A Novel Protective Mechanism for Melatonin Against Acute Lung Injury: Preserving Mitochondrial Dynamic Equilibrium of Lung Epithelial Cells Through SIRT3-Dependent Deacetylation of SOD2

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Research Article

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Abstract

Mitochondrial dynamic equilibrium of lung epithelial cells is disturbed during sepsis, which contributes to abnormal mitochondrial function and acute lung injury (ALI). Melatonin is one primary hormone secreted by the pineal gland, displaying favorable antioxidative actions in sepsis and cardiopulmonary disease. However, the potential roles and molecular basis of melatonin in lipopolysaccharide (LPS)-treated lung epithelial cells have not been explored and reported. Herein, we investigated whether melatonin could protect against sepsis-induced ALI and lipopolysaccharide (LPS)-treated lung epithelial cells through mitochondrial dynamic equilibrium as well as its possible molecular targets. Wild type and Sirt3 knockout mice were instilled with LPS intratracheally for 12 hours to construct an in vivo ALI model. And A549 lung epithelial cells were used to explore the possible roles of melatonin in vitro by incubating with small interfering RNA (siRNA) against Sirt3. To figure out the involvement of melatonin receptor, si Mtnr1b and luzindole were used in cells and mice. Melatonin pretreatment significantly inhibited pathological injury, inflammatory response, oxidative stress and apoptosis in LPS-treated lung tissues and LPS-treated lung epithelial cells. Meanwhile, melatonin also shifted the dynamic course of mitochondria from fission into fusion in LPS-treated lung epithelial cells in vivo and in vitro. However, SIRT3 inhibition abolished the protective roles of melatonin in ALI. Mechanistically, we found that melatonin increased the activity and expression of SIRT3, which further promoted the deacetylation of SOD2 at K122 and K68. More importantly, melatonin exerted pulmonary protection by activating MTNR1B but not MTNR1A in ALI. Collectively, melatonin could preserve mitochondrial dynamic equilibrium of lung epithelial cells through the deacetylation of SOD2 in a SIRT3-dependent manner, which eventually alleviated LPS-elicited injury, inflammation, oxidative stress, apoptosis. Thus, melatonin may serve as a promising candidate against ALI in the future.

1. Introduction

Acute lung injury (ALI) is a compelling clinical condition related with the development of multiple organ dysfunction, which could rapidly deteriorate into acute respiratory distress syndrome (ARDS) and even give rise to death unless treated properly and promptly[1–3]. ALI is featured by the activation of inflammatory cell, dysfunction/disruption of alveolar epithelial barrier, as well as edema, which is usually resulted from sepsis, mechanical ventilation, ischemia reperfusion, trauma and drug toxicity[4]. Among these conditions, sepsis is the most common and concerned cause. During sepsis, lipopolysaccharide (LPS) released from gram-negative bacterial cell wall directly activates toll-like receptor (TLR4) and triggers a cascade of signal transduction pathway, resulting inflammation, oxidative stress and various regulated cell death in inflammatory cells, lung epithelial cells and endothelial cells[5, 6]. Thus, molecules or drugs selectively blocking the above processes would be of critical therapeutic significance in sepsis-induced ALI.

Mitochondrion as a double-membrane organelle is mainly responsible for energy metabolism and the production of adenosine triphosphate[7, 8]. In recent years, the roles of mitochondrion in cell signaling
transduction, intracellular calcium homeostasis, gene expression, as well as regulated cell death involving apoptosis, pyroptosis, ferroptosis and autophagy have been well studied and explored[7, 9]. Consistent adverse stimulation can lead to mitochondrial dysfunction characterized by excessive reactive oxygen species (ROS) generation, cytochrome c release and ATP deficiency, which further impairs mitochondrial quality control capacity and triggers cell death[10]. Mitochondrial dynamics is one of the critical mechanisms contributing to mitochondrial quality control by maintaining the balance between fission and fusion, determining cell fate and homeostasis[11]. Emerging evidence has unveiled that mitochondrial dynamic equilibrium plays nonnegligible roles in sepsis and organ failure. In sepsis-induced ALI, mitochondrial abnormalities as well as dynamic dysfunction could trigger lung epithelial cell death and barrier breakdown by dysregulated dynamin-related protein 1 (Drp1), Fis1, mitofusin 1 (Mfn1), Mfn2 and optic atrophy 1 (Opa1)[12, 13]. Hence, endogenous or exogenous drugs which could reverse the abnormal expression of these proteins may act as possible candidates to combat ALI.

SIRT3 (sirtuin 3) serves as the critical mitochondrial acetyl-lysine deacetylase, which modulates mitochondrial function as well as biosynthetic pathways involving glucose and fatty acid metabolism, tricarboxylic acid cycle, oxidative stress, and mitochondrial dynamic equilibrium by deacetylating protein lysine. Full-length SIRT3 is distributed in the nucleus under physiological state while it could translocate to mitochondria as N-terminal SIRT3 once stimulated by external adverse factors[14, 15]. Mitochondrial antioxidant enzyme superoxide dismutase (SOD2) is mainly responsible for scavenging free radicals by converting free radicals into hydrogen peroxide and water[16]. Decreased SIRT3 expression and activity may give rise to SOD2 inactivation by deacetylating SOD2 at K68 and K122[17]. Previous study has figured out SIRT3 activation may mitigate sepsis-induced ALI by improving mitochondrial bioenergetic and redox homeostasis[18]. These facts hinted that targeting SIRT3-SOD2 axis is an important therapeutic strategy for ALI treatment.

