PROF. YAN QU (Orcid ID : 0000-0001-5434-7390)

Received Date : 15-Oct-2016
Revised Date : 09-Dec-2016
Accepted Date : 16-Dec-2016
Article type : Original Manuscript

Pre-ischemia melatonin treatment alleviated acute neuronal injury after ischemic stroke by inhibiting ER stress-dependent autophagy via PERK and IRE1 signalings

Dayun Feng[1#] | Bao Wang[1,2#] | Lei Wang[3,4#] | Neeta Abraham[2] | Kai Tao[1] | Lu Huang[1] | Wei Shi[5] | Yushu Dong[3*] | Yan Qu[1*]

[1]Department of Neurosurgery, Tangdu Hospital, Fourth Military Medical University, Xi'an, PR China

[2]Department of Neurology, Beth Isreal Deaconess Medical Center, Harvard medical school, Boston, MA, USA

[3]Department of Neurosurgery, The General Hospital of Shenyang Military Region, Shenyang, PR China

[4]Department of Neurosurgery, The 463rd Hospital of PLA, Shenyang, PR China

[5]Department of Urology surgery, Xijing Hospital, Fourth Military Medical University, Xi'an, PR China

# These authors contributed equally to this manuscript.

***CORRESPONDING AUTHORS:** Yan Qu, Department of Neurosurgery, Tangdu Hospital, Fourth Military Medical University, Xi'an, PR China; Yushu Dong, Department of

Neurosurgery, The General Hospital of Shenyang Military Region, Shenyang, PR China. Emails: yanqu0123@fmmu.edu.cn (YQ); dongyushu463@163.com (YD).

ABBREVIATED TITLE: Melatonin pretreatment alleviated cerebral IR via inhibiting ER stress-dependent autophagy

bstract

Melatonin has demonstrated a potential protective effect in central nervous system. Thus, it is interesting to determine whether pre-ischemia melatonin administration could protect against cerebral ischemia/reperfusion (IR) related injury and the underlying molecular mechanisms. In this study, we revealed that IR injury significantly activated endoplasmic reticulum (ER) stress and autophagy in a middle cerebral artery occlusion (MCAO) mouse model. Pre-ischemia melatonin treatment was able to attenuate IR-induced ER stress and autophagy. In addition, with tandem RFP-GFP-LC3 adeno-associated virus, we demonstrated pre-ischemic melatonin significantly alleviated IR-induced autophagic flux. Furthermore, we showed that IR-induced neuronal apoptosis through ER stress related signalings. Moreover, IR-induced autophagy was significantly blocked by ER stress inhibitor (4-PBA), as well as ER related signaling inhibitors (PERK inhibitor, GSK; IRE1 inhibitor, DBSA). Finally, we revealed that melatonin significantly alleviated cerebral infarction, brain edema, neuronal apoptosis and neurological deficiency, which were remarkably abolished by tunicamycin (ER stress activator) and rapamycin (autophagy activator), respectively. In summary, our study provides strong evidence that pre-ischemia melatonin administration significantly protects against cerebral IR injury through inhibiting ER stress-dependent autophagy. Our findings shed light on the novel preventive and therapeutic strategy of daily administration of melatonin, especially among the population with high risk of cerebral ischemic stroke.

KEY WORDS: Melatonin; ischemic stroke; ER stress; autophagy; PERK; IRE1

1 | INTRODUCTION

Ischemic stroke is a common disease of central nervous system and a frequent cause of death and disability all over the world.[1] Rapid vessel recanalization is often chosen as a therapeutic strategy whenever possible.[2,3] However, restoring cerebral blood supply can paradoxically result in reperfusion injury.[4] The mechanisms of ischemia/reperfusion (IR) injury are still far from clear and an effective prevention for cerebral IR injury has not been established yet.

Melatonin (N-acetyl-5-methoxytryptamine) has demonstrated diverse pharmacological capabilities, including anti-inflammation, anti-oxidative stress, anti-apoptotic, and analgesic properties.[5,6] Melatonin exerts beneficial effects in multiple central nervous conditions, including subarachnoid hemorrhage, Parkinson's disease (PD), Alzheimer's disease (AD), cognitive impairment and anxiety disorders.[7-11] Moreover, recent researches revealed that melatonin can protect against cerebral ischemic injury. Our report shows that melatonin modulates the SIRT1 pathway to protect mitochondrial function and prevent cell apoptosis after ischemia.[12] Melatonin also protects neuronal cells of newborn rats against hypoxia-ischemia induced toxicity.[13] In addition, melatonin alters the expression time course of Nox family to exert protection in a cerebral IR rat model.[14] These studies applied melatonin after or at same time as ischemia began. Besides, the patients in acute cerebral ischemic phrase usually could not get timely treatment. Thus, it is of high importance to evaluate the benefits of pre-ischemia melatonin administration and the underlying molecular mechanisms.

Recent studies have revealed that endoplasmic reticulum (ER) stress is related to the neuronal injury after cerebral IR. ER plays vital roles in protein translocation, modification and foldings. Accumulation of dysfolded proteins in ER can perturbate ER function and this process is defined as ER stress. Under physiological condition, ER stress is activated to promote protein folding with ER chaperones, reduce the load of newly-synthesized protein by shutting down protein translocation and accelerate the degradation of dysfolded proteins. By these processes, ER stress rebalances intercellular homeostasis and protects cells from various stimuli. However, overly-activated ER stress and unfolded protein response (UPR) can cause damage. Increased UPR and ER stress are observed in hippocampal CA1 and CA3 neurons in a global brain ischemia and reperfusion rat model.[15] Besides, inhibiting ER stress with compounds can significantly protect neurons against ischemic injury.[16-18] Even though evidence indicates that melatonin is involved in maintaining ER homeostasis and one study shows melatonin can reduce ER stress in hypoxia-ischemia.[13,19] However, whether ER stress is involved in the protective effects of melatonin against focal cerebral I/R injury is still unknown.

