SUPPLEMENTAL MATERIAL

Modulation of mitochondrial function and autophagy mediates carnosine neuroprotection against ischemic brain damage

Seung-Hoon Baek, PhD[1,5]; Ah Reum Noh, MSc[2,5]; Kyeong-A Kim, BSc[2]; Muhammad Akram, BPharm[2]; Young-Jun Shin, MSc[2]; Eun-Sun Kim, MSc[2]; Seong Woon Yu, PhD[3]; Arshad Majid, MD[4]*; and Ok-Nam Bae, PhD[2]*

[1]College of Pharmacy, Ajou University, Suwon, Republic of Korea
[2]College of Pharmacy Institute of Pharmaceutical Science and Technology, Hanyang University, Ansan, Republic of Korea
[3]Department of Brain Science, Daegu Gyeongbuk Institute of Science and Technology (DGIST), Daegu, Republic of Korea
[4]Sheffield Institute for Translational Neuroscience (SITraN), University of Sheffield, 385A Glossop Road, SHEFFIELD S10 2HQ, England

[5] These authors contributed equally to this work.

Corresponding Author: Ok-Nam Bae, PhD, onbae@hanyang.ac.kr
Co-corresponding Author: Arshad Majid, MD, majidarshad@msn.com

Supplemental Materials and Methods

Animal treatments

All animal experiments were conducted using adult male Sprague-Dawley rats weighing 250 to 300 g (Harlan) and performed in accordance with the NIH Policy and Animal Welfare Act under the approval by Institutional Animal Care and Use Committee (IACUC) at Hanyang University. Treatment groups were allocated in a randomized fashion using a Researcher Randomizer Program (http://www.randomizer.org/). Investigators were blind to the allocation of treatment during surgeries and outcome evaluations. Carnosine was obtained from Sigma and dissolved in saline. Carnosine (1,000 mg/kg) was administered into the lateral tail vein at 6 hr after ischemic onset both in permanent and transient models. The choice of this dose and time window is based on previous dose finding studies.[1-4]

Ischemic stroke in rats

Permanent or transient focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO) according to previous reports.[2] After induction of general anesthesia by isoflurane inhalation, rats were maintained under anesthesia throughout the surgical period. Rectal temperature was maintained at 37°C and the cerebral blood flow (CBF) was measured with laser Doppler (Perimed, North Royalton, OH). The left common carotid artery (CCA) and the external carotid artery (ECA) were exposed and ligated by a suture, and the occipital artery of the ECA was coagulated. The internal carotid artery (ICA) was exposed and the pterygopalatine artery was ligated. Ischemia was initiated by a silicone-coated 4-0 monofilament nylon suture (Doccol Co.) as described previously.[2,5] The monofilament was inserted into the CCA and advanced into the ICA to the origin of the MCA (18.0 mm from the bifurcation). The filament was left in place for the permanent model, while reperfusion was produced by withdrawal of the monofilament 6 hrs after occlusion in the transient model. Rats were excluded from the study when the CBF was not decreased below 30% of baseline after occlusion, or CBF was not restored above 80% of basal CBF after reperfusion.

Calculation of infarct volume

Rats were euthanized by isoflurane overdose, and the brains were rapidly isolated. Brains were cut into 2 mm sections, and stained with 2% triphenyltetrazolium chloride (TTC) .[6] After fixed in 4% paraformaldehyde, each section was scanned to a digital image, and analyzed using the NIH ImageJ software. The infarct volume for each section was calculated and edema correction was performed by the measurement of the ipsilateral and contralateral hemisphere.

Assessment of neurological function

Deficit in neurological function was evaluated by behavioral tests at 24 hr after

tMCAO (6 hr ischemia), based on the previous literature.[2,7] Somatosensory deficit was measured using the adhesive tape removal test before and after surgery. All rats were trained to the tests for 5 consecutive days before focal ischemia. As bilateral tactile stimuli, small pieces of adhesive were applied on the wrist of each forelimb. The time to remove each tape from forelimbs was recorded on 3 trials (maximum record of 180 sec). Motor–ambulatory function was assessed with a Rota Rod test. Rats were trained for 3 consecutive days before ischemia. Rats were placed on the Rota Rod and then tested with acceleration speeds. Latencies to fall off or the 395 sec cutoff were recorded for analysis with 3 trials.

