4-Acetoxyphenol Prevents RPE Oxidative Stress–Induced Necrosis by Functioning as an NRF2 Stabilizer

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PURPOSE. Oxidative stress has been suggested to be a major risk factor for the pathogenesis of AMD. Retinal pigment epithelial (RPE) cells are essential for maintaining the homeostasis of the retina, and RPE cell death and the resultant photoreceptor apoptosis have been observed in dry AMD, especially in geographic atrophy. The purpose of this article was to identify and repurpose the Food and Drug Administration–approved natural compound 4-Acetoxyphenol (4-AC), and to evaluate its effect and mechanism in protecting against oxidative stress–induced RPE necrosis.

METHODS. We exposed ARPE-19 cells to tert-Butyl hydroperoxide (tBHP) after pretreatment with 4-AC, and measured cell viability by MTT assay. Aggregation of RIPK3 and HMGB1 nuclear release were analyzed by transfected reporter genes. Reactive oxygen species (ROS) were measured using a commercially available ROS detection system. The importance of the NRF2/NQO1/HO-1 pathway in mediating 4-AC function was corroborated by siRNA studies, qRT-PCR, and immunostaining.

RESULTS. We have identified a natural antioxidant, 4-AC, which demonstrates strong abilities to protect RPE cells from oxidative stress–induced necrosis. Mechanistically, 4-AC blocked the increase of cellular ROS induced by oxidative stress, and upregulated *NQO1* and *HO-1* genes by stabilizing and inducing the nuclear translocation of NRF2 transcription factor. The NQO1, HO-1, and NRF2 were further shown to be required for 4-AC protection of RPE cells from death induced by tBHP. The tBHQ, an NRF2 stabilizer, consistently mimicked the protective effect of 4-AC against tBHP-induced RPE death.

CONCLUSIONS. The compound 4-AC protects ARPE-19 cells from oxidative stress–induced necrosis through upregulation of *NQO1* and *HO-1* genes by stabilization of NRF2.

Keywords: necrosis, age-related macular degeneration, geographic atrophy, 4-Acetoxyphenol (4-AC), NF-E2-related factor-2 (NRF2)

Age-related macular degeneration (AMD) is a degenerative disorder of the central retina and is the leading cause of irreversible central vision loss in elderly populations in developed countries, affecting 30 to 50 million people worldwide. Age-related macular degeneration is classified into early and late stages. Severe visual loss is usually associated only with the advanced (late) forms of the disease: geographic atrophy (GA) and choroidal neovascularization.¹

Geographic atrophy is characterized by scattered or confluent areas of degeneration of RPE cells and the overlying photoreceptors, which rely on the RPE for trophic support. Currently, the mechanism and pathogenesis of GA are still unclear, and no treatment currently exists for GA. Oxidative stress, together with advanced age, smoking, and genetic factors, are considered major contributors to AMD development.^{2,3} The retina is particularly vulnerable to oxidative damage because of its intensive oxygen metabolism, continual exposure to light, high concentrations of polyunsaturated fatty acids, and the presence of photosensitizers, which increase the production of reactive oxygen species (ROS) in the retina.^{4,5} An overload of ROS can lead to RPE cell death and chronic inflammation, which can lead to a pathological immune response in AMD. Consistent with involvement of oxidative stress in dry AMD, the Age-Related Eye Disease Study (AREDS)

formulation, which contains vitamins and zinc, has been shown to slow AMD progression.²

Currently, RPE cell death in AMD is mostly attributed to apoptosis,⁶ although there is some evidence from in vitro and in vivo studies supporting a necrotic nature of RPE cell death.^{7,8} By performing detailed morphological, biochemical, and molecular studies, we recently showed that necrosis is a major mechanism of RPE cell death in response to oxidative stress.⁹ The inflammatory nature of necrosis is consistent with the significant involvement of inflammation in AMD pathogenesis.

Cells have developed a complex system to protect them against oxidative stress. Initial exposure activates a battery of defensive genes that leads to both the detoxification of chemicals and ROS and the prevention of free radical generation so as to promote cell survival. The nuclear factor E2-related factor 2 (NRF2) pathway is a key cellular defense mechanism to combat oxidative stress. The NRF2 is a transcription factor and master regulator of many antioxidant/detoxification genes, including phase 2 antioxidant enzymes.¹⁰ The NRF2 functions by binding to a *cis*-acting element known as the antioxidant response element (ARE), and regulates expression and induction of antioxidant enzyme genes in response to a variety of stimuli, including oxidants, xenobiotics,

metals, and UV irradiation.^{11,12} The RPE of older mice demonstrated impaired induction of the NRF2 pathway following oxidative stress induced with sodium iodate, indicating that the aging RPE is vulnerable to oxidative damage due to impaired NRF2 signaling and that the NRF2 pathway may play an important role in AMD pathogenesis.¹³

Hydroquinone derivatives have established antioxidant properties characterized by different dynamic of NRF2 induction and have been shown to activate phase 2 enzymes.^{14,15}

Hydroquinone has the ability to induce HO-1 expression,¹⁶ whereas its derivatives, like quinone, quercetin, kaempferol, or tert-butylhydroquinone, activate HO-1 and NQO1 expression.¹⁷⁻¹⁹

