



Metformin protects retinal pigment epithelium cells against H₂O₂-induced oxidative stress and inflammation via the Nrf2 signaling cascade

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Abstract

Purpose Dysfunctions of retinal pigment epithelium (RPE) attributed to oxidative stress and inflammation are implicated with age-related macular degeneration (AMD). A debate on the curative role of metformin in AMD has been raised, though several recent clinical studies support the lower odds by using metformin. This study aimed to determine whether metformin could exert cytoprotection against RPE oxidative damages and the potential mechanisms.

Methods A cellular AMD model was established by treating ARPE-19 cells with hydrogen peroxide (H₂O₂) for 24 h. The reactive oxygen species (ROS) generation, expression of antioxidant enzymes, and levels of pro-inflammatory cytokines were monitored under administrations with H₂O₂ with/without metformin. The expression and DNA-binding activity of transcription factor erythroid-related factor 2 (Nrf2) were determined by western blot, immunofluorescence, and electrophoretic mobility shift assay. Knockout of Nrf2 was conducted by CRISPR/Cas9 gene deletion system.

Results Metformin pretreatment significantly improved the H₂O₂-induced low viability of ARPE-19 cells, reduced ROS production, and increased contents of antioxidative molecules. Concurrently, metformin also suppressed levels of pro-inflammatory cytokines caused by H₂O₂. The metformin-augmented nuclear translocation and DNA-binding activity of Nrf2 were further verified by the increased expression of its downstream targets. Genetic deletion of Nrf2 blocked the cytoprotective role of metformin.

Conclusion Metformin possesses antioxidative and anti-inflammatory properties in ARPE-19 cells by activating the Nrf2 signaling. It supports the potential use for the control and prevention of AMD.

Keywords Age-related macular degeneration · Metformin · Retinal pigment epithelial cells · Nrf2 · Oxidative stress · Inflammation

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Key messages

What is known:

- Age related macular degeneration (AMD) is a leading cause of severe vision loss among the elderly. The efficacy of current treatments for control and prevention of AMD remains unsatisfactory. Several recent clinical studies note the curative effect of metformin on AMD, however, some controversies emerge by showing no significant association with risk of developing AMD.
- Nuclear factor Nrf2 possesses great properties of antioxidation and anti-inflammation within cells, which has been considered a latent target for the treatment of neurodegenerative diseases.

What is new:

- Metformin pretreatment significantly suppressed oxidative stress and inflammatory response induced by H₂O₂ in ARPE-19 cells.
- Metformin enhanced nuclear translocation and DNA binding activity of Nrf2, which might play pivotal role in its cytoprotective effect, further identified by the genetic deletion of Nrf2.

Introduction

The retinal pigment epithelium (RPE) consists of highly specialized epithelial cells that interact with photoreceptors on the apical side and with Bruch's membrane on the basal side. Dysfunction and loss of RPE cells in the macula contribute to photoreceptor cell death and irreversible impairment of central visual acuity, which is postulated to play a major role in the pathogenesis of age-related macular degeneration (AMD) [1, 2]. AMD is a leading cause of vision loss among the elderly. As the population continues to age, the prevalence of AMD is projected to be 288 million in 2040 [3, 4]. While the advent of treatment with anti-vascular endothelial growth factor (anti-VEGF) has revolutionized the management of neovascular AMD, the efficacy is still unsatisfactory [5]. Moreover, this benefit does not extend to the atrophic changes which remain an unmet medical need [6]. Although these two forms of AMD present different clinical features, they share some common pathophysiological processes. Oxidative threats coupled with secondary immunity activation have been identified to be involved in the pathogenesis and progression of both forms of AMD [7–9], raising the possibility that antioxidative agents may be a new AMD therapeutic option.

Oxidative stress has long been considered a major factor in RPE degeneration in AMD [10]. To combat oxidative threats, RPE cells are equipped with strong antioxidant defenses, but these are found to be less functional and less adequate in patients with AMD [11]. A crucial consequence of oxidative stress is the oxidatively damaged protein, lipid, and DNA. Aside from the altering biological functions, these oxidative modifications can also induce inflammation. The initial inflammatory response is

intended to remove the triggers and prevent tissue injury. However, inadequate neutralization of the oxidative molecules can convert the protective immune reaction into a chronic pathologic response, which will eventually contribute to RPE and photoreceptor cell loss [9]. Inflammatory proteins, including a variety of pro-inflammatory cytokines, complements, and inflammasomes, have been identified in the drusen of AMD [12, 13]. Mechanisms between oxidative stress, inflammation, and AMD development are complex, albeit with a well-elaborated two-level model hypothesis of AMD [14].

Metformin was found to effectively control hyperglycemia as early as 1922 and has become the first-line medication for type 2 diabetes worldwide for decades. However, mechanisms of metformin have not been fully understood. Several clinical trials demonstrated that metformin can improve cardiovascular outcomes in type 2 diabetic patients, independent of its hypoglycemic effects [15, 16]. Beneficial effects have also been observed in patients with diabetic retinopathy [17]. It is indicated that metformin may exert cytoprotection via its anti-angiogenic, anti-inflammatory, and antioxidative mechanisms [18–20]. Some retrospective studies have suggested that metformin may have a role in treatment of AMD and may provide a prevention against progression to late stages [21–23]. However, most of studies have been based on the retrospective electronic medical records, which still have no conclusive results.

Nuclear factor erythroid-related factor 2 (Nrf2) is a key transcription factor maintaining redox balance in most cells (including RPE cells) by driving the expression of a set of antioxidant enzymes [24, 25]. The activity of Nrf2 is tightly controlled by the suppressor protein kelch-like ECH-associated protein 1 (Keap1). Under conditions of oxidative stress,

Nrf2 is released from Keap1 in the cytoplasm and enters the nucleus to transactivate its target genes. Heme oxygenase-1 (HO-1) and NAD(P)H quinone oxidoreductase 1 (NQO1) are representative target genes of Nrf2 that can convert heme to other products (e.g., carbon monoxide) to exert antioxidation and to exchange electrons between NADPH and NADH to keep the proteins in a reduced state [26, 27]. The antioxidative function of the Nrf2 signaling pathway has also been reported to contribute to the elimination of inflammatory responses [28]. Thus, Nrf2 is considered a latent target for the treatment of neurodegenerative diseases.

