Melatonin reduces oxidative damage in mouse granulosa cells via restraining JNK-dependent autophagy

Yan Cao*, Ming Shen* , Yi Jiang, Shao-chen Sun and Honglin Liu

College of Animal Science and Technology, Nanjing Agricultural University, Nanjing, China

Correspondence should be addressed to M Shen or H Liu; Email: shenm2015@njau.edu.cn or liuhonglin@njau.edu.cn

*(Y Cao and M Shen contributed equally to this work)

Abstract

Oxidative stress-induced granulosa cell (GCs) injury is believed to be a common trigger for follicular atresia. Emerging evidence indicates that excessive autophagy occurs in mammalian cells with oxidative damage. N-acetyl-5-methoxytryptamine (melatonin) has been shown to prevent GCs from oxidative injury, although the exact mechanism remains to be elucidated. Here, we first demonstrated that the suppression of autophagy through the JNK/BCL-2/BECN1 signaling is engaged in melatonin-mediated GCs protection against oxidative damage. Melatonin inhibited the loss of GCs viability, formation of GFP-MAP1LC3B puncta, accumulation of MAP1LC3B-II blots, degradation of SQSTM1 and the expression of BECN1, which was correlated with impaired activation of JNK during oxidative stress. On the other hand, blocking of autophagy and/or JNK also reduced the level of H₂O₂-induced GCs death, but failed to further restore GCs viability in the presence of melatonin. Particularly, the suppression of autophagy provided no additional protective effects when GCs were pretreated with JNK inhibitor and/or melatonin. Importantly, we found that the enhanced interaction between BCL-2 and BECN1 might be a responsive mechanism for autophagy suppression via the melatonin/JNK pathway. Moreover, blocking the downstream antioxidant system of melatonin using specific inhibitors further confirmed a direct role of melatonin/JNK/autophagy axis in preserving GCs survival without scavenging reactive oxygen species (ROS). Taken together, our findings uncover a novel function of melatonin in preventing GCs from oxidative damage by targeting JNK-mediated autophagy, which might contribute to develop therapeutic strategies for patients with ovulation failure-related disorders.

Reproduction (2018) 155 307–319

Introduction

Macroautophagy (referred to here as autophagy) is an evolutionary conserved catabolic pathway in eukaryotes that involves recycling of cellular materials, including long-lived proteins and organelles (Levine & Klionsky 2004). Autophagy is initiated by the formation of double-membrane vesicles called autophagosomes that sequester and deliver cytoplasmic components to the lysosome for degradation. Autophagy occurs at a basal rate in normal cells, removing damaged cellular content to maintain homeostasis (Levine & Kroemer 2008). During periods of starvation, stimulation of autophagy is an autonomous cell survival mechanism, which provides nutrients to maintain metabolism and ATP levels compatible with cell survival (Mehrpour *et al.* 2010). In the context of cancer, activation of autophagy by hypoxia or drug treatment has also been shown to promote tumor cell survival (Sui *et al.* 2013). However, there is considerable evidence suggesting the role of autophagy in repressing cell viability (Tsujimoto & Shimizu 2005). Almost paradoxically, autophagy has been described as a type of apoptosis-independent cell death (also known as type II programmed cell death),

which is characterized by autophagosome accumulation in the absence of apoptotic bodies and cleaved CASP3 (Kroemer & Levine 2008, Gustafsson & Gottlieb 2009, Nishida *et al.* 2009).

In mammalian ovaries, only a few follicles are selected for ovulation, while most of them ($\geq 99\%$) undergo a degenerative process termed follicular atresia (Kaipia & Hsueh 1997). Reactive oxygen species (ROS) are free radicals derived from cellular metabolism or environmental stimuli. Unlimited ROS generation shift cells into a state of oxidative stress, leading to destructive effects on cellular components (Birben *et al.* 2012). Previous studies indicated that increased oxidative damage in granulosa cells (GCs) is closely correlated to the occurrence of follicular atresia (Murdoch 1998), which may give rise to certain anovulatory disorders, such as polycystic ovary syndrome and premature ovarian failure (Agarwal *et al.* 2012). In contrast, impaired ROS production has been shown to alleviate GC injury in atretic follicles (Tilly & Tilly 1995, Gupta *et al.* 2006). Notably, recent evidence suggested that autophagy may be responsible for oxidative stress-induced GC damage during follicular atresia (Duerschmidt *et al.* 2006,

Serke *et al.* 2009). In fact, our previous work has provided more direct evidence demonstrating the role of oxidative stress in promoting autophagic GC death (Shen *et al.* 2017). Therefore, discovery and identification of an antioxidant agent by targeting autophagy may provide benefits to clinical therapy for anovulatory disorders caused by inappropriate follicular atresia.

N-acetyl-5-methoxytryptamine (melatonin), the main secretory product of the pineal gland, plays diverse functions in regulating circadian rhythms, inflammation, carcinogenesis, immune response and ROS clearance (Manchester *et al.* 2015, Reiter *et al.* 2016). Growing evidence suggested that melatonin also serves as a prosurvival factor for antral follicles (Tamura *et al.* 2009). It stimulates GCs to synthesize sex steroid hormones and promotes the progression of follicular maturation (Tamura *et al.* 2009). In contrast, inhibiting melatonin production by pinealectomy significantly increased the number of atretic follicles in mammalian ovaries (Soares *et al.* 2003b). Interestingly, it was claimed that melatonin in the follicular fluid may be directly produced by GCs, whereas melatonin in turn enhances the antioxidant capacity of GCs by upregulating gene expression of SOD, GPx and CAT (Tamura *et al.* 2009). As previously mentioned, excessive ROS induces GC death and the resultant follicular atresia. Increased levels of melatonin in follicular fluid would normally protect GCs from free radical damage and suppress atresia through its direct and indirect antioxidant actions (Tamura *et al.* 2013). However, there are few further reports investigating the downstream signaling of melatonin for GCs protection during oxidative stress.

BECN1, the mammalian homolog of yeast ATG6, is a platform protein that recruits several different partners to control the activity of PIK3C3 (class III phosphatidylinositol 3-kinase), which catalyzes the conversion of phosphatidylinositol into phosphatidylinositol 3-phosphate, a lipid required for autophagosome formation in response to ATG proteins (Levine & Yuan 2005). As a BH3-only protein, BECN1 also interacts with other BCL-2 family members, such as BCL-2, BCL-XL, BCL-W and MCL-1 (Erlich *et al.* 2007). Previous studies indicated that dissociation of BCL-2 from BECN1 may be an important mechanism for autophagy induction under stress conditions (Pattingre *et al.* 2005). JNK (also known as c-Jun N-terminal kinase), a member of the MAPKs (mitogen-activated protein kinases), regulates diverse biological processes including proliferation, differentiation, senescence, autophagy, metabolism and stress response (Weston & Davis 2007). Further investigations suggested that the activation of JNK by nutrient deprivation could inhibit the interactions between BCL-2 and BECN1 (Wei *et al.* 2008). Moreover, melatonin has been shown to reduce hepatic ischemia/reperfusion injury by suppressing the JNK pathway (Liang *et al.* 2009). These findings thus implicate a

possible relationship among melatonin, JNK and the autophagic machinery. Nevertheless, it remains unclear whether autophagy is correlated with JNK signaling in oxidative stress-induced GCs death and the protective role of melatonin in this process.

Herein, we investigated whether the preventive effects of melatonin on oxidative stress-induced GC injury is associated with autophagy signaling. We specifically focused on the BCL-2/BECN1 complex and upstream pathway activity, including JNK activation. Furthermore, we determined whether melatonin could attenuate oxidative stress-induced GCs toxicity via this signaling cascade.

Materials and methods

Chemicals and antibodies

PBS was purchased from Gibco. Pregnant mare serum gonadotropin (PMSG) was purchased from Ningbo Second Hormone Factory (Ningbo, Zhejiang, China). Melatonin, SP600125, 3-methyladenine (3-MA) and NSC 23766 were from Selleck Chemicals (Houston, TX, USA). H₂O₂, anti-MAP1LC3B and anti-TUBA1A were bought from Sigma-Aldrich. L-Buthionine sulfoximine, 3-amino-1,2,4-triazole, diethyldithiocarbamic acid sodium salt trihydrate, mercaptosuccinic acid, Luzindole and Map2k7 siRNA were purchased from Santa Cruz Biotechnology. Active Rac1 Detection Kit and antibodies against BCL-2, BECN1, Phospho-MAP2K7 and MAP2K7 were obtained from Cell Signaling Technology. SQSTM1 antibody was purchased from Abcam.