Melatonin (5 methoxy-N-acetyltryptamine) was firstly isolated and identified from bovine pineal in 1960s[19]. Apart from pineal gland, melatonin could also be secreted by cerebellum, bone marrow cells, retina, skin, platelets as well as lymphocytes in vertebrate species[20]. Melatonin displays vital pharmacological actions in various diseases including cardiovascular disease, respiratory disease, digestive disease, and cancers[21]. Many of these pharmacological effects from melatonin are attributed to SIRT3-mediated antioxidant activity. For example, melatonin could protect against cerebral and myocardial ischemia/reperfusion injury by activating SIRT3[22, 23]. In terms of sepsis-induced ALI, some studies have explored and reported the potential roles of melatonin. For instance, melatonin could not only directly block the activation of NLRP3 inflammasome in LPS-treated macrophages but also inhibit the release of extracellular histones[24]. In lung epithelial cells, melatonin effectively blocked LPS-induced epithelial-mesenchymal transition (EMT), by upregulating Nrf2 in a PI3K/GSK-3β-dependent manner[25]. Additionally, melatonin enhanced epithelial sodium channel-mediated alveolar fluid clearance by activating SIRT1 in LPS-treated lung epithelial cells[26]. However, these studies did not systematically analyze the potential mechanisms of melatonin against LPS-induced ALI using RNA-seq when exploring the possible roles of melatonin. Based on these realities, we aimed to explore the potential effects and
molecular basis of melatonin in LPS-induced ALI by combining bioinformatics analysis with experimental validation.

2. Materials And Methods

2.1 Regents and antibodies

Lipopolysaccharide (LPS) purified by phenol extraction (0111:B4) was obtained from Sigma-Aldrich Co., Ltd (#L2630, Shanghai, China). Melatonin (#HY-B0075) with the purity 99.47% and Luzindole (N-0774) (#HY-101254) with the purity 98.48% were purchased from MedChemExpress Co., Ltd (Shanghai, China). Cell counting kit-8 (CCK-8) was provided by Dojindo Laboratories (# CK04, Kumamoto, Japan). TUNEL assay kit was obtained from Beyotime Biotechnology (Shanghai, China). ROS fluorescence probe was provided by Servicebio (Wuhan, China). Primary antibodies against GAPDH (#ab8245), TNF-α (#ab183218), GPX4(#ab125066), Bax(#ab32503), Bcl-2(#ab32124), cleaved-caspase3 (C-Caspase3) (#ab32042), SOD2/MnSOD (acetyl K122) (#ab214675), SOD2/MnSOD (acetyl K68) (#ab137037), SIRT3 (#ab246522), Mfn1(#ab221661), Mfn2(#ab124773), Opa1(#ab157457), Drp1(#ab184247) and Fis1(#ab229969) were purchased from Abcam (Cambridge, UK). Primary antibodies against pan Acetyl-Lysine (#A2391), MTNR1A (#A13030) and MTNR1B (#A7823) were obtained from ABclonal (Wuhan, China). Secondary antibodies were provided by LI-COR Biosciences (Lincoln, United States). The assay kits for SIRT3 activity, SOD2 activity, SOD activity, TBARS content, and LDH content were obtained from Abcam (Cambridge, UK). NADPH oxidase activity assay kits were provided by Jihe Biotechnology Co., Ltd (Shanghai, China). Trypsin-EDTA (0.25%) phenol red, Ham’s F-12K medium containing 10% fetal bovine serum (FBS) was obtained from Invitrogen-Gibco (Grand Island, NY). All chemical reagents used are of analytical grade.

2.2 Animal experiments

Male C57BL/6 mice (8–10 weeks old, 23.3~25.5g) were provided by the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences (Beijing, China) and Sirt3 knockout mice possessing C57BL/6 background (8–10 weeks old, 22.4~24.2g) were purchased from Nanjing Gem Pharmatech (Nanjing, China). The mice were kept in the Animal Center of Renmin Hospital of Wuhan University, which was a specific pathogen-free (SPF) barrier system with humidity (45–55%) and temperature (20–25°C) on a regular 12 h light/dark cycle. All animal experiments carried out in the study were performed in compliance with the Guidelines for Care and Use of Laboratory Animals published by the US National Institutes of Health (revised 1996). To mimic the ALI upon LPS exposure, the mice were intratracheally injected with LPS (5mg/kg) dissolved in 50 µL sterile saline as reported previously[27]. Mice were received intraperitoneal injections of a high dosage of melatonin (30 mg/kg body weight/day) and a low dosage of melatonin (5 mg/kg body weight/day) for 1 week before LPS injection. To block the MTNR1B, luzindole (2-benzylN-acetyltryptamine, dissolved in DMSO) was injected intraperitoneally (5 mg/kg body weight/day) for 1 week before LPS injection as described[28]. The mice were sacrificed by cervical dislocation after LPS injection for 12h under deep anesthesia. Then the intact left lungs were excised and
instilled with 10% neutral buffer formalin to keep the alveoli intumescent. And the right lungs were quickly stored into liquid nitrogen for subsequent biochemical analysis after removed and cut into pieces.

### 2.3 Cell culture and treatment

The A549 lung epithelial cells provided by American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Ham’s F-12K medium containing 10% FBS in a 5% CO$_2$ incubator at 37°C. To downregulate SIRT3 or MTNR1B, the lung epithelial cells were infected with the small interfering RNA (siRNA) (GenePharma Co. Ltd., Shanghai, China). According to the manufacturer’s instructions, the lung epithelial cells were incubated with Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher Scientific, Waltham, MA, USA) before siRNA transfection, respectively. The A549 lung epithelial cells were incubated with LPS at the concentration of 100 µg/mL for 6 hours to establish an *in vitro* ALI model.

