Melatonin antagonizes oxidative stress-induced mitochondrial dysfunction in retinal pigmented epithelium cells via melatonin receptor 1 (MT1)

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ABSTRACT — High energy-consumption in retinal pigmented epithelium (RPE) cells poses oxidative stress (OS) and contributes to mitochondrial dysfunction (MD) for retinal degeneration-associated diseases. In the present study, we evaluated the protective role of Melatonin, a natural antioxidant, against the hydrogen peroxide (H2O2)-induced damage to RPE cells. The cellular viability, apoptosis, the expression of apoptosis-associated proteins and mitochondrial function were examined in the retinal ARPE-19 cells, post the treatment with H2O2 or (and) with Melatonin. The regulation by Melatonin receptor 1 (MT1) on the Melatonin-mediated protection was also examined via MT1 knockdown with siRNA. Results demonstrated that Melatonin significantly ameliorated cell viability reduction, reduced apoptosis and downregulated the apoptosis-associated proteins in H2O2-treated ARPE-19 cells. The H2O2-induced mitochondrial dysfunction was also significantly blocked by the Melatonin treatment, presenting as a reduced accumulation of reactive oxygen species (ROS) and mitochondrial superoxide and an ameliorated reduction of mitochondrial membrane potential (MMP). In addition, the knockdown of MT1 with MT1-specific siRNA inhibited the Melatonin-mediated protection against OS damage in ARPE-19 cells. In summary, we confirmed the protective role of Melatonin against H2O2-induced mitochondrial dysfunction in RPE cells. MT1 knockdown blocked such protective role of Melatonin. It is implied that Melatonin exerts a protective role against oxidative stress via Melatonin-MT1 signaling in RPE cells.

Key words: Melatonin, Mitochondrial dysfunction (MD), Oxidative stress (OS), Retinal pigmented epithelium (RPE) cells

INTRODUCTION

Due to the extremely high workload of retinal pigment epithelial (RPE) cells, enriched mitochondrial population and robust metabolic activity were required to meet the high-energy need in RPE cells (Plafker et al., 2012; Boulton et al., 2001). High local reactive oxygen species (ROS) and an amplified oxidative phosphorylation produce during the robust metabolic activity in RPE cells (King et al., 2004). Sustained high ROS production will eventually overcome host neutralizing capability, and then cause cytosolic or mitochondrial damage, and even result in cell apoptosis. Oxidative stress (OS), particularly, chronic intracellular OS, constantly threatens the structural and functional integrity of the RPE cells (Plafker et al., 2012). Particularly, age-related macular degeneration (AMD), which is a leading cause of blindness in the elderly population (Congdon et al., 2004; Mehta, 2015; Randolph, 2014), has been increasingly attributed to the intracellular ROS accumulation (Rózanowska et al., 1995; Jin et al., 2001). Therefore, countermeasures to antagonize the OS-mediated damage to RPE cells or to eliminate ROS might be alternative measures for AMD prevention and treatment.
ROS mainly targets mitochondrion, destroys its membrane integrity, causes a collapse in the mitochondrial membrane potential (ΔΨm, MMP), and thus results in mitochondrial dysfunction. Released mitochondrial mediators even activate apoptosis-associated caspases, such as cytochrome c (Cyt c) and induce cell apoptosis (Lieven et al., 2003; Sano et al., 2006; Jiang et al., 2005; Balaban et al., 2005). A reduced MMP, an activated complex IV and the release of Cyt c were found in the RPE cells under OS (Liu et al., 2015; Hanus et al., 2015a). Autophagy is a self-degradation process for non-essential or damaged cellular constituents, to keep the balance between organelle biogenesis, protein synthesis and their clearance. (De Duve, 1963; De Duve and Wattiaux, 1966). There is cross-talk with ROS in both cell signaling and protein damage, in response to ROS-mediated damage (Lee et al., 2012a). Mitochondrial ROS and oxidized lipids in mitochondria regulate autophagy (Kissová et al., 2006), and can be antagonized by antioxidants (Kissová et al., 2006). H2O2 also plays a regulatory role in autophagy in response to nutrient starvation (Djavaheri-Mergny et al., 2006).

In recent years, several antioxidants have been identified to antagonize OS in RPE cells, such as Melatonin (Ozdemir et al., 2014), Gossypol Acetic Acid (Hanus et al., 2015b), Lycium barbarum polysaccharides (Liu et al., 2015) and Epigallocatechin gallate (EGCG) (Cia et al., 2014). In the present study, we examined the antioxidant effect of Melatonin in retinal pigment epithelial (RPE) cells under oxidative stress. Then the induction of autophagy and the regulation by Melatonin on autophagy was also investigated. Our study indicated a promising anti-oxidative stress effect of Melatonin in retinal pigment epithelial cells.