Animals

All procedures with mice were conducted in accordance with the guidelines of the Animal Research Institute Committee at Nanjing Agricultural University. Three week-old female ICR mice (Qing Long Shan Co., Animal Breeding Center, Nanjing, China) were injected intraperitoneally with 10 IU PMSG. After 48 h, all mice were killed by cervical dislocation, and their ovaries were harvested for *in vitro* experiments. Prior to killing, the mice were housed five per cage in a temperature-controlled (22 ± 2°C) room with 12:12-h light:darkness cycles (lights on from 07:00h to 19:00h); they had ad libitum access to water and food. All experimental protocols were approved by the Committee of Animal Research Institute, Nanjing Agricultural University, China.

Cell collection and treatments

Primary GCs were collected as described (Shen *et al.* 2012). Briefly, superovulated mouse ovaries were harvested and individually transferred into 35 mm Petri dishes containing PBS, and then punctured with a syringe to release GCs (a mixture of mural and cumulus cells) from pre-ovulatory follicles under a surgical dissecting microscope (Olympus). For drug administration, GCs pretreated with melatonin (10 µM) for 24 h were washed in PBS and incubated

with medium containing 0.2 mM H₂O₂ for 2 h. In some experiments, GCs were treated with SP600125 (25 μM) or 3-MA (10 mM) 1 h before H₂O₂ exposure. For the suppression of melatonin-mediated antioxidative effects, a cocktail consists of inhibitors (hereafter referred to as antioxidant inhibitors or AOI) against glutathione/GSH (L-Buthionine sulfoximine; 1.5 mM), catalase/CAT (3-Amino-1,2,4-triazole; 10 mM), superoxide dismutase/SOD (diethyldithiocarbamic acid sodium salt trihydrate, 5 mM), glutathione peroxidase/GPx (mercaptosuccinic acid, 5 mM) and glutathione reductase/GR (mercaptosuccinic acid, 5 μM) were added 1 h prior to H₂O₂ incubation. For the inhibition of melatonin receptors, cells were incubated with 10 μM Luzindole for 2 h before melatonin treatment.

Cell transfection

Transfection was performed using Lipofectamine 3000 reagent (Invitrogen) according to supplier's instructions. Briefly, lipoplexes were prepared by combining 1 μL P3000 reagent with 0.5 μg of plasmid DNA suspended in 25 μL of serum-free media and incubated for 5 min at room temperature. This solution was then combined with 1.5 μL of Lipofectamine 3000 in 25 μL serum-free media and further incubated for 5 min at room temperature. The mixture was then diluted into serum-free media to produce the desired concentration in a total volume of 0.5 mL and incubated with the cells at 37°C with 5% CO₂ for 48 h.

Confocal imaging of autophagic vacuoles

The GFP-tagged microtubule-associated protein 1 light chain 3 beta (MAP1LC3B) expression vector was kindly provided by Prof. Jiyong Zhou (Zhejiang University, Hangzhou, China). The GFP-MAP1LC3B expression plasmid was transfected into GCs grown on coverslips. After 24 h, cells were cultured in medium with or without 10 μM melatonin for another 24 h, rinsed using PBS and then exposed to 2 h of H₂O₂ incubation. The distribution and fluorescence emitted by GFP-MAP1LC3B puncta were then observed under a laser-scanning confocal microscope (Carl Zeiss, Zeiss LSM 710 META). Experiments were repeated three times. The fields of each coverslip were divided into 9 component squares, and 3 of them were randomly selected for counting GFP-MAP1LC3B-stained puncta at a magnification of 400×.

Cell viability assay

Cell viability was assessed by measuring the conversion of tetrazolium salt (WST-8) to formazan according to the manufacturer's instructions (Dojindo Laboratories, Kumamoto, Japan). Briefly, cells seeded in 96-well plate were exposed to H₂O₂ incubation following melatonin, SP600125, AOI or 3-MA administration as indicated. After treatment, 10 μL of CCK-8 solution were added to each well containing 100 μL medium and incubated for an additional 2 h at 37°C. Cell viability was then determined by reading the optical density at 450 nm using a microplate reader (Thermo Fisher Scientific).

Measurement of JNK activity

The activity of total JNK and JNK1 was determined using a JNK Activity Assay Kit (Genmed, Shanghai, China) as previously described (Weng *et al.* 2016). Briefly, cytosolic extracts were centrifuged at 16,000 g at 4°C for 10 min, and the protein concentrations were determined using a Bradford Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). 50 μg of total protein was incubated with 10 μL chromophoric substrate at 37°C. The optical density of each sample was immediately detected at 340 nm using a microplate reader (Thermo Fisher Scientific) and recorded 2 times at 5-min intervals.

RAC1 activation assay

The levels of active RAC1 were measured using Active RAC1 Detection Kit (Catalog number 8815, Cell Signaling Technology) according to the manufacturer's instructions. Briefly, 500 μg of GCs lysates were incubated with GST-PAK1-PBD fusion protein beads to enrich active GTP-bound RAC1, which were then immunoprecipitated with glutathione resin. Additional lysates were used as control inputs to determine total RAC1 levels. The proteins on the beads or the total cell lysates were subjected to immunoblot analysis using kit-provided RAC1 primary antibody.

Detection of ROS generation

ROS levels were determined by measuring the oxidative conversion of dichlorodihydrofluorescein (DCFH) to dichlorofluorescein (DCF), which generates green fluorescence upon excitation at 488 nm. The experiments were conducted using the Intracellular ROS green Fluorescence Determination Kit (Beyotime Institute of Biotechnology) according to the manufacturer's directions. Fluorescent images were obtained using a Carl Zeiss laser-scanning confocal microscope (Carl Zeiss, Zeiss LSM 710 META). The optical density was evaluated in each GC with ImageJ 1.42q software (National Institutes of Health).

qRT-PCR assay

Total RNA was isolated with TRIzol reagent (Invitrogen) and reverse transcribed into cDNA using the Reverse Transcription System (TaKaRa) according to the manufacturer's instructions. Real-time PCR was performed with SYBR Premix Ex Taq (TaKaRa) and genespecific primers (Table 1 for primer sequences) on the ABI StepOne PCR system (Applied Biosystems). Melting curves were analyzed to verify amplification specificity. Expression data were normalized to the amount of *Actb* expressed.

Western blotting

Western blotting was performed as described previously (Shen *et al.* 2014). Briefly, total protein extracts were fractionated by electrophoresis on a 12% ExpressPlus PAGE gel (Genscript, Nanjing, China) and transferred to PVDF membranes (Millipore) by electroblotting. Nonspecific binding sites were

Table 1 The forward and reverse primers used for qRT-PCR.

Gene name	GenBank accession no.	Primer sequence (5'→3')	
		Forward	Reverse
<i>Bcl-2</i>	NM_009741.3	GTGGATGACTGAGTACCTGAACC	AGCCAGGAGAAATCAAACAGAG
<i>Becn1</i>	NM_019584.3	AGTTGCCGTTACTGTTCT	TCAATCTTGCCTTTCTCC
<i>Actb</i>	NM_007393.5	GCTGTCCCTGTATGCCTCT	GTCTTTACGGATGTCAACG

blocked with 5% bovine serum albumin in TBST (Solarbio, Beijing, China) for 1 h. The membranes were then incubated with primary antibodies (1:1000) against MAP1LC3B, SQSTM1, TUBA1A, BCL-2 and BECN1 in blocking solution overnight followed by a secondary antibody conjugated with horseradish peroxidase (1:2000) for another 1 h. Detection of the bands was performed using the SuperSignal West Pico chemiluminescent substrate (Pierce). The expression values for target proteins were normalized to TUBA1A as the control for loading.

Immunoprecipitation

After washing with PBS, GCs were lysed in 1 mL cold IP lysis buffer (Pierce) containing a complete protease inhibitor cocktail (Roche). Whole-cell lysates were used for immunoprecipitation with anti-BCL-2. We added 2–4 mg of antibody to 1 mL of cell lysate, which was incubated at 4°C overnight. After the addition of Protein A/G Plus Agarose beads (Beyotime Institute of Biotechnology), the incubation was continued for 1 h. Immunoprecipitates were extensively washed with lysis buffer, eluted with SDS loading buffer (SunShineBio, Nanjing, China) by boiling for 5 min and then processed for immunoblotting with the indicated antibodies. The amount of coimmunoprecipitated proteins for each IP reaction was normalized to TUBA1A content in the whole-cell lysates (input).