A hydroquinone derivative, 4-Acetoxyphenol (4-AC), has been shown to be a potent activator of the NRF2-ARE pathway in motor neurons.²⁰ It was also identified as an α -synuclein modulator that can possibly prevent or slow down the onset or progression of Parkinson's disease.²¹

We have identified several natural compounds approved by the US Food and Drug Administration (FDA) that protect oxidative stress-induced RPE death from a chemical library screening.²² Here we provide a detailed study on one of the top candidates, 4-AC. We found that 4-AC, a hydroquinone derivative, has potent antioxidation capability. It protects ARPE-19 cells from oxidative stress-induced necrosis by blocking an increase of intracellular ROS induced by oxidative stress through regulation of antioxidative genes *NQO1* and *HO-1*. Mechanistically, 4-AC induces NRF2 stabilization and nuclear translocation; and NRF2 is required for 4-AC function in preventing RPE cells from oxidative stress-induced necrosis.

METHODS

Cell Culture and Treatments

Human retina pigment epithelium cell lines (ARPE-19) (CLR-2302; ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's (DME/F12) medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone) and 1× penicillin-streptomycin solution (HyClone) at 37°C in 5% CO₂. Both nonconfluent and confluent RPE cells were used in the assays. For monolayer culture, ARPE-19 was seeded at high confluence and maintained for 3 days to form a monolayer. The human dermal fibroblast cell line (HDF) (PCS-201-012; ATCC) was cultured in Dulbecco's modified Eagle's medium/high glucose (HyClone) supplemented with 10% FBS (HyClone) and 1× penicillin-streptomycin solution (HyClone). Human choroidal endothelial cells (hCEC) were kindly provided by Ashwath Jayagopal from Vanderbilt University, and cultured in endothelial cell growth (EGM) medium (Lonza, Walkersville, MD, USA). Both cell types were cultured at 37°C in 5% CO₂. Tert-Butyl hydroperoxide (tBHP) (Sigma-Aldrich Corp., St. Louis, MO, USA) was freshly prepared in growth culture medium before adding to the cultured cells. Cells were treated with 4-AC (Haoyuan Chemexpress, Shanghai, China), ascorbic acid (Sigma-Aldrich Corp.), α -Tocopherol (Sigma-Aldrich Corp.), tBHQ (dissolved in 0.01% ethanol; Sigma-Aldrich Corp.), and Necrostatin 1, 5, and 7 (Enzo Life Sciences, Farmingdale, NY, USA) for 24 hours before induction of oxidative stress, unless stated otherwise.

Isolation of Human RPE Cells and Cell Culture

Human donor eyes were obtained from the Southern Eye Bank (Metairie, LA). Human RPE cells (hRPE) were harvested as described.²³ In brief, the anterior segment from each donor eye was removed and the neural retinas were carefully peeled

away from the RPE-choroid-sclera. The eyecup was rinsed with Ca²⁺ and Mg²⁺ free 1× PBS (Gibco, Grand Island, NY, USA) and treated with 0.25% trypsin (Gibco) for 1 hour at 37°C. The trypsin was neutralized with DME/F12 1:1 medium (Gibco) supplemented with 20% FBS (Atlanta Biologicals, Norcross, GA, USA). Medium was gently agitated to release RPE into the media.

The hRPE cells were cultured in DME/F12 1:1 medium supplemented with 20% FBS at 37°C and 5% CO₂. After reaching confluence, hRPE cells were subcultured in DME/F12 1:1 with 10% FBS at 37°C with 5% CO₂. The hRPE cells were characterized by RPE65 immunostaining and Western blot (anti-RPE65; Santa Cruz Biotechnology, Dallas, TX, USA).

Cell Viability Assay, ATP Level, and ROS Detection

Both MTT assay and CellTiter-Glo (Promega, Madison, WI, USA) were used to measure cell viability. The MTT assay and cellular ATP level detection were performed as described previously.⁹ CellTiter-Glo was used to measure cell viability of cells growing in monolayer. Equal volume of the reagent was added to the cell culture medium and mixed for 2 minutes on an orbital shaker. Then the plate was incubated for 10 minutes at room temperature to stabilize the luminescence. The fluorescence signal was read using a Perkin Elmer Victor X3 Multilabel Plate Reader (Waltham, MA, USA).

Detection of ROS was performed using the total ROS/Superoxide Detection Kit (Enzo Life Sciences). In brief, ARPE-19 cells were pretreated with 4-AC for 24 hours before inducing oxidative stress. Cells were then incubated with oxidative stress detection reagent for 1 hour, then washed three times with wash buffer before 150 μ M tBHP treatment for 30 minutes. After the treatment, growth medium was removed. Cells were then washed three times with wash buffer supplied by the manufacturer, dried briefly, and overlaid with mounting medium, and analyzed under fluorescence microscope (Nikon, Melville, NY, USA). Captured pictures were analyzed with ImageJ software (<http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) to quantify mean gray value after background subtraction.