### 2.4 Quantitative real-time PCR

The total RNA from the frozen lung tissues or cells was isolated using TRIZOL reagent according to the manufacturer’s instructions. To begin with, 1 ml of Trizol reagent was added per 5-10x10$^6$ cells after PBS washing, or per 100mg frozen lung tissues homogenate. After incubating at room temperature for 10 minutes, 0.2 ml of chloroform was added per 1 ml of TRizol, then the tubes were shaken vigorously for 15 seconds and incubated for 5 minutes. Next, the tubes were centrifuged at 4°C, 12000rpm for 15 minutes, after which the colorless upper aqueous phase, about 400 µl, was removed and kept. The colorless upper aqueous phase was then added with 0.4 ml of isopropyl alcohol, incubated 10 minutes at room temperature, and centrifuged at 4°C, 12000rpm for 10 minutes. The supernatant was removed, 1ml of 75% ethanol was added to the RNA pellet, and the sample was vortexed and centrifuged at 4°C, 12000rpm for 5 minutes. Finally, the RNA pellet was air-dried for 10 minutes, then 20 µl RNase free water was added to dissolve the total RNA. Reverse transcription was then performed using Transcriptor First Strand cDNA Synthesis Kit, then cDNA was subjected to quantitative real-time PCR with a Light Cycler 480 SYBR Green I Master Mix. Thermal cycling conditions were as follows: 5 minutes at 95°C, 40 cycles of 30 seconds at 95°C, and 60 seconds at 60°C. The expression levels of RNA were normalized to *Gapdh* expression and the obtained data was analyzed with the $2^{-\Delta\Delta Ct}$ method.

### 2.5 Western blot

To begin with, the mass of lung tissue was weighed and the number of cells in the culture dish was calculated. Then, an appropriate amount of RIPA lysate and protease inhibitor were added to lyse the lung tissue or cells to extract protein, the protein concentration of which was determined using BCA method. Then the samples were diluted to a certain concentration for subsequent electrophoresis. The concentration of separation gel and concentrated gel were prepared according to the molecular weight of the protein to be detected. After polyacrylamide gel electrophoresis, the protein was transferred to polyvinylidene fluoride (PVDF) membrane, and 5% of the skim milk powder was placed at room temperature for 2 hours. The primary antibodies were added and incubated at 4 °C overnight. The dilution ratio of primary antibodies was displayed as follows: Drp1(1:1000), Fis1(1:500), Mfn1 (1:300), Mfn2(1:400), Opa1(1:1000), BAX (1:500), BCL-2(1:500), C-Caspase3(1:400), SOD2-K22-Ac (1:400), SOD2-
K68-Ac (1:400), SOD2(1:1000), MTNR1A (1:1000), MTNR1B (1:1000), SIRT3(1:1000) and GAPDH (1:5000)). After washed by TBST 3 times, the corresponding secondary antibodies were added to incubate the bands at room temperature for 1.5 hours. and the dilution ratio was 1:10000. After that, ECL luminescent solution was added onto the protein bands away from light for 1 minutes, which were visualized via a ChemiDoc™ XRS + system. At last, the gray value of each band was semi-quantified by an Image Lab software (Bio-Rad Laboratories, Inc.).

2.6 Lung wet/dry ratio

After the left lung was excised from mice, intrapulmonary blood was removed at once. And the wet weight of each lung was detected and recorded. After that, these lung tissues were dried using an oven (80°C) for 4 days until the weight kept constant. Then, the dry weight of each lung was recorded and the lung wet/dry ratio was calculated.

2.7 Hematoxylin & eosin (H&E) staining

After dewaxed in xylene, the lung tissue sections were rehydrated using graded ethanol. Then, the sections were stained using hematoxylin for a few minutes and rinsed by tap water, followed by the differentiation with 1% hydrochloric acid alcohol (100 ml 75% ethanol+1 ml concentrated hydrochloric acid) for 3-5 seconds. The differentiation was stopped once dark blue discoloration occurred in the section. After rinsed with clean water, the sections were stained with eosin for 2-3 minutes. The lung tissue sections were then carefully observed and photographed under a microscope. Finally, the lung pathological injury was scored according to the previous description[29].

2.8 Immunohistochemical and immunofluorescence staining

For immunohistochemical staining, rehydrated lung tissue sections were subjected to antigen retrieval by microwave heating (10-15 minutes) in citrate buffer (pH 6), and endogenous peroxidases were blocked by incubation with 3% H$_2$O$_2$ for 10 minutes. Then, these sections were saturated in PBS supplemented with 5% goat serum for 1 hour at room temperature. After that, the sections were incubated with primary antibodies in blocking solution at 4°C overnight, which were further processed with biotinylated secondary antibodies at 37°C for 1 h. At last, these sections were incubated with diaminobenzidine for 5 minutes at room temperature. The lung tissue sections were observed under a microscope and were quantified using Image J software.

Immunofluorescence staining was carried out on the basis of previous description[30]. In brief, the paraffin-embedded lung tissue sections were fixed with 4% formaldehyde and permeabilized with Triton-X 100 (2%). Then the sections were incubated with 8-OHdG primary antibody after blocked with 10% goat serum. Subsequently, the target protein was probed with the Alexa Fluor 488-goat anti-rabbit secondary antibody and the cell nucleus was probed with DAPI.

2.9 Detection of oxidative stress
The levels of reactive oxygen species (ROS) in lung tissues and lung epithelial cells were detected using ROS probe and DCFH-DA probe, respectively. To detect the level of ROS in lung tissues, the frozen lung sections were incubated with AutoFluo quencher to block the autofluorescence. After that, the lung sections were incubated with ROS fluorescence probe at 37°C for 30 minutes in the dark. To detect the level of ROS in lung epithelial cells, 2,7-Dichlorodi-hydrofluorescein diacetate (DCFH-DA probe) (25µM) was added to mark intracellular ROS at 37°C for 30 minutes in the dark. The cell nucleus in both lung tissues and lung epithelial cells were stained with DAPI. Finally, the levels of ROS fluorescence intensity in lung tissues and lung epithelial cells were photographed via an Olympus DX51 fluorescence microscope (Tokyo, Japan).

Additionally, the markers of oxidative stress including SOD activity, TBARS content and NADPH oxidase activity were also detected using commercial assay kits according to the instructions.