**MATERIALS AND METHODS**

**Cell culture and treatment**

Human RPE cell line ARPE-19 (ATCC, Manassas, VA, USA) was cultured with Dulbecco’s modified Eagle’s medium (DMEM) (Gibco, Rockville, MD, USA), adding 10% fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA, USA) and 2 mM L-glutamine solution (Sigma-Aldrich, St. Louis, MO, USA) at 37°C with CO2 5%. For Melatonin treatment, ARPE cells with 85% confluence were transfected with 40 or 80 nM siRNA 1-MT1 or siRNA 2-MT1 (GenePharma, Shanghai, China), with Scramble RNA as negative control for 48 hr. The transfection was performed via Lipofectamine RNAiMax (Invitrogen), according to reagent manual.

**Protein sample preparation and western blotting**

ARPE cells were scratched and were collected for the protein isolation of mitochondrial and cytosol fractions with Mitochondria/Cytosol Fractionation Kit (BioVision, Milpitas, CA, USA), according to the kit’s manual. Protein concentration was titered using the BCA protein assay reagent (Sigma-Aldrich). Protein samples were separated with 12% SDS-PAGE gel, and were transferred to a PVDF Transfer Membrane (Thermo Fisher Scientific, Waltham, MA, USA). The membrane was inoculated with a rabbit polyclonal antibody to cytochrome c, caspase 3, caspase 9, Cox411 or β-actin (either with 1:1000, all from Sigma-Aldrich) for the antigen-antibody binding. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Pierce, Rockford, IL, USA) and enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Amersham, UK) were utilized to visualize the Ag-Ab binding.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining**

TUNEL staining was performed for apoptosis assay with TUNEL Apoptosis Detection Kit (Promega, Madison, WI, USA). ARPE-19 cells were fixed with 4% Parafomaldehyde, were washed with phosphate buffered solution (PBS) for three times, and then were incubated for 1 hr at room temperature 26°C with TUNEL and DAPI (4’, 6-Diamidino-2-Phenylindole, Dihydrochloride) solution respectively for segmented DNA and nucleus. Cells were observed and imaged with confocal laser microscopy (Olympus, Tokyo, Japan).

**Measurement of mitochondrial ROS, superoxide and MMP**

Cytosolic ROS was stained with DCFH-DA (2’,7’-Dichlorodihydro-fluorescein diacetate) (Invitrogen), which was converted to DCF (2,7-dichlorofluorescein) in the presence of ROS, according to the reagent manual. 2,7-dichlorofluor-rescin (DCF) fluorescence was measured with a luminescence spectrometer (Ex/Em = 488/530 nm). The mitochondrial superoxide was stained with MitosOX™ Red (Invitrogen Molecular Probes, Carlsbad, CA, USA),
which highly selectively binds to the mitochondrial superoxide. MitoSOX Red fluorescence was detected with a luminescence spectrometer (Ex/Em = 510 / 580 nm). MMP was quantified with MitoSensor red (Abcam, Cambridge, UK) according to the kit’s manual. MitoSensor red (Pierce) attaches to the mitochondrial membrane in live cells, presenting red in monomeric form, and detaches when the mitochondrial membrane is broken, presenting green in aggregated form. MitoSensor red fluorescence was observed with a luminescence spectrometer (Ex/Em = 579 / 599 nm) and was indicated as an OD value.

Statistical Analysis
Quantitative results are presented as mean ± S.D. of the results from at least triple independent experiments. Student’s t test or one-way ANOVA followed by Newman-Keuls test was performed to compare results from two or more groups. A p value < 0.05 or less was considered statistically significant.

RESULTS
Cytotoxicity of Melatonin in retinal pigmented epithelium ARPE-19 cells
Melatonin is chemically known as N-acetyl-5-methoxy tryptamine (Singh and Jadhav 2014) (Fig. 1A). It mainly regulates sleep and wakefulness in animals (Hardeland et al., 2006), and antagonizes OS in plants (Tan et al., 2012). Firstly, we examined the cytotoxic effect of Melatonin in ARPE-19 cells with MTT assay. It is shown in Fig. 1B that the treatment with 20, 50, 100 or 200 μM Melatonin did not downregulate the viability of ARPE-19 cells post the incubation for 48 hr. However, the cellular viability was markedly higher in the ARPE-19 cells with the treatment with 100 μM Melatonin for 48 hr (Fig. 1A). We then repeated the assay for various time points with 100 μM Melatonin; as indicated in Fig. 1C, significant cellular viability increase was found at 48 or 72 hr post Melatonin treatment. Therefore, Melatonin upregulates the viability of retinal pigmented epithelium ARPE-19 Cells with a concentration of 100 μM.

