Biochemical and Biophysical Research Communications xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Melatonin suppresses ischemia-induced fibrosis by regulating miR-149

Yong-Seok Han [a], Jun Hee Lee [a, b], Sang Hun Lee [a, b, *]

[a] Medical Science Research Institute, Soonchunhyang University Seoul Hospital, Seoul, 04401, Republic of Korea
[b] Department of Biochemistry, Soonchunhyang University College of Medicine, Cheonan, 31151, Republic of Korea

ARTICLE INFO

Article history:
Received 29 January 2020
Accepted 12 February 2020
Available online xxx

Keywords:
Fibrosis
Ischemia
miR-149
Myoblast
PGC-1α
Anti-inflammation

ABSTRACT

Ischemic injury is a major risk factor for fibrosis. However, the precise mechanisms by which fibrosis is regulated and induced under ischemic oxidative stress conditions are unknown. To address this, we investigated the effect of melatonin on ischemia-induced fibrosis. In a hindlimb ischemia mouse model, ischemia induced fibrosis by increasing inflammation and the expression of extracellular matrix (ECM) proteins. Melatonin prevented ischemia-induced fibrosis in the injured tissues. In particular, melatonin suppressed the fibrosis-mediated inflammatory reaction in myoblasts through the microRNA-149 (miR-149)/indoleamine 2,3-dioxygenase-1 (IDO-1) signaling pathway. The melatonin-induced increase in miR-149 inhibited the expression of the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) and ECM components, such as collagen I and fibronectin. In addition, melatonin increased antioxidative activity and mitochondrial function in myoblasts via the miR-149/peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) signaling axis, and the anti-fibrotic effects of melatonin were blocked by inhibition of miR-149. These findings indicate that melatonin is a key target molecule in fibrosis related to ischemic diseases and that miR-149 might be a novel target for the treatment of ischemia-induced fibrosis.

1. Introduction

Fibrosis and fibrosis-mediated organ dysfunction are responsible for at least one-third of all deaths worldwide [1]. Ischemic injuries, such as myocardial infarction, hindlimb ischemia, ischemia-reperfusion, and acute kidney injury, are a major risk factor for fibrosis [2]. Although a short-term fibrogenic response is an adaptive response, a prolonged fibrogenic response leads to tissue dysfunction and organ failure [2]. Since a reduction in fibrosis results in improved clinical outcomes in several ischemic diseases, suppression of the factors triggering fibrosis is an efficient approach for treating ischemic diseases.

Excessive accumulation of extracellular matrix (ECM) proteins is a major pathophysiological factor in fibrosis leading to organ malfunction. In addition, nutrient and oxygen limitations promote cell injury and activation of myofibroblasts, resulting in macrophage infiltration and subsequent inflammatory responses, which are involved in fibrosis [3]. Since an imbalance in tumor necrosis factor-α (TNF-α) and interleukin (IL)-10 affects the progression of fibrosis, regulation of TNF-α and IL-10 levels is important for controlling fibrosis in ischemic diseases.

Previous studies have shown that microRNA-149 (miR-149) plays a role in regulating the transforming growth factor-β (TGF-β)/small mothers against decapentaplegic homolog (Smad) signaling pathway, which is involved in the progression of fibrosis [3,4]. In TNF-α-induced endothelial dysfunction, miR-149 also regulated TNF-α [5]. Thus, we investigated the effect of the endogenous pineal gland hormone melatonin on fibrosis in ischemic diseases as well as the role of miR-149 in the mechanism by which melatonin reduced ischemic injury-induced fibrosis.

2. Materials and methods

2.1. Primary myoblast culture

Myoblasts were isolated from the dissected hindlimb muscles of a mouse under sterile conditions. The tissues were minced into a

* Corresponding author. Soonchunhyang Medical Science Research Institute, Soonchunhyang University Seoul Hospital, 59, Daesagwan-ro (657 Hannam-dong), Yongsan-gu, Seoul, 04401, Republic of Korea.
E-mail addresses: ykckss1114@nate.com, jhlee0407@sch.ac.kr (S.H. Lee).

https://doi.org/10.1016/j.bbrc.2020.02.090
0006-291X/

coarse slurry using sterile surgical scissors and then enzymatically dissociated with collagenase (0.2 mg/mL type IV) for 20 min at 37 °C. After filtration through an 80 μm nylon mesh (Millipore, Inc., Billerica, MA, USA) to remove larger tissue pieces, cells were plated in culture plates containing Dulbecco's modified Eagle's medium (DMEM; Hyclone, Logan, UT, USA) with 10% (v/v) FBS (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL penicillin (Hyclone), and 100 mg/mL streptomycin (Hyclone).

