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YY1/HIF-1α/NDUFA4L2 signaling axis mediates bisphenol A-induced mitochondrial dysfunction and apoptosis in human ovarian granulosa cells

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Abstract

Bisphenol A (BPA) is implicated in impairing ovarian function by disrupting the normal function of granulosa cells (GCs); however, the underlying molecular mechanisms remain unclear. Based on our previous results, this study used KGN cells as a model and combined functional assays with mechanistic analyses to investigate the role of the YY1/HIF-1α/NDUFA4L2 signaling axis in BPA-induced granulosa cell injury. The results showed that BPA treatment upregulated YY1, HIF-1α, and NDUFA4L2 expression, accompanied by decreased ATP levels, suppressed mitochondrial complex I activity, accumulation of mitochondrial reactive oxygen species, and increased apoptosis. Further analyses demonstrated that YY1 knockdown markedly attenuated BPA-induced mitochondrial dysfunction, oxidative stress, and apoptosis, while also suppressing HIF-1α and NDUFA4L2 expression. Mechanistically, YY1 promoted NDUFA4L2 expression mainly by enhancing HIF-1α protein stability. YY1 knockdown alleviated BPA-induced cellular injury, whereas restoration of HIF-1α substantially weakened this protective effect, and further silencing of NDUFA4L2 mitigated the injury phenotype again. Taken together, these findings indicate that BPA induces mitochondrial bioenergetic impairment, oxidative stress, and apoptosis in granulosa cells through activation of the YY1/HIF-1α/NDUFA4L2 signaling axis, and further suggest that YY1 serves as an important upstream regulatory node linking environmental exposure to mitochondrial injury.

Keywords: Bisphenol A; Granulosa cells; YY1; HIF-1α; NDUFA4L2; Mitochondrial dysfunction

Introduction

Bisphenol A (BPA) is an endocrine-disrupting chemical (EDC) widely present in plastic products and the environment, and chronic exposure to BPA can adversely affect the female reproductive system[1-3]. This is especially concerning at environmentally relevant concentrations because certain occupational groups, such as female cashiers

exposed to thermal receipt paper, have BPA exposure levels that are significantly higher than those of the general population[4]. Ovarian granulosa cells (GCs) are key somatic cells that support follicular development, steroid hormone synthesis, and oocyte maturation, and impairment of their function can directly lead to abnormal folliculogenesis and ovarian dysfunction[5-8]. Our previous studies have shown that BPA exposure is associated with poorer early outcomes of in vitro fertilization (IVF), including reduced oocyte retrieval rate, maturation rate, and embryo implantation rate[9], and can also induce autophagy, apoptosis, and disordered hormone secretion in granulosa cells both in vivo and in vitro[9], which are consistent with other studies [10-12]. In addition, BPA can directly disrupt granulosa cell function and alter estradiol and progesterone secretion[12, 13]. Using rat granulosa cells, Lee et al. reported that even a sublethal concentration (10 μM) of BPA can interfere with endocrine activity by affecting mitochondrial function[14]. These findings suggest that BPA-induced ovarian toxicity is not limited to a single phenotypic change, but may instead involve a deeper mechanistic basis characterized by disruption of mitochondrial homeostasis and reprogramming of cell fate.

Against this background, it is important to clarify the upstream regulatory factors that drive BPA-associated mitochondrial injury. Yin Yang 1 (YY1) is a multifunctional transcriptional regulator, and recent studies have shown that it can enhance hypoxia-inducible factor 1α (HIF-1α) stability and inhibit its degradation under hypoxia-related conditions, thereby amplifying downstream signaling responses[15]. On the other hand, HIF-1α can promote the transcription of NDUFA4L2 (NADH dehydrogenase (ubiquinone)-1α subcomplex 4-like 2)[16]. As a complex I-associated regulatory molecule, NDUFA4L2 can restrain complex I activity and regulate mitochondrial reactive oxygen species (mitoROS) generation, thereby influencing mitochondrial metabolic status and oxidative stress levels[17, 18].Although these lines of evidence were obtained from different disease models, together they form a coherent mechanistic cascade in which YY1 stabilizes HIF-1α upstream, HIF-1α subsequently transcriptionally activates NDUFA4L2, and NDUFA4L2 in turn modulates

mitochondrial respiration and oxidative stress. Combined with our previous findings on BPA-induced granulosa cell injury[9], it is reasonable to speculate that the YY1/HIF-1α/NDUFA4L2 axis may represent an important molecular pathway linking environmental exposure to mitochondrial dysfunction in granulosa cells and may therefore have potential therapeutic relevance.

Based on this rationale, the present study used the human ovarian granulosa cell line KGN as a model to investigate mitochondrial bioenergetic alterations, oxidative stress, and apoptosis following BPA exposure. By integrating YY1 knockdown, protein stability analysis, dual-luciferase reporter assays, and rescue experiments, we systematically examined the regulatory relationship within the YY1/HIF-1α/NDUFA4L2 axis and its functional significance in BPA-induced granulosa cell injury. Through this study, we aimed to elucidate the molecular basis of BPA-induced ovarian toxicity from the perspective of mitochondrial stress regulation and to provide a new theoretical basis for targeted intervention in environmentally related female reproductive injury.

Materials and methods

Cell culture

KGN cells were cultured in KGN cell-specific medium (Procell, CM-0603) supplemented with fetal bovine serum (VISTECH, SE100-011) at 37°C in a humidified incubator with 5% CO_2. Cells were routinely passaged using trypsin-EDTA and collected for subsequent assays.

ATP assay

Intracellular ATP levels were measured using an ATP chemiluminescence assay kit (ELabscience, E-BC-F002) according to the manufacturer's instructions. Briefly, collected cells were lysed at a ratio of 2×10^6 cells in 0.3 mL extraction reagent in a 2 mL tube. After mixing thoroughly, the samples were incubated in boiling water for 10

min, cooled under running water, and centrifuged at 10000 × g for 10 min at 4°C using a refrigerated microcentrifuge (Cylotech, CF1524R). The supernatants were collected for ATP measurement. For detection, 100 μL enzyme working solution was added to both standard and sample wells and allowed to stand for 5 min. Subsequently, 100 μL serially diluted standards were added to the standard wells, whereas 100 μL sample supernatant was added to the remaining wells and mixed immediately. Luminescence was recorded using a chemiluminescence detector.

Apoptosis analysis

Cell apoptosis was determined using an Annexin V apoptosis detection kit (Elabscience, E-CK-A217) followed by flow cytometry. Briefly, cells in the logarithmic growth phase were digested, resuspended in complete medium, and counted. Approximately 1×10^6 cells were resuspended in 500 μL 1×binding buffer and equally divided into four tubes, including an unstained control, Annexin V single-stained tube, PI single-stained tube, and Annexin V/PI double-stained tube. Annexin V and PI were added according to the experimental design. After gentle mixing, the cells were incubated for 15 min at room temperature in the dark. Then, 200 μL 1× binding buffer was added to each tube. Before flow cytometric analysis, stained cell suspensions were filtered through a 400-mesh cell strainer to obtain single-cell suspensions. The percentages of apoptotic cells were subsequently quantified.