### 2.10 RNA-sequence and analysis

Total RNA was isolated from LPS-treated murine lung tissues with or without melatonin (30 mg/kg body weight/day) using TRIzol Reagent and RNeasy min kits. RNA purity and integrity were assessed using 2100 Bioanalyzer. RNA-seq libraries were built on the basis of NEBNext Ultra II Directional RNA Library Prep Kit and sequenced via an Illumina HiSeq 2500 System. Subsequently, RNA-seq libraries and sequencing were carried out via the CNICs Genomics Unit. The web tool RaNA seq was used to process and analyze FastQ sequencing files. Then, the differentially expressed genes (DEGs) were determined via moderating t-test with Limma and adjusted by Benjamini-Hochberg \((P<0.05)\). And the DEGs were then analyzed for GO and KEGG.

### 2.11 Transmission electron microscopy

Fresh lung tissues (1mm x 1mm x 1mm) were fixed in certain solution containing 2.5% glutaraldehyde in 0.1 M phosphate buffer (PH 7.4) in beforehand tubes for 4 hours once they were excised from the mice. Subsequently, the lung tissues were permeated, dehydrated and embedded in acetone and sectioned approximately at 70nm as described[31]. At last, the ultrathin sections were stained with 3% uranyl acetate and lead citrate and viewed under a transmission electron microscope (HITACHI, Japan) at 80 KV. And the number of mitochondrial and mean mitochondrial size in lung epithelial cells were recorded.

### 2.13 Co-immunoprecipitation

After the lung tissue was weighed and ground, immunoprecipitation lysate was added and centrifuged at 13000rpm at 4°C for 10-15 minutes before supernatant was taken. The obtained supernatant was divided into a small part as an input, and the remaining target antibody was added and placed in a flip mixer for reaction overnight at 4°C. The above antibo-antigen binding complex was then mixed with the cleaned Protein A/G Plus MaqPoly Beads and reacted at 4°C overnight. After magnetic separation, the magnetic bead - antibody - antigen complex was added into 2X SDS-page Loading Buffer to mix evenly.
and heated at 95°C for 15min. After the second magnetic separation, the supernatant was taken and analyzed referring to the steps in western blot.

**2.14 Cell viability**

Cell viability was measured using a commercial CCK-8 assay kit on the basis of instruction. In brief, 10 µL of CCK-8 working solution was added to each well of a 96-well plate and incubated at 37°C for 2 hours. Cell viability was detected using an ELISA reader through measuring absorbance at 450 nm.

**2.15 JC-1 staining**

JC-1 fluorescent probe was used to assess the mitochondrial membrane potential in lung epithelial cells. The lung epithelial cells in each group were incubated with JC-1 working solution for 20 minutes at 37°C. Subsequently, the lung epithelial cells were washed using JC-1 buffer solution three times. The degree of mitochondrial membrane potential was expressed as the ratio of red to green fluorescence intensity.

**2.16 TUNEL staining**

Cell slides or frozen sections were fixed with 4% paraformaldehyde for 30 minutes and sealed with blocking solution from the same source as antibody at room temperature for 30 minutes. TUNEL apoptosis kit was used to determine the degree of cell death according to the instructions. The nuclei were stained with DAPI. The apoptotic nuclei and total nuclei were observed under the immunofluorescence microscopy.

**2.17 Statistical analysis**

All data in this study are expressed as the mean±standard deviation (SD) and analyzed using SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with Tukey post hoc test was used for comparisons among 3 or more groups while unpaired Student's t-test was used for comparisons between two groups. Statistical significance was set at $P<0.05$.

**3. Results**

**3.1 Melatonin alleviated LPS-induced inflammation, oxidative stress and apoptosis in murine lung tissues**

To begin with, the effects of a low (5 mg/kg) and a high (30 mg/kg) dosage of melatonin on LPS-induced lung pathological injury and edema were detected. As shown in Figure 1A-C, lung injury score and lung wet/dry ratio in both LPS+LD group and LPS+HD group were significantly lower than those in LPS group.
And both the low and the high dosages of melatonin could decrease LDH activity in serum from LPS-treated mice (Figure 1D). Meanwhile, two dosages of melatonin obviously decreased inflammatory response in LPS-induced murine lung tissues, as evidenced by decreased levels of IL-1β, TNF-α and IL-6 (Figure 1E-H). Additionally, LPS stimulation significantly reduced the level of antioxidant enzyme glutathione peroxidase 4 (GPX4) and SOD activity in lung tissues, apart from increasing the level of TBARS and NADPH oxidase activity (Figure 1I-L). As expected, melatonin pretreatment could decrease oxidative stress in lung tissues from LPS-treated mice. ROS probe staining further demonstrated the antioxidant effect from melatonin on LPS-induced ALI (Figure 1M-N). Finally, the levels of apoptosis in each group were also investigated. TUNEL staining and western blots showed that both the low and the high dosages of melatonin suppressed apoptosis in lung tissues from LPS-treated mice (Figure 1O-Q). Collectively, these results uncovered that melatonin could relieve LPS-induced inflammation, oxidative stress and apoptosis in murine lung tissues.

3.2 Mitochondrial quality control was involved in the protective roles of melatonin in LPS-induced ALI in vivo

To further explore potential mechanisms contributing to the protective roles of melatonin in LPS-induced ALI, the transcriptomics of each group was analyzed via RNA sequencing next. We first screened 596 DEGs between LPS group and LPS+HD group, followed by GO analysis for these DEGs. As shown in Figure 2A, GO analysis revealed these genes were associated with the mitochondrial fission, mitochondrial fusion, and oxidative stress. Hence, we next detected mitochondrial DNA damage in each group. The results showed that the levels of 8-hydroxy-deoxyguanosine (8-HOdG) in both LPS+HD group and LPS+LD group were significantly lower than that in LPS group, suggesting that melatonin owned the potential against LPS-induced mitochondrial DNA damage (Figure 2B-C). Next, we detected the mRNA levels and protein levels of genes associated with mitochondrial quality control (Figure 2D-I). As shown, melatonin treatment significantly increased the expression of Mfn1, Mfn2 and Opa1, but decreased the expression of Drp1 and Fis1 in LPS-treated mice, hinting that melatonin restrained mitochondrial fission and enhanced mitochondrial fusion. In line with these data, transmission electron microscopy further proved that melatonin treatment could shift the dynamic course of mitochondria from fission into fusion during ALI (Figure 2J-L). Taken together, all these findings suggested that melatonin could modulate mitochondrial quality control and protect against mitochondria damage in the context of LPS-induced ALI in vivo.

