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Original Article

Role of the aryl hydrocarbon receptor signaling pathway in promoting mitochondrial biogenesis against oxidative damage in human melanocytes

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ABSTRACT

Background: Reactive oxygen species (ROS)-induced mitochondrial damage aggravates oxidative stress and activates mitochondrial apoptosis pathway to mediate melanocyte death. However, the repair mechanisms underlying damaged mitochondria of melanocytes remain unclear. Accumulative evidence has revealed that the aryl hydrocarbon receptor (AHR), a ligand-activated transcription factor, plays a vital role in maintaining mitochondrial homeostasis.
Objective: To investigate whether the AHR signaling pathway could protect human melanocytes from oxidative damage through controlling mitochondrial quality.
Methods: We constructed an oxidative stress model of melanocytes with hydrogen peroxide (H_2O_2) in the human normal melanocyte PIG1 cell line, and detected ROS level, apoptosis, mitochondrial ROS level, mitochondrial membrane potential, ATP production, mitochondrial DNA and mitochondrial modulators after co-treatment with AHR ligand or antagonist and H_2O_2 in the PIG1 cells.
Results: In the present study, we found that H_2O_2-induced oxidative stress directly activated the AHR signaling pathway in melanocytes, whereas abnormal activation of AHR signaling pathway enhanced oxidative damage to mitochondria and melanocytes. Further studies showed that the AHR signaling pathway promoted mitochondrial DNA synthesis and ATP production probably by regulating the expression of nuclear respiratory factor 1 (NRF1) and its downstream targets.
Conclusion: Our findings reveal that the AHR signaling pathway might have a major role in protecting melanocytes against oxidative damage via inducing mitochondrial biogenesis, while impaired AHR activation could cause defective repair of mitochondria and exacerbate oxidative damage-induced apoptosis in melanocytes. Our data suggest that the AHR signaling pathway might be a novel mechanism of mitochondrial biogenesis involved in protecting melanocytes from oxidative stress.

1. Introduction

The skin interfaces with the environment and is the first line of defense against external factors [1]. Melanocytes located in the skin epidermis are vulnerable to exogenous reactive oxygen species (ROS) (produced by UV, electromagnetic radiation, chemical pollutants, drugs and microbial pathogens) and endogenous ROS (mainly derived from energy transformation in mitochondria) [2,3]. Meanwhile, melanocytes produce melanin through a series of complex oxidation-reduction reactions, which could inevitably form hydrogen peroxide (H_2O_2) and aggravate oxidative stress in melanocytes [4]. Thus, melanocytes are susceptible to ROS and are prone to severe oxidative damage in response to oxidative stress. Excessive ROS in melanocytes could

Abbreviations: AHR, aryl hydrocarbon receptor; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species; ARNT, AHR nuclear translocator; CYP1A1, cytochrome P450 family 1, subfamily A, polypeptide 1; CYP1B1, cytochrome P450 family 1, subfamily B, polypeptide 1; PGC1α, eroxisome proliferator-activated receptor gamma coactivator 1α; NRF1, nuclear respiratory factor 1; TFAM, transcription factor A mitochondrial; CYCS, cytochrome c; PGC-1β, peroxisome proliferator-activated receptor gamma coactivator 1-beta; CYTB, cytochrome b; COX1, cytochrome c oxidase I; COX3, cytochrome c oxidase subunit 3; CREB, cAMP response element-binding protein; YY1, yin yang 1; cAMP, cyclic adenosine monophosphate; mTOR, mechanistic target of rapamycin; ACTB, beta-actin; MMP, mitochondrial membrane potential; DMSO, dimethyl sulfoxide; PBS, phosphate-bufferedsaline; ATP, adenosine triphosphate; MT-ND4, NADH-ubiquinone oxidoreductase chain 4.

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primarily attack mitochondria and cause oxidative damage [5–7]. Mitochondrial damage could directly trigger the intrinsic apoptotic pathway and eventually mediate melanocytes death [8]. Therefore, mitochondrial oxidative damage plays a central role in oxidative stress-mediated cell death [8]. Mitochondrial regeneration is critical for protecting cells against oxidative stress [9,10]. However, the mechanisms responsible for repairing damaged mitochondria of melanocytes are unknown.

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor, belonging to the family of basic helix-loop-helix transcription factors [11]. AHR is normally inactive in cytoplasm, bound to several co-chaperones [12]. Upon ligand binding, the chaperones dissociate resulting in AHR translocating into the nucleus and dimerizing with AHR nuclear translocator (ARNT), leading to changes in AHR responsive gene transcription [12]. AHR is highly expressed in the skin, involved in toxin response, adaptive and innate immunity, melanogenesis and antioxidation of skin [13,14]. Recent studies highlight major roles of AHR in maintaining mitochondrial homeostasis. AHR could reduce mitochondrial ROS, stabilize mitochondrial membrane potential, and protect human lung fibroblasts against tobacco extract-induced oxidative damage [15]. AHR could protect mitochondrial function against starvation-induced oxidative stress in mouse liver cells [16]. AHR signaling pathway could reduce PM2.5-induced mitochondrial membrane potential loss, mitochondrial ROS level, mitochondrial membrane permeability and apoptosis of lung epithelial cells [17]. These studies strongly suggest that AHR plays pivotal roles in protecting cells against oxidative stress through regulating mitochondrial homeostasis, however, the functional role of AHR in melanocytes under oxidative stress and the related specific molecular mechanisms have never been described.

In the present study, we demonstrated that the AHR signaling pathway could exert antioxidantion function in maintaining mitochondrial homeostasis to protect melanocytes from oxidative damage through modulating mitochondrial biosynthesis.

2. Materials and methods

2.1. Cell culture and reagents

Human normal melanocyte PIG1 cell line [18] was cultured in Medium 254 with Human Melanocyte Growth Supplement (Cascade Biologics/Invitrogen, Portland, OR, USA) and 5% Fetal Bovine Serum (Invitrogen, Carlsbad, CA, USA) at 37 °C in the presence of 5% CO_2. FICZ (AHR physiological ligand), CH223191 (AHR antagonist) and analytical pure grade H_2O_2 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Real-time PCR (RT-PCR) analysis

The total RNA was extracted from the PIG1 cells with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and converted to cDNA using PrimeScript™ RT Master Mix (TaKaRa, Dalian, Liaoning, China). The mRNA levels of *CYP1A1*, *CYP1B1*, *PPARGC1A*, *NRF1*, *TFAM* and *ACTB* were measured by RT-PCR using specific primers (Supplementary Table 1).

