Resveratrol Protects Against Hydroquinone-Induced Oxidative Threat in Retinal Pigment Epithelial Cells

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Received: November 4, 2019
Accepted: March 2, 2020
Published: April 25, 2020

Citation: Neal SE, Buehne KL, Besley NA, et al. Resveratrol protects against hydroquinone-induced oxidative threat in retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci*. 2020;61(4):32. https://doi.org/10.1167/iovs.61.4.32

Purpose. Oxidative stress in retinal pigment epithelial (RPE) cells is associated with age-related macular degeneration (AMD). Resveratrol exerts a range of protective biologic effects, but its mechanism(s) are not well understood. The aim of this study was to investigate how resveratrol could affect biologic pathways in oxidatively stressed RPE cells.

Methods. Cultured human RPE cells were treated with hydroquinone (HQ) in the presence or absence of resveratrol. Cell viability was determined with WST-1 reagent and trypan blue exclusion. Mitochondrial function was measured with the XFe24 Extracellular Flux Analyzer. Expression of heme oxygenase-1 (HO-1) and glutamate cysteine ligase catalytic subunit was evaluated by qPCR. Endoplasmic reticulum stress protein expression was measured by Western blot. Potential reactions between HQ and resveratrol were investigated using high-performance liquid chromatography mass spectrometry with resveratrol and additional oxidants for comparison.

Results. RPE cells treated with the combination of resveratrol and HQ had significantly increased cell viability and improved mitochondrial function when compared with HQ-treated cells alone. Resveratrol in combination with HQ significantly upregulated HO-1 mRNA expression above that of HQ-treated cells alone. Resveratrol in combination with HQ upregulated C/EBP homologous protein and spliced X-box binding protein 1. Additionally, new compounds were formed from resveratrol and HQ coincubation.

Conclusions. Resveratrol can ameliorate HQ-induced toxicity in RPE cells through improved mitochondrial bioenergetics, upregulated antioxidant genes, stimulated unfolded protein response, and direct oxidant interaction. This study provides insight into pathways through which resveratrol can protect RPE cells from oxidative damage, a factor thought to contribute to AMD pathogenesis.

Keywords: AMD, RPE, resveratrol, hydroquinone

Retinal pigment epithelial (RPE) cell dysfunction and loss is a feature of the late stages of the nonneovascular ("dry") form of age-related macular degeneration (AMD) and also may occur in neovascular AMD (nAMD).[1] RPE damage is associated with photoreceptor cell death and irreversible vision loss.[2,3] Given their high oxygen consumption and continual exposure to light, RPE cells are equipped with strong natural antioxidant defenses to combat their inherent susceptibility to oxidative damage.[4] However, in patients with AMD, these systems are less functional and less abundant.[5] Accordingly, oxidative stress, both exogenous and endogenous, is thought to contribute to the development of AMD.[6] Related to this hypothesis and consistent with epidemiologic findings, cigarette smoking is a major environmental risk factor for the development of AMD.[7]

Hydroquinone (HQ) is an environmental toxin and an important component of cigarette smoke.[8] In mouse models, HQ given orally or as subconjunctival injection can induce sub-RPE deposits and RPE dysfunction that is similar to the pathology observed in AMD.[9] In in vitro experiments, HQ causes dose-dependent RPE cell death, decreased mitochondrial membrane potential, and triggers an endoplasmic reticulum (ER) stress-induced unfolded protein response (UPR).[10,11]

Resveratrol (3, 4', 5-trihydroxystilbene) is a polyphenol phytoalexin that is naturally present in grapes. Resveratrol has recently become a therapeutic agent of interest for many conditions because it possesses a range of biologic activities, including both proapoptotic anti-cancer effects[12,13] and survival-promoting antioxidant effects.[14] Although some common cellular targets and pathways of resveratrol have been identified, such as the ER stress response and mitochondrial function and biogenesis, the mechanism(s) by which resveratrol produces these divergent, potentially cell type–specific effects remains unclear.[15]

Currently, there is no standard treatment for non-nAMD or the atrophy and associated cell loss that may accompany nAMD, so there is a need to explore potential therapies

for these disease manifestations. As a result of the finding that antioxidant supplements may prevent further vision loss in patients with macular degeneration, many groups have investigated antioxidants for their effectiveness with a specific focus on their effects on RPE cell viability as a model for dry AMD.[7,16] However, there are limited data on the effect of resveratrol on HQ-induced cell injury. Wang et al.[17] showed that pretreatment of resveratrol protected mouse primary hepatocytes against HQ-induced damage. One abstract also demonstrated that resveratrol in the presence of HQ decreased apoptosis in the human RPE cell line ARPE-19 (Kaushal A, et al. *IOVS* 2010;51:ARVO E-Abstract 1430). Additionally, it is likely that in eyes with AMD, oxidative stress is already present at the time of therapeutic treatment, therefore a model in which resveratrol is coadministered with oxidants is clinically relevant. Our work, which used human donor RPE cells, expands on this finding and explores potential mechanisms for these effects.