In the present study, we investigated the cytoprotective role of metformin in an *in vitro* model of AMD consisting of oxidatively damaged RPE cells. Given the fact that the profound antioxidative function of Nrf2 might have an anti-inflammatory effect, we further studied the role of the Nrf2 signaling pathway in metformin's antioxidative and anti-inflammatory activities.

Materials and methods

Cell culture and treatments

ARPE-19 cells were incubated at 37 °C under a humidified 5% CO₂ atmosphere in Dulbecco's modified Eagle medium and Ham's F-12 nutrient mixture (Gibco BRL, NY, USA) with 10% fetal bovine serum (Gibco BRL) and 1% antibiotics. The cells were passaged through trypsinization every 3–4 days.

The *in vitro* oxidative stress model of AMD was established by treating ARPE-19 cells with various concentrations (125–1000 μM) of hydrogen peroxide (H₂O₂, Sigma-Aldrich, Shanghai, China) for 24 h. Metformin stock solution (Sigma-Aldrich, Shanghai, China) was prepared at 1 M for further dilution to final concentrations of 0.5–5 mM, which were used to pretreat ARPE-19 cells for 2 h before the H₂O₂ exposure.

Cell viability assay

A Cell Counting Kit-8 (CCK-8, APEX-BIO, USA) assay was used for cell viability assay. ARPE-19 cells were seeded on 96-well plates at a density of 5 × 10³/well and treated with metformin and H₂O₂ for the indicated times. The cell culture

medium was replaced with a new serum-free medium containing 10 μL CCK-8 solutions, and cells were incubated for 2 h at 37 °C. The optical density was measured with a microplate reader (BioTek, USA) at a 450-nm absorbance wavelength.

Hoechst fluorescent staining in living cells

ARPE-19 cells (2 × 10⁵/well) were incubated in 6-well plates at 37 °C and in 5% CO₂ for 24 h. The cells were pretreated with metformin for 2 h and then stimulated with 250 μM H₂O₂ for 24 h. Then the nuclear-specific fluorescent dye Hoechst 33342 (Sigma-Aldrich, Shanghai, China) was added to the culture medium for 10 min. The morphology of living cells and nuclei was observed under a confocal microscope (20 ×, Zeiss LSM 880, Germany).

Enzyme-linked immunosorbent assay

The levels of the pro-inflammatory cytokines interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-8 (IL-8) in the ARPE-19 cell-conditioned media were determined from medium samples using human enzyme-linked immunosorbent assay (ELISA) kits (Sino Biological, Beijing, China and Boster Biological Technology, Wuhan, China). All the ELISAs were performed according to the manufacturer's instructions.

Determination of intracellular reactive oxygen species

Intracellular reactive oxygen species (ROS) formation was detected using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probes (Beyotime, Shanghai, China). Cells were seeded on 96-well plates and incubated with 20 μM DCFH-DA at 37 °C for 30 min. Flow cytometry was utilized to determine the level of intracellular ROS.

Reduced and oxidized glutathione measurements

Reduced glutathione (GSH), the most important endogenous antioxidant, is oxidized to its oxidized form GSSG when reacting with ROS. Higher level of GSH or higher GSH/GSSG ratio provides a stronger antioxidative defense

Table 1 Specific primers

Gene	Forward primer	Reverse primer
SOD2	CTCAAGTCATCCACCCACCT	AGCTGATGACGTGGAGAGAG
CAT	GGCCTTTGGCTACTTTGAGG	GAACCCGATTCTCCAGCAAC
HO-1	AACTTTCAGAAGGGCCAGGT	GAAGACTGGGCTCTCCTTGT
NQO1	ATGGAAGAAACGCCTGGAGA	GTCAGTTGGGATGGACTTGC
GAPDH	ACCCAGAAGACTGTGGATGG	TCAGCTCAGGGATGACCTTG

capacity. The GSH and GSSG concentrations were determined using the GSH and GSSG Assay Kit (Beyotime, Shanghai, China). ARPE-19 cells (1×10^6 cells) were washed once with phosphate-buffered saline and then incubated with protein removal reagent at 4 °C for 5 min. Samples were centrifuged in 10,000 g at 4 °C for 10 min, and supernatants were collected to detect the total GSH level. To determine the GSSG level, we incubated the aforementioned samples with GSH removal agents at 25 °C for 1 h. The total GSH and GSSG levels were measured with the absorbance set at 412 nm.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from the cells with TRIzol lysis buffer. The purity and concentrations were measured using ultraviolet spectrophotometry (Aokai Biomedical, Beijing, China). First-strand complementary DNA (cDNA) was synthesized from 1 µg total RNA in 20 µg reaction mixture (Taraka, Beijing, China). The primers for superoxide dismutase 2 (SOD2), catalase (CAT), HO-1, NQO1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 1. They were synthesized by Synbio Technologies. cDNA was utilized as a template for PCR amplification using SYBR Green Real-Time PCR Master Mix (Taraka, Beijing, China) at 95 °C for 30 s for denaturing, followed by 40 cycles at 95 °C for 5 s, at 55 °C for 10 s, and at 72 °C for 15 s in an ABI 7500 (Life Technology, Shanghai, China). GAPDH was utilized for normalization.

