Redox Biology 18 (2018) 138–157

Contents lists available at ScienceDirect

Redox Biology

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

Melatonin protects mouse granulosa cells against oxidative damage by inhibiting FOXO1-mediated autophagy: Implication of an antioxidation-independent mechanism

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ARTICLE INFO

Keywords:
Melatonin
Autophagic death
FOXO1
Granulosa cells
Oxidative damage
Antioxidation-independent

ABSTRACT

Oxidative stress has been described as a prime driver of granulosa cell (GCs) death during follicular atresia. Increasing evidence suggests potential roles of melatonin in protecting GCs from oxidative injury, though the underlying mechanisms remain largely undetermined. Here we first proposed that the inhibition of autophagy through some novel regulators contributes to melatonin-mediated GCs survival under conditions of oxidative stress. Oxidant-induced loss of GCs viability was significantly reduced after melatonin administration, which was correlated with attenuated autophagic signals upon oxidative stimulation both in vivo and in vitro. Compared with melatonin treatment, suppression of autophagy displayed similar preventive effect on GCs death during oxidative stress, but melatonin provided no additional protection in GCs pretreated with autophagy inhibitors. Notably, we found that melatonin-directed regulation of autophagic death was independent of its antioxidation/radical scavenging ability. Further investigations identified FOXO1 as a critical downstream effector of melatonin in promoting GCs survival from oxidative stress-induced autophagy. Specifically, suppression of FOXO1 via the melatonin-phosphatidylinositol 3-kinase (PI3K)-AKT axis not only improved GCs resistance to oxidative stress, but also abolished the autophagic response, from genes expression to the formation of autophagic vacuoles. Moreover, the activation of SIRT1 signaling was required for melatonin-mediated deacetylation of FOXO1 and its interaction with ATG proteins, as well as the inhibition of autophagic death in GCs suffering oxidative stress. These findings reveal a brand new mechanism of melatonin in defense against oxidative damage to GCs by repressing FOXO1, which may be a potential therapeutic target for anovulatory disorders.

1. Introduction

In mammalian ovaries, more than 99% of the follicles are destroyed through a process known as atresia [1]. Previous studies suggested that granulosa cell (GCs) death dominates the progression of atretic degeneration [2]. Reactive oxygen species (ROS) are natural and unavoidable by-products of aerobic metabolism, but unlimited ROS generation gives rise to oxidative stress [3]. During follicular development, increased ROS levels are associated with accelerated metabolic rates in rapidly proliferating GCs [4]. ROS accumulation induces oxidative damage of ovarian GCs, hence setting the atretic program in motion, leading to pathogenesis of anovulatory disorders, such as polycystic ovary syndrome (PCOS) and premature ovarian failure (POF) [5]. Thus, elucidating the preventive mechanisms against oxidative stress-triggered GC death may provide plausible treatment strategies for reproductive failure caused by aberrant follicular atresia.

Abbreviations: Ac, acetylated; ADA, the 3 AKT phosphorylation sites of FOXO1 are mutated; AKT, thymoma viral proto-oncogene; ATG, autophagy-related; AVOs, acidic vesicular organelles; AOI, antioxidant inhibitors; BECN1, beclin 1; BP, blank plasmid; CAT, catalase; CCK-8, Cell Counting Kit-8; DBD, a FOXO1 mutant without DNA-binding activity, $FOXO1^{N208A,H212R}$; EP300, E1A binding protein p300; FOXO1, forkhead box O1; GCs, granulosa cell; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; MAP1LC3B, microtubule associated protein 1 light chain 3 beta; Mel, melatonin; MTOR, mechanistic target of rapamycin (serine/threonine kinase); NPC, non-plasmid control; 3-NP, 3-nitropropionic acid; PCOS, polycystic ovary syndrome; PCD, programmed cell death; P.E, pepstatin A and E64; PIK3C3, phosphoinositide-3-kinase, class 3; PMSG, pregnant mare serum gonadotropin; PI3K, class I phosphoinositide 3-kinase; POF, premature ovarian failure; ROS, reactive oxygen species; SOD, superoxide dismutase; SQSTM1, sequestosome 1; T-AOC, total antioxidation capability; TEM, transmission electron microscopy; WCL, whole-cell lysates; 3-MA, 3-methyladenine

