Full Paper

Inhibition of Autophagy Contributes to Melatonin-Mediated Neuroprotection Against Transient Focal Cerebral Ischemia in Rats

Yongqiu Zheng\textsuperscript{1,1,*a}, Jincai Hou\textsuperscript{1,1}, Jianxun Liu\textsuperscript{1,1,*b}, Mingjiang Yao\textsuperscript{1}, Lei Li\textsuperscript{1}, Bo Zhang\textsuperscript{2}, Hua Zhu\textsuperscript{2,1,*c}, and Zhong Wang\textsuperscript{3}

\textsuperscript{1}Research Center, Xiyuan Hospital, \textsuperscript{2}Institute of Basic Research in Clinical Medicine, China Academy of Chinese Medical Sciences, Beijing 100091, China
\textsuperscript{2}Department of Surgery, Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, Columbus, OH 43210, USA

Received October 30, 2013; Accepted December 24, 2013

Abstract. Melatonin, a natural product of the pineal gland, has been shown to protect against ischemic stroke, but the molecular mechanisms underlying its protective function are not fully understood. In the present study, we tested whether melatonin could protect against ischemia–reperfusion (I/R) injury to rat brain by targeting the autophagy pathway. The I/R brain injury was induced by the established rat transient middle cerebral artery occlusion model. We found intraperitoneal injection of melatonin can ameliorate rat brain injury as evidenced by multiple morphological and behavioral criteria, such as infarct size, neurological score, serum creatine kinase, and lactate dehydrogenase content, as well as pyknotic-positive cells. Further studies revealed that the beneficial effects of melatonin is through targeting the autophagy pathway by inhibiting expression of beclin-1 and conversion of LC3, as well as activating the PI3K/Akt pro-survival pathway. To further confirm this finding, the autophagy pathway was activated by lentiviral mediated beclin-1 delivery and the PI3K/Akt pathway was inhibited by a pharmacological inhibitor, LY294002. In both manipulations, the beneficial effects of melatonin were greatly abolished. Taken together, our study suggested melatonin plays a protective role against I/R brain injury by inhibiting autophagy and activating the PI3K/Akt pro-survival pathway.

Keywords: melatonin, autophagy, LC3-II/LC3-I, Beclin-1, Akt phosphorylation

Introduction

Neuronal fate after ischemic stroke is determined by a balance between cell survival and death signals. Autophagy is generally viewed as one of the cell survival mechanisms under various conditions of stress such as oxidative stress, endoplasmic reticulum stress, and protein aggregate accumulation. On the other hand, prolonged autophagy can be a non-apoptotic route of type II programmed cell death (1). Enhanced autophagy has been implicated in various neurological conditions including intracerebral hemorrhage, cerebral ischemia and spinal cord injury (2, 3). There are evidences that autophagy can promote either cell survival or cell death based on different situations (4, 5). It has been reported that suppression of autophagy by beclin-1 or LC3 knockdown, or by pharmacological inhibitors, promoted apoptosis and caspase-3 activation in starved HeLa cells (6, 7). Furthermore, hyperbaric oxygen preconditioning elevated autophagic activity, which elicits a neuroprotective effect against ischemic injury to the brains (8). These studies generally support a role for autophagy as a means for prolonging cell survival, particularly during starvation. On the other hand, it has also been well documented that autophagy is involved in the regulation of neuronal death following cerebral ischemia. Inhibition of autophagy with chemical inhibitors (i.e., 3-MA, bafilomycin A1) reduced neuronal injury in focal ischemia (9). Our previous report revealed that lentiviral-mediated knockdown of beclin-1 improved outcome of cerebral ischemic injury, with evidence for increased...
populations of progenitor cells and reduced neural cell apoptosis (10). On the basis of such evidences, many researchers accept that autophagy is a major mediator of cell death in cerebral ischemia (2). Despite current controversies on the possible role of autophagy in ischemic stroke progression, it is believed that investigations on therapies targeting the autophagic pathway may give new insights into stroke treatments.

