



Melatonin Inhibits Endoplasmic Reticulum Stress and Epithelial-Mesenchymal Transition during Bleomycin-Induced Pulmonary Fibrosis in Mice

Hui Zhao^{2,3}, Qing-Qing Wu³, Lin-Feng Cao³, Hou-Ying Qing³, Cheng Zhang¹, Yuan-Hua Chen¹, Hua Wang¹, Rong-Ru Liu^{2*}, De-Xiang Xu^{1*}

1 Department of Toxicology, Anhui Medical University, Hefei, China, **2** First Affiliated Hospital, Anhui Medical University, Hefei, China, **3** Second Affiliated Hospital, Anhui Medical University, Hefei, China

Abstract

Several reports indicate that melatonin alleviates bleomycin (BLM)-induced pulmonary fibrosis in rodent animals. Nevertheless, the exact mechanism remains obscure. The present study investigated the effects of melatonin on endoplasmic reticulum (ER) stress and epithelial-mesenchymal transition (EMT) during BLM-induced lung fibrosis. For the induction of pulmonary fibrosis, mice were intratracheally injected with a single dose of BLM (5.0 mg/kg). Some mice were intraperitoneally injected with melatonin (5 mg/kg) daily for a period of 3 wk. Twenty-one days after BLM injection, lung fibrosis was evaluated. As expected, melatonin significantly alleviated BLM-induced pulmonary fibrosis, as evidenced by Sirius red staining. Moreover, melatonin significantly attenuated BLM-induced EMT to myofibroblasts, as determined by its repression of α -SMA expression. Further analysis showed that melatonin markedly attenuated BLM-induced GRP78 up-regulation and elevation of the cleaved ATF6 in the lungs. Moreover, melatonin obviously attenuated BLM-induced activation of pulmonary eIF2 α , a downstream target of the PERK pathway. Finally, melatonin repressed BLM-induced pulmonary IRE1 α phosphorylation. Correspondingly, melatonin inhibited BLM-induced activation of XBP-1 and JNK, two downstream targets of the IRE1 pathway. Taken together, these results suggest that melatonin alleviates ER stress and ER stress-mediated EMT in the process of BLM-induced pulmonary fibrosis.

Citation: Zhao H, Wu Q-Q, Cao L-F, Qing H-Y, Zhang C, et al. (2014) Melatonin Inhibits Endoplasmic Reticulum Stress and Epithelial-Mesenchymal Transition during Bleomycin-Induced Pulmonary Fibrosis in Mice. PLoS ONE 9(5): e97266. doi:10.1371/journal.pone.0097266

Editor: Bernhard Ryffel, French National Centre for Scientific Research, France

Received: March 15, 2014; **Accepted:** April 16, 2014; **Published:** May 12, 2014

Copyright: © 2014 Zhao et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are included within the manuscript.

Funding: This work was supported by National Natural Science Foundation of China (30671786,31000664). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: xudex@126.com (D-XX); rongyuliu@gmail.com (R-RL)

Introduction

Idiopathic pulmonary fibrosis, characterized by fibroblast proliferation and extracellular matrix remodeling, is a chronic pulmonary disease of unknown origin ultimately leading to death [1,2]. Bleomycin (BLM), a drug widely used as an antineoplastic, causes a dose-dependent interstitial pulmonary fibrosis [3]. Intratracheal instillation of BLM into the lungs of rodent animals causes alveolar cell damage, an inflammatory response, epithelial-mesenchymal transition (EMT), fibroblast proliferation and subsequent extracellular matrix deposition, resembling human fibrotic lung disease [4]. BLM-induced pulmonary fibrosis is the most commonly used model of idiopathic pulmonary fibrosis for studying disease pathogenesis and testing of novel pharmaceutical compounds [5]. Nevertheless, the mechanisms of BLM-induced pulmonary fibrosis are not completely understood.

Endoplasmic reticulum (ER) is an important organelle required for normal cellular function. In the ER, nascent proteins are folded with the assistance of ER chaperones. If nascent proteins in the ER are excessive compared with the reserve of ER chaperones, ER stress occurs. Accumulation of unfolded and misfolded proteins aggregated in the ER lumen causes the activation of a signal

response termed the unfolded protein response (UPR) [6]. The UPR signaling is mediated by three transmembrane ER proteins: inositol requiring ER-to-nucleus signal kinase (IRE1), activating transcription factor (ATF)6 and double-stranded RNA-activated kinase (PKR)-like ER kinase (PERK) [7,8]. Active IRE1 cleaves *x-box binding protein-1* (*XBP-1*) mRNA in a site-specific manner to remove an intron, promoting its unconventional splicing to generate an active transcription factor [9]. Active ATF6 is a transcription factor that promotes the expression of genes that include glucose regulated protein 78 (GRP78), ATP-dependent protein chaperone localized within the ER lumen where it promotes protein folding [10]. PERK is an ER stress-sensitive eukaryotic initiation factor (eIF) 2 α kinase. Following ER stress, PERK phosphorylates and activates eIF2 α , thus attenuating cap-dependent translation [11]. An earlier report observed an increased expression of the processed p50 ATF6 and spliced XBP-1, a downstream molecule of IRE1 pathway, in the lungs of IPF patients [12]. Two recent studies have demonstrated that pulmonary ER stress is involved in the pathogenesis of BLM-induced lung fibrosis [13,14].

Melatonin is the major secretory product of the pineal gland. As a potent antioxidant, melatonin and its metabolites directly

