Biochemical and Biophysical Research Communications 444 (2014) 182–188

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Rapamycin attenuates mitochondrial dysfunction via activation of mitophagy in experimental ischemic stroke

Qiang Li [a,b,1], Ting Zhang [a,1], Jixian Wang [c,d], Zhijun Zhang [d], Yu Zhai [b], Guo-Yuan Yang [c,d,*], Xiaojiang Sun [a,*]

[a] Department of Neurology, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University, Shanghai 200233, China
[b] Department of Neurology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, China
[c] Department of Neurology, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China
[d] Med-X Research Institute and School of Biomedical Engineering, Shanghai Jiao Tong University, Shanghai 200030, China

ARTICLE INFO

Article history:
Received 28 December 2013
Available online 16 January 2014

Keywords:
Brain
Ischemia
Mitochondria function
Mitophagy
Rapamycin

ABSTRACT

Rapamycin has been demonstrated to exhibit neuroprotective functions via the activation of autophagy in a cerebral ischemia model. However, the involvement of mitophagy in this process and its contribution to the protection of mitochondrial function remains unknown. The present study explored the characteristics of mitophagy after cerebral ischemia and the effect of rapamycin on mitochondrial function. Male Sprague–Dawley rats underwent transient middle cerebral artery occlusion (tMCAO). Neurological deficits scores; infarct volumes; mitophagy morphology; and the levels of malondialdehyde (MDA), adenosine triphosphate (ATP) and mitochondrial membrane potentials ($\Delta\psi m$) were examined. The expression of LC3, Beclin-1 and p62 in the mitochondrial fraction combined with transmission electronic microscopy were used to explore mitophagic activity after ischemia. We also blocked autophagosome formation using 3-methyladenine (3-MA) to check the linkage between the mitochondrial protective effect of rapamycin and enhanced mitophagy. We observed that rapamycin significantly enhanced mitophagy, as evidenced by the increase in LC3-II and Beclin-1 expression in the mitochondria and p62 translocation to the mitochondria. Rapamycin reduced infarct volume, improved neurological outcomes and inhibited mitochondrial dysfunction compared with the control animals ($p < 0.05$). However, these protective effects were reversed by 3-methyladenine treatment after rapamycin. The present study indicates that rapamycin treatment attenuates mitochondrial dysfunction following cerebral ischemia, which is linked to enhanced mitophagy.

1. Introduction

Autophagy is an evolutionarily conserved process that is responsible for the degradation of some organelles and cytoplasm compositions via the lysosomal pathway [1]. As the power generator of the cell and a major source of reactive oxygen species (ROS), mitochondria are especially prone to ROS damage [2]. Mitochondria have been proposed to be the principal subcellular target of ischemic injury [3,4]. The important role of mitochondria provides a promising therapeutic target for pharmacological and biomedical research using experimentally ischemic strokes.

Ischemia reperfusion injury causes mitochondrial damage and dysfunction. The timely elimination of dysfunctional mitochondria is essential to protect cells from ischemia stress. Cells can develop a self-defense mechanism against aberrant mitochondria to maintain normal mitochondrial function; the term "mitophagy" has been suggested for this process [5,6]. Mitophagy can prevent damaged mitochondria accumulation and protect against cell death [7]. Several in vivo studies in experimental cardiac ischemic injury have reported that enhanced mitophagy promoted cell survival from ischemia stress [8,9]. However, the role of mitophagy in cerebral ischemic injury is unknown.

Targeting damaged mitochondria by activating mitophagy is a novel strategy for the treatment of ischemic strokes. Rapamycin, a macrolide antibiotic, can reduce injury in various models of

Abbreviations: ATP, adenosine triphosphate; DMSO, dimethyl sulfoxide; LC3, microtubule-associated protein A/B light chain 3; MDA, malondialdehyde; TEM, transmission electronic microscope; 3MA, 3-methyladenine; tMCAO, transient middle cerebral artery occlusion.

* Corresponding authors. Address: Med-X Research Institute, Shanghai Jiao Tong University, 1954 Hua Shan Road, Shanghai 200030, China. Fax: +86 21 62932302 (G.-Y. Yang). Address: Department of Neurology, Shanghai Sixth People's Hospital, Shanghai Jiao Tong University, 600 Yishan Road, Shanghai 200233, China (X. Sun).
E-mail addresses: gyyang0626@gmail.com (G.-Y. Yang), sunxj19@gmail.com (X. Sun).

[1] The authors contributed equally to this study.

0006-291X/$ - see front matter Crown Copyright © 2014 Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2014.01.032

neurodegenerative disorders by activating autophagy [10,11]. Rapamycin is also neuroprotective in the neonatal hypoxia–ischemia (HI) model, which is associated with increased autophagy and the inhibition of apoptosis [12]. Recently, one study has demonstrated the neuroprotective properties of rapamycin in rat focal cerebral ischemic preconditioning models [13]. However, it is not known how rapamycin regulates mitophagy and mitochondrial dysfunction in ischemic stroke. The present study was designed to investigate the effect of rapamycin on mitophagy and improvements in mitochondrial function after cerebral ischemia.

