Fullerenol Protects Retinal Pigment Epithelial Cells From Oxidative Stress–Induced Premature Senescence via Activating SIRT1

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Purpose. Oxidative stress–induced retinal pigment epithelium (RPE) senescence is one of the important factors in the pathogenesis of age-related macular degeneration (AMD). This study aimed to develop a new antisenescence-based intervention and clarify its possible molecular mechanism.

Methods. A cell premature senescence model was established in both primary RPE cells and ARPE-19 cells by exposure of the cells to pulsed H_2O_2 stress for 5 days, and confirmed with senescence-associated β-galactosidase (SA-β-gal) staining. The final concentration of fullerenol (Fol) in the cell culture system was 5 μg/mL. Cellular redox status was determined by the examination of cellular reactive oxygen species (ROS) staining, catalase activity, and the ratio of reduced to oxidized glutathione, respectively. Deoxyribonucleic acid double-strand breaks were determined by quantitative analysis of γH_2AX. Cell cycle analysis was performed with flow cytometry. SIRT1 activity was examined with SIRT1 Assay Kit. SIRT1 overexpression and knockdown in ARPE-19 cells were performed with lentiviral-mediated infection.

Results. Pulsed H_2O_2 exposure triggered the acetylation of p53 at lysine 382 (K382) and subsequent increase in its target $p21^{Waf1/Cip1}$. It also increased the number of accumulated phospho-γH_2AX foci and the level of phosphor-ATM in RPE cells. Fullerenol protected the RPE cells, as it reduced the number of positive SA-β-gal–staining cells, alleviated the depletion of cellular antioxidants, and reduced genomic DNA damage. Its mechanism might involve the activation of deacetylase SIRT1, resulting in decreased levels of acetyl-p53 and $p21^{Waf1/Cip1}$. The roles of SIRT1 in protecting cells in response to Fol were further confirmed by applications of SIRT1 activator (resveratrol) and inhibitors (nicotinamide and sirtinol), and through SIRT1 overexpression and knockdown.

Conclusions. Fullerenol could rescue RPE cells from oxidative stress–induced senescence through its antioxidation activity and the activation of SIRT1. The protective effect of Fol is useful for the development of new strategies to treat oxidative stress–related retinal diseases like AMD.

Keywords: fullerenol, RPE senescence, SIRT1, DNA damage, oxidative stress

Age-related macular degeneration (AMD) is the leading cause of blindness in the elderly population in developed countries and in the developed regions of some developing countries.[1–4] Clinically, AMD is classified into two types, atrophic (dry) AMD and exudative (wet) AMD. The two types share some common pathological characteristics, including the degeneration and death of retinal pigment epithelial (RPE) cells and secondary photoreceptor cell damage.

Retina has high metabolic activities, and oxidative stress has been reported as one of the possible causes of AMD.[5] Moreover, RPE cells generated reactive oxygen species (ROS) when exposed to light.[6] Protein adducts, such as the advanced glycation end-products (AGEs) malondialdehyde and carboxyethylpyrrole, also accumulated in degenerative retina,[7] causing senescence and/or death of RPE cells and then photoreceptor degeneration and vision loss.[8] Therefore, approaches that rescue RPE cells through antioxidation and/or cell protection would be helpful in intervention for AMD.

Fullerenol (Fol), the well-known polyhydroxylated fullerene derivative, is an effective free radical scavenger and antioxidant that has attracted researchers' interest in further drug development. Fullerenol is approximately 1 nm in diameter, with symmetrically arranged hydroxyl groups on the C_{60} sphere. The most important feature of Fol, considering the mechanism of its biology activity, is free radical scavenging. Several studies have revealed the therapeutic potential of Fol as a glutamate receptor antagonist, a neuroprotectant, and a cytotoxicity modulator, for example.[9–12] Here we demonstrate that Fol protected RPE cells from H_2O_2-induced senescence and might act through its antioxidation effect to block genomic DNA damage and activation of SIRT1 to reduce p53 acetylation as well as $p21^{Waf1/Cip1}$ expression. Since oxidative stress plays a central role in the alterations of structure and function of RPE cells,[13] Fol could be a potential solution for treating AMD.

Methods

Cell Culture and Premature Senescence Model

Primary porcine RPE (ppRPE) cells and primary human RPE (phRPE) cells were cultured as previously described, with slight modifications.[14] Briefly, primary RPE cells were plated onto fibronectin (BD Biosciences, San Jose, CA, USA)-coated dishes and maintained in Dulbecco's modified Eagle's medium: Nutrient Mixture F-12 (DMEM/F12) containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C and 5% CO_2. Cells at passages 2 to 5 were used. The ARPE-19 cell line was a gift from Gail M. Seigel (State University of New York, Buffalo, NY, USA). The cells were cultured under conditions similar to those described above. ARPE-19 cells were used to establish an oxidative stress–induced premature senescence model following the procedure described previously[15] with minor modifications. Briefly, for the H_2O_2 exposure group, ARPE-19 cells at 70% confluence were exposed daily to 200 μM H_2O_2 for 2 hours for 5 days. After each 2-hour H_2O_2 exposure, the cells were washed with PBS three times and cultured with complete culture medium or medium with Fol and/or other treatments for 22 hours. The cells were allowed to recover in complete culture medium for 3 days. The cells were then replated at various densities, 10,000 to 40,000 cells/cm^2, for subsequent experiments (Supplementary Fig. SA). For other experimental groups, Fol (5 μg/mL, equivalent to 5 μM), sirtinol (30 μM), nicotinamide (6 mM), and resveratrol (50 nM) were used. The cell senescence model in ppRPE and phRPE cells was generated in the same way as the model in ARPE-19 cells. Cell senescence was examined with the Senescence-Associated β-Galactosidase Staining Kit (Cat. No. 9860; Cell Signaling Technology, Beverly, MA, USA) according to the manufacturer's instructions. Cell viability was detected by MTT assay.

Redox Status Analysis

Cellular ROS were detected by 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. The cells were cultured at 37°C in medium containing 10 μM DCFH-DA for 20 minutes and washed twice with PBS. The DCFH-DA fluorescence was detected by microscope or fluorescence-activated cell sorter (FACs) with an excitation wavelength of 485 nm and emission wavelength of 522 nm. Catalase activity was measured with spectrophotometry at 520 nm (Beyotime, Haimen, China). Cell extract (20 μL) was used for the assay, and the activity was expressed as units per microgram proteins (U/mg). The ratio between reduced and oxidized glutathione (GSH and GSSG) was measured with a commercial kit (GSH&GSSG Assay Kit; Beyotime). The absorbance value was monitored at 415 nm on a microplate reader (model 680; Bio-Rad, Hercules, CA, USA).