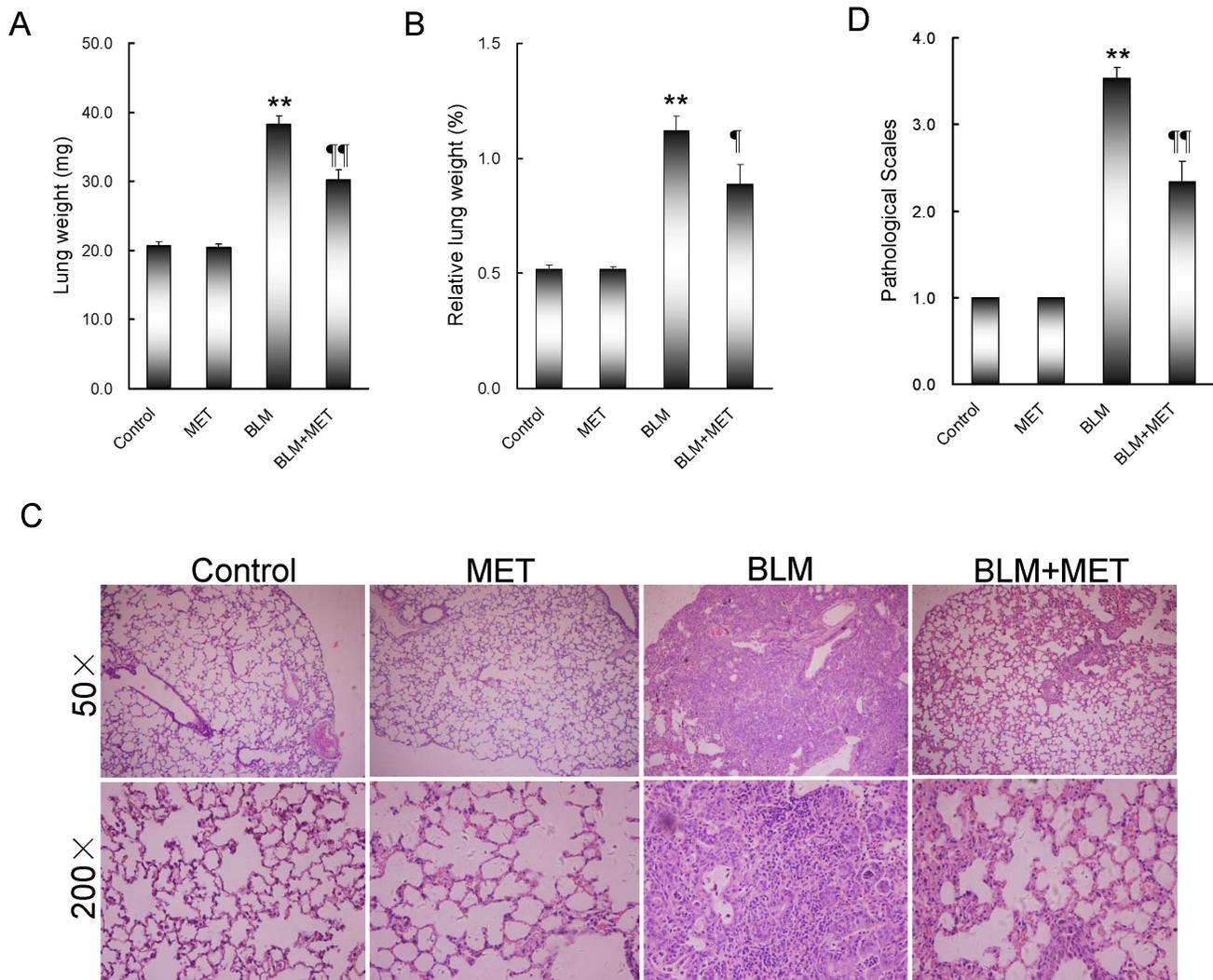


Figure 1. The effects of melatonin on BLM-induced pathohistological damage. All mice except controls were intratracheally injected with BLM (5.0 mg/kg). In BLM+melatonin group, mice were i.p. injected with melatonin (5 mg/kg) daily. Lungs were collected at 21 days after BLM. Lungs were weighed. (A) Absolute lung weight. (B) Relative lung weight. (C) Lung cross sections were stained with H & E. Original magnification: 50 \times (upper) and 200 \times (lower). (D) Pathohistological scores were evaluated according to pulmonary inflammation. All data were expressed as means \pm SEM (n = 12). ** $P < 0.01$ as compared with control group. ‡ $P < 0.05$, †† $P < 0.01$ as compared with BLM group. doi:10.1371/journal.pone.0097266.g001

scavenge a variety of free radicals [12,13]. In addition, melatonin reduces free radical levels via stimulating the activities of antioxidative enzymes [15–20]. Melatonin has an anti-inflammatory effect. Several studies have demonstrated that melatonin alleviates lipopolysaccharide (LPS)-evoked inflammatory cytokines/chemokines [21–26]. According to a recent report, melatonin attenuates LPS-induced acute lung inflammation in sleep-deprived mice [27]. Moreover, two studies found that melatonin protected against BLM-induced pulmonary fibrosis [28–30]. Nevertheless, the exact mechanism remains obscure.

In the present study, we investigated the effects of melatonin on BLM-induced pulmonary ER stress and the UPR in mice. We also investigated whether melatonin alleviates the EMT in the process of BLM-induced lung fibrosis. We show that melatonin protects against BLM-induced lung fibrosis in mice. We demonstrate for the first time that melatonin inhibits pulmonary ER stress and EMT during BLM-induced lung fibrosis.

Materials and Methods

Chemicals and reagents

BLM and melatonin were from Sigma Chemical Co. (St. Louis, MO). XBP-1, α -SMA, GAPDH and phosphor-JNK antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). GRP78, phosphor-IRE1 α , ATF6, and phosphor-eIF2 α antibodies were from Cell Signaling Technology (Beverly, MA). Chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) if not otherwise stated.

Animals

Adult male CD-1 mice (8 week-old, 28–32 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food and water at all times and maintained on a 12-h light/dark cycle in a controlled temperature