2. Material and methods

2.1. Experimental design

The experimental protocol was approved by the Institute of Animal Care and Use Community (IACUC), Shanghai Jiao Tong University, Shanghai, China. Adult male Sprague–Dawley rats (SLAC Inc., Shanghai, China) weighing 280–300 g were divided into eight groups. In the tMCAO experiment, four groups of rats ($n = 20$ per group) were subjected to 2 h of MCAO followed by 6, 24, 48 or 72 h of reperfusion; another group ($n = 20$) received the surgical procedure without tMCAO as a control. In the rapamycin treatment experiment, one group ($n = 16$) received rapamycin (8 ng, 2 μl in 0.1% DMSO), injected intraventricularly 30 min before MCAO. Another group ($n = 16$) received the same amount of 0.1% DMSO as a vehicle group before MCAO. The final group ($n = 16$) received rapamycin and 3-MA (100 μg, 2 μl in saline) before MCAO, 3-MA was injected intraventricularly 10 min after rapamycin treatment. All of the animals were sacrificed under deep anesthesia and their brains were removed at the designed point after reperfusion.

2.2. Transient middle cerebral artery occlusion (tMCAO)

Rats were anesthetized with ketamine/xylazine (100 mg/10 mg/kg) intraperitoneally. Body temperature was maintained at 37 ± 0.5 °C using a heating pad (RWD Life Science, Shenzhen, China). Animal surgery was performed as previously described [14]. Briefly, a 4–0 suture (Covidien, Mansfield, MA) with a round tip and silicon coating was inserted from the left external carotid artery (ECA) into the internal carotid artery (ICA). The suture reached the circle of Willis to occlude the origin of the middle cerebral artery (MCA). The success of occlusion was determined by monitoring the decrease in surface cerebral blood flow (CBF) to 20% of baseline CBF using a laser Doppler flow meter (Moor Instruments, Devon, UK). Reperfusion was performed via withdraw of the suture after 2 h of tMCAO.

2.3. Immunohistochemistry staining

Immunohistochemistry staining was performed as described previously [15]. Twenty-five micrometer sections from rat brain tissues were used for immunofluorescence analysis. Sections were blocked with 10% goat serum for 1 h at room temperature, followed by incubation with primary antibodies overnight at 4 °C and incubation for 60 min at 25 °C with secondary antibodies conjugated with FITC (Molecular Probes, Eugene, OR) or Cy-5 (Santa Cruz, Santa Cruz, CA). The compound 4′-6-diamidino-2-phenylindole (DAPI, Molecular Probes) was used to label cell nuclei. Primary antibodies were used in IHC as follows: LC3 (1:200 dilution, Sigma–Aldrich, St. Louis, MO), glial fibrillary acidic protein (GFAP, 1:500 dilution, Sigma–Aldrich) and NeuN (1:500 dilution, Millipore, Billerica, MA). Negative controls were performed without the primary antibody.

2.4. Mitochondria isolation

Purified mitochondria were obtained using an animal tissue active mitochondrial extraction kit according to manufacturer instructions (Genmed Scientifics Inc., Shanghai, China). Briefly, brain cortical tissue was homogenized with a glass homogenizer (25–30 strokes) and centrifuged at 1500*g* for 10 min at 4 °C. The supernatant was removed and centrifuged at 10,000*g* for 10 min. The pelleted materials were washed three times and resuspended in 10 mM Tris–HCl, pH 7.4, containing 10 mM KCl, 0.25 M sucrose and 5 mM $MgCl_2$. The protein concentration was measured using a Pierce BCA kit (Pierce, Rockford, IL). The isolated mitochondrial fraction was collected for western blot, MDA assay, ATP assay and JC-1 ($\Delta\psi m$) assay.

2.5. Western blot analysis

Equal amounts of protein per lane (30 μg) were subjected to electrophoresis on 10–12% SDS–PAGE gel. Proteins were electrotransferred onto a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% non-fat dry milk/0.1% Tween-20 in Tris-buffered saline for 2 h at room temperature. Thereafter, the membrane was incubated with different primary antibodies, including rabbit anti-Beclin-1 (1:500 dilution, Sigma–Aldrich), rabbit anti-LC3 (1:1000 dilution, Sigma–Aldrich), rabbit anti-p62 (1:800 dilution, Sigma–Aldrich) and mouse anti-cox4 (1:500 dilution, Santa Cruz). Subsequently, the membrane was treated with horseradish peroxidase-labeled secondary antibody for 2 h at room temperature. Immunoblots were probed using enhanced ECL substrate (Pierce). The chemiluminescence level was recorded using an imaging system (Bio-Rad, Hercules, CA). The results were normalized to loading control β-actin.

2.6. Transmission electronic microscope

Twenty-four hours after tMCAO, rats were sacrificed and perfused transcardially with 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 mol phosphate buffer. Coronal sections (100 μm) were cut by a vibratome, postfixed with 4% glutaraldehyde in 0.1 mmol cacodylate buffer (pH 7.4) for 1 h and incubated with 1% osmium tetroxide in 0.1 mmol cacodylate buffer for 2 h. Brain sections were dehydrated by an ascending series of ethanol and dry acetone and embedded in Durcupan ACM Fluka (Sigma–Aldrich). Ultrathin sections (0.1 μm) were stained with uranyl acetate and lead citrate and subsequently examined with a JEOL JEM-1230 transmission electron microscope (JEOL, Tokyo, Japan).