Methods

Antibodies and Reagents

Transresveratrol (resveratrol), HQ, benzoquinone (BQ), *tert*-butyl hydroperoxide (*t*-BHP), hydrogen peroxide (H_2O_2), and collagen type I were purchased from Sigma (St. Louis, MO, USA). Mouse monoclonal anti-C/EBP homologous protein (CHOP; cat# L63F7) antibody and rabbit monoclonal anti-spliced X-box binding protein 1 (XBP1s; cat# D2C1F) antibody were purchased from Cell Signaling Technology (Danvers, MA, USA); rabbit polyclonal anti-heme oxygenase-1 (HO-1; cat# ADI-OSA-150D) antibody was purchased from Enzo Life Sciences (Farmingdale, NY, USA); rabbit monoclonal anti-glutamate cysteine ligase catalytic subunit (GCLC; cat# ab190685) antibody, that detects total GCLC, was purchased from Abcam (Cambridge, MA, USA); and mouse anti-β-Actin (cat# sc-47778) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase conjugated donkey anti-mouse IgG and donkey anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Radioimmuno-precipitation assay (RIPA) lysis and extraction buffer (cat# 89900) and BCA protein assay kit (cat# 23227) were purchased from ThermoScientific (Chicago, IL, USA). The tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1.3-benzene disulfonate) was purchased from Roche Applied Science (Indianapolis, IN, USA). Trypan blue (0.4%) was purchased from Sigma (cat# T8154, St. Louis, MO, USA). Ultrapure water for LCMS analysis was obtained in-house using a Hydro Service & Supplies, Durham, NC, USA. Optima LC/MS grade acetonitrile, methanol, and formic acid were purchased from Fisher Scientific (Hampton, NH, USA). The Atmospheric Pressure interface Time of Flight (API-TOF) reference mass solution kit (P/N G1969-85001) was purchased from Agilent Technologies (Santa Clara, CA, USA).

RPE Cell Culture and Treatment

Human donor eyes from a 62-year-old man were obtained within 24 hours after death from the North Carolina Organ Donor and Eye Bank, Inc. (Winston-Salem, NC, USA) in accordance with the provisions of the Declaration of Helsinki for research involving human tissue. RPE cells from the eyes were harvested as previously described.[18] Cells were grown in Eagle's minimal essential medium (MEM; Invitrogen, Chicago, IL, USA) with 10% fetal bovine serum (FBS; Invitrogen) and with 1× penicillin/streptomycin (Pen/Strep; Invitrogen) at 37°C in a humidified environment containing 5% CO_2. The identity of RPE cells was confirmed by cytokeratin-18 and ZO-1 stain (not shown). RPE cells had apical nuclear localization and basal-lateral localization of phalloidin and ZO-1, as previously reported.[19]

Donor RPE cells were seeded on collagen-coated 96-well plates or 24-well plates (Corning-Costar Incorporated, Corning, NY, USA) as we have previously described.[20] For all experiments, cells were grown to confluence prior to treatment. Cells were washed once with 1% FBS/phenol free (PF)-MEM with 1× Pen/Strep and then treated in the same media for 4 hours (unless otherwise specified) with the following four treatments: 1% FBS/PF-MEM alone, resveratrol alone (15 and 30 µM), HQ alone (varying concentrations), and HQ in the presence of resveratrol. In preliminary experiments, the effects of 24 hour resveratrol pretreatment prior to HQ treatment were inconsistent (not shown). Therefore in subsequent experiments cells were coincubated with HQ and resveratrol.

WST-1 Assay

Cells ($1x10^4$/well) plated in triplicate wells of a 96-well plate were treated with oxidant in the presence or absence of resveratrol in 1% FBS/PF-MEM for 4 hours at 37°C. Cell viability was measured based on the cleavage of the tetrazolium salt WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1.3-benzene disulfonate) by mitochondrial dehydrogenases in viable cells (Roche Applied Science, Indianapolis, IN, USA). After treatment, the WST-1 solution (10 µL/well) was added, and cells were incubated for 30 minutes at 37°C. The plate was read using a spectrophotometer at 440 nm with a reference wavelength at 690 nm.

Trypan Blue Exclusion Assay

Cells ($6x10^4$/well) plated in triplicate wells of a 24-well plate were treated for 4 hours at 37°C with HQ (150 µM) in the presence or absence of resveratrol (30 µM) in 1% FBS/PF-MEM. Treatment media were collected, and cells were detached from the plate with TrypLE Express Enzyme (1x, 350 µL/well) (cat# 12604-013; ThermoScientific). A total of 700 µL of 10% FBS MEM was then added to stop the action of TrypLE, and collected treatment media were readded. Cell numbers were determined with trypan blue exclusion assay using a hemocytometer.

Measurement of Cellular Respiration

Analysis of mitochondrial function was performed on live cells using a Seahorse Biosciences XFe24 Flux Analyzer (Seahorse Biosciences, North Billerica, MA, USA). Additional materials involved in this assay included the Cell Mito Stress Test Kit (cat# 103015-100; Seahorse Biosciences), which contained reagents, and the FluxPak (cat# 102342-100; Seahorse Biosciences), which contained sensor cartridges, microplates, and calibrant solution. RPE cells ($2x10^4$/well) were seeded in triplicate wells of a XF 24-well plate and grown for 24 hours. Confluent RPE cells were washed with 1% FBS/PF-MEM and treated with HQ (175 µM) with or without resveratrol (30 µM) for 1.5 hours. The cells were washed twice with XF base medium supplemented

