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Molecular, Biological, Physiological and Clinical Aspects of Melatonin

Melatonin suppresses doxorubicin-induced premature senescence of A549 lung cancer cells by ameliorating mitochondrial dysfunction

Abstract: Melatonin is an indolamine that is synthesized in the pineal gland and shows a wide range of physiological functions. Although the anti-aging properties of melatonin have been reported in a senescence-accelerated mouse model, whether melatonin modulates cellular senescence has not been determined. In this study, we examined the effect of melatonin on anticancer drug-induced cellular premature senescence. We found that the doxorubicin (DOX)-induced senescence of A549 human lung cancer cells and IMR90 normal lung cells was substantially inhibited by cotreatment with melatonin in a dose-dependent manner. Mechanistically, the DOX-induced G2/M phase cell cycle arrest and the decrease in cyclinB and cdc2 expression were not affected by melatonin. However, the DOX-induced increase in intracellular levels of ROS, which is necessary for premature senescence, was completely abolished upon melatonin cotreatment. In addition, the reduction in mitochondrial membrane potential that occurs upon DOX treatment was inhibited by melatonin. An aberrant increase in mitochondrial respiration was also significantly suppressed by melatonin, indicating that melatonin ameliorates the mitochondrial dysfunction induced by DOX treatment. The treatment of A549 cells with luzindole, a potent inhibitor of melatonin receptors, failed to prevent the effects of melatonin treatment on mitochondrial functions and premature senescence in cells also treated with DOX; this suggests that melatonin suppresses DOX-induced senescence in a melatonin receptor-independent manner. Together, these results reveal that melatonin has an inhibitory effect of melatonin on premature senescence at the cellular level and that melatonin protects A549 cells from DOX-induced senescence. Thus, melatonin might have the therapeutic potential to prevent the side effects of anticancer drug therapy.

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Introduction

Cellular senescence was first described by Hayflick and Moorhead more than 50 yr ago as a state of irreversible growth arrest of normal human fibroblast after serial cultivation in vitro [1]. They proposed that normal cells are not capable of immortal proliferation because they are programmed with a specific proliferative lifespan. This phenomenon, known as replicative senescence, plays a central role in cellular aging and is also implicated in organismic aging [2]. The important role of replicative senescence is supported by evidence that shows the lifespan and the growth potential of normal fibroblasts are correlated with the lifespan or the age of the donor organism from which the fibroblasts are derived [3–5]. Recent studies have shown that various cellular stresses, such as oxidative stress, DNA damage, and anticancer drug treatment, induce another type of senescence termed premature senescence, in both normal and cancer cells [6]. Premature senescence is accompanied by dramatic physiological and phenotypic changes very similar to replicative senescence, including cell cycle arrest, enlarged and flattened morphology, and the appearance of senescence-associated β-galactosidase activity (SA β-gal) at pH 6.0 [7], implicating a common molecular mechanism in both types of cellular senescence. Thus, premature senescence could be a useful system for understanding the molecular mechanism of the aging process and for studying the effect of exogenous treatment on cellular longevity and senescence onset.

Melatonin (*N*-acetyl-5-methoxytryptamine) is a key regulator for the control of circadian rhythms in humans [8, 9]; in addition to this critical function, melatonin also has other biological functions, including an immunomodulatory effect and a cytoprotective role [8]. Melatonin exerts this wide range of physiological effects through its strong antioxidant ability, through specific plasma membrane receptors MT1 and MT2, or through lower affinity intracellular targets, such as quinone reductase and calmodulin [10, 11]. Melatonin also exerts pro-oxidant effect in some tumor cells [12–14]. Interestingly, previous studies have

shown that melatonin exhibited a protective effect on aging-related physiological defects. Long-term administration of melatonin has been shown to improve the age-related neurodegenerative defects in the accelerated senescence-prone mouse-8 (SAMP8) model [15–17]. Furthermore, long-term administration of melatonin increased the longevity of senescence-prone mice [18]. Although it has been suggested that melatonin has an anti-aging and anti-inflammatory effect in a senescence-accelerated mouse model, whether melatonin plays a role in cellular senescence has not been determined.