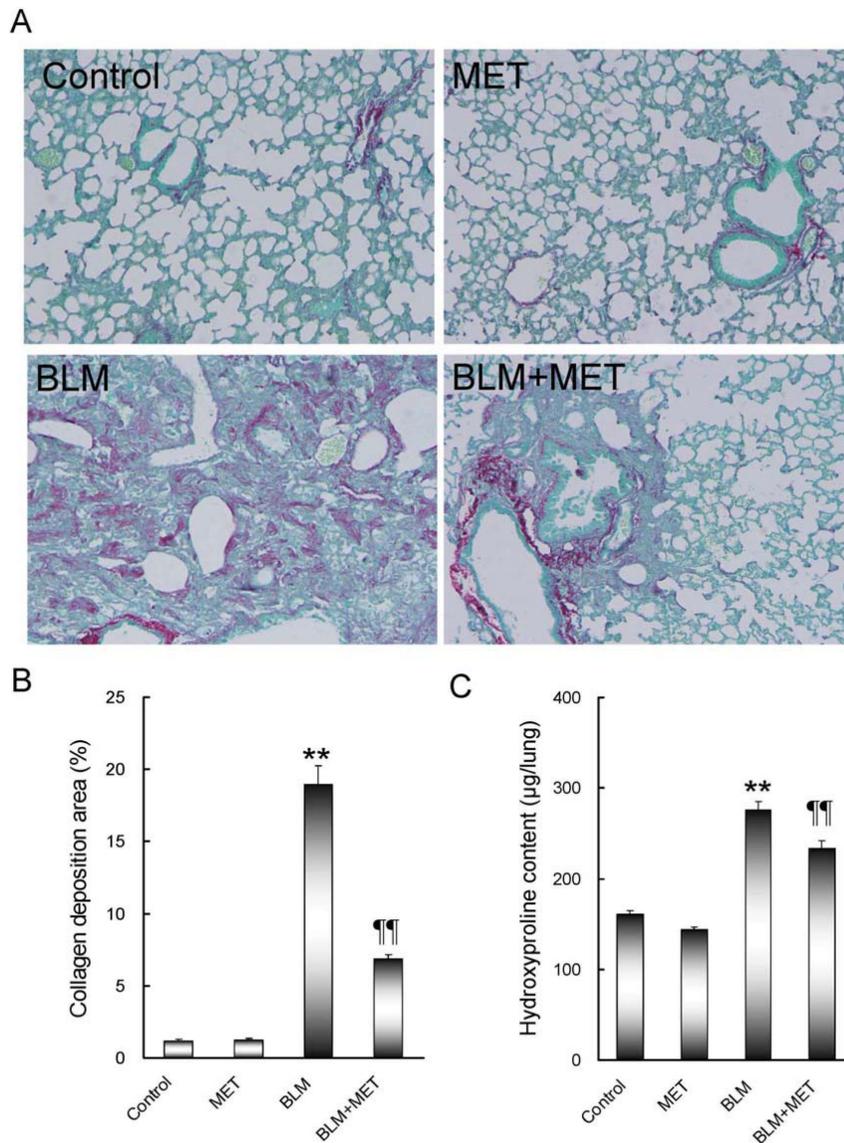


Figure 2. The effects of melatonin on BLM-induced pulmonary fibrosis. All mice except controls were intratracheally injected with BLM (5.0 mg/kg). In BLM+melatonin group, mice were i.p. injected with melatonin (5 mg/kg) daily. Lungs were collected at 21 days after BLM. (A) Lung fibrosis was evaluated by Sirius red staining. Original magnification: 100 \times . (B) Collagen deposition areas were quantified. (C) Hydroxyproline content in the lungs was analyzed. All data were expressed as means \pm SEM (n=12). ** $P<0.01$ as compared with control group. †† $P<0.01$ as compared with BLM group.

doi:10.1371/journal.pone.0097266.g002

(20–25°C) and humidity (50 \pm 5%) environment. This study was approved by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University (Permit Number: 12-0046). All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Animal model and experimental treatments

For the induction of pulmonary fibrosis, mice were intratracheally injected with BLM (5.0 mg/kg body weight in 50 μ L phosphate buffered saline). To investigate the effects of melatonin on BLM-induced pulmonary fibrosis, mice were intraperitoneally (i.p.) injected with melatonin (5 mg/kg) daily, beginning at 30 min

before BLM. Melatonin was dissolved in 10% ethanol and further diluted in saline (0.09% NaCl w/v) to give a final concentration of 1% ethanol. Thus, control mice received an i.p. injection of 1% ethanol daily as a control for the melatonin injections. All mice were euthanized by exsanguination during pentobarbital anesthesia (75 mg/kg, i.p.) 21 days after BLM injection. Lungs were weighed and relative lung weights were calculated. Lung fibrosis was assessed by pulmonary hydroxyproline content as well as lung histology. Some lung samples were collected and kept at -80°C for subsequent immunoblots.

Hydroxyproline assay

Pulmonary collagen content was determined by the measurement of hydroxyproline content. In brief, lung lobes were

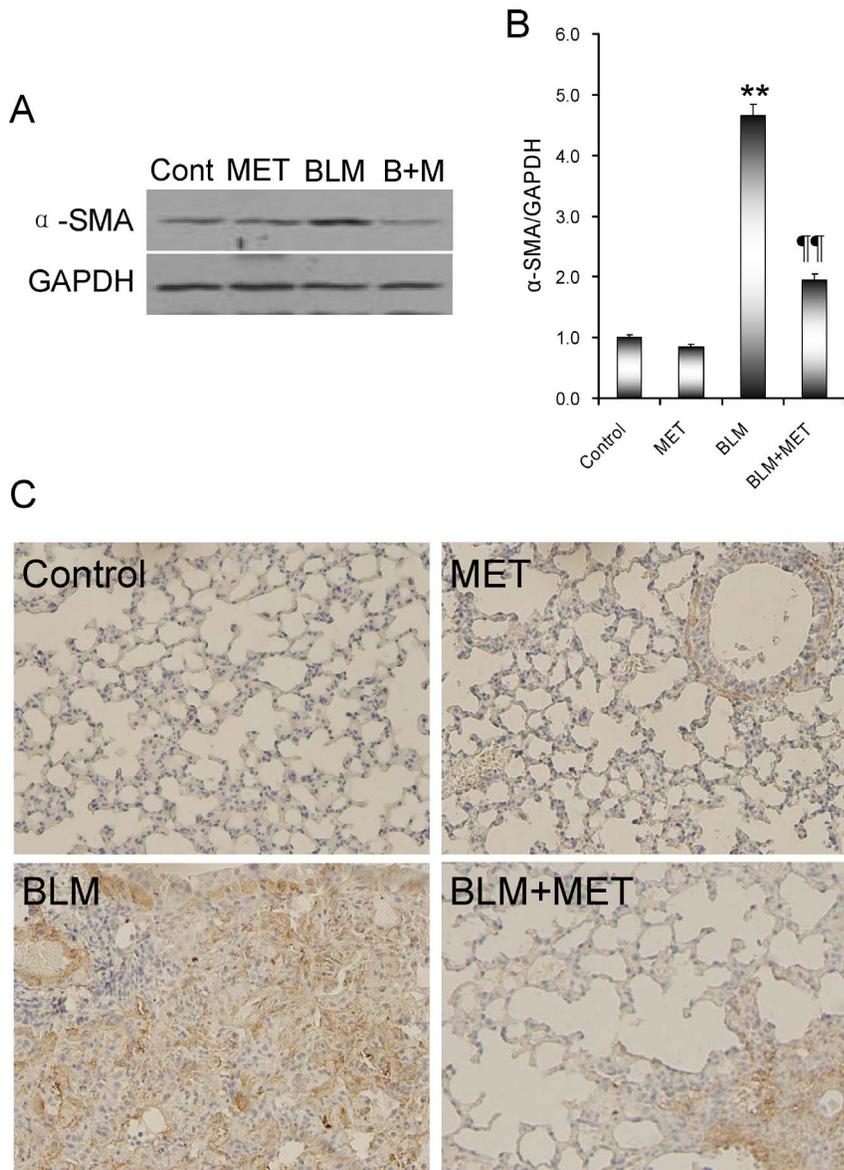


Figure 3. The effects of melatonin on BLM-induced pulmonary α -SMA expression. All mice except controls were intratracheally injected with BLM (5.0 mg/kg). In BLM+melatonin group, mice were i.p. injected with melatonin (5 mg/kg) daily. Lungs were collected at 21 days after BLM. (A and B) The expression of pulmonary α -SMA was detected using immunoblots. All experiments were replicated for four times. The data were expressed as means \pm SEM ($n=4$). ** $P<0.01$ as compared with control group. †† $P<0.01$ as compared with BLM group. (C) Pulmonary α -SMA was detected by immunohistochemistry. All experiments were replicated for four times. Original magnification: 200 \times . doi:10.1371/journal.pone.0097266.g003