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with 1 mM sodium pyruvate, 2 mM glutamine, and 8 mM glucose, pH 7.4, and incubated in a CO_2-free environment for 60 minutes at 37°C and then placed in the XFe24 Flux Analyzer. Oxygen consumption rate (OCR) was measured under basal conditions followed by the sequential addition of 1 µM oligomycin to inhibit adenosine triphosphatase (ATP) synthase activity, 1 µM tri-fluorocarbonylcyanide phenylhydrazone (FCCP) to uncouple mitochondrial oxidative phosphorylation, and 1 µM antimycin A and rotenone to inhibit electron transport in complex I and III, respectively. These mitochondrial inhibitors were used to determine a number of bioenergetic and mitochondrial function parameters, including basal respiration, ATP turnover rate, maximal respiration, and spare respiratory capacity. ATP turnover rate was the difference in OCR between the last measurement before the oligomycin injection and minimum OCR after the oligomycin injection. Maximal respiration was the difference in OCR between FCCP-induced respiration and OCR after the injection of antimycin A. Spare respiratory capacity was the difference between maximal respiration and basal OCR. After OCR measurements, media were removed, and the total proteins were extracted in RIPA lysis buffer and quantified with a BCA protein assay. OCRs were normalized to total protein content.

Real-Time RT-PCR Analysis

Cells ($6x10^4$/well) in triplicate wells of a 24-well plate were treated with HQ (150 µM) in the presence or absence of resveratrol (30 µM) in 1% FBS/PF-MEM for 4 hours at 37°C. Total RNA was isolated from cultured RPE cells using RNeasy Plus Mini Kit (Qiagen Inc., Valencia, CA), according to the manufacturer's protocols. Real-time quantitative reverse transcription-polymerase chain reaction (qPCR) was performed as we have described previously.[21] Primer pairs for GCLC, HO-1, and ribosomal protein, large, P0 (RPLP0) were as follows (5' to 3'): *GCLC*, forward: ATG GAG GTG CAA TTA ACA GAC; reverse: ACT GCA TTG CCA CCT TTG CA; *HO-1*, forward: CAG GAG CTG ACC CAT GA; reverse: AGC AAC TGT CGC CAC CAG AA; *RPLP0*, forward: GGA CAT GTT GCT GGC CAA TAA; reverse: GGG CCC GAG ACC AGT GTT.

Western Blot

RPE cells ($6x10^4$/well) in triplicate wells of a 24-well plate were treated with HQ (150–200 µM) in the presence or absence of resveratrol (30 µM) in 1% FBS/PF-MEM and incubated for 4 or 6 hours at 37°C. Following two washes with ice-cold PBS, cells were lysed with RIPA buffer supplemented with protease inhibitors for protein extraction. Aliquots of total cell lysate (30 µg) were subjected to SDS-PAGE and Western blot analysis as previously described.[21,22]

High-Performance Liquid Chromatography Mass Spectrometry (LCMS)

Samples of resveratrol (230 and 240 µM) in the presence or absence of HQ (490 µM), BQ (490 µM), *t*-BHP (5 mM), and H_2O_2 (5 mM) were prepared in ultrapure water at room temperature. The mixtures were analyzed by high-performance LCMS with ultraviolet (UV) detection after 30 minutes, 4 hours, and 24 hours of incubation. In preliminary LCMS experiments (not shown) samples were prepared in 1% FBS MEM and also in ultrapure water. There were no differences in major peaks. Therefore to simplify LCMS analysis, subsequent experiments were conducted in water.

LCMS analysis was performed on a 6224 TOF LC/MS system (Agilent Technologies), consisting of a 1200 HPLC (degasser, binary pump, thermostated column compartment, diode array detector [DAD]) coupled to a 6224 accurate-mass TOF mass spectrometer. The mass spectrometer was equipped with a Dual ESI source (Agilent, Santa Clara, CA, USA), and accurate mass data were obtained by internal calibration (reference ion 922.009798 m/z) using a secondary nebulizer to continuously deliver the reference solution. Positive-ion mass spectral data were acquired in full-scan mode over the range of 60 to 1700 m/z using the following source parameters: gas temperature 325°C, gas flow 11 L/min, nebulizer pressure 33 psig, VCap 3500 V, and fragmentor voltage 120 V. HPLC separations were achieved on a Phenomenex Kinetex C18 column (Torrance, CA, USA) (3 x 30 mm, 2.6 µM) using a linear gradient of mobile phase B in A, a flow rate of 0.4 mL/min, and a column temperature of 40°C. Mobile phase A was prepared by combining 400 mL ultrapure water with 12 mL methanol and 1.2 mL formic acid. Mobile phase B was prepared by combining 400 mL acetonitrile with 12 mL ultrapure water and 1.2 mL formic acid. The gradient program included an initial hold at 0% solvent B for 1 minute, followed by a linear increase to 95% solvent B from 1 to 9 minutes, a hold at 95% solvent B from 10 to 11 minutes, and re-equilibration back to 0% B for a total run-time of 20 minutes. In addition to MS detection, the DAD was used to acquire a UV chromatogram at 254 nm, which would allow visualization of compounds with aromatic character (resveratrol and HQ). Samples were analyzed using a 5-µL injection volume.