Brain homogenization and mitochondria isolation

Brain samples between bregma levels +2 and -4 mm, which include ischemic core and penumbra, were rapidly isolated at 24 hrs after MCAO, and brain homogenates were obtained by homogenization in isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, and 1 mM EGTA, pH 7.2). The protein concentrations were determined using BCA assay (Thermo Scientific, Rockford, IL) after brain tissue homogenate was collected, and used for western blot.

For further isolation of mitochondria, 30% Percoll in isolation buffer was added to brain homogenates. The resultant homogenate was layered on a discontinuous Percoll gradient with the bottom layer containing 40% Percoll solution, followed by a 24% Percoll solution, and finally the sample in a 15% Percoll solution.[8] The density gradients were centrifuged at 30,400g for 10 minutes. Following centrifugation, band 3 (non-synaptic mitochondria) were separately removed from the density gradient. The final mitochondrial pellet was re-suspended in isolation buffer without EGTA. Protein concentration was determined using the BCA protein assay.

Western Blot of brain homogenate or isolated brain mitochondria

Processed brain homogenates or brain mitochondria were separated in Tris-HCl SDS-PAGE Ready Gels (Bio Rad) and were transferred to PVDF membrane (Millipore).[2,8] After blocking with 5% BSA, membranes were incubated overnight at 4°C with primary antibodies. Primary antibodies against phospho-mTOR (#2971), mTOR (#2972), phospho-p70S6 kinase (#9205), p70S6 kinase (#9202), phospho-p44/42 MAPK (Erk1/2) (#9101), p44/42 MAPK (Erk1/2) (#9102), phospho-DRP1 (#3455), AIF (#4642), and parkin (#4211) were purchased from Cell Signaling Technology (Danvers, MA). Anti-LC-3B (L7543), anti-β-actin (A5441), and anti-β-tubulin (P4026) antibodies were from Sigma. Anti-VDAC1 (ab154846) and anti-cytochrome C (#556432) antibodies were obtained from Abcam (Cambridge, UK) and BD Bioscience (Franklin Lakes, NJ), respectively. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (MAB374) was purchased from Millipore. Horseradish peroxidase-conjugated antibodies were used as secondary antibodies. The immune complexes were visualized by enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate. Bands were quantified by NIH Image J program, and normalized by the corresponding loading controls. The extent of

phosphorylation was normalized by the total amount of the target proteins. All immunoblots were repeated for four independent experiments.

Complex I activity

Complex I activity was measured using colorimetric method as previously described with 2,6 dichloroindophenol (DCIP).[9] Isolated brain mitochondria was mixed in a reaction buffer containing 25 mM potassium phosphate, 3.5 g/L BSA, 60 μM DCIP, 70 μM decylubiquinone, 1.0 μM antimycine-A, and 0.2 mM NADH (pH 7.8). Complex I oxidizes NADH generating electrons which reduce decylubiquinone, and the reduced decylubiquinone subsequently delivers the electrons to DCIP. The extent of DCIP reduction was monitored spectrophotometrically at 600 nm.

***In vitro* culture of primary cortical neurons**

Primary cortical neuronal cultures were established as described previously.[1] Cell culture media and reagents including Neurobasal A, B27, DMEM, glutamine, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). At post-natal day 0, cerebral cortices were isolated from newborn C57BL/6J mice, and cells were dissociated in the presence of 4 mM L-cysteine, 10 U/ml papain (Worthington), and 1000 U/ml DNase (Roche) for 30 min at 37°C. Dissociated cells were washed and triturated with a pipette, and plated onto poly-D-lysine-precoated plates. Three days after plating, 50% of the medium was changed with Neurobasal A supplemented with 2% B27, and subsequently replaced every three days. Neuronal cultures were maintained in a CO_2 incubator at 37°C, and used between days *in vitro* (DIV) 7 and 11. These cultures contained >90% neurons as revealed by NeuN/ beta tubulin-immunohistochemistry.