Melatonin (5-methoxy-N-acetyltryptamine, MLT) is a natural product of the pineal gland. It has been shown that MLT and its metabolites are potent antioxidants and free radical scavengers with physiological activity to reduce DNA damage and infant volume after ischemic injury (11–15). Moreover, MLT has many protective effects against ischemic stroke (14–16). The efficacy of MLT treatment in experimental stroke has been established in the past. However, the underlying signaling mechanisms mediating MLT’s neuroprotective effects remained essentially uninvestigated. Some of MLT’s neuroprotective properties have been attributed to MLT’s anti-oxidant, anti-inflammatory, metabolism-modulating, and autophagy regulation effects (17–20). Our data provide the first evidence that MLT induces protective autophagy that prevents mouse hepatoma H22 cells from undergoing apoptosis (21). It has also been demonstrated that the antiapoptotic effects of MLT was related to the enhancement of autophagy in subarachnoid hemorrhage (17–20). There are two different pathways potentially linking autophagy and stroke. One is the dysregulation of Class I phosphatidylinositol 3-kinase (PI3K), which in turn activates the Akt/protein kinase B (PKB) kinase pathway. The other is the binding of Beclin-1 (Atg6 or BECN1) to Class III PI3Ks that regulates autophagosome formation (10, 22). Autophagy falls under negative regulation by a signaling pathway involving Class I PI3K/Akt, while Beclin-1 forms a protein complex with a Class III PI3Ks to promote autophagic vacuole formation (23, 24). Recently, it has been demonstrated that MLT prevents ischemic brain injury and this neuroprotective effect is mediated by the activation of the Class I PI3K/Akt signaling pathway (25–27). However, the exact role of MLT on the autophagic pathway in the process of ischemic stroke progression remains unelucidated.

Herein, we investigate whether MLT could inhibit autophagy in ischemic stroke in vivo. We used a rat transient middle cerebral artery occlusion (tMCAO) model to induce transient focal brain ischemia–reperfusion (I/R) injury. We found that MLT inhibited autophagy in tMCAO rat and such inhibition was associated with activation of the Class I PI3K/Akt signaling pathway. In addition, the injection of Beclin-1 overexpression lentiviral vectors into the lateral ventricle compromised the biological effects of MLT in tMCAO, suggesting that protective effects of MLT were, at least partly, dependent on Beclin-1–mediated autophagic pathway.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (220–230 g), provided by the Animal Facility, Health Science Center of Peking University (Beijing, China), were housed in the laboratory animal room and maintained at 25°C ± 1°C with 65% ± 5% humidity on a 12-h light/dark cycle (lights on from 07:30 to 19:30) for at least 1 week before the experiments. Animals were given food and water ad libitum. All experimental protocols described in this study were approved by the Ethics Review Committee for Animal Experimentation of Xiyuan Hospital, China Academy of Chinese Medical Sciences.

Transient MCAO

Rats were subjected to transient focal cerebral ischemia induced by right MCAO as previously described (28), with some modifications. In brief, rats were anesthetized with 10% chloral hydrate (360 mg/kg, i.p.), and arterial blood samples obtained via a femoral catheter were collected to measure \( pO_2 \), \( pCO_2 \) and pH with an AVL 998 Blood Gas Analyzer (Roche Co., Basel, Switzerland). The rectal temperature was maintained at 37°C ± 0.5°C during MCAO via a temperature-regulated heating lamp. A fiber-optic probe was attached to the parietal bone overlaying the middle cerebral artery territory 5-mm posterior and 5-mm lateral to the bregma and connected to a laser–Doppler flowmeter (Perifluxsystem 5000, Stockholm, Sweden) for continuous monitoring of the cerebral blood flow (CBF). A 4-0 nylon monofilament suture with a heat-blunted tip was introduced into the internal carotid artery through the stump of the external carotid artery and gently advanced for a distance of 18 mm from the common carotid artery bifurcation to block the origin of the middle cerebral artery for 90 min and then withdrawn to allow reperfusion. Only animals that exhibited a reduction in CBF of > 85% during right middle cerebral artery occlusion and a CBF recovery of > 80% after 10 min of reperfusion were included in the study. After the wound had been closed, the animals were allowed to recover from anesthesia before they were returned to their home cages.

