Original Paper

Melatonin Attenuates Myocardial Ischemia/Reperfusion Injury by Inhibiting Autophagy Via an AMPK/mTOR Signaling Pathway

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Key Words

Melatonin • Ischemia/reperfusion injury • Autophagy • AMPK • mTOR

Abstract

Background/Aims: Melatonin has been demonstrated to protect cardiac microvascular endothelial cells (CMECs) against ischemia/reperfusion injury (IRI). Autophagy plays different roles in the heart during ischemia and reperfusion. The AMP activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) pathway is associated with autophagy. This study sought to explore whether melatonin regulates CMEC autophagy through the AMPK/mTOR signaling pathway. **Methods:** The effects of melatonin in IRI were investigated *in vivo* rat models and *in vitro* neonatal CMECs. Myocardial infarct size was achieved by Evans blue and triphenyltetrazolium chloride staining. The severity of cell injury was evaluated by cell vitality and lactate dehydrogenase (LDH) release assays, and autophagy was evaluated by transmission electron microscopy and the assessment of autophagy-related gene expression, such as that of Beclin 1 and light chain 3-II. **Results:** *In vivo*, melatonin markedly reduced infarcted area, improved cardiac function and decreased LDH release. However, the AMPK activator AICAR and the mTOR inhibitor rapamycin reduced the protective effects of melatonin on IRI. *In vitro*, Beclin1 and light chain 3-II protein were found to be down-regulated and autophagosomes were found to be reduced in response to melatonin, together with an increase in cell vitality and a decrease in LDH. Treatment with AICAR or rapamycin ablated the benefit observed with melatonin treatment. **Conclusions:** Melatonin played an important and protective role in CMECs by inhibiting autophagy against IRI via the AMPK/mTOR system.

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Introduction

Cardiac microvascular endothelial cells (CMECs) are the predominant cell type damaged immediately after myocardial ischemia/reperfusion injury (IRI) [1]. Autophagy plays different roles in the heart during ischemia and reperfusion [2]. The AMP activated protein kinase/mammalian target of rapamycin (AMPK/mTOR) pathway is associated with autophagy [3, 4]. Melatonin is the main indoleamine produced by the pineal gland; it is a well-known antioxidant and free radical scavenger [5]. Several studies have shown that melatonin protects against IRI [6, 7]. In our previous study, melatonin protected CMECs against oxidative stress injury by activation of the MAPK/ERK signaling pathway [8]. The aim of the present study was to investigate whether melatonin regulates CMEC autophagy through the AMPK/mTOR signaling pathway.

Materials and Methods

Animal models of cardiac IRI

Sprague-Dawley (SD) rats (250-300 g) were purchased from the Experimental Animal Center of PLA General Hospital, Beijing, China (Approval No. SCXK 20120001). All animal procedures and experiments were conducted in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. SD rats were anesthetized with 10% chloral hydrate before endotracheal intubation. IRI was induced by ligating the left anterior descending artery (LAD) as previously reported [9]. Thirty rats were randomized into five groups: (i) sham group (thoracotomy without ligation); (ii) control group (subjected to ischemia for 30 min and reperfusion for 2 h (IRI)); (iii) melatonin group (treated with melatonin (20 mg/kg i.p.) 12 h before IRI) [10]; (iv) melatonin + 5-aminoimidazole-4-carboxamide-1- β -D-ribofuranoside (AICAR) group (melatonin (20 mg/kg i.p.) and AICAR (0.7 mg/g i.p.) 12 h before IRI) [11]; (v) melatonin + rapamycin group (treated with melatonin (20 mg/kg i.p.) and rapamycin (1mg/kg i.p.) 12 h before IRI) [12]. At the end of the reperfusion period, echocardiography was performed by echocardiogram (14.0 MHz, Sequoia C512; Acuson, Germany). Hearts were removed and stained with 2% Evans blue and 1% triphenyltetrazolium chloride. Infarct size was expressed as percentage of the total left ventricular (LV) area (n=6/group).

Lactate dehydrogenase release

Lactate dehydrogenase (LDH) is a fairly stable enzyme that is released from the cytosol into the culture medium as a consequence of cellular integrity damage. We used an LDH assay (Beyotime Institute of Biotechnology, Shanghai, China) to evaluate the presence of cell injury or damage.

