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Melatonin reduces OGD/R-induced neuron injury by regulating redox/inflammation/apoptosis signaling

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Abstract. – OBJECTIVE: To investigate the roles and underlying mechanisms of melatonin in oxygen-glucose deprivation/reoxygenation (OGD/R)-insulted SH SY5Y cells.

MATERIALS AND METHODS: SH SY5Y cells were cultured for OGD/R stimulation. Cell viability and cytotoxicity were measured by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, lactate dehydrogenase (LDH), and Hoechst 33258/propidium iodide (PI) staining assays. The mRNA levels of high mobility group box-1 (HMGB1), tumor necrosis factor α (TNF-α), and inducible nitric oxide synthase (iNOS) were analyzed by quantitative Real Time-PCR assays. Nitric oxide (NO) production was assessed by Griess reagent. Reactive oxygen species (ROS) production was detected by fluorescent probe. Malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and 8-hydroxy-2′-deoxyguanosine (8-OHdG) were examined by commercial kits. Cell apoptosis was analyzed by flow cytometry and caspase-3 activity. The protein levels were detected by Western blot.

RESULTS: Melatonin enhanced the viability and reduced the death and LDH release of OGD/R exposed SH SY5Y cells. Melatonin repressed the HMGB1, TNF-α, and iNOS mRNA expression, NO production, and nuclear factor κB (NF-κB) activation in OGD/R challenged SH SY5Y cells. Melatonin reduced the ROS, MDA, 4-HNE, and 8-OHdG contents but further enhanced the levels of the nuclear factor E2-related factor-2 (Nrf2) and heme oxygenase (HO-1). Melatonin-increased viability and melatonin-decreased LDH release were also mediated by the blockage of NF-κB or reversed by Nrf2 or HO-1 knockdown. Melatonin exerted antiapoptotic effect on OGD/R treated SH SY5Y cells partly by activating Akt signaling. OGD/R challenged SH SY5Y cell autophagy was also repressed by melatonin, as evidenced by the decreased levels of LC-II and beclin-1 and the increased phosphorylation of mammalian target of rapamycin (mTOR), p70 ribosomal protein S6 kinase (p70S6K), and eukaryotic initiation factor 4E binding protein 1 (4E-BP-1).

CONCLUSIONS: Melatonin protected SH SY5Y cells from OGD/R induced oxidative stress, inflammation, apoptosis, and autophagy by blocking NF-κB signaling and activating Nrf2/HO-1, Akt, and mTOR/p70S6K/4E-BP-1 pathways, thereby indicating that melatonin is a potential and novel therapeutic drug for ischemic stroke.

Key Words:

Melatonin, Oxygen-glucose deprivation/reoxygenation, SH-SY5Y cell, Oxidative stress, Inflammation, Autophagy.

Introduction

Ischemic stroke is a leading cause of disability and mortality in elderly people worldwide[1]. Stroke results from a transient or permanent reduction of cerebral blood flow, thereby leaving cerebral cells in hypoxic and ischemic states and leading to irreparable damages[2]. In subsequent reoxygenation, the restoration of blood supply can further aggravate neuronal injury, which is referred to as cerebral ischemia/reperfusion (I/R) injury[3]. Although many studies have investigated cerebral I/R lesion and explored neuroprotective agents, the underlying mechanisms of I/R damage remain poorly understood, and effective therapeutic drugs are still limited[4]. Thus, uncovering the detailed mechanisms and developing additional favorable drugs for cerebral I/R damnification are urgently needed.

Cerebral I/R induces a complex series of biochemical and molecular events in the brain, such as oxidative stress, inflammation, apoptosis, and autophagy, which ultimately result in irreversible brain injury[5-8]. The oxygen-glucose depri-

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vation/reoxygenation (OGD/R) cell model can successfully mimic the acute restriction of metabolites and oxygen supply that occurs during cerebral I/R. OGD/R can also cause oxidative stress by producing excessive reactive oxygen species (ROS) in SH-SY5Y cells[9]. Considering the importance of oxidative stress in OGD/R-led neuronal impairment, its alleviation by phase II antioxidant detoxifying enzymes, such as heme oxygenase (HO-1), is favorable to cerebral I/R lesion[10,11]. Nuclear factor E2-related factor-2 (Nrf2) is a transcription factor that binds to antioxidant response elements (AREs) in antioxidant and detoxification genes, including HO-1, and Nrf2/HO-1 signaling provides neuroprotection in ischemic stroke[12,13]. ROS overproduction during cerebral I/R triggers intensive inflammation and consequent neuronal death[14,15]. The inflammatory response to brain injury, especially nuclear factor κB (NF-κB) activation, strongly contributes to the pathogenesis of ischemic stroke[16]. Several downstream molecules of NF-κB increased by OGD/R, such as tumor necrosis factor α (TNF-α) and inducible NO synthase (iNOS), are heavily conducive to the loss of neurons[16]. NO synthesized by iNOS is a major proinflammatory mediator and a primary stimulus of neuronal apoptosis[17]. High mobility group box-1 (HMGB1) promotes the progression of cerebral I/R injury[18]. Programmed cell death, including apoptosis and autophagy, is highly involved in the pathogenesis of ischemic stroke[19]. In literature, phosphatidylitol-3-kinase (PI3K)/Akt signaling is involved in the inhibition of neuronal apoptosis during cerebral I/R[20]. The mammalian target of rapamycin (mTOR) signaling, which negatively regulates autophagy, is deactivated by OGD/R in neurons[21]. Therefore, the attenuation of inflammation, oxidative stress, apoptosis, and autophagy may be an effective therapeutic strategy for cerebral I/R injury.

Melatonin (N-acetyl-5-methoxytryptamine) is a secretary neurohormone that is primarily synthesized by the pineal gland[22]. Melatonin is a multifunctional agent with neuroprotective effects in cerebral I/R models *in vitro* and *in vivo*[23-26]. The neuroprotection of melatonin is associated with its highly potent free radical scavenging and anti-oxidative properties[27]. Melatonin also exerts anti-inflammatory effects by blocking NF-κB signaling in methamphetamine-treated or H_2O_2-treated SH-SY5Y cells[23,24]. Hu et al[25] reported that melatonin reduces hypoxic-ischemic brain injury-induced autophagy and apoptosis. However, the effects of melatonin on OGD/R-caused oxidative stress, inflammation, and cell death, as well as the underlying mechanisms, are still largely unclear.

In this study, we demonstrated that melatonin reduces OGD/R-induced toxicity and death in SH-SY5Y cells. OGD/R-augmented inflammation, oxidative stress, apoptosis, and autophagy of SH-SY5Y cells were attenuated by melatonin. The anti-inflammatory, antioxidative, antiapoptotic, and antiautophagic effects of melatonin were mediated by NF-κB, Nrf2/HO-1, PI3K/Akt, and mTOR signaling pathways, respectively. Overall, these findings revealed melatonin treatment as a novel strategy for abating neuronal oxidative stress, inflammation, apoptosis, and autophagy in cerebral I/R injury by regulating several signaling pathways.

Materials and Methods

Cell Culture

The human neuroblastoma SH-SY5Y cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in a 1:1 mixture of Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA) and Ham's F12 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (both from Sigma-Aldrich, St. Louis, MO, USA) in a humidified atmosphere at 37°C and 5% CO_2.