Western blot

The levels of cytoplasmic and nuclear proteins were determined through western blot. The protein extracts were measured using a Bicinchoninic Acid Protein Assay Kit (Beyotime, Shanghai, China). Equal numbers of protein samples were separated through SDS-PAGE and then incubated with primary antibodies against Nrf2, SOD2, CAT, HO-1, and NQO1 (Santa Cruz Biotechnology, Shanghai, China) and against GAPDH and Histone H3 (Cell Signaling Technology, USA) at 4 °C overnight. GAPDH and Histone H3 were used for cytosol and nuclear normalization, respectively. Horseradish peroxidase-conjugated secondary antibodies were administered after washing. The expression of each protein was determined using a chemiluminescence detection system.

Determination of SOD2 and CAT activities

Following treatments with H₂O₂ with or without metformin as described in the foregoing, the cells were washed and lysed with radioimmunoprecipitation assay buffer containing 1 mM phenylmethylsulfonyl fluoride and maintained on

ice for 30 min. Cell lysates were collected and centrifuged at 10,000 g for 5 min. Supernatants were used to detect the SOD2 and CAT activities through the Total Superoxide Dismutase Assay Kit and Catalase Assay Kit (Beyotime Biotechnology, Shanghai, China).

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was used to investigate the effect of metformin on the DNA-binding activity of Nrf2. After the treatments, nuclear extracts of ARPE-19 cells were prepared using a Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China). DNA-binding assay was performed on the nuclear extracts using a commercial kit (Beyotime, Nantong, China). The EMSA kit employed a 96-well plate to which an oligonucleotide containing the ARE consensus binding site (5'-TGG GGAACCTGTGCTGAGTCACTGGAG-3') had been immobilized. Briefly, complete binding buffer was added to each well, followed by 10 µg nuclear extract samples incubated at room temperature for 20 min. The mixture was loaded on 6% precast polyacrylamide gel in 0.5 × Tris–borate–ethylenediaminetetraacetic acid (TBE) at 100 V for 1 h and then transferred to a nylon membrane in 0.5 × TBE at 100 V for 30 min. The transferred DNA was cross-linked to the membrane and detected using horseradish peroxidase-conjugated streptavidin.

Immunofluorescence

Cells were grown on polylysine-coated glass chamber slides and fixed with 4% paraformaldehyde for 15 min at room temperature after indicated treatments with H₂O₂ and metformin. After treatment with 0.2% Triton X-100 at room temperature for 10 min, polyclonal anti-Nrf2 primary antibody (1:150) was incubated overnight at 4 °C. DAPI staining for 5 min and the fluorescence was visualized using a confocal microscope.

Nrf2 gene knockout by the CRISPR/Cas9-mediated genome

CRISPR small guide RNA (sgRNA) was designed on <http://crispor.tefor.net/>. The target sequence for human Nrf2 was TTGACTTCAGTCAGCGACGGA (exon 3), which was cloned into the lentiCRISPR plasmid. The single-vector lentiviral system also includes SpCas9 expressed from an elongation factor 1a short promoter with a FLAG octapeptide tag and a puromycin resistance selection marker linked by a 2A self-cleaving peptide. ARPE-19 cells were infected with the lentiCRISPR/Cas9-Nrf2 KO construct through a lentiviral transduction system (Life Technology, Austin, USA). Puromycin (EMD Millipore, Hayward, CA, USA) was subjected to the selection of Nrf2 knockout monoclonal cells (Sg-1).

The control cells were transfected with the lentiCRISPR/Cas9 construct with scrambled sgRNA (Sg-NC).

T7 endonuclease I (T7EI) assay was used to screen the mutants produced by the CRISPR/Cas9 system [29]. Genomic DNA from the infected cells was extracted with a commercial kit (Zymo Genomic DNA Isolation Kit, USA) following the manufacturer's protocol and then quantified using a spectrophotometer. The targeted regions in exon 3 of the Nrf2 gene were PCR-amplified with high-fidelity DNA polymerase (Takara, Japan) using primers flanking the target sites, and then purified with a PCR purification kit (Zymo PCR Clean Kit, USA). We denatured 200 ng of the PCR product and then slowly hybridized it to form heteroduplexes, which were digested with T7EI (Beyotime, Shanghai, China) at 37 °C for 15 min. The reaction was stopped with 0.02 M EDTA and the

digested products were separated in 1% Tris–acetate-EDTA agarose gel for analysis.

Statistical analysis

The data were presented as mean \pm standard deviation (SD) and analyzed using the SPSS package for Windows (version 19.0). The normality and homoscedasticity of the data were verified using the Shapiro–Wilk and Levene tests, respectively. The differences between the datasets were analyzed with one-way analysis of variance (ANOVA) or Kruskal–Wallis ANOVA when the data were ordinal, followed by the Bonferroni test for post hoc comparisons, and statistical significance was set at $P < 0.05$.