Statistical analysis

Statistical significance was determined using the SPSS, version 16.0 software (SPSS). All experiments were repeated at least three times. Data were presented as means $\pm$ s.e. The statistical significance between groups was analyzed using one-way ANOVA, and *P* values less than 0.05 were considered to be statistically significant.

Results

Melatonin counteracted oxidative injury in mouse granulosa cells (GCs) via inhibiting the JNK signaling

Using an established *in vitro* model for the induction of oxidative damage in GCs, we found that H₂O₂ significantly decreased cell survival in a dose-dependent manner (Fig. 1A). According to the results, H₂O₂ at 0.2 mM tended to provide a maximum useful dose for quantifying protective vs harmful response, and thus was chosen for subsequent experiments. To test whether melatonin protects GCs from oxidative injury, the CCK-8 assay was performed to measure cell viability. As shown

in Fig. 1B, GCs pretreated with 10 μ M melatonin for 24 h exhibited an apparent resistance to H₂O₂-induced cell death. Notably, melatonin did not significantly alter the viability of cells under normal growth conditions (Fig. 1B), indicating that melatonin itself had no side effects on GC survival.

Figure 1 JNK is involved in melatonin-mediated GC protection during oxidative stress. (A) Primary cultured GCs were incubated with H₂O₂ in various concentrations (from 0.05 mM to 1 mM) for 2 h. Cell viability was then determined using CCK-8 assay. Data represent mean $\pm$ s.e.; *n*=3 in each group. *Represents *P*<0.05 vs control group. **Represents *P*<0.01 vs control group. (B) GCs were pretreated with or without melatonin (10 μ M) for 24 h, rinsed in PBS, exposed to 2 h of H₂O₂ (0.2 mM) incubation, and then processed for the detection of cell viability (B), JNK activity (C) or JNK1 activity (D). Data represent mean $\pm$ s.e.; *n*=3. ***P*<0.01; NS, not significant, *P*>0.05. (E) GCs cultured in medium containing 10 μ M melatonin for 24 h were washed in PBS, and then subjected to 2 h of H₂O₂ (0.2 mM) exposure. For the inhibition of JNK activity, SP600125 (25 μ M) was added 1 h prior to H₂O₂ treatment. Data represent mean $\pm$ s.e.; *n*=3. **Represents *P*<0.01 vs control group. **Represents *P*<0.01 vs H₂O₂ group. NS, not significant, *P*>0.05.

c-Jun N-terminal kinase (JNK), a major effector of cellular stress response signaling, has been reported to be activated upon oxidative stimulation (Weston & Davis 2007). To evaluate whether the protective effect of melatonin on H_2O_2 -triggered GC injury is correlated with the inhibition of JNK, its activity was determined by measuring the oxidative conversion of NADH to NAD, which was detectable at 340nm under a microplate reader. Significantly, oxidative stress-induced activation of total JNK and JNK1 isoform was abolished in the presence of melatonin (Fig. 1C and D).

RAC1, a small GTPase, plays essential roles in the cellular response to multiple types of stresses (Ozaki *et al.* 2000). Here, we examined whether RAC1 might be involved in oxidative stress-induced JNK activation in GCs. As expected, cells with H_2O_2 incubation exhibited a significant increase in JNK activity (Fig. 2C). However, GCs pretreated with melatonin or RAC1 inhibitor NSC 23766 blocked H_2O_2 -induced JNK activation (Fig. 2C). Correspondingly, both melatonin and NSC 23766 repressed the upregulation of JNK

activity upon oxidative stimulation, but RAC1 showed no additional inhibitory effect on JNK activation in cell received melatonin treatment (Fig. 2C), indicating that the suppression of RAC1 might contribute to melatonin-mediated JNK inhibition. MAP2K7 has been reported to stimulate JNK activation in response to extracellular stimuli (Moriguchi *et al.* 1997). To explore any influence of MAP2K7 on JNK activity in response to H_2O_2 or melatonin treatment, we blocked MAP2K7 expression in cultured GCs using RNA interference (Fig. 2D). The H_2O_2 exposure facilitated MAP2K7 activation as suggested by increased phosphorylation, which was impaired in cells subjected to melatonin treatment or *Map2k7* siRNA transfection (Fig. 2D and Supplementary Fig. 1C, see section on supplementary data given at the end of this article). Consistently, both MAP2K7 knockdown or melatonin incubation significantly repressed H_2O_2 -stimulated JNK activation (Fig. 2F). However, *Map2k7* siRNA did not further inhibit JNK activity in GCs pretreated with melatonin (Fig. 2F). Therefore, these data suggested a possible regulation by H_2O_2 and melatonin

Figure 2 H_2O_2 activates JNK through the RAC1/MAP2K7 pathway, which is inhibited by melatonin treatment. (A) GCs grown in medium containing $10\mu M$ melatonin for 24 h were rinsed using PBS, and then incubated with 0.2 mM H_2O_2 for 2 h. For the inhibition of RAC1, the RAC1 specific antagonist NSC 23766 ($10\mu M$) was added 1 h prior to H_2O_2 exposure. The cell lysates were collected for GST-PAK1-PBD pulldown assay of RAC1 activation (GTP-bound RAC1 levels) and immunoblotting analysis of total RAC1. (B) Western blot analysis of phosphorylated MAP2K7 (p-MAP2K7) and total MAP2K7 in GCs with the indicated treatments as described above. TUBA1A served as the control for loading. (C) The detection of JNK activity in GCs subjected to the treatments as mentioned earlier. Data represent mean $\pm$ S.E.; $n=3$. **Represents $P<0.01$ vs control group. ##Represents $P<0.01$ vs H_2O_2 group. N, not significant, $P>0.05$. (D) GCs transfected with scrambled control siRNA (SC siRNA) or *Map2k7* siRNA were cultured with $10\mu M$ melatonin for 24 h, washed in PBS, and then grown in medium containing 0.2 mM H_2O_2 for 2 h. The expression of phosphorylated MAP2K7 (p-MAP2K7) and total MAP2K7 was determined by Western blotting. TUBA1A served as the control for loading. (E) GCs transfected with scrambled control siRNA (SC siRNA) or *Map2k7* siRNA for 48 h were cultured with or without 0.2 mM H_2O_2 for 2 h. Cell lysates were collected for Western blot analysis of RAC1 activation (GTP-RAC1 levels) by GST-PAK1-PBD pulldown and of total RAC1 levels. (F) GCs transfected with scrambled control siRNA (SC siRNA) or *Map2k7* siRNA for 24 h were cultured for another 24 h in the presence or absence of $10\mu M$ melatonin before 2 h of H_2O_2 (0.2 mM) incubation. Cells were then processed for JNK determination. Data represent mean $\pm$ S.E.; $n=3$. **Represents $P<0.01$ vs control group. ##Represents $P<0.01$ vs H_2O_2 group. N, not significant, $P>0.05$.

of JNK activity in GCs through the MAP2K7 pathway. We then examined the relationship between RAC1 and MAP2K7. It was found that blocking GTP-RAC1 formation with melatonin or NSC 23766 abrogated H₂O₂-induced MAP2K7 phosphorylation, whereas the RAC1 antagonist provided no further inhibitory effect on MAP2K7 activity in the presence of melatonin (Fig. 2A, B and Supplementary Fig. 1A, B). In contrast, MAP2K7 knockdown had little effect on H₂O₂-induced RAC1 activation (Fig. 2E and Supplementary Fig. 1D). Collectively, the RAC1/MAP2K7 pathway might be involved in melatonin-directed inhibition of JNK activation upon oxidative stimulation.

To determine whether the suppression of H₂O₂-triggered JNK activation by melatonin is a melatonin receptor-mediated event, we treated cells with Luzindole, a competitive antagonist of melatonin receptor. However, Luzindole did not significantly influence the inhibitory effects of melatonin on JNK activity upon H₂O₂ exposure (Supplementary Fig. 2).

To further assess whether JNK affected the prosurvival actions of melatonin under oxidative stress conditions, GCs were treated with JNK inhibitor SP600125. As shown in Fig. 1E, both melatonin and SP600125 displayed preventive effects on GC death upon H₂O₂ exposure. However, melatonin could not further restore the viability of cells pretreated with the JNK inhibitor. Based on these data, we proposed that the suppression of JNK is required for melatonin-mediated GC protection during oxidative stress.

