Melatonin enhances NF-κB deacetylation to induce autophagy in senescence-like SH-SY5Y cells via Sirtuin 1 activation

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Abstract
Autophagy is a major route for the bulk degradation of abnormal cytosolic macromolecules. This process plays a pro-survival role by maintaining cellular homeostasis. However, autophagy diminishes in a senescent cell which is considered as an aging characteristic. Melatonin is a hormone which plays a wide range of physiological functions. This hormone exerts its anti-aging effect, possibly through the regulation of Sirtuin1 (SIRT1) pathway. The deacetylation ability of SIRT1 is important for controlling several transcription factors function including NF-κB. Apart from inflammation, NF-κB can regulate autophagy. Although numerous studies have revealed the role of melatonin in regulation of autophagy, very limited experiments showed that melatonin could increase autophagic activity via SIRT1 in a senescent model. In this study, the pro-autophagic effect of melatonin through deacetylation activity of SIRT1 on RelA p65 was investigated in hydrogen peroxide (H_2O_2)-induced cellular senescent SH-SY5Y neuroblastoma cells. Our results demonstrated that melatonin could increase protein level of SIRT1 and LC3-II, a hallmark protein of autophagy, and reduced the level of acetylated-Lys310 in p65 subunit of NF-κB in SH-SY5Y cells treated with H_2O_2. The present data indicated that melatonin enhanced autophagy via SIRT1 pathway. Taken together, we suggest that melatonin might be therapeutically beneficial for antiaging process.

Keywords: autophagy, aging, NF-κB, melatonin, RelA p65, SIRT1

Background

Aging is the most common factor of neurodegenerative diseases such as Mild cognitive impairment, Alzheimer's disease, cerebrovascular disease, and Parkinson's disease. It is the natural phenomenon occurring when people reach advanced stage of their life. The aging process is associated with several structural, chemical, and functional changes in the brain as well as a host of neurocognitive changes (Kadakkuzha et al 2013) and increase risks of acquiring chronic diseases such as cardiovascular disease (North & Sinclair 2012). In addition, several studies have indicated that neuroinflammation is one of the fundamental causes of these diseases. The inflammation can be triggered by the accumulation of free radicals which leads to DNA damage. Cellular response to this unfavorable circumstance is becoming senescence, one of the features of aging.

Autophagocytosis is a major route for the bulk degradation of abnormal cytosolic macromolecules and organelles to maintain the homeostasis. The extension of lifespan has been linked to the efficient maintenance of autophagic degradation, a process which declines during aging. . Recent studies have indicated that autophagy is involved in several cellular functions regulated by NF-κB including cell survival, differentiation, senescence, inflammation, and immunity. On a molecular level, autophagy and NF-κB share common upstream signals and regulators and can control each other through positive or negative feedback loops. One of the key regulators isSIRT 1. SIRT1 is anenzyme that deacetylates proteins involved with cellular regulation, including NF-κB-mediated protein, a key transcription factors of inflammation. Overexpression of SIRT1 or activation of SIRT1 by resveratrol (RES) promotes deacetylation of RelA p65 subunit of NF-κB, results in suppression of transcriptional activation (Ashburner et al 2001, Kim et al 2012). Furthermore, under some stress condition such as age-related stresses, NF-κB-driven anti-apoptotic Bcl-2 proteins which repress the autophagy via beclin1 signaling. Suppression of autophagocytosis provokes inflammation, including NF-κB activation which further potentiates anti-apoptotic defense.

Melatonin is an endogenous hormone secreted by the pineal gland and shows a wide range of physiological functions. The level of melatonin and its precursors decrease with age and may be the cause of neurodegenerative diseases. Moreover, the anti-aging property of melatonin has been reported in senescence mouse models (Hardeland 2013). Melatonin and its metabolites scavenge free radicals directly or by induction of antioxidant enzymes (Reiter et al 2003). In addition, melatonin exerts its anti-inflammatory effects, possibly through the regulation of NF-κB pathway (Mauriz et al 2013). The anti-aging properties of melatonin also regulate epigenetic processes in neuronal cells. Recent studies indicated that melatonin associates with molecules and signaling pathways that sense and influence with FoxO and sirtuin pathways (Cristofol et al 2012, Gutierrez-Cuesta et al 2008, Hardeland 2013, Tajes et al 2009). These pathways are now implicated in the epigenetic processes of both young and aging brains and associated with neurodegenerative diseases. Melatonin not only associates with these pathways but also modulates autophagic activity. It can either induce or inhibit autophagy, depending on cellular requirements and oxidative stress levels (Jenwitheesuk et al 2014). However, very limited experiments showed the effect of melatonin on autophagy via pathway involved with SIRT1 and NF-κB. Therefore, this present study aimed to investigate the effect of melatonin on autophagy via RelA p65 deacetylation activity of Sirtuin1 in SH-SY5Y neuroblastoma cells induced by hydrogen peroxide.