Autophagy, as a self-degradation process, is responsible for the removal of proteins and organelles in a lysosome-dependent manner. Study indicates that autophagy can be compensatorily activated to remove the accumulated dysfunctional proteins resulting from ER stress, hinting that ER stress might be a trigger for autophagy induction.[20] In melatonin-mediated protection against brain IR injury, evidence about the role of autophagy is still limited and contradictory. One study indicates that melatonin protects neuronal cells against cerebral IR injury through activating autophagy.[21] While another study demonstrates that autophagy inhibition contributes to melatonin-mediated protection against brain IR

injury.[22] Moreover, the crosstalk between ER stress and autophagy in the melatonin-mediated protection agains cerebral IR injury is still unknown. Consequently, it is necessary to confirm the role of autophagy in the protective effects of melatonin against cerebral I/R injury and explore the relationship between ER stress and autophagy in the context of cerebral ischemia.

In this study, we investigated whether pre-ischemia melatonin treatment could protect against cerebral IR injury. Further, we verified that melatonin exerted its protective effects through inhibiting ER stress and autophagy. Finally, we explored the relationship between ER stress and autophagy in the context of IR injury.

2 | MATERIAL AND METHODS

2.1 | Ethics statement

This study was designed according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996) and approved by the Animal Ethics Committee of the Tangdu Hospital, Fourth Military Medical University (Xi'an, China). All male Sprague-Dawley mice (8-10 weeks old) were bought from the laboratory animal center of the Fourth Military Medical University (Xi'an, China) and kept in a pathogen-free environment with free access to water and food. Every effort was made to minimize the suffering of all the animals.

2.2 | Transient middle cerebral artery occlusion (MCAO)

MCAO mouse model was established with an intraluminal filament method as previously described.[23] Briefly, after the mouse was anesthetized with ketamine (10 mg/mL)/xylazine (5 mg/mL), a midline neck incision was made to allow the isolation of the right common carotid artery (CCA), internal carotid artery (ICA) and external carotid artery (ECA). A monofilament nylon suture with a round tip was inserted into the right ICA (the depth is

about 8-10 mm from the bifurcation of CCA) via right CCA to block the blood supply of the right middle cerebral artery. Laser Doppler flowmetry (PeriFlux 5000; Perimed AB, Stockholm, Sweden) was used to monitor the right middle cerebral artery blood flow. Over 25 % decrease in blood flow of right middle cerebral artery from baseline was considered as successful ischemia. After 2 h of ischemia, the filament was withdrawn to initiate reperfusion. The sham operation groups were objected to the same operation without inserting a monofilament. Throughout the procedure, rectal temperature was monitored and maintained between 37 and 37.8 °C with a feedback temperature maintaining system.

2.3 | Drug administration

Melatonin (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMSO and then diluted with 0.9 % saline to a final concentration of < 2 % DMSO. Seven days before ischemia, melatonin was intraperitoneally (*i.p.*) injected into the mouse at a dose of 10 mg/kg, daily, for consecutive 7 days. The control group received the same amount of vehicle for the same duration. Following the last administration of melatonin/vehicle, the mice underwent ischemia/reperfusion or sham operation.

ER stress inhibitor 4-phenyl butyric acid (4-PBA) (200 μM, 2 μl), ER stress activator tunicamycin (TM) (25 μM, 2 μl), autophagy inhibitor 3-Methyladenine (3-MA) (25 μM, 2 μl), autophagy activator rapamycin (RAPA) (10 μM, 2 μl), PERK inhibitor GSK2656157 (10 μM, 2 μl) and IRE1 inhibitor 3,5-dibromosalicylaldehyde (DBSA) (50 μM, 2 μl) were injection into the right cerebral ventricle (from the bregma: AP, −0.3 mm; ML 1.0 mm; DV 2.5 mm) with a Hamilton microsyringe immediately after the onset of ischemia. The injection rate is 0.5 μl/min and the cannula remained in place for 3 min after injection. 4-PBA, TM, 3-MA, RAPA and DSBA were bought from Sigma-Aldrich (St. Louis, MO, USA). GSK2656157 was purchased from EMD Millipore (Darmstadt, Germany).

2.4 | Neurological score and motor assessment scale

Neurological deficiency was evaluated one day after MCAO according to a 21-point Garcia test score system (including 7 individual tests: spontaneous activity (I), axial sensation (II), vibrissae proprioception (III), symmetry of limb movement (IV), lateral turning (V), forelimb outstretching (VI) and climbing (VII); each test received a score between 0 (worst) and 3 (best) and the total score was out of 21 points (maximum)) and a 10-point score system of forelimb motor test (expressed as the number of successful paw placements out of 10 consecutive vibrissae-elicited excitation) as reported previously.[24-26] The trained investigators were blinded to the animal groups in the test.

2.5 | TTC staining and quantification of infarct volume

At 24 h post reperfusion, the mice were anesthetized with ketamine (10 mg/mL)/xylazine (5 mg/mL) and then underwent quick decapitation. Immediately after being carefully removed, the brain was weighed. Then, the brain was sectioned coronally (thickness: 2 mm). After staining with 2 % 2, 3, 5-triphenyl tetrazolium chloride (TTC) for 0.5 h at room temperature in the dark, the brain slices were fixed with 10 % formalin at room temperature overnight. The viable part of brain slice was red, while the dead part was pale white. The infarct area and the whole area of each brain slice were measured with Image J (Version 1.49). The infarct volume was calculated by multiplying the added infarct areas of each slices by slice thickness (2 mm) and results were expressed as the ratio of (infarct volume / the whole brain volume) × 100 %.

2.6 | Assessment of brain water content

The weight of brain (wet weight) was assessed immediately after being removed. Then, after TTC staining, all the brain slices were dried at 110 ^{o}C for 48 h and weighed (dry weight). The brain water content was quantified as: (wet weight - dry weight) / wet weight × 100 %.