DNA Double-Strand Break Analysis

The ARPE-19 cells were cultured on coverslips coated with Matrigel (Cat. No. 356234; BD Biosciences) the day before assay. The cells were fixed with 4% PFA (paraformaldehyde in PBS, pH 7.4) at 4°C for 30 minutes. Ice-cold 90% methanol was gently dropped onto the coverslips and incubated for 10 minutes. After blocking with 5% BSA-phosphate buffered saline with 0.1% Tween-20, the coverslips were examined with the OxiSelect DNA Double-Strand Break (DSB) Staining Kit (STA-321; Cell Biolabs, USA). DAPI (4′,6-diamidino-2-phenylindole, Cat. No. D1306; Invitrogen, Carlsbad, CA, USA) was used to stain the nuclei. Flow cytometry analysis was used for the quantitative analysis of γH_2AX staining. The cells were prepared with the same protocol as above, but the tests were performed in a suspension system in centrifuge tubes. Propidium iodide (PI) with 1 mg/mL RNase A was used for staining of nuclei.

Cell Cycle Analysis

The cells were fixed in 70% cold ethanol and stored at −20°C. The cells were washed twice with chilled PBS and incubated with RNase A (1 mg/mL) and DNA intercalating dye 50 mg/mL PI for 30 minutes in the dark. Cell cycle analysis was performed with FACs (BD Biosciences).

Western Blot Analysis

The cells were lysed with NE-PER Nuclear and Cytoplasmic Extraction Reagents (Cat. No. 78833; Pierce, Rockford, IL, USA) plus a supplement of 1% phosphatase inhibitor cocktail (Cat. No. p2714; Sigma-Aldrich Corp., St. Louis, MO, USA) and radioimmunoprecipitation lysis buffer (P0013; Beyotime) containing 1% phenylmethylsulfonyl fluoride (ST506; Beyotime) and 1% phosphatase inhibitor cocktail 2 (p5726; Sigma-Aldrich Corp.), and stored at −80°C. Protein concentrations were determined with BCA assay kit (Cat. No. 23225; Pierce). Protein extracts (50 μg protein per each sample) were subjected to 12% SDS-polyacrylamide gel for electrophoresis and then transferred to nitrocellulose membrane (Millipore, Darmstadt, Germany). Immunoblot analyses were performed using specific antibodies: DSBs Repair Antibody Sampler Kit 9653, ATM rabbit mAb 2873, BRCA1 rabbit mAb 9010, cleaved caspase-3(Asp175) rabbit mAb 9664, p21 $Waf1^{/Cip1}$ rabbit mAb 2947, FoxO3a rabbit mAb 2497, SIRT1 Assay Kit (1F3) mAb 8549 (Cell Signaling Technology); catalase rabbit pAb sc50508 (Santa Cruz Biotechnology, Dallas, TX, USA); $p16^{ink4a}$ polyclonal antibody 10883-1-AP (Proteintech, Chicago, IL, USA). To evaluate protein modification, the samples were first detected with antibodies against specific modifications (antibody 2525 for acetyl-p53 Lys382 and antibody 9284 for phospho-p53 Ser15 [Cell Signaling Technology], antibody Ser139 2212-1 for phospho-γH_2AX [Abcam, Cambridge, UK]), and then reprobed with the antibody against total proteins (p53 mAb 2527 [Cell Signaling Technology], H_2AX mAb 3522-1 [Abcam]). Each blot

was stained with Amido Black for loading control as described previously.[16]

Real-Time PCR Assay

The human SIRT1 gene expression in ARPE-19 cells was qualified by SYBR Green real-time PCR using the sequence detection system (Prism 7900; Applied Biosystems, Inc. [ABI], Foster City, CA, USA). *SIRT1* forward primer was TGGGTCTTCCCTCAAAGTAAG; reverse primer was TTCACCA CCTAACCTATGACAC. *GAPDH* served as the reference gene. *GAPDH* forward primer was TCCACCCATGGCAAATTCC; reverse primer was TCGCCCCACTTGATTTTGG.

Sirtuin Activity Assay

SIRT1 activity was examined with *SIRT1* Assay Kit (Cat. No. CS1040; Sigma-Aldrich Corp.), and SIRT3 activity was evaluated with *SIRT3* Fluorometric Assay Kit (Cat. No. CY-1153; CycLex Co., Nagano, Japan), according to the manufacturers' instructions.

Lentiviral-Mediated *SIRT1* Overexpression and Knockdown in RPE Cells

Lentiviral vector was purchased from GeneCopoeia (SIRT1 overexpression lentiviral vector: EX-T8013-Lv105; GFP lentiviral vector: EX-GFP-Lv105; SIRT1 short hairpin RNA (shRNA) lentiviral vector: HSH006080-8-HIVH1 OS396339; Rockville, MD, USA). Viral supernatant was generated by 293FT cells transfected with EX-Lv105 plasmid and lentiviral packaging plasmids. Viral titers were determined with a quantity kit. The ARPE-19 cells (2×10^5) were seeded in 6-cm dishes with lentivirus (multiplicity of infection approximately 0.04) and 10 μg/mL polybrene, and cultured for 48 hours with the medium renewed after 24 hours. Then the cells were seeded in 10-cm dishes and cultured with complete DMEM/F12 medium (Invitrogen) containing 0.5 μg/mL puromycin for 48 hours. The surviving cells were SIRT1-overexpressing ARPE-19 cells or SIRT1 shRNA ARPE-19 cells (iSIRT1 ARPE-19).

Statistical Analysis

All values are given as means ± SEM; n represents the number of independent experiments. Statistical analyses were performed using one-way ANOVA and Student's t-test, with $P < 0.05$ considered statistically significant.

Results

Fullerenol Inhibits Oxidative Stress–Induced Premature Senescence in RPE Cells

To investigate the effect of Fol on RPE cell senescence, premature senescence was induced in both ARPE-19 cells and ppRPE cells by pulsed exposure to H_2O_2. On day 8 after H_2O_2 exposure, RPE cells were evaluated with cell morphology and senescence-associated β-galactosidase (SA-β-gal) activity. In normal control groups, there were few SA-β-gal–positive cells (0.4 ± 0.2% in ARPE-19 cells and 1.0 ± 0.6% in ppRPE cells, respectively). In phRPE cells, the average SA-β-gal–positive cell ratio was 14.4 ± 3.2%. In H_2O_2-treated groups, the positive SA-β-gal staining cell ratio significantly increased to 12.4 ± 2.5% in ARPE-19 cells, 16.6 ± 2.8% in ppRPE cells, and 46.0 ± 2.5% in phRPE cells. Fol significantly alleviated the damage of H_2O_2 and reduced the senescent cells to 4.9 ± 0.7% in ARPE-19 cells, 2.7 ± 0.5% in ppRPE cells, and 8.8 ± 2.4% in phRPE cells (Figs. 1A–D, Supplementary Fig. SB), indicating that Fol inhibited senescence in RPE cells caused by oxidative stress. Moreover, we found that the cell cycles were arrested at G_2/M phase after H_2O_2 exposure (G_2/M ratio raised from 16.4 ± 3.0% to 41.3 ± 4.0% in ARPE-19 cells, $P < 0.05$). Fol intervention significantly decreased the percentage of such G_2/M arrest (from 41.3 ± 4.0% to 25.1 ± 2.5% in ARPE-19 cells, $P < 0.05$.), but it did not affect the cell cycle distribution in the control cells (Figs. 1E, 1G). Similar results were obtained in the phRPE and ppRPE cells (Fig. 1H, Supplementary Fig. SC). Thus, our result revealed for the first time that Fol could be a useful agent to rescue oxidative stress–induced RPE cell senescence.