Materials and methods

Chemicals and reagents

Human dopaminergic neuroblastoma SH-SY5Y cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The chemicals used in this study were purchased from the following sources: Minimum essential medium (MEM), Ham's F-12 medium, fetal bovine serum (FBS), penicillin and streptomycin from Gibco BRL (Gaithersburg MD, USA), 30% hydrogen peroxide from MERCK (Darmstadt, Germany), melatonin (Cat. No. M5250). Rabbit polyclonal anti-LC3-II (#2775), Rabbit polyclonal anti-Acetyl-NF-κB p65 (Lys310) (#3045), mouse monoclonal anti-Sirt1 (#8469), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (#7074) and anti-mouse IgG (#7076) antibodies were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Mouse monoclonal anti-actin (MAB1501) were obtained from Millipore (Billerica, MA). ECL Prime Western Blotting Reagent® was purchased from GE Healthcare (Little Incorporated (Acton, MA, USA). Other chemicals used in this study are an analytical grade and were Chalfont, Buckinghamshire, UK). Culture plates and flasks were obtained from Corning obtained mostly from Sigma Aldrich (St Louis, MO, USA) or Lab-scan analytical science (Dublin, Ireland).

Cell culture and treatment

The human neuroblastoma SH-SY5Y celllines were cultured in media containing Eagle's minimum essential medium (MEM) and Ham's F-12 medium (1:1) supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/streptomycin in a humidified atmosphere at 37 ºC and 5% CO_2. In each experiment, cells were seeded in 60 mm petri dishes. After reaching 90% confluence, cells were treated with 100 µM of H_2O_2 prepared from 1M H_2O_2 in complete medium for 2 hours. Then, the treatment was removed and cells were washed twice with medium. 100 µM of melatonin prepared from 10 mM in 40% ethanol was applied to cells cultured in complete medium to make final concentration of 1 µM for 24 hours. The final concentration of ethanol in the cultured medium will be less than 0.01% that was included in the control group.

Western immunoblotting

The samples were denatured in sample buffer (125 mM Tris-HCl pH 8.5, 2% SDS, 20% glycerol, 2.5% mercaptoethanol and 2.5% bromophenol blue) at 95 °C for 5 min. The equal amount of protein samples were loaded onto a 12% polyacrylamide gel for SDS-PAGE electrophoresis, and then transferred to a nitrocellulose membrane at 120 V for 1.5 h. The transfer efficiency was detected by Ponceau-S red solution stained on the membranes which were washed out by distilled water until the red color of Ponceau-S red disappear. The blots were blocked with 5% non-fat dry milk in TBST (1% BSA in tris-buffered saline (TBS) containing 0.1% Tween-20)for 1 hour at room temperature and incubated overnight at 4 °C with anti-Acetyl-NF-κB p65 (1:1,000), anti-SIRT1(1:1,000), anti-LC3-II (1:1,000), and anti-actin (1:10,000). Subsequently, the membranes were washed with TBST for 5 min three times and incubated with these following secondary antibodies for 1.5 hour at room temperature. Finally, proteins on membranes were visualized by using enhanced chemiluminescence ECL Prime and exposed to X-ray film (Kodak). The immunoblot bands were quantified using Scion image software (National Institutes of Health, Bethesda, MD).

Statistical analysis

Data were represented as mean with standard error (mean ± S.E.M). Significance was assessed using a one-way analysis of variance (ANOVA) followed by Tukey–Kramer test using the scientific statistic software GraphPad Prism version 5. The significance was taken when P-values were less than 0.05.

Results

The effect of melatonin on SIRT1 protein expression in senescent human SH-SY5Y neuroblastoma cells.

To investigate the effect of melatonin on Sirtuin 1 protein expression in senescent human SH-SY5Y cells induced by H_2O_2, cells were treated with 100 µM of H_2O_2 for 2 hours and/or followed by 1 µM of melatonin and incubated for 24 hours. After treated with H_2O_2 and incubated for 24 hours, protein level of SIRT1 decrease significantly to 69.14 ± 2.33% ($P < 0.001$) (Fig.1) when compared with the control untreated cells. In the groups co-treatment with 1 µM melatonin, the results showed that melatonin increased SIRT1 protein level significantly to 91.34 ± 3.12% ($P < 0.05$) when compare with the group treated with only H_2O_2 and increase to 119.90 ± 5.45% ($P < 0.01$) in melatonin treatment alone when compared with the control untreated cells (Fig. 1).