2.7 | Western blot

The peri-infarct brain tissues were homogenized and lysed with RIPA buffer (89901, Thermo Scientific™, Chelmsford, MA, USA) supplemented with protease and phosphatase inhibitor cocktails (ab201119, abcam, Cambridge, MA, USA). Proteins (20 μg) were separated by sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to PVDF membrane (Millipore, Shanghai, China). After blocking with 5% non-fat milk, PVDF membrane was incubated with primary antibody in cold room overnight. After washing with tris buffered saline-tween (TBST) buffer three times, PVDF membrane was incubated with peroxidase-conjugated secondary antibody. Then, after washing with TBST three times, the protein was visualized with ECL detection solution (Millipore, Shanghai, China). The primary antibodies were listed as follows: anti-ATF6 (1:2000, Abcam), anti-p-PERK (Thr980) (1:1000, Cell Signaling Technology), anti-PERK (1:1000, Cell Signaling Technology), anti-p-IRE1 (Ser724) (1:1000, Abcam), anti-IRE1 (1:2000, Abcam), anti-LC3-I/II (1:1000, Cell Signaling Technology), anti-beclin-1 (1:1000, Cell Signaling Technology), anti-ATF4 (1:2000, Cell Signaling Technology), anti-CHOP (1:2000, Cell Signaling Technology), anti-Atg5 (1:1000, Cell Signaling Technology), anti-Atg12 (1:1000, Cell Signaling Technology), anti-Bcl-2 (1:1000, Cell Signaling Technology), anti-Bax (1:1000, Cell Signaling Technology), anti-Bim (1:1000, Cell Signaling Technology), anti-p-JNK (1:2000, Cell Signaling Technology), anti-JNK (1:2000, Cell Signaling Technology), anti-cleaved caspase-3 (1:1000, Cell Signaling Technology),

anti-cleaved caspase-9 (1:1000, Cell Signaling Technology) and anti-β-actin (1:5000, Abcam). Intensity of protein bands were quantified with Image J and normalized to that of β-actin.

2.8 | Double immuno-fluorescence staining

After fixing with 4 % formaldehyde solution, the brain sections were penetrated with 0.1 % Triton X-100 for 0.5 h and then blocked with 5 % goat serum for 0.5 h. Afterward, brain sections were incubated with the primary antibodies (anti-p-PERK and anti-p-IRE1) at 4 °C overnight. Then they were incubated with a secondary antibody at room temperature for 2 h after three washes with PBS. Samples were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (Dapi) for 5 min and photographed with a laser confocal microscope (Nikon, A1, Tokyo, Japan).

2.9 | TUNEL assay

TUNEL assay was used to detect neuronal apoptosis. Briefly, brain sections were fixed with 4 % paraformaldehyde solution for 0.5 h at room temperature. Then, in order to block endogenous peroxidase activity, the sections were incubated with a methanol solution containing 0.2 % H_2O_2 for 0.5 h. Afterward, treated with TUNEL reaction mixture (Millipore, Shanghai, China), the brain sections were maintained in an 37 °C incubator for 60 min. The fluorescence was captured with a laser confocal microscopy (Nikon, A1, Tokyo, Japan). The results were presented as apoptosis index which was quantified as the ratio of (TUNEL-positive cells)/ (total cells) × 100%.

2.10 | Autophagic flux measurement

Tandem RFP-GFP-LC3 adeno-associated virus was bought from Hanbio (Shanghai, China) and stereotactically injected into ipsilateral cortex of mouse (2 μl) before 24 h before ischemia. Briefly, the mouse was fixed with 4 % paraformaldenhyde at 24 h after reperfusion and the brain was carefully removed. Serial coronal sections (30 μm) were collected and photographed with a laser confocal microscope (Nikon, A1, Tokyo, Japan). The relative intensity of fluorescence was analyzed with ImageJ software (Version 1.49). GFP degrades in acidic environment while RFP does not. Thus, yellow spots (formed out of the overlap between red and green) indicate autophagosomes, while, red spots indicate autophagic lysosomes. If autophagy is activated, the red signal will dominate over yellow. If autophagy is suppressed, there will be more yellow signal than red signal.

2.11 | Statistical analysis

Data were presented as means ± standard error of the mean (SEM) from at least three independent experiments. Comparison between and within multiple groups was performed using one-way analysis of variance followed by Student-Newman-Keuls test and the statistical difference was analyzed by SPSS (version 19.0, Michigan Avenue, Chicago, USA). A value of $P<0.05$ was considered to be statistically significant.

3 | RESULTS

To determine whether levels of ER stress and autophagy were altered in response to ischemia/reperfusion (IR) injury, a transient middle cerebral artery occlusion (MCAO) mouse model was established. ATF6, phosphorlated-PERK (p-PERK) and phosphorylated-IRE1 (p-IRE1) are three main markers for ER stress activation. As compared with sham group, ATF6 as well as ratio of p-PERK/PERK and p-IRE1/IRE1 were significantly increased in IR

groups (P<0.05 at IR-6h; P<0.01 at IR-12h and 24h) (Fig. 1A). Autophagy markers (ratio of LC3II/I and Beclin-1) were also evaluated. Western blot showed that ratio of LC-3II/I and Beclin-1 were markedly augmented in IR groups relative to sham group (P<0.01 at IR-6h, 12h and 24h) (Fig. 1B). Based on the above data, we concluded that IR injury activated ER stress and autophagy. In addition, we chose IR-24h model for the subsequent experiments.

Furthermore, the effect of pre-ischemia melatonin treatment (10 mg/kg, daily, for consecutive 7 days) on IR-induced ER stress and autophagy was evaluated. Mice were randomly divided into three groups: (i) sham surgery group; (ii) ischemia/reperfusion (IR) group; (iii) IR with pre-ischemia melatonin treatment (IR + Mel) group. As shown in Fig. 2A, western blot revealed that IR precipitated significant ER stress as evidenced by the increase in levels of ATF6, p-PERK and p-IRE1 (P<0.01 versus with sham group). However, pre-ischemia melatonin administration dramatically attenuated IR-induced ER stress (P<0.05). In addition, results from immunofluorescence staining supported these findings. When compared with sham groups, IR injury enhanced the fluorescence intensity of p-PERK and p-IRE1 (P<0.01). However, pre-ischemia melatonin treatment significantly weakened the fluorescence intensity of p-PERK and p-IRE1 (P<0.05 *versus* with IR groups) (Fig. 2B). Together, the above data showed pre-ischemia melatonin treatment significantly attenuated IR-induced ER stress.