Preparation of drug for administration

Melatonin from Sigma-Aldrich (St. Louis, MO, USA) was dissolved in vehicle (1% ethanol in 1 mL saline) to yield a concentration of 5 or 10 mg/kg body weight during the treatment. The MT2 melatonin–receptor
antagonists 4-phenyl-2-propionamidotetralin (4P-PDOT) and luzindole were purchased from Tocris Bioscience Co. (Bristol, UK). LY294002, a PI3 kinase inhibitor, was purchased from Sigma-Aldrich Co. Except where indicated, all other chemicals used were of analytical grade purchased from Sigma-Aldrich Co.

Animal grouping and drug administration

The rats were randomly divided into 7 groups (n = 15 for each group): sham control, I/R, I/R plus melatonin (5 or 10 mg/kg, i.p., once daily), and I/R plus melatonin (10 mg/kg, i.p., once daily) after pretreatment with 4P-PDOT (10 mg/kg, i.p.), luzindole (30 mg/kg, i.p.) or LY294002 (10 mg/kg, i.p.) 30 min before MCA occlusion. Generally, the rats were treated with melatonin or a vehicle control (normal saline for sham and I/R groups) once daily 1 h after MCAO.

An additional group of rats pretreated with Beclin-1 over-expression transfectants followed by daily melatonin treatment (10 mg/kg, i.p.) was used. In brief, the rats were randomly divided into 5 groups (n = 15 for each group): sham control, I/R (stroke), I/R plus melatonin (10 mg/kg, i.p.), and I/R plus melatonin (10 mg/kg, i.p.) after pretreatment with PcDNA plenti6.3-Bec transfectants or empty vectors 30 min before MCAO. At 1 h after I/R induction, the rats were treated with melatonin or a vehicle control once daily for 3 days. All animals were allowed to move and take food freely (Fig. 1A).

Administration of Beclin-1 over-expression by lentiviral delivery

The cDNA encoding Beclin-1 was inserted into the plenti6.3-MCS vectors between the constitutively active cytomegalovirus promoter (pCMV) and the enhanced green fluorescent protein (EGFP) reporter gene. Lentiviral vectors plenty 6.3-LTR-RRE-U6-CMV EYFP-WRE-SV40 BSD-LTR were generated.

Following MCAO, rats were divided into PcDNA plenti6.3-Bec (Beclin-1 gene over-expressed) and control (pEGFP-empty vector) groups. The Lentiviral vectors mixture, 2 µl vectors (1 × 10^9 viral particles/ml) and 1 µl lipofectamine (Invitrogen, Carlsbad, CA, USA), were stereotaxically delivered into the ipsilateral lateral ventricle (coordinates from bregma: AP-0.8 mm, ML 1.4 mm, DV-3.6 mm from the pial surface). After recovering from anesthesia, rats were returned to their cages and given access to food and water ad libitum.

Assessment of neurological deficit score and analysis of survival rates

The neurological deficit score was assessed before rats were sacrificed 24 h after reperfusion as described previously (29). Each rat was scored by 2 examiners who were kept unaware of the identity of the rat and the treatment protocol. The following neurological deficit scoring (NDS) system was used: 0, no motor deficits (normal); 1, forelimb weakness and torso turning to the ipsilateral side when held by tail (mild); 2, circling to the contralateral side but normal posture at rest (moderate); 3, unable to bear weight on the affected side at rest (severe); and 4, no spontaneous locomotor activity or barrel rolling (critical). If no deficit was observed 2 h recovering from anesthesia, the animal was removed from further study.