In this study, we explored the effect of melatonin treatment on anticancer drug-induced premature senescence. Our data revealed that melatonin suppresses the onset of premature senescence of A549 cells upon treatment with the anticancer drugs doxorubicin (DOX) or camptothecin (CPT). In addition, melatonin significantly suppresses various mitochondrial defects that occur upon DOX treatment; this suggests that melatonin inhibits the onset of premature senescence by ameliorating the mitochondrial dysfunction caused by DOX treatment.

Materials and methods

Cell culture and treatments

A549 human lung cancer cells were maintained with DMEM containing 10% fetal bovine serum (FBS) (Sigma, St Louis, MO, USA). Melatonin was obtained from Sigma. It was dissolved in dimethylsulfoxide (DMSO) and added to the culture media at the indicated concentration. Doxorubicin (DOX) and camptothecin (CPT) were purchased from Sigma. To induce premature senescence, A549 cells were treated with 100 nM DOX for 2 days or 250 n CPT for 16 hr and then incubated with drug-free culture medium for the indicated time. 2-benzyl-*N*-acetyltryptamine (luzindole), a potent melatonin receptor antagonist, was purchased from Sigma.

Senescence-associated (SA)-β-galactosidase staining

Cells were fixed with 0.25% glutaraldehyde, and SA β-galactosidase staining was performed at pH 6.0 as described previously [7]. After staining, at least 300 cells in several fields were examined and SA β-gal positive cells were counted. These experiments were repeated three times, and the results were presented as the mean values with standard deviations.

Cell cycle analysis

To determine the cell cycle distribution, 1×10^6 cells were seeded into a 100-mm dish. After p53 adenovirus infection, cells were trypsinized at the indicated time points and fixed with 70% ethanol. The cells were subsequently stained with propidium iodide (PI), and flow cytometric analysis was performed using an EPICS XL cytometer and WINCYCLE software (Beckman Coulter, Inc., Brea, CA, USA). A total of 10,000 events were analyzed for each sample, and the experiment was repeated at least three times.

Western blot analysis and antibodies

Cells were lysed in RIPA buffer and subjected to western blot analysis as described previously [19]. Antibodies for cyclinA, cyclinB, and cdc2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Levels of actin were monitored as an internal loading control using anti-actin (Sigma) antibodies.

Measurement of mitochondrial membrane potential

To measure mitochondrial membrane potential, cells that had been treated with DOX alone or together with melatonin were incubated with 1 μg/mL of JC-1 dye (Invitrogen, Grand Island, NY, USA) for 20 min and analyzed by microscopy. Fluorescence images were captured with a Carl Zeiss LSM-700 confocal microscope (Carl Zeiss, Thornwood, NY, USA). To quantitate the mitochondrial membrane potential, cells were trypsinized, and 5×10^5 cells were stained with 1 μg/mL of JC-1 dye for 20 min. The red and green fluorescence intensities of JC-1 dye were measured by flow cytometry using an EPICS XL cytometer (Beckman Coulter Inc.). A total of 10,000 events were analyzed for each sample, and the results obtained from three experiments are presented as the mean values with standard deviations.

Measurement of ROS levels

To measure the intracellular levels of ROS, cells were stained with 50 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) (Sigma) for 30 min and then harvested. The fluorescence intensities were quantified using an EPICS XL cytometer (Beckman Coulter Inc.). Experiments were performed in triplicate, and the results are presented as the mean values with standard deviations.

Measurement of respiration rate

The measurement of the cellular respiration rate was performed in 24-well plate using the XF24 flux analyzer (Seahorse Bioscience Inc., North Billerica, MA, USA) according to manufacturer's instructions. A549 cells were plated at a density of 10,000 cells/well on an XF24 tissue culture plate. After overnight culture, 100 nM DOX and 2 mM melatonin were treated for 2 days. The oxygen consumption rate was measured under basal conditions, in the presence of DNP (40 μM), or rotenone (1 μM) to assess the maximal oxidative capacity. The oxygen consumption measurements were normalized to the number of A549 cells.