2.2. Murine hindlimb ischemia model

All animal care, procedures, and experiments were approved by the Institutional Animal Care and Use Committee of Soonchunhyang University Seoul Hospital (IACUC2013-5) and were performed in accordance with the National Research Council Guidelines for the Care and Use of Laboratory Animals. The experiments were performed using 8-week-old male BALB/c nude mice (Biogenomics, Seoul, Korea) maintained on a 12-h light/dark cycle at 25 °C. The murine hindlimb ischemia model was established as previously described, with minor modifications [6]. Briefly, ischemia was induced by ligation and excision of the proximal femoral artery and boundary vessels. No later than 6 h after surgery, melatonin (20 mg/kg/day), intraperitoneal administration was started and continued daily for either 3 or 28 days. Melatonin was dissolved in ethanol and filter sterilized through a 0.45-μm pore filter (Sartorius Biotech GmbH, Gottingen, Germany). The final ethanol concentration in the administered melatonin preparation was less than 1%.

2.3. Histological staining

At 28 days after surgery, the ischemic tissues were fixed with 4% paraformaldehyde. For the histological analysis, tissue sections were stained with Sirius red and hematoxylin and eosin (H&E) to assess fibrosis and necrosis, respectively.

2.4. Western blot analysis

Total cellular proteins were extracted with RIPA lysis buffer (Thermo Fisher Scientific) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to polyvinylidene fluoride membranes (Sigma-Aldrich, St. Louis, MO, USA), which were blocked with 5% skim milk and then incubated with primary antibodies against p-Smad2 (Novus Biological, Centennial, CO, USA), collagen I (Thermo Fisher Scientific), fibronectin (Thermo Fisher Scientific), indoleamine 2,3-dioxygenase-1 (IDO-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA), peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α; Novus), cyclin E (Santa Cruz Biotechnology), cyclin-dependent kinase 2 (CDK2; Santa Cruz Biotechnology), cyclin D1 (Santa Cruz Biotechnology), CDK4 (Santa Cruz Biotechnology), β-actin (Santa Cruz Biotechnology), and α-tubulin (Santa Cruz Biotechnology). After incubation with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology), the bands were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific) in a dark room.

2.5. Quantitative real-time PCR

Quantitative real-time PCR (qPCR) analysis was performed using a Rotor-Gene 6000 real-time thermal cycling system (Corbett Research, Mortlake, NSW, Australia) with the QuantiMix SYBR Kit (Phile Korea Technology, Daejeon, Korea). The reactions were performed under the following conditions: 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 60 s. The data were analyzed by the comparative threshold cycle method and normalized to *Gapdh*. The primer sequences were as follows: miR-149 forward, 5′-TCTGGCTCCGTGTCTTC-3′; miR-149 reverse, 5′-GAACATGTCTGCGTATCTC-3′ (OriGene, Rockville, MD, USA).

2.6. IDO enzymatic activity

To investigate the enzymatic activity of IDO, myoblasts were suspended in PBS containing 100 μmol/L tryptophan (Sigma-Aldrich) and incubated for 4 h. Then, the supernatant was examined for the presence of kynurenine, the first stable catabolite downstream of IDO, as follows. The culture supernatant (100 μL) was mixed with 50 μL of 30% trichloroacetic acid, vortexed, and centrifuged at 12,000 × g for 5 min. The supernatant (75 μL) was then mixed with an equal volume of Ehrlich reagent, consisting of 100 mg of p-dimethylbenzaldehyde in 5 mL of glacial acetic acid, in a 96-well microplate. IDO activity was quantified by measuring the absorbance at 492 nm using a microplate reader (BMG Labtech, Ortenberg, Germany).

2.7. Macrophage inflammation assay and TNF-α and IL-10 ELISAs

After a 48-h exposure to phorbol-12-myristate-13-acetate (PMA, 100 ng/mL) in RPMI 1640 medium in 12-well culture plates, THP-1 monocytic cells were differentiated into macrophages. To mimic the inflammatory conditions in ischemia, the macrophages were stimulated with lipopolysaccharide (LPS) at 37 °C for 10 min. The LPS-stimulated macrophages (1×10^5 cells) were then co-cultured with myoblasts (5×10^4 cells), and the levels of TNF-α and IL-10 were estimated using ELISA kits (Koma Biotechnology, Seoul, Korea), according to the manufacturer's instructions.

2.8. miRNA transfection

Myoblasts (2×10^5 cells) were seeded in 60 mm culture plates, grown to 70% confluence in serum-free DMEM (Hyclone), transfected with miR-149 inhibitor (miRNA-149 inhibitor Applied Biological Materials, Vancouver, Canada) using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA), incubated at 37 °C for 48 h, and then prepared for subsequent experiments.