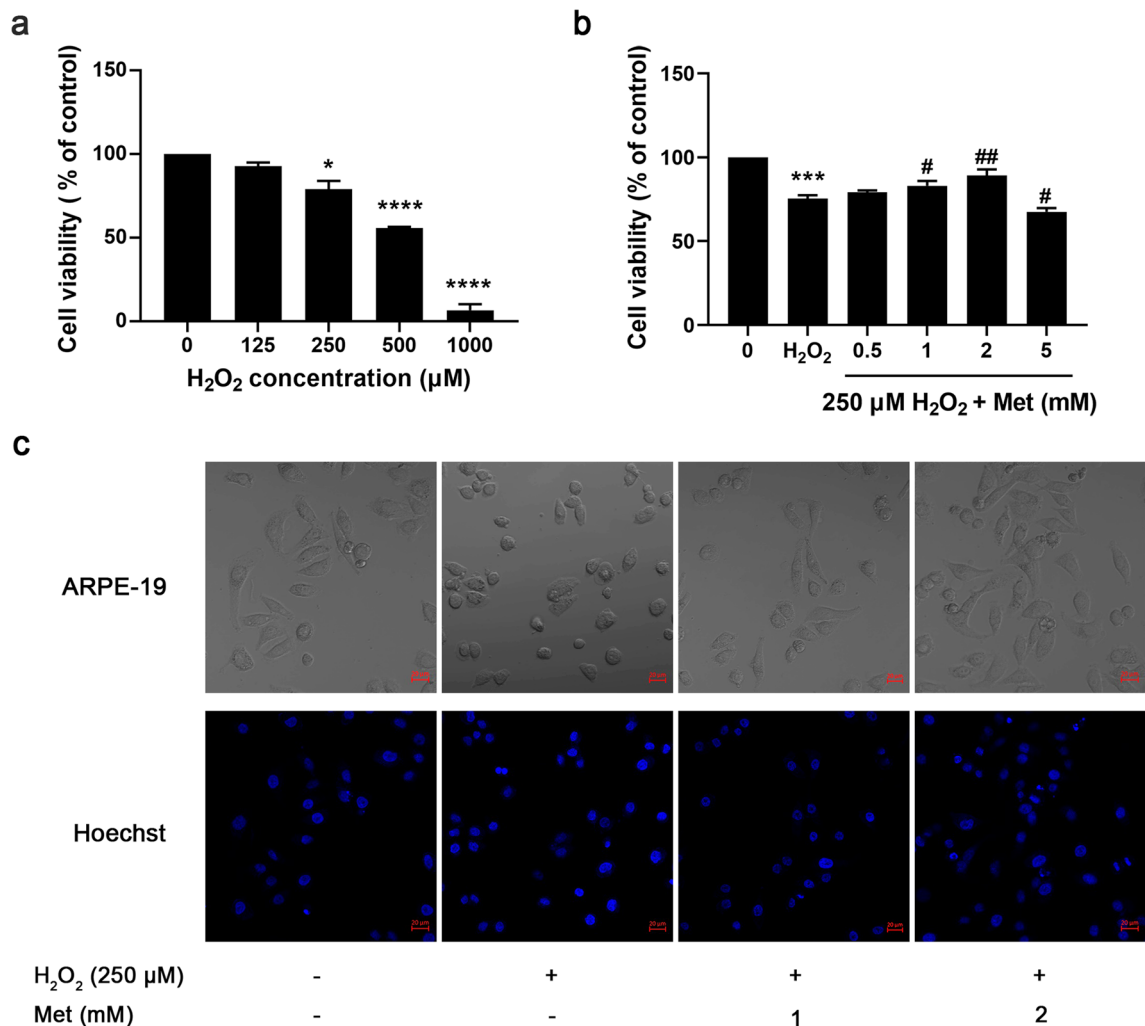


Fig. 1 Metformin restored cell viability and morphology of ARPE-19 cells impaired by H₂O₂. **a** Cell viability determined by CCK-8 assay was decreased by various H₂O₂ concentrations incubated for 24 h. **b** CCK-8 assay for cell viability after treatment with H₂O₂ and metformin. **c** The cell morphology of ARPE-19 cells and Hoechst 33,342

nuclear staining were undertaken after treatments with H₂O₂ with or without metformin (magnification: 20 \times ; scale bar: 20 μ m). Data presented as mean \pm SD. * $P < 0.05$, *** $P < 0.001$ and **** $P < 0.0001$ versus 0 concentration; # $P < 0.05$ and ## $P < 0.01$ versus H₂O₂ alone

Results

Metformin protected ARPE-19 cells against the H₂O₂-induced cell death and morphological changes

To identify the latent role of metformin in AMD treatment, ARPE-19 cells stimulated with H₂O₂ were employed to mimic oxidative stress in AMD pathogenesis. ARPE-19 cells were incubated with increasing concentrations of H₂O₂ (125–1000 μM) for 24 h. Figure 1a shows that cell viability was significantly decreased by H₂O₂ in a dose-dependent manner starting at 250 μM. Cells were then pretreated with

different concentrations of metformin (0.5–5 mM) for 2 h, prior to co-incubation with 250 μM H₂O₂ for another 24 h. Shown as Fig. 1b, the pretreatment of metformin markedly attenuated the cell loss caused by H₂O₂, with increased cell viability by ~13.73% at 2 mM concentration. The cell morphology was altered concomitant with exposure to H₂O₂ from a mosaic arrangement with only a few spindle-shaped cells to the appearance of more round shrinkage cells. Pretreatment with metformin restored the morphology of the oxidative ARPE-19 cells. Furthermore, the nucleus pyknosis and creased-like changes could be observed in the H₂O₂-treated group after Hoechst 33,342 staining, whereas the metformin administration improved it (Fig. 1c).

