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NAD+ administration decreases ischemic brain damage partially by blocking autophagy in a mouse model of brain ischemia

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ABSTRACT

Nicotinamide adenine dinuleotide (NAD+) plays critical roles in multiple biological functions. Previous studies have indicated that NAD+ treatment decreases oxidative stress-induced death of primary neurons and astrocytes. Intranasal administration of NAD+ also reduces brain damage in a rat model of transient focal brain ischemia. However, the mechanisms underlying this protective effect remain unknown. In this study, we used a mouse model of brain ischemia to test our hypothesis that NAD+ decreases ischemic brain damage partially by preventing autophagy. Adult male mice were subjected to transient middle cerebral artery occlusion (tMCAO) for 90 min, and NAD+ was administered intraperitoneally (i.p.) immediately after reperfusion started. We found that administration with 50 mg/kg NAD+ led to significant decreases in infarct size, edema formation, and neurological deficits at 48 h after ischemia. NAD+ administration also significantly decreased brain ischemia-induced autophagy in the cortex and hippocampus. We further found that prevention of autophagy by 3-methyladenine (3-MA), a selective autophagy inhibitor, significantly reduced ischemic brain damage, suggesting an important role of autophagy in the ischemic brain injury in our animal model. Collectively, our findings have suggested that NAD+ administration decreases ischemic brain damage at least partially by blocking autophagy. This is the first suggested mechanism regarding the protective effects of NAD+ in cerebral ischemia, which further highlights the promise of NAD+ for treating brain ischemia.

A large number of studies have indicated that NAD+ plays important roles in not only energy metabolism and mitochondrial functions, but also aging, gene expression, calcium homeostasis and immunological functions [16]. It has also been found that NAD+ treatment decreases oxidative stress-induced death of primary cultures of neurons, astrocytes, and myocytes [1,2,17]. Our previous study showed that intranasal NAD+ administration profoundly decreases the infarct volume in a rat model of transient focal ischemia [18]. However, the mechanisms underlying this protective effect remain unknown. It is necessary to elucidate the mechanisms underlying the protective effects of NAD+, which may not only expose novel mechanisms of brain protection, but also establish basis for potential applications of NAD+ for treating brain ischemia.

Autophagy is a catabolic process for degradation of cytoplasmic contents by using lysosomal degradation machinery. Although autophagy is activated in several brain ischemia models, it has not been conclusive yet if autophagy produces protective or damaging effects in brain ischemia [10]. Several studies have suggested that autophagy plays a detrimental role in brain ischemia: Inhibition of autophagy by 3-methyladenine (3-MA) led to decreased ischemic brain injury [12]; and downregulation of Beclin 1 by RNA interference also attenuated ischemic brain damage in rats [20].

We hypothesized that NAD+ reduces ischemic brain damage partially by decreasing autophagy for the following reasons: mitochondrial permeability transition (MPT) mediates autophagy under certain conditions [8]; and our previous studies have suggested that NAD+ treatment decreases the MPT induced by oxidative stress or alkylating agents in cell culture studies [1,2]. In our current study, we used a mouse model of brain ischemia to test the hypothesis that NAD+ decreases ischemic brain damage partially by blocking autophagy. Our results have provided evidence supporting the hypothesis.

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except where otherwise noted. CD-1 mice were purchased from Shanghai SLAC Laboratory Animal Corporation (Shanghai, China). Animal procedures for the use of laboratory animals were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University. The surgical procedure was

Abbreviations: NAD+, nicotinamide adenine dinuleotide; 3-MA, 3-methyladenine; tMCAO, transient middle cerebral artery occlusion; TTC, triphenyltetrazolium chloride.

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conducted as previously described [14]. Briefly, Adult male CD-1 mice (25–30 g) were subjected to tMCAO. After being anesthetized by intraperitoneal injection with ketamine (100 mg/kg) and xylazine (10 mg/kg), the mouse was mounted supinely on a heating pad. The left common carotid artery (CCA), the external carotid artery (ECA) and the internal carotid artery (ICA) were isolated under an operating microscope (Leica, Wetzlar, Germany). A 6-0 nylon suture was introduced into the ECA stump, and advanced from the ICA to the ostium of MCA until a slight resistance was felt. Successful occlusion, characterized as a reduction of blood flow down to 10% of baseline, was verified by laser doppler flowmetry (Moor Instruments, England). After 90 min, the suture was withdrawn back into the external carotid artery to restore ICA–MCA blood flow.

Mice were anesthetized and placed in a stereotaxic frame. NAD^+, dissolved in 0.9% saline solution, was administered intraperitoneally. 3-MA, after being dissolved in 0.9% saline solution that was heated to 65 °C, was injected by intracerebroventricular (i.c.v) approach into the left lateral ventricle using a 5 μL Hamilton syringe.