2.9. Small-interference RNA transfection

Myoblasts (2×10^5 cells) were seeded in 60 mm culture plates, grown to 70% confluence, and transfected with small-interfering RNA (siRNA) in serum-free DMEM (Hyclone) using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions. The siRNAs used to silence PGC-1α (*PGC-1α* siRNA no. 1: 5′-UCACCGAGACCGACGUUAA-3′, no. 2: 5′-GAUCGAGCAUGGUCCUCUU-3′, no. 3: 5′-AGAUGUGUAUCACCCAGUA-3′, and no. 4: 5′-GACCGUUACUAUCGUGAAA-3′) and the control scrambled siRNAs (scrambled siRNA no. 1: 5′-UGGUUUACAUGUCGACUAA-3′, no. 2: 5′-UGGUUUACAUGUUGUGUGA-3′, no. 3: 5′-UGGUUUACAUGUUUUCUGA-3′, and no. 4: 5′-UGGUUUACAUGUUUUCCUA-3′) were purchased from Dharmacon (Lafayette, CO, USA).

2.10. Dihydroethidium (DHE) staining

DHE (Sigma-Aldrich) was used to measure superoxide anion levels in myoblasts as follows. The cells were incubated with DHE (10 μM) for 30 min at 37 °C. After washing with PBS, the samples were analyzed with a Cyflow Cube 8 (Partec, Munster, Germany). The generated data were analyzed using standard FSC Express (De

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Novo Software, Los Angeles, CA, USA).

2.11. Catalase activity assay

Cell lysates (containing 40 μg of total protein) were incubated with 20 mM H_2O_2 in 0.1 M Tris–HCl for 30 min. Then, Amplex Red reagent (50 mM; Thermo Fisher Scientific) and horseradish peroxidase (0.2 U/ml; Sigma-Aldrich) were added to the lysates and incubated for 30 min at 37 °C. Substrate decomposition was recorded as the decrease in absorbance at 563 nm, which was used to calculate catalase activity.

2.12. Mitochondrial complex I and IV activity assays

Mitochondrial fractions isolated from myoblasts were incubated in assay medium (250 mmol/L sucrose, 50 mmol/L potassium phosphate, 1 mmol/L potassium cyanide, 50 μmol/L decylubiquinone, and 0.8 μmol/L antimycin, pH 7.4) for 3 min. Complex I activity was assessed by determining the rate of oxidation of nicotinamide adenine dinucleotide (100 mm) at 340 nM. Complex IV activity was analyzed by adding 75 mL of cytochrome *c* previously reduced with sodium borohydride and measuring the absorbance at 550 nm.

2.13. Single cell expansion assay

Myoblasts were dissociated into a single cell suspension using trypsin (Thermo Fisher Scientific). The cell density was adjusted to 1 cell per 100 μL via a limited-dilution assay, and then, single cells were seeded in 96-well plates and incubated in a humidified incubator at 37 °C with 5% CO_2 for 10 days.

2.14. Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). One-way analysis of variance followed by Tukey's post hoc test was used for multiple comparisons. Differences were considered to be statistically significant at p values less than 0.05.

3. Results

3.1. Melatonin inhibits ischemic injury-induced fibrosis in a murine hindlimb ischemia model

To determine whether ischemic injury induces fibrosis, we assessed fibrosis in ischemic-injured tissues in a murine model of hindlimb ischemia using Sirius red and H&E staining (Fig. 1A). The results showed that ischemic injury induced fibrosis, and melatonin administration reduced fibrosis (Fig. 1A). To further explore whether melatonin regulates fibrosis-related inflammatory reactions and the expression of ECM proteins, the secretion of two inflammation-related cytokines, the pro-inflammatory cytokine TNF-α and the anti-inflammatory cytokine IL-10, was assessed in ischemic-injured tissues. The results showed that ischemia increased the expression of TNF-α and decreased the expression of IL-10 in ischemic-injured tissues (Fig. 1B). However, administration of melatonin significantly reduced the expression of TNF-α and increased the expression of IL-10 (Fig. 1B). In addition, phosphorylation of Smad2, a protein that is involved in fibrosis, was significantly increased in ischemic-injured tissues, and this increase was significantly reduced by administration of melatonin (Fig. 1C). The expression levels of the ECM proteins collagen I and fibronectin were also increased in ischemic tissues, and these increases were inhibited by administration of melatonin (Fig. 1C). These results indicate that melatonin reduces ischemic injury-induced fibrosis.

