Original Article
Neuroprotective effects of resveratrol against cerebral ischemia/reperfusion injury in rats through attenuation of inflammation

Rong Zou, Zhaoxia Liu, Xiaoning Qu, Yan Zhan

Department of Neurology, Yantai Affiliated Hospital of Binzhou Medical University, Yantai, China

Abstract: Considerable evidence has been accumulated to suggest that blocking the inflammatory reaction promotes neuroprotection and shows therapeutic potential for clinical treatment of ischemic brain injury. Resveratrol (Res) has been demonstrated to exhibit neuroprotective functions in cerebral ischemia/reperfusion (I/R) injury. However, the underlying mechanisms in this process and its contribution to the protection function remain unknown. The current study examined the neuroprotective effects of Res after middle cerebral artery occlusion (MCAO) in rats. MCAO for 90 min was induced in male Sprague-Dawley rats. Resveratrol (30 mg/kg) pretreatment was injected intraperitoneally 10 days prior to I/R induction. Neurological score, pathological changes, infarct ratio, brain edema and inflammation-related cytokines and mediators were estimated after operation. We found that Res prevented the impairment of neurological function and decreased the infarct volume and brain edema, compared with the sham group. The inflammation-related molecules tumor necrosis factor α (TNF-α) and interleukin (IL)-6 levels usually caused by (I/R) injury were significantly ameliorated by Res. Meanwhile, treatment with Res inhibited the production of nitric oxide (NO) and prostaglandin E2 (PGE$_2$). Finally, Res also inhibited the upregulation of nuclear factor-kappa B (NF-κB). In conclusion, the neuroprotection of resveratrol preconditioning may be due in part to the suppression of the inflammatory response via regulation of NF-κB signaling pathway, which provided a strong evidence for a promising therapeutic agent for cerebral I/R injury through attenuation of inflammation.

Keywords: Resveratrol, inflammation, nuclear factor-kappa

Introduction

Cerebral ischemia/reperfusion (I/R) injury is closely related to stroke, which is a serious disease with poor blood flow to the brain. Stroke is the second leading cause of long-term disability and death [1], which affects 15 million people every year worldwide [2]. Ischemic stroke accounts for approximately 80% of all strokes, and occurs when a major cerebral artery is blocked by a thrombus or embolism [3]. This blockage leads to brain injury through a complex series of pathophysiological events leading to neuronal cell death and subsequent neurological dysfunction [4]. As brain-blood reperfusion is the standard treatment for stroke with no other effective replacement, there is a compelling need to develop novel therapeutic options for IR injury.

The brain's inflammatory responses to reperfusion are characterized by a rapid activation of resident cells (mainly microglia), production of pro-inflammatory mediators and infiltration of circulating inflammatory cells in the ischemic brain region, as demonstrated in animal models [5-7] and in stroke patients [8, 9]. Recent studies have demonstrated that nuclear factor-kappa B (NF-κB), a well-known transcription factor that functions in immune and inflammatory responses, play pivotal roles in global cerebral I/R injury [10-12]. It has been reported that pretreatment of NF-κB inhibitor peptide IKK-NBD, ischemic preconditioning or some pharmacological preconditioning can all reduce the neuronal death in cerebral ischemia associated with decreased expression of inflammatory cytokine [13, 14]. These inflammatory responses are believed to play a critical role in estab-

lishing tolerance because inhibiting their action or production eliminates protection. Consequently, anti-inflammatory therapies are being explored for prevention and treatment of these diseases.

Resveratrol (trans-3,4,5-trihydroxystilbene, Res) is a natural compound produced by a restricted number of plants (about 31 genera), primarily from root extracts of the oriental plant, Polygonum cuspidatum and from red grapes. Res is believed to have multiple bioactivities including anti-cancer, prevention of cardiovascular diseases, antioxidant and antiinflammatory [15-17]. The neuroprotective role of resveratrol was recently reported against ischemia and other neurodegenerative diseases [18, 19]. However, the molecular mechanisms underlying the neuroprotective effects of Res are not fully understood. Therefore, the major objective of this study was to assess whether the neuroprotection of Res pretreatment is also related to attenuation of the inflammatory response via regulation of NF-κB induced by I/R injury.