Statistical Analyses

Data are expressed as the mean ± SD. A Student's *t*-test was used to determine whether there were statistically significant differences between treatment groups as measured by XFe24 Flux Analyzer, qPCR, Western blot, trypan blue exclusion and WST-1 assay.

Results

Resveratrol Prevents HQ-Induced RPE Cell Viability Loss

To assess the effect of HQ on RPE cell viability, and the potential protective effect by resveratrol, cells were treated with HQ in the presence or absence of resveratrol for 4 hours. By light microscopy, RPE cells treated with HQ alone appeared more shriveled and less adherent (Fig. 1A). RPE cells treated with HQ in the presence of resveratrol remained confluent and appeared similar to cells treated with 1% FBS/PF-MEM or resveratrol alone. WST-1 (mitochondrial activity) and trypan blue exclusion (cell membrane integrity) assays were used to assess cell viability. Treatment with HQ alone significantly reduced RPE cell viability in a dose-dependent manner (Figs. 1B, 1C). Resveratrol (15 and 30 µM) significantly protected against these cytotoxic concentrations of HQ, which was replicated in at least three independent experiments. In subsequent assays described later, 30 µM resveratrol was used because this concentration reproducibly improved cell viability.

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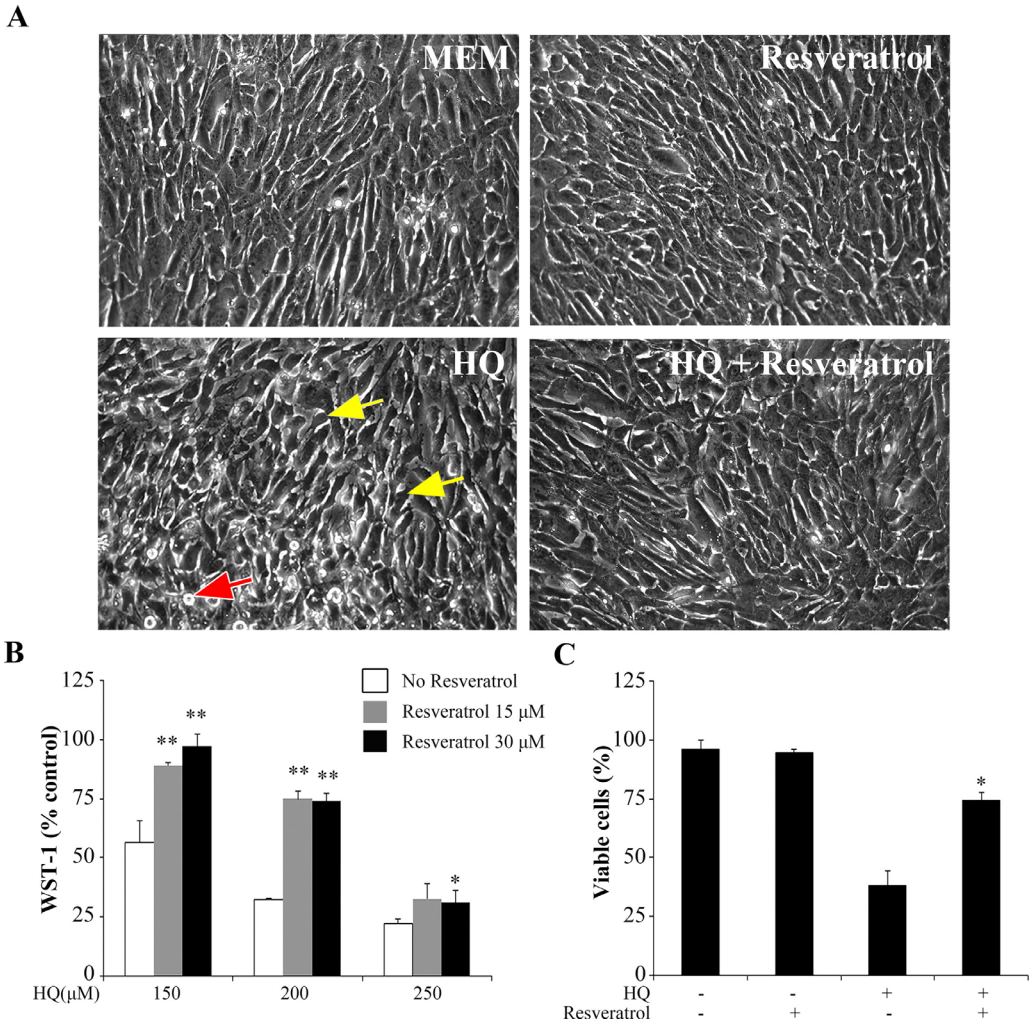

Fig. 1. Resveratrol protects RPE cells from HQ damage. RPE cells were treated with HQ in the presence or absence of resveratrol (30 µM) for 4 hours. In the HQ-treated group, some RPE cells detached from RPE monolayer (*red arrow*) and other cells appeared shriveled (*yellow arrows*) (**A**). Cell viability was determined using WST-1 reagent (*$P < 0.05$ compared with no resveratrol; **$P < 0.01$ compared with no resveratrol) (**B**) and trypan blue exclusion (*$P < 0.005$ compared with HQ [150 µM] alone) (**C**). Data are representative of three separate, repeated experiments.