3.2. Melatonin reduces fibrosis-related inflammatory reactions by upregulating miR-149

To evaluate whether melatonin regulates the expression of IDO-1, which plays a pivotal role in immunomodulation [7], we assessed the expression of IDO-1 after treating myoblasts with melatonin. IDO-1 levels in myoblasts were significantly increased after treatment with melatonin for 12 h (Fig. 2A and 2B). After melatonin treatment, miR-149 levels were also significantly increased. However, treatment with luzindole, which is an inhibitor of melatonin receptor, blocked this effect (Supplementary Fig. 1A and 1B, Fig. 2C). To explore whether melatonin-induced miR-149 regulates the expression of IDO-1 and the secretion of inflammatory cytokines, IDO-1 activity and TNF-α expression were assessed in myoblasts under oxidative stress conditions. After H_2O_2 exposure, treatment of myoblasts with melatonin significantly increased the expression of IDO-1, but inhibition of miR-149 blocked this effect (Fig. 2D and 2E, Supplementary Fig. 1C and 1D). IDO-1 activity was also significantly increased by treatment with melatonin and was dependent on miR-149 expression (Fig. 2F). TNF-levels α in myoblasts were significantly decreased by treatment with melatonin under oxidative stress conditions through upregulation of miR-149 expression (Fig. 2G). In a co-culture assay of myoblasts and macrophages, melatonin-treated myoblasts significantly suppressed TNF-α secretion and drastically increased IL-10 expression under oxidative stress conditions (Fig. 2H and 2I). These findings suggest that melatonin suppresses fibrosis-related inflammatory reactions under oxidative stress conditions through the IDO-1/miR-149 signaling axis.

3.3. Melatonin reduces the accumulation of ECM by upregulating miR-149

To verify whether melatonin suppresses the expression of ECM proteins in myoblasts under oxidative stress conditions, the phosphorylation of Smad2 and expression of collagen I and fibronectin were assessed in myoblasts after treatment with melatonin under H_2O_2 exposure. Under these oxidative stress conditions, treatment with melatonin for 24 h significantly increased p-Smad2 levels compared to those in H_2O_2-exposed cells, and inhibition of miR-149 blocked this effect (Supplementary Fig. 1B, Fig. 3A, and 3B). The levels of collagen I and fibronectin were also significantly decreased by administration of melatonin under oxidative stress conditions via upregulation of miR-149 (Fig. 3A, 3C, and 3D). Furthermore, the proliferative capacity of the myoblasts was significantly increased by treatment with melatonin under oxidative stress conditions (Supplementary Fig. 2A and 2B). This enhanced proliferative capacity was mediated by upregulation of miR-149-dependent cell cycle-associated proteins, such as cyclin E, CDK2, cyclin D1, and CDK4 (Supplementary Fig. 2C-2F). These results demonstrate that melatonin suppresses the expression of ECM proteins by upregulating miR-149.

3.4. Melatonin increases mitochondrial function under oxidative stress conditions by upregulating miR-149

Previous studies revealed that PGC-1α protects against fibrosis in the kidneys and the formation of fibrotic tissues in injured skeletal muscle [8,9]. In addition, our recent study showed that melatonin increased the level of PGC-1α in mesenchymal stem cells, resulting in enhanced mitochondrial function and angiogenic potential [10]. Thus, to explore the relationship between miR-149 and PGC-1α in melatonin-treated myoblasts, we assessed whether melatonin regulates mitochondrial function under oxidative stress conditions via the miR-149/PGC-1α signaling