The suppression of JNK by melatonin protects GCs from oxidative stress-induced autophagic death

Recent studies suggested that the antioxidative effect of melatonin is partly attributed to its role in autophagy regulation (Khalidy *et al.* 2003, Vega-Naredo *et al.* 2012). To test whether melatonin exerts any influence on autophagy in GCs with oxidative stress, we examined the levels of the phosphatidylethanolamine-conjugated microtubule-associated protein 1 light chain 3B-II (MAP1LC3B-II), which is a representative marker of autophagy (Klionsky *et al.* 2016). Immunoblotting results showed that treatment of melatonin for 24 h markedly reduced the accumulation of MAP1LC3B-II following 2 h of H₂O₂ (0.2 mM) exposure (Fig. 3A and B). Accordingly, it was accompanied by compromised degradation of SQSTM1, which functions as an adaptor protein for autophagy (Klionsky *et al.* 2016) (Fig. 3C). The inhibition of JNK with SP600125 also impaired H₂O₂-induced autophagy (Fig. 3A, B and C), but the inhibitor could not further restrain the autophagic response in cells pretreated with melatonin.

To obtain a better estimation of the cellular autophagic response to the melatonin-JNK signaling during oxidative stress, we next detected the autophagic flux by monitoring GCs transfected with GFP-MAP1LC3B-

expressing vector (Fig. 3D and E). The numbers of green puncta, which represent autophagic vacuoles, were markedly increased in GCs subjected to H₂O₂ incubation. In contrast, both melatonin and SP600125 blocked the formation of autophagic puncta upon oxidative stress. Moreover, no additional decline in autophagosome formation was observed in melatonin-treated cells despite JNK inhibitor administration. The results indicated that melatonin-induced deactivation of JNK might contribute to the prohibition of autophagy in GCs during oxidative stress.

Growing evidence reveals a close correlation between autophagy and cell death under stressful conditions (Levine & Kroemer 2008). To investigate whether the suppression of JNK-dependent autophagy is responsible for melatonin-mediated GC protection upon oxidative stress, cells were treated with melatonin, SP600125 or the autophagy inhibitor 3-methyladenine (3-MA) prior to H₂O₂ exposure. As shown in Fig. 3F, the cell viability loss during oxidative stress was reversed by the inhibition of autophagy and/or JNK. In accordance with this, melatonin displayed similar level of suppression in H₂O₂-induced cell death. Moreover, SP600125 and/or 3-MA did not significantly change cell viability in GCs treated with melatonin. In particular, the inhibition of autophagy failed to further improve GC survival following the co-treatment of melatonin and SP600125. Therefore, our data suggested that melatonin provides a preventive effect on oxidative stress-induced autophagic GC death by antagonizing the JNK signaling.

Melatonin inhibits JNK-dependent upregulation of BECN1 expression during autophagy

Considering the negative influence of melatonin on JNK function, we examined the expression of BCL-2 and BECN1, both of which belong to the downstream effectors of the JNK signaling (Wei *et al.* 2008). qRT-PCR analysis showed that *Becn1* mRNA levels were significantly increased upon oxidative stimulation, whereas both melatonin and SP600125 (JNK inhibitor) counteracted H₂O₂-induced transcriptional activation of *Becn1* (Fig. 4B). Notably, SP600125 provided no additional inhibitory effects on *Becn1* transcription in GCs pretreated with melatonin, indicating a potential downregulation of *Becn1* by melatonin through the JNK pathway (Fig. 4B). Western blotting results shown in Fig. 4C and E further confirmed that the melatonin-mediated JNK deactivation contributes to the suppression of BECN1, a critical platform protein for autophagosome formation (Levine & Yuan 2005). However, no evident induction of BCL-2 was observed under the same conditions (Fig. 4A, C and D). Nevertheless, these data suggested the possibility that the melatonin-JNK signaling might regulate autophagy with a BECN1-dependent manner.

Figure 3 Melatonin prevents GCs from oxidative injury by repressing JNK-dependent autophagy. (A) After culturing with 10 μ M melatonin for 24 h, GCs were rinsed in PBS and subjected to 2 h of H₂O₂ (0.2 mM) incubation. For the inhibition of JNK activity, SP600125 (25 μ M) was added 1 h before H₂O₂ treatment. The expression of MAP1LC3B and SQSTM1 in GCs was determined by Western blotting. (B and C) Quantification of MAP1LC3B-II accumulation and SQSTM1 degradation using densitometric analysis. TUBA1A served as the control for loading. Data represent mean $\pm$ S.E.; $n=3$. **Represents $P<0.01$ vs control group. ##Represents $P<0.01$ vs H₂O₂ group. NS, not significant, $P>0.05$. (D) GCs transfected with GFP-MAP1LC3B plasmid for 24 h were grown with or without 10 μ M melatonin for another 24 h before 2 h of H₂O₂ (0.2 mM) incubation. SP600125 (25 μ M) was added 1 h prior to H₂O₂ exposure. Laser confocal-scanning microscopy was employed to observe the GFP fluorescent puncta in GCs. Bar, 10 μ m. (E) Quantification of the punctate GFP-MAP1LC3B per cell. Experiments were repeated in triplicate, and three fields of each coverslip were selected in random for counting. Data represent mean $\pm$ S.E.; $n=3$. **Represents $P<0.01$ vs control group. ##Represents $P<0.01$ vs H₂O₂ group. NS, not significant, $P>0.05$. (F) GCs with 24 h of melatonin (10 μ M) treatment were rinsed in PBS, and then exposed to H₂O₂ (0.2 mM) for 2 h. The autophagy inhibitor 3-MA (10 mM) or the JNK inhibitor SP600125 (25 μ M) was added 1 h before H₂O₂ incubation. Cell viability was determined using the CCK-8 assay. Data represent mean $\pm$ S.E.; $n=3$ in each group. **Represents $P<0.01$ compared to non-treatment control group. ##Represents $P<0.01$ compared to H₂O₂-only-treated cells. &Represents $P>0.05$ compared to H₂O₂ + melatonin group. N, not significant, $P>0.05$.

Melatonin-mediated JNK inhibition facilitates the formation of BCL-2/BECN1 complex

Since the activation of JNK has been reported to promote the dissociation between BCL-2 and BECN1 (Wei *et al.* 2008), which is required for autophagy induction under stressful conditions (Pattingre *et al.* 2005), we wonder

whether melatonin-induced JNK suppression affects the interactions between BCL-2 and BECN1 in GCs suffering oxidative damage. To test this assumption, GCs exposed to H₂O₂ incubation following melatonin and/or SP600125 treatment were collected for immunoprecipitation with the antibody against BCL-2. As shown in Fig. 5A, similar levels of BCL-2 expression

Figure 4 Suppression of JNK by melatonin reduces BECN1 expression during autophagy. (A and B) Primary cultured GCs with 24 h of melatonin (10 μ M) pretreatment were rinsed in PBS and stimulated with H₂O₂ (0.2 mM) for 2 h. The JNK inhibitor SP600125 (25 μ M) was added into the culture medium 1 h before H₂O₂ exposure. The mRNA expression of *Bcl-2* (A) and *Becl1* (B) in GCs was determined by qRT-PCR. Expression data were normalized to that of *Actb*. Data represent mean $\pm$ S.E.; $n=3$. **Represents $P<0.01$ compared to control group. #Represents $P<0.05$ compared to H₂O₂ group. ##Represents $P<0.01$ compared to H₂O₂ group. NS, not significant, $P>0.05$. (C) Immunoblotting analysis of BCL-2 and BECN1 in GCs with the indicated treatments as described earlier. (D and E) Bar charts show the quantification of endogenous BCL-2 and BECN1. TUBA1A served as the control for loading. Data represent mean $\pm$ S.E.; $n=3$. **Represents $P<0.01$ compared to control group. #Represents $P<0.05$ compared to H₂O₂ group. ##Represent $P<0.01$ compared to H₂O₂ group. NS, not significant, $P>0.05$.

were detected in the whole-cell lysates harvested from each group. When the corresponding IP components were immunostained using anti-BCL-2, a single band of about 26kDa was observed, indicating that BCL-2 was successfully immunoprecipitated (Fig. 5A and B). Western blot analysis of the IP contents revealed that BECN1 was coprecipitated by the BCL-2 antibody (Fig. 5A), implying a physical interaction between BCL-2 and BECN1. However, the binding affinity of BECN1 to BCL-2 was markedly repressed upon oxidative stimulation (Fig. 5A and C). In contrast, both melatonin and SP600125 partly restored the formation of BCL-2/ BECN1 complex upon H₂O₂ exposure (Fig. 5A and C). Importantly, the JNK inhibitor failed to further improve BECN1-BCL-2 interaction in cells pretreated with melatonin (Fig. 5A and C). Therefore, the formation of BCL-2/BECN1 complex might be an essential step in melatonin-mediated suppression of autophagy through the JNK signaling pathway.