MitoSOX Red staining

Mitochondrial superoxide levels were detected using a mitochondrial superoxide detection kit (Beyotime, S0061S). Cells were seeded into 6-well plates at a density of 2×10^5 cells per well in 2 mL medium one day before staining. The working solution was freshly prepared according to the manufacturer's instructions. After removal of the culture medium, cells were incubated with 1 mL staining solution at 37°C for 30 min. The staining solution was then discarded, and cells were washed twice with PBS. Fluorescence images were acquired using a laser confocal microscope with excitation at 396 nm and emission at 610 nm.

Measurement of mitochondrial complex I activity

Mitochondrial complex I activity was determined using a mitochondrial respiratory chain complex I/NADH-CoQ reductase activity assay kit (Solarbio, BC0515) according to the manufacturer's instructions. Prior to measurement, the spectrophotometer or microplate reader (Thermo, MK3) was prewarmed for at least 30 min and set to 340 nm. Reagent I was preincubated at 37°C for 15 min. For each reaction in a 96-well plate, 10 μL sample, 154 μL reagent I, 20 μL working solution, and 16 μL reagent IV were added and mixed thoroughly. The absorbance at 340 nm was first recorded at 10 s as A1. The reaction mixture was then incubated at 37°C for 1 min, and the absorbance was measured again at 1 min 10 s as A2. The change in absorbance was calculated as $\Delta A = A1 - A2$. Complex I activity was calculated using the following formula: complex I activity ($U/10^6$ cells) = $3215.43 \times \Delta A / N$, where N represents the cell number in units of 10^6. One unit of enzyme activity was defined as the consumption of 1 nmol NADH per min per 10^6 cells in the reaction system.

Construction of YY1-knockdown cells

YY1-knockdown stable cell lines were established by lentiviral transduction. KGN cells were seeded into 6-well plates one day before infection. Lentiviruses carrying shRNAs targeting YY1 (sh-YY1) or the negative control (sh-NC) were added to the cells according to the experimental design. After infection, cells were further cultured under routine conditions, and stable YY1-knockdown cells were obtained by puromycin (MCE, HY-B1743) selection. Knockdown efficiency was verified by quantitative real-time PCR (qRT-PCR) and Western blot analysis. The sh-YY1 and sh-NC sequences are listed in Table S1.

Small interfering RNA (siRNA) transfection

KGN cells were seeded into 6-well plates at a density of 2×10^5 cells per well one day before transfection. siRNAs targeting NDUFA4L2 (si-NDUFA4L2) or the negative control (si-NC) were transfected using Lipofectamine 2000 (Invitrogen, 11668030) according to the manufacturer's instructions. Cells were collected at the indicated time

points for subsequent analyses. The si-NDUFA4L2 and si-NC sequences are listed in Table S2.

qRT-PCR analysis

Total RNA was extracted using RNAiso Plus (Takara, 9109). First-strand cDNA was synthesized using the Hifair II 1st Strand cDNA Synthesis Kit (Yeasen, 11119ES60). qRT-PCR was performed using Hieff qPCR SYBR Green Master Mix (Yeasen, 11202ES08) under standard cycling conditions of 95°C for 5 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. Melting curve analysis was subsequently performed. The primer sequences used for qRT-PCR are listed in Table S3.

Western blot analysis

Total protein was extracted using Western and IP lysis buffer (Beyotime, P0013) supplemented with protease inhibitor (Biosharp, BL612A) and phosphatase inhibitor mixture A (Beyotime, P1082). Protein concentration was determined using a BCA protein assay kit (UElandy, B6169). Equal amounts of protein were separated by SDS PAGE using precast FastPAGE gels (Tsingke, TSP024) and then transferred onto PVDF membranes. After blocking with 5% nonfat milk, the membranes were incubated overnight at 4°C with primary antibodies against YY1 (Proteintech, 22156-1-AP, 1:1500), HIF-1α (Proteintech, 20960-1-AP, 1:2000), NDUFA4L2 (Proteintech, 16480-1-AP, 1:2000), and β-actin (Proteintech, 20536-1-AP, 1:4000). The membranes were then incubated with the corresponding secondary antibodies for 1 h at room temperature. Protein bands were visualized using ECL substrate (Biosharp, BL520B).

Cycloheximide (CHX) chase assay

Protein stability was assessed by CHX chase assay. Cells were seeded into 12-well plates at a density of 5×10^5 cells per well in 1 mL medium one day before treatment. Cells were treated with 10 nM BPA (MCE, HY-18260) for 48 h, after which the medium was replaced with fresh complete medium containing 100 μg/mL CHX (MCE, HY-12320) and 10 nM BPA. Cells were harvested at 0, 30, 60, 90, and 120 min after

CHX treatment. Total protein was extracted at each time point, and HIF-1α protein levels were analyzed by Western blot.

Dual-luciferase reporter assay

The promoter region of NDUFA4L2 was cloned into the pGL3 basic vector to generate the wild type reporter plasmid, and a mutant reporter plasmid was generated by site directed mutagenesis of the predicted HIF-1α binding site, in which ACGTG was replaced with AAAAA. KGN cells were seeded into 96 well plates and co transfected with the reporter plasmids and HIF-1α expression plasmid or empty vector using Lipofectamine 2000. At 48 h after transfection, firefly and Renilla luciferase activities were measured sequentially according to the manufacturer's instructions. Relative luciferase activity was normalized to Renilla luciferase activity. The sequence of the 2000 bp region upstream of the transcription start site of NDUFA4L2 used for promoter analysis is provided in File S1.

Statistical analysis

Statistical analyses were conducted in GraphPad Prism (v10.4.1). Data are reported as mean ± SEM. Multiple-group comparisons were assessed with one-way or two-way ANOVA, as appropriate. A value of $P < 0.05$ was considered statistically significant.Statistical significance was defined as $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), and $P < 0.0001$ (****). All in vitro experiments included at least three biological replicates.

Results

BPA induces mitochondrial dysfunction and apoptosis

To investigate the effects of BPA on human ovarian granulosa cells (KGN), we first examined mitochondrial function and apoptosis after BPA treatment. As shown in Figure 1A, intracellular ATP levels decreased significantly in a time-dependent manner after 24, 48, and 72 h of BPA treatment. Mitochondrial oxidative stress was assessed

by MitoSOX Red staining, and the results showed that fluorescence intensity was significantly increased in the BPA-treated group (Figure 1B). Meanwhile, the activity of mitochondrial complex I was markedly reduced with prolonged treatment time (Figure 1C). Flow cytometric analysis of apoptosis revealed that the apoptotic rate began to increase after 24 h of BPA treatment, and the proportion of apoptotic cells reached its highest level at 72 h (Figure 1D). These results indicate that BPA induces mitochondrial dysfunction and promotes apoptosis in KGN cells.