Analyses of RNA

Quantitative (q)RT-PCR was used to test the effect of 4-AC on expression of genes involved in response to oxidative stress. We purified RNA from ARPE-19 cells using RNeasy Mini Kit (Qiagen, Waltham, MA, USA), followed by DNase I treatment (Thermo Scientific, Waltham, MA, USA); cDNA was synthesized with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Primers used were as follows: NQO1:

5'-GCACTGATCGTACTGGCTCA-3' and 5'-CATGGCATAGAGGTCCGACT-3'; HO-1:
5'-AACTTTCAGAAGGGCCAGGT-3' and 5'-GTAGACAGGGGCGAAGACTG-3'; NRF2:
5'-CCTGGGAACAGAACAGGAAA-3' and 5'-TTCTGTGCAACAGTTCAGG-3'; Cyclophilin A:
5'-CCAGTGCTCAGAGCACGAAA-3' and 5'-CCCACCGTGTTCCTCGACAT-3'.

Immunostaining

Immunostaining of NRF2 was performed as described previously.²⁴ Briefly, cells were washed three times with 1× PBS and fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich Corp.) for 30 minutes at room temperature. Next, cells were washed three times with PBS and permeabilized with PBS containing

0.1% Triton X-100 (Sigma-Aldrich Corp.) for 30 minutes at room temperature. In the next step, cells were blocked with 1× PBS containing 3% horse serum (Gibco) for 30 minutes. Anti-NRF2 antibody (Santa Cruz Biotechnology) was diluted 1:250 in 1× PBS containing 3% horse serum and incubated with cells overnight at 4°C, followed by washing with 1× PBS. Secondary antibody Alexa Fluor 594 rabbit anti-mouse (Life Technologies, Carlsbad, CA, USA) was prepared in 1:800 dilution in 1× PBS containing 3% horse serum and incubated with cells for 30 minutes at room temperature, followed by washing with 1× PBS. Next, cells were dried and mounted with 4',6-diamidino-2-phenylindole containing mounting medium for fluorescence (Vector, Burlingame, CA, USA) and analyzed under fluorescence microscope.

Luciferase Activity

The ARPE-19 cells, cultured in a 96-well plate, were transfected with 100 ng pTi-hQR41luc14 plasmid (kind gift from Jeffrey Johnson, University of Wisconsin)²⁵ and 20 ng pCMV-β-gal as normalization control. Next day after transfection, the medium was replaced with fresh medium containing 5 μM 4-AC. Luciferase assay was performed 24 hours later as described.²⁶

Plasmid and Small Interfering RNA (siRNA) Transfection

Plasmid and siRNA transfection was performed as described previously.⁹ The following siRNAs were used: *NRF2* (sense: 5'-GACUCUUAUUGGAUACAGU-3'; antisense:

5'-ACUGUAUCCAUAAGAGUC-3'), *NQO1* (sense: 5'-GAUGUAGAAAGAUGCUGA-3'; antisense: 5'-UCUAGCAUCUUCUACAUC-3'), *HO-1* (sense: 5'-CUGUGUCCUCUCUGGA-3'; antisense: 5'-UCCAGAGAGAGGGACACAG-3').

Protein Half-Life Measurement

To measure the half-life of NRF2, ARPE-19 cells were either treated or not treated with 5 μM 4-AC for 24 hours. Cycloheximide (40 μg/mL) was added to block protein synthesis. Total cell lysates were collected at different time points and subjected to immunoblot analysis with anti-NRF2 antibody.

Western Blot

The ARPE-19 cells were treated with 150 μM tBHP, 5 μM 4-AC, or 10 μM tBHQ for 30 minutes. Next, cells were trypsinized and collected by centrifugation, washed briefly with PBS, and resuspended in the lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Thermo Scientific). Antibodies used included rabbit polyclonal anti-NRF2 (1:1000; Santa Cruz Biotechnology) and mouse monoclonal anti-α-Tubulin (1:5000; Cell Signaling, Danvers, MA, USA). Following primary antibody incubation, membranes were probed with IRDye 800CW donkey-anti-mouse IgG (LiCOR) or IRDye 680RD goat-anti-rabbit IgG (LiCOR, Lincoln, NE, USA) secondary antibodies, and imaged and quantified using the LiCOR Odyssey system.

Statistics

Each experiment was repeated at least three times. Student's *t*-tests were used to determine statistical significance between

groups. *P* values of less than 0.05 were considered to be statistically significant.

RESULTS

The 4-AC Protects RPE Cells From Oxidative Stress-Induced Cell Death

In an effort to identify natural compounds that protect oxidative stress-induced RPE cell death, we conducted a chemical screening of a library with 1840 FDA-approved drugs and natural products (The Spectrum Collection; MicroSource Discovery Systems, Inc., Gaylordsville, CT, USA)²⁷ using tBHP as a stressor.²² One of the major compounds we identified was 4-AC (Fig. 1A), a hydroquinone derivative with the ability to drastically protect ARPE-19 cells from tBHP (150 μM)-induced cell death (Fig. 1B). The 4-AC itself did not impact cell morphology or cell viability when used at a broad range of concentrations (0.01–100 μM), suggesting the safe use of 4-AC in RPE cells (Fig. 1B, Supplementary Fig. S1A). We also tested the effect of 4-AC in protecting ARPE-19 monolayer. We found that 4-AC protected up to 89%, 92%, and 90% of ARPE-19 cells exposed to 100, 200, and 300 μM tBHP, respectively, compared with 66%, 19%, and 8% survival in the control (Fig. 1C). We also tested the effect of 4-AC on isolated human RPE cells, and observed that 4-AC protected up to 100% of hRPE from tBHP-induced cell death compared with 58% (400 μM tBHP) in the control (Fig. 1D).