Materials and methods

Animals

Adult male Sprague-Dawley (SD) rats, weighing 180 to 220 g, were obtained from Experimental Animal Center of Shandong Luye Pharmaceutical Co., Ltd (Yantai, China). The animals were kept in a controlled light room with a photoperiod of 12 h dark and 12 h light at a temperature of (22 ± 2)°C and 55 ± 5% relative humidity. All animal experiments were approved by the guidelines of Institute for Laboratory Animal Research of Cangzhou Central Hospital and were in strict accordance with US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 85-23, revised 1985).

Experimental design

Rats were randomly assigned into three experimental groups (n=10 for each group): sham operation (sham) group, middle cerebral artery occlusion (MCAO) group, Res-treated MCAO group (Res + MCAO). Resveratrol (30 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) which was dissolved in 2% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA), was intra-

peritoneally administered to rats of Res + MCAO groups for consecutive 10 days. Rats of the other three groups were injected solely with an equal concentration of DMSO. On the eleventh day, the last administration was performed 1 h before surgery. Transient focal cerebral ischemia was produced by the MCAO procedure as described previously [20]. Briefly, rats were anesthetized with chloral hydrate (400 mg/kg, i.p.). The right common carotid artery, external carotid artery, and internal carotid artery were isolated. A 4-0 monofilament nylon suture (Beijing Sunbio Biotech Co. Ltd., Beijing, China) with a rounded tip was introduced through the internal carotid artery until a slight resistance was felt, thereby to block the origin of the middle cerebral artery. After a 90-minute occlusion, the 4-0 filament was pulled out to permit reperfusion for 24 h. During the operation, the rectal temperature of rat was kept at 37°C by a heating pad. Rats in sham groups were subjected to the same procedure, but the middle cerebral artery was not occluded.

Neurobehavioral assessment

Neurological deficit was assessed on the basis of neurological scores obtained after 24 hours of reperfusion injury in all experimental groups [21]. The neurobehavioral deficit scores after ischemia were recorded on a 5-point: 0, No neurological deficit; 1, failure to extend opposite forepaw fully; 2, contralateral circling; 3, rat lost to grip the wire meshes and fell on the contralateral side of brain damage; 4, unable to walk spontaneously; 5, death.

Measurement of cerebral infarct volume

The animals were euthanized 24 h after reperfusion by chloral hydrate (350 mg/kg, i.p.) anesthesia overdose. The brains were rapidly dissected out and the forebrains were cut into six coronal sections (2 mm thick). The sections were stained by incubating them in a solution of 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) at 37°C for 30 min and then followed by overnight immersion in 4% paraformaldehyde. The cross-sectional areas with or without infarction in each brain slice were measured using Image J analysis software (version 1.6NIH).

Histological examination

As previously stated, the brain tissues were obtained and prepared for histological exami-
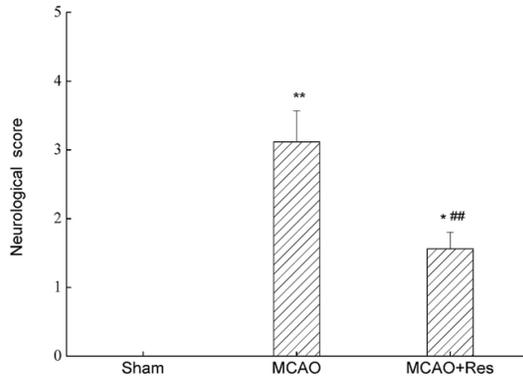
Figure 1. Effects of Res on neurological deficits. Data were expressed as mean ± SD; *$P<0.05$, **$P<0.01$ vs. sham group; #$P<0.05$, ##$P<0.01$ vs. MCAO group.

nation. Each brain was post-fixed in a 10% formalin solution. After that, brain tissue was embedded with paraffin and coronal sections were made into 5-μm thickness. To assess the histopathological change, the sections were further subjected to hematoxylin-eosin staining and then examined by light microscopy.