Measurement of infarct volume

At 24 and 72 h after reperfusion, the rats were deeply anesthetized with 3.5% chloral hydrate and then decapitated, after which the whole brains were rapidly removed. Coronal sections (n = 10 for each group) were cut into 2-mm slices and stained with standard 2% 2,3,5-triphenyltetrazolium chloride (TTC, Sigma-Aldrich) for 10 min at 37°C followed by overnight immersion in 4% formalin. Infarct volume, expressed as a percentage of whole-brain volume, was measured by an image processing and analysis system (1.25 × objective, Q570IW; Leica, Wetzlar, Germany) and was calculated by integration of the infarct area on each brain section along the rostral-caudal axis (30).

Immunohistochemistry and immunofluorescence staining

Rats were sacrificed 24 and 72 h after MCAO with an overdose of 3.5% chloral hydrate and transecardially perfused with 0.9% saline solution followed by 4% ice-cold phosphate-buffered paraformaldehyde (PFA). Brains were removed, postfixed overnight, and equilibrated in phosphate-buffered 30% sucrose. Coronal sections at 1.0 to 2.0 mm from the bregma were used for cutting on a cryostate (Leica CM3000, Leica) at a thickness of 25 µm and used for immunohistochemical staining.

Frozen sections were double-stained by phenotypic markers, using the following primary antibodies: rabbit polyclonal anti-autophagy APG8a (MAP1LC3A) antibody (1:100; Abgent, San Diego, CA, USA), mouse polyclonal anti-caspase-3 to label apoptosis of neurons (1:100; Chemicon Billerica, MA, USA); rabbit polyclonal anti-Beclin-1 antibody to label autophagy (1:100; Cell Signaling, Boston, MA, USA). Hoechst 33258 from Sigma-Aldrich Co. was used to label the nucleus. The following secondary antibodies were used: anti-rabbit and mouse IgG-fluorescein isothiocyanate (FITC) and IgG-Cy3 (1:200, Chemicon). Confocal images were taken using a Zeiss LSM-510 microscope.
Transmission electron microscopy (TEM)

TEM was used to evaluate the ultrastructural change of brain sections. Cerebral fragments were fixed with 2.5% glutaraldehyde solution overnight at 4°C; then they were washed with PBS and fixed with 1% osmic acid for 2 h. Tissues were embedded in an Epon/Araldite mixture. Ultra-thin sections were cut and stained with uranyl acetate and lead citrate. The samples were observed under a 1230 type transmission electron microscope (Electron Co., Tokyo) and photographed.

Western blotting

Rats were sacrificed 24 and 72 h after reperfusion with an overdose of 3.5% chloral hydrate. Cortical sections at 1.0 to 2.0 mm from infarcts were used for lactate dehydrogenase (LDH) and creatine kinase (CK) assay and western blotting analysis. Rat cerebral homogenates were collected and centrifuged for 4 min at 3000 × g and the resulting supernatants centrifuged at 10,000 × g for 30 min. Supernatants were diluted to 0.5 mg protein/ml for the measurement of Beclin-1 and LC3 and the phosphorylation of Akt. Protein content was determined with BSA as a standard according to Bradford (31). Proteins samples (20 μg/ lane) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred to PVDF membranes (Millipore, Billerica, MA, USA) by electroblotting. Blots were stained with rabbit polyclonal anti-autophagy APG8a (MAP1LC3A) antibody (Abgent), rabbit polyclonal anti-Beclin-1 antibody (Cell Signaling), phosphorylated-Akt (Ser473) antibody, and total Akt antibody (Cell Signaling). Then the blots were developed by enhanced chemiluminescence using SuperSignal West Femto maximum sensitivity substrate (Pierce, Rockford, IL, USA). Bio-Rad Image Lab™ Version 3.0 software was used to calculate the numerical value of every blot. The mean densitometric × area values were depicted as bar graphs. All the experiments reported in this study were performed 3 times and the results were reproducible.

Measurement of LDH and CK

Concentrations of LDH and CK in the supernatants of cerebral homogenates were measured by a colorimetric method, using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. The recorded values are presented in U/L.