Results

To determine the role of melatonin on cellular senescence, we studied the effect of melatonin on premature cellular senescence. Taking advantage of a premature senescence model of cancer cells, we first treated A549 human lung cancer cells with either the anticancer drug doxorubicin (DOX; 100 nM) alone or in combination with various doses of melatonin. Upon DOX treatment, A549 cells

became enlarged and flattened, which are characteristic morphological changes of premature senescence. In addition, senescence-associated β-galactosidase (SA β-gal) staining activity was observed in approximately 55% of the DOX-treated A549 cells, indicating that DOX treatment induces premature senescence in a substantial portion of A549 cells (Fig. 1A,B). Strikingly, melatonin cotreatment significantly reduced the SA-β gal staining activity of A549 cells in a dose-dependent manner. SA-β gal staining activity was reduced from 55% to 24% by 1 mM melatonin cotreatment and to 14% by 2 mM melatonin cotreatment ($P < 0.05$). Along with a decrease in SA-β gal staining activity, the morphology changes induced by DOX treatment were also remarkably inhibited in the melatonin-cotreated A549 cells, and the cells maintained normal morphology. In addition, we found that melatonin cotreatment also significantly inhibited camptothecin (CPT)-induced senescence of A549 cells (Figure S1). Moreover, melatonin cotreatment also inhibited DOX-induced premature senescence of IMR90 human normal lung cells in a dose-dependent manner (Fig. 1C,D). Thus, these results suggest that melatonin treatment efficiently inhibits anticancer drug-induced premature senescence in both cancer cells and normal cells.

Previous studies have shown that DOX treatment induced G2/M phase cell cycle arrest in various cancer cells [20, 21]. To investigate the mechanism by which melatonin inhibits DOX-induced premature senescence, we examined the cell cycle distribution of A549 cells after DOX and melatonin treatment. As shown in Fig. 2A, melatonin treatment (2 mM) did not significantly affect the cell cycle distribution. The proportion of cells in G1 phase was slightly increased at the 2-day time point, but this increase returned to normal 4 days after melatonin treatment (Fig. 2B). DOX treatment alone increased the G2/M portion to approximately 60% at the 2-day time point, and this increase was maintained until 4 days later, indicating that DOX treatment induced G2/M phase cell cycle arrest. Interestingly, this G2/M arrest was also observed in cells treated with DOX and melatonin together.