Fullerenol Reduces Cellular Oxidation Status in Premature Senescent ARPE-19 Cells

Since Fol serves as an antioxidant, we anticipated that it would inhibit oxidative stress–induced cell senescence through reducing intracellular ROS. To test this hypothesis, we examined the ROS levels in the cells under different treatments. As expected, ARPE-19 cells in the H_2O_2 treatment group had significantly higher cellular ROS levels than normal control cells, while Fol intervention evidently reduced the ROS level (Fig. 2A). In H_2O_2-treated senescent RPE cells, the glutathione peroxidase (GPx) activity was significantly decreased ($P < 0.05$), which was prevented by Fol treatment. Fol itself showed no effect on GPx in normal cells (Fig. 2B). Furthermore, catalase activity in Fol-treated ARPE-19 cells was around 2.5 times higher than that in the untreated cells. Under the same Fol treatment, the protein level of catalase was upregulated (Figs. 2C, 2D). Meanwhile, Fol displayed little effect on another antioxidant enzyme, superoxide dismutase (data not shown). Collectively, Fol could slightly activate the cellular antioxidant system in H_2O_2-induced senescence cells.

Fullerenol Decreases Senescence-Related DNA Damage in ARPE-19 Cells

Deoxyribonucleic acid damage often contributes to G_2/M cell cycle arrest in premature senescent cells. We examined whether DNA damage occurred in these cells through immunostaining of the p-γH_2AX (Ser139)-positive foci. As shown in Figure 3A, there was a substantially increased γH_2AX phosphorylation in the nucleus of H_2O_2-exposed ARPE-19 cells compared to the control [fluorescence density: $(9.8 \pm 0.1) \times 10^3$ vs. $(2.5 \pm 0.1) \times 10^3$, $P < 0.05$], indicating an accumulation of DNA damage in the senescent cells. With Fol intervention, the number of p-γH_2AX foci decreased significantly [$(6.9 \pm 1.5) \times 10^3$ vs. $(9.8 \pm 1.1) \times 10^3$, $P < 0.05$] by immunofluorescence staining and Western blot (Figs. 3A, 3B). Furthermore, Western blot analysis showed that the components of MRE11–RAD50–NBS1 complex and their regulators, such as phospho-ATM (Ser1981), phospho-BRCA1 (Ser1524), and Rad 50, were all upregulated in senescent ARPE-19 cells (Fig. 3B). These results support the notion that the DNA damage/repair pathway is activated in senescent ARPE-19 cells. Meanwhile, we detected that p16Ink4a, another cell senescence marker, was accumulated in H_2O_2-treated ARPE-19 cells and decreased following Fol treatment (Fig. 3B).

Fullerenol Protects ARPE-19 Cells From Senescence via Activation of SIRT1

The p53-p21$^{Waf1/Cip1}$ pathway is the major signal pathway of the DNA damage-related senescence response.[17] As expected, the p53 protein level was markedly elevated when RPE cells were exposed to H_2O_2, and this elevation was diminished by

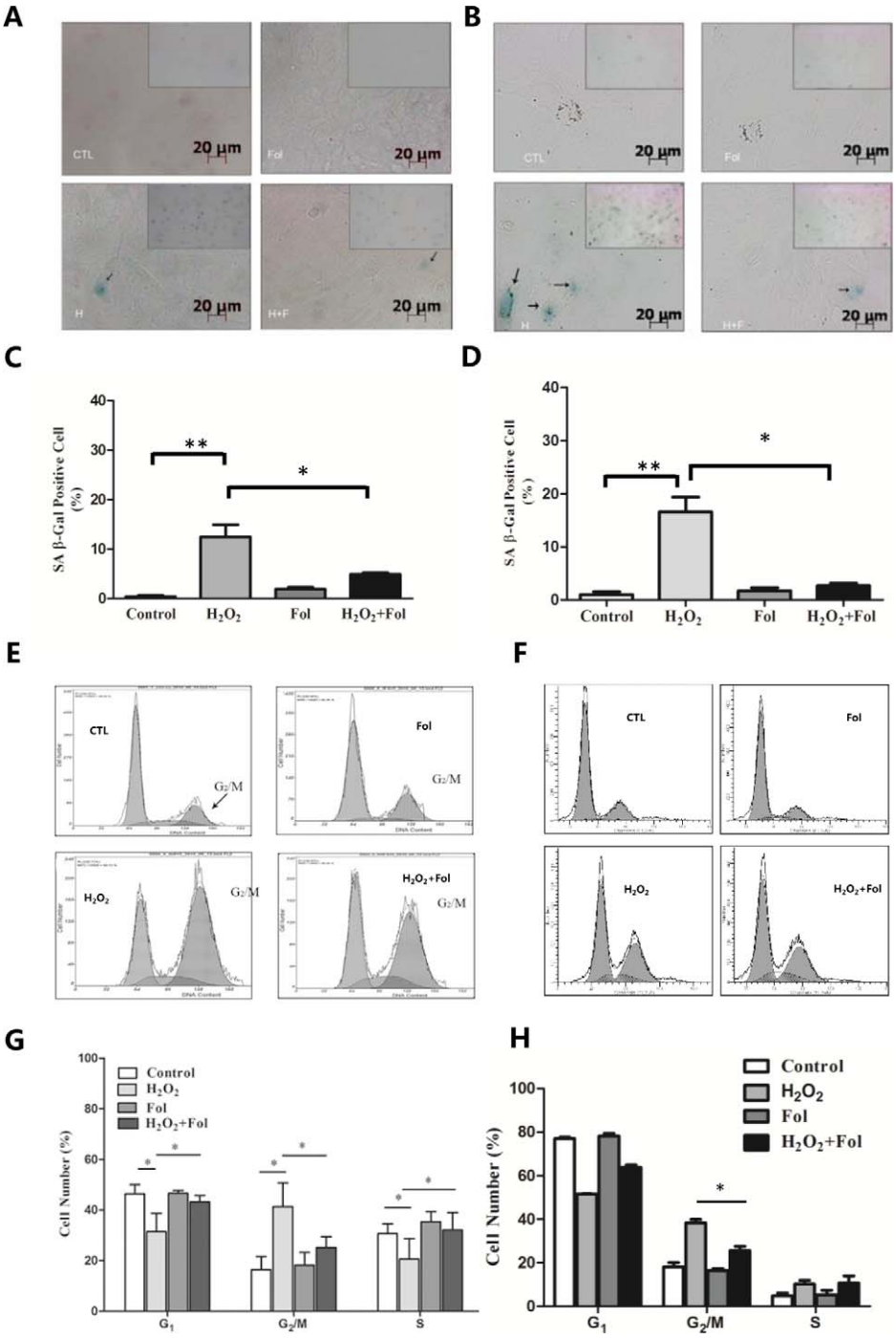

Figure 1. Fullerenol alleviates oxidative stress–induced cell senescence in RPE cells. (**A**) SA-β-gal staining of H_2O_2-treated ARPE-19 cells, with or without Fol intervention. *Arrows*: typical senescence cells. (**B**) Senescence-associated β-gal staining of H_2O_2-treated ppRPE cells, with or without Fol intervention. *Arrows*: typical senescence cells. (**C**, **D**) Quantitative analysis of SA-β-gal–positive staining of ARPE-19 cells and phRPE cells. (**E**, **F**) Flow cytometry of ARPE-19 and phRPE cell proliferation under different treatments using PI staining. *Arrows*: G_2/M phase cells. (**G**, **H**) Quantitative analysis of ARPE-19 and ppRPE cell proliferation by flow cytometry. CTL, control; Fol, cells treated with 5 μg/mL Fol only; H_2O_2, cells treated with 200 μM H_2O_2; H_2O_2 + Fol, cells treated with 200 μM H_2O_2 and 5 μg/mL Fol ($n = 3$; $^*P < 0.05$).