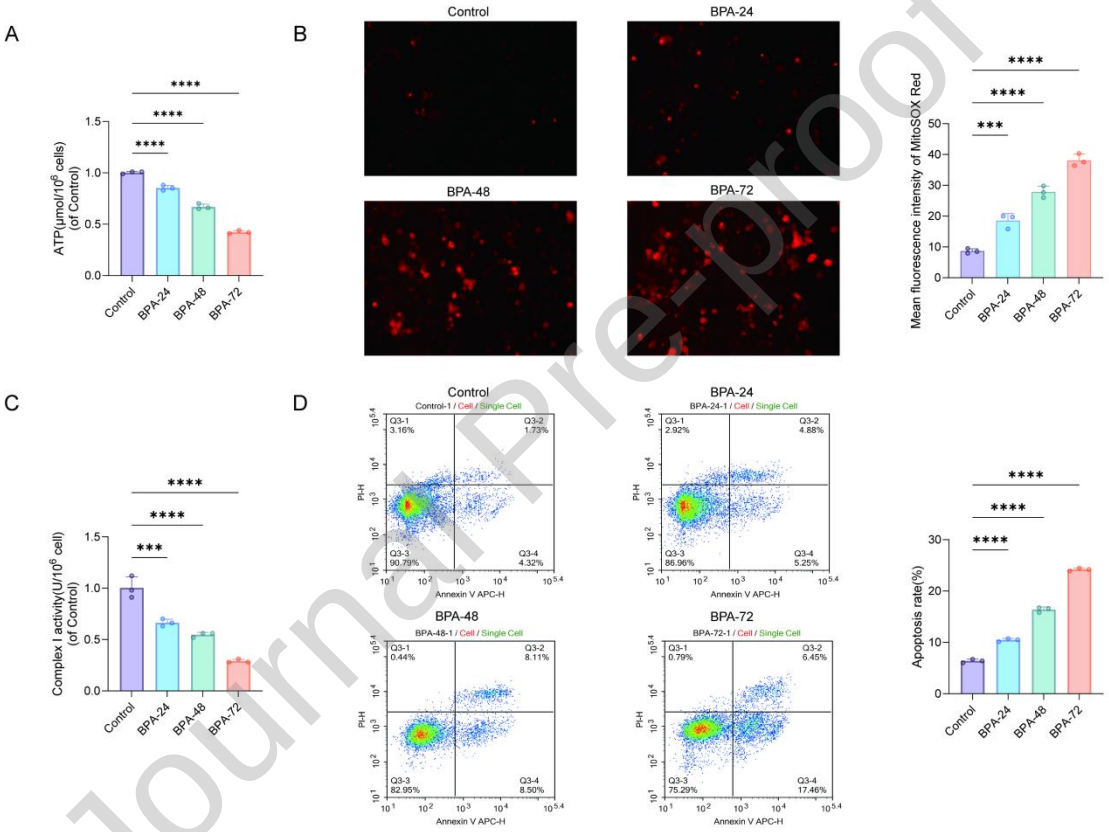

Figure 1. BPA induces mitochondrial dysfunction and apoptosis in KGN cells.

(A) ATP levels in KGN cells treated with 10 nM BPA for 0, 24, 48, and 72 h, measured using a luciferase-based assay.(B) MitoSOX Red staining showing mitochondrial reactive oxygen species levels in KGN cells after BPA treatment. Left, representative confocal images; right, quantification of fluorescence intensity. (C) Mitochondrial complex I activity in KGN cells after BPA treatment, determined using an NADH:ubiquinone oxidoreductase activity assay. (D) Apoptosis in KGN cells after BPA treatment, assessed by Annexin V-APC/PI staining followed by flow cytometry. Left, representative flow cytometric plots; right, statistical analysis. Data are from three

independent experiments (n=3).

BPA activates the YY1/HIF-1α/NDUFA4L2 signaling axis

To further investigate the potential molecular mechanism by which BPA affects mitochondrial function and apoptosis, we reviewed the literature and found that the transcription factor YY1 can stabilize HIF-1α protein by inhibiting proteasomal degradation[15]. Activated HIF-1α, in turn, promotes the transcription of NDUFA4L2, which functions as a negative regulator of mitochondrial complex I and can thereby affect mitochondrial function[17]. Based on these findings, we hypothesized that the YY1/HIF-1α/NDUFA4L2 signaling axis may be involved in the effects of BPA on KGN cells.

To test this hypothesis, we first examined the effect of BPA treatment on the expression of proteins in this signaling axis. Western blot analysis showed that the protein levels of YY1, HIF-1α, and NDUFA4L2 were all increased in a time-dependent manner after BPA treatment (Figure 2A). To further clarify the regulatory relationship among these three factors, we established a stable YY1-knockdown KGN cell line (sh-YY1), and the knockdown efficiency was confirmed by qRT-PCR and Western blot (Figure 2B). In sh-YY1 cells, the BPA-induced upregulation of HIF-1α and NDUFA4L2 proteins was significantly suppressed (Figure 2C), suggesting that YY1 acts upstream in this signaling axis.