OGD/R Cell Model and Drug Treatments

An OGD/R-treated SH-SY5Y cell model was established to mimic cerebral I/R injury. For the OGD procedure, after washing twice with PBS, SH-SY5Y cells were cultured with glucose-free and serum-free DMEM/F12 medium in an anaerobic chamber with 95% N_2 and 5% CO_2 at 37°C for 3 h. Then, the cells were incubated in a normal DMEM/F12 medium under normoxic conditions for certain indicated times (6, 12, 18, 24, and 30 h) as reperfusion. For melatonin (Sigma-Aldrich, St. Louis, MO, USA) administration, the cultures were treated with melatonin (0.01, 0.1, 1, and 10 mM) immediately before reoxygenation. To investigate the inhibitory effects, we obtained small interfering RNA (siRNA) targeting human Nrf2 (siNrf2), HO-1 (siHO-1), and negative control (NC) siRNAs from Santa Cruz Biotechnology,

Inc. (Santa Cruz, CA, USA) and transfected them into SH-SY5Y cells with Lipofectamine® 2000 reagent (Invitrogen, Carlsbad, CA, USA) for 24 h before OGD/R exposure. NF-κB inhibitor BAY 11-7082 (10 μM), PI3K/Akt inhibitor LY294002 (10 μM), mTOR inhibitor rapamycin (10 μM), and autophagy inhibitor 3-methyladenine (3-MA, 1 mM) were provided by Sigma-Aldrich (St. Louis, MO, USA) and added to the culture medium for 30 min before OGD/R exposure. Untreated cells were used as the control.

Cell Viability Assay

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) assay was conducted to measure cell viability. In brief, SH-SY5Y cells were seeded into 96-well plates at 2×10^4 cells/well and allowed to adhere overnight. After the cells were subjected to the indicated treatments, 100 μL of MTT solution (5 mg/mL) was added to each well and incubated at 37°C for 4 h in the dark. Following the removal of the media and the addition of 150 μL of dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) to dissolve the formazan crystals, the absorbance was measured at 570 nm with a microplate reader (BioTek Instrument, Winooski, VT, USA). Cell viability was determined by calculating the absorbance ratio of the control cells.

Lactate Dehydrogenase (LDH) Assay

LDH activity was measured to evaluate cytotoxicity by using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China) according to the manufacturer's instructions. SH-SY5Y cells were subjected to the indicated treatments, and the supernatants were collected for the determination of LDH release. The absorbance was read on a microplate reader (BioTek Instrument, Winooski, VT, USA) at 450 nm.

Hoechst 33258 and Propidium Iodide (PI) Staining

Cell death was assessed by staining SH-SY5Y cells with propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA). After the indicated treatments, Hoechst dye was added to the culture medium and the samples were incubated at 37°C for 30 min. A PI solution was added just before the cells were observed with a fluorescence microscope (Olympus, Tokyo, Japan). PI-positive cells were counted as dead cells.

Quantitative Real-Time PCR (qPCR) Assay

Total RNA was isolated from SH-SY5Y cells by using the TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). Complementary DNA was reversely transcribed from 1 μg RNA by using a PrimerScript™ RT reagent kit (TaKaRa, Dalian, Liaoning, China). qRT-PCR reactions were performed using the SYBR Green reagent (Applied Biosystems Life Technologies, Foster City, CA, USA) in accordance with the manufacturer's instructions. Each reaction was carried out on an ABI 7500 Real-Time RT-PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). The primers for qRT-PCR assays are as follows: for HMGB1 5′-GGAGATCCTAAGAAGCCGAGA-3′ (forward) and 5′-C ATGGTCT TCCACCTCTCTGA-3′ (reverse); for TNF-α, 5′-ATGAGCACTGAAAG CATGATC CGG-3′ (forward) and 5′-GCAATGATCCCAAAGTAGACCTGCCC-3′ (reverse); for iNOS, 5′-AGAGAGATCGGGTTCACA-3′ (forward) and 5′-CACAGA ACTGAGGGTACA-3′ (reverse); and for glycerinaldehyde-3-phosphate-dehydrogenase (GAPDH), 5′-AGTGGGGTGATGCTGGTGCTG-3′ (forward) and 5′-CGCCTGCTTCACCACCTTCTT-3′ (reverse). The mRNA levels were normalized to the GAPDH by using the $2^{-\Delta\Delta Ct}$ method.

NO Production Measurement

The level of nitrite, which is the stable reaction product generated from NO with molecular oxygen, was used as an indicator of NO production. After the indicated treatments, the culture supernatants were collected and the nitrite levels were measured using Griess reagent (Cayman Chemical Co., Ann Arbor, MI, USA) following the manufacturer's instructions.

ROS Generation Determination

The fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA) was procured to monitor intracellular ROS production. SH-SY5Y cells with the indicated treatments were incubated with 20 μM DCFH-DA in the dark for 30 min at 37°C. After washing with PBS, the fluorescence level was measured by flow cytometry (BD FACSCanto, BD Biosciences, San Jose, CA, USA). Fluorescent intensity was expressed as the relative value compared with that of the control.

Measurement of Malondialdehyde (MDA), 4-hydroxynonenal (4-HNE), and 8-hydroxy-2'-deoxyguanosine (8-OHdG)

Oxidative stress-associated indicators, including MDA, 4-HNE, and 8-OHdG, were detected using commercial kits as per the manufacturers' instructions. After the indicated treatments, the SH-SY5Y cell supernatants were collected. The MDA content was measured using the kit from Jiancheng Bioengineering Institute (Nanjing, China). The 4-HNE and 8-OHdG levels were measured by using specific ELISA kits (Cell Biolabs, San Diego, CA, USA).

Flow Cytometric Detection of Cell Apoptosis

Cell apoptosis was ascertained by using an Annexin V-fluorescein isothiocyanate (FITC)/PI kit (BD Biosciences, San Jose, CA, USA). After the indicated treatments, SH-SY5Y cells were harvested, washed twice with ice-cold PBS, and then, suspended in 300 μL of binding buffer. A total of 5 μL Annexin V-FITC were added to the cells and incubated for 15 min in the dark at room temperature, followed by treatment with 5 μL of PI in the dark for 30 min. The stained cells were immediately analyzed by using flow cytometry (BD FACSCanto, BD Biosciences, San Jose, CA, USA). The apoptotic cell percentage was calculated.

Caspase-3 Activity Assay

Caspase-3 activity was measured using the Caspase Activity Kit (Beyotime Institute of Biotechnology, Haimen, Jiangsu, China) in accordance with the manufacturer's protocol. After the different treatments, SH-SY5Y cells were lysed and centrifuged. The supernatants were collected, and the reaction buffer containing the substrate peptides (acetyl-Asp-Glu-Val-Asp p-nitroanilide) was added. After incubation at 37°C for 2 h, the enzymatic activity was measured at 405 nm with a microplate reader (BioTek Instrument, Winooski, VT, USA).

Western Blot Analysis

Cytoplasmic and nuclear proteins were extracted from SH-SY5Y cells with the indicated treatments by using the nuclear and cytoplasmic extraction kits (Pierce Biotechnology, Inc., Rockford, IL, USA), following the manufacturer's instructions. Protein concentration was determined using a bicinchoninic acid assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal amounts of protein (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skim milk in Tris-Buffered Saline (TBST) containing 0.5% Tween-20 for 1 h at room temperature, and then, incubated with primary antibodies against NF-κB p65, phosphorylated (p)-NF-κB p65, Akt, p-Akt (Ser473), mTOR, p-mTOR (Ser2448), ribosomal protein S6 kinase (p70S6K), p-p70S6K (Thr389), eukaryotic initiation factor 4E binding protein 1 (4E-BP-1), p-4E-BP-1 (Ser65/Thr70, all from Cell Signaling Technology, Inc., Danvers, MA, USA), Lamin B, TNF-α, HMGB1, iNOS, Nrf2, HO-1, Bcl-2, Bax (all from Abcam, Cambridge, UK), cleaved (cl)-caspase-3, caspase-3, LC3, beclin-1, and β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. The membranes were washed thrice with TBST and incubated with horseradish peroxidase-conjugated appropriate secondary antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence reaction reagents (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Statistical Analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using One-way analysis of variance, followed by Dunnett's post-hoc test for multiple comparisons with SPSS 16.0 statistical software (SPSS, Inc., Chicago, IL, USA). $p<0.05$ was considered as statistically significant.