Detection of inflammation cytokines

Each brain tissue sample was weighed using an analytical balance, and 100 mg tissue of each sample was homogenized at 0.01 M PBS buffer (pH 7.2). After the homogenate was centrifuged at 12,000×g for 30 min at 4°C, the supernatant was collected and quantitatively assayed for tumor necrosis factor α (TNF-α) and interleukin (IL)-6, using ELISA kits (Jiancheng Biological Engineering Institute, Nanjing, China) according to the manufacturers' instructions.

Determination of NO and PGE_2

The amount of nitrite (a stable metabolite of NO) in the brain tissues was detected by the Griess Reagent System (Beyotime, China) according to the manufacturer's recommendation. The level of PGE_2 in the brain tissues was detected by commercially available ELISA kit (Wanlei, China) according to the manufacturer's recommendation.

Western blot analysis

At 24 h after reperfusion, all the rats were euthanized and the infarct side of the cortex was harvested. The tissues were immediately frozen in liquid nitrogen and stored at -80°C. Total

protein lysates were extracted from brain tissues in NP-40 lysis buffer (Beyotime) containing 1% Triton X-100 with 1 mM PMSF. Protein extracts were centrifuged at 12,000×g for 10 min. and the supernatants were collected. Nuclear and cytosolic proteins were extracted using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) following the manufacturer's instruction. Total protein content was determined using BCA protein assay kit (Beyotime). Equal amounts of protein (40 μg) were electrophoresed and subsequently transferred onto PVDF membranes (Millipore, Boston, MA, USA). The blotted membranes were blocked with 5% skim dry milk for 1 h at room temperature then incubated with the corresponding primary antibodies in a blocking buffer overnight at 4°C. Blots were probed with antibodies recognizing NF-κB (Cell Signaling Technology, Beverly, MA, USA). The bands on the membranes were scanned and analyzed with an image analyzer (Labworks Software, UVP Upland, CA, USA).

Statistical analysis

The data are expressed as mean ± standard deviation. SPSS statistical software (version 16.0; SPSS, Inc., Chicago, IL, USA) was employed for statistical analysis. Significant differences between the treatment and control groups were determined using one-way analysis of variance, followed by a Student-Newman-Keuls test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Res treatment attenuated neurological deficits

No neurological deficits were seen in the sham group. The neurological deficit score was significantly increased to 3.1 ± 0.5 in the MCAO group compared with that of the sham operated group at 24 h after reperfusion ($P<0.01$; **Figure 1**). When pretreated with resveratrol, the score was significantly decreased to 1.8 ± 0.2 compared with that of the MCAO group ($P<0.01$, **Figure 1**).