To determine the effect of pre-ischemia melatonin treatment on autophagic flux, we stereotactically injected tandem RFP-GFP-LC3 adenovirus into the cortex of mouse (RFP-GFP-LC3-mouse). All these RFP-GFP-LC3-mice were randomly classified into three groups: (i) sham surgery group; (ii) ischemia/reperfusion (IR) group; (iii) IR with pre-ischemia melatonin treatment (IR + Mel) group. As shown in Fig. 3A, the principle of

this assay depends on the PH difference. During autophagic process, when an autophagosome (pH: neutral) fuses with lysosome to form autophagolysosome (pH: acidic), the green fluorescent protein (GFP) will be degraded while the red fluorescent protein (RFP) remains. Consequently, in the merged image, yellow punctates denote the autophagosomes while red punctates refer to autophagolysosomes. Compared with sham groups, the percentage of red punctate was significantly increased while the percentage of yellow punctate was decreased in IR groups (Fig. 3B), which denoted that ischemia/reperfusion led to increase in autophagolysosomes and decrease in autophagosomes (P<0.01 *versus* sham groups) (Fig. 3C). However, pre-ischemia melatonin treatment obviously reversed the augment in autophagolysosomes as well as the reduction in autophagosomes (P<0.01 *versus* IR groups) (Fig. 3B,C). In addition, western blot showed that, pre-ischemia melatonin treatment significantly reduced IR-induced increase in ratio of LC-3II/I and Beclin-1 (P<0.01 *versus* IR groups) (Fig. 3D,E). Together, the above data suggested that pre-ischemia melatonin treatment significantly alleviated IR-induced autophagy.

Studies report that prolonged activation of ER stress promotes cell death via transcriptional responses mediated by ATF4 and CHOP.[27] Therefore, we evaluated levels of ATF4 and CHOP. IR injury significantly enhanced expression of ATF4 and CHOP (P<0.01 *versus* sham groups), however, such increase was weakened by pre-ischemia melatonin treatment (P<0.05 *versus* IR groups) (Fig. 4A). In addition, evidence show that ER stress can induce the conversion of LC3 (II to I) through activation of Atg12-Atg5 complex. Thus, we further tested levels of Atg12-Atg5 complex. Compared with sham groups, IR injury augmented levels of Atg5 and Atg12 (P<0.01 *versus* sham groups). This increase was significantly inhibited by pre-ischemia melatonin treatment (P<0.05 *versus* IR groups) (Fig. 4B). We also evaluated the status of the JNK pathway. IR injury increased phosphorylated-JNK (P<0.01

versus sham groups), which was reversed by pre-ischemia melatonin treatment (P<0.05 *versus* IR groups) (Fig. 4C). Furthermore, a major consequence of sustained ER stress is activation of ATF4 and JNK signaling, thereby inhibiting prosurvival B cell lymphoma 2 (Bcl-2) as well as inducing Bcl-2-associated X protein (Bax) and Bcl-2-interacting mediator of cell death (Bim). As expected, IR injury remarkably inhibited Bcl-2 and activated Bax/Bim (P<0.01 *versus* sham groups). However, such effects were markedly abolished by pre-ischemia melatonin treatment (P<0.05 *versus* IR groups) (Fig. 4D). Together, the above data suggested that IR injury activated apoptosis through multiple ER related signaling pathways.

Reports indicate that ER stress can trigger autophagy through PERK pathway or IRE1 pathway.[28,29] In order to determine the relationship between ER stress and autophagy in context of IR injury, we applied specific ER stress inhibitor 4-PBA and specific autophagy inhibitor 3-MA. Results showed that, compared with IR groups, 4-PBA not only inhibited ER stress as evidenced by the decrease in p-PERK (P<0.01 *versus* IR groups) and p-IRE1 (P<0.01 *versus* IR groups), but also suppressed autophagy activity as indicated by the decline in ratio of LC3-II/I (P<0.01 *versus* IR groups) and beclin-1 (P<0.01 *versus* IR groups) (Fig. 5A,B). However, 3-MA can only restrain autophagy activity without significant effects on ER stress (p-PERK: P>0.05 *versus* IR groups; p-IRE1: P>0.05 *versus* IR groups) (Fig. 5A,B). The above data indicated that ER stress was the upstream event of autophagy during IR injury. Furthermore, in order to elucidate the roles of PERK and IRE1 pathway in ER stress-induced activation of autophagy during IR injury, we used the specific PERK inhibitor GSK2656157 and specific IRE1 inhibitor 3,5-dibromosalicylaldehyde (DBSA). Results showed that, compared with the IR groups, inhibiting either PERK or IRE1 could significantly alleviate ER stress-induced autophagy activity, as indicated by the decrease in

ratio of LC3-II/I (P<0.05 *versus* IR groups) and beclin-1 (P<0.05 *versus* IR groups) (Fig. 5A,B). Together, the above data demonstrated that ER stress triggered autophagy through both PERK and IRE1 pathway during IR injury.

Further, in order to evaluate whether pre-ischemia melatonin treatment protects against IR injury through inhibiting ER stress and autophagy, the specific ER stress activator tunicamycin (TM) and specific autophgy activator rapamycin (RAPA) were used. TUNEL assay showed that pre-ischemia melatonin treatment remarkably decreased IR-induced cell death (P<0.05 *versus* IR groups). However, both tunicamycin and rapamycin significantly attenuated the protective effects of melatonin against IR injury (P<0.05 *versus* IR+Mel groups) (Fig. 6A). Consistently, melatonin significantly mitigated IR-induced increase in cleaved caspase-3 and 9 (P<0.05 *versus* IR groups). Both tunicamycin and rapamycin abolished such effects of melatonin (P<0.05 *versus* IR+Mel groups) (Fig. 6B,C).