Results

Melatonin Protected SH-SY5Y Cells Against OGD/R-Induced Injury

To mimic neuronal insults during cerebral I/R, we exposed the SH-SY5Y cells to 3 h of OGD, followed by the indicated durations of reoxygenation. As shown in Figure 1A, MTT assay showed the progressive decline of cell viability over the reoxygenation compared with the control group. Meanwhile, LDH leakage significantly increased from 6 h to 30 h after reoxygenation (Figure 1B). We also conducted Hoechst/PI staining to check for OGD/R-induced SH-SY5Y

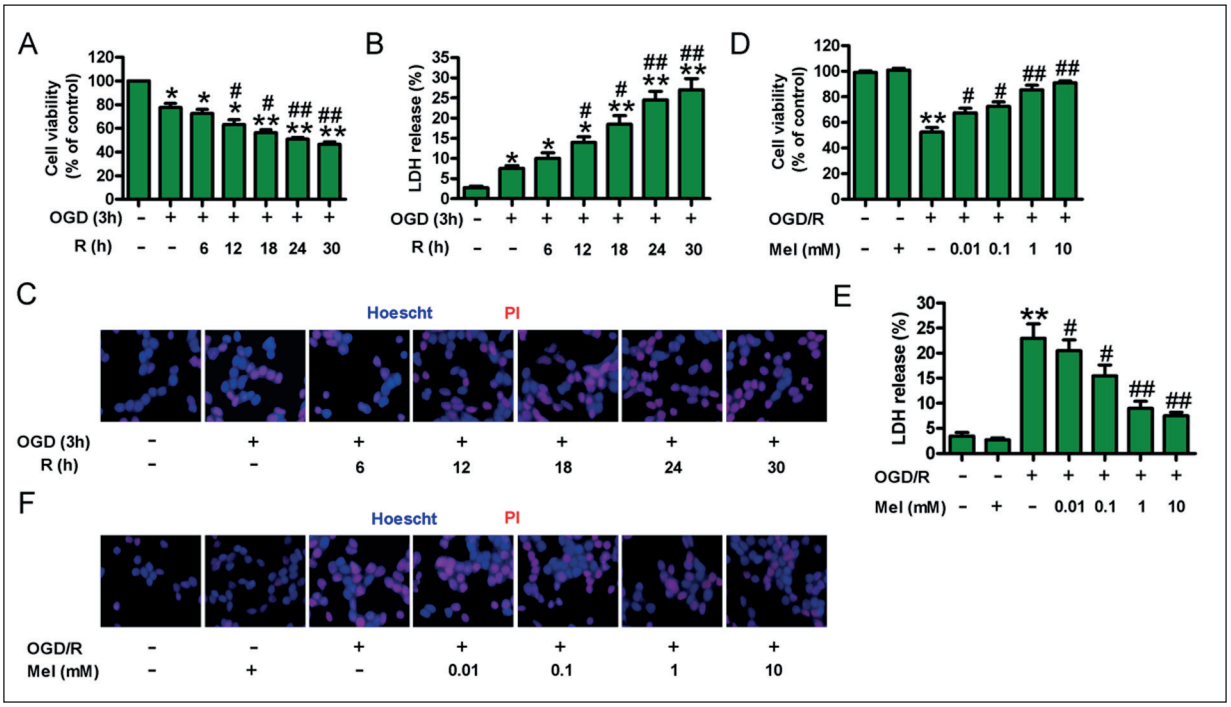

Figure 1. Melatonin improved viability and inhibited LDH release and apoptosis in OGD/R-insulted SH-SY5Y cells. **A-C**, SH-SY5Y cells were subjected to 3 h of OGD followed by reoxygenation for 6, 12, 18, 24, and 30 h. **A**, Cell viability was analyzed by MTT assay. **B**, Cytotoxicity was assessed by LDH release by using a commercial kit. **C**, Cell death was determined by Hoechst/PI staining. (50× magnification). **D-F**, SH-SY5Y cells were subjected to 3 h of OGD, followed by reoxygenation for 24 h. Melatonin (0.01, 0.1, 1, and 10 mM) was added to the culture medium immediately before reoxygenation. **D**, MTT, (**E**) LDH release, and (**F**) Hoechst/PI staining (50× magnification) assays were performed to detect cell viability, cytotoxicity, and cell death, respectively. The data expressed as mean ± SD from three independent experiments. *p<0.05, **p<0.01 *vs.* control group. $^{\#}p$<0.05, $^{\#\#}p$<0.01 *vs.* OGD group. OGD/R: oxygen-glucose deprivation/reperfusion; R: reoxygenation; Mel: melatonin.

cell death. The staining images revealed that PI-positive cells (dead cells) were evidently higher in OGD/R-exposed groups than in the control group (Figure 1C). Reoxygenation for 24 h was selected for the following experiments because the difference in cell viability, LDH leakage, and PI-positive cells among the groups reoxygenated for 24 h and 30 h was insignificant. To investigate the protective effects of melatonin on cell damage caused by OGD/R, we added different concentrations of melatonin (0.01, 0.1, 1, and 10 μM) to the culture medium immediately before reoxygenation. Melatonin significantly enhanced the viability (Figure 1D) and reduced the LDH leakage (Figure 1E) and death (Figure 1F) of the OGD/R-treated SH-SY5Y cells. Melatonin at 1 mM was selected for the subsequent studies due to its considerable beneficial effects against OGD/R-induced damages in SH-SY5Y cells and insignificant difference in 1 and 10 μM groups. These results demonstrated that melatonin attenuated OGD/R-induced injuries in SH-SY5Y cells.

Melatonin Inhibited OGD/R-Caused Inflammation via Inactivation of NF-κB in SH-SY5Y Cells

Inflammatory mediators, such as HMGB1 and TNF-α, play important roles in brain I/R injury[28,29]. Melatonin reduces proinflammatory factors in methamphetamine-challenged or H_2O_2-challenged SH-SY5Y cells[23,24]. To examine whether melatonin abates OGD/R-led inflammation in SH-SY5Y cells, we measured the HMGB1, TNF-α, and iNOS levels by qRT-PCR assays. The mRNA levels of HMGB1 (Figure 2A), TNF-α (Figure 2B), and iNOS (Figure 2C) were much higher in the OGD/R-stimulated SH-SY5Y cells than in the control cells. However, melatonin evidently attenuated the increase in HMGB1, TNF-α, and iNOS induced by OGD/R (Figure 2A-2C). Moreover, melatonin reduced OGD/R-enhanced NO production in SH-SY5Y cells (Figure 2D). NF-κB activation is necessary for the induction of proinflammatory mediators, including TNF-α and iNOS[30]. Melatonin can suppress metham-