To further characterize the potency of 4-AC on ARPE-19 confluent cells, we compared its activity to other antioxidants that are included in the AREDS, such as α-Tocopherol (vitamin E) and ascorbic acid (vitamin C).²⁸ The 4-AC protected up to 98% of cells in comparison with average 70% rescue when vitamins E and C were used at their published concentrations (100 μM) from tBHP-induced cell death, and average of 60% when both vitamins E and C were used at 5-μM concentration. Interestingly, when subconfluent ARPE-19 cells were tested, vitamin E at 100 μM rescued cells from tBHP-induced RPE death at a similar level to 5 μM 4-AC, whereas vitamin C (100 μM) failed to protect RPE cells under the same setting (Supplementary Fig. S1D). When used at the same lower concentrations as 4-AC (5 μM), neither vitamin E nor vitamin C demonstrated the capability to protect ARPE-19 from tBHP-induced cell death (Supplementary Fig. S1D).

We also tested the effect of different concentrations of 4-AC in protecting ARPE-19 monolayer from tBHP-induced cell death. We found that 4-AC at concentrations of 5 μM had the highest efficiency in protecting ARPE-19 cells (up to 98%), which gradually decreased to 87% and 70% with 2.5 μM and 1 μM 4-AC pretreatment, respectively (Fig. 1F). However, in subconfluent ARPE-19 cells, the level of cell protection was comparable when 4-AC was used at 1.0, 2.5, or 5.0 μM, with no statistical significance between them (Supplementary Fig. S1E).

To test whether 4-AC protects against oxidative stress-induced cell death in other cell types, we analyzed its effect on human choroidal endothelial cells (hCECs) and human dermal fibroblasts (HDEFs). Both cell types grown in monolayer were sensitive to tBHP-induced cell death. Pretreatment with 4-AC for 24 hours stimulated growth of hCECs and effectively protected them from oxidative stress-induced cell death (Supplementary Fig. S1B). The 4-AC protected up to 93% of HDEF cells from tBHP-induced death in comparison with 32% in the control (Supplementary Fig. S1C).

To conclude, we have identified an FDA-approved natural compound, 4-AC, that potently protects cells from tBHP-induced cell death without detectable toxicity.

FIGURE 1. The 4-AC protects ARPE-19 cells from oxidative stress-induced cell death. **(A)** Chemical structure of 4-AC. **(B)** Light microscopy revealed that pretreatment with 4-AC for 24 hours protects ARPE-19 cells from 150 μM tBHP-induced cell death as shown by cell density and morphology. **(C)** The ARPE-19 cultured in monolayer was pretreated with 5 μM 4-AC for 24 hours, followed by exposure to different concentrations of tBHP. Cell viability was measured 24 hours later by CellTiter-Glo assay. **(D)** The 4-AC protects hRPE cells from 400 μM tBHP-induced cell death measured by CellTiter-Glo assay. **(E)** Comparison of 4-AC antioxidant activity at 5 μM concentration with other well-established antioxidants: vitamin E (α -Tocopherol) and vitamin C (ascorbic acid) used at 100- μM or 5- μM concentration. **(F)** Protection of ARPE-19 monolayer by 4-AC in low concentration range (1–5 μM); ARPE-19 viability was measured by CellTiter-Glo assay. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Scale bar: 25 μM .

FIGURE 2. The 4-AC inhibits induction of intrinsic necrotic pathway. (A) Protection of ARPE-19 cells from ATP depletion induced by 150 μ M tBHP by 4-AC pretreatment. (B) Activation of RIPK3 (arrow points at RIPK3 aggregates) by tBHP in RIPK3-GFP-transfected ARPE-19 cells (a, b) was inhibited by 24-hour pretreatment with 5 μ M 4-AC (c, d). (C) Passive release of HMGB1 (marked by the arrow) is a result of increased permeability of the nuclear envelope and biochemical hallmark of necrosis in HMGB1-YFP-transfected ARPE-19 (a, b); pretreatment with 5 μ M 4-AC for 24 hours inhibited HMGB1 release (c, d). (D) Comparison of biological properties of 4-AC and necrostatins in protecting ARPE-19 cells from oxidative stress-induced necrosis. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Scale bar: 50 μ M.