2.7. Mitochondria functional assay

2.7.1. MDA level measurement

The MDA level was examined using commercial kits (Jiancheng Bioengineering Inc., Nanjing, China) according to the manufacturer's directions with minor modifications. Briefly, the supernatant of mitochondria lysis was incubated with the MDA reagent for 40 min at 95 °C. The mixture was detected by a Synergy HT multi-mode microplate reader (Biotek, Winooski, VT, USA) at a 532 nm absorbance. Data were expressed as nmol per mg protein.

2.7.2. ATP level measurement

The ATP level was examined by a firefly luciferase-based ATP assay kit (Beyotime, Haimen, China). The assay was conducted according to the manufacturer's instructions. Briefly, after mitochondrial lysis, mitochondrial fraction was centrifuged at 10,000*g* for 10 min, and the supernatant was removed for the ATP assay. ATP reagents (100 μl) were added into a microwell for 5 min at 37 °C. The samples (50 μl) were then added, and the contents were

mixed for 1–10 s and measured by a Synergy HT multi-mode microplate reader. The ATP concentrations were calculated from standard curve data and expressed as nmol per mg protein.

2.7.3. Mitochondrial membrane potential ($\Delta\psi m$) assessment

Mitochondrial membrane potential was determined by a fluorescent probe JC-1 according to the manufacturer's directions (Genmed Scientifics Inc.). Briefly, JC-1 reagent B was completely thawed and warmed to room temperature. Reagent B was diluted by JC-1 reagent C, mixing well to make sure there were no particles or flakes in the solution. The final JC-1 staining working solution was 5 μg/ml. Isolated mitochondrial (100 μg) were incubated with 10 μl JC-1 staining working solution for 10 min at 37 °C. Fluorescence was determined using a Synergy HT multi-mode microplate reader at 560 nm excitation/590 nm emission. Data were expressed as fluorescence units per μg protein.

2.8. Infarct volume measurements

Twenty-four hours after tMCAO, animals were killed, and their brains were removed. The brains were then sliced into six coronal sections with 2-mm thickness each. The sections were stained with 2% TTC (Sigma–Aldrich) at 37 °C for 20 min. The slices were subsequently stored in 10% phosphate-buffered formalin. Infarct volume was calculated as described previously [16].

2.9. Neurological deficit evaluation

To evaluate the impairment of neuronal function after stroke, a neurological deficit grading system with a scale of zero to five was performed on each animal as described previously [17]: grade 0: normal behavior; grade 1: rats exhibited contralateral forelimb consistently flexed during the suspension from the tail; grade 2: rats moved spontaneously in all directions but exhibited a monodirectional circling toward the paretic side; grade 3: rats exhibited a consistent spontaneous contralateral circling; grade 4: rats were very weak and walked only when stimulated; and grade 5: rats died by the day of assessment. Higher scores indicated more severe impairment of neuronal function.

2.10. Statistical analysis

Parametric data in different groups were compared using a one-way ANOVA followed by Bonferroni *t*-test (GraphPad Software, San Diego, CA). Significant differences between two groups were determined by two-tailed unpaired Student's *t*-test. The survival rates were compared using the Chi-Square test. Data presented as the mean ± SD. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Transient ischemia induced autophagy and mitophagy

To explore the involvement of autophagosomes, we used electron microscopy, western blots and immunohistochemistry staining. LC3 is distributed in the cytoplasm under normal conditions (LC3-I), but when autophagy is induced, LC3-I is modified to become LC3-II, which integrates into the autophagosome membrane. LC3-II is a relatively sensitive biochemical marker of autophagy [18]. In this study, numerous cells exhibited strong LC3-positive staining in the peri-ischemic cortex at 24 h after tMCAO (Fig. 1A-b, c). Double-staining experiments indicated that LC3 is distributed selectively in neurons, as shown by numerous colocalizations with NeuN (Fig. 1A-f) in contrast to the absence of colocalization with GFAP (Fig. 1A-i).

Mitophagy is one of the most important patterns of autophagy and plays a critical role during ischemia. To determine the characteristics of neuronal mitophagy after cerebral ischemia, we examined autophagosomes and autolysosome structures in neurons, which are different stages of the autophagy process [18]. At 24 h, electron microscopy revealed that neurons in the border of the lesion contained numerous autolysosomes filled with membranous whorls (Fig. 1B-b, white arrows). Furthermore, we clearly observed some partially degraded mitochondria (Fig. 1B-b, black arrows) and some mitophagy structures that abnormal mitochondria were fused with autophagic vesicles (Fig. 1B-g) or surrounded by double membranes of typical autophagosomes (Fig. 1B-h). We further examined LC3-II formation in mitochondrial fractions to determine mitophagy level in the ischemic cortex from 6 to 72 h post reperfusion. As shown in Fig. 1C, ischemia reperfusion induced a time-dependent accumulation of LC3-II in mitochondria, beginning at 6 h and increasing markedly 24 h after ischemia. The expression of LC3-II could not be detected in the cytoplasmic fraction after tMCAO, and the expression of LC3-I in the cytoplasmic fraction increased only 24 h after tMCAO. These results indicate the presence of active mitophagy after cerebral ischemia.