The protective effect of melatonin on GC survival by inhibiting JNK-induced autophagy is independent of ROS scavenging

Melatonin can protect cells directly as an antioxidant or indirectly through activation of several antioxidative components, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione (GSH) (Shen *et al.* 2014). Our current treatment procedure eliminated the direct antioxidative action of melatonin, because GCs incubated with this indoleamine for 24h were rinsed in PBS before H₂O₂ exposure. To verify whether the prosurvival role of melatonin-JNK signaling is achieved by removing oxidative stress, cells were treated with specific antagonists targeted to the downstream antioxidants (hereafter referred as antioxidant inhibitors, or AOI) of melatonin. The results of ROS detection showed that AOI abolished melatonin-mediated inhibition of oxidative stress despite SP600125 administration (Fig. 6A and B), indicating that JNK is not necessary for the antioxidation capacity of melatonin. However, AOI did not significantly influence the inhibitory effects of melatonin on GC death (Fig. 6C) or JNK activity (Supplementary Fig. 3) upon oxidative stimulation. Additionally, after AOI and SP600125 treatment, no apparent changes in cell viability were observed in GCs subjected to melatonin incubation during oxidative stress. Moreover, when cells were treated with both melatonin and AOI, the JNK inhibitor could not further improve GC resistance to oxidative damage. These findings thus suggested a potential role of melatonin-JNK signaling in preventing oxidative damage without eliminating oxidative stress itself.

We next investigated whether a nonspecific effect of melatonin might influence autophagy by antagonizing oxidative stress. By determining the autophagic flux

Figure 5 Melatonin maintains the association of BCL-2 and BECN1 by inhibiting JNK in H₂O₂-treated GCs. (A) Primary cultured GCs pretreated with or without 10 μ M melatonin were washed in PBS, and then grown in medium containing 0.2 mM H₂O₂ for 2 h. To block the JNK signaling, SP600125 (25 μ M) was added 1 h before H₂O₂ incubation. After the indicated treatment, cell lysates were collected for co-immunoprecipitation with anti-BCL-2, followed by probing with anti-BECN1. WCL, whole-cell lysates. IP, immunoprecipitation. (B and C) The amount of co-immunoprecipitated BCL-2 (B) and BECN1 (C) was normalized to TUBA1A content in the whole-cell lysates (input). Data represent mean $\pm$ s.e.; $n=3$ in each group. ** $P<0.01$ vs control group; ## $P<0.01$ vs H₂O₂ group. NS, not significant, $P>0.05$.

Figure 6 The melatonin-JNK signaling directly attenuates oxidative injury in GCs. (A) GCs grown in medium containing 10 μ M melatonin for 24 h were then rinsed in PBS and exposed to 2 h of H₂O₂ (0.2 mM) incubation. The JNK antagonist SP600125 (25 μ M) and inhibitors of melatonin downstream antioxidants (AOI) were added 1 h prior to H₂O₂ treatment. The ROS levels were detected by dichlorofluorescein fluorescence (green), and nuclei were counterstained with DAPI (blue). Bar, 20 μ m. (B) The optical density of intracellular ROS was quantified using ImageJ software. Experiments were repeated in triplicate, and three fields of each coverslip were selected in random for counting. Data represent mean $\pm$ s.e.; $n=3$. * $P<0.05$, ** $P<0.01$; N, not significant, $P>0.05$. (C) The measurement of cell viability by CCK-8 assay. GCs were treated as above. Data represent mean $\pm$ s.e.; $n=3$ in each group. **Represents $P<0.01$ compared to non-treatment control group. ##Represents $P<0.01$ compared to H₂O₂-only-treated cells. &Represents $P>0.05$ compared to H₂O₂+melatonin group. N, not significant, $P>0.05$.

in GCs transfected with GFP-MAP1LC3B plasmid, we found that AOI exerts no evident influence on melatonin-induced suppression of autophagosome

Figure 7 The inhibitory effect of melatonin on JNK-dependent autophagic GC death is independent of ROS elimination. (A) GCs transfected with GFP-MAP1LC3B plasmid for 24 h were cultured with or without 10 μ M melatonin for another 24 h before 2 h of H₂O₂ (0.2 mM) incubation. The JNK antagonist SP600125 (25 μ M) and inhibitors of melatonin downstream antioxidants (AOI) were added 1 h before H₂O₂ exposure. Formation of GFP-MAP1LC3B vacuoles (puncta) was determined in GCs as described above. Bar, 10 μ m. (B) Quantification of GFP-MAP1LC3B puncta per cell. Experiments were repeated in triplicate, and 3 fields of each coverslip were selected in random for counting. Data represent mean $\pm$ s.e.; $n=3$. ** $P<0.01$; N, not significant, $P>0.05$. (C) GCs with 24 h of melatonin (10 μ M) treatment were rinsed in PBS, and then subjected to 2 h of H₂O₂ (0.2 mM) exposure. The inhibitors against JNK (SP600125), autophagy (3-MA) or melatonin downstream antioxidants (AOI) were added into the culture medium 1 h before H₂O₂ incubation. Cell viability was examined as described above. **Represents $P<0.01$ compared to non treatment control group. ##Represents $P<0.01$ compared to H₂O₂-only-treated cells. &Represents $P>0.05$ compared to H₂O₂+melatonin group. &Represents $P>0.05$ compared to H₂O₂+AOI+melatonin group. N, not significant, $P>0.05$.

formation in cells with H₂O₂ exposure (Fig. 7A and B). In particular, when GCs were pretreated with melatonin and AOI, the inhibition of JNK provided no additional preventive effects on autophagy activation (Fig. 7A and B). Correspondingly, loss of cell viability induced by H₂O₂ incubation was markedly reduced following melatonin, SP600125 and/or 3-MA treatment, but AOI did not seem to affect this process (Fig. 7C). Moreover, inhibitors of autophagy and/or JNK could not further restore cell viability following the treatment of melatonin and AOI (Fig. 7C). On the other hand, when JNK was blocked in cells treated with melatonin and AOI, the autophagy inhibitor 3-MA also failed to provide a greater protection to GCs upon oxidative stimulation (Fig. 7C). Collectively, these data further confirmed that the melatonin-JNK signaling directly inhibits autophagic death in GCs suffering oxidative damage.

Discussion

In response to metabolic or environmental stimuli, an excess production or decreased scavenging of reactive oxygen species (ROS) destroys cellular lipids, carbohydrates, proteins and DNA in a situation termed as oxidative stress. The adverse effects of oxidative stress on female reproduction have been well documented (Agarwal *et al.* 2012). For example, exposure to ionizing radiation, tobacco smoke or alcohol causes GC death and subsequent follicle loss in the ovary, leading to the pathogenesis of anovulatory infertility (Agarwal *et al.* 2012). Moreover, recent evidence reveals a potential correlation between GC autophagy and follicular degeneration in mice with cigarette smoke exposure (Gannon *et al.* 2012, 2013, Furlong *et al.* 2015). Melatonin, an indoleamine with antioxidant properties, has been implicated in preserving normal functions of GCs and follicles during oxidative stress (Tamura *et al.* 2009); however, the underlying mechanisms were still largely undetermined. In this study, we show for the first time that melatonin relieved oxidative damage in ovarian GCs by inhibiting JNK-mediated autophagy.

The present findings demonstrate that melatonin (1) prevents GC injury by restraining JNK activity, (2) inhibits JNK-dependent autophagy, (3) decreases autophagic GCs death by antagonizing the JNK pathway, (4) counteracts JNK-induced dissociation of BCL-2/BECN1 complex during the autophagic process and (5) provides an antioxidation-independent GC protection by targeting the JNK-autophagy signaling under oxidative stress conditions. Taken together, suppressing autophagy through the melatonin/JNK/BCL-2/BECN1 axis might constitute part of a survival mechanism for maintaining GC viability upon oxidative stimulation.