3.3 Melatonin inhibited the deacetylation of SOD2 by activating SIRT3 in LPS-induced ALI in vivo

Next, the top 20 DEGs with the highest fold change between LPS group and LPS+HD group were analyzed. Among these DEGs, SIRT3 served the most critical gene associated with mitochondrial quality control (Figure 3A). Previous studies have unveiled that SIRT3 in the mitochondrial matrix could deacetylate SOD2 at K68 and K122, thus participating in mitochondrial quality control[32, 33].
Subsequently, we detected the expression and activity of SIRT3 and SOD2 in each group. The results showed that LPS stimulation significantly decreased the expression as well as the activity of SIRT3, which could be partially reversed after melatonin treatment (Figure 3B-C). And melatonin also inhibited the LPS-induced acetylation of SOD2 at K68 and K122 in murine lung tissues. Although the total protein level of SOD2 in lung tissues was not affected by LPS or melatonin, SOD2 activity was significantly enhanced after melatonin treatment in LPS-treated mice (Figure 3D-E). Further, co-immunoprecipitation showed that LPS stimulation significantly decreased the interaction between SOD2 and SIRT3 compared to the control group. After melatonin treatment, the interaction between SOD2 and SIRT3 was significantly enhanced in LPS-treated mice (Figure 3F). The above results suggested that melatonin may exert pulmonary protection by promoting the deacetylation of SOD2 at K68 and K122, which may be mediated by SIRT3 activation.

3.4 Melatonin prevented LPS-induced lung epithelial cell damage through SIRT3-dependent deacetylation of SOD2

To further confirm the protective effects of melatonin in LPS-induced ALI, the A549 lung epithelial cells were employed to mimic in vitro ALI model. As shown in Figure 4A-B, melatonin treatment enhanced cell viability and blocked LDH release in a dose-dependent manner in LPS-treated lung epithelial cells. Next, we selected 5µM and 20µM as low dose and high dose to treat cells. In line with in vivo data, melatonin treatment could significantly enhance the expression of SIRT3 in LPS-treated lung epithelial cells and decrease the protein levels of SOD2-K122-Ac and SOD2-K68-Ac (Figure 4C). Meanwhile, melatonin treatment also reversed LPS-induced inactivity of SIRT3 and SOD2 (Figure 4D-E). To figure out whether the protective effects of melatonin in LPS-induced ALI were mediated by SIRT3 activation, the lung epithelial cells were subjected to siRNA to knockdown the expression of SIRT3. In keeping with our hypothesis, SIRT3 knockdown abolished the anti-apoptosis and anti-oxidative effects from melatonin in LPS-treated lung epithelial cells. Meanwhile, in LPS-treated lung epithelial cells transfected with SIRT3 siRNA, melatonin lost its anti-apoptosis and anti-oxidative effects (Figure 4F-H).

3.5 Melatonin maintained mitochondrial quality control in a SIRT3-dependent manner in LPS-induced lung epithelial cells

Next, mitochondrial fission and fusion were assessed in each group. In accordance with animal experiments, melatonin also preserved the balance between mitochondrial fission and fusion in LPS-induced lung epithelial cells. As expected, SIRT3 inhibition significantly counteracted the effects from melatonin on mitochondrial quality control in LPS-induced lung epithelial cells, which further proved that the benefit from melatonin was mediated in a SIRT3-dependent manner in ALI in vitro (Figure 5A-F). Also, we detected the mitochondrial membrane potential (MMP) in each group. The results indicated that melatonin also maintained the MMP homeostasis in LPS-induced lung epithelial cells in a SIRT3-dependent manner (Figure 5G). In brief, the data in this section unveiled that SIRT3 was essential for mitochondrial quality control preserved by melatonin in LPS-induced lung epithelial cells.
3.6 SIRT3 knockout partially abolished the protective effects of melatonin in LPS-induced ALI in vivo

Next, SIRT3-deficient mice were used to verify the mechanisms contributing to protective effects of melatonin in ALI. As shown in Figure 6A-C, lung injury score and lung wet/dry ratio of SIRT3-deficient mice were significantly lower in LPS+melatonin group than those in LPS group, indicating that SIRT3 was involved in the protective effects of melatonin in LPS-induced ALI in vivo. Intriguingly, compared with LPS-treated wild type mice, melatonin could still to some extent relieve lung injury and edema in LPS-treated SIRT3-deficient mice. Similarly, melatonin also partially inhibited the mRNA levels of Il-1β and Tnf-α in LPS-treated SIRT3-deficient mice (Figure 6D-E). Meanwhile, we detected the levels of oxidative stress in the indicated groups. The data also disclosed that SIRT3 knockout could partially abolished the anti-oxidative effects of melatonin in LPS-treated murine lung tissues, as evidenced by lower levels of ROS, TBARS and NADPH oxidase activity (Figure 6F-I). Western blots showed that melatonin displayed limited inhibiting effects on LPS-induced apoptosis after SIRT3 inhibition (Figure 6J). These data suggested that SIRT3 was critical to melatonin-mediated pulmonary protection but it was not the only target of melatonin during ALI.