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https://doi.org/10.1016/j.redox.2018.07.004
Received 4 June 2018; Received in revised form 24 June 2018; Accepted 6 July 2018
Available online 07 July 2018

Autophagy, a highly conserved self-renewal process in eukaryotic cells, is characterized by the engulfment of cytoplasmic materials into double-membrane vesicles (autophagosomes) for subsequent degradation in lysosomes [6]. Generally, the autophagic machinery is necessary for the clearance of dysfunctional proteins and organelles [7]. However, under certain stressful conditions, autophagy is overstimulated to the extent that essential components for cell survival are digested [8]. For example, mammalian cells with normal ROS production usually display basal autophagic activity, whereas excessive autophagy has been suggested to induce self-destruction of cells suffering oxidative injury [9]. Recent observations from rodent ovaries showed elevated autophagic signals in GCs during follicular atresia [10,11]. These findings were further confirmed by several reports which revealed a close correlation between oxidized low-density lipoprotein (oxLDL)-induced autophagy and GC death [12–14]. Notably, obese women with high levels of oxLDL exhibited ROS accumulation in the ovary, along with increased incidence of anovulatory infertility [12]. In fact, our earlier study has demonstrated a critical role of autophagy in promoting GC death upon oxidative stress [15]. Therefore, the discovery and identification of an antioxidant by targeting autophagy may provide benefits to GC survival against oxidative injury.

N-acetyl-5-methoxytryptamine (melatonin), an indoleamine originally discovered to be secreted by the pineal gland, performs versatile functions in regulating circadian rhythms, immune response, inflammation, carcinogenesis, and ROS scavenging [16,17]. Recent evidence indicated potential effects of melatonin on autophagy suppression through its antioxidant properties [18,19]. Further researches suggested that melatonin-mediated inhibition of autophagic death improves cellular resistance to noxious stimuli [20,21]. Melatonin is ubiquitously distributed in every bodily compartment including in follicular fluid where its concentration is significantly higher than that in blood (36.5 ± 4.8 pg/ml vs. 10.0 ± 1.4 pg/ml) [22]. Correspondingly, the expression of melatonin receptors is detectable throughout the ovary [23,24], but melatonin-binding sites have been observed more frequently in the granulosa layers of antral follicles [25]. Increased levels of follicular melatonin might protect GCs from free radical cytotoxicity, and thus maintaining the growth and development of healthy follicles [26]. In contrast, blocking melatonin production via pinealectomy accelerated the atretic process in mammalian ovaries [27]. Moreover, the reduction of follicular melatonin concentrations has been reported to induce anovulation in patients with PCOS [26]. However, few further clues exist regarding the role of autophagy in melatonin-mediated GC protection during oxidative stress.

FOXO1/FKHR is a pleiotropic transcription factor that modulates diverse cellular and physiological processes including proliferation, metabolism, differentiation, cell cycle, cell death, stress response and longevity [28–32]. The specific functions of FOXO1 are controlled by post-translational modifications (phosphorylation, acetylation, ubiquitination, and methylation), which in turn regulates its subcellular localization, protein-protein interactions, DNA-binding properties, protein stability and transcriptional activity in response to a wide range of external stimuli, such as growth factors, hormones, nutrients, cytokines and oxidative stress [33,34]. Recently, evidence has emerged regarding the novel roles of FOXO1 in autophagy regulation upon stressful conditions, not only because FOXO1 promotes the expression of several autophagy-related genes, but also FOXO1 post-translational modifications are required for triggering the autophagic process [35–37]. Indeed, FOXO1 has been identified as a key inductor of autophagic death in GCs with oxidative damage [15]. Considering the capability of melatonin to inhibit autophagy upon oxidative stress [18,19], we wonder whether melatonin-induced GC survival is correlated with the downregulation of FOXO1-dependent autophagy.

The present study suggested a primary role for autophagy suppression rather than antioxidation in melatonin-mediated GC protection through coordinating the PI3K-AKT-FOXO1 signaling cascades and SIRT1-FOXO1-ATG7 pathway. Our findings may provide new insights into the defense mechanisms of melatonin against oxidative injury.