The 4-AC Inhibits Oxidative Stress-Induced RPE Necrosis

We have recently shown that RPE cells die primarily from necrosis in response to oxidative stress.⁹ We asked whether 4-AC protects RPE cells from tBHP-induced cell death by preventing the activation of the necrotic pathway. In contrast to apoptosis, necrosis is associated with dramatic ATP reduction in cells. We analyzed the effect of 4-AC on intracellular ATP level in cells exposed to oxidative stress. The ARPE-19 cells exposed to tBHP showed rapid ATP depletion within the first 3 hours. With 4-AC pretreatment, the ATP level in ARPE-19 cells was maintained when treated with tBHP, suggesting necrosis was prevented by 4-AC in RPE cells (Fig. 2A).

We also examined the effect of 4-AC on other hallmarks of necrosis. When RIPK3-GFP expression plasmid was transfected

into confluent ARPE-19 cells, formation of discrete punctuations was observed in control cells after exposure to 200 μ M tBHP, which indicated the activation of RIPK3 and induction of necrosis (Figs. 2Ba, 2Bb). Pretreatment with 4-AC prevented RIPK3 activation when cells were exposed to tBHP (Figs. 2Bc, 2Bd). Passive release of HMGB1 from the nucleus to the cytoplasm is another hallmark of necrosis.²⁹ In the control confluent ARPE-19 cells transfected with HMGB1-YFP expressing plasmid, HMGB1 tightly binds to the chromatin. The tBHP treatment led to permeability of the nuclear envelope and passive release of HMGB1 from the chromatin to the cytoplasm (Figs. 2Ca, 2Cb). Pretreatment with 4-AC inhibited the HMGB1 release caused by tBHP treatment (Figs. 2Cc, 2Cd).

We also compared the potency of 4-AC with inhibitors of necrosis. Necrostatins (Nec) are inhibitors of receptor-interacting protein (RIP) kinase. Necrostatin-1 (Nec-1) is a small

FIGURE 3. The 4-AC activates phase 2 antioxidant enzymes in ARPE-19 cells. (A) Pretreatment with 4-AC inhibited ROS accumulation in cells exposed to oxidative stress induced by 150 μ M tBHP. (B) Pretreatment for 24 hours with 5 μ M 4-AC induced the expression of *NQO1* and *HO-1* as measured by qRT-PCR. Exposure of ARPE-19 to 150 μ M tBHP for 30 minutes does not result in induction of *NQO1* or *HO-1* expression and did not affect upregulation of these genes by 4-AC. (C) Pretreatment for 24 hours with 5 μ M 4-AC induced the expression of *NQO1* and *HO-1* in hRPE cells measured by qRT-PCR. (D) Downregulation of *NQO1* abolished the ability of 4-AC to protect ARPE-19 cells from oxidative stress-induced cell death. (E) Downregulation of *HO-1* abolished the ability of 4-AC to protect ARPE-19 cells from oxidative stress-induced cell death. ** P < 0.01; *** P < 0.001.

molecule that specifically inhibits RIP1 function.³⁰ Necrostatin-5 is a necrosis inhibitor that inhibits RIP1 kinase indirectly,³¹ whereas Nec-7 targets other elements of the necrosis pathway but does not inhibit RIP1 kinase activity.³² We show that 4-AC protects ARPE-19 cells at a comparable level when used at a 6-fold lower concentration compared with Nec (5 μ M for 4-AC versus 33 μ M for Nec), indicating that 4-AC has strong capability in protecting against oxidative stress-induced RPE cell necrosis (Fig. 2D).

Taken together, our observations indicate that 4-AC has the ability to inhibit oxidative stress-induced necrosis by preventing the activation of RIP3 kinase and therefore inhibiting downstream events in the necrotic pathway.

The 4-AC Blunts Cellular ROS Increase in Response to Oxidative Stress and Upregulates the Expression of Cytoprotective Genes

Hydroquinone derivatives are known for their antioxidant properties. We first investigated whether 4-AC, as a hydroqui-

none derivative, has an effect on intracellular ROS level induced by oxidative stress. Treatment with 150 μ M of tBHP for 1 hour increased ROS level by 2-fold, which was blunted by 4-AC pretreatment (Fig. 3A). However, 4-AC treatment alone did not affect ROS level at the baseline. This finding confirms that 4-AC has antioxidative property in RPE cells.

Having established that 4-AC regulates intracellular ROS level, we next used qRT-PCR to analyze whether 4-AC regulates the expression of an array of the genes involved in response to oxidative stress. We found that the expression of two cytoprotective enzymes (or phase 2 enzymes), *NQO1* and *HO-1*, was significantly upregulated by 4-AC by approximately 3.3-fold and approximately 2.5-fold, respectively (Fig. 3B). Interestingly, the expression of these two genes was not affected by half-hour tBHP treatment, indicating that they are regulated by 4-AC independently of tBHP. A similar effect was observed in isolated hRPE cells ex vivo. Pretreatment with 4-AC induced upregulation of *NQO1* and *HO-1* by approximately 3.6- and approximately 1.8-fold, respectively (Fig. 3C). Further

tBHP treatment did not affect the expression level of these genes.