3.2. Rapamycin enhanced mitophagy

To investigate the effect of rapamycin on stimulating focal mitophagy, we measured LC3-II expression in the mitochondria isolated from the cortices of rapamycin-treated ischemic rats. As shown in Fig. 2A,B, treatment with rapamycin significantly increased LC3-II and Beclin-1 expression in the mitochondria. This result suggests that rapamycin can enhance cell mitophagy. 3-methyladenine (3MA), an autophagy inhibitor, reversed the rapamycin-mediated induction of LC3-II and Beclin-1 in mitochondria. We next assessed the mechanism by which rapamycin enhances mitophagy. p62, which can bind ubiquitinated mitochondria to direct phagophore through a LC3 binding domain, is a sensitive marker for mitophagy [19]. We detected the expression of p62 in mitochondrial fractions and cytosolic fractions using western blot assays. We observed that the expression of p62 increased in the mitochondrial fractions, in contrast to the decrease in the cytosolic fractions (Fig. 2C) after rapamycin treatment. As shown in Fig. 2D, these results indicated that rapamycin enhanced p62 translocation from the cytosol to the mitochondria, thereby demonstrating that rapamycin significantly enhances mitophagy.

3.3. Rapamycin improved mitochondrial function

Protecting mitochondrial function is critical in attenuating ischemic brain injury. To further assess the effects of rapamycin on mitochondrial function, indicators of mitochondrial activity such as membrane potential ($\Delta\psi$) and levels of mitochondrial ATP and MDA were determined. After 24 h of ischemic reperfusion, the $\Delta\psi$ decreased to 42.1% compared with the control group, but rapamycin significantly recovered mitochondrial $\Delta\psi$ levels (Fig. 3A). The MDA levels in rats undergoing ischemic injury were 206% greater compared with the control group; rapamycin treatment significantly reversed the increase of MDA levels (Fig. 3B). Mitochondrial ATP levels are an indicator of cell death. The ATP level decreased significantly after cerebral ischemia, and rapamycin treatment significantly prevented the decrease in ATP levels compared with the control group (Fig. 3C). However, treatment combined with 3MA attenuated the protective effect of rapamycin on mitochondria function. This indicates that the protective role of rapamycin on mitochondrial function is related to the activation of mitophagy.