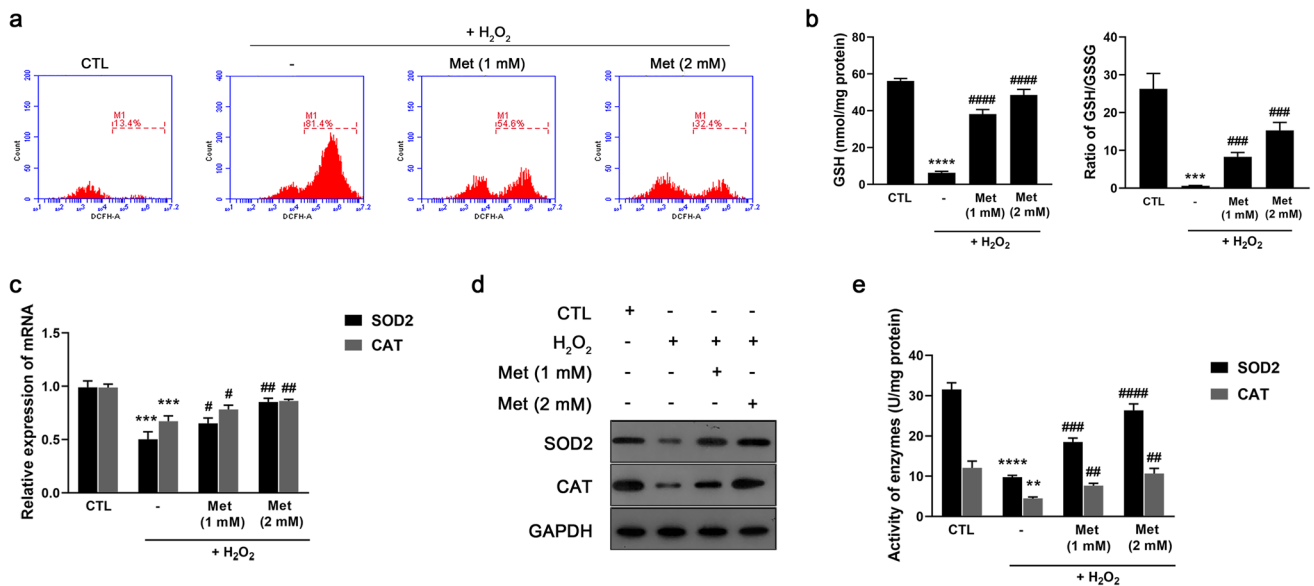


Fig. 2 Metformin ameliorated H₂O₂-induced oxidative stress in ARPE-19 cells. **a** DCFH-DA was used for detection of intracellular ROS. **b** The GSH level detected at 412 nm wavelengths and the GSH/GSSG ratio represented redox state within the cells. **c–e** The expres-

sion and activity of SOD2 and CAT were determined by qRT-PCR, western blot and commercial kits. Data presented as mean ± SD. ****P* < 0.01, *****P* < 0.001, and *****P* < 0.0001 versus CTL; #*P* < 0.05, ##*P* < 0.01, ###*P* < 0.001, and ####*P* < 0.0001 versus H₂O₂ alone

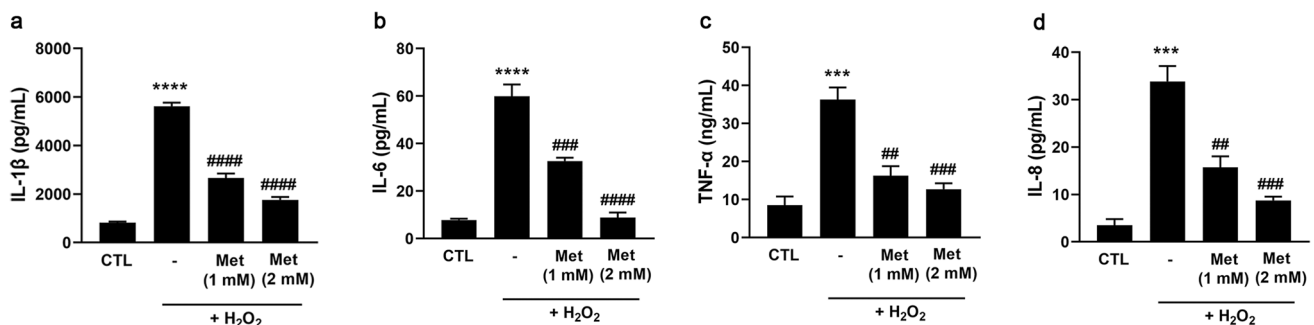


Fig. 3 Metformin attenuated the elevation of pro-inflammatory cytokines induced by H₂O₂ in ARPE-19 cells. **a–d** ARPE-19 cells were pretreated with 1 mM and 2 mM metformin for 2 h plus

250 μM H₂O₂ for 24 h. Data presented as mean ± SD. ****P* < 0.001, *****P* < 0.0001 versus CTL; ##*P* < 0.01, ###*P* < 0.001, *****P* < 0.0001 versus H₂O₂ alone

Metformin attenuated oxidative stress in ARPE-19 cells

Intracellular ROS is an important component of oxidative stress, shown as Fig. 2a; H₂O₂ induced a significant ROS production in ARPE-19 cells. However, the administration of metformin elicited inhibition, and the level of ROS was decreased to ~31.97% at 2 mM concentration. To further identify the oxidative state within ARPE-19 cells, we assessed the non-enzymatic and enzymatic antioxidants. As a pivotal and well-known antioxidant, level of reduced GSH and its ratio with GSSG were alleviated by H₂O₂ exposure, which were markedly reversed by metformin pretreatment (Fig. 2b). Concomitantly, metformin redeemed the oxidative-impaired expression and activity of SOD2 and CAT (Fig. 2c–e).

Metformin inhibited the H₂O₂-induced inflammation in ARPE-19 cells

Oxidative damages can ultimately lead to inflammation which has been well-characterized in the dysfunction of RPE. Protein expression of several pro-inflammatory cytokines in oxidative ARPE-19 cells was explored using ELISA. As shown in Fig. 3a–d, the H₂O₂-induced elevated levels of IL-1 β , IL-6, TNF- α , and IL-8 were obviously suppressed by metformin in a dose-dependent manner.

Metformin pretreatment activated Nrf2 signaling pathway

To identify whether metformin could regulate expression of Nrf2, we carried out the immunofluorescence and western blot. Figure 4a and b show that, after H₂O₂ exposure, the nuclear fraction of Nrf2 was decreased and sequestered in the cytoplasm, compared with the control group. However, these changes were remarkably reversed by the pretreatment of metformin and presented a better nuclear translocation at higher concentration. As a transcription factor, the DNA-binding activity of Nrf2 was also evaluated. The results of EMSA showed that metformin concentration-dependently boosted the binding between Nrf2 and downstream DNA in ARPE-19 cells (Fig. 4c). Expectedly, the expression of Nrf2 target genes (HO-1 and NQO1) was restored by metformin (Fig. 4d, e).