homogenized in 1 mL of phosphate buffered saline (PBS, pH = 7.4) and then hydrolyzed in 1 mL of 6 N hydrochloric acid for 16 hours at 110°C, and neutralized to pH 7.0 with NaOH. Chloramines T reagent (1 mL of 0.5 mol/L) was then added and the samples were left at room temperature for 20 minutes. Then 20% p-Dimethylaminobenzaldehyde solution (dissolved in 3.15 N perchloric acid) was added to each sample, and the mixture was incubated at 60°C for 15 minutes. Absorbance was measured at 550 nm. Pulmonary hydroxyproline content was expressed as mg/lung.

Pulmonary histology

Lung tissues were fixed in 4% formalin and embedded in paraffin according to the standard procedure. Paraffin-embedded lung

tissues were serially sectioned. At least five consecutive longitudinal sections were stained with hematoxylin and eosin (H&E) and scored for the extent of pathology on a scale of 0 to 5, where 0 was defined as no lung abnormality, and 1, 2, 3, 4, and 5 were defined as the presence of inflammation involving 10%, 10–30%, 30–50%, 50–80%, or >80% of the lungs, respectively. Lung fibrosis was evaluated by Sirius red staining for collagen accumulation. The percentages of collagen deposition areas were quantified using NIH ImageJ software (<http://rsb.info.nih.gov/ij/>).

Immunoblots

Total pulmonary lysate was prepared by homogenizing 50 mg lung tissue in 300 μ l lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium

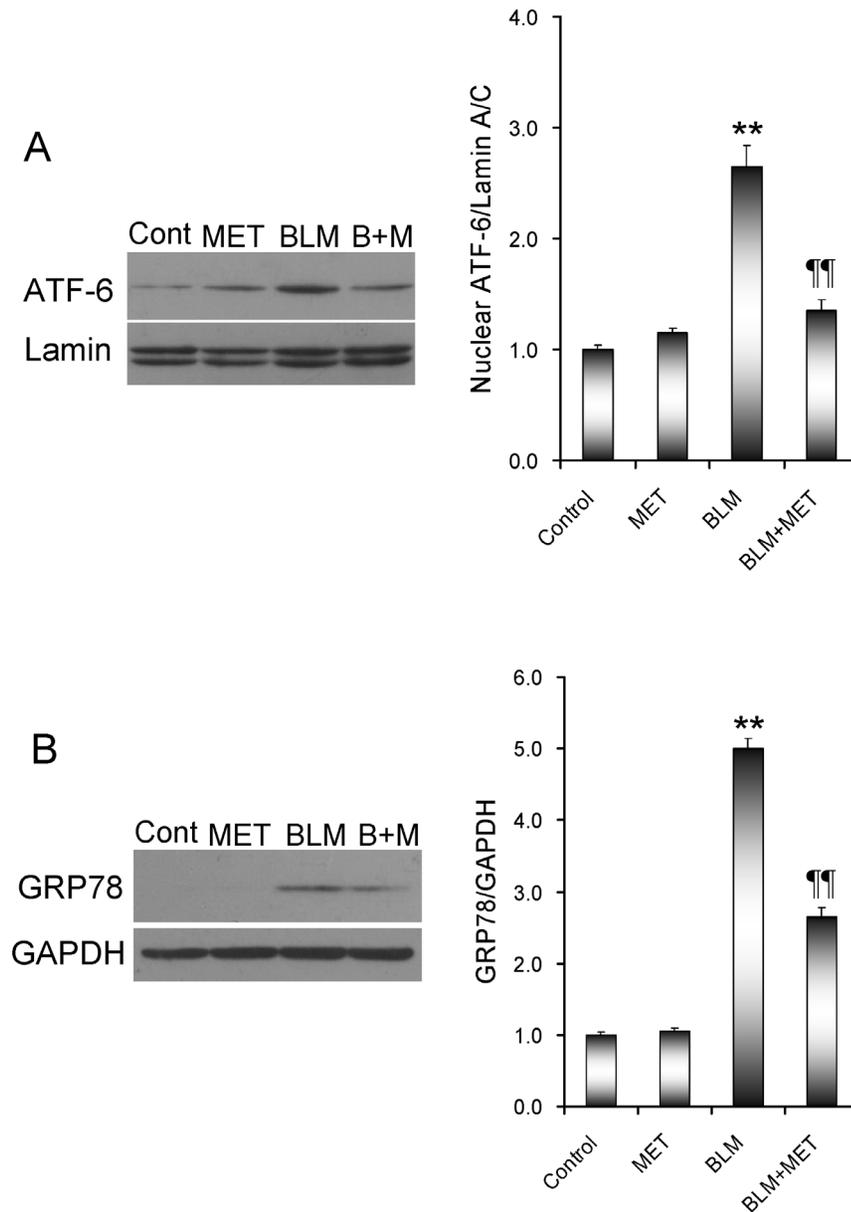


Figure 4. The effects of melatonin on BLM-induced activation of ATF6 pathway in the lungs. All mice except controls were intratracheally injected with BLM (5.0 mg/kg). In BLM+melatonin group, mice were i.p. injected with melatonin (5 mg/kg) daily. Lungs were collected at 21 days after BLM. (A) The cleaved ATF6 in the nuclei was detected using immunoblots. (B) GRP78 in the lungs was detected using immunoblots. All experiments were replicated for four times. The data were expressed as means \pm SEM (n = 4). ** $P < 0.01$ as compared with control group. ‡‡ $P < 0.01$ as compared with BLM group.

doi:10.1371/journal.pone.0097266.g004

deoxycholate, 0.1% sodium dodecylsulfate, 1 mM phenylmethylsulfonyl fluoride) supplemented with a cocktail of protease inhibitors (Roche). For nuclear protein extraction, total pulmonary lysate was suspended in hypotonic buffer and then kept on ice for 15 min. The suspension was then mixed with detergent and centrifuged for 30 s at $14,000 \times g$. The nuclear pellet obtained was resuspended in complete lysis buffer in the presence of the protease inhibitor cocktail, incubated for 30 min on ice, and centrifuged for 10 min at $14,000 \times g$. Protein concentrations were determined with the bicinchoninic acid (BCA) protein assay reagents (Pierce, Rockford, IL) according to manufacturer's instructions. For immunoblots, same amount of protein (30–60 μ g) was separated electrophoretically by SDS-PAGE and transferred to a polyvinyl-

lidene fluoride membrane. The membranes were incubated for 2 h with the following antibodies: XBP-1, α -SMA, GAPDH, phosphor-JNK, GRP78, phosphor-IRE1 α , ATF6, and phosphor-eIF2 α . For total proteins, GAPDH was used as a loading control. For nuclear protein, lamin A/C was used as a loading control. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit IgG or goat anti-mouse antibody for 2 h. The membranes were then washed for four times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an ECL detection kit. The density of the specific bands was quantified using NIH ImageJ software (<http://rsb.info.nih.gov/ij/>).