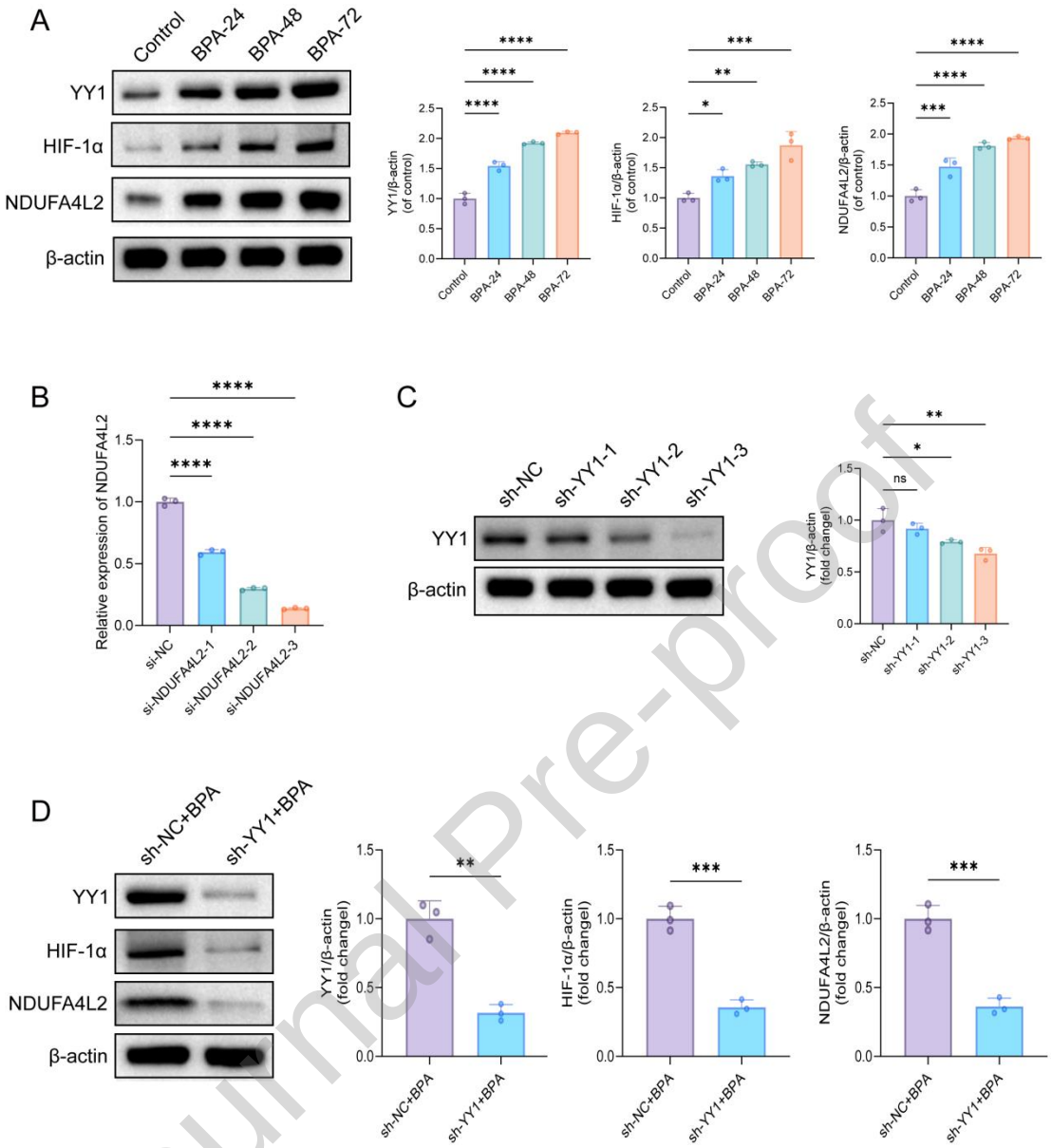

Figure 2. BPA activates the YY1/HIF-1α/NDUFA4L2 signaling axis.

(A) Western blot analysis of YY1, HIF-1α, and NDUFA4L2 protein levels in KGN cells after BPA treatment for the indicated times. Quantification of band intensity is shown on the right. (B) YY1 mRNA knockdown efficiency in stable sh-NC and sh-YY1 KGN cells, as determined by qRT-PCR.(C) YY1 protein knockdown efficiency in stable sh-NC and sh-YY1 KGN cells, as determined by Western blot. Quantification of band intensity is shown on the right.(D) Western blot analysis of HIF-1α and NDUFA4L2 protein expression in stable sh-NC and sh-YY1 KGN cells after BPA treatment for 48 h. Quantification of band intensity is shown on the right. All Western

blots were repeated three times (n=3). qRT-PCR data represent mean ± SEM from three independent experiments.

YY1 stabilizes HIF-1α and promotes NDUFA4L2 transcription

To clarify the mechanism by which YY1 regulates HIF-1α, CHX chase assays were performed to determine the half-life of HIF-1α protein. Compared with control cells, stable YY1-knockdown cells exhibited a markedly accelerated degradation rate and a shortened half-life of HIF-1α protein (Figure 3A), indicating that YY1 stabilizes HIF-1α protein. We next examined the effects of YY1 knockdown on the transcriptional levels of related genes by qRT-PCR. YY1 mRNA levels were significantly reduced in stable YY1-knockdown cells, confirming the knockdown efficiency, whereas no significant difference in HIF-1α mRNA levels was observed between the two groups. When combined with the CHX chase results, these findings indicate that YY1 regulates HIF-1α mainly at the level of protein stability rather than transcription. In contrast, NDUFA4L2 mRNA levels were significantly decreased after YY1 knockdown, suggesting that NDUFA4L2 expression may be regulated by upstream YY1 signaling (Figure 3B). To further determine whether HIF-1α directly regulates NDUFA4L2 transcription, luciferase reporter plasmids containing either the wild-type NDUFA4L2 promoter or a mutant promoter with a disrupted HIF-1α-binding site were generated. Dual-luciferase reporter assays showed that HIF-1α overexpression significantly increased the activity of the wild-type promoter, but had no effect on the mutant promoter (Figure 3C), indicating that HIF-1α directly activates NDUFA4L2 transcription by binding to its promoter.

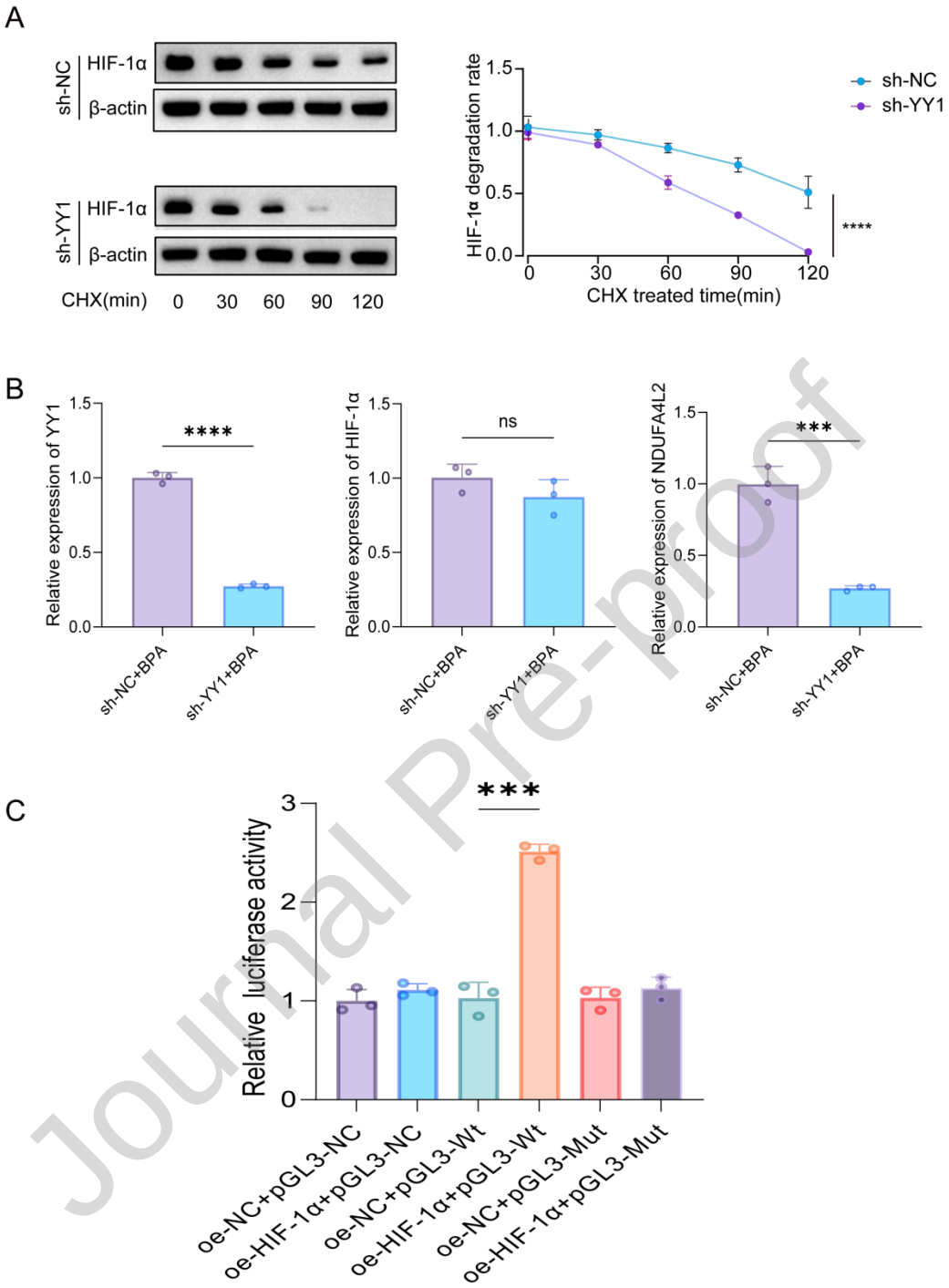

Figure 3. YY1 stabilizes HIF-1α and promotes NDUFA4L2 transcription.