2.3. Western blot analysis

The protein expressions of CYP1A1, CYP1B1, PGC1α, NRF1 and TFAM in the PIG1 cells were examined by Western blots analysis using specific polyclonal antibodies for these proteins (Abcam, Cambridge, MA; ProteinTech Group, Chicago, IL, USA) (More details in Supplementary Data).

2.4. Immunofluorescence confocal microscopy

PIG1 cells were cultured in the 15 mm-glass bottom cell culture dishes (Nest Biotechnology, Wuxi, Jiangsu, China) for 24 h. PIG1 cells were treated with 1.0 mM H_2O_2 for 15 min, pretreated with 10 μM CH223191 for 1 h followed by stimulation with 1.0 mM H_2O_2 for 15 min, or treated with 10 nM FICZ for 15 min as positive control, respectively. More details of procedure were shown in Supplementary Data. Specific polyclonal antibodies for AHR (Abcam, Cambridge, MA) were applied to cell incubation.

2.5. Annexin V-FITC/propidium iodide apoptosis assay

PIG1 cells were treated with H_2O_2 (0.25 mM, 0.5 mM, 0.75 mM, 1.0 mM or 1.25 mM) for 24 h, or pretreated with CH223191 (10 μM, 25 μM or 50 μM) or FICZ (10 nM) for 1 h and followed by stimulation with H_2O_2 (1.0 mM) for 24 h. Apoptotic and necrotic cells were identified using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, San Diego, California, USA) following the manufacturer's instructions (More details in Supplementary Data).

2.6. Measurement of intracellular ROS

PIG1 cells were pretreated with 10 μM CH223191 or 10 nM FICZ for 1 h, followed by stimulation with 1.0 mM H_2O_2 for 24 h. The production of intracellular ROS was monitored by flow cytometry using CM-H2DCFDA (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions (More details in Supplementary Data).

2.7. Measurement of mitochondrial superoxide

PIG1 cells were pretreated with 10 μM CH223191 or 10 nM FICZ for 1 h, followed by stimulation with 1.0 mM H_2O_2 for 24 h. Mitochondrial superoxide was measured using mitoSOXred (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions (More details in Supplementary Data).

2.8. Measurement of mitochondrial membrane potential (MMP)

Melanocytes were seeded at a density 1×10^5 cells per well in 6-well plates and exposed to vehicle (0.01%, DMSO), H_2O_2 alone or cotreatment with CH223191 (10 μM) or FICZ (10 nM) for 24 h. MMP was estimated using JC-1 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions (More details in Supplementary Data).

2.9. ATP determination

Melanocytes were seeded at a density 1×10^5 cells per well in 6-well plates and exposed to vehicle (0.01%, DMSO), H_2O_2 alone or cotreatment with CH223191 (10 μM) or FICZ (10 nM) for 24 h. ATP levels were measured using ENLITEN® ATP Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions (More details in Supplementary Data).

2.10. Measurement of mitochondrial DNA content

PIG1 cells were pretreated with 10 μM CH223191 or 10 nM FICZ for 1 h, followed by stimulation with 1.0 mM H_2O_2 for 48 h. Genomic DNA was extracted using a DNA isolation kit (Taigen, Beijing, Taiwan). The mitochondrial DNA content was analyzed by RT-PCR using primers amplifying *MT-ND4* (Supplementary Table 1) normalized to *ACTB*.

2.11. In silico promoter analysis

The UCSC Genome Bioinformatics website (http://www.genome.ucsc.edu/) was used to predict the sequence of the promoter region of *PPARGC1A*, *NRF1* and *TFAM*. The CLC Main Workbench software (CLC bio, Aarhus, Denmark) was used to predict the putative AHR binding sites (core sequence: 5′-GCGTG-3′) in the promoter of *PPARGC1A*, *NRF1* and *TFAM*. The Deepview (Swiss Pdp Viewer) and Hyperchem softwares (Hypercube Inc. version 8) were used to predict the functional domains of AHR protein.

2.12. RNA interference and transfection

PIG1 cells were seeded at a density 1×10^5 cells per well in 6-well plates for 24 h and transiently transfected with siRNAs for NRF1 and negative control (Santa Cruz Biotechnology, Santa Cruz, CA, USA) following the Lipofectamine 3000 protocol (Invitrogen, Carlsbad, CA, USA). At 24 h after transfection, the cells were subjected to 1.0 mM H_2O_2 for 24 and 48 h to assess mRNA and protein changes, respectively.

2.13. Chromatin immunoprecipitation (ChIP)

PIG1 cells were treated with 1.0 mM H_2O_2 for 24 h, pretreated with 10 μM CH223191 for 1 h followed by stimulation with 1.0 mM H_2O_2 for 24 h, or treated with 10 nM FICZ for 24 h as positive control. ChIP experiments were performed using the EZ ChIPTM chromatin immunoprecipitation kit (Millipore, Billerica, MA) according to the manufacturer's protocol. Immunoprecipitation was performed with AHR antibody (ProteinTech Group, Chicago, IL, USA). Rabbit IgG (Abcam, Cambridge, MA) was used as an isotype control antibody. The purified DNA and input genomic DNA were analyzed by real time PCR using primers for the NRF1 promoters (−34∼−30 bp, Supplementary Table 1) (More details in Supplementary Data).

2.14. Statistical analysis

Each experiment was performed at least three times, and statistical analysis of the data was performed using one-way ANOVA analysis or unpaired two-tailed Student's *t*-test by SPSS software (Version 13.0, SPSS Inc., Chicago, USA). All data are expressed as mean ± SD. Values of $P < 0.05$ were considered to be statistically significant.