Res treatment reduced the cerebral infarction

TTC staining showed that pretreatment with Res reduced the infarct volumes. Compared with MCAO group, Res treatment markedly decreased the infarct volume from 43.29 ±
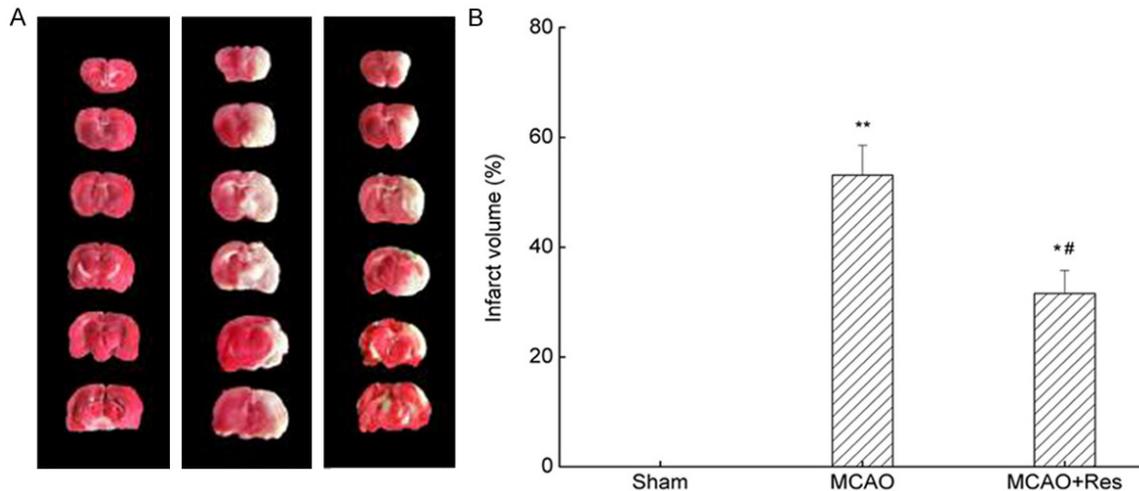
Figure 2. Effects of Res on infarction caused by MCAO in rats. A: Representative photographs of brain sections stained with 2% TTC of sham, MCAO and Res + MCAO groups. B: Measurement of infarct volume. *$P<0.05$ compared with sham group; #$P<0.05$ compared with MCAO group.
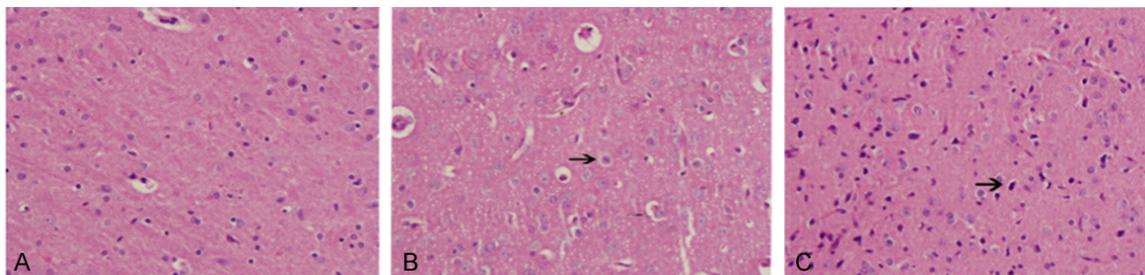
Figure 3. Histological examinations of cortex neurons by hematoxylin and eosin staining (×10 magnification). A: Sham group; B: MCAO group; C: MCAO + Res group. As indicated by the arrows: in MCAO group, appearance of shrunken cytoplasm and pyknotic nuclei and significant recovery in Res-treated group.

2.826% to 33.00 ± 2.760% ($P<0.05$, **Figure 2A, 2B**).

Res alleviated the pathological damage of cerebral tissue

To evaluate the effects of Res on cerebral IR injury, histological examination in each group was performed in HE stained sections. The sham group showed normal neurons with no pathological change (**Figure 3A**). In the MCAO group, neuronal loss was severe and dying neurons showed shrunken cytoplasm and pyknotic nuclei (**Figure 3B**). In the MCAO + Res group, the extent of damage was significantly ameliorated (**Figure 3C**).