NMDA-induced excitotoxicity

Ischemic neuronal damage was examined by N-methyl-d-aspartate (NMDA)-induced excitotoxicity.[10] Primary neuronal cells were treated with NMDA-containing media and incubated at 37°C for 20 min on DIV 9. Exposure to NMDA was terminated by replacement with the original media collected before NMDA treatment. NMDA-induced cytotoxicity was measured at 24 hr after NMDA exposure by leakage of lactate dehydrogenase (LDH). Alterations in cellular proteins were assessed by western blot as described earlier, with cell lysates extracted from neuronal cells using RIPA buffer (Thermo Scientific). To examine carnosine protection, cells were pretreated with carnosine for 30 min prior to NMDA stimulation.

Statistics

We calculated the means and standard errors of means (SEM) for all treatment groups. Differences in values were analyzed using Student t-test or analysis of variance (ANOVA), as appropriate, using SPSS software (Chicago, IL). Multiple comparisons were made using one-way ANOVA followed by Tukey test. Two-tailed Student's t-test analysis

was used for comparing values between two groups. In all cases, a p value of < 0.05 was considered significant.

Citations in the Supplemental Material

1. Bae ON, Majid A. Role of histidine/histamine in carnosine-induced neuroprotection during ischemic brain damage. *Brain Res*. 2013;1527:246-254.
2. Bae ON, Serfozo K, Baek SH, Lee KY, Dorrance A, Rumbeiha W, et al. Safety and efficacy evaluation of carnosine, an endogenous neuroprotective agent for ischemic stroke. *Stroke*. 2013;44:205-212.
3. Min J, Senut MC, Rajanikant K, Greenberg E, Bandagi R, Zemke D, et al. Differential neuroprotective effects of carnosine, anserine, and N-acetyl carnosine against permanent focal ischemia. *J Neurosci Res*. 2008;86:2984-2991.
4. Rajanikant GK, Zemke D, Senut MC, Frenkel MB, Chen AF, Gupta R, et al. Carnosine is neuroprotective against permanent focal cerebral ischemia in mice. *Stroke*. 2007;38:3023-3031.
5. Lee KY, Bae ON, Serfozo K, Hejabian S, Moussa A, Reeves M, et al. Asiatic acid attenuates infarct volume, mitochondrial dysfunction, and matrix metalloproteinase-9 induction after focal cerebral ischemia. *Stroke*. 2012;43:1632-1638.
6. Yang G, Park D, Lee SH, Bae DK, Yang YH, Kyung J, et al. Neuroprotective Effects of a Butanol Fraction of Rosa hybrida Petals in a Middle Cerebral Artery Occlusion Model. *Biomol Ther (Seoul)*. 2013;21:454-461.
7. Wang X, Fan X, Yu Z, Liao Z, Zhao J, Mandeville E, et al. Effects of tissue plasminogen activator and annexin A2 combination therapy on long-term neurological outcomes of rat focal embolic stroke. *Stroke*. 2014;45:619-622.
8. Baek SH, Bae ON, Kim EK, Yu SW. Induction of mitochondrial dysfunction by poly(ADP-ribose) polymer: implication for neuronal cell death. *Mol Cells*. 2013;36:258-266.
9. Janssen AJ, Trijbels FJ, Sengers RC, Smeitink JA, van den Heuvel LP, Wintjes LT, et al. Spectrophotometric assay for complex I of the respiratory chain in tissue samples and cultured fibroblasts. *Clin Chem*. 2007;53:729-734.
10. Panickar KS, Nonner D, White MG, Barrett JN. Overexpression of Cdk5 or non-phosphorylatable retinoblastoma protein protects septal neurons from oxygen-glucose deprivation. *Neurochem Res*. 2008;33:1852-1858.