The effect of melatonin on acNF-κB protein expression in senescent human SH-SY5Y neuroblastoma cells.

To investigate the effect of melatonin on the activation of acNF-κB in senescence-like human SH-SY5Y cells induced by H_2O_2, cells were treated with 100 µM H_2O_2 for 2 hr and/or followed by 1 µM melatonin. Then, cells were further incubated for 24 hr before western blot analysis of acetylated NF-κB (acNF-κB). After treated with 100 µM H_2O_2 and incubated for 24 hr, the level of acNF-κB increased significantly to 131.70 ± 2.22% ($p < 0.001$) when compared with the control untreated cells. In the groups treated with both H_2O_2 and melatonin, the result demonstrated that melatonin reduced acNF-κB level significantly to 100.50 ± 2.96% ($p < 0.001$), when compared with the group treated with H_2O_2 (Fig. 2).

The effect of melatonin on LC3-II protein expression in senescent human SH-SY5Y neuroblastoma cells.

To investigate the effect of melatonin on the LC3-II, the autophagic marker, expression in senescence-like human SH-SY5Y cells induced by H_2O_2, cells were treated with 100 µM H_2O_2 for 2 hr and/or followed by 1 µM melatonin. Then, cells were further incubated for 24 hr before western blot analysis of LC3-II. After treated with 100 µM H_2O_2 and incubated for 24 hr, the level of LC3-II reduced statistically to 69.95 ± 3.98 % ($p < 0.001$) when compared with the control untreated cells. In the group co-treated melatonin, the result demonstrated that melatonin increased LC3-II level significantly to 100.36 ± 5.41 % ($p < 0.01$), when compared with the group treated with H_2O_2 (Fig. 3).

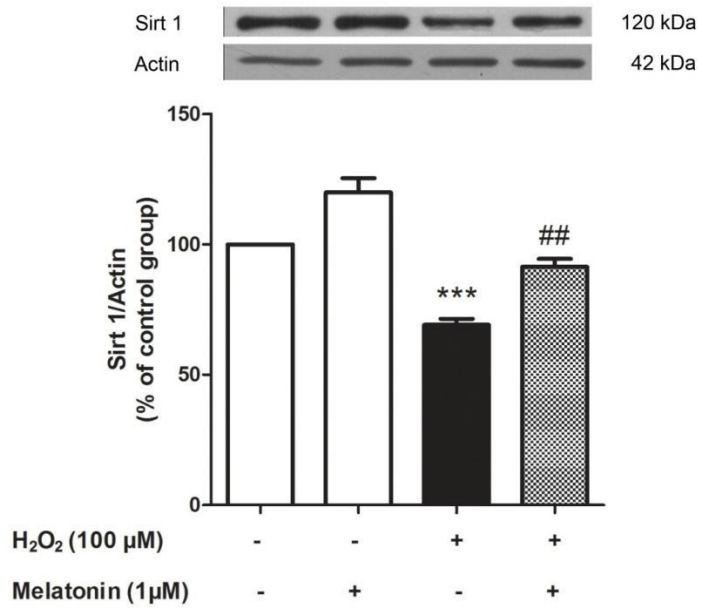

Fig. 1 Effect of melatonin on SIRT1 activation in SH-SY5Y cells. Cells were treated with 100 µM of H_2O_2 prepared from 1M H_2O_2 in complete medium for 2 hours. Then, the treatment was removed and cells were washed twice with medium. 100 µM of melatonin prepared from 10 mM in 40% ethanol was applied to cells cultured in complete medium to make final concentration of 1 µM for 24 hours. The results are expressed as mean ± S.E.M. of 3 independent experiments. The ANOVA was performed for statistical analysis. Values represent the mean ± S.E.M. of three separate determinations. $^{***}P < 0.001$ significance compared with untreated controls and $^{\#\#}P < 0.01$ significance compared with H_2O_2-treated groups.