Res treatment ameliorates inflammatory response

To analyze the inflammatory response in the mice, we measured the levels of TNF-α (**Figure 4A**) and IL-6 (**Figure 4B**) in cerebral tissue by ELISA. Compared with the sham group, TNF-α ($P<0.01$) and IL-6 ($P<0.01$) levels were found to be dramatically increased in the MCAO group, whereas Res pretreatment noticeably prevented TNF-α ($P<0.05$) and IL-6 ($P<0.05$) production.
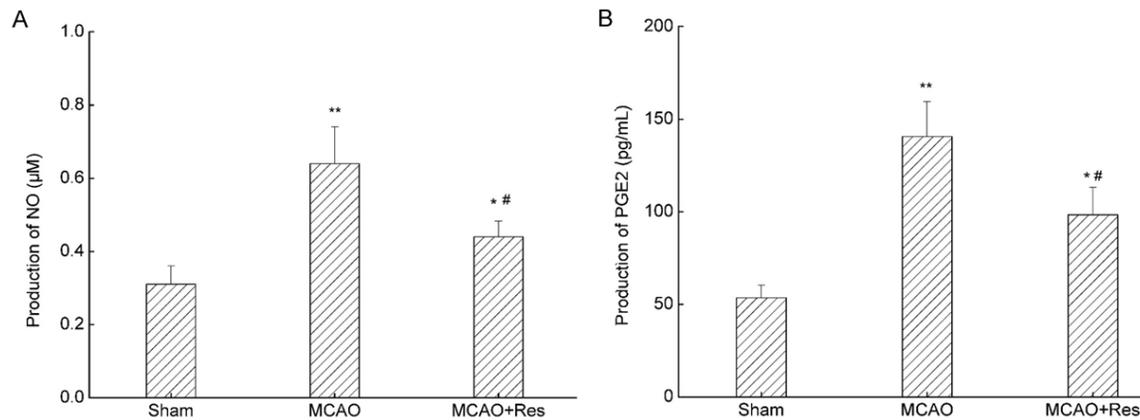
Res inhibited the productions of NO and PGE$_2$

To further justify the effect of Res on inflammatory reactions, the inflammatory mediators productions were detected. As shown in **Figure 5**, MCAO resulted in a significant increase in NO production in cerebral tissue compared with sham group ($P<0.01$), whereas Res significantly inhibited production of NO ($P<0.05$) (**Figure 5A**). Moreover, Res markedly inhibited I/R-induced production of PGE$_2$ in the same manner as it inhibited the production of NO in corresponding groups ($P<0.05$) (**Figure 5B**).
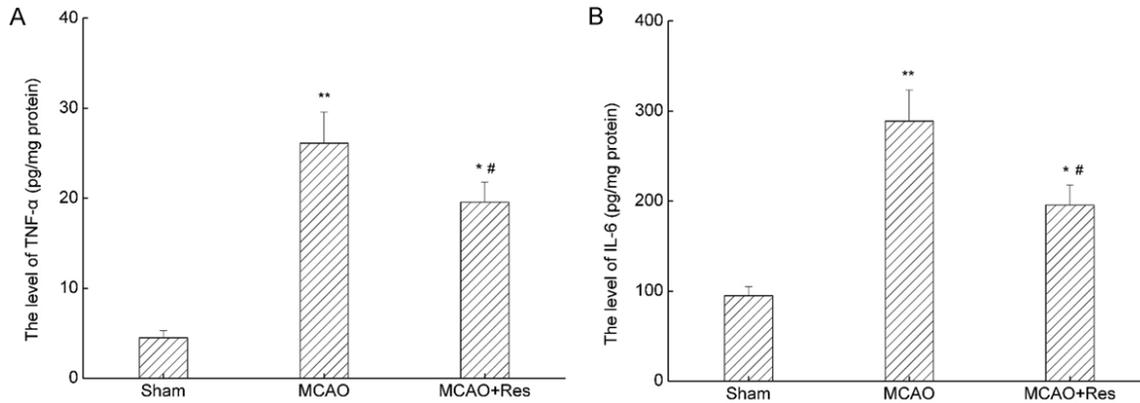
Figure 4. Effects of Res on the inflammatory cytokine. A: The level of TNF-α; B: The level of IL-6. Data were expressed as mean ± SD; *$P<0.05$, **$P<0.01$ vs. sham group; #$P<0.05$, ##$P<0.01$ vs. MCAO group.

Figure 5. Effects of Res on the productions of NO and PGE$_2$. A: Productions of NO; B: Productions of PGE$_2$. Data were expressed as mean ± SD; *$P<0.05$, **$P<0.01$ vs. sham group; #$P<0.05$, ##$P<0.01$ vs. MCAO group.

Res decreases the expression of NF-κB

To further evaluate the anti-inflammatory mechanism of Res, we compared the levels of NF-κB in cytoplasm and nucleus extracts from rats with and without resveratrol pretreatment. As shown in **Figure 6**, the level of NF-κB on nucleus was significantly increased and in brain tissue subjected to I/R and the level of NF-κB in cytoplasm was evidently decreased in the MCAO group. High level of NF-κB was observed in rats in cytoplasm and decreased on nucleus after Res pretreatment.