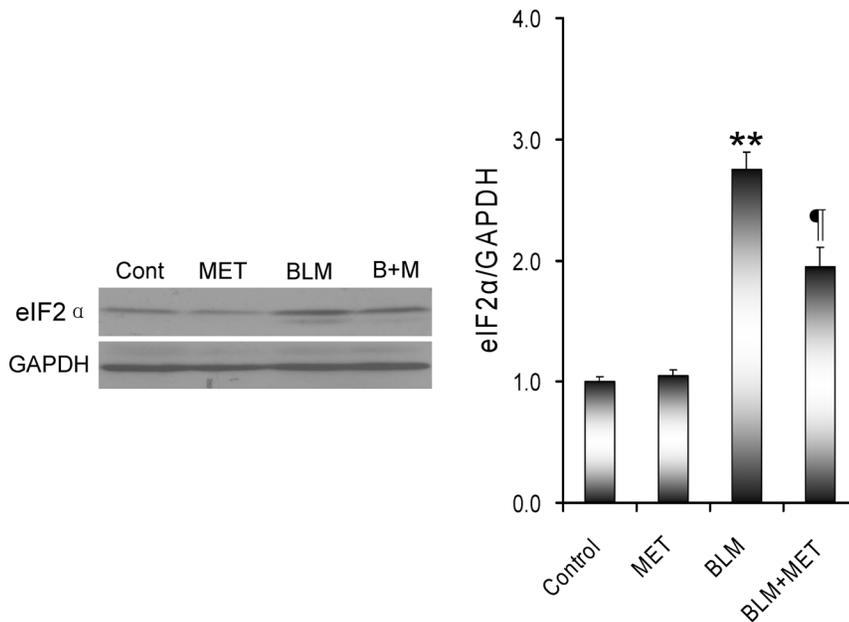


Figure 5. The effects of melatonin on BLM-induced eIF2 α phosphorylation in the lungs. All mice except controls were intratracheally injected with BLM (5.0 mg/kg). In BLM+melatonin group, mice were i.p. injected with melatonin (5 mg/kg) daily. Lungs were collected at 21 days after BLM. Pulmonary p-eIF2 α was detected using immunoblots. All experiments were replicated for four times. The data were expressed as means \pm SEM (n=4). ** $P < 0.01$ as compared with control group. ‡ $P < 0.05$ as compared with BLM group. doi:10.1371/journal.pone.0097266.g005

Immunohistochemistry

For immunohistochemistry, paraffin-embedded lung sections were deparaffinized and rehydrated in a graded ethanol series. After antigen retrieval and quenching of endogenous peroxidase, sections were incubated with α -SMA monoclonal antibodies (1:200 dilution) at 4°C overnight. The color reaction was developed with HRP-linked polymer detection system and counterstaining with hematoxylin.

Statistical analysis

Normally distributed data were expressed as means \pm SEM. ANOVA and the Student-Newmann-Keuls post hoc test were used to determine differences among different groups. Data that were not normally distributed were assessed for significance using non-parametric tests techniques (Kruskal-Wallis test and Mann-Whitney U test). $P < 0.05$ was considered to indicate statistical significance.

Results

Effects of melatonin on BLM-induced pulmonary fibrosis

An obvious pulmonary edema was observed in BLM-treated mice. Consistent with pulmonary edema, the absolute and relative weights of the lungs were significantly increased in BLM-treated mice (Figures 1A and 1B). Of interest, melatonin significantly attenuated BLM-induced pulmonary edema. Correspondingly, melatonin significantly alleviated BLM-induced elevation of the absolute and relative lung weight (Figure 1A and 1B). Histological examination showed that melatonin significantly alleviated BLM-induced infiltration of inflammatory cells in the lungs (Figure 1C and 1D). The hallmark characteristic of BLM-induced pulmonary fibrosis is the excessive deposition of an extracellular matrix, such as collagen. As shown in Figures 2A and 2B, an obvious matrix protein deposition, as evidenced by Sirius red staining, was observed in the lungs of BLM-treated mice. As expected,

melatonin significantly attenuated BLM-induced matrix protein deposition in the lungs. Moreover, melatonin significantly attenuated BLM-induced elevation of hydroxyproline content in the lungs (Figure 2C).

Effects of melatonin on BLM-induced epithelial-mesenchymal transition

Alpha-SMA is a hallmark of myofibroblasts and is also accepted as a marker of pulmonary fibrosis. As shown in Figures 3A and 3B, α -SMA was up-regulated in the lungs of BLM-treated mice. Immunohistochemistry showed that α -SMA was expressed in the area of pulmonary fibrosis in BLM-treated mice (Figure 3C). Of interest, melatonin significantly attenuated BLM-induced up-regulation of α -SMA in the lungs (Figures 3A, 3B and 3C).

Effects of melatonin on BLM-induced pulmonary ER stress

The effects of melatonin on BLM-induced pulmonary ER stress were analyzed. As shown in Figure 4A, the cleaved ATF6 in the nuclei was significantly increased in the lungs of mice treated with BLM. Correspondingly, pulmonary GRP78, an ER chaperone and the target of ATF6 pathway, was up-regulated in BLM-treated mice (Figure 4B). Of interest, melatonin markedly attenuated BLM-induced elevation of the cleaved ATF6 in the lungs. Moreover, melatonin inhibited BLM-induced up-regulation of pulmonary GRP78. Eukaryotic initiation factor 2 α (eIF2 α) is a downstream target of the PERK pathway. As shown in Figure 5, phosphorylated eIF2 α in the lung was increased in BLM-treated mice. Of interest, melatonin significantly attenuated BLM-induced eIF2 α phosphorylation in the lungs. The effects of melatonin on pulmonary IRE1 pathway were then analyzed. As shown in Figure 6A, the level of phosphorylated IRE1 α in the lungs was significantly increased in BLM-treated mice. Consistent with IRE1 α phosphorylation, nuclear XBP-1, a downstream target of