Finally, the effects of pre-ischemia melatonin treatment on cerebral infarction, brain edema, neurological function were assessed. IR injury significantly increased infarct volume (58.2 ± 4.93 mm^3) (Fig. 7A,B), brain water content (84.3 ± 2.3 %) (Fig. 7C) and decreased neurological score (13.8 ± 1.2) (Fig. 7D) and motor assessment scale (3.2 ± 0.78) (Fig. 7E) (P<0.01 versus sham groups). Pre-ischemia melatonin treatment significantly reduced infarct volume (37.2 ± 5.15 mm^3), brain water content (77.1 ± 1.2 %) and increased neurological score (18.8 ± 0.81) and motor assessment scale (6.89 ± 1.13) (P<0.01 *versus* IR groups). However, both tunicamycin and rapamycin remarkably attenuated the effects of melatonin on infarct volume, brain edema, neurological score and motor assessment scale (P<0.05 *versus* IR+Mel groups). The above data demonstrated that pre-ischemia melatonin treatment protects against IR injury through inhibiting ER stress and autophagy.

4 | DISCUSSION

Ischemic stroke is a common disorder of central nervous system without effective therapeutic strategy. Ischemic stroke causes about 4.4 million deaths per year worldwide and the lifetime costs per patient are assessed at between 59 and 230 thousand dollars, which put enormous emotional and economic burdens on patients, family and state health services.[22] Previous studies showed that post-ischemia melatonin treatment immediately after IR exerted a potential protective effect in cerebral ischemic stroke.[24,30,31] Actually, most patients in acute cerebral ischemia phase usually could not get timely treatment clinically. Thus, it is necessary to evaluate the benefits of pre-ischemia melatonin administration and the underlying molecular mechanisms. Thus far, only one paper from Kilic E et al reported that prophylactically administration of melatonin (4 mg/kg/day, orally, 9 weeks) reduced the infarct size.[32] However, their study indicates that, compared with acute post-ischemia melatonin treatment, chronic prophylactic melatonin administration shows less protective effects, especially in neurological deficit score assessment. Compared with their report, our study used a different regimen for melatonin pre-ischemia treatment (10 mg/kg/day, intraperitoneally, 7 days). Our study showed that prophylactic melatonin administration could significantly alleviated ischemic injury. More importantly, compared with acute post-ischemic melatonin treatment, prophylactic melatonin administration (10 mg/kg/day, intraperitoneally, 7 days) can significantly augment the survival rate during 2 weeks post-ischemia (supplemental figure 1). The difference might be mainly due to variance in route of melatonin administration. Furthermore, our present study provided first evidence that prophylactic melatonin administration exerts its protective effects through inhibiting ER stress and autophagy, activating ER stress and autophagy with compounds can significantly abolished the protective effects of prophylactic melatonin treatment *in vitro* and *in vivo*. More interestingly, we identified that ER stress triggered autophagy through both PERK and IRE1

pathway during IR injury. Our findings shed light on the novel preventive and therapeutic strategy of daily administration of melatonin for the population with high risk of cerebral ischemic stroke, which still needs further clinical studies.

ER stress is one of the main molecular events underlying the pathology of cerebral ischemia/reperfusion injury. ER stress can activate multiple signaling pathways to remove the unfolded/misfolded proteins and rebalance the intercellular homeostasis, which collectively is termed as unfolded protein response (UPR). UPR is characterized by the activation of three main sensors: PERK, IRE1 and ATF6. Under physiological condition, these three sensors were inhibited by binding with GRP78.[33] When ER stress is induced, GRP78 will be released from the association with PERK, IRE1 and ATF6 to bind the accumulated dysfunctional proteins, which lead to activation of these sensors.[34] In our present study, we showed that IR significantly activated ER stress evidenced by the increase in ATF6 as well as the hyper-phosphorylation of PERK and IRE1, which was consistent with the previous studies. However, one recent study reported global brain ischemia activated PERK but not IRE1and ATF6.[35] The discrepancy might be ascribed to the difference in animal models. Once activated, PERK can enhance expression of its downstream target transcription factor 4 (ATF4), which in turn induces the pro-apoptotic protein CHOP. In the present study, we showed that IR injury activated PERK/ATF4/CHOP pathway, which was markedly reversed by pre-ischemia melatonin treatment. In addition, activation of IRE1 can promote the downstream JNK signaling pathway to induce cell death.[36] Our present results indicated that melatonin exerted its protection via suppression of JNK signaling pathway. ER stress was also demonstrated to induce mitochondrial dysfunction via phosphorylation of PERK and eIF2α signaling.[37] The present study showed that melatonin significantly reversed

ischemia-induced decrease of Bcl-2 and increase of Bax/Bim. Together, the present study demonstrated that pre-ischemia melatonin treatment can rescue neurons against IR injury through inhibiting ER stress-associated death pathways and alleviating mitochondrial dysfunction.

Autophagy is fundamentally active to maintain cellular homeostasis. While certain extent of autophagy is necessary for cell survival, excessive autophagic activation can result in neuronal injury. Currently, the role of autophagy in cerebral ischemic stroke is still inconclusive. Some studies indicate that autophagy is detrimental to neurons during cerebral ischemic injury and inhibiting autophagy can decrease the infarct size and augment neurological scores.[38,39] However, other studies show that autophagy activation or enhancing mitophagy can protect against IR induced injury.[40] The present study added evidence that autophagy was significantly activated and was detrimental to neurons in the acute stage of ischemia injury. Pre-ischemia melatonin administration could protect against IR injury via inhibiting autophagy. However, since mitophagy (the removal of mitochondria by autophgy) is considered to be an adaptive mechanism in response to hypoxia,[41] whether mitophagy is involved in the protective effects of melatonin warrants further investigation.