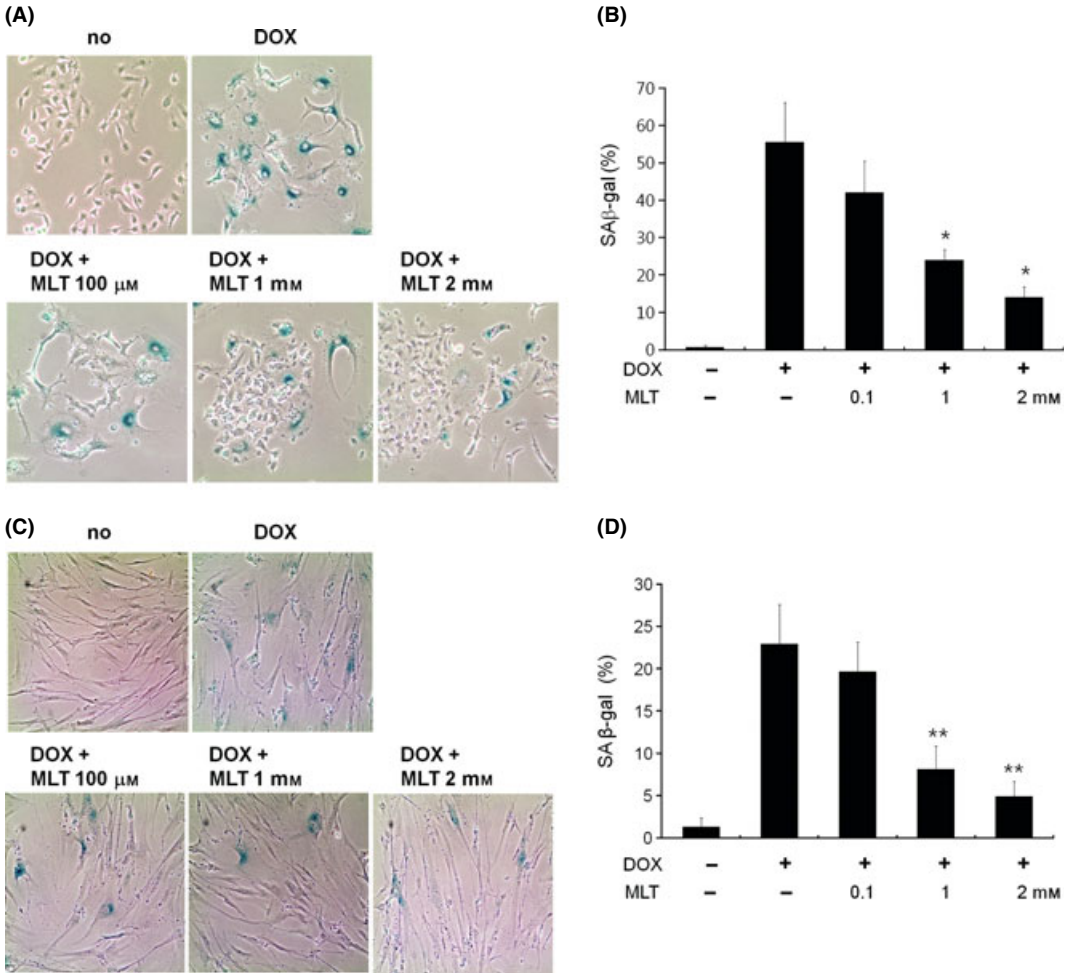

Fig. 1. Inhibition of DOX-induced premature senescence by melatonin in A549 cells. (A) A549 cells were treated with either DOX (100 nM) alone or together with melatonin at the indicated concentration for 2 days. After 6 days of culture in a drug-free medium, cells were stained with freshly prepared SA β-gal staining solution. Cell images were captured with a microscope. The data shown are representative of three independent experiments. (B) SA β-gal positive values that were counted in three independent experiments are presented as the mean values with standard deviations. $*P < 0.05$; $**P < 0.01$ using Student's *t*-test.

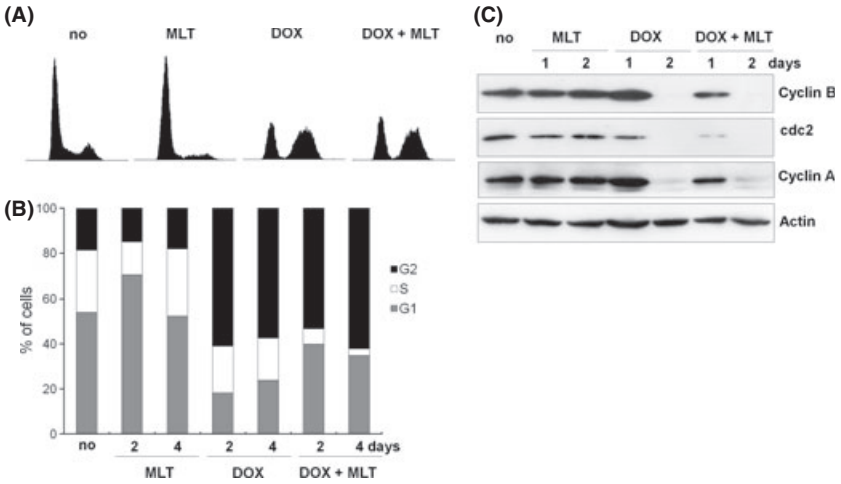

Fig. 2. DOX-induced G2/M cell cycle arrest is not inhibited by melatonin. (A) A549 cells were treated with DOX alone, melatonin (MLT) alone (2 mM), or DOX and melatonin together for 2 days and then harvested. Cells were fixed, stained with PI, and then analyzed using flow cytometry as described in Materials and methods. The data shown are representative of three independent experiments. (B) A549 cells were treated with DOX and melatonin as in (A), and cell cycle distributions obtained at the indicated time points in three independent experiments are presented as the mean values. (C) A549 cells were treated with DOX and melatonin as in (A) and harvested at the indicated time points. Cell lysates were subjected to western blotting using the indicated antibodies.

A decrease in the protein levels of cyclinB and cdc2, which are critical regulators for G2/M cell cycle progression, was also observed in cells cotreated with DOX and melatonin (Fig. 2C). These data suggest that DOX-induced cell cycle arrest was not affected by the addition of melatonin treatment.