Fol treatment (Fig. 4A). Since DNA damage increases p53 acetylation and reduces cell survival and proliferation,[18] we examined the K382 acetylation and S15 phosphorylation of p53, and found that H_2O_2 exposure increased both modifications of p53 proteins. The levels of p53 modifications recovered to near normal under Fol treatment (Figs. 4D, 4E). Furthermore, the transcriptional activity of p53 was activated by oxidative stress, as evidenced by the accumulation of $p21^{Wafl/Cip1}$ protein in the senescent RPE cells, which was abrogated by Fol intervention (Fig. 4A). These results indicated that p53-$p21^{Wafl/Cip1}$ pathway was activated by oxidative stress and that Fol played an effective role in controlling p53 activities in senescent cells.

Acetyl-p53 has been reported as the substrate of SIRT1, a senescence regulator in cells.[19,20] It led us to explore whether SIRT1 was associated with the effects of Fol. SIRT1

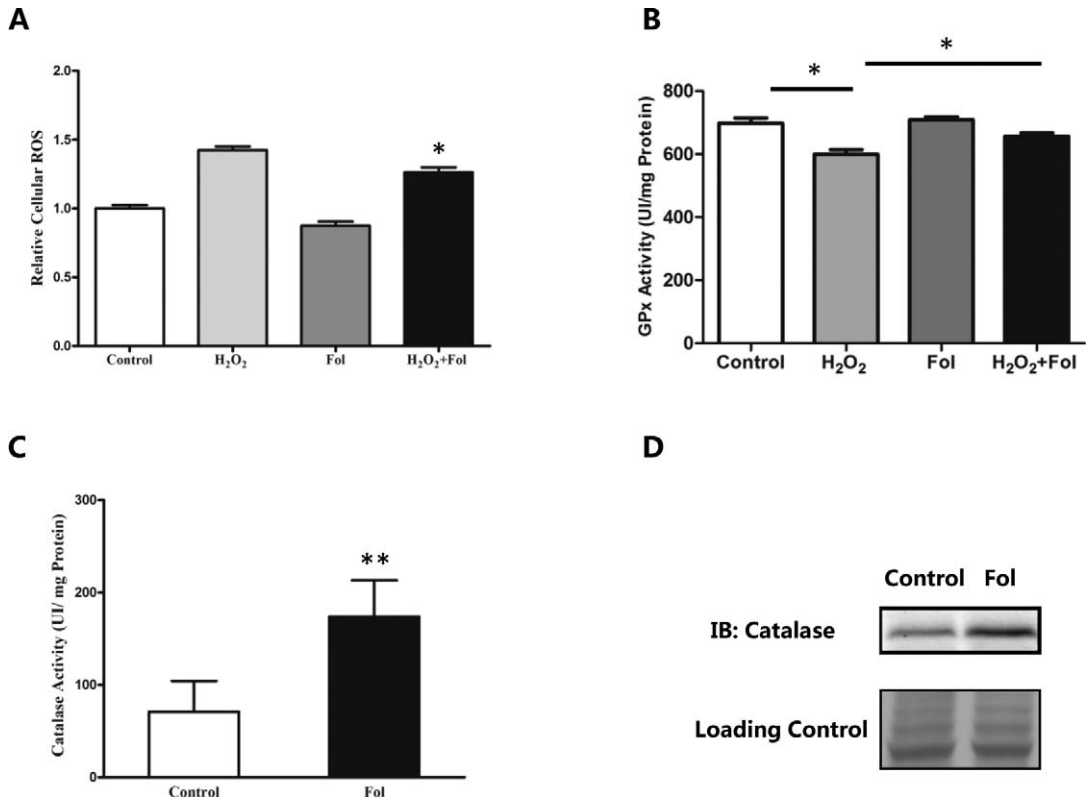

FIGURE 2. Fullerenol reduces cellular oxidation in senescent cells. (**A**) Quantitative analysis of cellular ROS in ARPE-19 cells under different treatments. (**B**) Quantitative analysis of cellular GSH to GSSG ratio in ARPE-19 cells under different treatments ($n = 3$; $^{*}P < 0.05$). (**C**) Quantitative analysis of cellular catalase activity in ARPE-19 cells under different treatments ($n = 3$; $^{**}P < 0.01$). (**D**) Catalase expression was upregulated by Fol treatment.