Studies show that ER stress can trigger autophagy.[42-44] In addition, ER stress can induce the formation of autophagosome through the IRE1/JNK signaling pathway and perturbation of autophagy makes neurons vulnerable to ER stress.[45] In embryonic fibroblasts, a defect of IRE1 decreases the accumulation of LC3-positive vesicles.[46] Furthermore, the activation of PERK/eIF2α pathway induced after UPR is considered to be mediator of ER stress-induced autophagy.[28] Mutations of eIF2α at the phosphorylation site of PERK impedes Atg complex

formation and LC3 conversion.[28] In the present study, IR-induced autophagy was significantly blocked by ER stress inhibitor (4-PBA), as well as PERK and IRE1inhibitors. However, autophagy inhibitor (3-MA) can only restrain autophagy activity without significant effects on ER stress. Thus, our data suggested that ER stress was the upstream event of autophagy during IR injury and ER stress triggered autophagy through both the PERK and IRE1 pathway, hinting that ER stress might induce autophagy to tip the balance towards IR injury associated cellular death.

In summary, our study suggests that pre-ischemia melatonin administration significantly protects against cerebral IR injury. This protective effect is through inhibiting ER stress-dependent autophagy and the related mechanisms, which are summarized in Fig. 8. Our findings shed light on the novel preventive and therapeutic strategy of daily administration of melatonin, especially among the population with high risk of cerebral ischemic stroke.

ACKNOWLEDGEMENTS

This study was supported by grants of the National Natural Science Foundation of China (No. 81500909, 81630027, 81671313) and Postdoctoral Science Foundation of China (2015M44926).

DISCLOSURE

The authors declare that no competing interests exist.

AUTHOR CONTRIBUTIONS

Qu Y and Dong YS designed the study, conducted the experimental research and wrote the protocol. Feng DY, Wang B and Wang L completed the animal experiments and statistical analysis. Tai K, Huang L and Shi W accomplished the morphology experiments. Abraham N managed the literature edition. All authors read and approved the final manuscript.

REFERENCES

1. Strong K, Mathers C , Bonita R. Preventing stroke: saving lives around the world. *Lancet Neurol*. 2007; **6**:182-7.
2. Rha JH , Saver JL. The impact of recanalization on ischemic stroke outcome: a meta-analysis. *Stroke*. 2007; **38**:967-73.
3. Goyal M, Demchuk AM, Menon BK, et al. Randomized assessment of rapid endovascular treatment of ischemic stroke. *N Engl J Med*. 2015; **372**:1019-30.
4. Duehrkop C , Rieben R. Ischemia/reperfusion injury: effect of simultaneous inhibition of plasma cascade systems versus specific complement inhibition. *Biochem Pharmacol*. 2014; **88**:12-22.
5. Reiter RJ, Mayo JC, Tan DX, et al. Melatonin as an antioxidant: under promises but over delivers. *J Pineal Res*. 2016; **61**:253-78.
6. Fernandez A, Ordonez R, Reiter RJ, et al. Melatonin and endoplasmic reticulum stress: relation to autophagy and apoptosis. *J Pineal Res*. 2015; **59**:292-307.
7. Dong Y, Fan C, Hu W, et al. Melatonin attenuated early brain injury induced by subarachnoid hemorrhage via regulating NLRP3 inflammasome and apoptosis signaling. *J Pineal Res*. 2016; **60**:253-62.
8. Naskar A, Prabhakar V, Singh R, et al. Melatonin enhances L-DOPA therapeutic effects, helps to reduce its dose, and protects dopaminergic neurons in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinsonism in mice. *J Pineal Res*. 2015; **58**:262-74.
9. Stefanova NA, Maksimova KY, Kiseleva E, et al. Melatonin attenuates impairments of structural hippocampal neuroplasticity in OXYS rats during active progression of Alzheimer's disease-like pathology. *J Pineal Res*. 2015; **59**:163-77.
10. Ali T , Kim MO. Melatonin ameliorates amyloid beta-induced memory deficits, tau hyperphosphorylation and neurodegeneration via PI3/Akt/GSk3beta pathway in the mouse hippocampus. *J Pineal Res*. 2015; **59**:47-59.
11. Manchester LC, Coto-Montes A, Boga JA, et al. Melatonin: an ancient molecule that makes oxygen metabolically tolerable. *J Pineal Res*. 2015; **59**:403-19.
12. Yang Y, Jiang S, Dong Y, et al. Melatonin prevents cell death and mitochondrial dysfunction via a SIRT1-dependent mechanism during ischemic-stroke in mice. *J Pineal Res*. 2015; **58**:61-70.
13. Carloni S, Albertini MC, Galluzzi L, et al. Melatonin reduces endoplasmic reticulum stress and preserves sirtuin 1 expression in neuronal cells of newborn rats after hypoxia-ischemia. *J Pineal Res*. 2014; **57**:192-9.