3. Results

3.1. H_2O_2-mediated oxidative stress induced AHR activation in melanocytes

To characterize the effect of H_2O_2-mediated oxidative stress on the AHR signaling pathway in melanocytes, the human normal melanocyte PIG1 cells were treated with H_2O_2 (1.0 mM) alone, pretreated with CH223191 (10 μM, 1 h) followed by stimulation

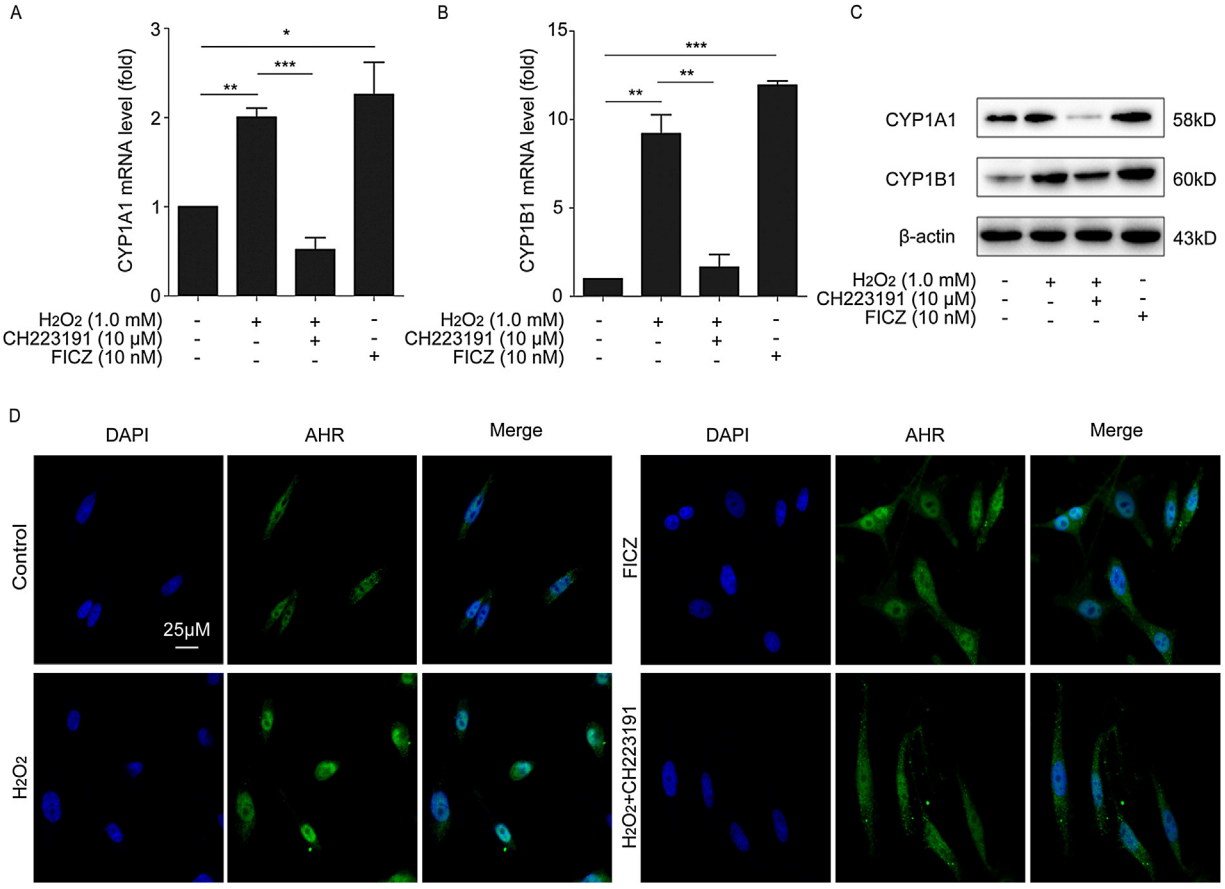

Fig. 1. Characterization of the AHR signaling pathway in normal human melanocytes under H_2O_2-induced oxidative stress. The human normal melanocyte PIG1 cells were treated with 1.0 mM H_2O_2, pretreated with 10 μM CH223191 for 1 h followed by stimulation with 1.0 mM H_2O_2, or treated with 10 nM FICZ as positive control, respectively. (A, B, C) After treatment for 24 and 48 h of H_2O_2 or FICZ, the mRNA and protein level of AHR classical target genes including CYP1A1 and CYP1B1 in the PIG1 cells was measured by real-time PCR (RT-PCR) and western blots, respectively. (D) AHR cellular localization was assessed by immunofluorescence confocal microscopy after treatment with H_2O_2 or FICZ for 15 min in PIG1 cells. Data are presented as mean ± SD of triplicate samples. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to vehicle-treated cells or H_2O_2-treated cells.

with H_2O_2 (1.0 mM), or treated with FICZ (10 nM) as positive control. The PIG1 cells were harvested 24 and 48 h with H_2O_2 or FICZ to assess mRNA and protein changes, respectively. The expression of AHR classical target genes including CYP1A1 and CYP1B1 was induced after treatment with H_2O_2 at both mRNA and protein level, which was significantly attenuated by CH223191 pre-treatment (H_2O_2/CH223191 vs. H_2O_2: $P = 0.0006$ for CYP1A1, $P = 0.0021$ for CYP1B1, Fig. 1A–C).

We further investigated whether H_2O_2-mediated AHR target genes expression is dependent on translocation of AHR protein into the nucleus, and therefore examined the subcellular localization of AHR protein by immunofluorescence assay. Our data showed that AHR was predominantly localized in the cytoplasm of untreated PIG1 cells, and addition of H_2O_2 (1.0 mM) or FICZ (10 nM) resulted in substantial nuclear translocation of AHR within 15 min (Fig. 1D). Furthermore, CH223191 pre-treatment inhibited a large proportion of H_2O_2-induced AHR nuclear translocation (Fig. 1D). Collectively, these data indicate that melanocytes expressed a functional AHR signaling pathway, and H_2O_2-mediated oxidative stress induced the activation of AHR signaling pathway in melanocytes.