CMEC isolation, culture and in vitro IRI assay

CMECs were isolated from the hearts of neonatal SD rats (age: 5-7 days, weight: 12-16 g) using an enzyme dissociation method described in our previous study [8]. Cultured cell purity was evaluated using morphological characteristics and immunohistochemistry. CMEC monolayers displayed uniform 'cobblestone' morphology and demonstrated positive (>95%) staining for factor VIII (Abcam, #ab61910) and CD31 (Xinxingtang Biotechnology, #Xt-0468R) by immunohistochemistry [13]. *In vitro* IRI was simulated by 30 min hypoxia with serum starvation followed by 2 h of reoxygenation [14]. Hypoxic conditions were created by replacing culture medium with Hanks solution and exposing the cells to 95% N₂ and 5% CO₂. The pH was adjusted to 6.8 with lactate to mimic ischemic conditions. For drug treatment, cultured CMECs were pre-incubated with 1 μ M, 5 μ M or 10 μ M melatonin for 12 h before inducing IRI. To evaluate whether the AMPK/mTOR pathway was involved in the protective effect of melatonin, CMECs were treated with AICAR (0.5 mM) 24 h before inducing IRI or with rapamycin (50 ng/mL) addition 30 min before inducing IRI [14, 15]. The MTT assay was used to evaluate cell viability. Absorbance was measured at 450 nm using a microtiter plate reader (Bio-Rad, USA). Cellular ATP levels were determined using an ATP Assay Kit (Beyotime Institute of Biotechnology, China) according to the manufacturer's protocol [16].

Western blots

Following experimental treatment, CMECs were lysed with RIPA lysis buffer (Beyotime, China) for 30 min and centrifuged at 14,000 $\times$ g for 30 min. Equal amounts of protein were loaded into wells of a 10%

sodium dodecyl sulfate-polyacrylamide gel. Proteins were separated by gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). Membranes were blocked with 5% milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) at room temperature for 1 h followed by overnight incubation at 4°C with the following primary antibodies: anti-Becn1 (1:1000), anti-light chain 3 (LC3) I/II (1:1000), anti-AMPK (1:1000), anti-phospho-AMPK- α (1:1000), anti-mTOR (1:1000), anti-phospho-mTOR (1:500) and anti- β -actin (1:1000). After overnight incubation, membranes were washed with TBST and further incubated with the appropriate secondary antibody at 37°C for 60 min. Membranes were developed with an enhanced chemiluminescence reagent.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse transcribed with the one-step RT-PCR kit (TransGen Biotech Co., Ltd., China) according to the manufacturer's instructions. The primers used for polymerase chain reaction were as follows: Becn1, forward - 5'-GGCAGTGGCTCCTATT-3', reverse - 5'-GGCGTGTCTGTCTCTGAAAA-3'; β -actin, forward - 5'-GATGGTGGGTATGGGTCAGAAGGAC-3', reverse - 5'-GCTCATTTGCCGATAGTGATGACT-3'. Quantification of gene expression was performed using the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Green (TransGen Biotech Co., Ltd., China). mRNA levels were determined by qRT-PCR in triplicate for each of the independently prepared RNAs and were normalized to β -actin expression.

Detection of autophagosomes by transmission electron microscopy

Transmission electron microscopy, the standard method to detect autophagy, was used to further clarify the effects of melatonin on autophagy. CMECs were collected, fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4°C for 2 h then post-fixed in 1% osmic acid at 4°C for 1.5 h. Cells were washed with PBS, dehydrated by a serial gradient ethanol and embedded in Embed-812 medium (Electron Microscopy Sciences). The resulting blocks were cut into ultrathin sections using an Ultramicrotome (Leica, Reichert Ultracuts) then stained with 0.2% lead citrate/1% uranyl acetate. The ultrastructure of the cells was then observed under a transmission electron microscope (JEM1230; JEOL, Japan).

Statistical analysis

Data are described as the mean $\pm$ standard deviation (SD) of at least three independent experiments and were analyzed by one-way analysis of variance (ANOVA). The limit of statistical significance between treated and control groups was $P < 0.05$.

Results

Melatonin reverses IRI in vivo

As shown in Fig. 1A, mice treated with melatonin developed smaller infarcts (expressed as a percentage of the total LV). However, the beneficial effects were not observed when melatonin was used with AICAR or rapamycin. LDH release was significantly increased in response to IRI and melatonin significantly reduced IRI-induced LDH release. Melatonin improved cardiac function in the context of IRI as evidenced by an improvement in left ventricular ejection fraction (LVEF). However, AICAR and rapamycin reduced the protective effects of melatonin on cardiac function (Fig. 1B).

Becn1 mRNA and the ratio of LC3-II/I protein in IRI were downregulated by melatonin in vivo

Becn1 mRNA was upregulated in the infarcted myocardium after IRI, however, this IRI-induced upregulation was blocked in the group of rats that were pretreated with melatonin (Fig. 1C). After IRI, the ratio of LC3-II/I protein in the infarcted myocardium of the control group was significantly increased (vs. non-IRI rats). In the group pre-treated with melatonin, this ratio was significantly lower compared with the control group. However, treatment with AICAR or rapamycin ablated the benefit observed with melatonin treatment (Fig. 1D).