Nrf2 depletion blocked the cytoprotective effect of metformin

To further investigate the role of Nrf2 in metformin cytoprotection, we conducted gene deletion by infecting ARPE-19 cells with Cas9-expressing lentiviral vectors to target Nrf2. Figure 5a shows the frequency of indel formation for sgRNA targeting Nrf2 (Sg-1) using T7EI mismatch detection assay. The protein level of Nrf2 was decreased by ~94% (Fig. 5b). Shown as Fig. 5c, knock-out of Nrf2 further repressed the H₂O₂-induced low expression of

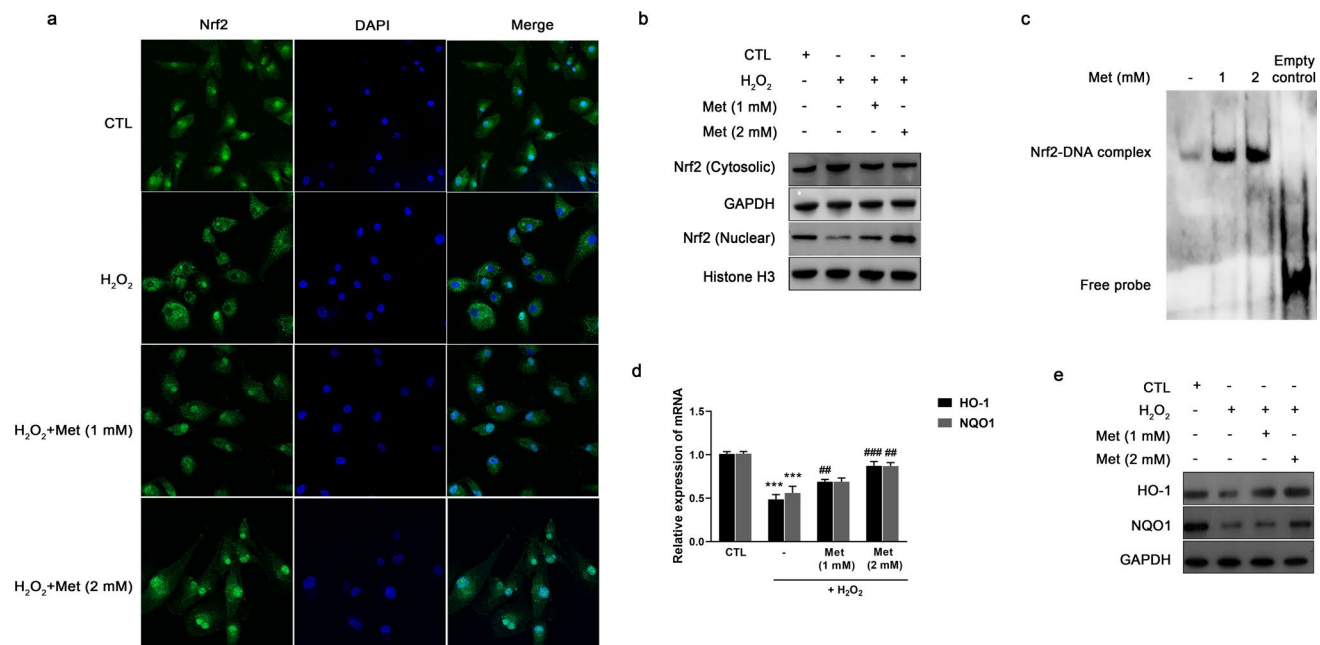


Fig. 4 Effects of metformin on the expression of Nrf2 in ARPE-19 cells. **a**, **b** The nuclear and cytoplasmic fractions of Nrf2 were measured under conditions of metformin+H₂O₂ using immunofluorescence and western blot. **c** EMSA identified the DNA-binding activity of Nrf2 after metformin treatment. **d**, **e** qRT-PCR and western blot

showed the expression of HO-1 and NQO1 with indicated treatments. GAPDH and Histone H3 served as the internal controls. Data presented as mean \pm SD. *** P < 0.001 versus CTL; ## P < 0.01 and ### P < 0.001 versus H₂O₂ alone