3.2. Impaired activation of AHR signaling pathway increased sensitivity to oxidative stress in melanocytes

To explore the relevance of AHR signaling pathway to oxidative damage of melanocytes, apoptosis and intracellular ROS level were examined. Exposure of PIG1 cells to 1.0 mM H_2O_2 for 24 h resulted in typical apoptotic features and increased intracellular ROS level (Fig. 2). Additionally, CH223191 (10 μM, 1 h) pretreatment significantly aggravated H_2O_2-mediated apoptosis and intracellular ROS of PIG1 cells ($P = 0.0483$ for apoptosis, $P = 0.0125$ for intracellular ROS, Fig. 2D, F). FICZ pretreatment decreased the intracellular ROS level in the H_2O_2-treated PIG1 cells ($P = 0.0452$, Fig. 2F), however, it seems not to have a significant impact on H_2O_2-mediated apoptosis of PIG1 cells ($P = 0.9397$, Fig. 2D). The results imply that abnormal activation of AHR signaling pathway could exacerbate oxidative stress in melanocytes, suggesting that AHR might be involved in the regulation of oxidant-antioxidant balance of melanocytes.

3.3. Abnormal AHR signaling pathway aggravated oxidative stress induced mitochondrial damage in melanocytes

Considering that H_2O_2 induces oxidative damage of mitochondria and therefore activating apoptosis [5–8], we assessed the role of AHR in mitochondrial oxidative damage by estimating mitochondrial superoxide and mitochondrial membrane potential of PIG1 cells. H_2O_2 treatment obviously increased mitochondrial ROS level and declined mitochondrial membrane potential in the PIG1 cells (Fig. 3). CH223191 pretreatment significantly enhanced the effect of H_2O_2-induced oxidative stress on the mitochondrial ROS level and mitochondrial membrane potential of the H_2O_2-treated PIG1 cells ($P = 0.0496$ for mitochondrial ROS, $P = 0.0210$ for mitochondrial membrane potential, Fig. 3D, E). FICZ pretreatment appeared not to have much impact on the mitochondrial ROS and mitochondrial membrane potential in the H_2O_2-treated PIG1 cells ($P = 0.9717$ for mitochondrial ROS, $P = 0.6176$ for mitochondrial membrane potential, Fig. 3D, E). These results suggest that abnormal AHR signaling pathway could enhance the oxidative stress-triggered mitochondrial damage in melanocytes.

3.4. AHR signaling pathway modulated mitochondrial biogenesis in melanocytes

To determine whether AHR signal pathway have an influence on mitochondrial function, we detected the ATP level of PIG1 cells under H_2O_2-induced oxidative stress. There were no obvious changes in the ATP level of H_2O_2-treated PIG1 cells within 24 h. CH223191 significantly decreased the ATP level of H_2O_2-treated PIG1 cells ($P = 0.0163$, Fig. 4A). Conversely, FICZ obviously increased the level of ATP in the H_2O_2-treated PIG1 cells ($P = 0.0386$, Fig. 4A). These results indicate that AHR could regulate mitochondrial ATP synthesis and cellular energy metabolism in melanocytes.

As mitochondrial biogenesis is a prominent process that increases mitochondrial mass and copy number to increase the production of ATP [19], we therefore measured the effect of AHR signal pathway on mitochondrial biogenesis through observing mitochondrial DNA levels. H_2O_2 treatment resulted in significantly decreased levels of mitochondrial DNA in the PIG1 cell ($P < 0.0001$, Fig. 4B), which was worse when pretreated with CH223191 ($P = 0.0244$, Fig. 4B). In contrast, FICZ pretreatment significantly increased the number of mitochondrial DNA copies in the H_2O_2-treated PIG1 cells ($P = 0.0061$, Fig. 4B). These data imply that AHR might be involved in the regulation of mitochondrial DNA replication, and be closely associated with mitochondrial biogenesis.

Since AHR regulates biological responses through modulating gene transcription [12], we next predicted whether the promoter regions of mitochondrial biogenesis regulators [19], including peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC1α), nuclear respiratory factor 1 (NRF1) and transcription factor A mitochondrial (TFAM), contains the core sequence of AHR binding site (5′-GCGTG-3′) by using CLC Main Workbench bioinformatics software (Supplementary Table 2). We identified two putative AHR binding sites in the NRF1 promoter segment, indicating that AHR might play regulatory roles in the NRF1 transcription (Supplementary Fig. 1). We further assessed the expression levels of mitochondrial biogenesis regulator and found that H_2O_2 greatly enhanced expression of NRF1 and downstream TFAM at both mRNA and protein level in the PIG1 cells (Fig. 4C: $P = 0.0137$ for NRF1, $P = 0.0023$ for TFAM; Fig. 4E: $P < 0.0001$ for NRF1; Fig. 4F: $P < 0.0001$ for TFAM; Fig. 4C–G), whereas CH223191 or NRF1 knockdown by si-NRF1 significantly attenuated the NRF1 and TFAM expression at both mRNA and protein level in the H_2O_2-treated PIG1 cells (Fig. 4C: $P = 0.0098$ for NRF1, $P = 0.0080$ for TFAM; Fig. 4E: $P < 0.0001$ for NRF1; Fig. 4F: $P < 0.0001$ for TFAM; Fig. 4C–G). Next, we used ChIP to investigate the interaction between AHR and the NRF1 promoter (Fig. 4H). AHR was obviously bound to the NRF1 promoter in H_2O_2 and FICZ conditions, whereas decreased binding was observed in CH223191/H_2O_2 treatment, indicating that AHR positively regulates NRF1 transcription under oxidative stress through binding −34∼−30 bp of the NRF1 promoter. These data suggest that AHR activation might positively regulated mitochondrial biogenesis probably through its effects on the expression of NRF1 and its downstream targets.