3.7 SIRT3 knockout counteracted the regulatory effects of melatonin on mitochondrial quality control in LPS-induced ALI in vivo

Whether SIRT3 inhibition could affect melatonin on mitochondrial quality maintained by melatonin during ALI was investigated. Firstly, we observed the number and the size of mitochondria in each group. As shown, melatonin had no effects on the number or the size of mitochondrial of lung epithelial cells in SIRT3-deficient mice challenged with LPS (Figure 7A-C). Likewise, after SIRT3 was knocked out, melatonin lost its regulatory effects on the protein levels of Drp1, Fis1, Mfn1, Mfn2 and Opa1 in lung tissues from LPS-treated mice (Figure 7D). At last, we detected the acetylation level and activity of SOD2. The results further showed that the protein levels of acetylated SOD2 as well as SOD2 activity in SIRT3-deficient mice from LPS group and LPS+ melatonin group displayed no significance (Figure 7E-F). These data further hinted that melatonin preserved the mitochondrial dynamic equilibrium in a SIRT3-dependent manner during ALI.

3.8 Melatonin receptor 2 (MTNR1B) but not MTNR1A was involved in the melatonin-mediated cell protection in LPS-induced lung epithelial cells

To figure out which membrane melatonin receptor (MTNR1A and MTNR1B) was involved in the protection from melatonin. We next detected the mRNA and protein levels of MTNR1A and MTNR1B in
l lung epithelial cells. LPS stimulation significantly increased the expression of MTNR1B but showed no effects on the expression of MTNR1A (Figure 8A-B), suggesting that MTNR1B but not MTNR1A may participate in the protection of melatonin during ALI. To verify this hypothesis, we next knocked down the expression of MTNR1B using siRNA. As expected, MTNR1B inhibition blocked the protective roles of melatonin, as evidenced by impaired cell viability and increased LDH content in medium in LPS-treated lung epithelial cells (Figure 8C-D). Also, we detected the TUNEL positive cells and apoptosis-related proteins in the indicated groups. Similarly, compared with LPS group, the levels of apoptosis showed no significance in LPS+Melatonin group after MTNR1B was knocked down (Figure S1A-B). DCFH-DA probe staining also demonstrated that MTNR1A inhibition abolished anti-oxidative effects of melatonin in LPS-induced lung epithelial cells (Figure S1C). In addition, melatonin displayed no effects on the SIRT3 activity and SOD2 activity in LPS-treated lung epithelial cells transfected with Mtnr1b siRNA (Figure 8E-F). Western blots disclosed that the protein levels of SIRT3 and acetylated SOD2 of lung epithelial cells transfected with Mtnr1b siRNA in LPS group and LPS+ Melatonin group showed no significance (Figure 8G). We also detected the mRNA levels of Mfn1, Mfn2, Opa1, Fis1 and Drp1 in each group. The results unveiled that Mtnr1b siRNA completely abolished melatonin-mediated mitochondrial dynamic equilibrium in LPS-induced lung epithelial cells (Figure S2A-E). These data in this section proved that MTNR1B was essential for melatonin-mediated pulmonary protection during ALI.

3.9 Luzindole partially offset melatonin-mediated pulmonary protection in LPS-treated mice

The role of MTNR1B in animal experiments was also verified. Luzindole is a selective antagonist of melatonin receptor, which preferentially targets MTNR1B over MTNR1A[34]. Herein, luzindole was used to decrease the activity of MTNR1B in mice. The results demonstrated that luzindole intervention aggravated lung pathological injury and inflammatory response in lung tissues from melatonin-treated mice with ALI (Figure A-E). Compared with LPS+Melatonin group, the levels of apoptosis and oxidative stress significantly decreased in LPS+Melatonin+ Luzindole group (Figure F-H). The protein levels as well as activity of SIRT3 and SOD2 were also detected. As shown in Figure I, luzindole intervention also decreased the protein level of SIRT3 and increased the acetylation level of SOD2 in lung tissues from melatonin-treated mice with ALI. Meanwhile, luzindole intervention decreased SIRT3 activity and SOD2 activity despite of melatonin treatment in mice with ALI (Figure J-K). Quantitative real-time PCR showed that mitochondrial dynamic equilibrium was disturbed after luzindole intervention in lung tissues from melatonin-treated mice with ALI (Figure L). These results further proved the critical roles of MTNR1B in melatonin-mediated pulmonary protection in LPS-treated mice.

4. Discussion

In the present study, we uncovered that melatonin protected against ALI induced by LPS in mice.

Melatonin preserved mitochondrial dynamic equilibrium of lung epithelial cells challenged with LPS. The protection of melatonin was mediated by the activation of the SIRT3/SOD2 signaling pathway. SIRT3
deficiency or inhibition in lung epithelial cells could abolish melatonin-elicited protection. Also, we found that MTNR1B but not MTNR1A in lung epithelial cells was involved in the pulmonary protection of melatonin in LPS-induced ALI.

Mitochondria, serving as the most capital organelle with elongated double-membrane-bound organelles, own independent self-replicating genome[35]. Under normal physiologic condition, mitochondria could energize cells through generating ATP. In addition, mitochondria are also associated with redox signaling, autophagy, ferroptosis, apoptosis as well as calcium flux[36, 37]. In recent years, a great many studies have disclosed that alterations in mitochondrial function were closely related with the induction and propagation of sepsis-induced ALI and ARDS[38–40]. To our knowledge, the delicate alveolar epithelium could be disrupted by alveolar inflammatory cells and the storm of chemokines, cytokines, ROS and nitrogen species. The impairment of alveolar membrane integrity leads to the occurrence of protein-rich pulmonary edema[38]. In injured cells, the removal of damaged mitochondria as well as maintenance of ATP production are essential for the prevention of cycles of ROS overproduction and energy compromise[40]. For the reason that lung epithelial cells could not synthesize mitochondria de novo, they have to keep mitochondrial turnover and network balance through continual fission and fusion[41]. The balance between mitochondria fission and fusion provides an equilibrium of long interconnected mitochondrial as well as small fragmented mitochondria, which is critical for mitochondrial homeostasis, cell stability and survival[42]. During our exploration into the mechanism by which melatonin prevented sepsis-associated ALI, we for the first time unveiled the regulatory effects on mitochondrial dynamic equilibrium by melatonin following LPS stimulation in lung epithelial cells. During ALI, the dynamic course of mitochondria was shifted from fusion into fission. However, melatonin treatment reversed the alteration of mitochondrial morphology, maintaining normal function of mitochondria to the maximum extent.