Discussion

The present study demonstrated that treatment with Res in rat model with cerebral I/R injury could alleviated the pathological damage of cerebral tissue, attenuated cerebral infarc-

tion and inhibited the release of inflammatory cytokines and mediators, showing profoundly impact on the severity of cerebral I/R injury. To shed light on the underlying mechanisms, we focused on the NF-κB pathway. Our results showed that Res significantly inhibited the activation of NF-κB in cerebral tissue from MCAO challenged. These results indicated that the anti-inflammatory effects of Res act at least in part through inhibition the activation of NF-κB signaling pathway.

In basic sciences, middle cerebral artery occlusion (MCAO) followed by reperfusion is a widely accepted animal model of focal cerebral ischemia; it resembles the scenario of human ischemic stroke and has been widely used to study ischemia mechanisms and potential interventions [22]. Cerebral damage was assessed by the examination of percentage of infarction in
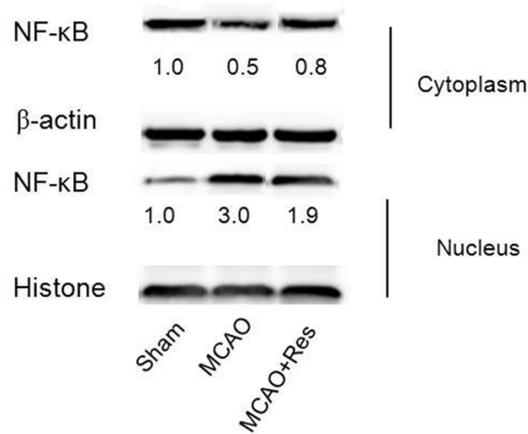
Figure 6. Representative the protein expression of NF-κB in cytoplasmic and nuclear following global cerebralI/R. β-actin and Histone were used as a loading control.

the injured brain, an important determinant in assessing the consequences of cerebral ischemia which leads to neurological impairment [23]. In the present study, we noticed a significant increase in percentage of infarction in I/R rats. In contrast, significant decreased infarction percent was noticed in resveratrol treated rats. Meanwhile, pretreatment with Res significantly reduced brain edema compared with that in the MCAO group. What's more, pathologic abnormalities were observed in the MCAO group. These changes were diminished in resveratrol-treated rats. These results suggest that resveratrol has neuroprotection by limiting the cerebral infarction and brain edema. Therefore, we assumed that resveratrol has neuroprotection effect.

Accumulating evidence suggested that post-ischemic inflammation contributes to the development of neuronal injury and cerebral infarction [24]. The release of inflammatory cytokines and the aggregation and infiltration of inflammatory cells are the key steps in inflammation. TNF-α, IL-1β, and IL-6 are the impotent cytokines which initiate inflammatory mediator and inflammatory reactions and induce expression of other cytokines after ischemia-reperfusion injury [25, 26]. The ischemic brain was observed with increased levels of TNF-α, IL-1β, and IL-6. They are considered as a part of tissue damaging response in ischemia and reperfusion injury [25, 26]. In the present study, we noticed that the IL-6 and TNF-α levels were significantly increased in rats with I/R injury. In contrast, the

levels of TNF-α and IL-6, were significantly decreased a significantly increased in resveratrol treated rats. Based on the above outcome, we demonstrated that resveratrol may be having neuroprotection action through anti-inflammatory effect.

NO is derived from the oxidation of L-arginine, which is catalyzed by nitric oxide synthase (NOS). After ischemia-reperfusion injury, the expression of inducible nitric oxide synthase (iNOS) induced by inflammatory mediators and cytokines is significantly increased in immunocytes, such as neutrophils and macrophages. In the present study, the production of NO was significantly increased in MCAO group, which was suppressed by the treatment of Res. PGE$_2$ is an important inflammatory mediator and plays an important role in the inflammatory response. Similarly, in our study, the production of PGE$_2$ was significantly increased in MCAO group and was suppressed by the treatment of Res. we further verified that Res exerts these anti-inflammatory effects on cerebral I/injury.