(A) CHX chase assay. sh-NC or sh-YY1 KGN cells were treated with 100 μg/mL CHX for the indicated times, and HIF-1α protein levels were analyzed by Western blot. Left, representative immunoblots; right, protein degradation curves. (B) qRT-PCR analysis of YY1, HIF-1α, and NDUFA4L2 mRNA levels in sh-NC and sh-YY1 cells after BPA treatment for 48 h. (C) Dual-luciferase reporter assay. KGN cells were co-transfected

with luciferase reporter plasmids containing the Wt or Mut NDUFA4L2 promoter, together with an HIF-1α overexpression plasmid or control vector, followed by measurement of relative luciferase activity. Data are from three independent experiments (n=3).

YY1 regulates NDUFA4L2 expression and mitochondrial bioenergetics through HIF-1α

To determine whether YY1 regulates NDUFA4L2 through HIF-1α, rescue experiments were performed in stable YY1-knockdown cells. Under BPA treatment, YY1 knockdown reduced the protein levels of HIF-1α and NDUFA4L2. MG-132 treatment restored HIF-1α expression and concomitantly increased NDUFA4L2 expression. Further silencing of NDUFA4L2 suppressed its expression again without affecting HIF-1α levels, indicating that YY1 regulates NDUFA4L2 expression by stabilizing HIF-1α rather than by directly targeting NDUFA4L2 (Figure 4A).

We then examined the effects of this axis on mitochondrial bioenergetic function. BPA treatment decreased ATP levels, whereas YY1 knockdown restored them. MG-132 treatment in YY1-knockdown cells reduced ATP levels again, while further silencing of NDUFA4L2 restored them once more (Figure 4B). A similar trend was observed for complex I activity. BPA treatment suppressed complex I activity, YY1 knockdown reversed this effect, MG-132 weakened the protective effect of YY1 knockdown, and further silencing of NDUFA4L2 restored complex I activity again (Figure 4C). Collectively, these findings demonstrate that YY1 regulates NDUFA4L2 through HIF-1α and thereby mediates BPA-induced mitochondrial bioenergetic impairment.

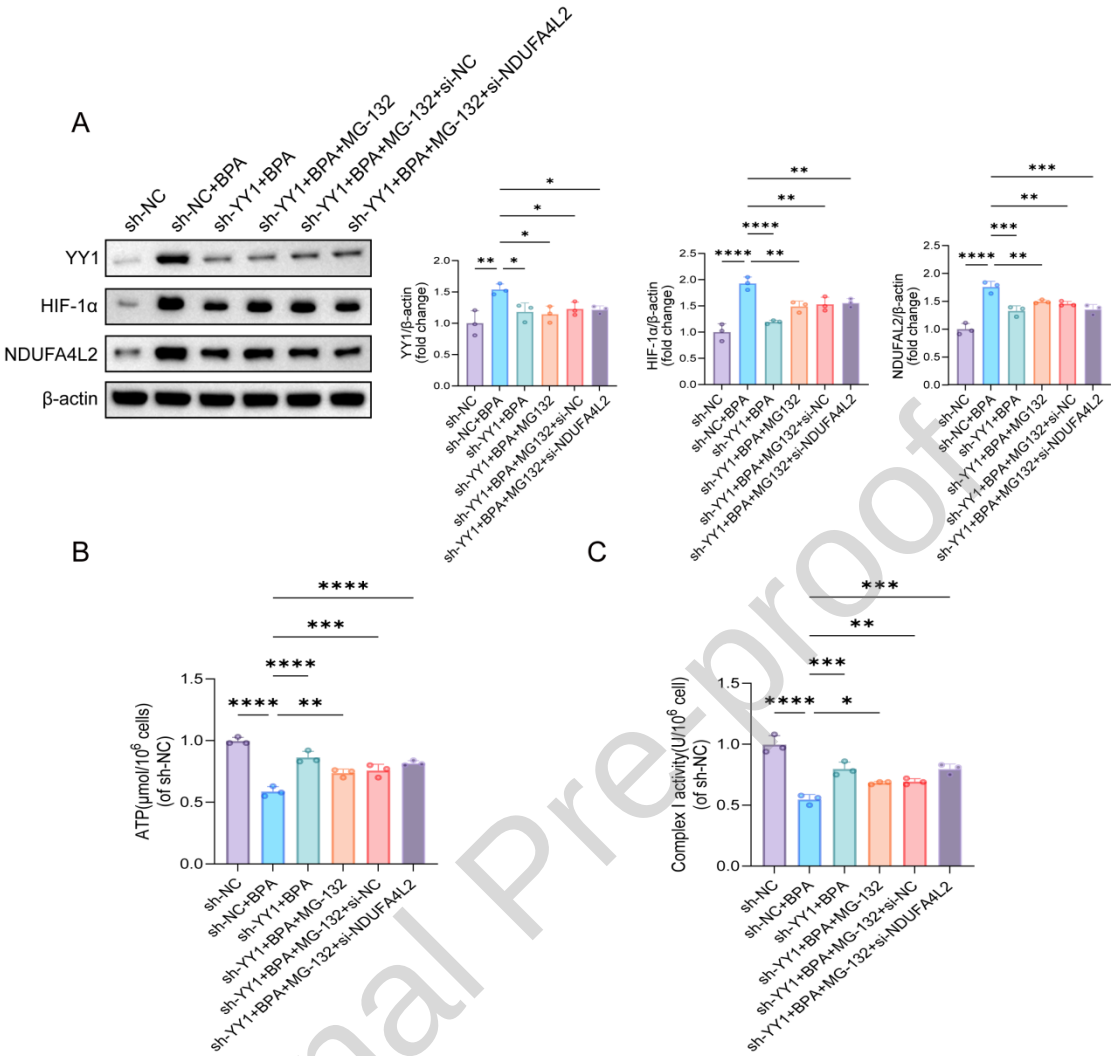

Figure 4. YY1 regulates NDUFA4L2 expression and mitochondrial bioenergetics through HIF-1α.

(A) Western blot analysis of HIF-1α and NDUFA4L2 protein expression in KGN cells under the indicated treatment conditions. Quantification of band intensity is shown on the right. (B) ATP levels measured under the same treatment conditions. (C) Mitochondrial complex I activity measured under the same treatment conditions. All experiments were performed three times (n=3). Data are mean ± SEM.

YY1/HIF-1α/NDUFA4L2 axis mediates BPA-induced apoptosis and oxidative stress

Finally, we assessed whether this signaling axis is functionally required for BPA-induced mitochondrial oxidative stress and apoptosis. As shown in Figure 5A, BPA treatment markedly increased mitoROS levels. This effect was reversed in YY1-knockdown cells, whereas MG-132 treatment restored mitoROS levels. Further silencing of NDUFA4L2 again reduced oxidative stress. A similar pattern was observed for apoptosis (Figure 5B). The increase in apoptosis induced by BPA was suppressed by YY1 knockdown, restored by MG-132 treatment, and attenuated again by si-NDUFA4L2, indicating that NDUFA4L2 is required for the pro-apoptotic effect downstream of HIF-1α. Together, these findings demonstrate that YY1 mediates BPA-induced mitochondrial oxidative stress and apoptosis through the HIF-1α/NDUFA4L2 axis.