Melatonin is ubiquitously distributed in every bodily compartment including in follicular fluid. The physiological melatonin concentrations measured in follicular fluid are almost three times higher than that

measured in the blood (36.5 ± 4.8 pg/mL vs 10.0 ± 1.4 pg/mL) (Brzezinski *et al.* 1987). It is possible that melatonin may be produced by GCs (Tamura *et al.* 2009). However, it is currently believed that the bulk of melatonin detected in the ovary, and follicular fluid is derived from the circulation (Tamura *et al.* 2009). The presence of high melatonin levels in follicular fluid and the presence of melatonin receptors in GCs (Lee *et al.* 2001, Soares *et al.* 2003a) suggest a potential beneficial property of this indoleamine on ovarian function. Actually, melatonin has been implicated in preventing the pathogenesis of polycystic ovary syndrome (PCOS) and premature ovarian failure (POF) (Tamura *et al.* 2009). In contrast, low intrafollicular melatonin concentrations in patients with PCOS were associated with anovulation and poor oocyte quality (Tamura *et al.* 2009). In the present study, data obtained from cultured GCs demonstrate a potential role for melatonin in protecting against autophagic GC death upon oxidative stress. Therefore, melatonin might be beneficial for the therapy for stress-related anovulatory infertility, although more *in vivo* experiments should be performed to investigate the underlying mechanism of melatonin for maintaining the well-being of GCs and ovarian follicles in future studies.

The c-Jun N-terminal kinase (JNK) branch of the mitogen-activated protein plays a critical role in cellular responses to mitogenic stimuli and environmental stresses (Weston & Davis 2007). Persistent JNK activation by UV radiation or H₂O₂ exposure has been reported to induce cell death (Vallyathan 2002). However, there is no definite evidence about what response JNK will take when melatonin-treated ovarian GCs experience oxidative stress. To determine the mechanism by which melatonin protects GCs from oxidative injury, the effect of JNK on cell death was investigated. Here, we found that H₂O₂ incubation improved JNK activation as suggested by increased cellular conversion of NADH to NAD, which was associated with elevated ROS production upon oxidative stress (Fig. 1). In contrast, melatonin exhibited an inhibitory effect on JNK activation during oxidative stimulation (Fig. 1). The protein kinases RAC1 and MAP2K7 have been implicated in cellular responses to various types of external stimuli (Moriguchi *et al.* 1997, Ozaki *et al.* 2000). In this study, it was found that both RAC1 and MAP2K7 were activated by H₂O₂ in GCs to promote JNK activation, but all three kinases were inhibited when cells were pretreated with melatonin before H₂O₂ exposure (Fig. 2). Further experiments using antagonists against RAC1, MAP2K7 or JNK identified RAC1 as an upstream activator of MAP2K7, which in turn stimulated the activation of JNK during oxidative stress. Conversely, melatonin might repress H₂O₂-induced JNK activation through the RAC1/MAP2K7 pathway (Fig. 2). We also tested the possible regulation of melatonin on JNK activity by examining the direct action of melatonin as an antioxidant to scavenge free radicals (Supplementary Fig. 3) or indirect action of melatonin

to bind its receptors (Supplementary Fig. 2). However, no definitive evidence was observed for the involvement of either the direct or indirect actions of melatonin in preventing oxidative stress-triggered JNK activation. Therefore, RAC1/MAP2K7 signaling might be one of the major pathways required for melatonin-mediated inhibition of JNK activation upon oxidative stimulation. We then determined whether JNK exerts any influence on the prosurvival effects of melatonin under oxidative stress conditions. Notably, JNK serves as a critical mediator of H_2O_2 -induced GC death, since the decline in cell viability was reversed in the presence of JNK inhibitor SP600125 (Fig. 1). Importantly, melatonin exhibited an inhibitory effect on both JNK activity and GC death; however, melatonin provided no additional protection to GCs when JNK was blocked (Fig. 1). Collectively, these data first demonstrate that JNK suppression is required for melatonin-mediated GC protection.

As a stress-activated protein kinase, JNK has been linked to autophagy induction upon oxidative stimulation (Zhou *et al.* 2015). Earlier evidence from neuronal cells suggested that ROS accumulation resulting from neurotoxin treatment led to JNK-induced activation of autophagy (Rodriguez-Blanco *et al.* 2012), whereas inhibitors of JNK and its downstream substrates antagonized the autophagic response in the hippocampus of mice (Borsello *et al.* 2003). Oxidative stress has been shown to induce autophagy in ovarian GCs (Shen *et al.* 2016, 2017), but it remains elusive whether JNK plays a role in this process. On the other hand, growing evidence in recent years indicates a potential function of melatonin in autophagy regulation during oxidative stress (Coto-Montes *et al.* 2012). Particularly, melatonin may serve primarily as a mitigator when autophagy is over-stimulated (Coto-Montes *et al.* 2012). The current study showed that both JNK inhibitor SP600125 and melatonin inhibited the autophagic flux in H_2O_2 -incubated GCs, but SP600125 could not further repress autophagy when cells were pretreated with melatonin. Thus, melatonin may suppress oxidative stress-induced autophagy in GCs through a JNK-dependent manner.

Autophagy is a process of catabolism that controls cell homeostasis. Basal level of autophagy acts as a cytoprotective mechanism through orderly clearance and recycling of dysfunctional cytoplasmic components, but autophagy may also culminate into a cell death modality when the intracellular constituents are excessively degraded (Tsujimoto & Shimizu 2005, Levine & Kroemer 2008). Research on acute toxic neuronal death showed that autophagic PCD (programmed cell death) induced by N-methyl-D-aspartate treatment was accompanied with JNK pathway activation, c-Jun-selective phosphorylation and c-Fos-specific upregulation in hippocampal neurons. By contrast, the induction of autophagy was markedly repressed by JNK antagonist, indicating that the autophagic death of neuronal cells observed here was regulated by JNK

pathway (Borsello *et al.* 2003). Considering the role of melatonin in preventing JNK-dependent autophagy as mentioned earlier, we examined the interplay between autophagy, JNK and cell death in H_2O_2 -treated GCs following melatonin administration. To our knowledge, this is the first evidence demonstrating that the melatonin-mediated JNK suppression prevents a detrimental form of autophagy in GCs during oxidative stress.

There have been multiple lines of evidence suggesting that the anti-apoptotic protein BCL-2 negatively regulates autophagy by blocking BECN1, an essential component of the autophagy initiation complex (Patingre *et al.* 2009). BECN1 serves to recruit autophagy/endosomal regulatory proteins, such as PIK3C3, UVRAG and ATG14, all of which are required for autophagosome biogenesis (Levine & Yuan 2005). BECN1 also binds to BCL-2 family members (Erlich *et al.* 2007). This binding decreases the association of PIK3C3 with BECN1, and thereby inhibiting the autophagic process (Maiuri *et al.* 2007). Here, we found that autophagy is correlated with the BCL-2/BECN1 system in GCs suffering oxidative stress. H_2O_2 incubation upregulated BECN1 expression and increased the level of free form BECN1, which had been detached from BCL-2/BECN1 complex. In contrast, JNK inhibitor (SP600125) enhanced the interaction between BCL-2 and BECN1. The results are supported by a previous report (Park *et al.* 2009), which showed that the elevated expression of BECN1 and its dissociation from BCL-2 are responsible for JNK-mediated autophagic cell death. Notably, melatonin also enhanced the interaction between BCL-2 and BECN1, but SP600125 did not further restore the formation of BCL-2/BECN1 complex in the presence of melatonin. These data suggest that BCL-2/BECN1 disassembly occurs in oxidative stress-induced GC injury, whereas melatonin attenuates this process via suppressing JNK. However, the exact downstream signaling of melatonin-JNK pathway in preventing oxidative injury via autophagy is yet to be investigated in GCs.

Since the BCL-2/BECN1 complex regulates both apoptosis and autophagy by modulating of the counterbalance between cell survival and cell death (Patingre *et al.* 2005), a possible interaction between apoptotic cell death and autophagic PCD might exist in melatonin-mediated GC protection upon oxidative stimulation. However, our earlier reports showed that no evident apoptotic signals occur in the early stage of oxidative stress-induced GCs damage (Shen *et al.* 2016, 2017). Further investigations revealed that autophagic PCD represents a major form of GC death, which is independent of apoptosis in GCs suffering short-term (2 h of H_2O_2 exposure) oxidative stress (Shen *et al.* 2016, 2017). Therefore, apoptosis may not be active in our current model. Nevertheless, this situation enables us to specify the effects of autophagic PCD without the interference from apoptosis.

