in vitro studies showing that NAMPT protein triggers TNF-α production in neuronal cultures and aggravates ischemic neuronal injury by acting as a pro-inflammatory factor [19]. Pro-inflammatory microglia/macrophages are known to release inflammatory cytokines that accelerate cell death and aggravate local inflammation [21, 22] while activated anti-inflammatory microglia/macrophages protect ischemic brain by removing cell debris and releasing trophic factors for brain repair [23, 24]. Given the potent neuroprotective effects against ischemic brain injury, delivery of NAMPT by lumbar puncture rather than ICV injection should also be tested, as the lumbar route of administration might be more clinically translatable. For stroke patients requiring lumbar puncture for CSF sampling, NAMPT could be administered immediately after CSF withdrawal.

In the present study, we found that NAMPT treatment significantly decreased the infiltration of neutrophils into the brain. Neutrophils are among the first immune cells to infiltrate the ischemic brain, peaking at days 2–3 post-injury, and decreasing thereafter [25, 26]. In our study, we chose 72 h after MCAO, a peak time for infiltration/activation of many inflammatory cells, including neutrophils. Our results indicate massive infiltration of neutrophils in vehicle-treated ischemic brains. Infiltrated neutrophils are short-lived cells and die rapidly through apoptosis. Therefore, neutrophil counts in the ischemic brain are influenced by a dynamic equilibrium between infiltration and apoptosis. It is possible that apoptosis also contributed to reduced neutrophils in the NAMPT-treated brain. However, the inclusion of the PBS vehicle control group supports our conclusion that decreased neutrophil counts in the NAMPT-treated group are mainly caused by reduced infiltration of neutrophils. ICV NAMPT is unlikely to act directly on circulating neutrophils, and it seems more likely that NAMPT reduces neutrophil infiltration by maintaining the integrity of the BBB. In line with this hypothesis, NAMPT treatment significantly decreased the leakage of small-molecule tracer Alexa Fluor 555 Cadaverine and larger-molecule IgGs into the brain parenchyma. Although the mechanism underlying BBB preservation by NAMPT is out of the scope of the present study, it is possible that NAMPT acts directly on the endothelial lining to reduce cell death, or that it reduces the number of pro-inflammatory microglia/macrophages and inhibits the infiltration of neutrophils. It is well recognized that proteases secreted by activated leukocytes are one of key pathologic factors contributing to BBB leakage [27–29]. Activated microglia/macrophages can also produce matrix metalloproteinases [30, 31], which degrade the extracellular matrix and damage the BBB.

In conclusion, we explored the neuroprotective effects of exogenously administered recombinant NAMPT protein in an animal model of ischemic brain injury. In addition to reducing infarct volumes and improving neurobehavioral outcomes, NAMPT exerted novel anti-inflammatory effects by reducing pro-inflammatory microglia/macrophages, promoting anti-inflammatory microglia/macrophage polarization, inhibiting neutrophil infiltration, and maintaining BBB integrity. These findings indicate that NAMPT is an endogenous protective molecule with manifold functions and might be useful as a new therapeutic agent for the treatment of ischemic stroke. The mechanism whereby NAMPT modulates the inflammatory response and preserves ischemic tissue warrants further investigation, as does exploration of the potential cell surface receptor/ligand for NAMPT. Furthermore, more work needs to be done to identify the mechanism underlying NAMPT-mediated microglial/macrophage polarization toward anti-inflammatory phenotypes, including the identification of the pro- and anti-inflammatory factors regulated by NAMPT in the context of brain ischemia.

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Compliance with Ethical Standards All animal procedures used in this study were conducted in strict compliance with The National Institue of Health Guide for Use and Care of Laboratory Animals and approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Conflict of Interest The authors declare that they have no conflict of interest.

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