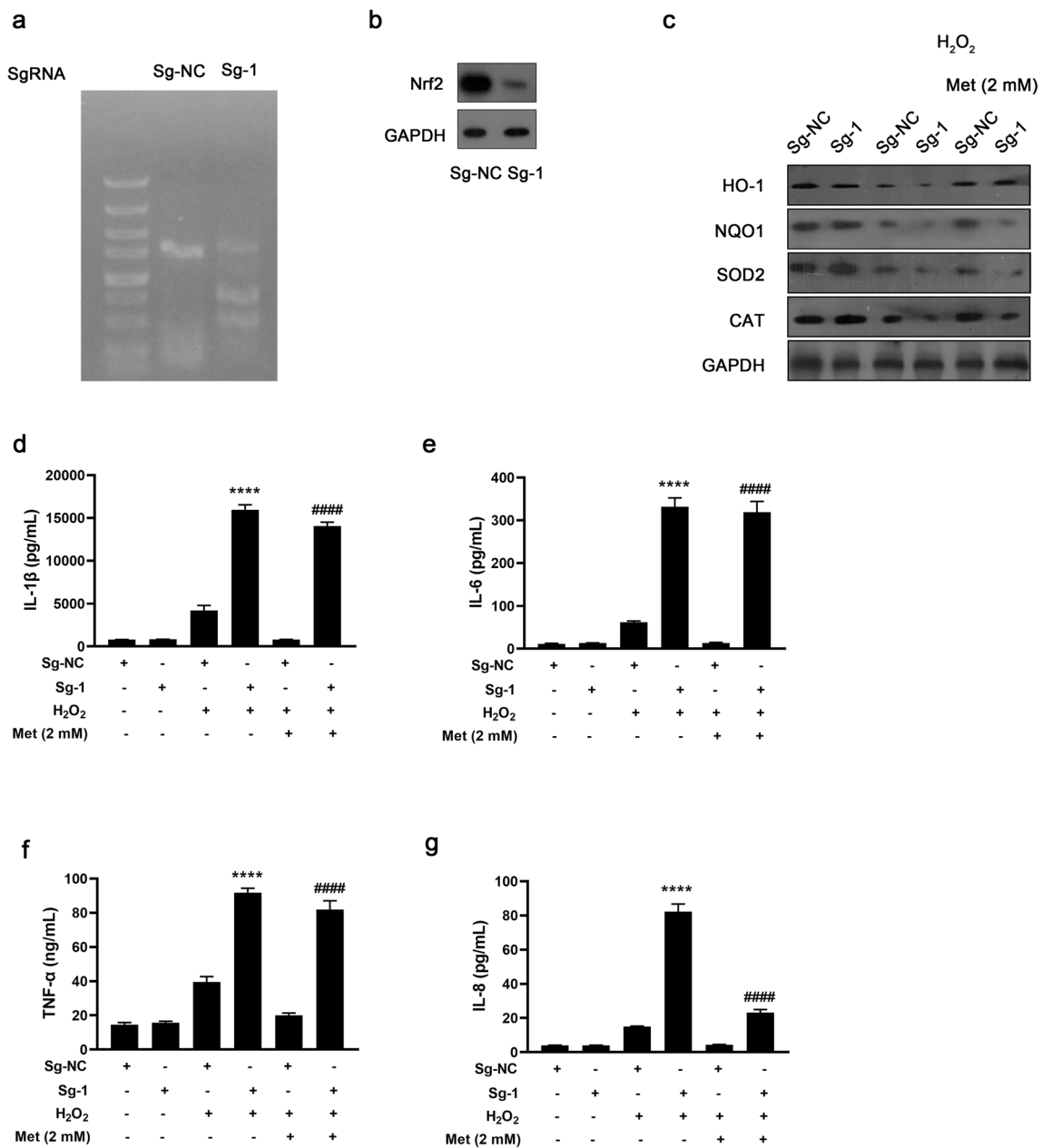


Fig. 5 Knockout of Nrf2 abolished the antioxidative and anti-inflammatory effects of metformin in ARPE-19 cells. ARPE-19 cells transfected with Cas9-expressing lentiviral vectors were incubated with a high concentration of metformin (2 mM) prior to 250 μ M H₂O₂. **a** T7EI mismatch detection assay showed the frequency of indel formation of Sg-1. **b** The protein level of Nrf2 detected by western blot

demonstrated the efficacy of the CRISPR/Cas9 genome knockout system. **c** Changes in the levels of HO-1, NQO1, SOD2, and CAT after Nrf2 knockout. **d–g** Changes in the levels of IL-1 β , IL-6, TNF- α , and IL-8 induced by the genomic disruption of Nrf2. Data presented as mean \pm SD. **** P < 0.0001 versus Sg-NC + H₂O₂; ##### P < 0.0001 versus Sg-NC + H₂O₂ + metformin

several enzymatic antioxidants in ARPE-19 cells when compared with Sg-NC + H₂O₂. Furthermore, administration of metformin could not boost expression of enzymes in the Sg-1 group. Similarly, the H₂O₂-induced inflammation was also exacerbated by the knockout, and the anti-inflammatory effect of metformin was blunted, as indicated by the higher levels of pro-inflammatory cytokines in the Sg-1 + H₂O₂ + metformin group (Fig. 5d–g).

Discussion

Clinical management of AMD is a great challenge with the increasing prevalence of patients worldwide, especially in the developed countries. Currently approved therapies for AMD target only the neovascular stage, whereas there is no treatment available for the other advanced form.

Metformin may be a promising therapeutic candidate due to its well-established safety and the pleiotropic effects in delaying aging and age-related diseases. A large, national-sample analysis also suggests a ~5–10% reduction of the odds ratio of AMD with low to moderate metformin dosage over 2 years [30]. Jiang et al. [31] further reveal that the preventive role of metformin is more efficacious in early-stage AMD. In a recent research in diabetic macular edema patients, metformin enhances the therapeutic effects of anti-VEGF agents to improve visual acuity, which reduces the central macular thickness and the number of intravitreal injections [32]. However, while increasing evidences support the beneficial effect of metformin on AMD, some controversies have emerged. A retrospective cohort study in the UK shows that metformin has no significant association with risk of any AMD [33]. Moreover, diabetic individuals taking metformin do not have a lower cumulative lifetime risk of AMD, but those taking other diabetes medication do [34]. Similar with it, a latest report from The Diabetes Prevention Program concludes that neither metformin usage nor duration of metformin use are associated with risk and severity of AMD [35]. There is still no conclusive result concerning the effectiveness of metformin on control and prevention of AMD.

In the present study, we provided evidence that metformin could prevent oxidative stress and inflammatory damage in the human RPE cell line. Pretreatment with metformin not only boosted both the expression and activity of key antioxidative enzymes but also reduced the levels of pro-inflammatory cytokines. These cytoprotective effects might be associated with the enhanced nuclear translocation and DNA-binding activity of Nrf2, which were further confirmed by the genetic depletion of Nrf2 via the CRISPR/Cas9 genome. Our findings support the clinical observation that metformin use may lower the risk of developing AMD.