DOX treatment leads to the production of reactive oxygen species (ROS) in many cancer cells [21, 22]. Induction of intracellular reactive oxygen species (ROS) plays a critical role in the onset of premature senescence of cancer cells upon various genotoxic stresses [6]. To examine whether melatonin affected ROS induction triggered by DOX treatment, we monitored the intracellular levels of ROS after treatment with DOX and melatonin. DOX treatment substantially increased the ROS levels of A549 cells approximately 2.8 fold at the 2-day time point and 6.4 fold at the 4-day time point (Fig. 3). This DOX-induced increase in intracellular ROS levels was completely abolished by cotreatment with melatonin (2 mM); cellular ROS levels in A549 cells were not affected by melatonin treatment without the addition of DOX (Fig. 3). These results suggest that melatonin suppresses the premature senescence of A549 cells by inhibiting the increase in intracellular ROS levels induced by DOX treatment.

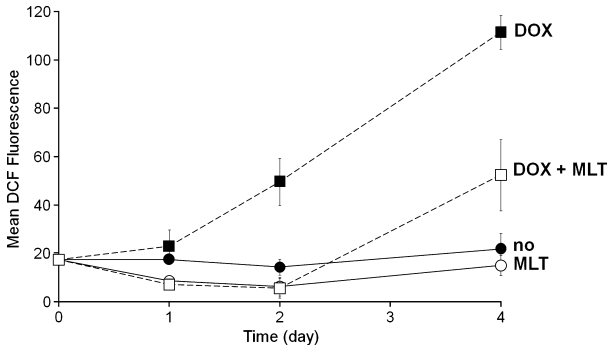

Fig. 3. Melatonin inhibits the DOX-induced increase in intracellular ROS levels. A549 cells were treated with DOX alone, melatonin (MLT) alone (2 mM), or DOX and melatonin together, and intracellular levels of ROS were determined at the indicated time points as described in Materials and methods. Experiments were repeated three times, and the results are presented as the mean values with standard deviations.

Previous studies have shown that DOX treatment induces mitochondrial dysfunction [22–25]. Mitochondria are the center of cellular metabolism and the primary site for ROS generation [26, 27]. Thus, it is possible to speculate that melatonin may suppress the DOX-induced ROS increase by inhibiting the effect of DOX on mitochondria. To examine whether melatonin inhibits the effect of DOX on mitochondria, we monitored the mitochondrial membrane potential, which is a reliable parameter for the integrity of mitochondrial function, using the mitochondrial membrane potential-dependent JC-1 fluorescent dye. As shown in Fig. 4, control A549 cells displayed a strong red fluorescence, which indicates a normal mitochondrial membrane potential, while DOX treatment induced a dramatic increase in green fluorescence, which indicates a collapse of mitochondrial membrane potential. Interestingly, the DOX-induced increase in green fluorescence was considerably suppressed by cotreatment with melatonin (2 mM) (Fig. 4A). Quantitative JC-1 fluorescence intensity analysis also indicates that melatonin significantly inhibits the decrease in mitochondrial membrane potential induced by DOX ($P < 0.05$) (Fig. 4B). These results suggest that melatonin prevents the loss of mitochondrial membrane integrity that is induced by DOX treatment.

Alteration in the rate of oxygen consumption is an informative indicator of mitochondrial dysfunction. To further confirm the effect of melatonin on the mitochondrial dysfunction induced by DOX treatment, we measured the oxygen consumption rate, which is an indicator of