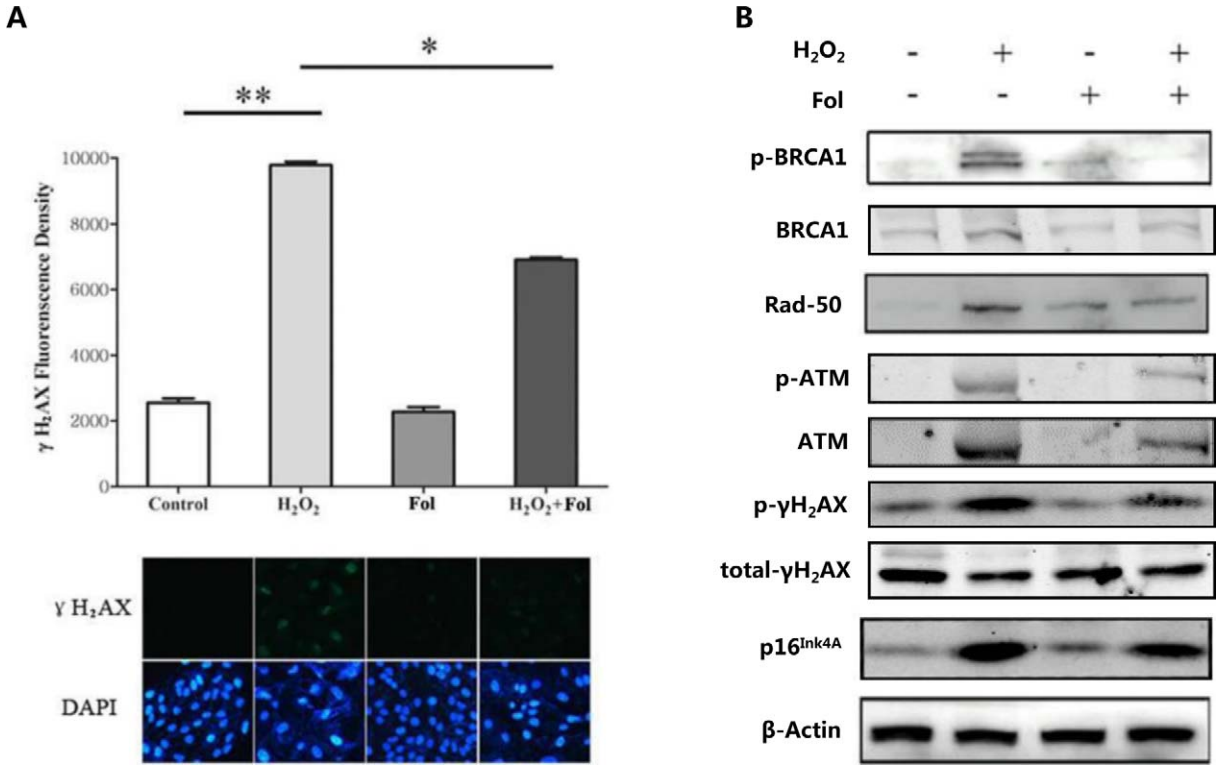

FIGURE 3. Fullerenol reduces cellular DNA damage in premature senescence cells. (**A**) Quantitative analysis of γH_2AX expression in ARPE-19 cells under different treatments with flow cytometry ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$) (*upper*) and a representative image (*lower*). (**B**) Immunoblot with DNA damage-related gene-specific antibodies showed that fullerenol reduced the DNA double-strand breakdown-related protein expression.

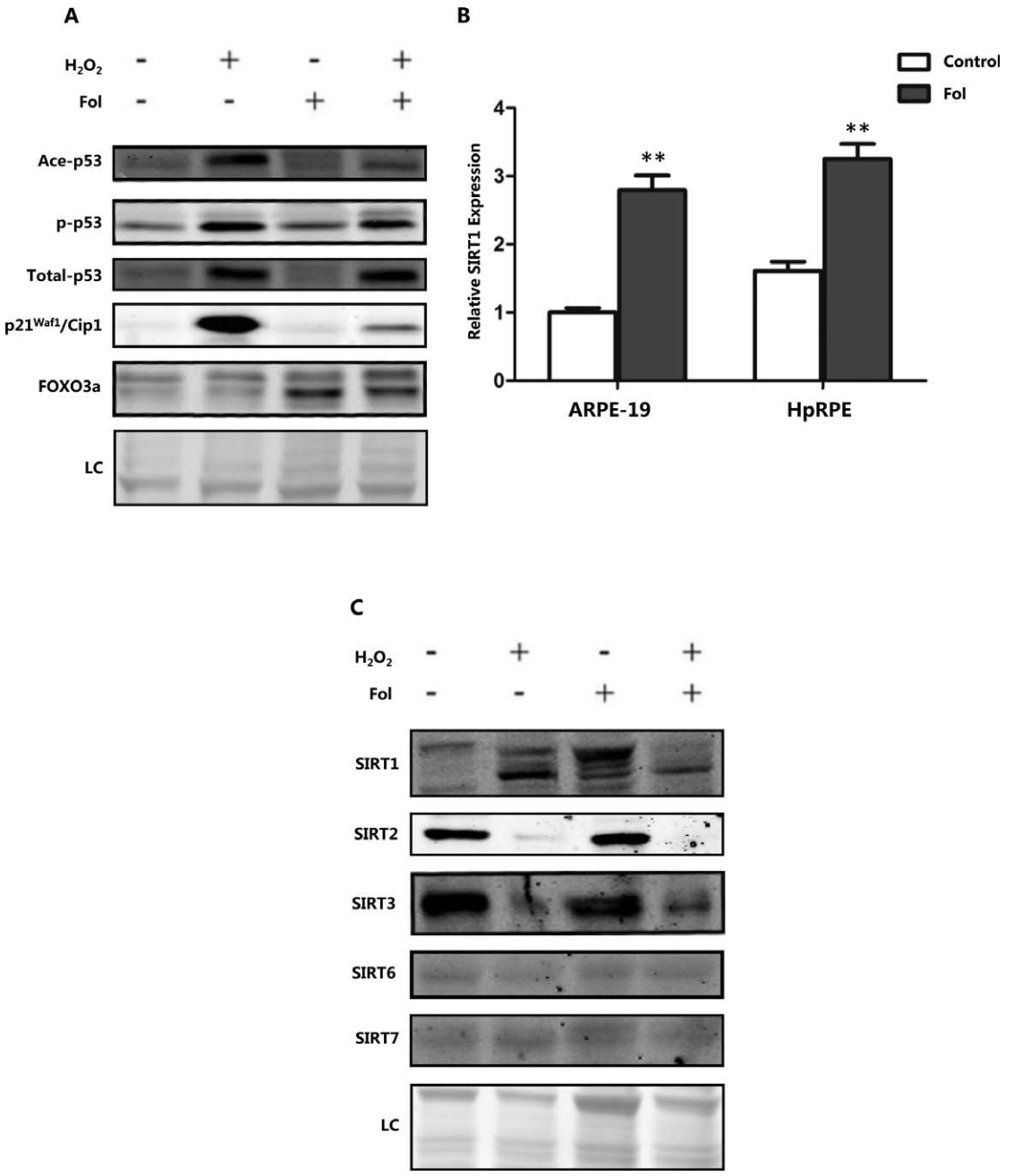

FIGURE 4. Effects of fullerenol on expressions of SIRT1, acetyl-p53, p21, and FoxO3a in senescent ARPE-19 cells. (**A**) Expression of SIRT1, p21, and different p53 modifications in ARPE-19 cells under different treatments. (**B**) Quantitative PCR showed that SIRT1 transcription level was induced by fullerenol treatment in both ARPE-19 and phRPE cells. (**C**) Immunoblot with sirtuin-specific antibodies indicated SIRT1 as the molecule that was the target of fullerenol ($n = 3$; $^{**}P < 0.01$).

transcription was increased with Fol treatment (Fig. 4B). The protein level of SIRT1 was depressed in the H_2O_2 group and partly recovered with Fol treatment. Other sirtuin members did not show such change with Fol treatment (Fig. 4C). In vitro assays further showed that Fol enhanced the deacetylase activity of SIRT1, but not SIRT3, suggesting that Fol might be a specific activator of SIRT1 (Figs. 5A, 5B). To test whether Fol's effect on SIRT1 activities could account for its protective effect in senescent cells, two sirtuin inhibitors, nicotinamide (NAM) and sirtinol, were employed. Both abolished the protective effect of Fol, as demonstrated by significantly increased SA-β-gal–positive cells in the HFN group (H_2O_2-exposed cells treated with Fol and NAM, 19.2 ± 3.0%) and the HFS group (H_2O_2-exposed cells treated with Fol and sirtinol, 45.8 ± 1.7%) compared to the HF group (H_2O_2 + Fol) (4.9 ± 0.7%, $P < 0.01$ for both), as shown in Figure 5C. Similar results were obtained when γH_2AX expression levels were compared between HFN cells, HF cells, and HFS cells (Fig. 5D). These data lead to the conclusion that the Fol protection is SIRT1 activity dependent.