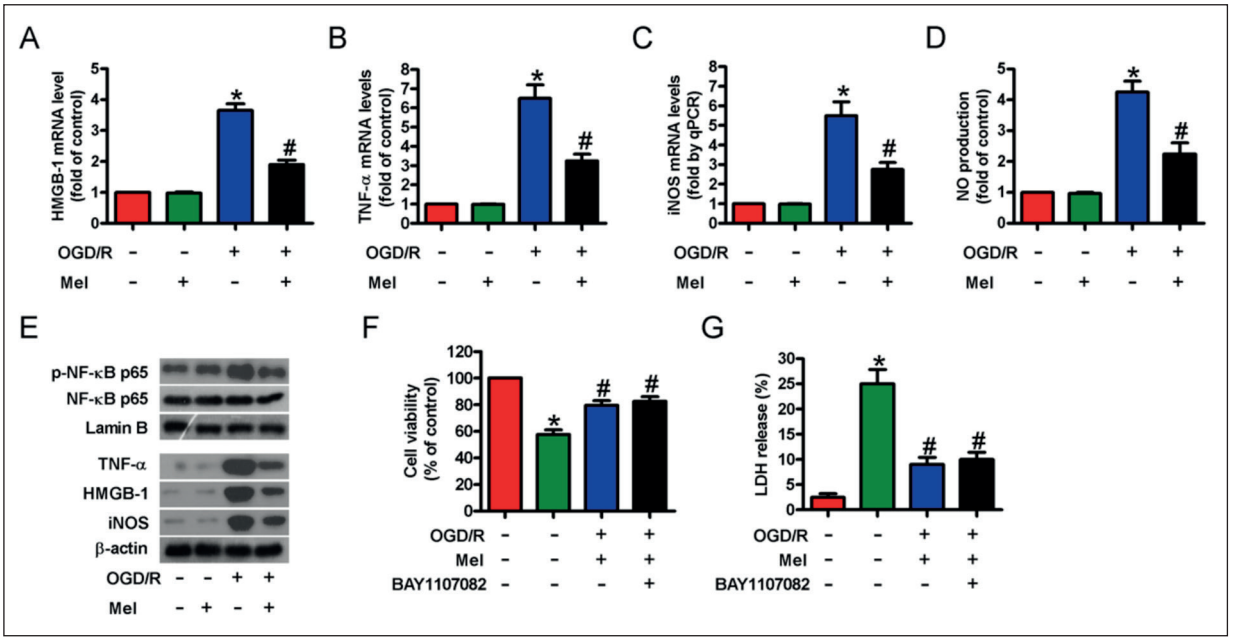

Figure 2. Melatonin reduced OGD/R-increased inflammation by blocking NF-κB activation in SH-SY5Y cells. **A-E**, SH-SY5Y cells were subjected to 3 h of OGD followed by reoxygenation for 24 h. Melatonin (1 mM) was added to the culture medium immediately before reoxygenation. qPCR assays were carried out to analyze the **A**, HMGB1, **B**, TNF-α, and **C**, iNOS mRNA levels. **D**, NO production was measured using Griess reagent. **E**, Representative Western blots of p-NF-κB p65, NF-κB p65, HMGB1, TNF-α, and iNOS. Lamin B and β-actin were used as the endogenous controls. **F**, **G**, SH-SY5Y cells were incubated with BAY 11-7082 (10 μM) for 30 min prior to exposure to OGD for 3 h, followed by 24 h of reperfusion. Melatonin (1 mM) was added to the culture medium at the initiation of reperfusion. **F**, Cell viability and **G**, cytotoxicity were evaluated by MTT and LDH release assays, respectively. The data are expressed as mean ± SD from three independent experiments. *$p<0.05$ *vs*. control group. $^{\#}p<0.05$ *vs*. OGD/R group. OGD/R: oxygen-glucose deprivation/reoxygenation; Mel: melatonin.

phetamine-induced or H_2O_2-induced NF-κB activation in SH-SY5Y cells[23,24]. As shown in Figure 2E, OGD/R considerably augmented NF-κB phosphorylation and the HMGB1, TNF-α, and iNOS protein expression levels compared with the control. The coincubation of OGD/R-insulted SH-SY5Y cells with NF-κB inhibitor BAY 11-7082 and melatonin significantly improved viability and diminished LDH release similar to the effect of melatonin alone (Figure 2F, 2G). These data indicated that melatonin hindered NF-κB activation to suppress OGD/R-triggered inflammation in SH-SY5Y cells.

Melatonin Decreased OGD/R-Induced Oxidative Stress by Activating Nrf2/HO-1 Signaling in SH-SY5Y Cells

To observe OGD/R-induced oxidative stress, we detected ROS production in SH-SY5Y cells. As shown in Figure 3A, OGD/R significantly increased ROS generation, which was reduced by melatonin. The levels of oxidative damage indicators, including MDA, 4-HNE, and 8-OHdG, were measured. The increased MDA (Figure 3B), 4-HNE (Figure 3C), and 8-OHdG contents (Figure 3D) in OGD/R-treated SH-SY5Y cells were significantly decreased by melatonin. Nrf2 signaling is activated and exerts antioxidative effects during OGD/R[13]. The Western blot analysis of SH-SY5Y cells showed that the Nrf2 and HO-1 levels significantly increased after OGD/R exposure, which were further increased by melatonin (Figure 3E). Nrf2 or HO-1 knockdown significantly counteracted the enhanced viability (Figure 3F) and decreased the LDH release (Figure 3G) caused by melatonin in OGD/R-exposed SH-SY5Y cells. These findings suggested that melatonin hampers OGD/R-induced oxidative stress depending on the activated Nrf2/HO-1 signaling in SH-SY5Y cells.

Melatonin Reduced OGD/R-Led SH-SY5Y Cell Apoptosis via Akt Signaling Activation

Apoptosis is involved in OGD/R-induced neuronal death[31]. Flow cytometry assay demonstrat-

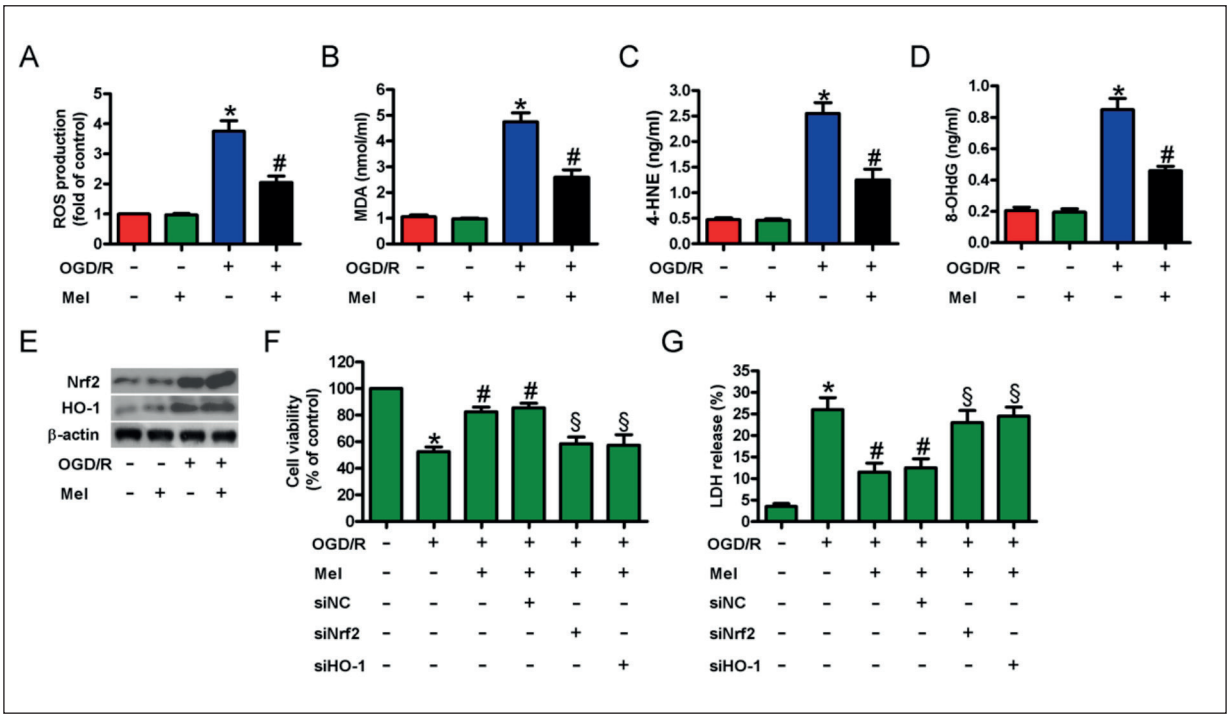

Figure 3. Melatonin repressed OGD/R-induced oxidative stress depending on Nrf2/HO-1 activation in SH-SY5Y cells. **A-E**, SH-SY5Y cells were subjected to 3 h of OGD, followed by reoxygenation for 24 h. Melatonin (1 mM) was added to the culture medium immediately before reoxygenation. **A**, ROS production was measured using DCFH-DA fluorescent probe. The **B**, MDA, **C**, 4-HNE, and **D**, 8-OHdG contents were assessed using commercial kits. **E**, Western blot was conducted to analyze the Nrf2 and HO-1 expression level. β-Actin was used as the endogenous control. **F**, **G**, SH-SY5Y cells were pretransfected with 100 μM of siNrf2, siHO-1, or NC for 24 h prior to treatment with OGD for 3 h, followed by 24 h of reperfusion. Melatonin (1 mM) was added to the culture medium at the initiation of reperfusion. **F**, MTT and **G**, LDH release assays were performed to evaluate cell viability and cytotoxicity, respectively. The data are expressed as mean ± SD from three independent experiments. *$p<0.05$ *vs.* control group. $^{\#}p<0.05$ *vs.* OGD/R group. $^{\S}p<0.05$ *vs.* melatonin group. OGD/R: oxygen-glucose deprivation/reoxygenation; Mel: melatonin.