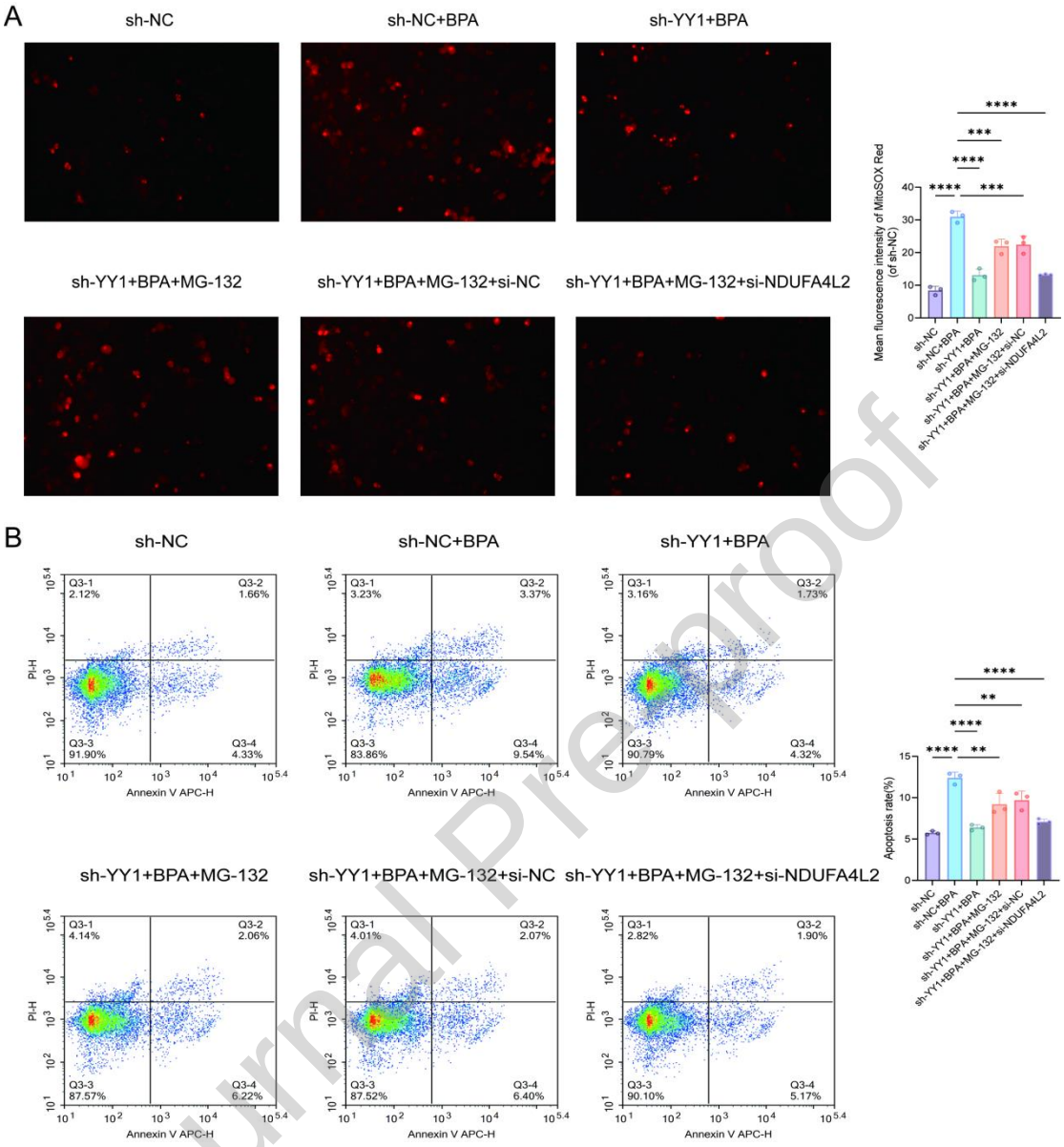

Figure 5. The YY1/HIF-1α/NDUFA4L2 axis mediates BPA-induced mitochondrial oxidative stress and apoptosis.

(A) Representative confocal microscopy images of KGN cells stained with mitoSOX under the indicated treatment conditions, showing mitoROS levels. Quantification of mitoROS fluorescence intensity is shown on the right. (B) Representative flow cytometric scatter plots of KGN cells stained with Annexin V-APC/PI under the indicated treatment conditions for apoptosis analysis. Quantification of apoptotic rates is shown on the right. Representative images and plots from three independent experiments (n=3) are shown.

Discussion

Although BPA-related ovarian toxicity has been extensively studied, previous work has largely focused on phenotypes such as oxidative stress[19], abnormal steroidogenesis[12], and enhanced apoptosis in granulosa cells[20], while a more integrated mechanistic explanation of how these alterations are regulated upstream and subsequently translated into mitochondrial dysfunction remains lacking.The emphasis of the present study was therefore not to simply reiterate that BPA can damage granulosa cells, but rather to propose and validate a relatively well-defined molecular pathway underlying this process. We found that BPA treatment reduced ATP levels in KGN cells, suppressed mitochondrial complex I activity, promoted mitoROS accumulation, and increased apoptosis, while simultaneously upregulating YY1, HIF-1α, and NDUFA4L2. More importantly, through YY1 knockdown, CHX chase assays, dual-luciferase reporter assays, and MG-132 rescue experiments, we further established and verified a mechanistic cascade in which YY1 enhances HIF-1α protein stability, thereby promoting NDUFA4L2 expression and subsequently mediating mitochondrial bioenergetic impairment and cellular injury. These findings indicate that BPA-induced granulosa cell injury is not an isolated oxidative stress event, but rather a process of mitochondrial metabolic imbalance driven by a specific transcriptional regulatory network, which represents the major innovation of this study. This finding has important practical implications. It suggests that targeting the YY1/HIF-1α/NDUFA4L2 signaling axis could enable effective intervention in female ovarian function impaired by environmental chemical exposure. This axis not only explains how environmental toxicants disrupt granulosa cell mitochondrial function at the molecular level, but also provides a novel and actionable therapeutic target for the clinical management of female reproductive disorders closely linked to environmental exposure, including polycystic ovary syndrome (PCOS), premature ovarian insufficiency, and infertility.