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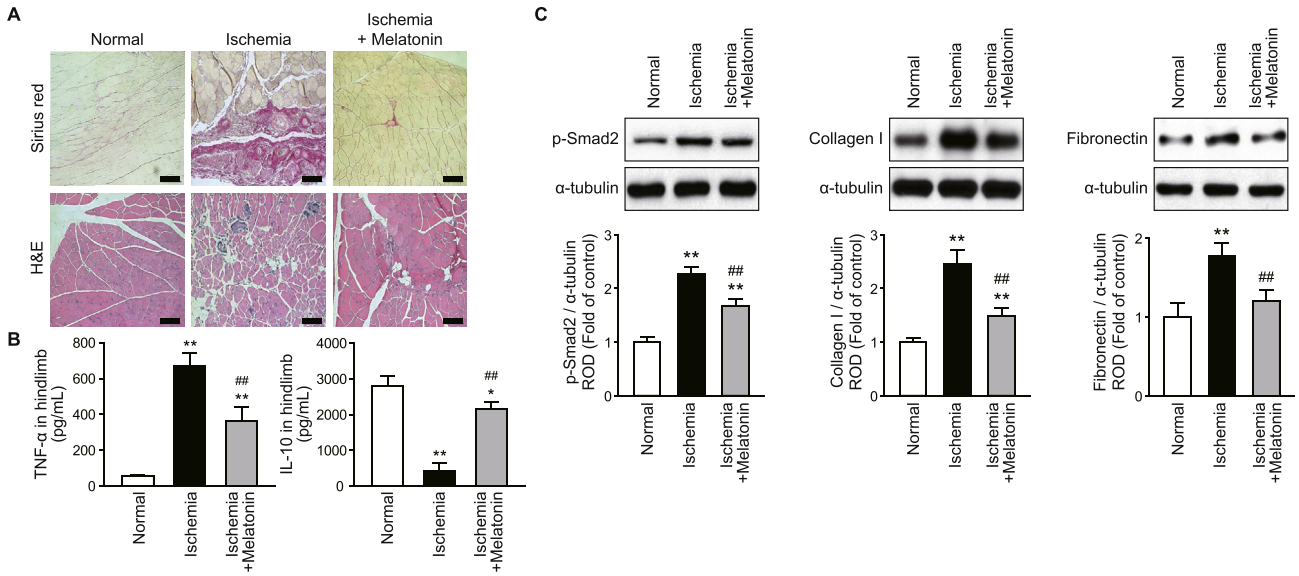

Fig. 1. Melatonin protects against ischemia-induced fibrosis in a murine model of hindlimb ischemia. (A) Sirius Red and H&E staining of ischemic hindlimbs on postoperative day 28 (n = 3). Scale bar = 100 μm. (B) TNF-α and IL-10 levels in ischemic tissues 3 days after surgery as assessed by ELISA (n = 3). (C) Expression levels of p-Smad2, collagen I, and fibronectin in ischemic tissues 3 days after surgery (n = 3). Protein levels relative to α-tubulin were determined by densitometry analysis. Values represent the means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Normal; $^{\#\#}p < 0.01$ vs. Ischemia. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

pathway. PGC-1α expression was significantly increased by treatment with melatonin for 24 h (Fig. 4A), and this melatonin-induced increase in PGC-1α levels was abrogated by inhibition of miR-149 (Fig. 4B). A DHE staining assay showed that melatonin significantly decreased ROS generation under oxidative stress conditions, whereas inhibition of miR-149 or silencing of PGC-1α blocked the anti-oxidative effects of melatonin (Fig. 4D and 4E). Catalase activity was also increased by treatment with melatonin under H_2O_2 exposure, whereas inhibition of miR-149 and/or knockdown of PGC-1α blocked this effect (Fig. 4F). Furthermore, mitochondrial oxidative phosphorylation in myoblasts was significantly increased by melatonin treatment under oxidative stress conditions through the miR-149/PGC-1α signaling axis (Fig. 4G and 4H). These results suggest that melatonin-induced PGC-1α modulates mitochondrial