Fig. 1. Melatonin reduced infarct size and preserved cardiac function following ischemia/reperfusion injury in vivo which was performed by 30 min of ischemia followed by 2 h reperfusion (n=6). (A) Results of infarct size and lactate dehydrogenase (LDH). (B) Result of left ventricular ejection fraction (LVEF). (C) Result of Beclin1 mRNA expression (infarcted myocardium). (D) Results of LC3-I and LC3-II protein expression, and the ratio of LC3-II/I was calculated to demonstrate the autophagy level (infarcted myocardium). *P<0.05 vs Sham, # P<0.05 vs Con, &P<0.05 vs Mel.

Fig. 2. Effects of melatonin on cell vitality and autophagy in CMECs (n=3). (A) Cell vitality was measured with MTT method. (B) LDH level of culture serum. (C) Result of Beclin1 mRNA expression. (D) Result of Beclin1 protein expression. (E) Results of LC3-I and LC3-II protein expression. *P<0.05 vs Con, # P<0.05 vs IRI.

Melatonin treatment attenuated IRI-induced injury in vitro

CMECs exposed to simulated IRI demonstrated a significant decrease in cell viability and a significant increase in LDH release compared with CMECs not exposed to IRI. To evaluate the protective effect of melatonin, CMECs were treated with melatonin before IRI. Cell viability was increased and LDH release was decreased after IRI in the melatonin treated group compared with the untreated group (Fig. s 2A and 2B). Cell viability in the 10 μ M melatonin group was not significantly higher than in the 5 μ M melatonin group ($P > 0.05$). Therefore, subsequent experiments were performed using melatonin at a concentration of 5 μ M.

IRI induced CMEC autophagy was attenuated by melatonin

CMECs were subjected to simulated IRI and autophagy was evaluated using autophagosome markers Beclin 1 and LC3-II/I ratio. Beclin 1 mRNA levels were determined by qRT-PCR and protein levels of Beclin 1 and LC3-I and -II were determined by western blotting. The ratio of LC3-II/I was used to measure the relative autophagy level of CMECs. Beclin 1 mRNA and protein along with the ratio of LC3-II/I were found to be increased by IRI in untreated cells but in CMECs treated with melatonin prior to IRI, the levels were decreased relative to the untreated cells (Fig. s 2C-E).

Effect of melatonin on AMPK/mTOR signaling

Western blot was performed to determine whether melatonin induced the expression and phosphorylation of AMPK and mTOR in CMECs. As shown in Fig. 3A, AMPK and mTOR signaling was detected in CMECs. Melatonin treatment significantly decreased AMPK phosphorylation and increased mTOR phosphorylation without inducing the expression of AMPK or mTOR. The AMPK activator AICAR (0.5 mM) was applied with and without melatonin for 24 h. A significant increase in AMPK phosphorylation was observed, as well as inhibition of the effects of melatonin on the phosphorylation of AMPK and mTOR in the presence of IRI ($P < 0.05$). We also investigated the effects of rapamycin on the phosphorylation of AMPK. We found that 50 ng/mL rapamycin activated the phosphorylation of AMPK (Fig. 3B). These data indicate that melatonin modulates the AMPK/mTOR signaling pathway.

Melatonin inhibited CMEC autophagy via AMPK/mTOR activation

To examine the effect of AMPK or mTOR on CMECs, the cells were pretreated with AICAR or rapamycin, with or without IRI. The ratio of LC3-II/I was increased by AICAR or rapamycin,

Fig. 3. Effects of melatonin on the phosphorylation of AMPK and mTOR in CMECs ($n=3$). (A) The effects of AICAR on the phosphorylation of AMPK and mTOR. Cells were pre-treated with or without AICAR (0.5 mM) for 24 h and stimulated with or without melatonin (5 μ M) before IRI ($n=3$). * $P < 0.05$ vs Con (without IRI), # $P < 0.05$ vs the values of cells treated with melatonin alone, $\Delta P < 0.05$ vs the values of cells after IRI, & $P < 0.05$ vs the values of cells treated with melatonin alone after IRI. (B) The effects of rapamycin on the phosphorylation of AMPK and mTOR ($n=3$). * $P < 0.05$ vs Con (without IRI), $\Delta P < 0.05$ vs the values of cells after IRI.