ed that OGD/R induced more apoptotic SH-SY5Y cells than in the control group. However, melatonin substantially decreased OGD/R-triggered apoptosis of SH-SY5Y cells (Figure 4A, 4B). The increase in the activity and cleavage of caspase-3 in OGD/R-stimulated SH-SY5Y cells was also significant, whereas melatonin significantly inhibited caspase-3 activation after OGD/R treatment (Figure 4C, 4D). Western blot analysis showed a decreased Bcl-2 expression level and an increased Bax expression level in OGD/R-insulted SH-SY5Y cells, and these alterations were reversed by melatonin (Figure 4D). Akt signaling activation prevents OGD/R-caused neuronal apoptosis[20]. Melatonin remarkably enhanced the phosphorylation of Akt in OGD/R-exposed SH-SY5Y cells (Figure 4D). The preincubation of SH-SY5Y cells with PI3K/Akt inhibitor LY294002 neutralized melatonin-induced increase in p-Akt and Bcl-2 and decrease in Bax and cl-caspase-3 (Figure 4D). Melatonin also cannot enhance the viability (Figure 4E) and inhibit LDH release (Figure 4F) in OGD/R-challenged SH-SY5Y cells in the presence of LY294002. These results revealed that the abatement of OGD/R-induced apoptosis of SH-SY5Y cells by melatonin partly relied on Akt pathway activation.

Melatonin Activated mTOR Signaling to Abolish OGD/R-Induced Autophagy in SH-SY5Y Cells

OGD/R induces the autophagic death of SH-SY5Y cells[32]. mTOR signaling is involved in the inhibitory effect of melatonin on autophagy during liver I/R injury[33]. To elucidate whether melatonin suppresses the autophagy of OGD/R-challenged SH-SY5Y cells in an mTOR-dependent pathway, we analyzed the level of p-mTOR and the

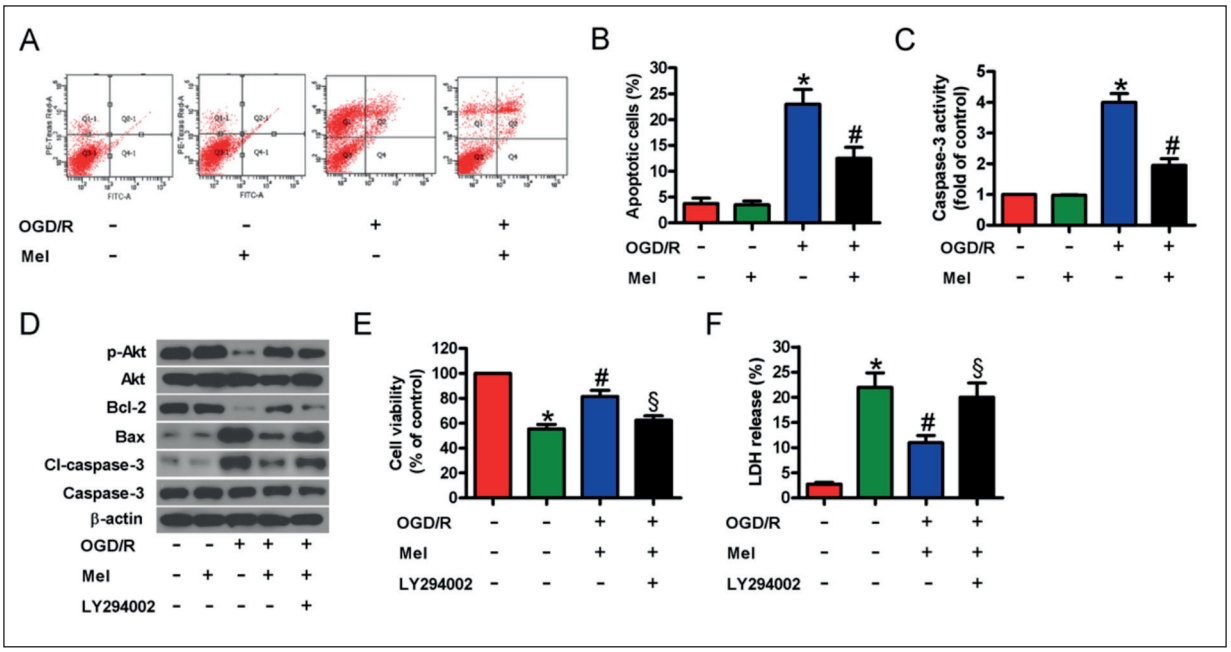

Figure 4. Melatonin suppressed OGD/R-triggered SH-SY5Y cell apoptosis by activating Akt signaling. **A-C**, SH-SY5Y cells were subjected to 3 h of OGD, followed by reoxygenation for 24 h. Melatonin (1 mM) was added to the culture medium immediately before reoxygenation. **A**, Flow cytometry assay was conducted to analyze cell apoptosis. **B**, Apoptotic cell percentage was calculated. **C**, Cell apoptosis was evaluated by caspase-3 activity assay. **D-F**, SH-SY5Y cells were pre-incubated with LY294002 (10 μM) for 30 min prior to exposure to OGD for 3 h, followed by 24 h of reperfusion. Melatonin (1 mM) was added to the culture medium at the initiation of reperfusion. **D**, Representative Western blots of p-Akt, Akt, Bcl-2, Bax, cl-caspase-3, and caspase-3. β-Actin was used as the endogenous control. **E**, Cell viability and **F**, cytotoxicity were evaluated by MTT and LDH release assays, respectively. The data are expressed as mean ± SD from three independent experiments. *$p<0.05$ *vs*. control group. $^{\#}p<0.05$ *vs*. OGD/R group. $^{\S}p<0.05$ *vs*. melatonin group. OGD/R: oxygen-glucose deprivation/reoxygenation; Mel: melatonin.

phosphorylation of p70S6K and 4E-BP-1, which are well-known downstream targets of mTOR, by Western blot assays. As shown in Figure 5A, mTOR, p70S6K, and 4E-BP-1 phosphorylation significantly decreased in OGD/R-exposed SH-SY5Y cells compared with the control cells, but this effect was attenuated by melatonin. OGD/R evidently increased the levels of LC3-II and beclin-1, which are indicators of autophagy; however, the enhancement was reduced by melatonin (Figure 5B). The incubation of OGD/R-exposed SH-SY5Y cells with the mTOR inhibitor rapamycin restored melatonin-decreased autophagy as revealed by the increased levels of LC3-II and beclin-1 (Figure 5B). Autophagy inhibitor 3-MA and melatonin co-treatment increased the viability (Figure 5C) and decreased LDH release (Figure 5D) of OGD/R-treated SH-SY5Y cells, paralleling with the effect of melatonin alone. These results revealed that melatonin hampered the OGD/R-induced autophagy of SH-SY5Y cells by activating mTOR signaling.