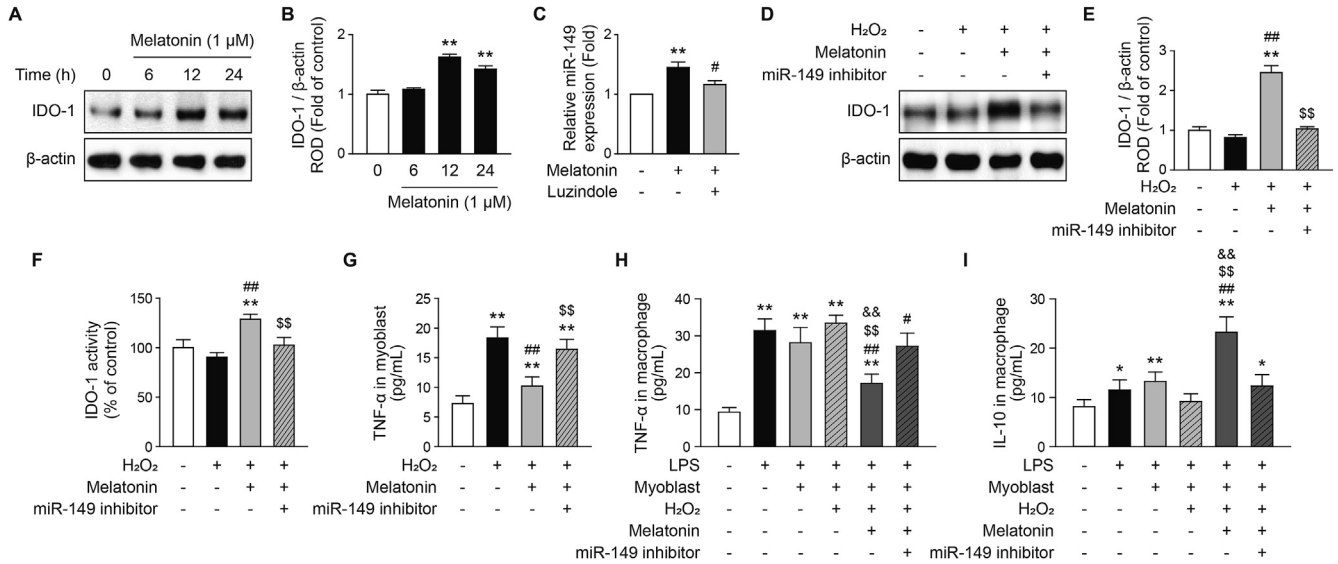

Fig. 2. Melatonin inhibits oxidative stress-induced inflammatory reactions in myoblasts via the miR-149/IDO signaling pathway. (A) Expression of IDO-1 in myoblasts after treatment with melatonin (1 μM; n = 3). (B) IDO-1 levels relative to that of β-actin were determined by densitometry. Values represent the means ± SEM. $^{**}p < 0.01$ vs. control (0 h). (C) miR-149 expression in myoblasts treated with melatonin (1 μM) for 12 h with or without luzindole (1 μM; n = 3). Values represent the means ± SEM. $^{**}p < 0.01$ vs. untreated; $^{\#}p < 0.05$ vs. melatonin only. (D) Expression of IDO-1 in melatonin-pretreated myoblasts (1 μM for 24 h) after treatment with H_2O_2 for 4 h (200 μM; n = 3) (E) IDO-1 levels relative to β-actin were determined by densitometry. Values represent the means ± SEM. $^{**}p < 0.01$ vs. untreated; $^{\#\#}p < 0.01$ vs. H_2O_2 only; $^{\$\$}p < 0.01$ vs. H_2O_2 + Melatonin. (F) IDO-1 activity in melatonin-pretreated myoblasts (1 μM for 24 h; n = 3). Values represent the means ± SEM. $^{**}p < 0.01$ vs. untreated; $^{\#\#}p < 0.01$ vs. H_2O_2 only; $^{\$\$}p < 0.01$ vs. H_2O_2 + Melatonin. (G) TNF-α expression in melatonin-pretreated myoblasts (1 μM for 24 h) as assessed by ELISA (n = 3). Values represent the means ± SEM. $^{**}p < 0.01$ vs. untreated; $^{\#\#}p < 0.01$ vs. H_2O_2 only; $^{\$\$}p < 0.01$ vs. H_2O_2 + Melatonin. (H and I) Expression levels of TNF-α (H) and IL-10 (I) Co-culture of macrophages and myoblasts pretreated with melatonin (1 μM for 24 h; n = 3). Values represent the means ± SEM. $^{**}p < 0.01$ vs. untreated; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs. LPS; $^{\$\$}p < 0.01$ vs. LPS + myoblasts; $^{\&\&}p < 0.01$ vs. LPS + myoblasts + H_2O_2.

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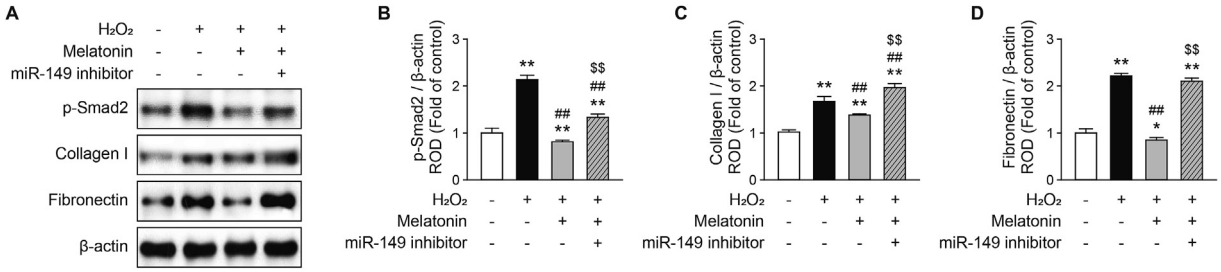

Fig. 3. Melatonin suppresses the expression of ECM proteins in myoblasts. (A) The expression levels of p-Smad2, collagen I, and fibronectin in melatonin-pretreated myoblasts (1 μM for 24 h) exposed to H_2O_2 for 4 h (200 μM; n = 3). (B-D) The levels of p-Smad2 (B), collagen I (C), and fibronectin (D) relative to β-actin were determined by densitometry. Values represent the means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. untreated; $^{\#\#}p < 0.01$ vs. H_2O_2; $^{\$\$}p < 0.01$ vs. H_2O_2 + Melatonin.

function by regulating miR-149 expression.