4. Discussion

Oxidant-antioxidant balance is critical for survival and function of melanocytes. The elevating production of ROS or abnormal antioxidant system could lead to excessive accumulation of ROS in melanocytes [20]. Oxidative stress could gravely damage mitochondria, and even directly mediate apoptosis of melanocytes through activating intrinsic apoptotic pathway [5–8]. However, the mechanism of mitochondrial regeneration in melanocytes remains obscure. In the present study, we found that H_2O_2-induced oxidative stress could activate the AHR signaling pathway in melanocytes, however, impaired AHR activation could aggravate oxidative damage in mitochondria and melanocytes. Further studies showed that the AHR signaling pathway could promote mitochondrial biogenesis by increasing mitochondrial DNA synthesis and ATP production, which might be tightly related to

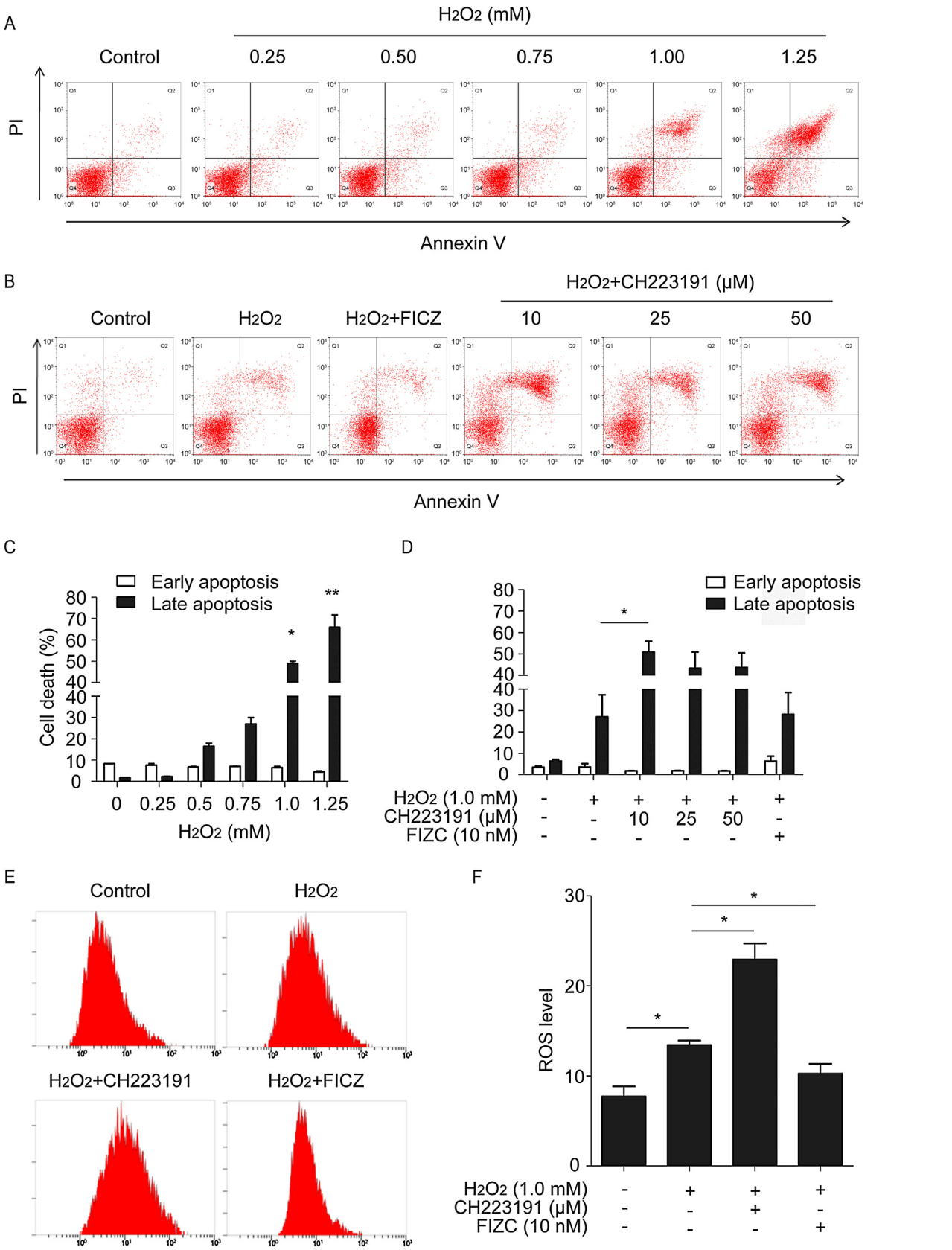

Fig. 2. Determination of the effect of AHR signaling pathway on apoptosis and intracellular ROS level in normal human melanocytes under H_2O_2-induced oxidative stress. (A, B) The human normal melanocyte PIG1 cells were treated with H_2O_2 (0.25 mM, 0.5 mM, 0.75 mM, 1.0 mM or 1.25 mM) for 24 h, or pretreated with CH223191 (10 μM, 25 μM or 50 μM) or FICZ (10 nM) for 1 h and followed by stimulation with 1.0 mM H_2O_2 for 24 h. Apoptotic and necrotic cells were identified using the annexin V and propidium iodide and analyzed by flow cytometry (FCM). (E) The PIG1 cells were pretreated with 10 μM CH223191 or 10 nM FICZ for 1 h, followed by stimulation with 1.0 mM H_2O_2 for 24 h. The production of intracellular ROS was monitored by FCM using CM-H2DCFDA. (**C, D, F**) Bar graphs represent the mean values of FCM data (n = 3). Data are presented as mean ± SD of triplicate samples. $^*P < 0.05$, $^{**}P < 0.01$, compared to vehicle-treated cells or H_2O_2-treated cells.