Discussion

In this study, melatonin prevented the OGD/R-induced damage of SH-SY5Y cells. The key findings are as follows. First, melatonin improved the survival and inhibited LDH release of OGD/R-exposed SH-SY5Y cells. Second, melatonin decreased the OGD/R-induced enhancement of HMGB1, TNF-α, and iNOS and the production of NO by blocking NF-κB signaling in SH-SY5Y cells. Third, melatonin suppressed the OGD/R-led oxidative stress in SH-SY5Y cells, as shown by the reduced levels of ROS, MDA, 4-HNE, and 8-OHdG involved in the activation of the Nrf2/HO-1 pathway. Fourth, melatonin repressed OGD/R-caused SH-SY5Y cell apoptosis, as shown by the reduction of caspase-3 activity, the decrease in the levels of cl-caspase-3 and Bax, and the increase in the expression of Bcl-2, by activating the PI3K/Akt pathway. Finally, melatonin inhibited the OGD/R-triggered autophagy of SH-SY5Y cells, as indicated by the decreased

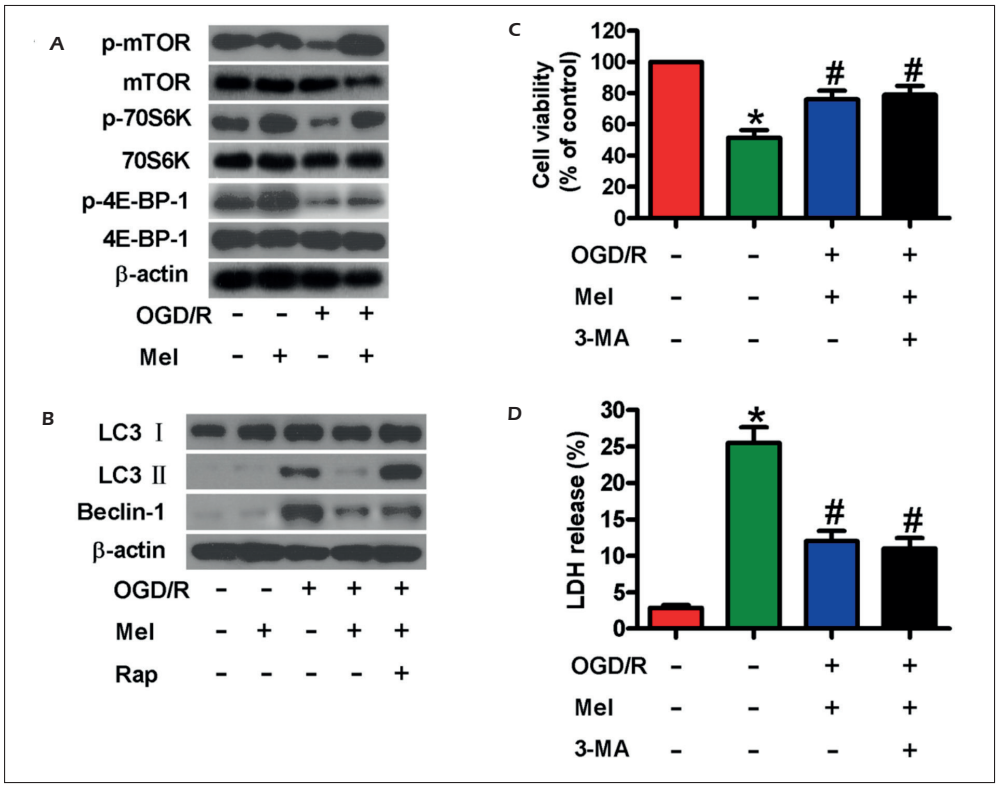

Figure 5. Melatonin abolished OGD/R-augmented autophagy of SH-SY5Y cells via mTOR signaling activation. **A**, SH-SY5Y cells were subjected to 3 h of OGD followed by reoxygenation for 24 h. Melatonin (1 mM) was added to the culture medium immediately before reoxygenation. Western blots were conducted to detect the p-mTOR, mTOR, p-p70S6K, p70S6K, p-4E-BP-1, and 4E-BP-1 expression level. β-Actin was used as the endogenous control. **B-D**, SH-SY5Y cells were preincubated with **B**, rapamycin (10 μM) or **C**, **D**, 3-MA (1 mM) for 30 min prior to treatment with OGD for 3 h, followed by 24 h of reperfusion. Melatonin (1 mM) was added to the culture medium at the initiation of reperfusion. **B**, Representative Western blots of LC3 and Beclin-1. β-Actin was used as the endogenous control. **C**, Cell viability and **D**, cytotoxicity were evaluated by MTT and LDH release assays, respectively. The data are expressed as mean ± SD from three independent experiments. *$p<0.05$ *vs.* control group. $^{\#}p<0.05$ *vs.* OGD/R group. OGD/R: oxygen-glucose deprivation/ reoxygenation; Mel: melatonin; Rap: rapamycin; 3-MA: 3-methyladenine.

levels of LC3-II and beclin-1 in the activation of the mTOR/p70S6K/4E-BP-1 signaling pathway. Collectively, melatonin attenuated the OGD/R-induced inflammation, oxidative stress, apoptosis, and autophagy of SH-SY5Y cells.

Inflammatory response plays an important role in the pathogenesis of neuronal injury and can be triggered after the onset of ischemic stroke[18]. Although microglia and astrocytes are the predominant sources of proinflammatory cytokines and chemokines in the central nervous system, neurons can also highly express these mediators in ischemic stroke[34,35]. Among these proinflammatory factors, HMGB1 promotes severe inflammatory reactions and is involved in the progression of brain I/R injury[28]. NO and TNF-α are the primary stimuli that induce neuronal apoptosis[17,36]. NF-κB is a pleiotropic transcription factor that encodes a diverse group of proinflammatory cytokines and iNOS expression. Melatonin also inhibits methamphetamine-induced or H_2O_2-induced inflammatory reactions *via* the deactivation of NF-κB signaling in SH-SY5Y cells[23,24]. Consistently, the results of the present study demonstrated that melatonin decreased the HMGB1, TNF-α, and iNOS expression levels; NO production; and NF-κB activation, thereby suggesting that NF-κB signaling inactivation was involved in the suppression of inflammation by melatonin in OGD/R-exposed SH-SY5Y cells.

The ROS generation during cerebral I/R can lead to severe brain damage[5]. The MDA produced in lipid peroxidation caused by ROS reflects oxidative injury of plasma membrane[37]. 4-HNE is one of the reaction products of lipid hydroperoxide break down, that occurs in

response to oxidative stress[38]. 8-OHdG, which is another marker of DNA damage, is produced when nucleic acid is frequently exposed to ROS[39]. Guo et al[40] reported increased ROS, 4-HNE, and 8-OHdG contents in the cortex of I/R-treated rats. Phase II enzymes, such as HO-1, can be induced by various oxidative agents for promoting anti-oxidative activities[10,11]. HO-1 knockout in mice results in severe cerebral damage compared with wild-type mice, thereby suggesting the neuroprotective role of HO-1 in cerebral I/R injury[41]. HO-1 expression is mainly controlled by Nrf2 by binding to its ARE in the promoter[42]. The involvement of Nrf2/HO-1 in neuroprotection has been found in I/R-treated rats and in OGD/R-exposed SH-SY5Y cells, and the silencing of Nrf2 or HO-1 reduces protocatechualdehyde-increased SH-SY5Y cell viability under OGD/R exposure[40]. Melatonin can activate Nrf2 in SH-SY5Y cells, and the protective effect of melatonin is significantly decreased in Nrf2-deficient mice[27]. In the present study, melatonin decreased the ROS, MDA, 4-HNE, and 8-OHdG levels but increased the Nrf2 and HO-1 expression level in OGD/R-exposed SH-SY5Y cells. This result indicated that Nrf2/HO-1 in the inhibitory effects of melatonin on OGD/R-induced oxidative stress in SH-SY5Y cells.

Apoptosis is implicated in ischemic brain damage and is a major mechanism of cell death resulting from cerebral I/R injury[7,43]. The Bax/Bcl-2 ratio is an important determinant of cell apoptosis after brain ischemia in senescence-accelerated mice[44]. Bax promotes cell death, whereas Bcl-2 hampers cell apoptosis. Caspase-3, which is the executor of apoptosis, can be activated by Bax homodimers[45]. When caspase-3 is activated, procaspase-3 is cut into an active fragment cleaved caspase-3, which plays the role of proteolytic enzymes to promote apoptosis. Cl-caspase-3, as a representative apoptosis marker, appears frequently in the cytosol during apoptosis[46]. PI3K/Akt signaling is associated with cell apoptosis during cerebral I/R injury[20]. In the present study, OGD/R significantly promoted the apoptosis of SH-SY5Y cells. Caspase-3 activity and cl-caspase-3 and Bax expression levels significantly increased, and Bcl-2 protein expression significantly decreased in OGD/R-exposed SH-SY5Y cells. However, these effects were remarkably reversed by melatonin treatment. The preincubation of OGD/R-treated SH-SY5Y cells with PI3K/Akt inhibitor LY294002 counteracted the effects of melatonin on the protein expression mentioned above, reduced the enhanced viability, and the decreased LDH release by melatonin. This result suggested that melatonin inhibited the apoptosis of OGD/R-exposed SH-SY5Y cells through the Akt-dependent caspase-3 activity reduction, cl-caspase-3, Bax downregulation, and Bcl-2 upregulation.