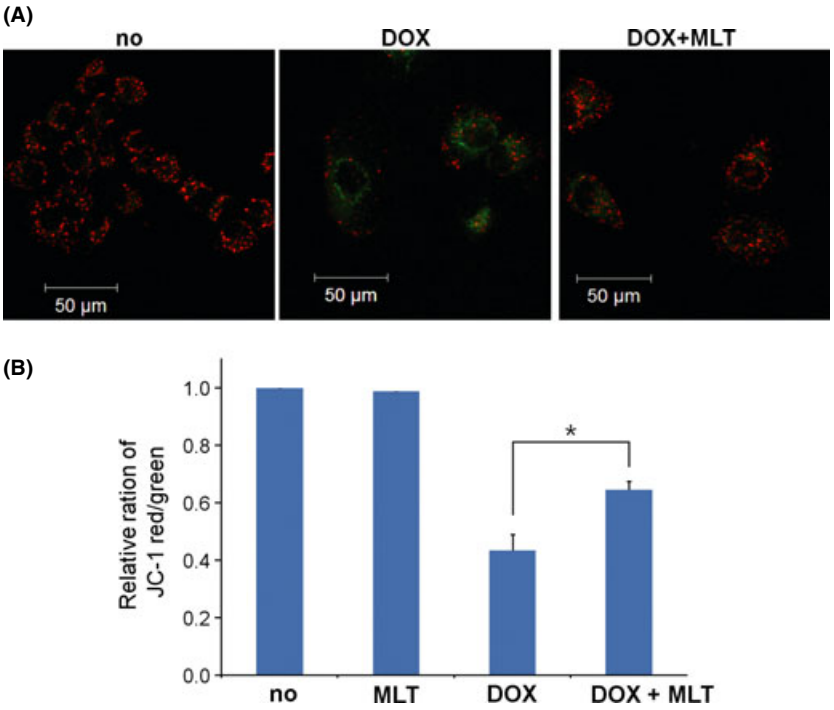

Fig. 4. Melatonin prevents a decrease in mitochondrial membrane potential upon DOX treatment. (A) A549 cells were treated with DOX alone (2 mM) or together with melatonin for 2 days and then stained with 1 μg/mL of JC-1 fluorescent dye for 20 min. JC-1 red and green overlay fluorescent images were captured with confocal microscopy. Red fluorescence indicates normal membrane potential, and green fluorescence indicates mitochondria membrane depolarization. The images shown are representative of three independent experiments. (B) For quantification, A549 cells were treated with DOX alone (2 mM), or together with melatonin for 2 days; they were then trypsinized and stained with JC-1. The red and green fluorescence intensity was analyzed by flow cytometry in three independent experiments, and the results are presented as the mean values with standard deviations. $^*P < 0.05$ using Student's *t*-test.

mitochondrial respiration. It has been reported previously that DOX treatment induced an aberrant increase in mitochondrial oxygen consumption in Jurkat and HL-60 cells [25]. Consistent with this previous report [25], DOX treatment increased the basal respiration rate of A549 cells approximately 2.7 fold (Fig. 5A,B). In addition, the maximal mitochondrial oxidative capacity was also remarkably increased upon DOX treatment, as assessed by the levels of 2,4-dinitrophenol (DNP)-stimulated respiration. These results indicate that DOX treatment induces mitochondrial uncoupling. Importantly, melatonin cotreatment (2 mM) completely inhibited the DOX-induced increase in the basal respiration rate (Fig. 5A,B). These results further confirm that melatonin prevents the mitochondrial dysfunction induced by DOX treatment.

Previous studies have shown that some functions of melatonin are attributed to its interaction with the plasma membrane receptors MT1 and MT2 [11]. To address whether the melatonin effect on DOX-induced premature senescence requires MT1 and MT2, we employed a specific MT1/MT2 antagonist luzindole. We pretreated cells with 50 μM luzindole, which is a concentration that has been shown to antagonize the effect of 1 mM melatonin on MT1 and MT2 receptors [28], for 1 h before adding DOX and melatonin. As shown in Fig. 6A, luzindole did not inhibit the melatonin effect on DOX-induced premature senescence. Consistent with this result, the melatonin effect on the expression of cell cycle regulators was not influenced by luzindole cotreatment (Fig. 6B). Moreover, melatonin inhibited the aberrant increase in mitochondrial respiration and intracellular ROS even in the presence of 50 μM luzindole (Fig. 6C). These results indicate that melatonin suppresses DOX-induced premature senescence in a manner that is melatonin receptor independent.

Discussion

In this study, we showed that melatonin suppresses the DOX-induced premature senescence of A549 human lung cancer cells. Our results showed that melatonin inhibited DOX-induced morphology changes and reduced SA β-gal staining activity markedly (Fig. 1). A mechanistic analysis revealed that the DOX-induced ROS increase was suppressed by melatonin (Fig. 3), whereas the DOX-induced G2/M cell cycle arrest was not changed by the addition of melatonin (Fig. 2). Importantly, we noted that the DOX-induced decrease in mitochondrial membrane potential and the aberrant increase in mitochondrial respiration was significantly suppressed with melatonin cotreatment (Figs 4 and 5), suggesting that the mitochondrial dysfunction induced by DOX treatment was ameliorated by melatonin cotreatment.