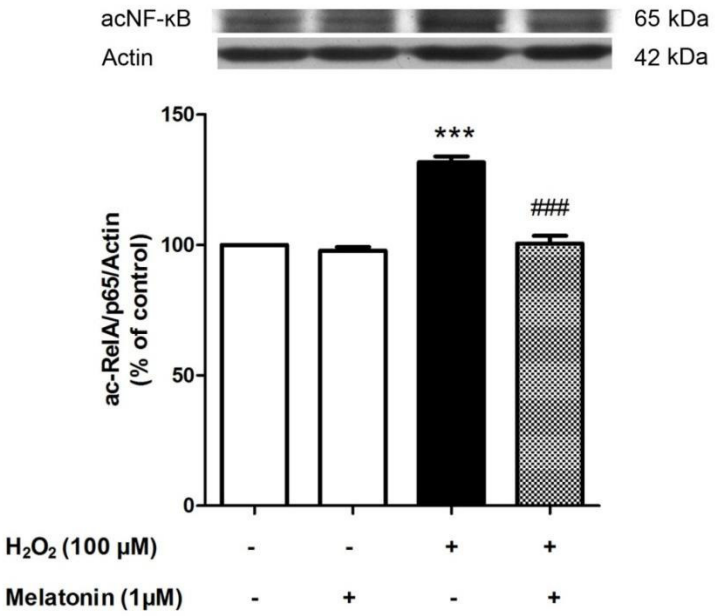

Fig.2 Effect of melatonin on acNF-κB expression in SH-SY5Y cells. Cells were treated with 100 μM of H_2O_2 prepared from 1M H_2O_2 in complete medium for 2 hours. Then, the treatment was removed and cells were washed twice with medium. 100 μM of melatonin prepared from 10 mM in 40% ethanol was applied to cells cultured in complete medium to make final concentration of 1 μM for 24 hours. The results are expressed as mean ± S.E.M. of 3 independent experiments. The ANOVA was performed for statistical analysis. Values represent the mean ± S.E.M. of three separate determinations. ***$P < 0.001$ significance compared with untreated controls and $^{###}P < 0.001$ significance compared with H_2O_2-treated groups.

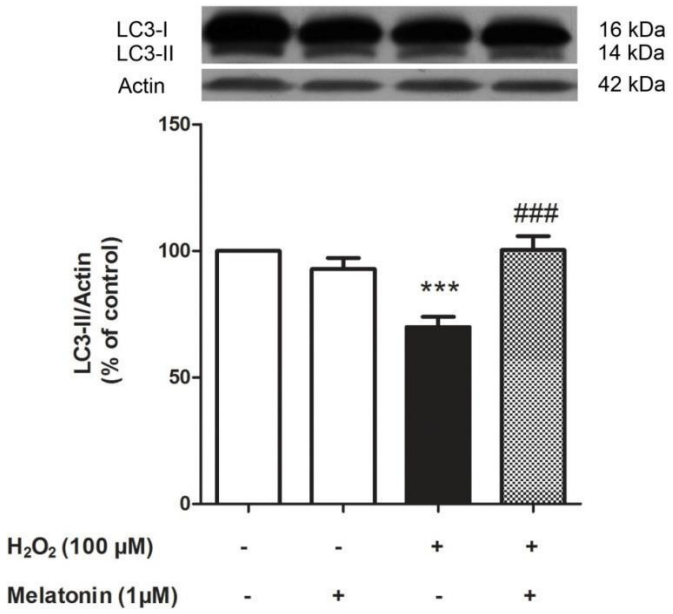

Fig. 3 Effect of melatonin on LC3-II activation in SH-SY5Y cells. Cells were treated with 100 μM of H_2O_2 prepared from 1M H_2O_2 in complete medium for 2 hours. Then, the treatment was removed and cells were washed twice with medium. 100 μM of melatonin prepared from 10 mM in 40% ethanol was applied to cells cultured in complete medium to make final concentration of 1 μM for 24 hours. The results are expressed as mean ± S.E.M. of 3 independent experiments. The ANOVA was performed for statistical analysis. Values represent the mean ± S.E.M. of three separate determinations. ***$P < 0.001$ significance compared with untreated controls and $^{###}P < 0.001$ significance compared with H_2O_2-treated groups.

Discussion

The "free-radical theory of aging" was proposed by Denham Harman in 1956. This theory pointed out that free radicals produced as the normal metabolism accumulated and caused damage to cellular components (Harman 1956). There are many types of free radicals can stimulate cellular senescence by multiple methods. However, the most well-accepted method is using the hydrogen peroxide (H_2O_2) to induce oxidative stress in vitro senescent model. Numerous studies revealed that H_2O_2 could induce DNA damage in different cell types (Atamna et al 2000, Schraufstatter et al 1986) including neurons (Pizarro et al 2009, Ramos-Espinosa et al 2012) and astrocytes (Cemeli et al 2003). In our study, SH-SY5Y neuroblastoma cell was induced to senescence-like state by H_2O_2 treatment.