RPE Mitochondrial Bioenergetics were Significantly Improved by Resveratrol

To understand the mechanism of the observed resveratrol protection, we investigated some potential biologic targets that could be affected by resveratrol. We used the XFe24 Extracellular Flux Analyzer (Seahorse Biosciences, North Billerica, MA, USA) to obtain real-time measurements of RPE cell mitochondrial function, specifically ATP turnover rate, basal respiration, spare respiratory capacity, and maximal respiration. When cultured donor RPE cells were treated with HQ (175 µM), mitochondrial function across all four cellular respiration parameters was significantly decreased. Treatment with HQ + resveratrol prevented these decreases, and these cells had significantly increased OCR in these four measured respiration functions. For ATP turnover rate, basal respiration, and maximal respiration, resveratrol treatment maintained OCR at levels that did not differ significantly from untreated cells. These experiments were repeated three times with similar results; averaged data from three independent experiments are shown (Fig. 2), and corresponding oxygen consumption traces are shown in Supplementary Figure S1.

Resveratrol Upregulated Antioxidant mRNA and Protein Expression

Induction of antioxidant enzymes is a significant component of the cellular defense against oxidative stress; considering that resveratrol is thought to function as an antioxidant, we investigated different antioxidant genes that may be affected by this treatment. HO-1 is the rate-limiting enzyme in heme degradation to biliverdin, which is then converted to bilirubin, a molecule shown to prevent lipid peroxidation.[23,24] GCLC is the catalytic subunit of the rate-limiting enzyme in the synthesis of reduced glutathione, an important antioxidant in a variety of human tissues.[25] Accordingly, we investigated mRNA expression and protein levels of HO-1 and GCLC after cells were treated with HQ in the presence

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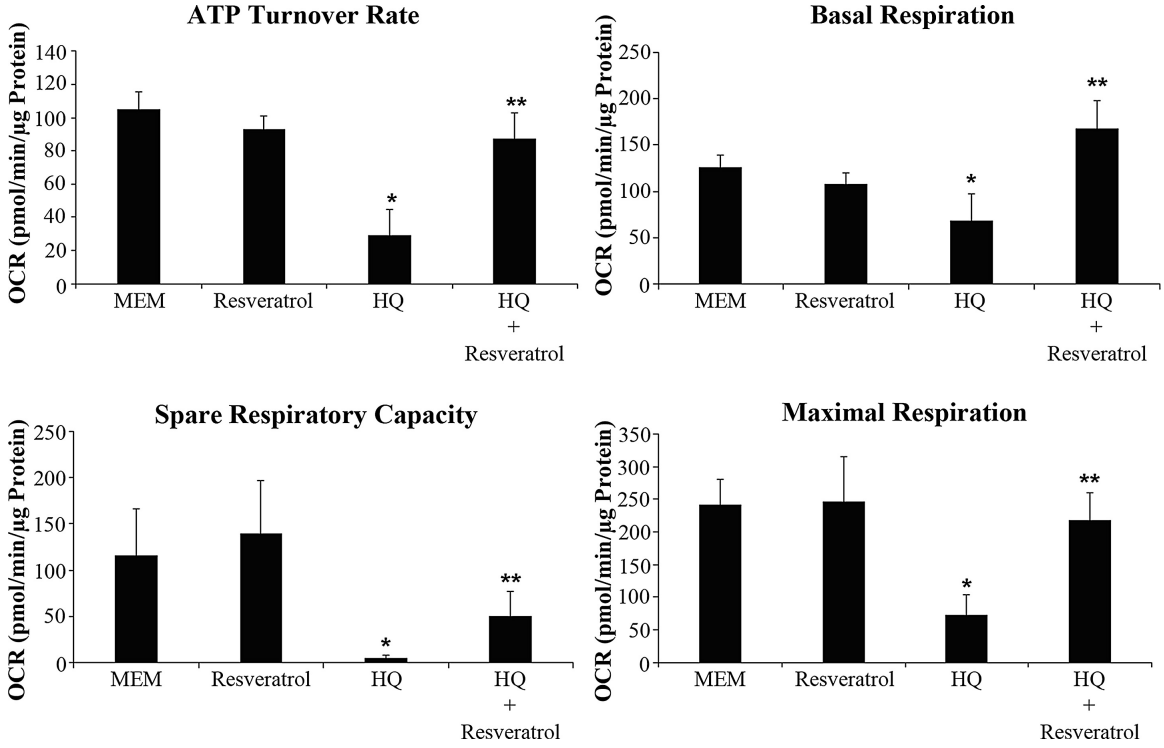

Fig. 2. Resveratrol improves mitochondrial bioenergetics. RPE cells were treated on an XF 24-well plate with serum-free MEM, resveratrol (30 µM), HQ (175 µM), HQ (175 µM) + resveratrol (30 µM) for 1.5 hours. Measurements of OCR were taken in real-time using an XFe24 Flux Analyzer. OCRs were normalized to total protein content. Data are an average of three separate experiments. (*$P < 0.05$ compared with MEM; **$P < 0.05$ compared with HQ).

and absence of resveratrol. HQ significantly upregulated steady-state *HO-1* and, to a lesser extent, *GCLC* mRNA expression ($P < 0.001$ for each gene). In the presence of resveratrol, expression of each of these molecules was even further increased ($P < 0.001$) (Figs. 3A, 3B). These results were repeated with similar fold changes relative to housekeeping gene *RPLP0* in two independent experiments.