Data quantification and statistical analyses

All data were presented as the mean ± S.D. Statistical significance was analyzed by a one-way analysis of variance (ANOVA) followed by Tukey’s test for multiple comparisons. The nonparametric test was used for the band density values comparisons between groups. \( P < 0.05 \) was considered statistically significant.

Results

Melatonin reduces cerebral infarction and ameliorates the neuronal death in cerebral ischemia (CI) / reperfusion (R) rats

Transient focal ischemia for 90 min caused infarction in the striatum and frontoparietal cortex 24 h after reperfusion. In parallel with the cerebral infarction, 90-min cerebral ischemia followed by 24-h reperfusion injury also induced severe neurological deficits compared to the sham-operated rats (Fig. 1B). Treatment with melatonin (5 and 10 mg/kg, i.p.) significantly reduced these CI/R-induced neurological deficits as well as cerebral infarction in ischemic-stroke rats (Fig. 1B). In the present experiments, the CI/R injury also induced neuronal death as demonstrated by a progressive increase in the number of pyknotic nuclei (Fig. 1D) or increased lactate dehydrogenase (LDH) and creatine kinase (CK) release into the cerebral tissues (Fig. 1C). Melatonin (5 and 10 mg/kg, i.p.) significantly ameliorated the neuronal death caused by CI/R injury and significantly diminished LDH and CK production 24 h after reperfusion (Fig. 1: C and D).

MLT inhibits autophagy in CI/R rats

The levels of Beclin-1 and LC3-II were significantly evaluated in the ipsilateral cortex from 90-min ischemia / 24-h reperfusion rats. Treatment of melatonin (5 and 10 mg/kg, i.p.) significantly reduced Beclin-1 and LC3-II expression at 24 h after reperfusion (Fig. 2D). LC3 immunofluorescence usually shows two staining patterns: diffuse cytoplasmic staining (basal) and punctate staining (AV-related) (32). The redistribution of LC3 from diffused cytosolic staining to punctated staining is a reliable marker of autophagosome formation. As shown in Fig. 2C, only a few LC3-positive punctuates were observed in the ipsilateral cortex from MLT-treated rat. Interestingly, along with the increased LC3-positive punctate, cortical neurons in CI/R brains showed increased autophagic vacuoles demonstrated by TEM (Fig. 2A), whereas treatment with melatonin (5 and 10 mg/kg, i.p.) showed decreased levels of autophagosomes (Fig. 2: A – C).

Disruption of the PI3K/Akt pathway reversed the biological effects of MLT on cerebral infarction and Beclin-1 expression

To investigate the signaling pathways that mediate the anti-autophagic and cell survival effect of MLT, chemical inhibitors of melatonin receptor and the PI3/Akt pathway were used. Phospho-Akt kinase was induced
Fig. 1. Effects of melatonin on rat middle cerebral ischemic/reperfusion injury. A) Protocols for animal treatment. B) Effects of melatonin on cerebral infarct and neurological deficit score. Left part: upper, representative TTC staining of the cerebral infarct. Lower, quantification of infarct volume. Right part: neurological deficit score. ANOVA test was used for statistical analysis. *P < 0.05 vs. I/R + Saline, n = 9. Data are shown as the mean ± S.D. C) At 24 h after I/R, LDH and CK release in cerebral homogenates. Values are reported as the mean ± S.D. (*P < 0.05, **P < 0.01 vs. I/R + Saline, n = 9). D) Melatonin decreases pyknotic nuclei. Left part: representative images of Hoechst-stained nuclei from the ipsilateral cortex. Arrows indicate pyknotic nuclei. Scale bar = 10 µm. Right part: quantification of pyknotic nuclei as a percentage of all nuclei, following 24 h after reperfusion, showing a progressive increase in neuronal death. Melatonin (5 and 10 mg/kg, i.p.) significantly ameliorated the neuronal death caused by I/R injury (*P < 0.05, **P < 0.01 vs. I/R + Saline, n = 5).
Melatonin Protects Ischemic Brain Injury