The Western blot data reinforced the conclusion by showing that Fol increased SIRT1 protein levels and decreased p53 K382 acetylation and $p21^{Wafl/Cip1}$ accumulation in senescent RPE cells. SIRT1 inhibitors such as NAM and sirtinol

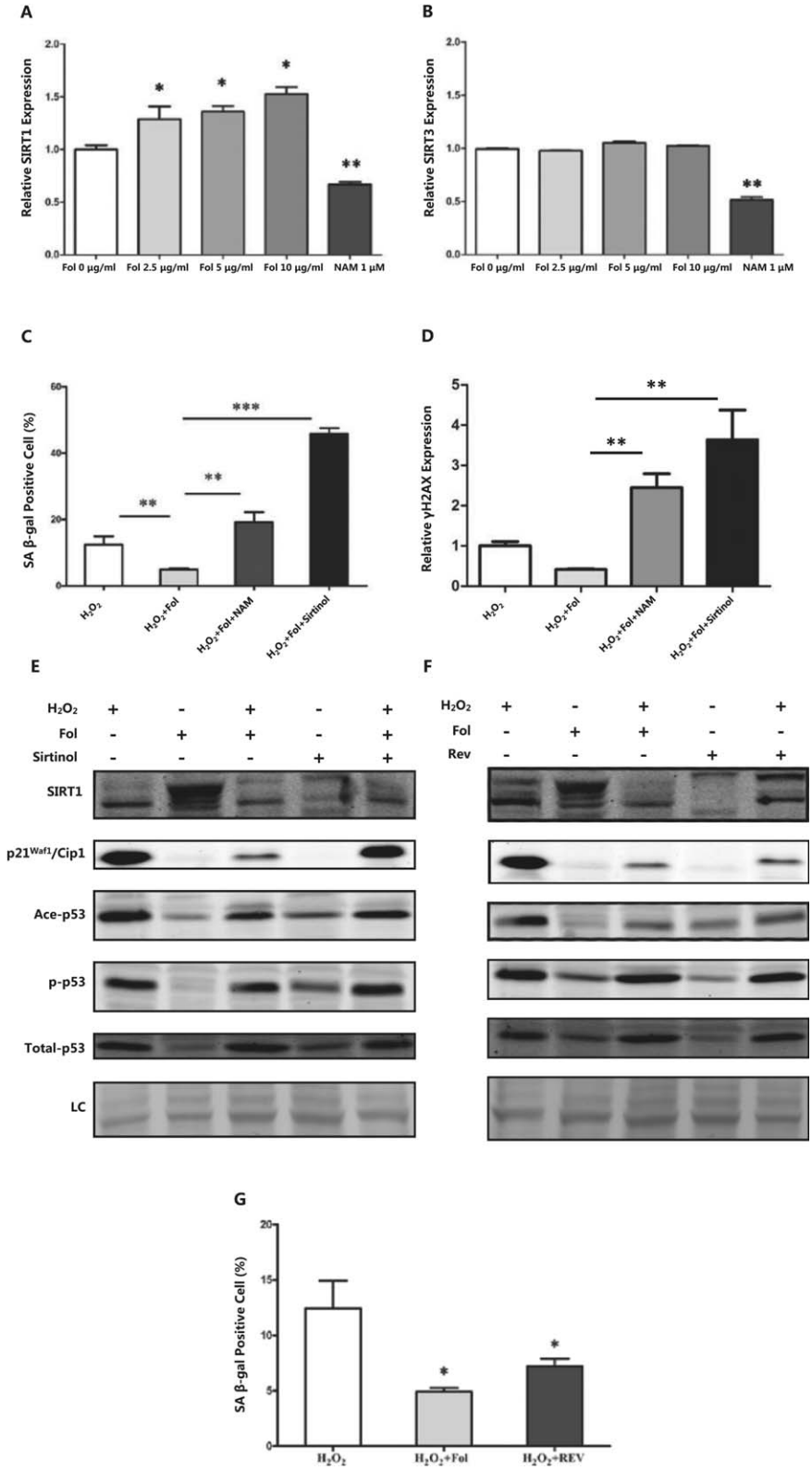

Figure 5. Effects of fullerenol, sirtinol, NAM, and resveratrol on cell senescence, sirtuin activity, and γH_2AX. (**A**, **B**) Effect of Fol on the deacetylase catalytic activity of SIRT1 and SIRT3 when nicotinamide (NAM) served as an inhibitor ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$). (**C**) Quantitative analysis of SA-β-gal staining of ARPE-19 cells under different treatments. NAM, 6 mM. (**D**) Quantitative analysis with flow cytometry of γH_2AX expression in ARPE-19 cells under different treatments ($n = 3$; $^{**}P < 0.01$, $^{***}P < 0.001$). (**E**, **F**) Effects of Fol and sirtinol on SIRT1-p53-p21$^{Waf1/Cip1}$ signaling. (**G**) Quantitative analysis of SA-β-gal staining of ARPE-19 cells treated with resveratrol ($n = 3$; $^*P < 0.05$). Sirtinol, 30 μM; LC, loading control; Rev, 50 nM resveratrol.