It has been shown that NF-κB mediates the transcription of a large number of genes, such as TNF-α, IL-1β, IL-6 and iNOS, the products of which are known to play important roles in the pathogenesis of cerebral ischemia [12, 27]. In the cytoplasm, NF-κB is bound to inhibitor kB (IkB) when inactive; however, when cells are stimulated, NF-κB translocates to the nucleus by separating from IkB, functioning as a transcription activity factor for the target gene. Therefore, we investigated whether resveratrol can protect the brain through inhibiting NF-κB activated after global ischemic insult. As expected, we have found that resveratrol blocked post-ischemic reduction of NF-κB in the cytoplasm and attenuated the increase of NF-κB level in the nucleus, which suggesting that resveratrol prevented translocation of NF-κB from the cytoplasm to the nucleus and thereby reduced NF-κB activation.

Taken together, our data indicate that Res may significantly ameliorate cerebral I/R injury through limiting the cerebral infarction and brain edema, inhibited the release of inflammatory cytokines and mediators, suggesting that Res has a protective effect on cerebral I/R injury. This neuroprotection may be due to its anti-inflammatory properties. We also provide evidence that the mechanism of action may be

through the inhibition of the NF-κB signaling pathway. These results provide evidence that resveratrol preconditioning induces its effect in the protection against ischemic brain injury with a more complex mechanism that also involves anti-inflammatory properties.

Acknowledgements

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yan Zhan, Department of Neurology, Yantai Affiliated Hospital of Binzhou Medical University, No. 717 Jinbu Street, Muping District, Yantai 264100, Shandong, China. Tel: 86-05354770721; E-mail: kuoli2016@126.com

References

[1] Di Carlo A. Human and economic burden of stroke. Age Ageing 2009; 38: 4-5.

[2] Vivien D, Gauberti M, Montagne A, Defer G and Touzé E. Impact of tissue plasminogen activator on the neurovascular unit: from clinical data to experimental evidence. J Cereb Blood Flow Metab 2011; 31: 2119-2134.

[3] Durukan A and Tatlisumak T. Acute ischemic stroke: overview of major experimental rodent models, pathophysiology, and therapy of focal cerebral ischemia. Pharmacol Biochem Behav 2007; 87: 179-197.

[4] Fang L, Gao H, Zhang W, Zhang W and Wang Y. Resveratrol alleviates nerve injury after cerebral ischemia and reperfusion in mice by inhibiting inflammation and apoptosis. Int J Clin Exp Med 2015; 8: 3219-3226.

[5] Alessandrini A, Namura S, Moskowitz MA and Bonventre JV. MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. Proc Natl Acad Sci U S America 1999; 96: 12866-12869.

[6] Kriz J. Inflammation in ischemic brain injury: timing is important. Crit Rev Neurobiol 2006; 18: 145-157.

[7] Schilling M, Besselmann M, Leonhard C, Mueller M, Ringelstein EB and Kiefer R. Microglial activation precedes and predominates over macrophage infiltration in transient focal cerebral ischemia: a study in green fluorescent protein transgenic bone marrow chimeric mice. Exp Neurol 2003; 183: 25-33.

[8] Gerhard A, Neumaier B, Elitok E, Glatting G, Ries V, Tomczak R, Ludolph AC and Reske SN.

[9] In vivo imaging of activated microglia using [11C]PK11195 and positron emission tomography in patients after ischemic stroke. Neuro Report 2000; 11: 2957-2960.

[9] Price CJ, Menon DK, Peters AM, Ballinger JR, Barber RW, Balan KK, Lynch A, Xuereb JH, Fryer T, Guadagno JV and Warburton EA. Cerebral neutrophil recruitment, histology, and outcome in acute ischemic. Stroke 2004; 35: 1659-1664.