Mitochondria have long been implicated in the aging process and the control of life span. Since Harman proposed his free radical theory of aging over 50 yr ago, mitochondria, as both the main generators and the main

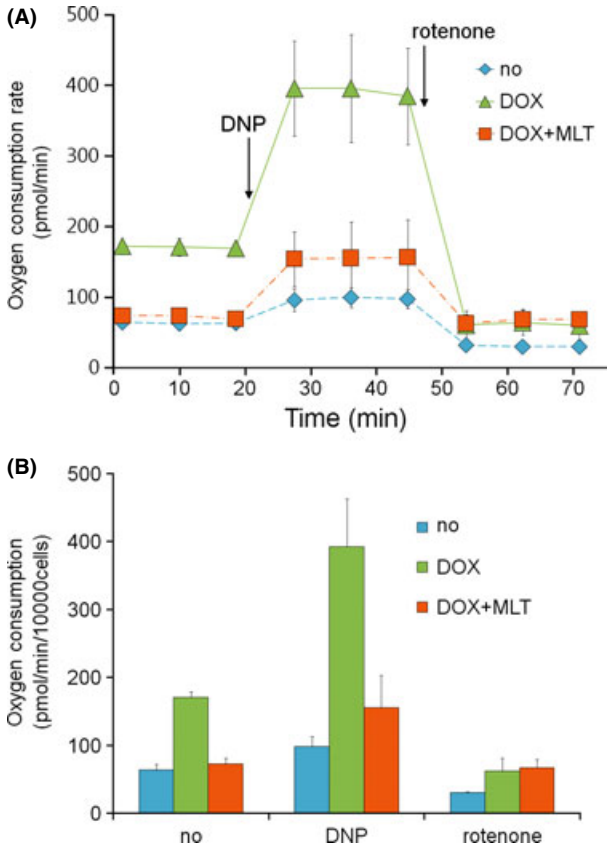

Fig. 5. DOX-induced aberrant increase in the oxygen consumption rate is inhibited by melatonin. (A) A549 cells were treated with DOX (100 nM) alone or together with melatonin (2 mM) for 2 days, and the respiration rate was measured with a XF24 flux analyzer as described in Materials and methods. Shown is a representative analysis of oxygen consumption performed in triplicate under basal conditions following the addition of 2,4-dinitrophenol (DNP; 40 μM) or rotenone (1 μM). (B) Averaged metabolic profile from three repeated experiments upon DOX treatment alone or melatonin cotreatment for 2 days. The results are presented as the mean values with standard deviations.

targets of free radicals, have generally been viewed as critical regulators of the aging process [29, 30]. The increased ROS generated by defective mitochondria can lead to a vicious cycle of exponentially increasing levels of mitochondrial DNA mutations and oxidative stress to the cell [31, 32]. Experimentally increased mitochondrial DNA mutations have been shown to result in premature aging phenotypes in mouse models [33, 34]. Moreover, the recent observations that the life span of most organisms is roughly proportional to their generation rate of mitochondrial ROS provide more support for the mitochondrial free radical theory of aging. In addition to organismic aging, recent studies have also demonstrated that mitochondria play an important role in cellular life span and the senescence process [35, 36]. Interestingly, previous studies have shown that melatonin improves mitochondrial function in both normal and pathological conditions [26, 27]. Long-term administration of melatonin improves mitochondrial function in senescence-accelerated mice models [18, 37]. Dragicevic et al. [38] showed that melatonin treatment restores both dysregulated mitochondrial membrane potential and respiration rate in an Alzheimer mouse model. It has been well documented that melatonin protects mitochondria from oxidative damage [39, 40]. Acuna-Castroviejo et al. [41] showed that melatonin protects lung mitochondria in older mouse. Our results show that melatonin treatment significantly prevented mitochondrial dysfunction upon DOX treatment, suggesting that melatonin also plays a protective role in the cellular senescence process.

One important question that arises from this study is how melatonin suppresses mitochondrial dysfunction upon DOX treatment. It is well documented that melatonin executes its physiological role mainly through its strong antioxidant activity. Melatonin and its metabolites have been shown to directly scavenge free radicals [42–46]. Previous studies have demonstrated that melatonin has stronger ROS-scavenging ability than many known classic antioxidants, such as glutathione and vitamin C [47, 48]. Because of its lipophilic nature, melatonin has the ability to reach and then accumulate at high concentrations in mitochondria [49, 50]. Martin et al. [49] used isolated mitochondria to show that melatonin prevents oxidative stress induced by t-butyl hydroperoxide (t-BHP). These results suggest that the antioxidant activity of melatonin plays an important role in protecting mitochondria from oxidative stress. Consistent with this notion, aged animals and humans that have deficient melatonin synthesis are more sensitive to oxidative stress [51]. There is a diminished production of melatonin with aging [52, 53], and this reduction in melatonin seems to be strongly associated with increased oxidative damage in aged individual [54] and may play a causative role in aging.