Several studies have revealed the anti-aging property of melatonin in many types of senescent model. Melatonin acts as anti-aging property via several signaling pathway including Sirtuin1, also known as longevity gene. Recent study has indicated that melatonin promotes the expression of sirtuin 1in neocortical neurons (Cristofol et al 2012). Melatonin also acts as an effective antioxidant to preserve NAD levels under oxidative stress (Tan et al 2005). These findings suggest that melatonin may have a neuroprotective role by acting on SIRT1 modulation via the NAD system. However, the level of SIRT1 expression is depended on age. Recent study has indicated that SIRT1 levels decreased in aging microglia (Cho et al 2015). In our study, we showed that the level of SIRT1 was reduced in H_2O_2-induced SH-SY5Y cell group (Fig.1). This finding suggests the SIRT1 level is age-dependent decline. On the other hand, the level of SIRT1 showed highly expression after treated with melatonin (Fig.1). This finding indicates that melatonin can enhance the SIRT1 expression in senescent SH-SY5Y cell.

NF-κB is a key signal molecule that regulates chronic inflammation during aging. It has five subunits that involves in transcription process. RelA/p65 is a key subunit that can regulate the transcriptional activity via phosphorylation and acetylation. Acetylation of Lys310 is required for the full transcriptional activity of RelA (Lanzillotta et al 2010). However, acetylated RelA/p65 is subsequently deacetylated by HDACs, especially, HDAC3, which promotes binding to IκBα and lead to export to outside (Chen et al 2002, Chen et al 2001). In the present study, the level of acetylated RelA was increase in H_2O_2-treated group (Fig.2.). This data suggests that the acetylation of NF-κB is related with aging. However, RelA can be deacetylated by HDAC3 including SIRT1. Previous study explored that SIRT1 attenuated Aβ-induced toxicity in rat primary neuronal cultures by the inhibition of NF-κB signaling (Chen et al 2005). Marwarha and coworker demonstrated that high level of SIRT1 resulting in decreased BACE1 expression through deacetylation of RelA/p65 subunit (Marwarha et al 2014). In our results, the level of acetylated RelA decreased after melatonin was added (Fig.2). From this data, we suggested that melatonin could enhance the deacetylating activity of SIRT1, then, result in decreased acetylated RelA/p65 protein.

Autophagy is the basic mechanism that maintains cellular homeostasis. Autophagy is activated during stressful conditions such as amino acid starvation, however, under some stress conditions; autophagy turned into death role and led to cell death type2. On the other hand, multiple reports indicate that essential proteins required for autophagy induction have reduced expression in aged tissues and that autophagy diminishes with aging. In addition to, several cellular functions regulated by the NF-κBsignaling is cooperated with autophagy. However, the role of NF-κBsignaling in autophagic degradation is unclear now. Recent study reported that NF-κB activation mediated repress autophagic activity in TNFα-treated Ewing sarcoma cells (Djavaheri-Mergny et al 2006) and in macrophages (Schlottmann et al 2008). In the present study, the level of LC3-II, the autophagic marker, was diminished in H_2O_2-treated group (Fig.3). The possible mechanism of autophagy inhibited by NF-κB could be lacking or suppression of essential proteins such as Beclin1, the autophagy induction protein, via NF-κB signaling pathway. Schlottmann and coworker demonstrated that NF-κB signaling pathway downregulating Atg5 and Beclin1 expression, leading to the autophagy interrupted and promotion of apoptosis and inflammation processes (Schlottmann et al 2008). To support this notion, the inhibition of NF-κBactivation could block the autophagic response and increased cell death after exposure to heat shock stress (Nivon et al 2009). On the other hand, the reversion of LC3-II level in co-treated group (Fig.3.) could result from melatonin interrupted NF-κBactivation via SIRT1 deacetylating activity. Therefore, further studies are warranted to determine the involvement of SIRT1, acetylated RelA/p65 and Beclin1 in the autophagic activity regulated by NF-κB signaling pathway.

Conclusions

This study demonstrated that melatonin could increase protein level of SIRT1 and LC3-II, a hallmark protein of autophagy, and reduced the level of acetylated-Lys310 in p65 subunit of NF-κB in SH-SY5Y cells treated with H_2O_2. The present data indicated that melatonin enhanced autophagy via inhibiting NF-κB signaling pathway. Taken together, we provided a novel mechanism of melatonin in modulating autophagy which could be a potent target for anti-aging therapy.

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