Autophagy is a process of self-degradation through an autophagosomal-lysosomal pathway. Autophagy is essential for neuronal development and remodeling, and defective autophagy can be critical in neurodegenerative diseases[47]. Autophagy has protective roles when it is activated by mild hypoxia or ischemia, whereas excessive autophagy caused by severe hypoxia or ischemia could be detrimental[48]. Some studies[49-51] suggested that autophagy plays a detrimental role during reperfusion in *in vitro* and *in vivo* models of brain I/R. In OGD/R-treated primary neurons, LC3-II and beclin-1 significantly increase after reoxygenation[21]. During autophagy, the cytosolic form LC3-I is converted to the phosphatidylethanolamine-conjugated form LC3-II to promote autophagosome formation[52]. The increased expression of LC3-II can be used as an indicator of autophagy activation[53]. Beclin-1, which is an autophagy-related protein, can mediate other autophagic proteins attached to autophagosome membranes and decrease LC3-II accumulation[54]. Therefore, beclin-1 is another key indicator of autophagy[55]. Previous studies state that mTOR/p70S6K/4E-BP-1 signaling contributes to the reduction of autophagy during neuronal OGD/R or liver I/R[21,33]. mTOR is implicated in the regulation of metabolic events, including autophagy[56]. mTOR stimulates protein synthesis by phosphorylating key translation regulators, such as p70S6K and 4E-BP-1[57]. The present study made clear that melatonin markedly enhances the phosphorylation of mTOR, p70S6K, and 4E-BP-1 and reduces the expression of LC3-II and beclin-1 in OGD/R-insulted SH-SY5Y cells. However, rapamycin preincubation abrogates the reduction of LC3-II and Beclin-1 induced by melatonin. The coincubation of OGD/R-exposed SH-SY5Y cells with autophagy inhibitor 3-MA and melatonin also increased cell viability and decreased LDH release, which was similar to the effect of melatonin alone. These results indicated that the antiautophagic effect of melatonin may be reliant on the activation of mTOR/p70S6K/4E-BP-1 signaling in OGD/R-exposed SH-SY5Y cells.

Conclusions

In summary, melatonin ameliorated OGD/R-induced inflammation, oxidative stress, apoptosis, and autophagy in SH-SY5Y cells simultaneously *via* the deactivation of NF-κB signaling and by activating the Nrf2, Akt, and mTOR pathways. The present findings highlighted an effective and novel strategy to ameliorate OGD/R-caused neuronal damage and provide favorable evidence for the development of melatonin as a therapeutic agent for cerebral I/R injury.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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References

1) Powers WJ, Rabinstein AA, Ackerson T, Adeoye OM, Bambakidis NC, Becker K, Biller J, Brown M, Demaerschalk BM, Hoh B, Jauch EC, Kidwell CS, Leslie-Mazwi TM, Ovbiagele B, Scott PA, Sheth KN, Southerland AM, Summers DV, Tirschwell DL. 2018 guidelines for the early management of patients with acute ischemic stroke: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke 2018; 49: e46-e110.
2) Zhao LP, JI C, Lu PH, Li C, Xu B, Gao H. Oxygen glucose deprivation (OGD)/re-oxygenation-induced in vitro neuronal cell death involves mitochondrial cyclophilin-D/P53 signaling axis. Neurochem Res 2013; 38: 705-713.
3) Gomis M, Davalos A. Recanalization and reperfusion therapies of acute Ischemic stroke: what have we learned, what are the major research questions, and where are we headed? Front Neurol 2014; 5: 226.
4) Kaur H, Prakash A, Medhi B. Drug therapy in stroke: from preclinical to clinical studies. Pharmacology 2013; 92: 324-334.
5) Crack PJ, Taylor JM. Reactive oxygen species and the modulation of stroke. Free Radic Biol Med 2005; 38: 1433-1444.
6) Zipp F, Aktas O. The brain as a target of inflammation: common pathways link inflammatory and neurodegenerative diseases. Trends Neurosci 2006; 29: 518-527.
7) Broughton BR, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. Stroke 2009; 40: e331-e339.
8) Ginet V, Puyal J, Clarke PG, Truttmann AC. Enhancement of autophagic flux after neonatal cerebral hypoxia-ischemia and its region-specific relationship to apoptotic mechanisms. Am J Pathol 2009; 175: 1962-1974.
9) Cheng MC, Pan TM. Glyceryl 1,3-dipalmitate produced from Lactobacillus paracasei subspecies. paracasei NTU 101 inhibits oxygen-dlucose deprivation and reperfusion-induced oxidative stress via upregulation of peroxisome proliferator-activated receptor gamma in neuronal SH-SY5Y cells. J Agric Food Chem 2017; 65: 7926-7933.
10) Shah ZA, Li RC, Ahmad AS, Kensler TW, Yamamoto M, Biswal S, Dore S. The flavanol (-)-epicatechin prevents stroke damage through the Nrf2/HO1 pathway. J Cereb Blood Flow Metab 2010; 30: 1951-1961.
11) Gao Y, Xu X, Chang S, Wang Y, Xu Y, Ran S, Huang Z, Li P, Li J, Zhang L, Saavedra JM, Liao H, Pang T. Totarol prevents neuronal injury in vitro and ameliorates brain ischemic stroke: potential roles of Akt activation and HO-1 induction. Toxicol Appl Pharmacol 2015; 289: 142-154.
12) Alfieri A, Srivastava S, Siow Rc, Cash D, Modo M, Duchen MR, Fraser PA, Williams SC, Mann GE. Sulforaphane preconditioning of the Nrf2/HO-1 defense pathway protects the cerebral vasculature against blood-brain barrier disruption and neurological deficits in stroke. Free Radic Biol Med 2013; 65: 1012-1022.
13) Park SY, Choi YW, Park G. Nrf2-mediated neuroprotection against oxygen-glucose deprivation/reperfusion injury by emodin via AMPK-dependent inhibition of GSK-3beta. J Pharm Pharmacol 2018; 70: 525-535.
14) Iadecola C, Alexander M. Cerebral ischemia and inflammation. Curr Opin Neurol 2001; 14: 89-94.
15) Sugawara T, Chan PH. Reactive oxygen radicals and pathogenesis of neuronal death after cerebral ischemia. Antioxid Redox Signal 2003; 5: 597-607.
16) Wang Q, Tang XN, Yenari MA. The inflammatory response in stroke. J Neuroimmunol 2007; 184: 53-68.
17) Brown GC. Nitric oxide and neuronal death. Nitric Oxide 2010; 23: 153-165.
18) Jiang WL, Xu Y, Zhang SP, Zhu HB, Hou J. Tricin 7-glucoside protects against experimental cerebral ischemia by reduction of NF-kappaB and HMGB1 expression. Eur J Pharm Sci 2012; 45: 50-57.
19) Mo ZT, Li WN. Icariin attenuates OGD/R-induced autophagy via Bcl-2-dependent cross talk between apoptosis and autophagy in PC12 cells. Evid Based Complement Alternat Med 2016; 2016: 4343084.
20) Shioda N, Han F, Fukunaga K. Role of Akt and ERK signaling in the neurogenesis following brain ischemia. Int Rev Neurobiol 2009; 85: 375-387.

21) Qin H, Tan W, Zhang Z, Bao L, Shen H, Wang F, Xu F, Wang Z. 15d-prostaglandin J2 protects cortical neurons against oxygen-glucose deprivation/reoxygenation injury: involvement of inhibiting autophagy through upregulation of Bcl-2. Cell Mol Neurobiol 2015; 35: 303-312.

22) Reiter RJ. Melatonin synthesis: multiplicity of regulation. Adv Exp Med Biol 1991; 294: 149-158.