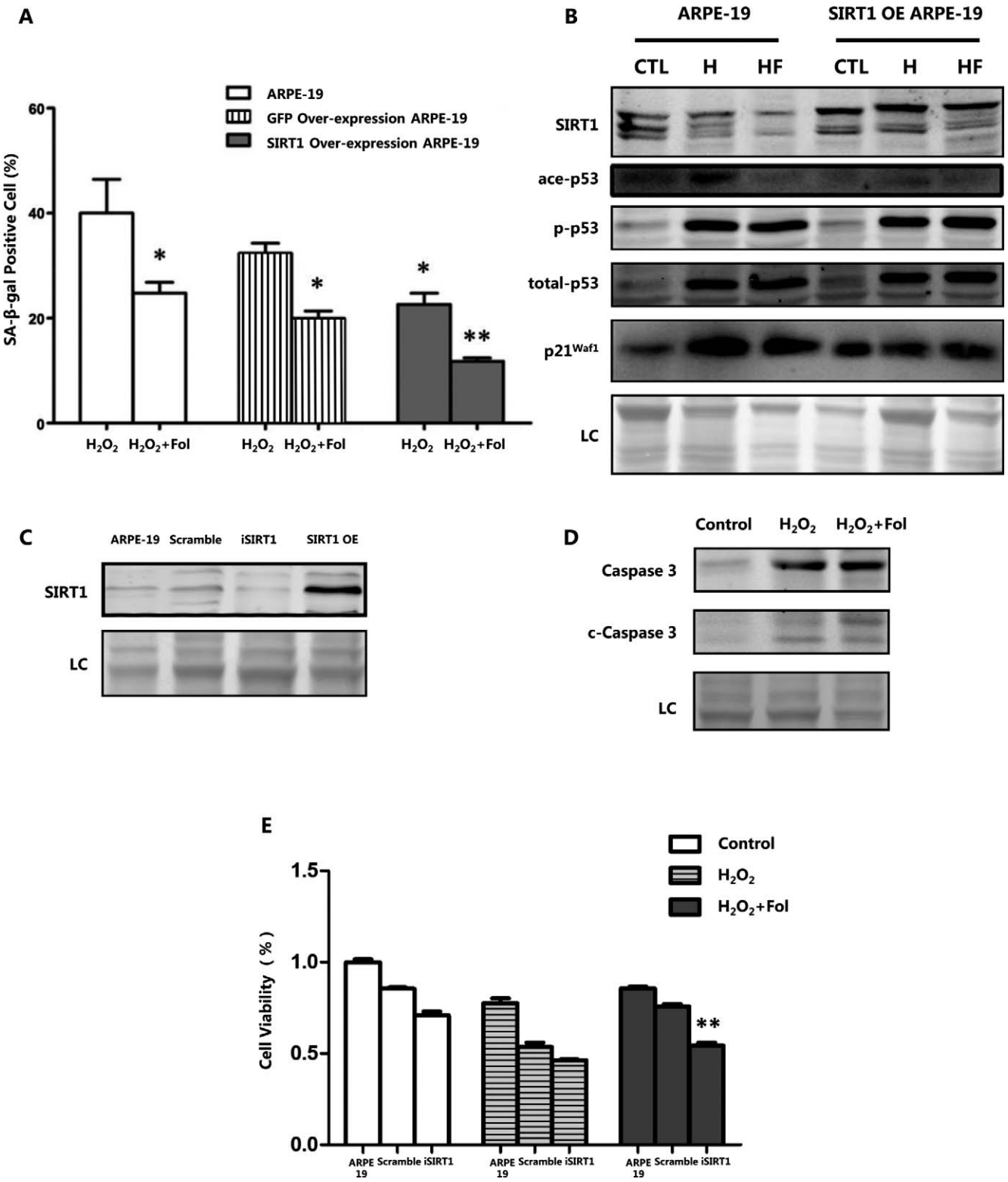

FIGURE 6. Effects of SIRT1 gene modification on RPE cell senescence model. (**A**) Cell senescence in H_2O_2-treated SIRT1-overexpressing ARPE-19 cell line, with or without Fol intervention. The H_2O_2-treated ARPE-19 cells were used as control. Retinal pigment epithelium senescence was evaluated by positive SA-β-gal staining cell ratio, which is presented as a *column graph* ($n = 3$; $^*P < 0.05$, $^{**}P < 0.01$). (**B**) SIRT1 and senescence-related protein expression in both ARPE-19 cells and SIRT1 OE ARPE-19 cells under different treatments. SIRT1 OE ARPE-19, SIRT1-overexpressing ARPE-19 cells; CTL, normal ARPE-19 cells; H, H_2O_2-treated cells; HF, H_2O_2+Fol-treated cells; LC, loading control. (**C**) SIRT1 was knocked down in iSIRT1 ARPE-19 cells. LC, loading control. (**D**) Immunoblot with caspase-3-specific antibodies suggested induced apoptosis in iSIRT1 RPE cells. c-Caspase3, cleaved caspase-3. (**E**) MTT assay indicated the hypersensitivity of iSIRT1 RPE to H_2O_2, and Fol had less effect on these SIRT1 knockdown cells. iSIRT1, SIRT1 shRNA ARPE-19 cells; Scramble, shRNA negative control ARPE-19 cells.

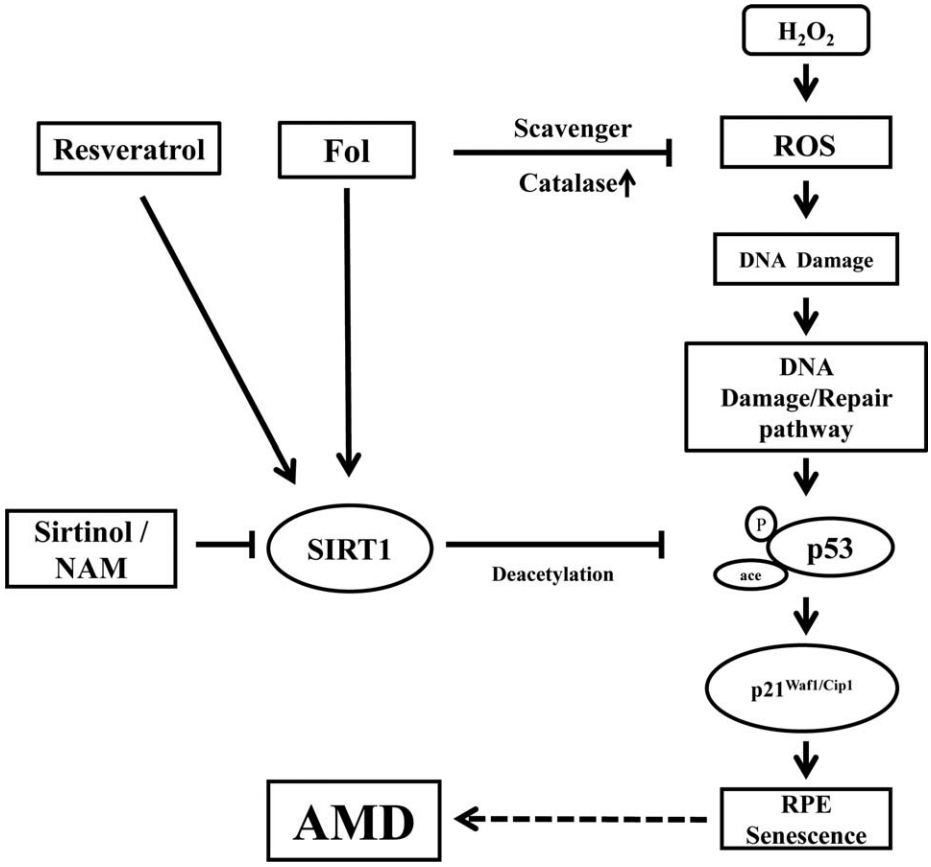

FIGURE 7. Schematic diagram of the proposed mechanisms for fullerenol protection against H_2O_2-induced senescence in RPE cells and in AMD development. Fullerenol protects RPE cells from senescence through two possible pathways: (1) It acts directly against oxidative injury as a scavenger and thus reduces p53-p21 signaling via regulating DNA damage/repair molecules; (2) it activates SIRT1 and thus reduces the p53-p21 signaling via p53 deacetylation. The p53-p21 signaling inhibition slowed down the process of senescence in RPE cells and might prevent or delay the development of AMD.

could counteract the effect of Fol, as manifested by the decreased SIRT1 level, increased p53 K382 acetylation, and $p21^{Waf1/Cip1}$ accumulation (Fig. 5E). On the other hand, when resveratrol (50 nM) was used, the SA-β-gal–positive cell number was similar to that in Fol-treated cells (Fig. 5G). Moreover, resveratrol activated SIRT1 expression and reduced the levels of p53 K382 acetylation and $p21^{Waf1/Cip1}$ accumulation similarly to Fol treatment (Fig. 5F). It should be mentioned that FoxO3a, the molecule that could induce catalase transcription, was upregulated by Fol treatment (Fig. 4A).