To test whether NQO1 is required for 4-AC function, ARPE-19 cell survival was analyzed after NQO1 knockdown by NQO1 siRNA transfection and treatment with 150 μ M tBHP. Silencing of *NQO1* was verified by qRT-PCR (Supplementary Fig. S2A). Downregulation of *NQO1* did not affect the survival of ARPE-19 cells at baseline or in response to oxidative stress ($39.1\% \pm 2.1\%$ cell survival). However, *NQO1* silencing blunted the ability of 4-AC to protect ARPE-19 cells and reduced cell viability to $46.5\% \pm 3.2\%$ (Fig. 3D). Similarly, downregulation of *HO-1* (Supplementary Fig. S2B) abolished ability of 4-AC to protect ARPE-19 cells from oxidative stress-induced necrosis (Fig. 3E). Taken together, both NQO1 and HO-1 are needed for 4-AC function in protecting against tBHP-induced RPE death.

Requirement of NRF2 in Mediating 4-AC Function

The NRF2 is a transcription factor that protects against oxidative stress by binding to the ARE in the promoter of its target genes, including NQO1 and HO-1.³³ We next studied whether NRF2 activity is regulated by 4-AC in ARPE-19 cells by examining its effect on ARE activity using luciferase reporter assay. Pretreatment with 4-AC for 24 hours induced significant increase in luciferase activity by 2.7 ± 0.6 -fold, indicating activation of NRF2 transcription factor (Fig. 4A). NRF2 mRNA level was not affected by 4-AC, measured by (q)RT-PCR, suggesting 4-AC functions at the NRF2 protein level (Supplementary Fig. S3). We next tested whether the increased NRF activity correlated with the nuclear localization of NRF2. Normally, NRF2 is localized in the cytoplasm in ARPE-19 cells (Fig. 4Ba). Treatment with 200 μ M tBHP for 30 minutes only occasionally induced the translocation of NRF2 to the nucleus (Fig. 4Bb). Pretreatment with 4-AC for 24 hours resulted in robust translocation of NRF2 to the nucleus with or without induction of oxidative stress (Figs. 4Bc, 4Bd). We also examined the NRF2 stability in response to 4-AC treatment. The half-life of NRF2 in ARPE-19 cells in the presence of 4-AC was evaluated by Western blot. The amount of NRF2 was decreased to approximately 25% after treatment with cycloheximide for 30 minutes. However, pretreatment with 4-AC significantly extended NRF2 protein half-life, as approximately 96% of NRF2 protein was detected at 30 minutes and approximately 83% of NRF2 protein was detected at 45 minutes after cycloheximide treatment (Fig. 4C).

To further examine whether NRF2 is required for mediating 4-AC function in protecting ARPE-19 from oxidative stress-induced cell death, NRF2 was silenced in ARPE-19 cells by a specific siRNA (Supplementary Fig. S2C). Knockdown of *NRF2* did not significantly reduce ARPE-19 cell survival under baseline or on tBHP treatment. However, it blunted the protective effect of 4-AC in RPE cells (Fig. 4D). To test whether NRF2 is required for NQO1 and HO-1 expression induced by 4-AC, *NQO1* and *HO-1* expression was examined after NRF2 knockdown and tBHP treatment. Expression of *NQO1* was dramatically reduced with or without 4-AC treatment (Fig. 4E). However, *HO-1* expression increased approximately 10-fold as a result of NRF2 downregulation (Fig. 4F), and 4-AC treatment surprisingly blunted the *HO-1* upregulation by NRF2 knockdown. In summary, these results proved that NRF2 is required for mediating 4-AC protective activity in RPE cells and 4-AC-regulated antioxidant gene expression. Additionally we conclude that NQO1 is regulated by NRF2 to mediate 4-AC antioxidant response, whereas HO-1 may be regulated by a compensatory or NRF2-independent mechanism.

Effect of NRF2 Stabilizer in RPE Cell Survival in Response to Oxidative Stress

Having shown 4-AC as an NRF2 stabilizer in RPE cells, we asked whether a known NRF2 stabilizer can mimic 4-AC function and prevent RPE cells from oxidative stress-induced cell death. Tert-Butylhydroquinone (tBHQ) is a highly effective antioxidant and a stabilizer of NRF2.^{34,35} First we compared protective capability of 4-AC and tBHQ. The tBHQ at 10 μ M or 4-AC at 5 μ M showed similar protection of ARPE-19 cells from tBHP-induced oxidative stress cell death (Fig. 5A). We further asked if the stabilization of NRF2 by tBHQ upregulates the expression of NRF2 target gene NQO1. By qRT-PCR, *NQO1* was upregulated to similar levels by 30-minute treatment of either 4-AC or tBHQ (Fig. 5B). By immunostaining, both compounds induced the translocation of NRF2 to the nucleus in ARPE-19 cells (Fig. 5C).

Taken together, our experiments suggest that NRF2 stabilization is a critical mechanism for protecting RPE from necrotic cell death in response to oxidative stress.

DISCUSSION

In the present study, we provided evidence that 4-AC, an FDA-approved natural compound, functions as a novel potent inhibitor of tBHP-induced RPE cell necrosis. The mechanism of 4-AC action is reflected at least partially by upregulating the expression of two antioxidative enzymes NQO1 and HO-1 through stabilizing NRF2 transcription factor and inducing its nuclear translocation. The 4-AC may represent an ideal repurposed drug for dry AMD, especially GA.