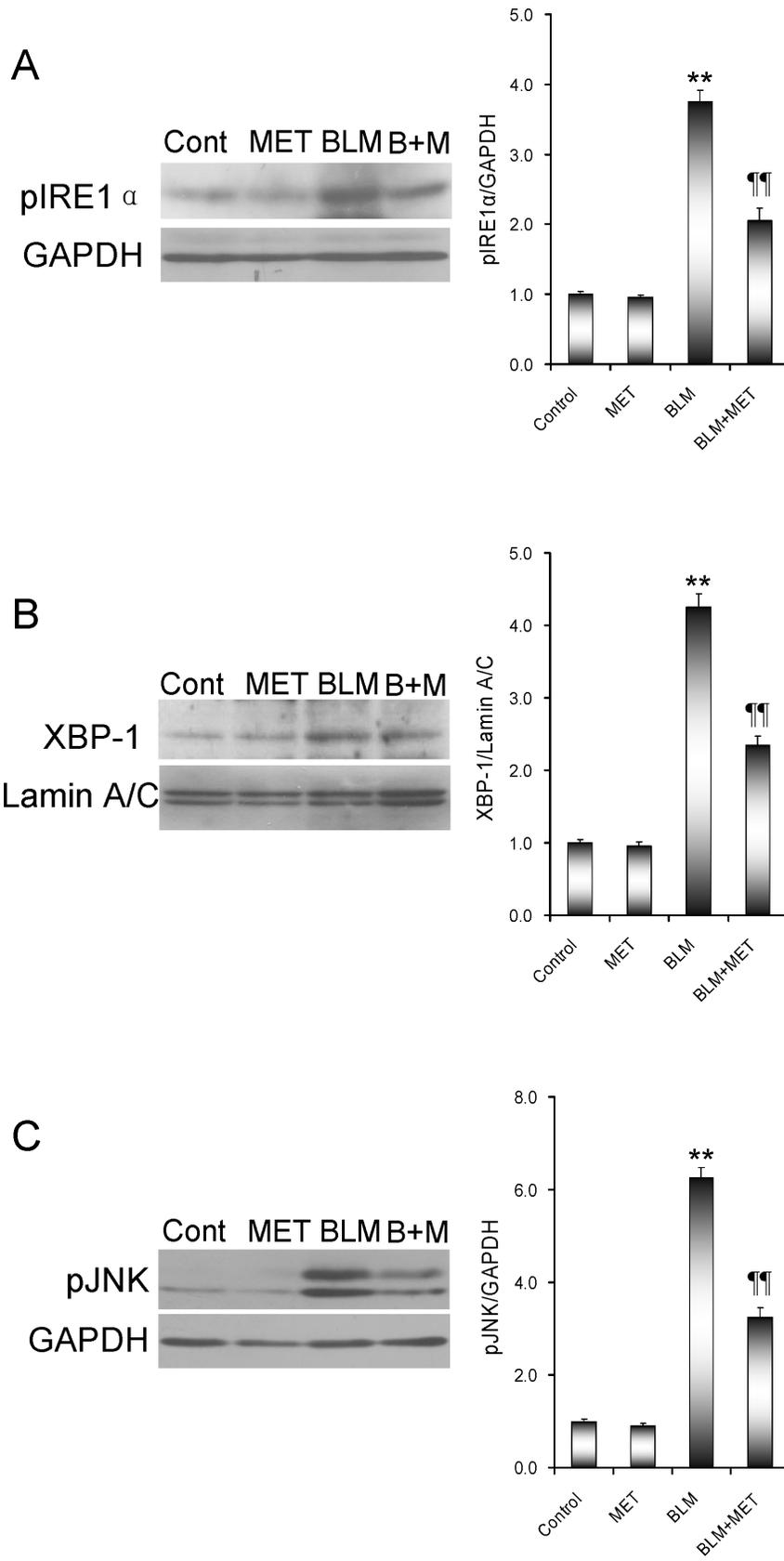


Figure 6. The effects of melatonin on BLM-induced activation of IRE1 pathway in the lungs. All mice except controls were intratracheally injected with BLM (5.0 mg/kg). In BLM+melatonin group, mice were i.p. injected with melatonin (5 mg/kg) daily. Lungs were collected at 21 days after BLM. (A) Pulmonary pIRE1 α was detected using immunoblots. (B) Nuclear XBP-1 was detected using immunoblots. (C) Pulmonary pJNK was detected

using immunoblots. All experiments were replicated for four times. The data were expressed as means \pm SEM ($n=4$). ** $P<0.01$ as compared with control group. †† $P<0.01$ as compared with BLM group.
doi:10.1371/journal.pone.0097266.g006

the IRE1 α pathway, was elevated in the lungs of BLM-treated mice (Figure 6B). JNK, another downstream target of the IRE1 signaling, was activated in the lungs of BLM-treated mice (Figure 6C). As expected, melatonin significantly attenuated BLM-induced pulmonary IRE1 α phosphorylation (Figure 6A). Moreover, melatonin significantly alleviated BLM-evoked activation of XBP-1 and JNK in the lungs (Figure 6B and 6C).

Discussion

The present study showed an obvious matrix protein deposition, as determined by Sirius red staining, in the lungs of BLM-treated mice. Moreover, an obvious rise in hydroxyproline content was observed in the lungs when mice were injected with BLM. Two earlier reports demonstrate that melatonin alleviates BLM-induced lung fibrosis in rats [28,30]. The present study investigated the effects of melatonin on BLM-induced lung fibrosis in mice. We showed that melatonin significantly alleviated BLM-induced matrix protein deposition in the lungs. Moreover, melatonin obviously attenuated BLM-induced elevation of pulmonary hydroxyproline content. These results suggest that melatonin protects against BLM-induced lung fibrosis in mice.

The ER is an important organelle required for normal cellular function. In the ER, nascent proteins are folded with the assistance of ER chaperones. The ER is sensitive to alterations in cellular homeostasis. When unfolded and misfolded proteins are retained in the ER lumen, ER stress occurs and the unfolded protein response (UPR) is activated [31–33]. Several studies demonstrate that ER stress and activation of the UPR signaling are involved in the pathogenesis of idiopathic pulmonary fibrosis [34]. According to several earlier reports, ER stress and UPR activation are observed in the alveolar epithelium in patients with idiopathic pulmonary fibrosis [12,35]. A recent study indicates that ER stress enhances BLM-evoked fibrotic remodeling in the lungs [14]. In the present study, we showed that a single dose of BLM injection caused pulmonary ER stress and activation of the UPR signaling in the lungs. First, the cleaved ATF6 in the nuclei was activated in the lungs of BLM-treated mice. GRP78, an ER chaperone and the target of ATF6 pathway, was up-regulated in the lungs of BLM-treated mice. Next, phosphorylated eIF2 α , a downstream target of the PERK signaling, was markedly increased in the lungs of BLM-treated mice. Finally, phosphorylated IRE1 α in the lungs was significantly increased when mice were injected with BLM. Correspondingly, pulmonary XBP-1 and JNK, two downstream targets of the IRE1 α signaling, were activated by BLM.