2. Materials and methods

2.1. Reagents and antibodies

PBS (20012) was purchased from Gibco (Grand Island, NY, USA). Pregnant mare serum gonadotropin (PMSG) was purchased from Ningbo Second Hormone Factory (Ningbo, Zhejiang, China). Melatonin (S1204), pepstatin A (S7381), E64 (S7379), LY294002 (S1105), perifosine (S1037), Sirtinol (S2804), SRT1720 (S1129), 3-methyladenine (3-MA; S2767), and Z-VAD-FMK (S7023) were from Selleck Chemicals (Houston, TX, USA). 3-nitropropionic acid (3-NP; N5636), H_2O_2 (216763-100 ml), Tiron (89460), anti-MAP1LC3B (L7543) and anti-TUBA1A (T5168) were bought from Sigma-Aldrich (St. Louis, MO, USA). L-Buthionine sulfoximine (sc-200824), 3-Amino-1,2,4-triazole (sc-202016), diethyldithiocarbamic acid sodium salt trihydrate (sc-202576), mercaptosuccinic acid (sc-250305), carmustine (sc-204671), Luzindole (sc-202700), anti-SIRT1 (sc-74465), anti-EP300 (sc-585) and acetylated FOXO1 antibody (sc-49437) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Plasmids for FLAG-tagged FOXO1, including FOXO1-WT (Addgene, 12148), FOXO1-ADA/$FOXO1^{T24A,\ S253D,S316A}$ (Addgene, 12149), and FOXO1-DBD/$FOXO1^{N208A,H212R}$ (Addgene, 17555) were kindly contributed from Prof. Domenico Accili (Columbia University Medical Center). Antibodies against AKT (9272), phospho-AKT (4060), FOXO1 (2880), phospho-FOXO1 (9461), FLAG (2908), BECN1 (3495), MTOR (2983), ATG3 (3415), ATG5 (8540), ATG7 (2631), and ATG12 (4180) were obtained from Cell Signaling Technology (Beverly, MA, USA). SQSTM1 antibody (ab56416) was purchased from Abcam (Cambridge, MA, USA).

2.2. Animals and ethics

All mice procedures were performed 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 group-housed in a temperature-controlled (22 ± 2 ℃) room with a 12/12 h light/dark cycle (lights on from 7:00 a.m. to 7:00 p.m.), and had ad libitum access to water and food. Mice were divided into Control group, melatonin group, 3-NP group, and melatonin + 3-NP group. Melatonin and 3-NP were dissolved in 0.9% saline containing 0.5% ethanol; 0.5% ethanol saline (v/v) is regarded as vehicle. Female ICR mice were injected intraperitoneally with vehicle or melatonin (15 mg/kg) at 8:00 a.m. once a day for 2 consecutive days, followed by 5 days of oxidative stimulation in ovarian GCs using a well-established in vivo model [38]. Briefly, from day 3 to day 7, mice received an additional intraperitoneal injection of 3-NP (50 mg/kg) or 0.5% ethanol saline at 8:00 p.m. each day, along with the daily administration of melatonin (15 mg/kg) or vehicle at 8:00 a.m. 24 h after the final injection, ovaries were collected for subsequent immunohistochemical staining, western blotting analysis or CCK-8 assay. The protocols of all animal experiments were approved by the Committee of Animal Research Institute, Nanjing Agricultural University, China.

2.3. Cell culture and treatments

Primary GCs were isolated from ovarian follicles and cultured as described previously [15,38–40]. For drug administration, GCs pretreated with melatonin (10 μM) for 24 h were washed in PBS, and incubated with medium containing 200 μM H_2O_2 for 0, 1, or 2 h as indicated. In some experiments, GCs were treated with pepstatin A (10 μg/ml), E64 (10 μg/ml), LY294002 (20 μM), perifosine (10 μM), Tiron (10 mM), Sirtinol (100 μM), SRT1720 (100 μM), 3-MA (10 mM), or Z-VAD-FMK (50 μM) 1 h before H_2O_2 exposure. For the suppression