The 4-AC Protects Oxidative Stress-Induced RPE Necrosis

Oxidative stress has been suggested to be a major factor for AMD pathogenesis. To study the mechanism of how oxidative stress induces RPE cell death in GA, we recently demonstrated that necrosis is a major type of RPE cell death in vitro.⁹ To identify chemical compounds that repress oxidative stress-induced RPE cell death, we performed a library screening of 1840 FDA-approved natural compounds, with hope to find a repurposed drug that prevents oxidative stress-induced RPE death for AMD and/or GA treatment.²² We used tBHP as an inducer of oxidative stress in RPE cells. The tBHP is an organic peroxide that induces lipid peroxidation, a self-propagating form of oxidative injury that damages cell membranes and is a particular risk to RPE and the lipid-rich photoreceptor cells.²⁸ The 4-AC stood out as one of the top candidate compounds that showed potent protection of RPE cell death from tBHP-induced oxidative stress. The 4-AC is a hydroquinone derivative. To date, no reports have been found regarding the function or biological properties of 4-AC. Pretreatment with 4-AC rescued ARPE-19 cell survival up to 90% when confluent cells were exposed to 100 to 300 μ M tBHP, and it also had a protective effect on hRPE cells, protecting up to 100% of cells from 400 μ M tBHP-induced cell death. Protective abilities of 4-AC were also tested on different cell types, showing that both hCEC and HDeF cells were protected from tBHP-induced cell death. Protective ability of 4-AC persisted when used at as low as 1 μ M, and no apparent toxicity was observed when used as high as 250 μ M. Importantly, 4-AC had a better protective effect on confluent ARPE-19 cells than subconfluent cells, with on average an 80% cell survival rate. To confirm that 4-AC protects against oxidative stress-induced RPE cell necrosis, we found 4-AC prevented the depletion of cellular ATP induced by tBHP, as well as RPE necrosis shown by the lack of RIPK3 activation and

FIGURE 4. The NRF2 mediates antioxidant properties of 4-AC. (A) Pretreatment with 4-AC for 24 hours induced activity of ARE-Luc, as shown by luciferase assay. (B) Pretreatment with 4-AC resulted in translocation of NRF2 to the nucleus as detected by immunostaining. (C) The half-life of NRF2 was extended by 4-AC pretreatment. The ARPE-19 cells were treated with cycloheximide for the indicated times. Protein level of NRF2 was tested by Western blot. (D) Downregulation of NRF2 abolished the ability of 4-AC in protecting ARPE-19 cells from oxidative stress-induced cell death. (E) Downregulation of NRF2 by siRNA inhibited 4-AC-mediated upregulation of *NQO1*. (F) Downregulation of NRF2 by siRNA induced the expression of *HO-1* and did not affect 4-AC-mediated upregulation of *HO-1*. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Scale bar: 25 μ M.

FIGURE 5. The 4-AC induces stabilization and activation of NRF2. (A) Comparison of 5 μM 4-AC and 10 μM tBHQ in protecting ARPE-19 cells from oxidative stress-induced necrosis. The ARPE-19 cells were pretreated for 24 hours before induction of oxidative stress. Viability of ARPE-19 was measured 24 hours later with MTT assay. (B) Expression level of *NQO1* was measured by qRT-PCR 24 hours after treating ARPE-19 cells with 5 μM 4-AC or 10 μM tBHQ. (C) Activation of NRF2 in ARPE-19 cells was analyzed with immunostaining 24 hours after treating cells with 5 μM 4-AC (c) or 10 μM tBHQ (d) in comparison with control cells (a) or cells treated with 200 μM tBHP for 30 minutes (b). *** $P < 0.001$.

HMGB1 release. These findings put oxidative stress upstream of the RIPK3 activation in the intrinsic necrotic pathway, further confirming necrosis as a major pathway downstream of oxidative stress in RPE cells.

The 4-AC is an Antioxidant That Functions by Upregulating NQO1 and HO-1 Gene Expression Through Stabilizing NRF2 Transcription Factor

To define the mechanism whereby 4-AC protects RPE cell death, we found 4-AC blunted cellular ROS increase induced by oxidative stress. This is consistent with our data showing upregulation of the expression of two antioxidative and/or detoxification enzyme genes NQO1 and HO-1 by 4-AC from our small-scale gene expression profiling. The NQO1 is an NAD(P)H quinone oxidoreductase that catalyzes the transformation of quinones into antioxidative forms; HO-1 is a hemeoxygenase that catalyzes heme degradation and has been recognized as an important antioxidant enzyme of the cells against the stress response. Both are members of phase 2 detoxifying enzymes that counteract oxidative stress insults. The importance of NQO1 and HO-1 in mediating antioxidant and anti-inflammatory effects has been well documented.^{36,37} Although there is no report about 4-AC, consistent with our results, other hydroquinone derivatives have been shown to function as antioxidants by activating phase 2 enzymes.^{14,15} By

knocking down of NQO1 or HO-1 in ARPE-19 cells, we found both NQO1 and HO-1 are required for mediating the protective effect of 4-AC, because the 4-AC protective effect was lost when either of the two genes was silenced in ARPE-19 cells.