23) Permpoonputtana K, Govitrapong P. The anti-inflammatory effect of melatonin on methamphetamine-induced proinflammatory mediators in human neuroblastoma dopamine SH-SY5Y cell lines. Neurotox Res 2013; 23: 189-199.

24) Nopparat C, Chantadul V, Permpoonputtana K and Govitrapong P. The anti-inflammatory effect of melatonin in SH-SY5Y neuroblastoma cells exposed to sublethal dose of hydrogen peroxide. Mech Ageing Dev 2017; 164: 49-60.

25) Hu Y, Wang Z, Liu Y, Pan S, Zhang H, Fang M, Jiang H, Yin J, Zou S, Li Z, Zhang H, Lin Z, Xiao J. Melatonin reduces hypoxic-ischaemic (HI) induced autophagy and apoptosis: an in vivo and in vitro investigation in experimental models of neonatal HI brain injury. Neurosci Lett 2017; 653: 105-112.

26) Kongsuphol P, Mukda S, Nopparat C, Villarroel A, Govitrapong P. Melatonin attenuates methamphetamine-induced deactivation of the mammalian target of rapamycin signaling to induce autophagy in SK-N-SH cells. J Pineal Res 2009; 46: 199-206.

27) Parada E, Buendia I, Leon R, Negredo P, Romero A, Cuadrado A, Lopez Mg, Egea J. Neuroprotective effect of melatonin against ischemia is partially mediated by alpha-7 nicotinic receptor modulation and HO-1 overexpression. J Pineal Res 2014; 56: 204-212.

28) Qiu J, Nishimura M, Wang Y, Sims JR, Qiu S, Savitz SI, Salomone S, Moskowitz MA. Early release of HMGB-1 from neurons after the onset of brain ischemia. J Cereb Blood Flow Metab 2008; 28: 927-938.

29) Yang QW, Lu FL, Zhou Y, Wang L, Zhong Q, Lin S, Xiang J, Li JC, Fang CQ, Wang JZ. HMBG1 mediates ischemia-reperfusion injury by TRIF-adaptor independent Toll-like receptor 4 signaling. J Cereb Blood Flow Metab 2011; 31: 593-605.

30) Beinke S, Ley SC. Functions of NF-kappaB1 and NF-kappaB2 in immune cell biology. Biochem J 2004; 382: 393-409.

31) Nakka VP, Gusain A, Mehta SL, Raghubir R. Molecular mechanisms of apoptosis in cerebral ischemia: multiple neuroprotective opportunities. Mol Neurobiol 2008; 37: 7-38.

32) Luo T, Liu G, Ma H, Lu B, Xu H, Wang Y, Wu J, Ge P, Liang J. Inhibition of autophagy via activation of PI3K/Akt pathway contributes to the protection of ginsenoside Rb1 against neuronal death caused by ischemic insults. Int J Mol Sci 2014; 15: 15426-15442.

33) Kang JW, Cho HI, Lee SM. Melatonin inhibits mTOR-dependent autophagy during liver ischemia/reperfusion. Cell Physiol Biochem 2014; 33: 23-36.

34) Liu T, Clark RK, McDonnell PC, Young PR, White RF, Barone FC, Feuerstein GZ. Tumor necrosis factor-alpha expression in ischemic neurons. Stroke 1994; 25: 1481-1488.

35) Janelsins MC, Mastrangelo MA, Park KM, Sudol KL, Narrow WC, Oddo S, Laferla FM, Callahan LM, Federoff HJ, BOWERS WJ. Chronic neuron-specific tumor necrosis factor-alpha expression enhances the local inflammatory environment ultimately leading to neuronal death in 3xTg-AD mice. Am J Pathol 2008; 173: 1768-1782.

36) Wajant H, Pfizenmaier K, Scheurich P. Tumor necrosis factor signaling. Cell Death Differ 2003; 10: 45-65.

37) Murakami K, Kondo T, Kawase M, Li Y, Sato S, Chen SF, Chan PH. Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency. J Neurosci 1998; 18: 205-213.

38) Cindric M, Cipak A, Zapletal E, Jaganjac M, Milkovic L, Waeg G, Stolc S, Zarkovic N, Suzana Borovic S. Stobadine attenuates impairment of an intestinal barrier model caused by 4-hydroxynonenal. Toxicol In Vitro 2013; 27: 426-432.

39) Valavanidis A, Vlachogianni T, Fiotakis C. 8-hydroxy-2'-deoxyguanosine (8-OHdG): a critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2009; 27: 120-139.

40) Guo C, Wang S, Duan J, Jia N, Zhu Y, Ding Y, Guan Y, Wei G, Yin Y, Xi M, Wen A. protocatechualdehyde protects against cerebral ischemia-reperfusion-induced oxidative injury via protein kinase cepsilon/Nrf2/HO-1 pathway. Mol Neurobiol 2017; 54: 833-845.

41) Kim YM, Pae HO, Park JE, Lee YC, Woo JM, Kim NH, Choi YK, Lee BS, Kim SR, Chung HT. Heme oxygenase in the regulation of vascular biology: from molecular mechanisms to therapeutic opportunities. Antioxid Redox Signal 2011; 14: 137-167.

42) Ishii T, Itoh K, Takahashi S, Sato H, Yanagawa T, Katoh Y, Bannai S, Yamamoto M. Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J Biol Chem 2000; 275: 16023-16029.

43) Kalogeris T, Baines CP, Krenz M, Korthuis RJ. Cell biology of ischemia/reperfusion injury. Int Rev Cell Mol Biol 2012; 298: 229-317.

44) Lu G, Wu Y, Mak YT, Wai SM, Feng ZT, Rudd JA, Yew DT. Molecular evidence of the neuroprotective effect of Ginkgo biloba (EGb761) using bax/bcl-2 ratio after brain ischemia in senescence-accelerated mice, strain prone-8. Brain Res 2006; 1090: 23-28.

45) Cregan SP, Maclaurin JG, Craig CG, Robertson GS, Nicholson DW, PARK DS, SLACK RS. Bax-depen-

dent caspase-3 activation is a key determinant in p53-induced apoptosis in neurons. J Neurosci 1999; 19: 7860-7869.

46) Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. Nat Rev Mol Cell Biol 2004; 5: 897-907.

47) Wong E, Cuervo AM. Autophagy gone awry in neurodegenerative diseases. Nat Neurosci 2010; 13: 805-811.

48) Xu M, Zhang HL. Death and survival of neuronal and astrocytic cells in ischemic brain injury: a role of autophagy. Acta Pharmacol Sin 2011; 32: 1089-1099.

49) Puyal J, Vaslin A, Mottier V, Clarke PG. Postischemic treatment of neonatal cerebral ischemia should target autophagy. Ann Neurol 2009; 66: 378-389.

50) Shi R, Weng J, Zhao L, Li XM, Gao TM, Kong J. Excessive autophagy contributes to neuron death in cerebral ischemia. CNS Neurosci Ther 2012; 18: 250-260.

51) Mehta SL, Kumari S, Mendelev N, Li PA. Selenium preserves mitochondrial function, stimulates mitochondrial biogenesis, and reduces infarct volume after focal cerebral ischemia. BMC Neurosci 2012; 13: 79.

52) Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J 2000; 19: 5720-5728.

53) Zhang Z, Singh R, Aschner M. Methods for the detection of autophagy in mammalian cells. Curr Protoc Toxicol 2016; 69: 20.12.21-20.12.26.

54) Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, Hibshoosh H, Levine B. Induction of autophagy and inhibition of tumorigenesis by beclin 1. Nature 1999; 402: 672-676.

55) Mei Y, Glover K, Su M, Sinha SC. Conformational flexibility of BECN1: essential to its key role in autophagy and beyond. Protein Sci 2016; 25: 1767-1785.

56) Yang Z, Klionsky DJ. Mammalian autophagy: core molecular machinery and signaling regulation. Curr Opin Cell Biol 2010; 22: 124-131.

57) Asnaghi L, Bruno P, Priulla M, Nicolin A. mTOR: a protein kinase switching between life and death. Pharmacol Res 2004; 50: 545-549.