To determine whether these mRNA changes correlated with protein levels, we extracted total protein from treated RPE cells after 6 hours of treatment with HQ in the presence or absence of resveratrol. Protein expression was normalized to β-actin levels, which served as a loading control. Consistent with the mRNA expression data, HQ significantly upregulated HO-1 protein levels compared with MEM alone and resveratrol-treated cells alone. However, there was a variable effect of resveratrol on HQ upregulated HO-1 levels; in some experiments, there was significant upregulation of HO-1 above that of HQ alone (as shown in Figs. 3C, 3D), and in other experiments, there was no further upregulation above that observed with HQ alone. Neither HQ nor resveratrol significantly altered the level of expression of GCLC protein, which remained unchanged from basal levels regardless of treatment (Figs. 3C, 3D). Full blots for each antibody are shown in Supplementary Figure S2.

Downstream ER Stress Elements Affected by Resveratrol

Activation of the ER-mediated UPR, which consists of several sensors and downstream elements, is another important component of the cellular response to oxidative stress. We studied the effects of HQ and resveratrol on two downstream proteins (CHOP and XBP1s). Protein levels were normalized to β-actin levels, which served as a loading control. Minimal CHOP protein was produced by cells treated with resveratrol or MEM alone. However, HQ significantly increased CHOP protein levels compared with those of untreated cells and cells treated with resveratrol alone (Fig. 4B), an effect that was not altered by addition of resveratrol. In contrast, resveratrol alone significantly upregulated XBP1s protein levels compared with that of untreated cells, and HQ alone produced even higher XBP1s levels. When cells were treated with the combination of HQ and resveratrol, XBP1s protein levels were reduced to that of cells treated with resveratrol alone. The effects of resveratrol and HQ on XBP1s protein levels were observed when cells were treated with resveratrol and HQ for either 4 or 6 hours (Figs. 4C, 4D). Full blots for each antibody are shown in Supplementary Figure S3.

Degree of Protection by Resveratrol is Oxidant-Dependent

Considering that resveratrol treatment resulted in improved morphology and viability in cells exposed to HQ, we wondered whether resveratrol would have a similar effect with different oxidants. To examine the effects of different oxidants, we first determined ranges of oxidant concentrations that would reduce viability to levels similar to that of HQ (not shown). Based on these preliminary experiments, in subsequent experiments, we treated cells with BQ (75–125 µM), *t*-BHP (500–900 µM), and H_2O_2 (700–1100 µM).

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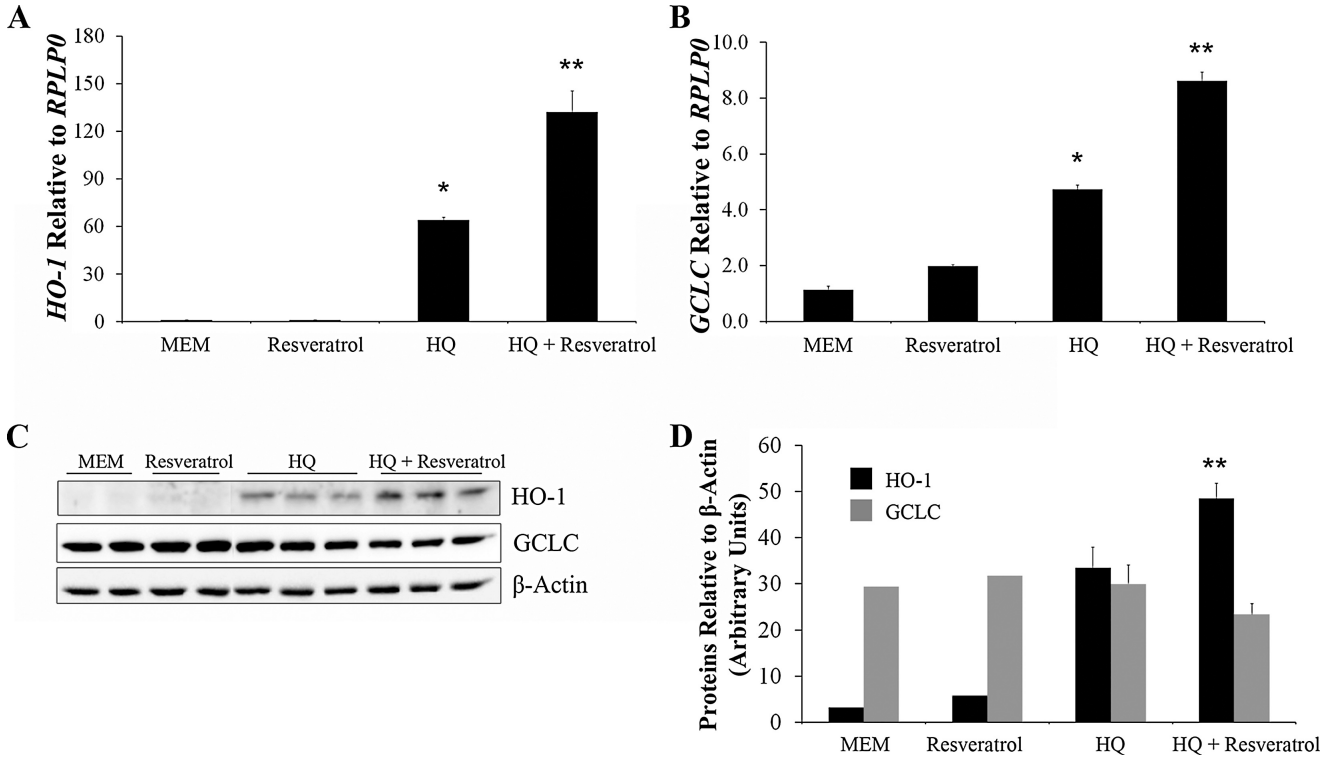