Melatonin blocks the H_{2}O_{2}-induced apoptosis in ARPE-19 cells
To explore the influence of Melatonin on oxidative stress-induced damage to retinal pigmented epithelium cells, we examined the viability and apoptosis induction in the H_{2}O_{2}-treated ARPE-19 cells. It was found that H_{2}O_{2} treatment significantly reduced the cellular viability of ARE-19 cells with a concentration of 400 μM, at 12, 24 or 48 hr post treatment (p < 0.05, p < 0.01 or p < 0.001, Fig. 2A). However, such viability reduction was blocked by 200 μM Melatonin at 24 or 48 hr post
Melatonin antagonizes H2O2-induced apoptosis in ARPE-19 cells. To further investigate the inhibition by Melatonin on the H2O2-induced damage to ARPE-19 cells, we performed TUNEL staining for apoptosis in the H2O2-treated (48 hr) ARPE-19 cells. Compared to normal ARPE-19 cells (First column, Fig. 2B; p < 0.0001, Fig. 2C). We also examined the influence of Melatonin on the H2O2-induced apoptosis in ARPE-19 cells. It was found that 100 μM Melatonin reduced the H2O2-promoted TUNEL-positive cells (48 hr), however not significantly (Third column, Fig. 2B). Such reduction of H2O2-promoted TUNEL-positive cells was significant when 200 μM Melatonin (48 hr) (Fourth column, Fig. 2B; p < 0.01, Fig. 2C).

In addition, western blotting was performed to evaluate apoptosis-associated proteins in H2O2 or (and) Melatonin-treated ARPE-19 cells. Compared to mitochondrial cytochrome c (Cyto c) (Cox4I1 as loading control for mitochondria) (Fig. 3A and 3B), significantly more cytochrome c (Cyto c) was released in cytosol in response to H2O2 treatment (400 μM) (p < 0.001, Fig. 3C). Such promotion was markedly blocked by 100 or 200 μM Melatonin (p < 0.01 or p < 0.001), dose-dependently (p < 0.01). The H2O2-induced promotion to cleaved caspase 3 (Cleaved CASP 3) and Cleaved CASP 9 was also significant (p < 0.0001, Fig. 3D and 3E), and was also blocked by Melatonin treatment (p < 0.001 or p < 0.0001), dose-dependently (p < 0.001). Taken together, these results indicate that Melatonin blocks the H2O2-induced apoptosis in ARPE-19 cells.

Melatonin antagonizes H2O2-induced mitochondrial dysfunction in ARPE-19 cells

Retinal pigmented epithelium cell is one type of high energy-consuming cell, in which the mitochondrion is the energy plant (Barot et al., 2011). Accumulating evidence supports the contribution of mitochondrial dysfunction to many retinal degenerations (Barot et al., 2011). To evaluate the oxidative stress-exerted damage to and a possible protection by Melatonin to such damage in retinal pigmented epithelium cells, we examined mitochondrial function in the H2O2-treated ARPE-19 cells, with or without Melatonin treatment. As indicated in Fig. 4A, compared with normal ARPE-19 cells, more DCFH-DA was oxidized to highly fluorescent compound DCF, presenting green under fluorescence microscope, in the ARPE-19 cells, post H2O2 treatment (400 μM for 48 hr) (p < 0.0001, Fig. 4B). Though the Melatonin treatment (100 μM for 48 hr) did not pose a significant effect on the conversion of DCFH-DA to DCF in normal ARPE-19 cells, markedly less DCF was found in the ARPE-19 cells, post the treatment with both 400 μM H2O2 and 100 μM Melatonin, than in the ARPE-19 cells, post the treatment only with 400 μM H2O2 (p < 0.01, Fig. 4A and 4B). In addition, theH2O2-promoted (400 μM for 48 hr) mitochondrial superoxide (p < 0.001, Fig. 4C) was inhibited by Melatonin (100 μM for 48 hr) (p < 0.01) in ARPE-19 cells. The H2O2-mediated MMP reduction and the Melatonin-mediated blockage of such reduction were also observed in ARPE-19 cells (p < 0.01 or p < 0.001, Fig. 4D and 4E). Taken together, our results confirmed Melatonin’s antagonism of the H2O2-induced mitochondrial dysfunction in ARPE-19 cells.