In addition to its direct ROS-scavenging activity, melatonin has been shown to increase the expression of antioxidant enzymes such as MnSOD and catalase [55–58]; we did not observe that melatonin increased the MnSOD and catalase protein levels upon DOX treatment (data not shown). Melatonin also exerts some function through the membrane receptors MT1 and MT2 [11]. However, a potent melatonin receptor antagonist, luzindole, failed to inhibit the effect of melatonin on DOX-induced premature senescence and mitochondrial dysfunction (Fig. 6). Therefore, it is possible that melatonin protects mitochondrial function upon DOX treatment through its direct antioxidant activity. Further studies will be important for the mechanistic understanding of melatonin in cellular senescence.

In conclusion, our results show that melatonin suppresses the onset of DOX-induced premature senescence and that melatonin can prevent the increase in ROS levels by protecting mitochondria from genotoxic stress-induced dysfunction. We used a premature senescence model in this study, but it is important to determine whether melatonin could also prevent replicative senescence. Although the detailed mechanism remains to be elucidated in further studies, our study revealed that melatonin has an inhibitory role on cellular senescence as well as on organismic aging.

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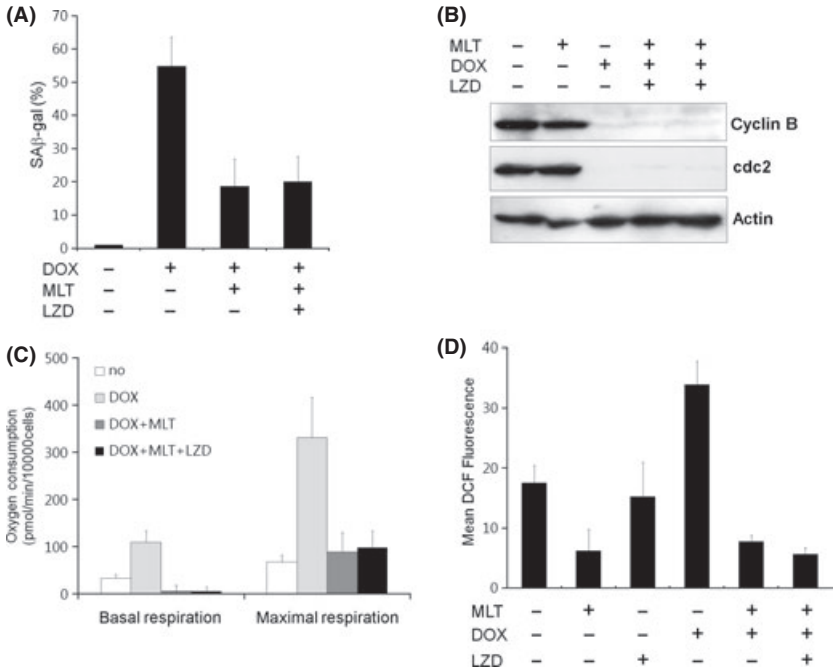

Fig. 6. Melatonin receptor antagonist does not inhibit melatonin effect on DOX-induced senescence. (A) A549 cells were treated with DOX (100 nM) alone or together with melatonin (2 mM) or luzindole (LZD; 50 μM) for 2 days. After 6 days of culture in a drug-free medium, cells were stained with freshly prepared SA β-gal staining solution. SA β-gal positive values that were counted in three independent experiments are presented as the mean values with standard deviations. (B–D) A549 cells treated as in (A) were harvested at a 2-day time point and subjected to western blotting using the indicated antibodies (B). (C) Oxygen consumption rate was measured with a XF24 flux analyzer. (D) Intracellular levels of ROS were determined with DCF-DA staining using flow cytometry. Experiments were repeated three times, and the results are presented as the mean values with standard deviations.

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Author contributions

NS, AK, YY, and JY gave the concept and designed the experiments; NS, AK, HK, HJ, and MK performed the experiments; NS, YY, and JY drafted the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Melatonin inhibits Camptothecin (CPT)-induced premature senescence of A549 cells.

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