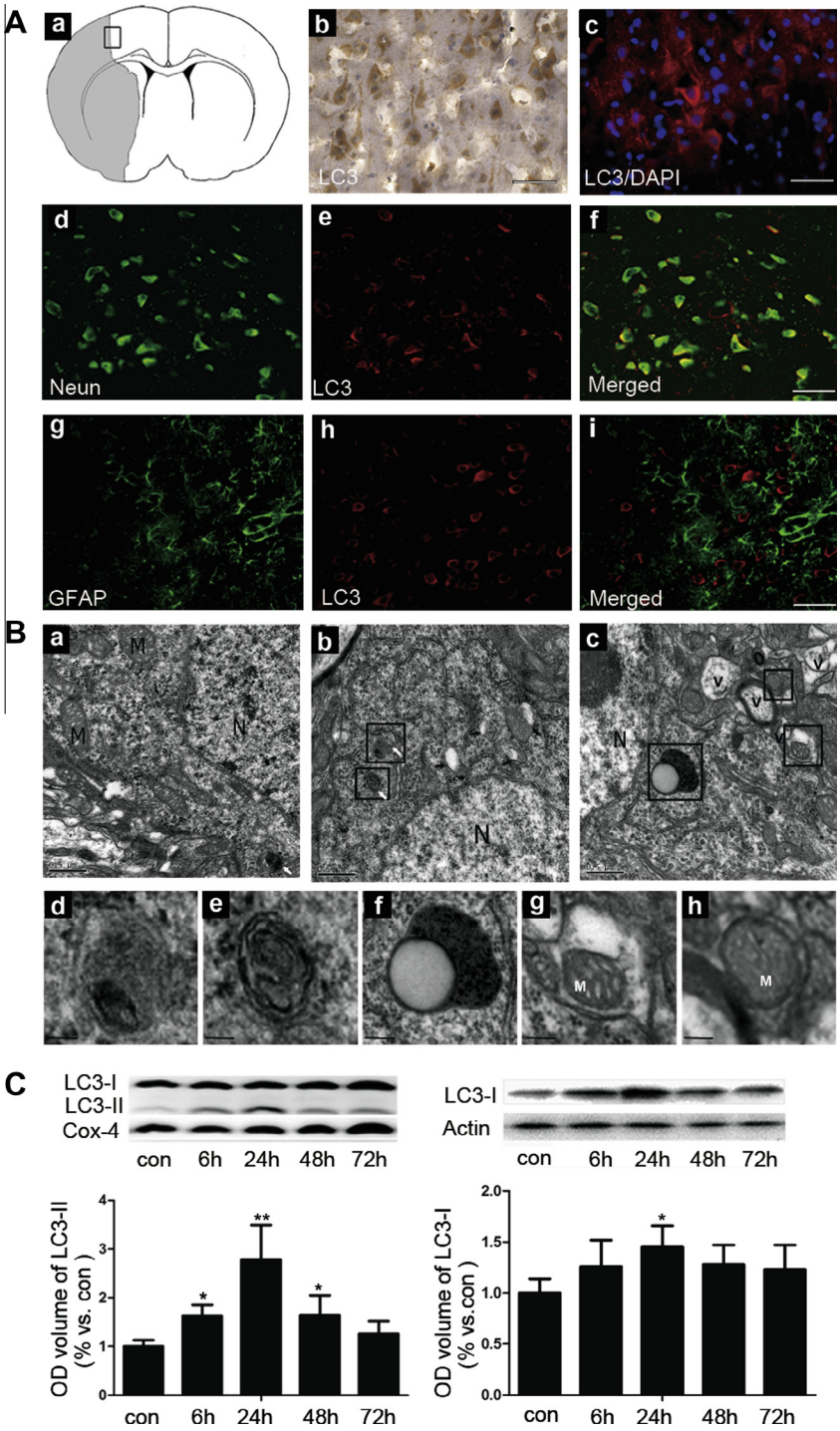

Fig. 1. Ischemia induced neuron autophagy and mitophagy. (A) LC3-positive cells were detected in the peri-infarct area at 24 h after ischemia. LC3 (red) immunostaining localized in NeuN (green)-positive cells but not in GFAP-positive cells. Scale bar = 40 μm. (B) (a) Electron micrographs demonstrating normal mitochondrial structure in control rats. (b) The characteristics of autophagy structure (white arrow) and partially degraded mitochondria (black arrow) were observed in neurons 24 h after tMCAO. The rectangle is high magnification and demonstrates a degradative autophagic vacuole (Avd) that includes a partially degraded mitochondrion (d). Another Avd was identified by two bilayers separated by a narrow electron-lucent cleft (e). (c) Autophagic vacuoles, autolysosomes (AL) and typical mitophagy structure were observed in neurons 24 h after tMCAO. The rectangle is high magnification. High magnifications revealed that ALs (f) and typical mitophagy structure (g, h). M-mitochondrial, N-nuclear, V-vacuole, scale bar = 0.5 μm (a–c), 0.1 μm (d–h). (C) Representative western blots demonstrate expression of LC3 in mitochondria fraction and cytosol fraction following 6, 24, 48, and 72 h of tMCAO. Bar graphs demonstrate quantitative expression of LC3-II in mitochondria fraction and LC3-I in cytosol fraction. Data are presented as the mean ± SD. $N = 6$ per group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Rapamycin attenuated brain injury after tMCAO

The effect of rapamycin on ischemic brain injury was determined using neurological scores, infarct volume and survival rates. Rapamycin significantly reduced infarct volumes compared with the control group (37.98 ± 4.87% vs. 49.84 ± 8.36%, $p < 0.05$). Ischemia-induced neurological deficits were also greatly attenuated in the rapamycin-treated animals (1.89 ± 0.36 vs. 2.67 ± 0.52, $p < 0.05$). In addition, the survival rate was higher in the rapamycin-treated group compared with the control group (91.6% vs.