Influence of the knockdown of MT1 melatonin receptor on the Melatonin-mediated mitochondria protection in ARPE-19 cells

To reconfirm the Melatonin-mediated mitochondria protection in ARPE-19 cells, we knocked down the MT1, and then re-evaluated the mitochondrial function in the ARPE-19 cells, post treatment with H2O2 or (and) Melatonin. We firstly evaluated the MT1 expression in the ARPE-19 cells, post treatment with both 400 μM H2O2 and (and) Melatonin. Moreover, there was a significant difference in the relative DCF level (p < 0.05 for siRNA 2-MT1 with 40 nM; p < 0.05 for siRNA 1-MT1 and p < 0.01 for siRNA 2-MT1 with 80 nM, Fig. 5C), in the relative mitochondrial superoxide accumulation (p < 0.05, p < 0.01 or p < 0.001 for 40 nM; p < 0.05 or p < 0.0001 for 80 nM); and siRNA 2-MT1 was more efficient than siRNA 1-MT1 (either p < 0.01 for 40 or 80 nM). Moreover, the Melatonin-mediated inhibition to the H2O2-mediated mitochondrial dysfunction (DCF conversion, mitochondrial superoxide accumulation and MMP reduction) was blocked by the MT1 knockdown. There was a significant difference in the relative DCF level (p < 0.05 for siRNA 2-MT1 with 40 nM, p < 0.05 for siRNA 1-MT1 and p < 0.01 for siRNA 2-MT1 with 80 nM, Fig. 5C), in the relative mitochondrial superoxide accumulation (p < 0.05, p < 0.01 or p < 0.001, Fig. 5D) and in the relative MMP reduction (p < 0.05, p < 0.01 or p < 0.001, Fig. 5E) between siRNA-transfected (siRNA 1-MT1 and siRNA 2-MT1) and scramble RNA-transfected ARPE-19 cells, post the treatment with both the treatment with H2O2 and Melatonin. Moreover, there was marked difference in the influence on both mitochondrial superoxide accumulation and MMP reduction between
Fig. 2. MTT assay for viability and TUNEL staining for apoptosis in the H₂O₂- or (and) Melatonin-treated ARPE-19 cells. A: Relative vitality of ARPE-19 cells, post the treatment with 0 or 400 μM H₂O₂, or (and) with 0, 100 or 200 μM Melatonin for 0, 12, 24 or 48 hr, via MTT assay. B: Apoptosis in the four groups (0 μM H₂O₂ + 0 μM Melatonin, 400 μM H₂O₂ + 0 μM Melatonin, 400 μM H₂O₂ + 100 μM Melatonin, 400 μM H₂O₂ + 200 μM Melatonin, treatment for 48 hr for each group of cells) of ARPE-19 cells, by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining. Nucleus was stained with DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride), and two types of staining was merged. C: Relative apoptotic ARPE-19 cells, presenting as percentage of TUNEL-stained to total cells, with the percentage of apoptotic cells in H₂O₂ treatment group as 100%. * p < 0.05, ** p < 0.01 or **** p < 0.0001, ns: no significance.
siRNA 1-MT1 and siRNA 2-MT1 (either \( p < 0.05 \) for relative mitochondrial superoxide or relative MMP, with 80 nM, Fig. 5D or 5E), being consistent with the knockdown efficiency of both siRNAs. Therefore, Melatonin antagonizes the \( \text{H}_2\text{O}_2 \)-induced mitochondrial dysfunction in ARPE-19 cells.

**DISCUSSION**

Melatonin, chemically named as N-acetyl-5-methoxytryptamine, is a natural antioxidant with a circadian secretion pattern (Colin-Gonzalez et al., 2015). Deregulation of Melatonin with a disrupted circadian rhythm is associated with oxidative stress and inflammatory processes for several central nervous system (CNS) disor-
It was found in multiple organs (Reiter, 1991), including in the retina, particularly during night (Tosini et al., 2012). An important role of Melatonin has recently been recognized in retinal physiology and pathophysiology (Tosini et al., 2012; Wiechmann and Sherry, 2013). Melatonin antagonizes OS-induced MD in RPE cells (Colin-Gonzalez et al., 2015; Espino et al., 2012). It was found in multiple organs (Reiter, 1991), including in the retina, particularly during night (Tosini et al., 2012). An important role of Melatonin has recently been recognized in retinal physiology and pathophysiology (Tosini et al., 2012; Wiechmann and Sherry, 2013). Mela-
Melatonin protects such retinal cells as retinal pigment epithelial cells and photoreceptors against oxidative stress- or ischemia-induced cell death (Osborne et al., 1998; Liang et al., 2004; Fu et al., 2012). Daily melatonin administration also protects the retina against progression of retinal age-related macular degeneration (AMD) (Rosen et al., 2009). Knockout of MT1 accelerates the age-related loss of photoreceptors cells (Baba et al., 2009). In the present study, oxidative stress model in RPE cells was constructed by the treatment with 400 μM $H_2O_2$ on ARPE-19 cells.
The H₂O₂ treatment markedly reduced the cellular viability, induced apoptosis and promoted apoptosis-associated proteins in ARPE-19 cells. However, these pathological changes were blocked by the melatonin with 100 μM, such concentration was also confirmed to be protective in neural cells in previous study (Su et al., 2015). Thus, we confirmed the protective role of Melatonin against H₂O₂ treatment. However, it is not known whether such concentration was physiologically and pharmacologically safe for treatment.