As one of the members of the Sir2 family of NAD$^+$-dependent protein deacetylases, SIRT3 is mainly distributed in mitochondria, knockout of which could give rise to a strong hyperacetylation of the mitochondrial-associated proteins[43]. In the past several decades, many SIRT3 substrates have been identified, most of which are responsible for the modulation of mitochondrial dynamism and biogenesis[44, 45]. Superoxide anion ($\text{O}_2^-.$) is one of the by-products of oxidative phosphorylation in mitochondria. These radicals can impair mitochondrial respiratory function by damaging the electron transport chain as well as some other cellular constituents. Fortunately, eucaryon has evolved defensive system to prevent such damaging moieties. One such member in this defensive system is mitochondrial antioxidant enzyme superoxide dismutase SOD2, which could convert superoxide to hydrogen peroxide with less reactivity efficiently by diffusing cross the mitochondrial membrane freely[46]. The critical roles of SOD2 in regulating mitochondrial dynamic equilibrium have also been extensively reported[47]. Previous studies have unveiled that SIRT3 can directly bind and regulate the acetylation of SOD2, increasing SOD2 activity and giving rise to a significant effect on mitochondria quality control. Two highly conserved catalytic centers of SOD2 include lysines 68 (K68) and lysines 122 (K122), the acetylation of which represents the inhibition of enzymatic activity[48, 49]. Therefore, targeting
SIRT3/SOD2 signaling pathway acts as a promising strategy to combat diseases featured with abnormal mitochondrial function. For instance, hydrogen sulfide pretreatment can enhance SIRT3 promoter activity and increase the level of SOD2 in cardiomyocytes, thus alleviating cardiac hypertrophy by improving mitochondrial function[47]. Additionally, diminished expression as well as redox inactivation of SIRT3 resulted in the increased acetylation of SOD2, giving rise to vascular oxidative stress and hypertension[49]. We herein found that SIRT3 in lung epithelial cells were upregulated and activated by melatonin. Once SIRT3 was activated, it further deacetylated SOD2 at lysines 68 and lysines 122 directly, showing no effects on total protein level of SOD2. Meanwhile, we found that SIRT3 was essential for the protection from melatonin in lung epithelial cells, the inhibition of which completely abolished melatonin-mediated effects on mitochondrial quality control. Intriguingly, in animal experiments, SIRT3 knockout could not completely contract melatonin-mediated pulmonary protection, hinting that there may exist other mechanisms by which melatonin exerts protective effects in ALI. Indeed, many other studies also reported that melatonin could prevent ALI by multiple mechanism including the inhibition of NLRP3 inflammasome and EMT[24, 25]. These studies explained why the protective effects of melatonin in vitro replied on SIRT3 while pulmonary protection of melatonin was not completely mediated by SIRT3 in vivo.

In fact, the beneficial effects of melatonin on mitochondrial dynamics and function have been broadly studied in many diseases. In LPS-induced cardiomyocytes, melatonin could modulate mitochondrial dynamics by decreasing the expression of Ripk3, thus reducing cell death[50]. In rat, melatonin also alleviated paclitaxel-induced mitochondrial dysfunction and prevented paclitaxel-induced neuropathic pain[51]. One of the critical reasons for mitochondrial protection of melatonin gives the credit to its higher concentrations in mitochondria than in other subcellular locations[52, 53]. As mentioned above, SIRT3 is also distributed in mitochondria and shares many similar functions with melatonin. Whether melatonin and SIRT3 interact with each other in a certain way to regulate mitochondrial physiology needs to be explored in the future.

In summary, the present study unveiled the positive roles of melatonin in ALI treatment. Melatonin preserved mitochondrial dynamic equilibrium of lung epithelial cells through SIRT3-dependent deacetylation of SOD2. MTNR1B but not MTNR1A was involved in the protection of melatonin in LPS-induced lung epithelial cells. Our study has proved that melatonin may serve as a promising candidate for the future treatment of sepsis-induced ALI.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication
The authors declared that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All authors consent for publication.

Authors’ contributions

Li Ning, Xiong Rui and Li Guorui contributed equally to this work. Li Ning, Xiong Rui and Li Guorui designed, performed research, analyzed data and wrote the paper. Fu Tinglv, Li Donghang and Xu Chenzhen performed experiments, analyzed data. Wu Xiaojing and Geng Qing helped to design experiments and reviewed the data. Li Ning, Wu Xiaojing and Geng Qing helped to design the research, analyzed data and reviewed the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

Figure 1

Melatonin alleviated LPS-induced inflammation, oxidative stress and apoptosis in murine lung tissues. (A-B). Histological analyses of the H&E staining and lung injury score of melatonin-treated and control-treated mice 12 hours after LPS stimulation (n = 5). (C). Lung wet/dry ratio (n = 5). (D). LDH activity in
serum (n=5). (E). Representative images of immunohistochemical staining of TNF-α in lung tissues (n = 5). (F-H). The mRNA levels of Il-1β, Tnf-α, and Il-1β in lung tissues (n = 5). (I). Representative images of immunohistochemical staining of GPX4 in lung tissues (n = 5). (J-L). SOD activity, TBARS content and NADPH oxidase activity in lung tissues (n = 5). (M-N). Representative images of ROS staining and fluorescent quantitation in lung tissues (n = 5). (O-P). Representative images of TUNEL staining and statistical results of apoptotic cells in lung tissues (n = 5). (Q). Representative western blot images and the corresponding quantitative results (n = 6). *P < 0.05 versus Control group; #P < 0.05 versus LPS group.