Fig. 2. Autophagic vacuoles observed by a transmission electron microscope. A) Formation of autophagic vacuoles. Ipsilateral cortical neurons in infarct sections from rats treated with a) sham, b) I/R model, c) I/R + MLT (5 mg/kg), d) I/R + MLT (10 mg/kg); arrows indicate multiple autolysosome-like vesicles in the cytoplasm. The formation of autophagic vacuoles was indicated by several autophagic profiles. Stage 1: an early stage of autophagic vacuoles formation, identified as membranes from the rough endoplasmic reticulum containing cytosolic components (1). This process will lead to the formation of the autophagosomes, which are characterized by double membrane vesicles (2). Stage 2: autophagolysosomes contain residues of membranes and lytic organelles (3). Stage 3: autophagosomes (4), adjacent to lysosomes (5), contain some vacuoles bounded by a small inner vesicle enclosing residues of the lytic organelles. Scale bar: 0.2 μm for the first panels.

B) Number of autophagic vacuoles per cell profile. Results shown in are the mean ± S.D. of 6 profiles for each condition. (**P < 0.01 vs. I/R + Saline).

C) Upper: the LC3-FITC fluorescence and Hoechst 33258 staining were observed under a Zeiss LSM-510 microscope (magnification: 400 ×), scale bar = 10 μm. Lower: The percentage of cells with LC3-punctuated staining per total LC3 cells was scored. Values are the mean ± S.D. of 5 independent experiments (***P < 0.01 vs. I/R + Saline).

D) Beclin-1 and LC3-II expression. Left part: representative images of Beclin-1 and LC3-II expression. An antibody for DAPDH was used to show equal protein loading. Right part: bar graphs show quantitative evaluation of Beclin-1 expression and LC3-II/LC3-I in the ipsilateral hemisphere, n = 3. Data are reported as the mean ± S.D. A nonparametric test was used for statistical analysis; *P < 0.05, compared with I/R + Saline.
by 90-min ischemia / 24-h reperfusion injury. Melatonin prevented the injury-induced decrease in Akt activation (Fig. 3A). Furthermore, we determined if LY294002 could reverse the protective effects of MLT. The effects of melatonin on reperfusion induced infarction was significantly reduced when rats were pretreated with LY294002 (10 mg/kg, i.p.), 4P-PDOT (10 mg/kg, i.p.), or luzindole (30 mg/kg, i.p.) (Fig. 3B). Moreover, Beclin-1 expression increased in CI/R rats and melatonin inhibited injury-induced Beclin-1 expression significantly. The effect of melatonin on Beclin-1 expression was abrogated by LY294002, 4P-PDOT, and luzindole (Fig. 3C). Interestingly, melatonin had no effects on Akt phosphorylation in contralateral brain samples (Fig. 3A) and Beclin-1 expression in sham groups. (Fig. 3C), indicating the effects of melatonin was injury-dependent.