Both NQO1 and HO-1 have been shown to be regulated by the NRF2-ARE pathway.^{38–40} The NRF2 is a master regulator of the endogenous antioxidative protection program by binding to the ARE element of genes encoding phase 2 enzymes, including NQO1 and HO-1, therefore controlling their expression. Interestingly, *Nrf2* knockout mice display an ocular phenotype similar to human AMD.⁴¹ To further determine whether NRF2 is mediating the effect of 4-AC in RPE survival and the regulation of *NQO1* and *HO-1* expression, we examined the effect of 4-AC on NRF2 by immunostaining and Western blot analysis. Activation and stabilization of NRF2 results in its translocation to the nucleus, where it forms a heterodimer with one of the small MAF proteins and binds to the ARE element to activate transcription.⁴² We found that 4-AC induced NRF2 translocation to the nucleus regardless of tBHP treatment. Those findings are consistent with a significant increase in ARE activity by luciferase assay. We further studied the stability of NRF2, and found that 4-AC stabilizes NRF2 in ARPE-19 cells by extending its half-life time. The NRF2 mRNA level was not affected by either 4-AC or tBHP treatment, confirming that 4-AC regulates NRF2 at the protein level. To confirm whether NRF2 stabilization by 4-AC leads to its nuclear translocation and target gene activation, an

established NRF2 stabilizer tBHQ was used to see if it could mimic the effect of 4-AC. We found tBHQ at 10 μ M can protect against RPE cell death induced by oxidative stress as efficient as 5 μ M 4-AC, therefore mimicking the effect of 4-AC. Furthermore, we found tBHQ also increases NRF2 nuclear translocation and activates the expression of its target genes, suggesting NRF2 stabilization is sufficient to induce NRF2 nuclear translocation and increases expression of its target gene. Several natural compounds have been shown to activate NRF2-dependent response in RPE cells. Salvianolic acid A and pinosylvin have been shown to activate HO-1 expression.^{43,44} Zeaxanthin, a major carotenoid pigment in human retina, has been shown to induce NRF2-dependent activation of glutathione.⁴⁵ Stabilization of NRF2 has been extensively studied in neurons where NRF2 stabilization resulted in protection against oxidative stress-induced apoptosis and necrosis, and also decreased amyloid beta formation.^{46,47} Natural compounds that stabilize NRF2 could protect against oxidative stress-induced RPE cell death and may have implications in AMD therapeutics. The significance of NRF2 in mediating the effect of 4-AC was further corroborated by an NRF2 silencing experiment. When NRF2 was silenced in ARPE-19 cells, the protective effect of 4-AC was lost. When NQO1 and HO-1 gene expression was examined, we found NQO1 gene expression was inhibited when NRF2 was silenced regardless of 4-AC treatment. However, there was a significant increase in HO-1 expression on NRF2 knockdown. Moreover, the effect of 4-AC on HO-1 expression was not affected by NRF2 knockdown. These results indicate that the effect of 4-AC on NQO1 but not HO-1 expression is mediated by NRF2, suggesting an additional mechanism regulating HO-1 expression by 4-AC. Interestingly, we observed a significant increase of HO-1 on NRF2 silencing, which could be explained by compensatory mechanisms. In this regard, AP-1 has been shown to regulate HO-1 expression independent of NRF2.⁴⁸

Implication of 4-AC in the Therapeutics of AMD and Other Diseases Involving Oxidative Stress

Oxidative damage has been suggested to be involved in the etiology of numerous chronic diseases, including AMD, cataracts, cancer, and cardiovascular disease.⁴⁹ Particularly in the eye, oxidative stress has been associated with a wide variety of eye diseases, such as AMD, glaucoma, cataracts, uveitis, retinopathies, and ocular surface diseases.⁵⁰ The retinal cells, especially the RPE cells, are equipped with an antioxidant defense system that responds to high oxidative stress environment. The NRF2 has been shown to be a master regulator in regulating the antioxidative stress response.^{51–53} Harnessing and reinforcing the cellular defenses that protect the retina and RPE against oxidative stress has been proposed to be a viable option for reducing the progression of AMD in patients.²⁸ In this regard, 4-AC or other NRF2 stabilizers could have therapeutic implications in AMD and other diseases involving oxidative stress. Because 4-AC is an FDA-approved natural compound with almost no toxicity to the RPE cells, it could be readily repurposed for AMD or GA treatment. Current AREDS formulation has shown efficacy in slowing down AMD progression. The formulation contains zinc and various antioxidants. Zinc has been shown to activate the NRF2-dependent antioxidant system in RPE.^{54,55} Current AREDS formulation contains 400 international units of vitamin E.⁵⁶ Our results indicate that 4-AC performs much better than vitamin E and vitamin C in protecting RPE cells. Particularly, NQO1 is known to maintain both α -tocopherol (vitamin E) and coenzyme Q10 in their reduced antioxidant state, therefore making it an even more potent antioxidant.⁵⁷ In this regard, as an FDA-approved natural compound, 4-AC should be further

evaluated for its potential use independent of AREDS formulation or in combination with AREDS formulation in preventing AMD progression.

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