Melatonin is an inhibitor of ER stress. Previous report from our laboratory showed that melatonin obviously attenuated lipopolysaccharide-induced placental ER stress in mice [36]. Indeed, melatonin significantly repressed arsenite-induced elevations in activating transcription factor-4, CCAAT/enhancer binding protein homologues protein (CHOP) and GRP78 and activation

of XBP-1 in rat brain [37]. According to a recent report, melatonin attenuates cadmium-induced activation of XBP-1, up-regulation of GRP78 and CHOP, and phosphorylation of eIF2 α and JNK in testes [38]. Another recent report demonstrated that melatonin significantly alleviated ER stress and modulates the UPR signaling in rabbits with lethal fulminant hepatitis [39]. In the present study, we investigated the effects of melatonin on pulmonary ER stress and the UPR signaling during BLM-induced lung fibrosis. We found that melatonin alleviated BLM-induced elevation of the cleaved ATF6 in the lungs. In addition, melatonin attenuated BLM-induced up-regulation of GRP78 in the lungs. Moreover, melatonin significantly attenuated BLM-evoked pulmonary eIF2 α phosphorylation. Finally, melatonin markedly alleviated BLM-induced pulmonary IRE1 α phosphorylation. Correspondingly, melatonin significantly attenuated BLM-induced elevation of nuclear XBP-1 and JNK phosphorylation in the lungs. These results suggest that melatonin inhibits pulmonary ER stress and activation of the UPR signaling during BLM-induced lung fibrosis. Melatonin-mediated protection against BLM-induced lung fibrosis might be associated with its alleviation of ER stress in the lungs.

Increasing evidence demonstrates that EMT of alveolar epithelial cells to myfibroblasts plays a potential role in the pathogenesis of idiopathic pulmonary fibrosis [40,41]. Indeed, pulmonary α -SMA, a hallmark of EMT to myfibroblasts, was up-regulated during BLM-induced pulmonary fibrosis in rodent animals [42–44]. According to two recent reports, pulmonary ER stress contributes to EMT in alveolar epithelial cells as a possible mechanism for fibrotic remodeling [13,34]. In the present study, we showed that melatonin significantly alleviated BLM-induced pulmonary ER stress and activation of the UPR signaling. Correspondingly, melatonin blocked BLM-induced EMT to myfibroblasts in the lungs, as evidenced by its repression of pulmonary α -SMA. Taken together, these results suggest that melatonin protects against BLM-induced pulmonary fibrosis through its inhibition of ER stress-mediated EMT in the lungs.

In summary, the present study indicates that ER stress and activation of the UPR signaling in the lungs are involved in the pathogenesis of BLM-induced lung fibrosis. We demonstrate for the first time that melatonin inhibits BLM-induced pulmonary ER stress and ER stress-mediated EMT in the lungs. Importantly, melatonin protects against BLM-induced pulmonary fibrosis in mice. Therefore, melatonin may be useful as pharmacological agents to protect against idiopathic pulmonary fibrosis.

Author Contributions

Conceived and designed the experiments: DXX HZ RRL. Performed the experiments: HZ QQW LFC HYQ YHC. Analyzed the data: HW CZ. Contributed reagents/materials/analysis tools: DXX HZ. Contributed to the writing of the manuscript: DXX HZ.

References

- King TE, Jr, Pardo A, Selman M (2011) Idiopathic pulmonary fibrosis. *Lancet* 378: 1949–1961.
- Borchers AT, Chang C, Keen CL, Gershwin ME (2011) Idiopathic pulmonary fibrosis—an epidemiological and pathological review. *Clin Rev Allergy Immunol* 40: 117–134.
- Adams IY, Bowden DH (1974) The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. *Am J pathol* 77: 185–197.
- Moore BB, Hogaboam CM (2008) Murine models of pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 294: L152–160.
- Moeller A, Ask K, Warburton D, Gaudie J, Kolb M (2008) The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? *Int J Biochem Cell Biol* 40: 362–382.
- Wu J, Kaufman RJ (2006) From acute ER stress to physiological roles of the Unfolded Protein Response. *Cell Death Differ* 13: 374–384.
- Ron D, Walter P (2007) Signal integration in the endoplasmic reticulum unfolded protein response. *Nat Rev Mol Cell Biol* 8: 519–529.
- Kohno K (2007) How transmembrane proteins sense endoplasmic reticulum stress. *Antioxid Redox Signal* 9: 2295–2303.

9. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* 107: 881–891.
10. Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D (2000) Perk is essential for translational regulation and cell survival during the unfolded protein response. *Mol Cell* 5: 897–904.
11. Yoshida H, Haze K, Yanagi H, Yura T, Mori K (1998) Identification of the cis-acting endoplasmic reticulum stress response element responsible for transcriptional induction of mammalian glucose-regulated proteins. Involvement of basic leucine zipper transcription factors. *J Biol Chem* 273: 33741–33749.
12. Lawson WE, Crossno PF, Polosukhin VV, Roldan J, Cheng DS, et al. (2008) Endoplasmic reticulum stress in alveolar epithelial cells is prominent in IPF: association with altered surfactant protein processing and herpesvirus infection. *Am J Physiol Lung Cell Mol Physiol* 294: L1119–L1126.
13. Zhong Q, Zhou B, Ann DK, Minoo P, Liu Y, et al. (2011) Role of endoplasmic reticulum stress in epithelial-mesenchymal transition of alveolar epithelial cells: effects of misfolded surfactant protein. *Am J Respir Cell Mol Biol* 45: 498–509.
14. Lawson WE, Cheng DS, Degryse AL, Tanjore H, Polosukhin VV, et al. (2011) Endoplasmic reticulum stress enhances fibrotic remodeling in the lungs. *Proc Natl Acad Sci U S A* 108: 10562–10567.
15. Galano A, Tan DX, Reiter RJ (2011) Melatonin as a natural ally against oxidative stress: a physicochemical examination. *J Pineal Res* 51: 1–16.
16. Galano A, Tan DX, Reiter RJ (2013) On the free radical scavenging activities of melatonin's metabolites, AFMK and AMK. *J Pineal Res* 54: 245–257.
17. Rodriguez C, Mayo JC, Sainz RM, Antolin I, Herrera F, et al. (2004) Regulation of antioxidant enzymes: a significant role for melatonin. *J Pineal Res* 36: 1–9.
18. Richter HG, Hansell JA, Raut S, Giussani DA (2009) Melatonin improves placental efficiency and birth weight and increases the placental expression of antioxidant enzymes in undernourished pregnancy. *J Pineal Res* 46: 357–364.
19. Crespo I, Miguel BS, Laliena A, Alvarez M, Culebras JM, et al. (2010) Melatonin prevents the decreased activity of antioxidant enzymes and activates nuclear erythroid 2-related factor 2 signaling in an animal model of fulminant hepatic failure of viral origin. *J Pineal Res* 49: 193–200.
20. Wu CC, Lu KC, Lin GJ, Hsieh HY, Chu P, et al. (2012) Melatonin enhances endogenous heme oxygenase-1 and represses immune responses to ameliorate experimental murine membranous nephropathy. *J Pineal Res* 52: 460–469.
21. Xu DX, Wang H, Ning H, Zhao L, Chen YH (2007) Maternally administered melatonin differentially regulates lipopolysaccharide-induced proinflammatory and anti-inflammatory cytokines in maternal serum, amniotic fluid, fetal liver, and fetal brain. *J Pineal Res* 43: 74–79.
22. Park HJ, Kim HJ, Ra J, Hong SJ, Baik HH, et al. (2007) Melatonin inhibits lipopolysaccharide-induced CC chemokine subfamily gene expression in human peripheral blood mononuclear cells in a microarray analysis. *J Pineal Res* 43: 121–129.
23. Choi EY, Jin JY, Lee JY, Choi JI, Choi IS, et al. (2011) Melatonin inhibits *Prevotella intermedia* lipopolysaccharide-induced production of nitric oxide and interleukin-6 in murine macrophages by suppressing NF- κ B and STAT1 activity. *J Pineal Res* 50: 197–206.
24. Min KJ, Jang JH, Kwon TK (2012) Inhibitory effects of melatonin on the lipopolysaccharide-induced CC chemokine expression in BV2 murine microglial cells are mediated by suppression of Akt-induced NF- κ B and STAT/GAS activity. *J Pineal Res* 52: 296–304.
25. Shi D, Xiao X, Wang J, Liu L, Chen W, et al. (2012) Melatonin suppresses proinflammatory mediators in lipopolysaccharide-stimulated CRL1999 cells via targeting MAPK, NF- κ B, c/EBP β , and p300 signaling. *J Pineal Res* 53: 154–165.
26. Xia MZ, Liang YL, Wang H, Chen X, Huang YY, et al. (2012) Melatonin modulates TLR4-mediated inflammatory genes through MyD88- and TRIF-dependent signaling pathways in lipopolysaccharide-stimulated RAW264.7 cells. *J Pineal Res* 53: 325–334.
27. Lee YD, Kim JY, Lee KH, Kwak YJ, Lee SK, et al. (2009) Melatonin attenuates lipopolysaccharide-induced acute lung inflammation in sleep-deprived mice. *J Pineal Res* 46: 53–57.
28. Arslan SO, Zerim M, Vural H, Coskun A (2002) The effect of melatonin on bleomycin-induced pulmonary fibrosis in rats. *J Pineal Res* 32: 21–25.
29. Genovese T, Di Paola R, Mazzon E, Muia C, Caputi AP, et al. (2005) Melatonin limits lung injury in bleomycin treated mice. *J Pineal Res* 39: 105–112.
30. Yildirim Z, Kotuk M, Erdogan H, Iraz M, Yagmurca M, et al. (2006) Preventive effect of melatonin on bleomycin-induced lung fibrosis in rats. *J Pineal Res* 40: 27–33.
31. Hetz C, Martinon F, Rodriguez D, Glimcher LH (2011) The unfolded protein response: integrating stress signals through the stress sensor IRE1 α . *Physiol Rev* 91: 1219–1243.
32. Walter P, Ron D (2011) The unfolded protein response: from stress pathway to homeostatic regulation. *Science* 334: 1081–1086.
33. Hetz C (2012) The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 13: 89–102.
34. Tanjore H, Cheng DS, Degryse AL, Zoz DF, Abdolrasulnia R, et al. (2011) Alveolar epithelial cells undergo epithelial-to-mesenchymal transition in response to endoplasmic reticulum stress. *J Biol Chem* 286: 30972–30980.
35. Korfei M, Ruppert C, Mahavadi P, Henneke I, Markart P, et al. (2008) Epithelial endoplasmic reticulum stress and apoptosis in sporadic idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 178: 838–846.
36. Wang H, Li L, Zhao M, Chen YH, Zhang ZH, et al. (2011) Melatonin alleviates lipopolysaccharide-induced placental cellular stress response in mice. *J Pineal Res* 50: 418–426.
37. Lin AM, Fang SF, Chao PL, Yang CH (2007) Melatonin attenuates arsenite-induced apoptosis in rat brain: involvement of mitochondrial and endoplasmic reticulum pathways and aggregation of alpha-synuclein. *J Pineal Res* 43: 163–171.
38. Ji YL, Wang H, Meng C, Zhao XF, Zhang C, et al. (2012) Melatonin alleviates cadmium-induced cellular stress and germ cell apoptosis in testes. *J Pineal Res* 52: 71–79.
39. Tunon MJ, San-Miguel B, Crespo I, Laliena A, Vallejo D, et al. (2013) Melatonin treatment reduces endoplasmic reticulum stress and modulates the unfolded protein response in rabbits with lethal fulminant hepatitis of viral origin. *J Pineal Res* 55: 221–228.
40. Willis BC, Liebler JM, Luby-Phelps K, Nicholson AG, Crandall ED, et al. (2005) Induction of epithelial-mesenchymal transition in alveolar epithelial cells by transforming growth factor- β 1: potential role in idiopathic pulmonary fibrosis. *Am J Pathol* 166: 1321–1332.
41. Vyas-Read S, Shaul PW, Yuhanna IS, Willis BC (2007) Nitric oxide attenuates epithelial-mesenchymal transition in alveolar epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 293: L212–L221.
42. Muro AF, Moretti FA, Moore BB, Yan M, Atrasz RG, et al. (2008) An essential role for fibronectin extra type III domain A in pulmonary fibrosis. *Am J Respir Crit Care Med* 177: 638–645.
43. Hu B, Gharraee-Kermani M, Wu Z, Phan SH (2011) Essential role of McCP2 in the regulation of myofibroblast differentiation during pulmonary fibrosis. *Am J Pathol* 178: 1500–1508.
44. Huang LS, Fu P, Patel P, Harijith A, Sun T, et al. (2013) Lysophosphatidic acid receptor-2 deficiency confers protection against bleomycin-induced lung injury and fibrosis in mice. *Am J Respir Cell Mol Biol* 49: 912–922.