4. Discussion

Fibrosis is a hallmark of pathologic remodeling in several tissues and organs. Due to the adverse effects of fibrosis, it is important to identify therapeutic targets for fibrosis. Although the factors that reverse fibrosis are not yet clear, it has been shown that regression of fibrosis improves clinical outcomes in cardiac, hepatic, renal, and pulmonary fibrosis [2]. In particular, elimination of the major cellular elements and molecular processes related to fibrogenesis is the most efficacious approach. The present study showed that administration of melatonin protected against ischemia-induced fibrosis by regulating the secretion of inflammatory cytokines and inhibiting the accumulation of ECM proteins. Our findings also indicate that melatonin regulates fibrogenesis through the IDO-1/miR-149 signaling pathway.

TGF-β is a pro-inflammatory cytokine that functions as a master regulator of pathophysiological tissue remodeling [11]. Activation of TGF-β signaling induces progressive fibrosis in multiple tissues by activating the downstream Smad signaling pathway [12]. One Smad family member, Smad2, plays a role in several biological activities, including cell growth, cell differentiation, and ECM production [13]. In the context of fibrosis, activation of Smad2 induces the expression of ECM proteins, resulting in increased fibrogenesis [14]. Our findings indicated that hindlimb ischemia induced the phosphorylation of Smad2 and the expression of ECM proteins, and administration of melatonin blocked ischemia-induced fibrosis in a murine model of hindlimb ischemia. In particular, melatonin suppressed fibrosis-mediated inflammatory reactions by upregulating IDO-1. Melatonin is an endogenous hormone produced by the pineal gland that regulates several physiological functions, including sleep, circadian rhythms, and immunity, and has anti-inflammatory, anti-apoptosis, and anti-oxidative effects [15,16]. Several studies have suggested that administration of melatonin enhances the therapeutic efficacy of treatments for ischemic