We first demonstrated that BPA treatment induced marked mitochondrial bioenergetic dysfunction in KGN cells, as reflected by reduced ATP production and

decreased complex I activity, accompanied by elevated mitoROS levels and increased apoptosis. Previous studies have shown that BPA and its analogs can induce mitochondrial damage, ROS accumulation, and apoptosis in granulosa cells, suggesting that mitochondrial abnormalities are a key basis of BPA-induced reproductive toxicity[19, 21].Building on this evidence, the study further narrowed the focus to dysfunction of complex I, suggesting that BPA does not merely trigger a nonspecific stress response, but instead causes a metabolic crisis centered on disruption of the electron transport chain and insufficient energy supply. For granulosa cells, which play a critical role in supporting follicular development, such changes are particularly likely to directly compromise cell survival and the stability of the local follicular microenvironment. The 10 nM BPA concentration used in this study was selected based on our previous work[9], in which this concentration reproducibly induced cellular phenotypes in KGN cells without immediate cytotoxicity. Importantly, this concentration is also environmentally relevant. Ikezuki et al. measured BPA levels in human follicular fluid from healthy women and found an average concentration of 2.4 ± 0.8 ng/mL[22], which corresponds to approximately 10.5 nM. Thus, the 10 nM dose reflects actual human exposure levels and is appropriate for mechanistic studies of BPA-induced granulosa cell injury.

Compared with previous studies, a more important advance of the present work lies in the clarification of the upstream regulatory logic responsible for BPA-induced mitochondrial injury. Previous studies have shown that BPA-induced granulosa cell injury is closely associated with disruption of mitochondrial homeostasis. For example, BPA can promote apoptosis in KGN cells by activating the apoptosis signal-regulating kinase 1 (ASK1)/c-Jun JNK pathway through G protein-coupled estrogen receptor (GPER)-dependent ROS and Ca^{2+} signaling[23].Consistently, improvement of peroxisome proliferator-activated receptor gamma coactivator 1α (PGC1α)-related mitochondrial function can partially alleviate BPA-induced apoptosis in KGN cells[24, 25].Taken together, these studies support, from both the perspectives of injury phenotypes and intervention effects, the important role of mitochondrial dysfunction in

BPA toxicity; however, the upstream molecular drivers of this process remain to be further elucidated. In the present study, BPA treatment induced coordinated upregulation of YY1, HIF-1α, and NDUFA4L2, whereas YY1 knockdown not only suppressed HIF-1α and NDUFA4L2 expression, but also simultaneously improved ATP levels, complex I activity, mitoROS accumulation, and apoptosis. These findings indicate that YY1 is not merely a concomitant change, but rather an important upstream node linking environmental exposure to mitochondrial injury. Accordingly, the question of what type of damage BPA causes naturally progresses to the deeper question of how BPA organizes and amplifies the injury process through specific regulatory factors.

Further CHX chase and MG-132 rescue experiments suggested that YY1 regulates HIF-1α primarily at the level of protein stability. Previous studies have shown that YY1 can inhibit HIF-1α degradation and enhance its stability, a phenomenon initially described mainly in the contexts of hypoxia and tumor biology[15, 26]. The present study extends this regulatory mode to BPA-induced granulosa cell injury, suggesting that BPA exposure does not simply trigger passive oxidative insult, but may also induce an abnormal hypoxia-like stress program in which HIF-1α remains persistently stabilized under nonphysiological conditions and activates downstream metabolic targets. This point is particularly important, because it allows the present study to move beyond a descriptive account of environmental toxicant-induced injury and instead connect environmental exposure, stress-related transcriptional regulation, and metabolic remodeling within a unified mechanistic framework, thereby deepening the mechanistic insight provided.

On this basis, we further confirmed that NDUFA4L2 is an important downstream effector of this pathway. Dual-luciferase assays showed that HIF-1α directly enhanced NDUFA4L2 promoter activity, and restoration of HIF-1α led to a corresponding recovery of NDUFA4L2 expression, indicating that NDUFA4L2 acts downstream of YY1 and HIF-1α. Classical studies have established that NDUFA4L2 is one of the key HIF-1α-dependent metabolic target genes and can reduce oxygen consumption and

modulate oxidative phosphorylation by inhibiting complex I activity[27-29]. In this study, NDUFA4L2 upregulation was consistent with decreased complex I activity, reduced ATP production, and aggravated oxidative stress, suggesting that it serves as an important effector molecule in BPA-induced mitochondrial injury. Notably, NDUFA4L2 may exert context-dependent functions in different biological settings. In granulosa cells already subjected to BPA-induced toxic stress, sustained elevation of NDUFA4L2 is more likely to further suppress complex I function, impair ATP generation, and drive the cells from compensation toward decompensation. Rescue experiments support this interpretation, as restoration of HIF-1α aggravated injury after YY1 knockdown had improved mitochondrial function, whereas further targeting of NDUFA4L2 attenuated this process. Thus, this pathway is not merely a molecular signature following BPA exposure, but a true mediator of the injury output.

Overall, the key advance of this study lies in integrating environmental exposure, stress-related transcriptional regulation, and mitochondrial metabolic remodeling into a single mechanistic axis. Previous studies have already shown that BPA can induce oxidative stress and apoptosis in granulosa cells[23, 30-33]. This study further demonstrates that these phenotypes are not simply the sum of several isolated alterations, but rather constitute a continuous pathological process initiated by YY1, amplified through HIF-1α, and ultimately executed through NDUFA4L2-mediated dysfunction of mitochondrial complex I. This mechanistic framework not only expands the molecular basis of BPA-induced ovarian toxicity, but also highlights the potential importance of the YY1/HIF-1α/NDUFA4L2 axis as a key pathway in environmentally related granulosa cell injury. Nevertheless, this study has several limitations. First, the experiments were performed mainly in the KGN cell model. KGN cells are derived from an ovarian granulosa cell tumor and are widely used for mechanistic studies because they retain key features such as hormone responsiveness and steroidogenic capacity. However, unlike primary human granulosa cells which are non-proliferative and have a limited culture lifespan, KGN cells are immortalized and proliferate rapidly, but the core regulatory relationships within the YY1/HIF-1α/NDUFA4L2 axis are

cell-intrinsic and unlikely to be fundamentally altered. Nevertheless, while the KGN model is appropriate for mechanistic dissection, validation in primary granulosa cells and in vivo models remains necessary. Second, the present work did not incorporate animal models or clinical samples to evaluate the direct association of this pathway with abnormal follicular development and reproductive outcomes. Future studies validating this pathway in primary granulosa cells, animal exposure models, and clinically derived samples, together with more systematic analyses of mitochondrial metabolism, will help clarify its actual pathological significance in environmentally related ovarian injury.

In conclusion, the present study not only demonstrates that BPA induces mitochondrial dysfunction, oxidative stress, and apoptosis in granulosa cells, but also further reveals a key mechanism whereby YY1 promotes NDUFA4L2 expression by stabilizing HIF-1α, thereby suppressing complex I function and driving bioenergetic injury. This finding highlights the central role of the YY1/HIF-1α/NDUFA4L2 axis in BPA-related granulosa cell injury and provides a new entry point for understanding the molecular basis of environmentally related female reproductive toxicity.