Fig. 4. The effects of melatonin and AMPK/mTOR pathway inhibitors on cell vitality, autophagy and cellular ATP levels in CMECs (n=3). (A) Cell vitality was measured with MTT method. (B) LDH level of culture serum. (C) Comparing with melatonin group, the ratio of LC3-II/I was increased by AICAR or rapamycin. (D) Result of cellular ATP level. *P<0.05 vs Con (without IRI), #P<0.05 vs the values of cells treated with melatonin alone, ΔP<0.05 vs the values of cells after IRI, &P<0.05 vs the values of cells treated with melatonin alone after IRI.

together with an increase in LDH release and a decrease in cell viability (without IRI). To demonstrate whether melatonin inhibited CMEC autophagy via AMPK or mTOR, the CMECs were treated with AICAR or rapamycin before IRI. In that experiment, LDH was increased and cell viability was decreased compared with the melatonin group. In addition, the ratio of LC3- II/I was increased by AICAR or rapamycin, which attenuated the inhibitory effect of melatonin on autophagy (with IRI) (Fig. 4A-C). Autophagosomes observed by transmission electron microscopy were significantly reduced by melatonin (Fig. 5).

Effects of melatonin and AMPK/mTOR pathway inhibitors on CMEC ATP levels

AMPK is a metabolic regulator that balances net energy production with the prevailing metabolic demands in eukaryotic cells. AMPK is activated by a decrease in intracellular ATP levels under different stress conditions (e.g., glucose deprivation, hypoxia, ischemia) [17]. Our experiments demonstrated that melatonin increased cellular ATP levels after IRI. AICAR and rapamycin inhibited the melatonin induced increase in cellular ATP levels. These results suggest that the effect of melatonin on cellular ATP levels might be dependent on AMPK/mTOR signaling (Fig. 4D).

Fig. 5. The effects of melatonin and AMPK/mTOR pathway inhibitors on autophagosomes. (A) Autophagosomes were observed by transmission electron microscope without IRI. (B) Autophagosomes were increased after IRI. (C) Autophagosomes were decreased by melatonin. (D) The inhibitory effect of melatonin on autophagy was attenuated by AICAR. (E) The inhibitory effect of melatonin on autophagy was attenuated by rapamycin. Magnification, $\times 30000$. Red arrows indicate autophagosomes.

Discussion

In the present study, we investigated the effects of melatonin on CMEC autophagy after IRI and the molecular mechanism by which these effects were produced. Our results suggest that the decrease of CMEC autophagy by melatonin is mediated, at least in part, by AMPK/mTOR signaling.

Autophagy has been demonstrated to be involved in many physiological and pathological processes. The effect of melatonin on autophagy has been investigated more recently [18–20]. Yoo and Jeung showed that melatonin suppresses CsA-mediated autophagy in rat pituitary GH3 cells [18]. San-Miguel et al. demonstrated that melatonin inhibits autophagy and endoplasmic reticulum stress in mice with carbon tetrachloride-induced fibrosis [19].

Autophagy is promoted by AMPK, which is a key energy sensor and regulates cellular metabolism to maintain energy homeostasis [21]. Conversely, autophagy is inhibited by mTOR, a central cell-growth regulator that integrates growth factor and nutrient signals [22]. Xie et al. found that melatonin ameliorates cardiac hypertrophy caused by chronic intermittent hypoxia by inducing autophagy via AMPK activation [23]. However, Kwon et al. showed that melatonin treatment inhibited AMPK activity and exerted synergistic effects on their neuroprotective properties [24]. The phosphorylation of AMPK was blocked by treatment with melatonin and the AMPK-activating compound, AICAR, induced neuronal cell death in HT22 hippocampal cells. Our experiments demonstrated that melatonin increased cell viability and inhibited the phosphorylation of AMPK. Furthermore, we used AICAR to enhance the phosphorylation of AMPK. We found that AICAR reduced the effects of melatonin on AMPK phosphorylation and inhibited the effects of melatonin on CMEC autophagy in the context of IRI response. Our data suggest that melatonin inhibits CMEC autophagy through AMPK signaling.

A study by Behram et al. showed that melatonin may have a significant protective effect on primordial follicles via activation of the mTOR pathway [25]. The findings of Kang, et al. suggest that melatonin downregulates autophagy via activation of mTOR signaling which may in turn contribute to its protective effects in liver IRI [26]. Our data show that melatonin activated mTOR. The AMPK/mTOR pathway antagonists, AICAR and rapamycin,

Fig. 6. Schematic representation showing that melatonin inhibited CMEC autophagy through an AMPK/mTOR signaling pathway. Melatonin increased the ATP/AMP ratio, which in turn inhibited AMPK and subsequently activated mTOR. These effects subsequently inhibited CMEC autophagy.

decreased the melatonin-induced phosphorylation of mTOR and increased CMEC autophagy. Taken together, melatonin increased the ATP/AMP ratio, which in turn inhibited AMPK and subsequently activated mTOR. These effects subsequently inhibited CMEC autophagy (Fig. 6).

Conclusion

In summary, our study demonstrated that melatonin played an important and protective role in CMECs by inhibiting autophagy against IRI via the AMPK/mTOR system.

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Disclosure Statement

The authors declared no potential conflicts of interest with respect to the research, authorship, or publication of this article.

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