Overexpression of SIRT1 in ARPE-19 Cells Enhances the Antisenescence Effect of Fullerenol

To further ascertain the role of SIRT1, we overexpressed SIRT1 in ARPE-19 cells (SIRT1 OE cells). Under H_2O_2 treatment, there were significantly fewer SA-β-gal–positive cells in the SIRT1 OE cells than in ARPE-19 cells or GFP OE ARPE-19 cells. Furthermore, Fol still enhanced the protective effect in SIRT1 OE cells as it did in other groups (Fig. 6A). In SIRT1 OE cells, SIRT1 expression was approximately six times higher than in normal ARPE-19 cells under the same H_2O_2 treatment. Consistently, SIRT1 overexpression reduced p53 acetylation at K382 remarkably, thus decreasing the level of $p21^{Waf1/Cip1}$. With Fol treatment, the levels of $p21^{Waf1/Cip1}$ and aK382 p53 in SIRT1 OE cells were significantly lower than those in ARPE-19 cells while pS15 p53 was not affected (Fig. 6B). Hence, SIRT1 is a key player in the Fol-mediated protective effect against oxidative stress–induced RPE cell senescence.

SIRT1 Knockdown in APRE-19 Cells Amplifies the Toxicity of H_2O_2

In addition to overexpression of SIRT1, the antisenescence effect of Fol was also confirmed in SIRT1 knockdown ARPE-19 cells (iSIRT1) (Fig. 6C). While stimulated with H_2O_2, iSIRT1 ARPE-19 cells were hypersensitive to oxidative stress compared to other cells (normal cells, SIRT1 OE cells, and scramble cells). The cell viability dropped dramatically within 24 hours under H_2O_2 treatment even when Fol was added to the culture medium, while the control cells survived well under the same stress (Figs. 6D, 6E).

DISCUSSION

Our results clearly demonstrate that Fol protects RPE cells from senescence induced by oxidative stress. With H_2O_2 exposure, there was an obvious increase in positive SA-β-gal–stained cells, cells arrested at the G_2/M phase and DSBs occurred. Fol attenuated all these cell senescence phenotypes and consequently prolonged the cell life span. The mechanism by which Fol protected H_2O_2-treated RPE cells is illustrated in Figure 7. In RPE cells, oxidative stress impairs the genomic DNA and triggers the accumulation of γH_2AX foci, which activates the DNA repair complex. The massive DNA damage induces

hyperacetylation of p53, which drives the cells into senescence through $p21^{Waf1/Cip1}$.[21] Fol, on one hand, can scavenge cellular ROS and reduce genomic DNA damage as has been shown previously and also in this paper.[22] On the other hand, it activates SIRT1, leading to decreases in the levels of acetyl-p53 and $p21^{Waf1/Cip1}$. Therefore, the antioxidative effect and SIRT1 activation coordinately mediate the function of Fol in protecting RPE cells from senescence under oxidative stress.

Previous studies showed that Fol had antioxidative ability in radioactively and chemically induced oxidative injury models.[23–25] In this H_2O_2-induced RPE senescence model, Fol reduced cellular ROS level, maintained near-normal GPx activity, and stimulated catalase, demonstrating that Fol's antioxidation effect is at least partially responsible for its protective effect in RPE cells.

While Fol showed convincing antioxidant ability, its toxicity was still a concern. One report has demonstrated that Fol could injure RPE cells even at a low concentration.[26] However, in our study, cell viability examination showed that a relatively high dose (20 μg/mL, approximately 20 μM) of Fol was still safe for ARPE-19 and phRPE cells (Supplementary Figs. SD, SE).

In addition, our study indicates that activation of SIRT1-p53-p21 signaling also accounts for the antisenescence function of Fol. Fol is a selective activator of the class III histone deacetylase SIRT1. It is well known that SIRT1 facilitates DNA damage repair,[27] and its homologues are important aging regulators in different organisms.[28] In mice, SIRT1 plays a critical role in both the development and aging of the retina.[29] In the process of aging, SIRT1 activity in the retina decreased and was accompanied by DNA damage accumulation.[30] Our results showed that Fol could specifically promote the expression and activity of SIRT1, but not SIRT3. As a SIRT1 substrate, p53 acetylation was modulated by Fol. Acetyl-p53 is one of the active forms of p53 that play an important role in cell cycle arrest.[31] It is thus easier to understand that Fol broke cell cycle arrest in the cell senescence model via activating SIRT1, followed by deacetylation of acetyl-p53. Further downstream to p53, $p21^{Waf1/Cip1}$, a cell senescence biomarker, is accumulated in the nuclei.[32] Resveratrol, a known SIRT1 activator, conferred protection similar to that of Fol, both with respect to phenotype and at the molecular level. In contrast, SIRT1 inhibitors like NAM and sirtinol blocked the protective effect of Fol, verifying that SIRT1 activity is required for Fol to protect RPE cells. In addition, Fol's antisenescence effect was maintained at the same level in the SIRT1-overexpressing RPE cells, but completely lost in the SIRT1 knockdown RPE cells, whereas the iSIRT1 cells were highly sensitive to oxidative stress and Fol showed no protective effect on them. All these data confirmed SIRT1, shown to be involved in the human RPE aging process,[33] as the pivotal factor in the antisenescence effect of Fol in RPE cells.

Water-soluble Fol serves as an effective free radical scavenger and antioxidant and thus could be therapeutically applied in AMD. Chronic and cumulative oxidative stress plays an important role in the pathogenesis of AMD.[13] Among the three major pathological changes seen in AMD, lipofusion and drusen are believed to be the result of oxidative damage in the retina, especially in RPE cells.[34–36] Researchers have also found that SIRT1 activity in retina decreased during the aging process and that such decrease was accompanied by DNA damage accumulation.[30] Based on our findings in this study, Fol should be a potential drug for age-related retinal diseases like AMD. Its nanosize allows it to reach the retina and RPE cells easily via intravitreal injection.[37]

Phosphorylation is another type of posttranslational modification of p53 in response to cellular stress,[38] and for p53 protein, S15 phosphorylation and K382 acetylation are two independent events.[39] In the present study, H_2O_2 treatment induced S15 phosphorylation of p53, which was slightly suppressed by Fol (Fig. 4). Meanwhile, H_2O_2 treatment increased p-BRCA1 and p-ATM in cultured cells; both could induce p53 phosphorylation in response to DNA damage.[40] Fol significantly reduced the levels of p-BRCA1 and p-ATM (Fig. 3B), which may explain the decrease of p-p53 in Fol-treated cells. Hence, Fol may modulate the posttranslation modification of p53 via both deacetylation and reduction of phosphorylation, while deacetylation is the major pathway. Other molecules in the DNA damage/repair pathway, like BRCA1 and Rad 50, were also examined, and their changes also confirmed the involvement of this pathway in RPE senescence and Fol protection.

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