Fig. 3. Antioxidant genes and protein upregulated by HQ and resveratrol. RPE cells in triplicate wells were treated with HQ (150 µM) in the presence or absence of resveratrol (30 µM) for 4 hours. RNA was extracted, reverse transcribed to cDNA, amplified with HO-1 or GCLC specific primer pairs, and quantified by qPCR (**A, B**). Protein samples were extracted from the RPE cells treated with HQ (175 µM) in the presence or absence of resveratrol (30 µM) after 6 hours, subjected to SDS-PAGE, and analyzed by Western blot (**C, D**). HO-1 and GCLC levels relative to β-actin were determined, and data are representative of two separate experiments with similar results (*$P < 0.01$ compared with MEM; **$P < 0.01$ compared with HQ).

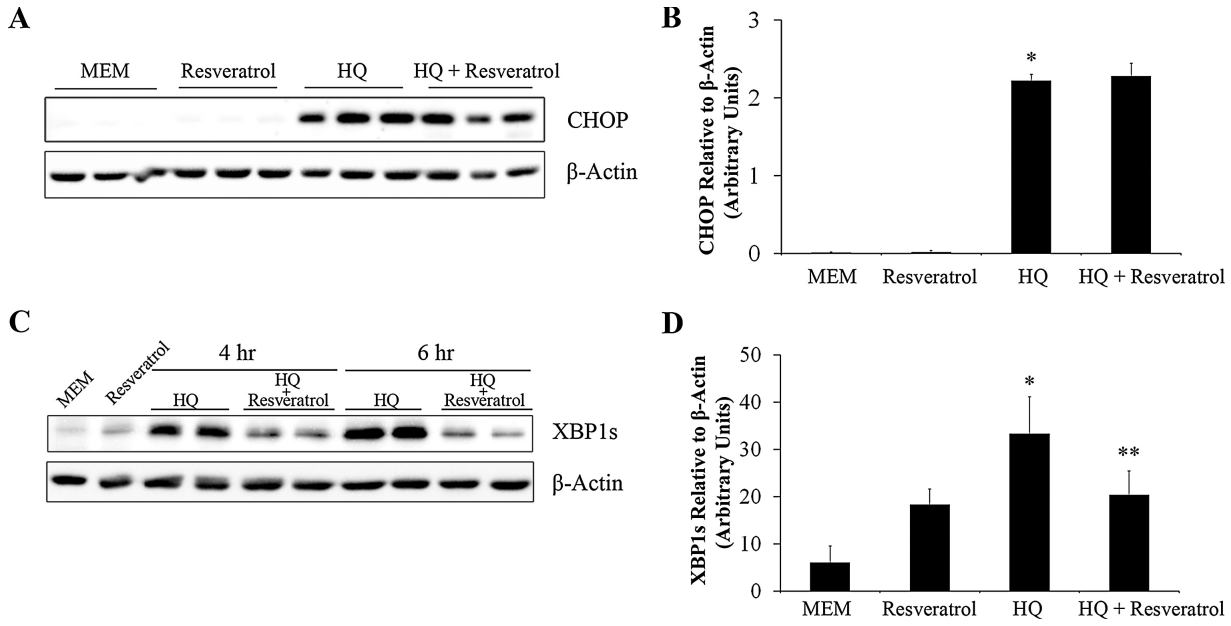

Fig. 4. Resveratrol alters downstream ER stress signaling. RPE cells were treated with HQ 150 µM for 4 hours (**A**) or HQ 175 µM for 4 and 6 hours (**C**) in the presence or absence of resveratrol (30 µM). Total cell lysates (30 µg) were subjected to SDS-PAGE and analyzed by Western blot. Protein expression of CHOP and XBP1s was normalized to β-actin expression. The amount of CHOP protein on the blot was quantified and is shown in the graph (**B**) and is representative of three separate experiments (*$P < 0.01$ compared with MEM). XBP1s protein levels from the 4 hour treatment on the blot shown was averaged with data from a second experiment and are displayed in the graph ($n = 4$ for MEM and resveratrol; $n = 5$ for HQ and HQ + resveratrol) (**D**). (*$P < 0.05$ compared with resveratrol; **$P < 0.05$ compared with HQ).