**Beclin-1 overpression compromises the protective effects of MLT in CI/R rats**

Our next question was whether Beclin-1 overpression prevented the protective effects of MLT in the ischemic brain. We injected Beclin-1–expressing lentiviral vectors or empty vectors into the lateral ventricle 30-min before MCAO and rats were sacrificed 72 h after MCAO. Immunohistochemical staining for EGFP, a reporter gene, was performed to determine the expression of the transcripts in the brain after injection of the vectors. As expected, EGFP-Beclin-1 immunostaining was visible...
Fig. 4. Beclin-1 overexpression in the brain compromises the protective effects of MLT. A) The photographs showed that EGFP was expressed in the ipsilateral cortex at 3 days after reperfusion. In the rats injected with the Beclin-1–expressing lentiviral vectors, EGFP-positive cells were co-labeled with Beclin-1 in the ipsilateral cortex. Scale bar = 20 μm. B) Beclin-1 expressing in rats treated with p-Beclin1 and empty vectors. Upper: representative immunoblots for Beclin-1 in the ipsilateral hemisphere. C: saline control, M: melatonin (10 mg/kg), p-Beclin1: Beclin-1–expressing lentiviral vectors. Lower: Bar graphs show quantitative evaluation of Beclin-1 expression. n = 3. Data are reported as the mean ± S.D. A nonparametric test was used for statistical analysis; *P< 0.05, compared with I/R + Saline. C) Cerebral infarct in rats treated with p-Beclin1 and empty vectors. Upper: representative TTC staining of the cerebral infarct. Lower: quantification of infarct volume. The ANOVA test was used for statistical analysis; **P< 0.01 vs. I/R + Saline, ##P< 0.01 vs. I/R + MLT (10 mg/kg, i.p.), &&P< 0.01 vs. I/R + MLT + P-Beclin-1. D) Beclin-1/cleaved caspase-3 double staining in rats treated with p-Beclin-1 and empty vectors. Left part: images of the FITC-Beclin-1 fluorescence and cleaved CY3-caspase-3 staining were observed under a Zeiss LSM-510 microscope (magnification: 400 ×). Scale bar = 20 μm. Right part: the percentage of Beclin-1/cleaved caspase-3 double–positive cells was scored. Values are each the mean ± S.D. of 5 independent experiments. **P< 0.01 vs. I/R + Saline, +++P< 0.01 vs. I/R + MLT (10 mg/kg, i.p.), &&&P< 0.01 vs. I/R + MLT + P-Beclin-1.
in the ipsilateral cortex at 3 days following MCAO, and it was increased by transient MCAO (Fig. 4A). Melatonin inhibited injury-induced Beclin-1 expression, but had no effects on Beclin-1 overexpression by lentiviral vectors (Fig. 4B). Moreover, immunobLOTS showed that Beclin-1 overexpression did not obviously increase infarct volume but blocked the melatonin-mediated inhibition of cerebral infarction in CI/R rats (Fig. 4C). In the ipsilateral hippocampus, the numbers of apoptotic and autophagic cells were then quantified by double immunostaining of Beclin-1 and activated caspase-3 (Beclin-1+–Casp-3+). Treatment with Beclin-1 expressing lentiviral vectors significantly increased the number of Beclin-1+–Casp-3+ cells that decreased by melatonin (Fig. 4D).

Discussion

In the present study, we provide the evidence that melatonin could prevent I/R-induced autophagy as well as improve neurological function in tMCAO rats. Moreover, Beclin-1 overexpression compromises the biological effects of melatonin in ischemic stroke. These findings suggest that inhibition of autophagy by melatonin prevents cerebral I/R-induced injury, partly by reducing Beclin-1 expression, and this effect requires the involvement of the PI3K/Akt signaling pathway.

It is well known that autophagy is involved in the regulation of neuronal death following cerebral ischemia (2, 3, 10). During autophagy, cytoplasmic components are sequestered into double-membrane vesicles called autophagosomes, which fuse with lysosomes and are degraded by lysosomal hydrolases. Melatonin is a natural compound that is produced in the pineal gland and has been proven to have neuroprotective activity (18, 33). It has been shown that spinal cord injury induced increased autophagy, which perhaps correlates with a decrease in MLT (33). LC3 and Beclin-1 are two pacemakers in the autophagic cascade. LC3, the microtubule-associated protein 1A light chain 3, exists in a cytosolic form (LC3-I) and a membrane-bound form (LC3-II). The ratio of conversion from LC3-I to LC3-II is closely correlated with the extent of autophagosome formation (34). Beclin-1, the homologue of the yeast autophagy genes 6 (Atg6), is essential for the recruitment of other autophagic proteins during the expansion of pre-autophagosomal membrane (35, 36). In the present study, we showed not only a decrease in autophagosome formation following MLT treatment but also an inhibition of autophagy-associated proteins Beclin-1 and LC3-II expression, thereby demonstrating a decrease in autophagic response. Moreover, reduced neurological deficits, alleviated cerebral infarction and palliated neuronal death were observed in the ipsilateral hemisphere from tMCAO rats treated with melatonin, suggesting that autophagy might be involved in the neuroprotective function of melatonin (37).