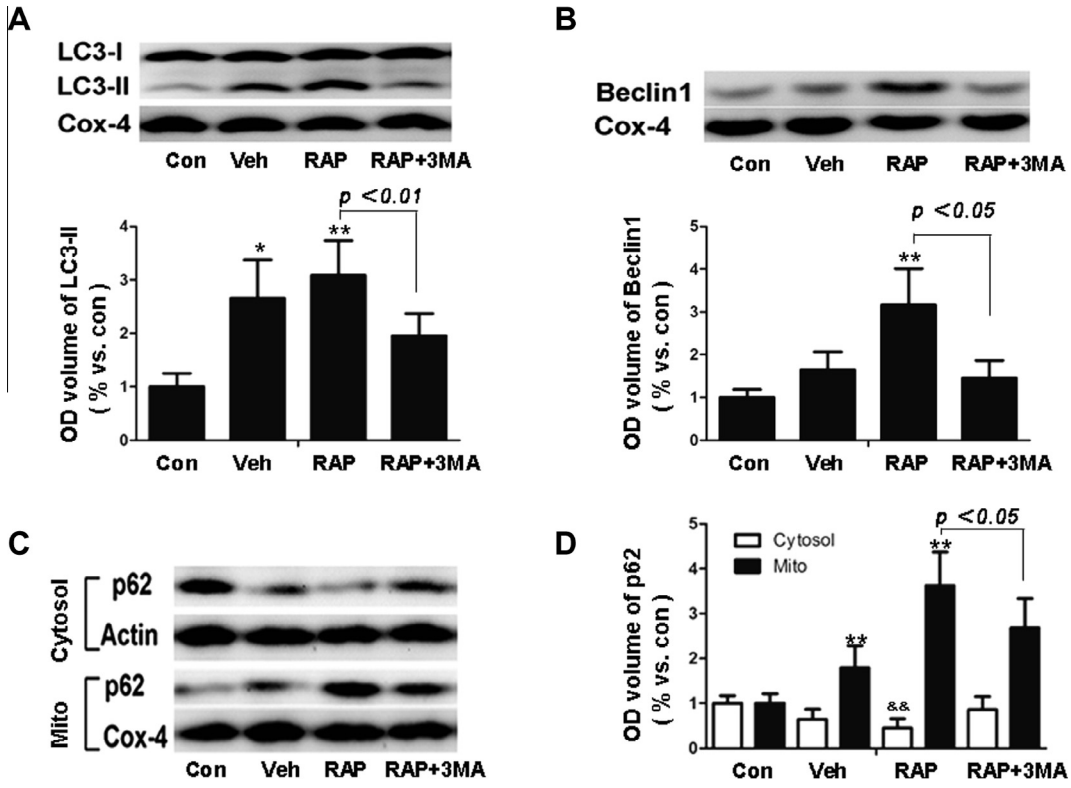

Fig. 2. Rapamycin enhanced mitophagy. (A) Representative western blots and quantitative graphs demonstrate the expression of LC3-I and LC3-II in the mitochondrial fractions of control (Con), vehicle-treated (Veh), rapamycin-treated (RAP) or rapamycin plus 3MA-treated ischemic animals (RAP plus 3MA). (B) Representative western blots and quantitative graphs demonstrate the expression of Beclin-1 in the mitochondrial fractions of control (Con), vehicle-treated (Veh), rapamycin-treated (RAP) or rapamycin plus 3MA-treated ischemic animals (RAP plus 3MA). (C, D) Representative western blots and quantification of p62 expression in cytosolic and mitochondrial fractions of control (Con), vehicle-treated (Veh), rapamycin-treated (RAP) or rapamycin plus 3MA-treated ischemic animals (RAP plus 3MA). $N = 6$ per group. Data are presented as the mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group (mitochondrial fraction); $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs. control group (cytosolic fraction).

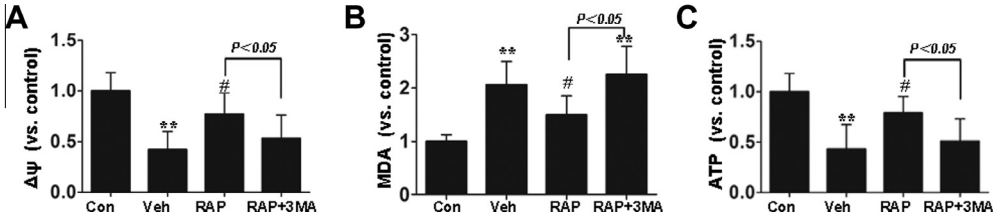

Fig. 3. Rapamycin improved mitochondrial function. Bar graphs demonstrate Δψ (A), MDA (B) and ATP levels (C) in the control (Con), vehicle-treated (Veh), rapamycin-treated (RAP) or rapamycin plus 3MA-treated ischemic animals (RAP plus 3MA) 24 h after tMCAO. $N = 6$ per group. Data are mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. vehicle group.

83.3%, $p > 0.05$). The 3MA blocked the protective effect of rapamycin on infarct volume and neurological deficit (Fig. 4). These results further demonstrate that rapamycin attenuated ischemic brain injury through enhanced mitophagy.

4. Discussion

The present study demonstrates the following: (1) Rapamycin enhances mitophagy by increasing p62 translocation to the mitochondria after ischemia. (2) Rapamycin attenuates brain ischemic damage and improves mitochondrial function. (3) The protection of mitochondrial function induced by rapamycin is linked to enhanced mitophagy. Enhanced mitophagy by rapamycin plays an important protective role in ischemic brain injury.

The crucial role of Parkin-mediated mitophagy in cardioprotection was also recently investigated in a cardio-ischemic preconditioning study [8]. This is the first study in which the involvement of mitophagy in cerebral ischemia reperfusion has been investigated. In the present study, mitophagy was examined during brain ischemia. Mitophagy was characterized by abnormal mitochondria within autophagosomes or fused with autophagic vesciles in ischemic perifocal areas under TEM. The morphological features suggested that the activation of mitophagy occurs in the ischemic brain. Western blotting revealed that LC3-II expression was increased in the mitochondria fraction. This is evidence that brain ischemia induces autophagy and mitophagy.

The next question we addressed was whether rapamycin enhanced mitophagy after cerebral ischemia. Rapamycin is known to stimulate autophagy in cancer, aging, mental disorders, neurodegenerative disorders and cerebral ischemia [20,21]. Increasing data suggests that appropriate autophagy and autophagy preactivation by rapamycin can protect cells from ischemic stress