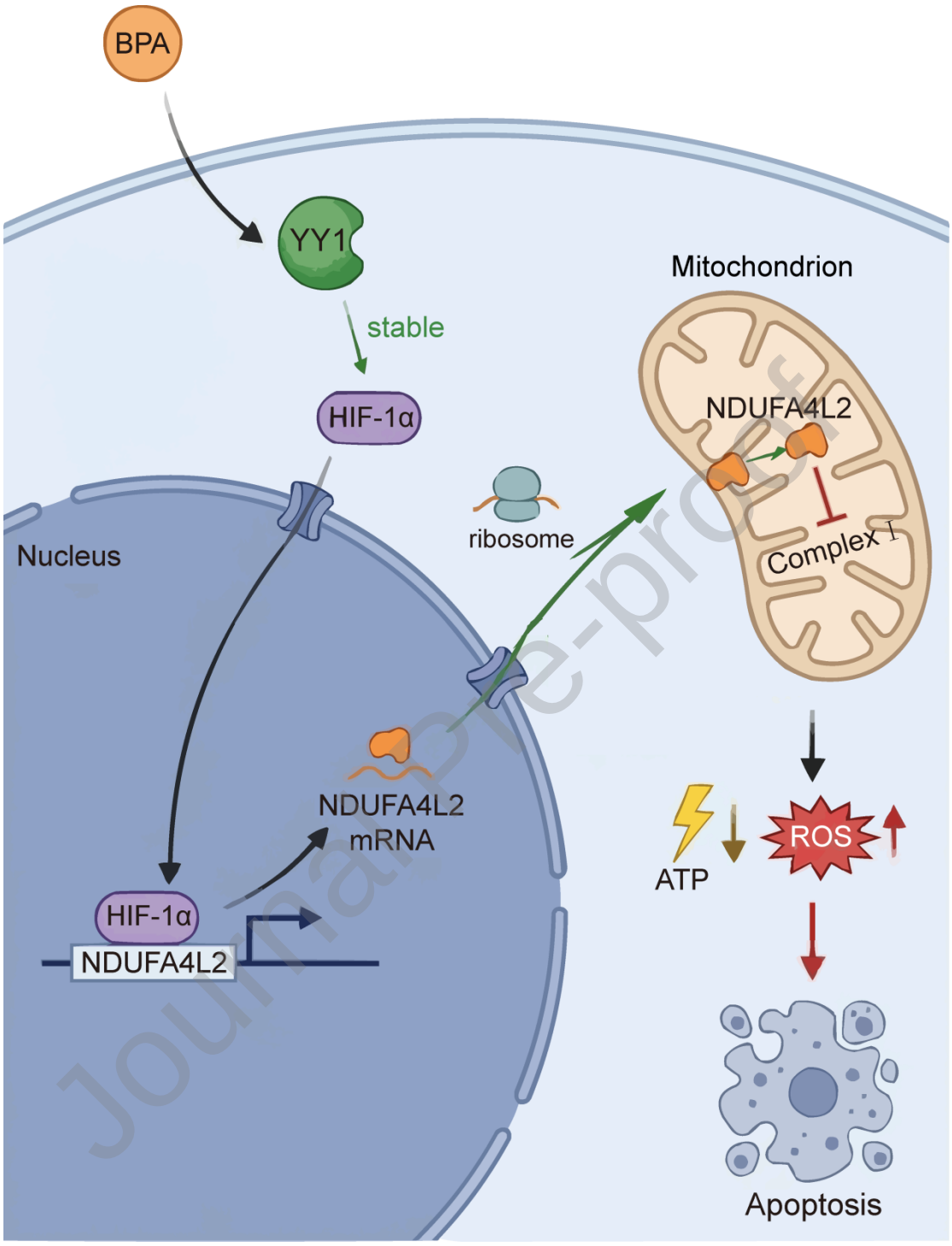

Figure 6. Schematic diagram of the YY1/HIF-1α/NDUFA4L2 signaling axis mediating BPA-induced mitochondrial dysfunction and apoptosis in granulosa cells.

BPA enters the granulosa cell and upregulates YY1 expression. YY1 enhances HIF-1α protein stability (green arrow). HIF-1α translocates to the nucleus, where it activates transcription of NDUFA4L2. Newly synthesized NDUFA4L2 mRNA is exported from the nucleus via nuclear pores and translated in the cytoplasm. The NDUFA4L2 protein

is then transported to the mitochondrial inner membrane, where it inhibits complex I activity (red "T"-shaped arrow). This leads to reduced ATP production, increased mitochondrial reactive oxygen species, and ultimately apoptosis. Upward and downward arrows indicate increases and decreases, respectively. Abbreviations: BPA, bisphenol A; YY1, Yin Yang 1; HIF-1α, hypoxia-inducible factor 1α; NDUFA4L2, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2; ROS, reactive oxygen species.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The data presented in this study are available on request from the corresponding authors.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Libin Xue: Conceptualization, Methodology, Investigation, Writing – original draft; **Cairong Chen**: Conceptualization, Investigation, Data curation, Writing – review & editing, Funding acquisition; **Yinghe Zhao**: Methodology, Investigation, Formal analysis; **Shengtao Ma**: Funding acquisition, Resources, Writing – review & editing; **Yingxin Yu**: Resources, Supervision, Writing – review & editing; **Song Quan**: Funding acquisition, Supervision, Project administration; **Rui Hua**: Conceptualization, Funding acquisition, Supervision, Project administration, Writing – review & editing. All authors contributed to manuscript revision, read, and approved the submitted version.

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Graphical abstract

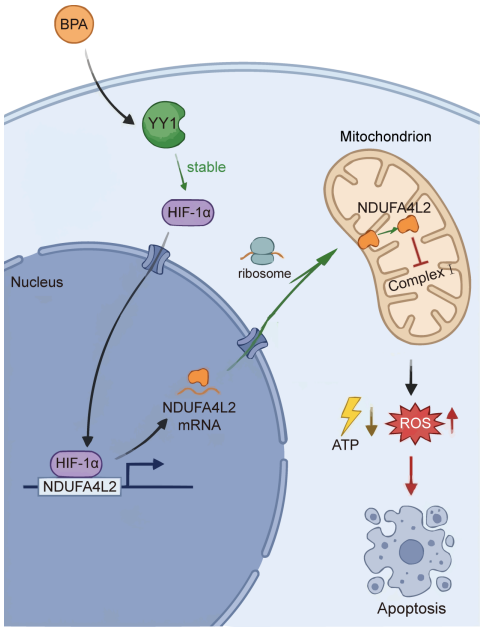

Figure. Schematic diagram of the YY1/HIF-1α/NDUFA4L2 signaling axis mediating BPA-induced mitochondrial dysfunction and apoptosis in granulosa cells.

BPA enters the granulosa cell and upregulates YY1 expression. YY1 enhances HIF-1α protein stability (green arrow). HIF-1α translocates to the nucleus, where it activates transcription of NDUFA4L2. Newly synthesized NDUFA4L2 mRNA is exported from the nucleus via nuclear pores and translated in the cytoplasm. The NDUFA4L2 protein is then transported to the mitochondrial inner membrane, where it inhibits complex I activity (red "T"-shaped arrow). This leads to reduced ATP production, increased mitochondrial reactive oxygen species, and ultimately apoptosis. Upward and downward arrows indicate increases and decreases, respectively. Abbreviations: BPA, bisphenol A; YY1, Yin Yang 1; HIF-1α, hypoxia-inducible factor 1α; NDUFA4L2, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 4-like 2; ROS, reactive oxygen species.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Highlights

- BPA activates the YY1/HIF-1α/NDUFA4L2 axis in granulosa cells.
- YY1 stabilizes HIF-1α to promote NDUFA4L2 expression.
- NDUFA4L2 inhibits complex I causing mitochondrial dysfunction.
- Targeting this axis alleviates BPA-induced apoptosis.