In summary, our findings showed excessive activation of autophagy induced by oxidative stress through the JNK

pathway in GCs. The detrimental effects of autophagic induction could be counteracted by melatonin-mediated JNK suppression, which protected GCs from oxidative damage. Moreover, we demonstrated a potential role for melatonin in protecting against autophagic GC death via the JNK/BCL-2/BECN1 signaling axis. Therefore, targeting this pathway using small molecular inhibitors may provide novel therapy for stress-related anovulatory infertility.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0002>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Natural Science Foundation of China (No. 31601939), the Fundamental Research Funds for the Central Universities (No. KJQN201705), Natural Science Foundation of Jiangsu Province (No. BK20150664), Key Program of National Natural Science Foundation of China (No. 31630072), the key Project of Chinese National Programs for Fundamental Research and Development (973 program no. 2014CB138502), China Postdoctoral Science Special Foundation (No. 2016T90476), China Postdoctoral Science Foundation (No. 2015M581818) and Postdoctoral Fund in Jiangsu Province (No. 1501047A).

Author contribution statement

M S conceived the project. M S designed the study. M S and H L L supervised the entire research. M S and Y C performed the experiments. Y J contributed to some technical supports. M S, H L L and S-C S discussed the study. M S contributed to data analysis, figure preparation and manuscript writing. All the authors reviewed the manuscript.

Acknowledgments

The authors thank Prof. Jiyong Zhou (Zhejiang University) for provision of the green fluorescent protein (GFP)-tagged microtubule-associated protein 1 light chain 3 beta (MAP1LC3B) expression plasmid. Co-authors: Yan Cao, Yi Jiang, Shao-chen Sun.

References

Agarwal A, Aponte-Mellado A, Premkumar BJ, Shaman A & Gupta S 2012 The effects of oxidative stress on female reproduction: a

- review. *Reproductive Biology and Endocrinology* **10** 49. (<https://doi.org/10.1186/1477-7827-10-49>)
- Birben E, Sahiner UM, Sackesen C, Erzurum S & Kalayci O 2012 Oxidative stress and antioxidant defense. *World Allergy Organization Journal* **5** 9–19. (<https://doi.org/10.1097/WOX.0b013e3182439613>)
- Borsello T, Croquelois K, Hornung JP & Clarke PG 2003 N-methyl-D-aspartate-triggered neuronal death in organotypic hippocampal cultures is endocytic, autophagic and mediated by the c-Jun N-terminal kinase pathway. *European Journal of Neuroscience* **18** 473–485. (<https://doi.org/10.1046/j.1460-9568.2003.02757.x>)
- Brzezinski A, Seibel MM, Lynch HJ, Deng MH & Wurtman RJ 1987 Melatonin in human preovulatory follicular fluid. *Journal of Clinical Endocrinology and Metabolism* **64** 865–867. (<https://doi.org/10.1210/jcem-64-4-865>)
- Coto-Montes A, Boga JA, Rosales-Corral S, Fuentes-Broto L, Tan DX & Reiter RJ 2012 Role of melatonin in the regulation of autophagy and mitophagy: a review. *Molecular and Cellular Endocrinology* **361** 12–23. (<https://doi.org/10.1016/j.mce.2012.04.009>)
- Duerschmidt N, Zabirnyk O, Nowicki M, Ricken A, Hmeidani FA, Blumenauer V, Borlak J & Spaniel-Borowski K 2006 Lectin-like oxidized low-density lipoprotein receptor-1-mediated autophagy in human granulosa cells as an alternative of programmed cell death. *Endocrinology* **147** 3851–3860. (<https://doi.org/10.1210/en.2006-0088>)
- Erllich S, Mizrachy L, Segev O, Lindenboim L, Zmira O, Adi-Harel S, Hirsch JA, Stein R & Pinkas-Kramarski R 2007 Differential interactions between Beclin 1 and Bcl-2 family members. *Autophagy* **3** 561–568. (<https://doi.org/10.4161/auto.4713>)
- Furlong HC, Stampfli MR, Gannon AM & Foster WG 2015 Cigarette smoke exposure triggers the autophagic cascade via activation of the AMPK pathway in mice. *Biology of Reproduction* **93** 93. (<https://doi.org/10.1095/biolreprod.115.132183>)
- Gannon AM, Stampfli MR & Foster WG 2012 Cigarette smoke exposure leads to follicle loss via an alternative ovarian cell death pathway in a mouse model. *Toxicological Sciences* **125** 274–284. (<https://doi.org/10.1093/toxsci/kfr279>)
- Gannon AM, Stampfli MR & Foster WG 2013 Cigarette smoke exposure elicits increased autophagy and dysregulation of mitochondrial dynamics in murine granulosa cells. *Biology of Reproduction* **88** 63. (<https://doi.org/10.1095/biolreprod.112.106617>)
- Gupta RK, Miller KP, Babus JK & Flaws JA 2006 Methoxychlor inhibits growth and induces atresia of antral follicles through an oxidative stress pathway. *Toxicological Sciences* **93** 382–389. (<https://doi.org/10.1093/toxsci/kfl052>)
- Gustafsson AB & Gottlieb RA 2009 Autophagy in ischemic heart disease. *Circulation Research* **104** 150–158. (<https://doi.org/10.1161/CIRCRESAHA.108.187427>)
- Kaipia A & Hsueh AJ 1997 Regulation of ovarian follicle atresia. *Annual Review of Physiology* **59** 349–363. (<https://doi.org/10.1146/annurev.physiol.59.1.349>)
- Khalidy H, Escames G, Leon J, Bikjdaouene L & Acuna-Castroviejo D 2003 Synergistic effects of melatonin and deprenyl against MPTP-induced mitochondrial damage and DA depletion. *Neurobiology of Aging* **24** 491–500. ([https://doi.org/10.1016/S0197-4580\(02\)00133-1](https://doi.org/10.1016/S0197-4580(02)00133-1))
- Klionsky DJ, Abdelmohsen K, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM, Adams PD, Adeli K *et al.* 2016 Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* **12** 1–222. (<https://doi.org/10.1080/15548627.2015.1100356>)
- Kroemer G & Levine B 2008 Autophagic cell death: the story of a misnomer. *Nature Reviews Molecular Cell Biology* **9** 1004–1010. (<https://doi.org/10.1038/nrm2529>)
- Lee CJ, Do BR, Lee YH, Park JH, Kim SJ, Kim JK, Roh SI, Yoon YD & Yoon HS 2001 Ovarian expression of melatonin Mel(1a) receptor mRNA during mouse development. *Molecular Reproduction and Development* **59** 126–132. (<https://doi.org/10.1002/mrd.1015>)
- Levine B & Klionsky DJ 2004 Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Developmental Cell* **6** 463–477. ([https://doi.org/10.1016/S1534-5807\(04\)00099-1](https://doi.org/10.1016/S1534-5807(04)00099-1))
- Levine B & Kroemer G 2008 Autophagy in the pathogenesis of disease. *Cell* **132** 27–42. (<https://doi.org/10.1016/j.cell.2007.12.018>)