To test this possibility of this mechanism, we chose to interrupt the process of autophagy. The available pharmacological inhibitors of autophagy are PI3K inhibitors. Autophagy falls under negative regulation by a signaling pathway involving Class I PI3K (38), which mediates the stimulation of cell survival and suppression of cell death in a variety of cells. Melatonin protects the brain from ischemic injury through the enhancement of the survival signaling pathway (26, 37, 39) including the PI3-K/Akt signal pathway. It has been reported that PI3K/Akt signaling played roles in acute melatonin-induced neuroprotection (37). Moreover, the neuroprotective effects of melatonin against ischemic brain injury are mediated through the activation of PI3K/Akt (37). Previous study has noted that melatonin plays a potent neuroprotective role by preventing the injury-induced reduction of Akt and Bad phosphorylation (26). Other studies also showed that melatonin prevented the down-regulation of pAkt decrease and blocked neuron death in cases of brain injury (40, 41). These studies support the neuroprotective effects of melatonin that result from the prevention of injury-induced Akt inactivation. In the present study we used LY294002 (LY), which is in many cases a very effective inhibitor of Class I PI3K/Akt, to promote autophagy (23). Indeed, we demonstrated that melatonin prevented injury-induced decreases of Akt activation in the ipsilateral hemisphere from MCAO/reperfusion rats. Furthermore, melatonin-mediated inhibition of cerebral infarction and Beclin-1 expression is blocked by LY294002.

Sustained activation of neuronal Akt by melatonin is achieved through two distinct G protein–coupled receptors, which are high-affinity membrane-bound receptors, the MT1 and MT2 receptors (42, 43). Both melatonin receptors have been reported to be present in hippocampal areas such as the dentate gyrus, CA1, and CA3 (44). In the present study, melatonin-mediated inhibition of cerebral infarction and Beclin-1 expression is blocked by the MT2 melatonin–receptor antagonists, namely, 4P-PDOT and luzindole, as well as LY294002.

In contrast to Class I PI3Ks, Class III PI3Ks stimulate autophagy (45). An integral protein in the Class III PI3K pathway is Beclin-1, whose knockdown inhibits autophagy and sensitizes to starvation-induced cell death (46, 47). Meanwhile even though many pharmacological inhibitors such as 3-MA, wortmannin, and LY294002 were rigorously specific for PtdIns3Ks, they might not be specific modulators of autophagy (48). In this study, we injected the lentiviral vectors into the cerebral
ventricle to induce the overexpression of Beclin-1 in the rat brain. Expression of the reporter gene EGFP confirmed that the DNA supplied by the vectors was taken in and expressed within the adult rat brain (Fig. 4A). As predicted, treatment with Beclin-1—expressing lentiviral vectors compromises the biological effects of melatonin including inhibition of infarct volume and decreasing cell apoptosis marked by cleaved caspase-3 following a tMCAO (Fig. 4: C and D).

Furthermore, because it has been demonstrated that autophagic and apoptotic mechanisms could occur in the same injured neurons (49), autophagy may inhibit apoptosis (50, 51) or autophagy may promote apoptosis (52). In our study, the Beclin-1 and cleaved caspase-3 double-positive cells were increased in the peri-ischemic hippocampus after tMCAO. Beclin-1 overexpression significantly increased cleaved caspase-3 in the peri-ischemic area, which was inhibited by melatonin. This result suggests that melatonin mediates neuroprotection by inhibiting different forms of cell death such as autophagy and apoptosis, partially by reducing Beclin-1 expression.

In conclusion, our study highlights the importance of autophagy inhibition by the biological effects of melatonin in ischemic stroke. These data suggest that melatonin may inhibit the detrimental autophagic reaction at least partially through the Class I PI3K/Akt pathway in cerebral ischemia/reperfusion. The results expand our understanding of the neuroprotective mechanism of melatonin against ischemic brain injury, which may be a strategy for the treatment of the brain after a stroke.

Acknowledgments

This work was supported by the National Science Foundation of China (No. 81073087) and American Heart Association (12SDG12070174).

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