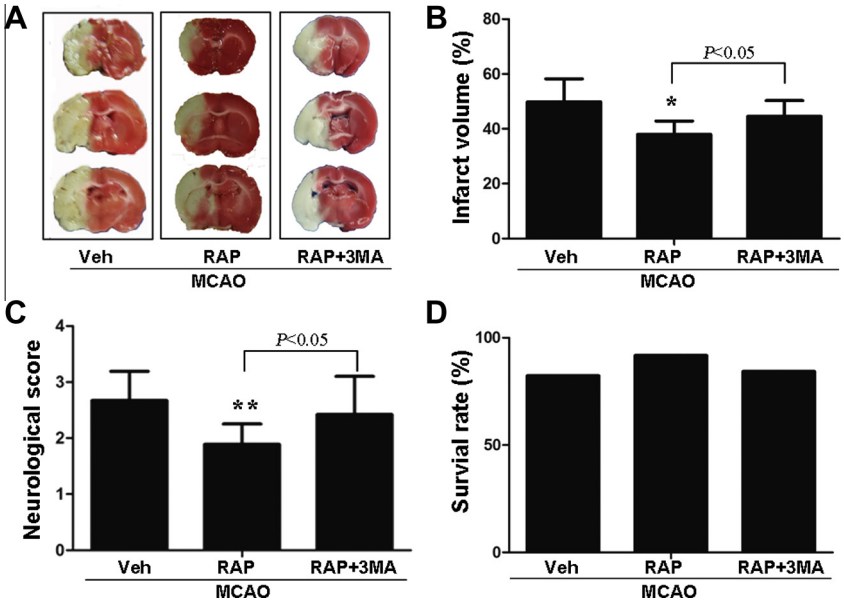

Fig. 4. Rapamycin attenuated brain injury after tMCAO. (A, B) TTC-stained sections demonstrated decreased infarct volume in rapamycin-treated rats 24 h after tMCAO (A). The white color indicates the infarct area. The bar graph represents quantitative infarct volume from the TTC staining (B). $N = 8$ per group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. vehicle group. (C, D) Bar graphs indicate neurological function deficit scores (C) and survival rates (D) in the rapamycin-treated group at 24 h after tMCAO. $N = 18$ per group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control group, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. vehicle group.

[22–24]. However, the ability of rapamycin to reduce brain injury by promoting mitophagy was unknown. Our data established that rapamycin also up-regulates mitochondrial LC3-II and Beclin-1, suggesting increased mitophagy in ischemic brain tissue. However, the mechanism by which rapamycin enhances mitophagy requires further investigation. The identification of the autophagy adaptor protein p62 has provided important insights into the process of mitophagy [19,25,26]. p62 can bind ubiquitinated mitochondria through its ubiquitin-binding domain and can recruit phagophores through an LC3-binding domain to degrade the damaged mitochondria. We demonstrated that rapamycin upregulated p62 translocation to the mitochondria in response to ischemia injury. This is the first study to clarify the effect of rapamycin on enhanced mitophagy by upregulating p62 translocation to the mitochondria in response to ischemic stress in the brain.

The removal of damaged mitochondria is beneficial in the setting of ischemia–reperfusion injury because damaged mitochondria could result in the robust increase in ROS after I/R. Although we did not measure ROS production in this study, many studies previously reported that ischemia–reperfusion injury results in ROS overproduction, cellular component damage (damaged lipids, proteins and nucleic acids) and triggers mitochondrial dysfunction [2,27]. Clearance of damaged and aging mitochondria is a critical process for neuron survival. Focal mitophagy can eliminate damaged mitochondria and reduce ROS-mediated neuronal death ROS [7–9]. The present results demonstrate that ischemia-induced oxidative stress injuries are attenuated in rapamycin-treated rats. Rapamycin also recovers mitochondrial $\Delta\psi$ and ATP content after tMCAO. Thus, rapamycin-induced mitochondrial protection is related to the mitophagy activation.

The evidence presented in this study indicates that rapamycin triggers mitophagy and mediates mitochondrial protection during ischemic stress. One can conclude that rapamycin enhances mitophagy by facilitating the recruitment of p62 to the damaged mitochondria. The autophagic removal of injured mitochondria attenuates mitochondrial dysfunction. The close relationship between mitophagy and mitochondrial protection merits further study.

Disclosure of potential conflicts of interest

None.

Acknowledgments

The study is supported by the Chinese Ministry of Science and Technology, 973 Program, 2011CB504405 (G.Y.Y.), the National Natural Science Foundation of China, 81070939 (G.Y.Y.), the Science and Technology Commission of Shanghai Municipality 10JC1408100 (G.Y.Y.), The National Natural Science Foundation 31100783 (T.Z.) and the National Natural Science Foundation 31171014 (X.J.S.). The authors thank Ms. Xiaoyan Chen for editorial assistance.

References

[1] N. Mizushima, B. Levine, A.M. Cuervo, D.J. Klionsky, Autophagy fights disease through cellular self-digestion, Nature 451 (2008) 1069–1075.
[2] K. Niizuma, H. Yoshioka, H. Chen, G.S. Kim, J.E. Jung, M. Katsu, N. Okami, P.H. Chan, Mitochondrial and apoptotic neuronal death signaling pathways in cerebral ischemia, Biochim. Biophys. Acta 1802 (2010) 92–99.
[3] M.A. Moskowitz, E.H. Lo, C. Iadecola, The science of stroke: mechanisms in search of treatments, Neuron 67 (2010) 181–198.
[4] M. Christophe, S. Nicolas, Mitochondria: a target for neuroprotective interventions in cerebral ischemia-reperfusion, Curr. Pharm. Des. 12 (2006) 739–757.
[5] G. Ashrafi, T.L. Schwarz, The pathways of mitophagy for quality control and clearance of mitochondria, Cell Death Differ. 20 (2013) 31–42.
[6] R.J. Youle, D.P. Narendra, Mechanisms of mitophagy, Nat. Rev. Mol. Cell Biol. 12 (2011) 9–14.
[7] D.A. Kubli, A.B. Gustafsson, Mitochondria and mitophagy: the yin and yang of cell death control, Circ. Res. 111 (2012) 1208–1221.
[8] C. Huang, A.M. Andres, E.P. Ratliff, G. Hernandez, P. Lee, R.A. Gottlieb, Preconditioning involves selective mitophagy mediated by Parkin and p62/SQSTM1, PLoS ONE 6 (2011) e20975.
[9] A. Hoshino, S. Matoba, E. Iwai-Kanai, H. Nakamura, M. Kimata, M. Nakaoka, M. Katamura, Y. Okawa, M. Ariyoshi, Y. Mita, K. Ikeda, T. Ueyama, M. Okigaki, H. Matsubara, P53-TIGAR axis attenuates mitophagy to exacerbate cardiac damage after ischemia, J. Mol. Cell. Cardiol. 52 (2012) 175–184.
[10] S. Erlich, A. Alexandrovich, E. Shohami, R. Pinkas-Kramarski, Rapamycin is a neuroprotective treatment for traumatic brain injury, Neurobiol. Dis. 26 (2007) 86–93.