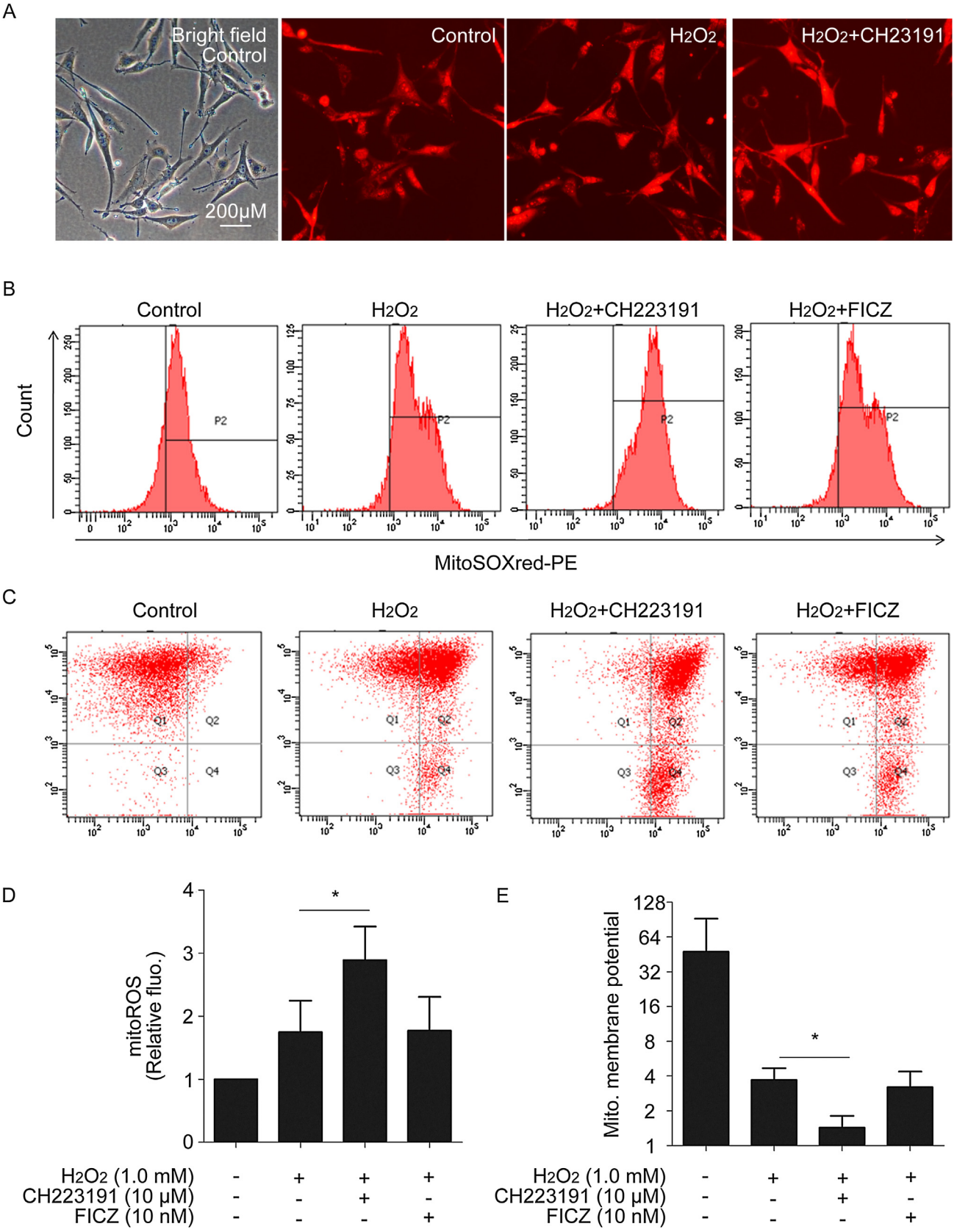

Fig. 3. Investigation of the role of AHR signaling pathway in mitochondrial damage in H_2O_2-treated melanocytes. (A, B) Mitochondrial superoxide was measured by immunofluorescence confocal microscopy and flow cytometry (FCM) using mitoSOXred in the human normal melanocyte PIG1 cells treated with vehicle (0.01%, DMSO, bright field and control), H_2O_2 (1.0 mM) alone or a combination of H_2O_2 (1.0 mM) and CH223191 (10 μM) or FICZ (10 nM) for 24 h. (C) Mitochondrial membrane potential was measured by FCM using JC-1 in the PIG1 cells treated with H_2O_2 (1.0 mM) or a combination of H_2O_2 (1.0 mM) and CH223191 (10 μM) or FICZ (10 nM) for 24 h. (**D, E**) Bar graphs represent the mean values of FCM data (n = 3). Data are presented as mean ± SD of triplicate samples. $^*P < 0.05$, compared to H_2O_2-treated cells.