The protective roles of metformin have been well-studied in many aging and senescent models [36, 37]. In a diabetic mice model, metformin protected retinal cell death by reducing level of pro-apoptotic factor thioredoxin-interacting protein and inactivating nuclear factor kappa B (NF- κ B) and poly (ADP-ribose) polymerase which were implicated with inflammatory processes and diabetic complications [38]. Coincidentally, metformin pretreatment prevented cell loss and mitochondrial damage by enhancing autophagy within oxidative RPE cells [39]. The antioxidative and anti-inflammatory effects of metformin were also observed in our study. Administration of metformin significantly reversed the H₂O₂-induced low cell viability and high ROS production, and improved the impaired GSH/GSSG ratio and expression of enzymes with a broad spectrum of antioxidant and detoxification. In addition, levels of inflammatory cytokines stimulated by H₂O₂ were dramatically decreased by metformin. As a matter of fact, chronic inflammation is one of

the cellular damages caused by ROS in the pathogenesis of AMD [40]. An environment rich in ROS may induce oxidative modification of phospholipids, nuclear and mitochondrial damage, and activation of programmed cell death of photoreceptors and RPE cells. These can be recognized by immune system and activate the inflammatory responses by promoting secretion of cytokines and chemokines. IL-1 β and TNF- α play a predominant role in the beginning of inflammation via activation of endothelial cells to restrict spreading of damages [41], while IL-8 recruits neutrophils to the site of inflammation to phagocytose [42]. Expression of IL-6 can be elevated directly by the oxidative stress and is postulated to be associated with incidence and progression of AMD [43]. Hence, we considered that the antioxidative role of metformin may be, at least in part, the mechanism of its inhibition of inflammation. Aside from it, numerical evidence also reveals that metformin can suppress degradation of inhibitor of NF- κ B and inactivate cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase, which are well-known factors involved in inflammatory processes, and boost expression of several anti-inflammatory cytokines, via AMP-activated protein kinase (AMPK)-dependent or independent pathways [44–46]. With these great properties of antioxidation and anti-inflammation, we do believe that metformin can be a promising therapeutic candidate for AMD.

Nrf2 is a master regulator of cellular antioxidative defense. The comprehensive properties against oxidative damage include directly or indirectly increasing the levels of enzymes with antioxidation, detoxification, and reduction abilities, promoting GSH synthesis, and regulating the mitochondrial electron-transport chain [25, 47]. Nevertheless, RPE of aging mice showed evidence of an impaired Nrf2 signaling, indicating with a poorer induction under oxidative stress and higher production of superoxide anion and malondialdehyde [48]. Indeed, the downregulated expression of Nrf2 pathway after H₂O₂ treatment was observed in our present study. To further explore mechanisms of metformin, we found that in the metformin treatment groups, the nuclear translocation of Nrf2 and DNA-binding activity were significantly enhanced. With the augmented expression of downstream targets, our results indicated a metformin-induced activation of Nrf2 cascade. The underlying molecular processes that metformin regulates Nrf2 pathways require to be further unveiled, though there may be a crosstalk between the AMPK pathway and Nrf2 [49]. As an activator of AMPK, metformin may upregulate Nrf2 in an AMPK-dependent way, validated by experiments with pharmacological inhibition of AMPK [50, 51]. However, in a primary mouse brain endothelial cell model, pretreatment of AMPK inhibitor did not inhibit the upregulation of Nrf2 pathway upon metformin treatment [52]. This AMPK-independent pathway of metformin was also indicated in cancer cells [53], suggesting there might be a tissue-specific influence.

Although the regulatory process of metformin on Nrf2 remains to be illustrated, the role of Nrf2 in metformin cytoprotection is indisputable. Nrf2 deficiency weakens the protective effects of metformin [54]. Concomitant with it, our study showed that genetic deletion of Nrf2 within ARPE-19 cells not only deteriorated the impaired expression of antioxidative enzymes caused by H₂O₂ but also blunted the antioxidative and anti-inflammatory effects of metformin. It supported that Nrf2 conferred a potent and fundamental defense within cells and was a significant intermedia of metformin treatment. Concerning the mechanism of Nrf2 combating against inflammation, we believed that the inhibition of oxidative stress might be a partial reason. NF-κB is a redox regulated factor, regulating inflammatory responses and cellular injury [55]. Nrf2 can suppress NF-κB activation via decreasing ROS production and increasing antioxidative defenses, while it also can upregulate expression of HO-1 and indirectly prevent degradation of inhibitor protein of NF-κB [56]. Despite these, expression of Nrf2 target gene NQO1 was proved to be negatively associated with levels of TNF-α and IL-1 which was independently from NF-κB [57]. Results of our study confirmed this negative tendency between NQO1 and inflammatory cytokines.

In this study, we used the H₂O₂-cultured ARPE-19 cells as an in vitro model of AMD. Pathophysiological changes mimicked by it provide important molecular basis for investigations of therapeutic effects of metformin, at some extent. However, as a transformed cell line, ARPE-19 cells have their limitations that there may be some alterations in genetics and phenotypes from the original cells after a long period of time and multiple passages. For better simulating AMD in vitro, the primary cultured RPE cells can be used. Zhao et al. [39] did verify the protective effects of metformin on primary human RPE cells showing a dose-dependent way on the prevention of H₂O₂-induced cell viability loss, which was consistent with our findings. It is also of great importance to identify the in vivo roles of metformin. Establishment of animal models of AMD and exploration of metformin's internal application are our next steps. Aside from it, the correlation between the antioxidative and anti-inflammatory pathways has not been fully understood; more details should be provided to better comprehend the pathophysiology of AMD and the therapeutic effects of metformin.

To conclude, the present study demonstrates that metformin has potent antioxidative and anti-inflammatory effects on oxidative stress-induced RPE cells by repairing the damaged expression of antioxidants and reducing levels of inflammatory cytokines via activation of Nrf2 pathway. It raises great potential of metformin for controlling and preventing visual impairment caused by AMD-like retinopathy.

Author contribution Qiting Feng and Yuehong Zhang designed the research; Qiting Feng, Xiangcai Ruan, and Min Lu performed experiments and collected data; Qiting Feng, Xiangcai Ruan, and Shimiao Bu analyzed data; Qiting Feng, Xiangcai Ruan, Min Lu, Shimiao Bu, and Yuehong Zhang wrote the paper. All authors have read and agreed to the published version of the manuscript.

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Data availability All the data is included in this article.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

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