[10] Desai A, Singh N and Raghubir R. Neuroprotective potential of the NF-κB inhibitor peptide IKK-NBD in cerebral ischemia-reperfusion injury. Neurochem Int 2010; 57: 876-883.

[11] Ji L, Nazarali AJ and Paterson PG. Protein-energy malnutrition increases activation of the transcription factor, nuclear factor kappaB, in the gerbil hippocampus following global ischemia. J Nutr Biochem 2008; 19: 770-777.

[12] Ridder DA and Schwaninger M. NF-κB signaling in cerebral ischemia. Neuroscience 2009; 158: 995-1006.

[13] Bahjat FR, Williams-Karnesky RL, Kohama SG, West GA, Doyle KP, Spector MD, Hobbs TR and Stenzel-Poore MP. Proof of concept: Pharmacological preconditioning with a Toll-like receptor agonist protects against cerebrovascular injury in a primate model of stroke. J Cereb Blood Flow Metab 2011; 31: 1229-1242.

[14] Kitagawa K. Ischemic tolerance in the brain: Endogenous adaptive machinery against ischemic stress. J Neurosci Res 2012; 90: 1043-1054.

[15] Athar M, Back JH, Tang X, Kim KH, Kopelovich L, Bickers DR and Kim AL. Resveratrol: a review of preclinical studies for human cancer prevention. Toxicol Appl Pharmacol 2007; 224: 274-283.

[16] Delmas D, Jannin B and Latruffe N. Resveratrol: preventing properties against vascular alterations and ageing. Mol Nutr Food Res 2005; 49: 377-395.

[17] Smoliga JM, Baur JA and Hausenblas HA. Resveratrol and health-a comprehensive review of human clinical trials. Mol Nutr Food Res 2011; 55: 1129-1141.

[18] Gao D, Zhang X, Jiang X, Peng Y, Huang W, Cheng G and Song L. Resveratrol reduces the elevated level of MMP-9 induced by cerebral ischemia-reperfusion in mice. Life Sci 2006; 78: 2564-2570.

[19] Huang SS, Tsai MC, Chih CL, Hung LM and Tsai SK. Resveratrol reduction of infarct size in Long-Evans rats subjected to focal cerebral ischemia. Life Sci 2001; 69: 1057-1065.

[20] Mulcahy NJ, Ross J, Rothwell NJ and Loddick SA. Delayed administration of interleukin-1 receptor antagonist protects against transient cerebral ischaemia in the rat. Br J Pharmacol 2003; 140: 471-476.

[21] Longa EZ, Weinstein PR, Carlson S and Cummins R. Reversible middle cerebral artery occlusion without craniectomy in rats. Stroke 1989; 20: 84-91.

[22] Simão F, Matté A, Pagnussat AS, Netto CA and Salbego CG. Resveratrol preconditioning modulates inflammatory response in the rat hippocampus following global cerebral ischemia. Neurochem Int 2012; 61: 659-665.

[23] Prabhakar O. Cerebroprotective effect of resveratrol through antioxidant and anti-inflammatory effects in diabetic rats. Naunyn Schmiedebergs Arch Pharmacol 2013; 386: 705-710.

[24] Ren J, Fan C, Chen N, Huang J and Yang Q. Resveratrol pretreatment attenuates cerebral ischemic injury by upregulating expression of transcription factor Nrf2 and HO-1 in rats. Neurochem Res 2011; 36: 2352.

[25] Yasuda Y, Shimoda T, Uno K, Tateishi N, Furuya S, Tsuchihashi Y, Kawai Y, Naruse S and Fujita S. Temporal and sequential changes of glial cells and cytokine expression during neuronal degeneration after transient global ischemia in rats. J Neuroinflammation 2011; 8: 70.

[26] Lakhan SE, Kirchgessner A and Hofer M. Inflammatory mechanisms in ischemic stroke: therapeutic approaches. J Transl Med 2009; 7: 97.

[27] Yenari MA and Han HS. Influence of hypothermia on post-ischemic inflammation: role of nuclear factor kappa B (NFκB). Neurochem Int 2006; 49: 164-169.