The mitochondrion is the energy plant (Barot et al., 2011) for the high energy-consuming retinal pigment-ed epithelium cells. And mitochondrial dysfunction has been implicated by accumulating evidence in many retinal degeneration-associated diseases (Barot et al., 2011). Chronic overproduction of ROS has been found in the retina and results in abnormal mitochondrial functions in diabetes (Kowluru, 2005; Madsen-Bouterse and Kowluru, 2008), in Glaucoma (Lee et al., 2012b; Kong et al., 2009) and in age-related macular degeneration (AMD) (Hageman et al., 2005). In our study, we are interested in whether the mitochondrion is the target for Melatonin against oxidative stress-posed damage to ARPE-19 cells. The mitochondrial dysfunction-associated pathological changes, such as the accumulation of ROS and superoxide in mitochondria and the MMP reduction, were found in H₂O₂-treated ARPE-19 cells, however, were inhibited by Melatonin. Therefore, we speculated that the mitochondrion may be the target for Melatonin against oxidative stress in RPE cells.

Melatonin receptor (MT) is located widely in multiple organs, including the retina (Uz et al., 2005). MT1 and MT2 are expressed in many types of cells within eye (Tosini et al., 2013). It was found that mice lacking MT1 receptors have elevated intraocular pressure (IOP), significantly reduced retinal ganglion cells, implying dysfunctional melatonin signaling as a possible risk factor in the pathogenesis of glaucoma (Tosini et al., 2013). It has also been confirmed that Melatonin modulates visual function in the mouse retina via the MT1 melatonin receptor (Baba et al., 2009). It has been confirmed that the Melatonin-MT1 signaling involves in the anti-oxidative stress procession in many types of cells or organs, such as the heart (Yu et al., 2014), germ cells (Mukherjee et al., 2015) and corneal fibroblasts (Choi et al., 2011). However, it is not clear whether the Melatonin-MT1 signaling is involved in the Melatonin-mediated protection against oxidative stress in RPE cells. We reconfirmed the protective role of Melatonin in RPE cells with gene knockdown method. The MT1-specific siRNA markedly blocked the Melatonin-mediated protection against oxidative stress in RPE cells. The Melatonin-mediated amelioration of mitochondrial dysfunction in the H₂O₂-treated ARPE-19 cells was blocked by the MT1 knockdown.

Up to now, the antagonization by melatonin against oxidative stress was not well understood. Melatonin reduces reactive oxygen species formation in normal human epidermal keratinocytes (NHEK), not only by directly scavenging ROS, but also by promoting anti-oxidative enzyme activity (Kleszczyński et al., 2016). The direct scavenging effect against ROS was also observed in aged seed (Deng et al., 2017). We did not examine a possible direct anti-oxidative stress effect in RPE cells. However, the loss-of-function results confirmed the MT-dependence, indicating the involvement of Melatonin-MT signaling in such effect. The activated signaling pathway then could mediate the translocation of the nuclear erythroid 2-related factor 2 resulting in activation of phase-2 antioxidant enzymes (γ-GCS, HO-1, NQO1) (Kleszczyński et al., 2016), and triggering the transcriptional upregulation of double-stranded RNA-activated protein kinase-like ER kinase (PERK) and CHOP.

Though various studies confirmed the protective effect of melatonin against oxidative damage and mitochondrial damage (Suofu et al., 2017; Ding et al., 2014), such kind of antagonizing role of melatonin has never been reported against oxidative stress-induced mitochondrial dysfunction in retina pigmented epithelium cells. However, in the present study, we did not know about the downstream involvement, post the MT1 activation.

In summary, we confirmed the protective role of Melatonin against H₂O₂-induced apoptosis and mitochondrial dysfunction in RPE cells. MT1 knockdown blocked such protective role of Melatonin against the H₂O₂-mediated damage to RPE cells. This suggests that Melatonin exerts a protective role against oxidative stress via Melatonin-MT1 signaling in RPE cells.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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