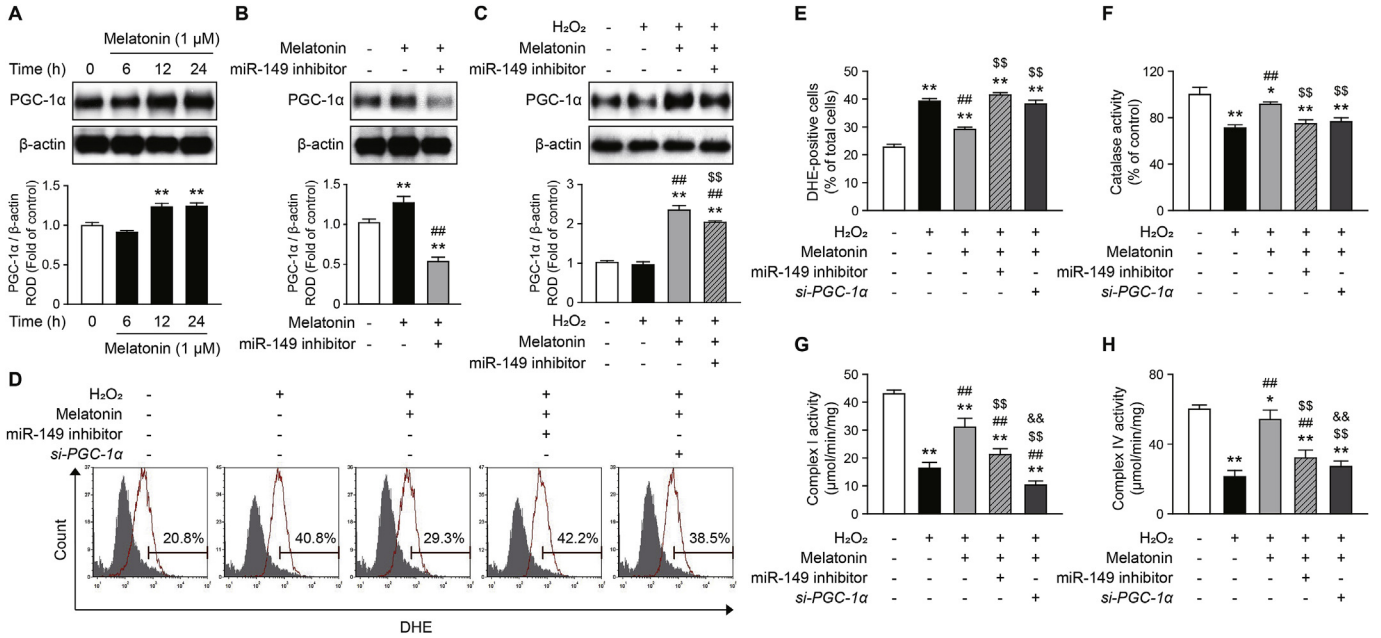

Fig. 4. Melatonin promotes mitochondrial function under oxidative stress conditions through the miR-149/PGC-1α signaling axis. (A) PGC-1α expression in myoblasts treated with melatonin (1 μM) for 0, 6, 12, and 24 h (n = 3). PGC-1α levels relative to β-actin were determined by densitometry. Values represent the means ± SEM. $^{**}p < 0.01$ vs. control (0 h). (B) PGC-1α expression in myoblasts treated with melatonin (1 μM) for 24 h (n = 3). PGC-1α levels relative to β-actin were determined by densitometry. Values represent the means ± SEM. $^{**}p < 0.01$ vs. untreated; $^{\#\#}p < 0.01$ vs. Melatonin. (C) PGC-1α expression in melatonin-pretreated myoblasts (1 μM for 24 h) exposed to H_2O_2 for 4 h (200 μM; n = 3). PGC-1α levels relative to β-actin were determined by densitometry. Values represent the means ± SEM. $^{**}p < 0.01$ vs. untreated; $^{\#\#}p < 0.01$ vs. H_2O_2; $^{\$\$}p < 0.01$ vs. H_2O_2 + Melatonin. (D) DHE staining of myoblasts as measured by flow cytometry (n = 5). (E) Quantification of the percentage of DHE-positive cells. (I) Catalase activity in myoblasts (n = 3). (G and H) Activity of mitochondrial complexes I (G) and IV (H) in myoblasts (n = 5). (E-H) Values represent the means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. untreated; $^{\#\#}p < 0.01$ vs. H_2O_2; $^{\$\$}p < 0.01$ vs. H_2O_2 + Melatonin; $^{\&\&}p < 0.01$ vs. H_2O_2 + Melatonin + miR-149 inhibitor.

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diseases, such as myocardial infarction, acute lung ischemia-reperfusion injury, and hindlimb ischemia [6,17,18]. Our previous study also showed that melatonin enhances the anti-inflammatory reaction against oxidative stress by regulating IDO-1 expression. These findings suggest that melatonin is a potential therapeutic agent for ischemia-induced fibrosis.

Our results showed that the anti-fibrogenic effect of melatonin during ischemia was dependent on the level of miR-149. Accumulating evidence indicates that miR-149 controls the expression of TNF-α, IL-6, and iNOS in the pathogenesis of osteoarthritis and endothelial dysfunction [5,19]. Furthermore, elevated miR-149 attenuates injury-induced inflammatory responses, resulting in improved wound healing [20]. miR-149 also enhances mitochondrial biogenesis in skeletal muscle [21]. Our study reported, for the first time, that melatonin increased the expression of miR-149 in ischemia-induced fibrogenesis, and the increased miR-149 suppressed fibrosis under oxidative stress conditions. These results suggest that miR-149 could be a powerful therapeutic target for pathologic fibrosis in ischemic diseases.

PGC-1α is a key regulator of several biological processes, including mitochondrial biogenesis, muscle function, and fiber repair and regeneration [9,22]. Recent studies have shown that PGC-1α regulates the development of fibrosis by modulating the inflammatory response [8,9]. This study showed that melatonin increased PGC-1α expression and melatonin-stimulated PGC-1α protected against oxidative stress and enhanced anti-oxidative enzyme activities and mitochondrial oxidative phosphorylation. Moreover, PGC-1α expression was regulated by miR-149. A previous study revealed that miR-149 improves mitochondrial function through the SIRT-1/PGC-1α signaling pathway [21]. Furthermore, our previous findings verified that melatonin promotes mitochondrial function by upregulating PGC-1α [10]. These findings indicate that melatonin protects mitochondria against ischemia-induced fibrosis-like conditions via the miR-149/PGC-1α signaling axis.

Taken together, our results indicate that melatonin protects against ischemia-induced fibrosis by regulating miR-149. In particular, melatonin prevents fibrosis-mediated inflammatory reactions and the accumulation of ECM proteins in ischemic-injured tissues. These findings suggest that melatonin could be a powerful physiological molecule for inhibiting fibrosis in patients with ischemic diseases. Furthermore, regulation of miR-149 provide an important clue regarding the reversibility of fibrogenesis.

Declaration of competing interest

The authors declare no conflict of interests.

Acknowledgments

This study was supported by a grant from the National Research Foundation funded by the Korean government (2016R1D1A3B01007727, 2017M3A9B4032528, 2019M3A9H1103495). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.02.090.

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