14. Li H, Wang Y, Feng D, et al. Alterations in the time course of expression of the Nox family in the brain in a rat experimental cerebral ischemia and reperfusion model: effects of melatonin. *J Pineal Res*. 2014; **57**:110-9.
15. Roberts GG, Di Loreto MJ, Marshall M, et al. Hippocampal cellular stress responses after global brain ischemia and reperfusion. *Antioxid Redox Signal*. 2007; **9**:2265-75.
16. Srinivasan K , Sharma SS. Edaravone offers neuroprotection in a diabetic stroke model via inhibition of endoplasmic reticulum stress. *Basic Clin Pharmacol Toxicol*. 2012; **110**:133-40.
17. Srinivasan K , Sharma SS. Sodium phenylbutyrate ameliorates focal cerebral ischemic/reperfusion injury associated with comorbid type 2 diabetes by reducing endoplasmic reticulum stress and DNA fragmentation. *Behav Brain Res*. 2011; **225**:110-6.
18. Srinivasan K , Sharma SS. 3-Bromo-7-nitroindazole attenuates brain ischemic injury in diabetic stroke via inhibition of endoplasmic reticulum stress pathway involving CHOP. *Life Sci*. 2012; **90**:154-60.
19. Hu W, Ma Z, Di S, et al. Snapshot: implications for melatonin in endoplasmic reticulum homeostasis. *Br J Pharmacol*. 2016; **173**:3431-3442.
20. Ding WX , Yin XM. Sorting, recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome. *Autophagy*. 2008; **4**:141-50.
21. Guo Y, Wang J, Wang Z, et al. Melatonin protects N2a against ischemia/reperfusion injury through autophagy enhancement. *J Huazhong Univ Sci Technolog Med Sci*. 2010; **30**:1-7.
22. Zheng Y, Hou J, Liu J, et al. Inhibition of autophagy contributes to melatonin-mediated neuroprotection against transient focal cerebral ischemia in rats. *J Pharmacol Sci*. 2014; **124**:354-64.
23. Wang S, Xu H, Xin Y, et al. Neuroprotective effects of Kaempferide-7-O-(4"-O-acetylrhamnosyl)-3-O-rutinoside on cerebral ischemia-reperfusion injury in rats. *Eur J Pharmacol*. 2016; **788**:335-42.
24. Tai SH, Chen HY, Lee EJ, et al. Melatonin inhibits postischemic matrix metalloproteinase-9 (MMP-9) activation via dual modulation of plasminogen/plasmin system and endogenous MMP inhibitor in mice subjected to transient focal cerebral ischemia. *J Pineal Res*. 2010; **49**:332-41.
25. Bederson JB, Pitts LH, Tsuji M, et al. Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke*. 1986; **17**:472-6.
26. Zhang Y, Chen Y, Wu J, et al. Activation of dopamine D2 receptor suppresses neuroinflammation through alphaB-crystalline by inhibition of NF-kappaB uuclear translocation in experimental ICH mice model. *Stroke*. 2015; **46**:2637-46.
27. Ohoka N, Yoshii S, Hattori T, et al. TRB3, a novel ER stress-inducible gene, is induced via ATF4-CHOP pathway and is involved in cell death. *EMBO J*. 2005; **24**:1243-55.
28. Kouroku Y, Fujita E, Tanida I, et al. ER stress (PERK/eIF2alpha phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. *Cell Death Differ*. 2007; **14**:230-9.
29. Lee H, Noh JY, Oh Y, et al. IRE1 plays an essential role in ER stress-mediated aggregation of mutant huntingtin via the inhibition of autophagy flux. *Hum Mol Genet*. 2012; **21**:101-14.
30. Parada E, Buendia I, Leon R, et al. Neuroprotective effect of melatonin against ischemia is partially mediated by alpha-7 nicotinic receptor modulation and HO-1 overexpression. *J Pineal Res*. 2014; **56**:204-12.

31. Koh PO. Melatonin regulates the calcium-buffering proteins, parvalbumin and hippocalcin, in ischemic brain injury. *J Pineal Res*. 2012; **53**:358-65.
32. Kilic E, Kilic U, Reiter RJ, et al. Prophylactic use of melatonin protects against focal cerebral ischemia in mice: role of endothelin converting enzyme-1. *J Pineal Res*. 2004; **37**:247-51.
33. Bertolotti A, Zhang Y, Hendershot LM, et al. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. *Nat Cell Biol*. 2000; **2**:326-32.
34. Lai E, Teodoro T , Volchuk A. Endoplasmic reticulum stress: signaling the unfolded protein response. *Physiology (Bethesda)*. 2007; **22**:193-201.
35. Sanderson TH, Deogracias MP, Nangia KK, et al. PKR-like endoplasmic reticulum kinase (PERK) activation following brain ischemia is independent of unfolded nascent proteins. *Neuroscience*. 2010; **169**:1307-14.
36. Nishitoh H, Matsuzawa A, Tobiume K, et al. ASK1 is essential for endoplasmic reticulum stress-induced neuronal cell death triggered by expanded polyglutamine repeats. *Genes Dev*. 2002; **16**:1345-55.
37. Hacki J, Egger L, Monney L, et al. Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. *Oncogene*. 2000; **19**:2286-95.
38. Dong F, Yao R, Yu H, et al. Neuroprotection of Ro25-6981 against ischemia/reperfusion-induced brain injury via inhibition of autophagy. *Cell Mol Neurobiol*. 2016; 10.1007/s10571-016-0409-5.
39. Wu Z, Zou Z, Zou R, et al. Electroacupuncture pretreatment induces tolerance against cerebral ischemia/reperfusion injury through inhibition of the autophagy pathway. *Mol Med Rep*. 2015; **11**:4438-46.
40. Zhang X, Yuan Y, Jiang L, et al. Endoplasmic reticulum stress induced by tunicamycin and thapsigargin protects against transient ischemic brain injury: Involvement of PARK2-dependent mitophagy. *Autophagy*. 2014; **10**:1801-13.
41. Semenza GL. Mitochondrial autophagy: life and breath of the cell. *Autophagy*. 2008; **4**:534-6.
42. Chandrika BB, Yang C, Ou Y, et al. Endoplasmic reticulum stress-induced autophagy provides cytoprotection from chemical hypoxia and oxidant injury and ameliorates renal ischemia-reperfusion injury. *PLoS One*. 2015; **10**:e0140025.
43. Hong SH, Chang SH, Cho KC, et al. Endoplasmic reticulum-Golgi intermediate compartment protein 3 knockdown suppresses lung cancer through endoplasmic reticulum stress-induced autophagy. *Oncotarget*. 2016; 10.18632/oncotarget.11678.
44. Ji C, Zhang Z, Chen L, et al. Endoplasmic reticulum stress-induced autophagy determines the susceptibility of melanoma cells to dabrafenib. *Drug Des Devel Ther*. 2016; **10**:2491-8.
45. Ogata M, Hino S, Saito A, et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. *Mol Cell Biol*. 2006; **26**:9220-31.
46. Hoyer-Hansen M , Jaattela M. Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. *Cell Death Differ*. 2007; **14**:1576-82.