**Figure 2**

**Mitochondrial quality control was involved in the protective roles of melatonin in LPS-induced ALI in vivo.** (A). Gene ontology (GO) analysis results of the DEGs between LPS group and LPS+HD group (n = 5). (B-C). Representative images of 8-OHdG immunofluorescence staining and quantitation in lung tissues (n=5). (D-H). The mRNA levels of Mfn1, Mfn2, Opa1, Drp1 and Fis1 in lung tissues (n = 5). (I). Representative western blot images and the corresponding quantitative results (n=5). (J). The morphology of mitochondria by transmission electron microscopy (n=5). Mitochondria were indicated with yellow, while fusing mitochondria were indicated with red. (K). The number of mitochondria in lung epithelial cells (n = 5). (L). Mean mitochondria size in lung epithelial cells (n = 5). *P < 0.05 versus Control group; #P < 0.05 versus LPS group.

**Figure 3**

**Melatonin inhibited the deacetylation of SOD2 by activating SIRT3 in LPS-induced ALI in vivo.** (A). The heatmap displaying top20 DEGs with the highest fold change between LPS group and LPS+HD group (n = 5). (B). Representative western blot images and the corresponding quantitative results (n = 6). (C). SIRT3 activity in lung tissues (n=5). (D). Representative western blot images and the corresponding quantitative results (n = 6). (E). SOD2 activity in lung tissues (n=5). (F). Representative images and quantitative results of co-immunoprecipitation of SOD2 and SIRT3 in lung tissues (n =5). *P < 0.05 versus Control group; #P < 0.05 versus LPS group.

**Figure 4**

**Melatonin prevented LPS-induced lung epithelial cell damage through SIRT3-dependent deacetylation of SOD2.** (A). Cell viability was detected CCK-8 in indicated groups (n = 5). (B). LDH content in medium in indicated groups (n = 5). *P < 0.05 versus the indicated group. (C). Representative western blot images and the corresponding quantitative results in vitro (n = 6). (D-E). SIRT3 activity and SOD2 activity in vitro
(n = 5). *P < 0.05 versus Control group; #P < 0.05 versus LPS group. (F). Representative western blot images and the corresponding quantitative results in indicated groups (n = 6). (G). Representative images of TUNEL staining and statistical results of apoptotic cells in lung epithelial cells (n = 5). (H). Representative images and statistical results of DCFH-DA staining in lung epithelial cells (n = 5). *P < 0.05 versus the indicated group.

Figure 5

Melatonin maintained mitochondrial quality control in a SIRT3-dependent manner in LPS-induced lung epithelial cells. (A-E). The mRNA levels of Mfn1, Mfn2, Opa1, Drp1 and Fis1 in lung epithelial cells in indicated groups. (n=5). (F). Representative western blot images and the corresponding quantitative results in lung epithelial cells in indicated groups (n=6). (G). Mitochondrial membrane potential in lung epithelial cells determined by JC-1 probe staining in indicated groups (n = 5). *P < 0.05 versus the indicated group; ns, no significance.

Figure 6

SIRT3 knockout partially abolished the protective effects of melatonin in LPS-induced ALI in vivo. (A-B). Histological analyses of the H&E staining and lung injury score of wild type mice and Sirt3 knockout mice 12 hours after LPS stimulation (n = 5). (C). Lung wet/dry ratio (n=5). (D-E). The mRNA levels of Il-1β and Tnf-α in lung tissues (n = 5). (F-G). Representative images of ROS staining and fluorescent quantitation in lung tissues (n=5). (H-I). TBARS content and NADPH oxidase activity in lung tissues (n = 5). (J). Representative western blot images and the corresponding quantitative results in lung tissues (n = 6). *P < 0.05 versus the indicated group; ns, no significance.

Figure 7

SIRT3 knockout counteracted the regulatory effects of melatonin on mitochondrial quality control in LPS-induced ALI in vivo. (A). The morphology of mitochondria by transmission electron microscopy (n=5). (B). The number of mitochondria in lung epithelial cells (n=5). (C). Mean mitochondria size in lung epithelial cells (n=5). (D). Representative western blot images and the corresponding quantitative results in lung tissues (n = 6). (E). Representative western blot images and the corresponding quantitative results of acetylated SOD2 and total SOD2 in lung tissues (n = 6). (F). SOD2 activity in lung tissues (n=6). *P < 0.05 versus the indicated group; ns, no significance.
Figure 8

Melatonin receptor 2 (MTNR1B) but not MTNR1A was involved in the melatonin-mediated cell protection in LPS-induced lung epithelial cells. (A). Representative western blot images and the corresponding quantitative results of MTNR1A and MTNR1B in lung epithelial cells (n = 5). (B). The mRNA levels of Mtnr1a and Mtnr1b in lung epithelial cells (n = 5). *P < 0.05 versus the indicated group; ns, no significance. (C). Cell viability was detected CCK-8 in indicated groups (n =5). (D). LDH content in medium in indicated groups (n = 6). (E-F). SIRT3 activity and SOD2 activity in indicated groups (n=5). (G). Representative western blot images and the corresponding quantitative results in indicated groups (n = 6). *P < 0.05 versus the indicated group; ns, no significance.

Figure 9

Luzindole partially offset melatonin-mediated pulmonary protection in LPS-treated mice. (A-B). Histological analyses of the H&E staining and lung injury score of mice in indicated groups (n = 5). (C). Lung wet/dry ratio in indicated groups (n=5). (D-E). The mRNA levels of Il-1β and Tnf-α in lung tissues in indicated groups (n=5). (F). Representative western blot images and the corresponding quantitative results in indicated groups (n = 6). (G-H). TBARS content and NADPH oxidase activity in lung tissues (n=5). (I). Representative western blot images and the corresponding quantitative results in lung tissues in indicated groups (n = 6). (J-K). SIRT3 activity and SOD2 activity in lung tissues in indicated groups (n = 5). (L). The mRNA levels of Mfn1, Mfn2, Opa1, Drp1 and Fis1 in lung tissues in indicated groups (n =5). *P < 0.05 versus Control group; #P < 0.05 versus LPS group.

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