As described previously [3], brain tissues from the ischemic cortex, hippocampus and striatum of the left middle cerebral artery territory (parietal cortex) and the corresponding area of sham-operated mice were homogenized in RIPA buffer (Millipore, Temecula, CA, USA) containing Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany), 2 mM PMSF, and 0.1% SDS. The lysates were centrifuged at 12,000 × *g* for 30 min at 4 °C. Protein concentrations were determined by using a BCA Protein Assay kit (Pierce Biotechonology, Rockford, IL, USA). Thirty μg of total protein was electrophoresed through a 12% SDS–polyacrylamide gel and then transferred to a 0.2 μm PVDF membrane (Millipore, Billerica, MA, USA). Blots were incubated overnight at 4 °C with a rabbit polyclonal LC3 antibody at dilution of 1:1000 (Sigma, St. Louis, MO, USA), then incubated with goat anti-rabbit HRP-conjugated secondary antibody at dilution of 1:4000 (Epitomics, Hangzhou, Zhejiang Province, China). Protein signals were detected using an ECL detection system (Pierce Biotechonology, Rockford, IL, USA). An anti-β-actin antibody (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) was used to normalize sample loading and transfer. The intensity of the bands was quantified by densitometry using a Gel-Pro Analyzer.

Twenty-four hours after reperfusion, the mice were sacrificed with an overdose of chloral hydrate (300 mg/kg). The brains were removed and sliced into 2-mm coronal sections in a matrix. The brain sections were incubated in 0.9% saline solution containing 2% triphenyltetrazolium chloride (TTC) at 37 °C for 20 min, and subsequently immersed in 10% phosphate-buffered formalin. When TTC reacts with intact mitochondrial respiratory enzymes, it generates a bright red color that contrasts with the pale color of the infarction. The infarction area of the left cerebral hemisphere was measured using Image J (NIH), and the infarction volume (in mm^3) was calculated from the infarction area.

As described previously [6], brain swelling was calculated according to the following formula: (infarct volume + ipsilateral undamaged volume − contralateral volume) × 100/contralateral volume (%).

The neurobehavioral functions of the mice were evaluated using a modified neurological severity score (NSS) at 24 or 48 h after reperfusion, as described previously [11]. The NSS is a composite of motor (muscle status, abnormal movement), sensory (visual, tactile, proprioceptive), reflex, and balance tests. Neurobehavioral functions were graded on a scale of 0–18 (normal score, 0; maximal deficit score, 18).

All data are presented as means ± S.E. Data were assessed by one-way ANOVA, followed by Tukey *post hoc* test. *P* values less than 0.05 were considered statistically significant.

To determine the effects of NAD^+ administration on the ischemic brain injury in the mouse model of tMCAO, we assessed the effects of administration with 50 mg/kg NAD^+ on ischemic brain damage 48 h after ischemia. The NAD^+ administration did not affect the cerebral blood flow of the mice that were subjected to tMCAO (data not shown). We found that at 48 h after tMCAO, the infarct volume in saline-treated and NAD^+-treated mice were 46.97 mm^3 and 20.84 mm^3, respectively (Fig. 1A and B), suggesting significant protection by the NAD^+ administration. The NAD^+ administration also significantly decreased tMCAO-induced brain swelling (Fig. 2A) and impairments of the neurobehavioral functions (Fig. 2B).

We determined if tMCAO induces autophagy in our mouse model of brain ischemia by assessing the levels of microtubule-associated protein 1 light chain 3 (LC3), a marker of autophagy. LC3 has two isoforms: LC3-I and LC3-II. During the elongation of autophagosome, the cytosolic isoform LC3-I is conjugated to

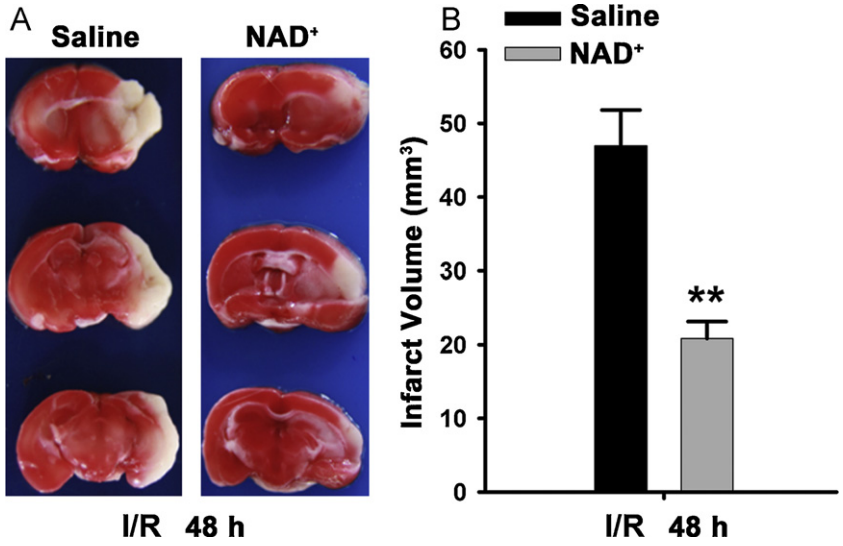

Fig. 1. NAD^+ administration significantly decreased the infarct formation in a mouse model of transient brain ischemia. Mice were subjected to 90-min middle cerebral artery occlusion (MCAO). 50 mg/kg NAD^+ was administered intraperitoneally immediately after reperfusion started. Forty-eight hours after reperfusion, the coronal brain sections were obtained from the mice and stained with TTC, showing NAD^+-produced protection against infarct formation (A). Quantifications of the infarct volume showed that administration with 50 mg/kg NAD^+ significantly decreased infarct formation (B). $N = 6$ for both of the groups. All values are expressed as mean ± S.E. $^*p < 0.05$, $^{**}p < 0.01$.