FIGURE LEGENDS

FIGURE 1 Ischemia/reperfusion (IR) injury activated ER stress and autophagy. (A) A transient middle cerebral artery occlusion (MCAO) mouse model was established and the peri-infarct brain tissues were lysed for analysis at 6 h, 12h and 24h post-reperfusion. Levels of ATF6, phosphorlated-PERK (p-PERK), PERK, phosphorylated-IRE1 (p-IRE1) and IRE1 were evaluated with western blot. (B) Levels of LC3-I/II and beclin-1 were also determined with western blot. The relative value of band density was measured with Image J (1.49V) and normalized to that of β-actin. Data were presented as mean ± SEM from at least three independent experiments. **P*<0.05 and ***P*<0.01 compared with sham group.

FIGURE 2 Pre-ischemia melatonin treatment significantly attenuated IR-induced ER stress. (A) Mice were randomly divided into three groups: (i) sham surgery group; (ii) ischemia/reperfusion (IR) group; (iii) IR with pre-ischemia melatonin treatment (IR + Mel) group. At 24 h post-reperfusion, levels of ATF6, phosphorlated-PERK (p-PERK), PERK, phosphorylated-IRE1 (p-IRE1) and IRE1 were evaluated with western blot. The relative value of band density was measured with Image J (1.49V) and normalized to that of β-actin. (B) The levels of p-PERK and p-IRE1 were also determined with double-immunofluorescence staining. Images were captured with laser confocal microscope (A1, Nikon, Japan). Green: p-PERK. Red: p-IRE1. Scale bar = 50 μm. The relative density of fluorescence was measured with Image J (1.49V). Data were presented as mean ± SEM from at least three independent experiments. ***P*<0.01 compared with sham group and $^{\#}P$<0.05 compared with the indicated group.

FIGURE 3 Pre-ischemia melatonin treatment significantly alleviated IR-induced autophagic flux. (A) A schematic illustrating the principal of this assay. (B) Representative images of the punctate RFP-GFP-LC3 staining. Scale bar = 40 μm. (C) Quantification of autophagolysomes (red punctate) was measured as the percentage of (punctate red/total punctate signals) in the

merged images. Likewise, quantification of autophagosmes (yellow) was measured as the percentage of (punctate yellow/total punctate signals) in the merged images. A minimum of 100 cells per sample were counted. (D) Representative western images for LC3-I/II and beclin-1. (E) Relative band density of LC3II/I and beclin-1 were measured with with Image J (1.49V) and normalized to that of β-actin. Data were presented as mean ± SEM from at least three independent experiments. *P<0.05 and **P<0.01 compared with sham group, $^{\#\#}P$<0.01 compared with the indicated group.

FIGURE 4 IR injury activated apoptosis through multiple ER related signaling pathway. (A-D) The representative western blot results of ATF4, CHOP, Atg5, Atg12, Bcl-2, Bax, Bim, p-JNK and JNK were shown. Relative band density was measured with with Image J (1.49V) and normalized to that of β-actin. Data were presented as mean ± SEM from at least three independent experiments. **P<0.01 compared with sham group and $^{\#}P$<0.05 compared with the indicated group.

FIGURE 5 ER stress triggered autophagy through both PERK and IRE1 pathway during IR injury. (A) 4-PBA, 3-MA, GSK and DBSA were used to specifically inhibit ER stress, autophagy, PERK and IRE1, respectively. Representative western blot results of p-PERK, PERK, p-IRE1, IRE1, LC-3I/II and beclin-1 were shown. (B) Relative band density was measured with with Image J (1.49V) and normalized to that of β-actin. Data were presented as mean ± SEM from at least three independent experiments. *P<0.05 and **P<0.01 compared with sham group, $^{\#}P$<0.05 and $^{\#\#}P$<0.01 compared with the IR group.

FIGURE 6 Pre-ischemia melatonin treatment protects against IR injury through inhibiting ER stress and autophagy. (A) Tunicamycin (TM) and rapamycin (RAPA) were used to specifically activate ER stress and autophagy, respectively. TUNEL assay was performed to detect apoptosis and the representative images were shown. Green: apoptotic cells. Blue: nucleus. Scale bar = 100 μm. Apoptosis index was expressed as the ratio of (apoptotic cells) /

(total cells) × 100%. (B, C) Representative western blot results of cleaved caspase-3 and 9 were shown. Relative band density was measured with with Image J (1.49V) and normalized to that of β-actin. Data were presented as mean ± SEM from at least three independent experiments. *P<0.05 and **P<0.01 compared with sham group, $^{\#}P$<0.05 compared with the indicated group.

FIGURE 7 The effects of pre-ischemia melatonin treatment on infarct volume, brain water content, neurological score and motor assessment scale of IR-injured brains. (A) Tunicamycin (TM) and rapamycin (RAPA) were used to specifically activate ER stress and autophagy, respectively. Infarct volume was determined with TTC staining. The white area defined the infarct area. (B) The infarct volume was expressed as the ratio of (infarct volume / the whole brain volume) × 100%. (C) Brain water content. (D) Neurological score. (E) Motor assessment scale. Data were presented as mean ± SEM from at least three independent experiments. *P<0.05 and **P<0.01 compared with sham group, $^{\#}P$<0.05 and $^{\#\#}P$<0.01 compared with the indicated group.

FIGURE 8 Schematic diagram of the molecular mechanisms underlying the protective effects of pre-ischemia melatonin treatment against cerebral IR injury. Pre-ischemia melatonin treatment alleviated the IR-induced activation of ER stress, thereby inhibiting multiple downstream pathways, including ATF4/CHOP, Atg5/Atg12/LC3 and JNK/Bax/Bcl2 signals. Pre-ischemia melatonin treatment also inhibited autophagy through PERK and IRE1 signalings.