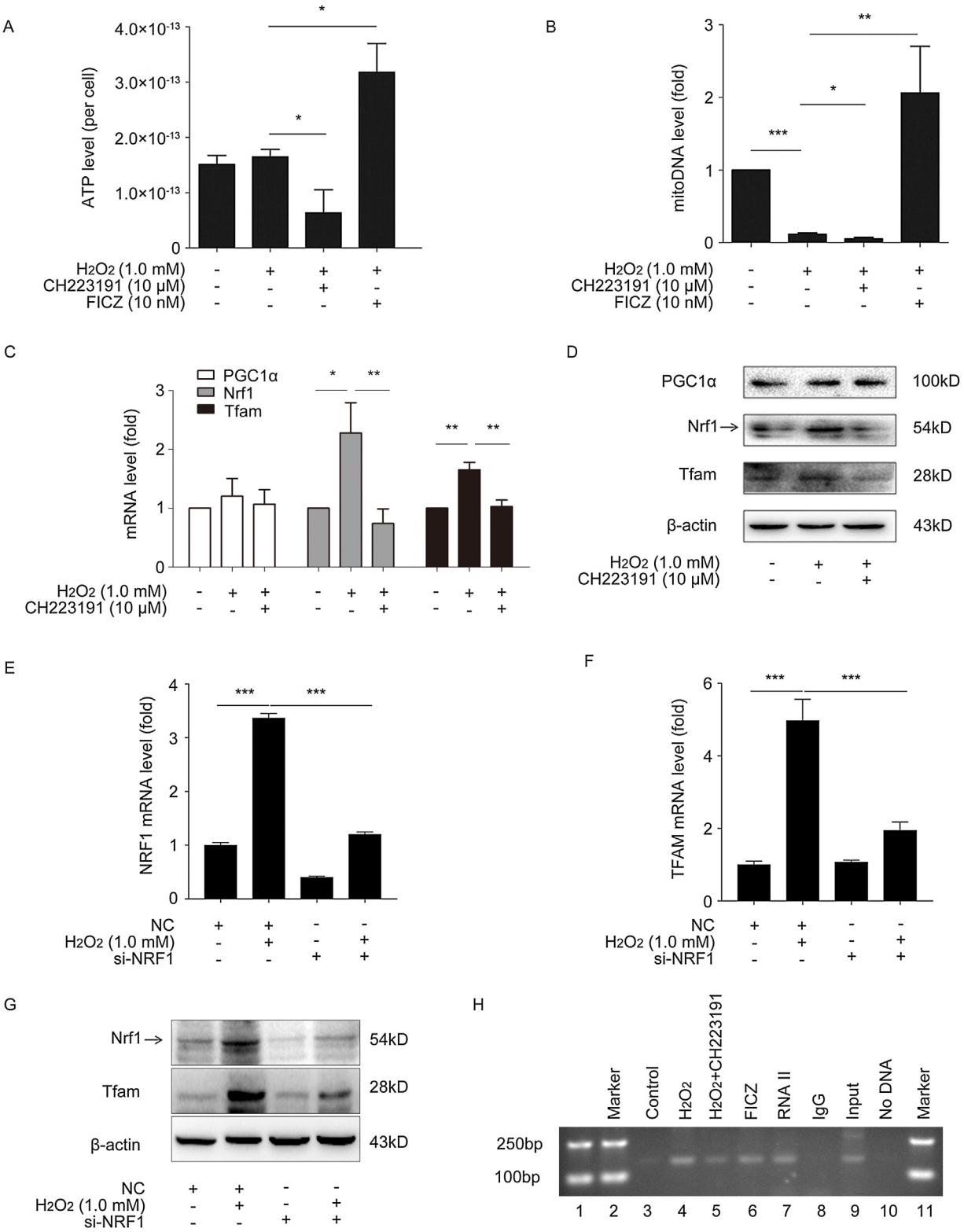

Fig. 4. Analysis of the influence of AHR signaling pathway on mitochondrial biogenesis in H_2O_2-treated melanocytes. (A, B) The human normal melanocyte PIG1 cells were treated with 1.0 mM H_2O_2, or pretreated with 10 μM CH223191 or 10 nM FICZ for 1 h and followed by stimulation with 1.0 mM H_2O_2. After treatment mentioned above for 24 h, the ATP mass of PIG1 cells was measured in a luminometer using ATP Assay System. After treatment mentioned above for 48 h, the mitochondrial DNA content of PIG1 cells was analyzed by real-time PCR (RT-PCR). (C, D) PIG1 cells were treated with 1.0 mM H_2O_2, or pretreated with 10 μM CH223191 for 1 h and followed by stimulation with 1.0 mM H_2O_2. After treatment with H_2O_2 for 24 and 48 h, mitochondrial biogenesis regulators mRNA and protein levels were measured by RT-PCR and western blots, respectively. (E, F, G) The PIG1 cells were transiently transfected with siRNAs (siNRF1 or negative control siRNA) for 24 h and then subjected to 1.0 mM H_2O_2 for 24 and 48 h to assess mRNA and protein changes of NRF1 and TFAM by RT-PCR and western blots, respectively. (H) The PIG1 cells were treated with 1.0 mM H_2O_2 for 24 h, pretreated with 10 μM CH223191 for 1 h followed by stimulation with 1.0 mM H_2O_2 for 24 h, or treated with 10 nM FICZ for 24 h as positive control. Chromatin was immunoprecipitated with anti-AHR antibodies. Rabbit IgG was a negative control, and anti-polymerase II antibody was positive control. Precipitated DNA or 1% of the chromatin input was amplifed with primers of NRF1 promoter (−34∼−30 bp). Data are presented as mean ± SD of triplicate samples. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to vehicle-treated cells, negative control siRNA-treated cells or H_2O_2-treated cells.

NRF1 and the downstream targets. These results demonstrated that the AHR signaling pathway might have a pivotal role in protecting melanocytes against oxidative damage via maintaining mitochondrial homeostasis, while the abnormal AHR signaling pathway could lead to defective mitochondria regeneration and further aggravate oxidative damage-induced apoptosis in melanocytes (Fig. 5).

AHR has an important function in defense against oxidative stress, which is involved in regulating the expression of multiple antioxidant molecules [21]. However, the molecular mechanisms of AHR activation under oxidative stress are not clear. We found that human melanocyte cell line PIG1 cells expressed a functional AHR signaling pathway. This is the first study to show that H_2O_2-induced oxidative stress could result in AHR translocating into the nucleus within 15 min, leading to changes in target genes transcription. We predicted the functional domains of AHR protein using the Deepview and Hyperchem software and found that 294–427 amino acid residues contain multiple oxidative modification sites. Previous study has reported that changes in this region could promote the dissociation of AHR from chaperone Hsp90 complex leading to AHR nuclear translocation [22]. We therefore hypothesized that H_2O_2 might alter the protein conformation of AHR by oxidative modification of AHR amino acid residues, and then promote cytosolic complex dissociation resulting in AHR activation. Other studies have shown that H_2O_2 could improve the formation of FICZ and might be involved in the regulation of AHR signaling pathway activation [23]. These results suggest that oxidative stress might activate the AHR signaling pathway of melanocytes in a non-ligand or ligand dependent manner.

Recent studies have shown that the AHR signaling pathway contributes to maintaining mitochondrial homeostasis against oxidative stress. Genetic ablation or lack of the AHR enhanced oxidative stress-induced mitochondrial dysfunction, including mitochondrial membrane permeabilization and subsequent loss of mitochondrial membrane potential, and cellular apoptosis [15,16]. The high-throughput researches suggest that AHR alters the expression of genes associated with mitochondrial function, such as electron transport chain complexes [24–26]. In addition, AHR activation has an effect on cellular respiration in an AHR-dependent manner to maintain respiratory efficiency [27]. Aspirin could regulate mitochondrial metabolism and energy conversion process in hepatocytes via AHR [28]. AHR controls mitochondrial enzyme expression (e.g., PGC-1β, ND4, Cytb, Cox1 and Cox3) and might be required for mitochondrial biogenesis in osteoclasts [29]. In the present study, inhibition of AHR signaling pathway was found to enhance H_2O_2-induced oxidative damage and apoptosis in melanocytes, suggesting that AHR is involved in modulating the oxidant-antioxidant balance of melanocytes. Moreover, we discovered that inhibition of AHR signaling pathway increased mitochondrial ROS level and mitochondrial membrane damage in melanocytes, leading to mitochondrial membrane depolarization and decreased mitochondrial membrane potential. We also found that activation of AHR by FICZ decreased the H_2O_2 induced-intracellular ROS level in melanocytes. Unfortunately, we did not observe the obvious effect of FICZ on reversing H_2O_2-induced oxidative damage of mitochondria or H_2O_2-induced apoptosis of melanocytes. Therefore, these data at least indicate that abnormal activation of AHR signaling pathway could worsen H_2O_2-induced oxidative damage of mitochondria and melanocytes.