- Levine B & Yuan J 2005 Autophagy in cell death: an innocent convict? *Journal of Clinical Investigation* **115** 2679–2688. (<https://doi.org/10.1172/JCI26390>)
- Liang R, Nickkholgh A, Hoffmann K, Kern M, Schneider H, Sobirey M, Zorn M, Buchler MW & Schemmer P 2009 Melatonin protects from hepatic reperfusion injury through inhibition of IKK and JNK pathways and modification of cell proliferation. *Journal of Pineal Research* **46** 8–14. (<https://doi.org/10.1111/j.1600-079X.2008.00596.x>)
- Maiuri MC, Le Toumelin G, Criollo A, Rain JC, Gautier F, Juin P, Tasdemir E, Pierron G, Troulinaki K, Tavernarakis N *et al.* 2007 Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. *EMBO Journal* **26** 2527–2539. (<https://doi.org/10.1038/sj.emboj.7601689>)
- Manchester LC, Coto-Montes A, Boga JA, Andersen LP, Zhou Z, Galano A, Vriend J, Tan DX & Reiter RJ 2015 Melatonin: an ancient molecule that makes oxygen metabolically tolerable. *Journal of Pineal Research* **59** 403–419. (<https://doi.org/10.1111/jpi.12267>)
- Mehrpour M, Esclatine A, Beau I & Codogno P 2010 Overview of macroautophagy regulation in mammalian cells. *Cell Research* **20** 748–762. (<https://doi.org/10.1038/cr.2010.82>)
- Moriguchi T, Toyoshima F, Masuyama N, Hanafusa H, Gotoh Y & Nishida E 1997 A novel SAPK/JNK kinase, MKK7, stimulated by TNF α and cellular stresses. *EMBO Journal* **16** 7045–7053. (<https://doi.org/10.1093/emboj/16.23.7045>)
- Murdoch WJ 1998 Inhibition by oestradiol of oxidative stress-induced apoptosis in pig ovarian tissues. *Journal of Reproduction and Fertility* **114** 127–130. (<https://doi.org/10.1530/jrf.0.1140127>)
- Nishida K, Kyo S, Yamaguchi O, Sadoshima J & Otsu K 2009 The role of autophagy in the heart. *Cell Death and Differentiation* **16** 31–38. (<https://doi.org/10.1038/cdd.2008.163>)
- Ozaki M, Deshpande SS, Angkeow P, Suzuki S & Irani K 2000 Rac1 regulates stress-induced, redox-dependent heat shock factor activation. *Journal of Biological Chemistry* **275** 35377–35383. (<https://doi.org/10.1074/jbc.M005287200>)
- Park KJ, Lee SH, Lee CH, Jang JY, Chung J, Kwon MH & Kim YS 2009 Upregulation of Beclin-1 expression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death. *Biochemical and Biophysical Research Communications* **382** 726–729. (<https://doi.org/10.1016/j.bbrc.2009.03.095>)
- Pattfingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD & Levine B 2005 Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell* **122** 927–939. (<https://doi.org/10.1016/j.cell.2005.07.002>)
- Pattfingre S, Bauvy C, Carpentier S, Levade T, Levine B & Codogno P 2009 Role of JNK1-dependent Bcl-2 phosphorylation in ceramide-induced macroautophagy. *Journal of Biological Chemistry* **284** 2719–2728. (<https://doi.org/10.1074/jbc.M805920200>)
- Reiter RJ, Mayo JC, Tan DX, Sainz RM, Alatorre-Jimenez M & Qin L 2016 Melatonin as an antioxidant: under promises but over delivers. *Journal of Pineal Research* **61** 253–278. (<https://doi.org/10.1111/jpi.12360>)
- Rodriguez-Blanco J, Martin V, Garcia-Santos G, Herrera F, Casado-Zapico S, Antolin I & Rodriguez C 2012 Cooperative action of JNK and AKT/mTOR in 1-methyl-4-phenylpyridinium-induced autophagy of neuronal PC12 cells. *Journal of Neuroscience Research* **90** 1850–1860. (<https://doi.org/10.1002/jnr.23066>)
- Serke H, Vilser C, Nowicki M, Hmeidan FA, Blumenauer V, Hummitzsch K, Losche A & Spanel-Borowski K 2009 Granulosa cell subtypes respond by autophagy or cell death to oxLDL-dependent activation of the oxidized lipoprotein receptor 1 and toll-like 4 receptor. *Autophagy* **5** 991–1003. (<https://doi.org/10.4161/auto.5.7.9507>)
- Shen M, Lin F, Zhang J, Tang Y, Chen WK & Liu H 2012 Involvement of the up-regulated FoxO1 expression in follicular granulosa cell apoptosis induced by oxidative stress. *Journal of Biological Chemistry* **287** 25727–25740. (<https://doi.org/10.1074/jbc.M112.349902>)
- Shen M, Liu Z, Li B, Teng Y, Zhang J, Tang Y, Sun SC & Liu H 2014 Involvement of FoxO1 in the effects of follicle-stimulating hormone on inhibition of apoptosis in mouse granulosa cells. *Cell Death and Disease* **5** e1475. (<https://doi.org/10.1038/cddis.2014.400>)
- Shen M, Jiang Y, Guan Z, Cao Y, Sun SC & Liu H 2016 FSH protects mouse granulosa cells from oxidative damage by repressing mitophagy. *Scientific Reports* **6** 38090. (<https://doi.org/10.1038/srep38090>)
- Shen M, Jiang Y, Guan Z, Cao Y, Li L, Liu H & Sun SC 2017 Protective mechanism of FSH against oxidative damage in mouse ovarian granulosa cells by repressing autophagy. *Autophagy* **13** 1364–1385. (<https://doi.org/10.1080/15548627.2017.1327941>)
- Soares JM Jr, Masana MI, Ersahin C & Dubocovich ML 2003a Functional melatonin receptors in rat ovaries at various stages of the estrous cycle. *Journal of Pharmacology and Experimental Therapeutics* **306** 694–702. (<https://doi.org/10.1124/jpet.103.049916>)
- Soares JM Jr, Simoes MJ, Oshima CT, Mora OA, De Lima GR & Baracat EC 2003b Pinelectomy changes rat ovarian interstitial cell morphology and decreases progesterone receptor expression. *Gynecological Endocrinology* **17** 115–123. (<https://doi.org/10.1080/gye.17.2.115.123>)
- Sui X, Chen R, Wang Z, Huang Z, Kong N, Zhang M, Han W, Lou F, Yang J, Zhang Q *et al.* 2013 Autophagy and chemotherapy resistance: a promising therapeutic target for cancer treatment. *Cell Death and Disease* **4** e838. (<https://doi.org/10.1038/cddis.2013.350>)
- Tamura H, Nakamura Y, Korkmaz A, Manchester LC, Tan DX, Sugino N & Reiter RJ 2009 Melatonin and the ovary: physiological and pathophysiological implications. *Fertility and Sterility* **92** 328–343. (<https://doi.org/10.1016/j.fertnstert.2008.05.016>)
- Tamura H, Takasaki A, Taketani T, Tanabe M, Kizuka F, Lee L, Tamura I, Maekawa R, Asada H, Yamagata Y *et al.* 2013 Melatonin as a free radical scavenger in the ovarian follicle. *Endocrine Journal* **60** 1–13. (<https://doi.org/10.1507/endocrj.EJ12-0263>)
- Tilly JL & Tilly KI 1995 Inhibitors of oxidative stress mimic the ability of follicle-stimulating hormone to suppress apoptosis in cultured rat ovarian follicles. *Endocrinology* **136** 242–252. (<https://doi.org/10.1210/endo.136.1.7828537>)
- Tsujimoto Y & Shimizu S 2005 Another way to die: autophagic programmed cell death. *Cell Death and Differentiation* **12** (Supplement 2) 1528–1534. (<https://doi.org/10.1038/sj.cdd.4401777>)
- Vallyathan V 2002 Third International Conference on Oxygen/Nitrogen Radicals: cell injury and disease. Morgantown, West Virginia, June 1–5, 2002. *Antioxidants and Redox Signaling* **4** 957–960. (<https://doi.org/10.1089/152308602762197498>)
- Vega-Naredo I, Caballero B, Sierra V, Garcia-Macia M, de Gonzalo-Calvo D, Oliveira PJ, Rodriguez-Colunga MJ & Coto-Montes A 2012 Melatonin modulates autophagy through a redox-mediated action in female Syrian hamster Harderian gland controlling cell types and gland activity. *Journal of Pineal Research* **52** 80–92. (<https://doi.org/10.1111/j.1600-079X.2011.00922.x>)
- Wei Y, Sinha S & Levine B 2008 Dual role of JNK1-mediated phosphorylation of Bcl-2 in autophagy and apoptosis regulation. *Autophagy* **4** 949–951. (<https://doi.org/10.4161/auto.6788>)
- Weng Q, Liu Z, Li B, Liu K, Wu W & Liu H 2016 Oxidative stress induces mouse follicular granulosa cells apoptosis via JNK/FoxO1 pathway. *PLoS ONE* **11** e0167869. (<https://doi.org/10.1371/journal.pone.0167869>)
- Weston CR & Davis RJ 2007 The JNK signal transduction pathway. *Current Opinion in Cell Biology* **19** 142–149. (<https://doi.org/10.1016/j.ceb.2007.02.001>)
- Zhou YY, Li Y, Jiang WQ & Zhou LF 2015 MAPK/JNK signalling: a potential autophagy regulation pathway. *Bioscience Reports* **35** e00199. (<https://doi.org/10.1042/BSR20150167>)

Received 21 October 2017

First decision 6 November 2017

Revised manuscript received 20 January 2018

Accepted 23 January 2018