[11] C. Malagelada, Z.H. Jin, V. Jackson-Lewis, S. Przedborski, L.A. Greene, Rapamycin protects against neuron death in in vitro and in vivo models of Parkinson's disease, J. Neurosci. 30 (2010) 1166–1175.
[12] S. Carloni, G. Buonocore, W. Balduini, Protective role of autophagy in neonatal hypoxia-ischemia induced brain injury, Neurobiol. Dis. 32 (2008) 329–339.
[13] R. Sheng, L.S. Zhang, R. Han, X.Q. Liu, B. Gao, Z.H. Qin, Autophagy activation is associated with neuroprotection in a rat model of focal cerebral ischemic preconditioning, Autophagy 6 (2010) 482–494.
[14] F. Yuan, Y. Tang, X. Lin, Y. Xi, Y. Guan, T. Xiao, J. Chen, Z. Zhang, G.Y. Yang, Y. Wang, Optimizing suture middle cerebral artery occlusion model in C57BL/6 mice circumvents posterior communicating artery dysplasia, J. Neurotrauma 29 (2012) 1499–1505.
[15] Y. Fan, F. Shen, T. Frenzel, W. Zhu, J. Ye, J. Liu, Y. Chen, H. Su, W.L. Young, G.Y. Yang, Endothelial progenitor cell transplantation improves long-term stroke outcome in mice, Ann. Neurol. 67 (2010) 488–497.
[16] G.Y. Yang, Y.J. Zhao, B.L. Davidson, A.L. Betz, Overexpression of interleukin-1 receptor antagonist in the mouse brain reduces ischemic brain injury, Brain Res. 751 (1997) 181–188.
[17] K.P. Loh, S.H. Huang, B.K. Tan, Y.Z. Zhu, Cerebral protection of purified Herba Leonuri extract on middle cerebral artery occluded rats, J. Ethnopharmacol. 125 (2009) 337–343.
[18] D.J. Klionsky, F.C. Abdalla, H. Abeliovich, R.T. Abraham, A. Acevedo-Arozena, K. Adeli, et al., Guidelines for the use and interpretation of assays for monitoring autophagy, Autophagy 8 (2012) 445–544.
[19] T.H. Pankiv, T. Clausen, A. Lamark, J.A. Brech, H. Bruun, A. Outzen, G. Overvatn, T. Bjorkoy, Johansen, p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy, J. Biol. Chem. 282 (2007) 24131–24145.
[20] M. Laplante, D.M. Sabatini, MTOR signaling in growth control and disease, Cell 149 (2012) 274–293.
[21] K.J. Hughes, B.K. Kennedy, Cell biology. Rapamycin paradox resolved, Science 335 (2012) 1578–1579.
[22] T. Ma, C.A. Hoeffer, E. Capetillo-Zarate, F. Yu, H. Wong, M.T. Lin, D. Tampellini, E. Klann, R.D. Blitzer, G.K. Gouras, Dysregulation of the mTOR pathway mediates impairment of synaptic plasticity in a mouse model of Alzheimer's disease, PLoS ONE 5 (2010).
[23] L. Yin, S. Ye, Z. Chen, Y. Zeng, Rapamycin preconditioning attenuates transient focal cerebral ischemia/reperfusion injury in mice, Int. J. Neurosci. 122 (2012) 748–756.
[24] B. Gabryel, A. Kost, D. Kasprowska, Neuronal autophagy in cerebral ischemia–a potential target for neuroprotective strategies?, Pharmacol Rep. 64 (2012) 1–15.
[25] W.X. Ding, H.M. Ni, M. Li, Y. Liao, X. Chen, D.B. Stolz, G.W. Dorn 2nd, X.M. Yin, Nix is critical to two distinct phases of mitophagy, reactive oxygen species-mediated autophagy induction and Parkin-ubiquitin-p62-mediated mitochondrial priming, J. Biol. Chem. 285 (2010) 27879–27890.
[26] M.L. Seibenhener, J.R. Babu, T. Geetha, H.C. Wong, N.R. Krishna, M.W. Wooten, Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation, Mol. Cell. Biol. 24 (2004) 8055–8068.
[27] T.H. Sanderson, C.A. Reynolds, R. Kumar, K. Przyklenk, M. Huttemann, Molecular mechanisms of ischemia-reperfusion injury in brain: pivotal role of the mitochondrial membrane potential in reactive oxygen species generation, Mol. Neurobiol. 47 (2013) 9–23.