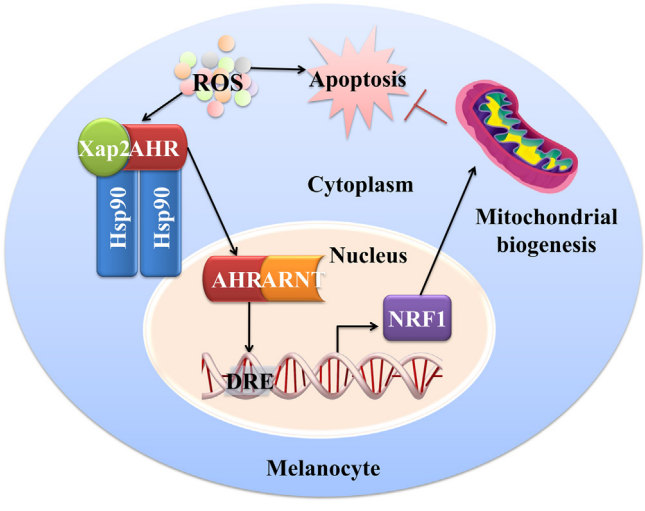

Fig. 5. A brief overview of the protective role of AHR signaling pathway in human melanocytes under oxidative stress. H_2O_2-induced oxidative stress activates the AHR signaling pathway in melanocytes. AHR regulates NRF1 transcription to promote mitochondrial biogenesis against oxidative damage in mitochondria and melanocytes. AHR, aryl hydrocarbon receptor; ARNT, AHR nuclear translocator; NRF1, nuclear respiratory factor 1; ROS, reactive oxygen species; Xap2, hepatitis B virus X-associated protein 2; DRE, dioxin-responsive element; Hsp90, heat shock protein 90.

Mitochondria are not only the main source of intracellular ROS, but also the main target of oxidative stress. Mitochondria are susceptible to various exogenous and endogenous ROS, which could further induce oxidative damage to mitochondria [30]. Mitochondrial biogenesis and mitophagy coordinately control mitochondrial quality that determine repair of oxidative stress induced mitochondrial dysfunction [31]. Our study showed that the levels of ATP and mitochondrial DNA were progressively increased during AHR activation by FICZ, whereas the levels of ATP and mitochondrial DNA were substantially reduced during AHR inhibition in the H_2O_2-treated melanocytes. However, in our system, we did not observe H_2O_2-induced decline of ATP level in the melanocytes. Previous studies as mentioned above suggest that AHR activation could alter the genes expression of mitochondrial energy metabolism including ATP synthase and maintain respiratory efficiency. In our study, we found that H_2O_2 could induce AHR activation in the melanocytes. We thus speculated that the ATP level might not be significantly decreased in the H_2O_2-treated melanocyte within a relatively short treatment duration, which might be probably due to the compensatory increase of ATP level induced by AHR activation in the H_2O_2-treated melanocytes [24–27]. These results imply that AHR could regulate mitochondrial ATP synthesis and mitochondrial DNA replication closely related to mitochondrial biogenesis. Mitochondrial biogenesis is a key process to govern mitochondrial content, distribution and activity, which has a crucial importance to maintaining normal cellular functions and cell survival [19].

Mitochondrial biogenesis includes mitochondrial DNA transcription and translation, translation of nuclear DNA-encoded transcripts, membrane recruitment, protein import and assembly of the oxidative phosphorylation complexes [19,32], which is tightly controlled by diverse mechanisms [19,33,34]. PGC-1α, NRF1 and TFAM are the major players of mitochondrial biogenesis [19]. PGC1-1α is a transcriptional coactivator and promotes gene transcription by enhancing the activity of DNA-binding transcription factors [34]. NRF-1 controls the expression of a significant number of the proteins that make up the five respiratory complexes, as well as proteins integral to mitochondrial import and heme biosynthesis [35]. NRF-1 is also able to integrate nuclear control of the transcriptional and replicative activity of the mitochondrial genome through the direct modulation of TFAM expression [36]. Mitochondrial biogenesis and its regulatory mechanisms are essential for the maintenance of normal cell function. We applied CLC main workbench software to predict the promoter regions of the major regulatory molecules PGC-1α, NRF1 and TFAM, and found that the

NRF1 promoter contains putative AHR binding sites and might be a novel target of the AHR signaling pathway. Further studies have shown that H_2O_2-induced AHR activation upregulated the expression of NRF1 and its downstream TFAM, oppositely, inhibition of the AHR signaling pathway or knockdown of NRF1 downregulated the H_2O_2-induced NRF1 and TFAM expressions. Moreover, we observed the directly interaction between AHR and the NRF1 promoter in the melanocytes under H_2O_2-induced oxidative stress. These data indicate that the AHR signaling pathway might play a positive role in regulating NRF1 transcription, representing a new mechanism of mitochondrial biogenesis.

In summary, this study clearly suggests that the AHR signaling pathway could be activated by oxidative stress and be essential for protecting melanocytes against oxidative damage through promoting mitochondrial biogenesis and controlling mitochondrial quality, whereas the impaired AHR signaling pathway could result in defective mitochondrial regeneration and further aggravate oxidative damage-induced apoptosis in melanocytes. AHR signaling pathway might be a key mechanism of mitochondrial biogenesis in melanocytes defending against oxidative damage. Further studies should be performed to determine its biological efficacy and precise mechanisms in vitro and in vivo.

Declaration of Competing Interest

The authors confirm that there are no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jdermsci.2019.09.001.

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