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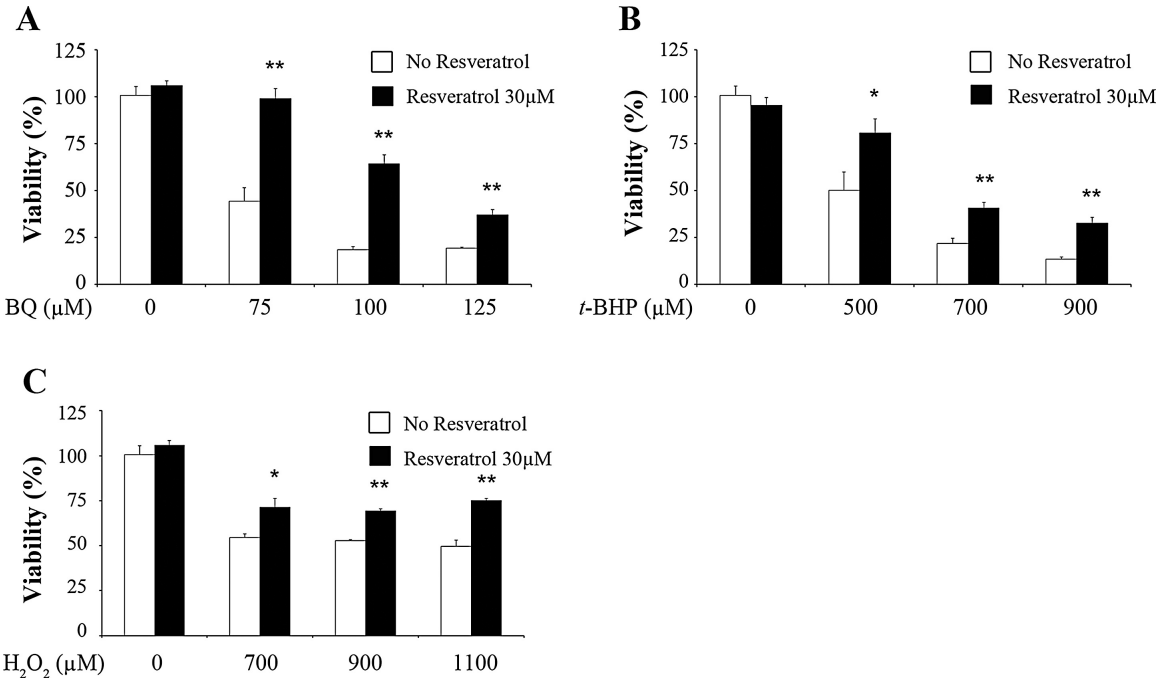

Fig. 5. Resveratrol protects against several different oxidants. RPE cells in triplicate wells were treated for 4 hours with BQ (**A**), *t*-BHP (**B**), or H_2O_2 (**C**) in the presence or absence of resveratrol. Cell viability was assessed using WST-1 reagent. Data are representative of three separate experiments, repeated with each oxidant with similar results. (*$P < 0.05$ vs. no resveratrol; **$P < 0.01$ vs. no resveratrol).

We treated cells with each oxidant for 4 hours in the presence or absence of resveratrol and assessed cell viability with WST-1 reagent.

BQ, *t*-BHP, and H_2O_2 all decreased cell viability in a dose-dependent manner (Figs. 5A–5C). We found that resveratrol significantly protected RPE cells from all oxidants, but the magnitude of the protective effect varied among the oxidants. At oxidant doses that produced similar levels of RPE cell viability loss as HQ, resveratrol protected best against BQ-induced viability loss, and improved viability by 54.3%, 45.7%, and 17.9% in cells treated with 75, 100, and 125 μM BQ, respectively ($P < 0.01$). For *t*-BHP, resveratrol provided a moderate level of protection and improved cell viability by 30.5%, 18.9%, and 19.1% for cells treated with 500, 700, and 900 μM *t*-BHP, respectively ($P < 0.05$). Cells treated with H_2O_2 in the presence of resveratrol were modestly protected against oxidative damage induced by H_2O_2 and improved cell viability by 16.9%, 16.5%, and 25.7% for cells treated with 700, 900, and 1100 μM H_2O_2, respectively ($P < 0.05$). Similar results were reproduced for each of the oxidants at these concentrations in three independent experiments.

Resveratrol and HQ Form Novel Products

To investigate potential chemical interactions in solution between resveratrol and the tested oxidants, we analyzed binary mixtures of resveratrol with the various oxidants in water using LCMS after 30 minutes and 4 hours at room temperature in a light-protected autosampler. As shown in Figure 6A, resveratrol by itself eluted as a single peak near 7.9 minutes. Three additional chromatographic peaks started to form within 30 minutes and had retention times in close proximity to resveratrol when either HQ (Fig. 6B) or BQ (Fig. 6C) was mixed with resveratrol. In contrast, *t*-BHP and H_2O_2 in the presence of resveratrol did not form any additional reaction products, as we did not observe any new chromatographic peaks (Figs. 6D, 6E). These peaks were also observed with mixtures in 1% FBS/PF-MEM (not shown). However, to minimize background, these solutions were prepared in water, which had no chromatographic peak (Supplementary Fig. S4A). At 30 minutes, HQ alone and BQ alone show a large peak near 3.19 minutes (Supplementary Figs. S4B, S4C), but the identity of this peak was undetermined because neither HQ nor BQ ionize by electrospray ionization under these experimental conditions. The *t*-BHP alone (Supplementary Fig. S4D) did not show a chromatographic peak, and H_2O_2 alone showed a small peak near 1.1 minutes (Supplementary Fig. S4E) that was unaffected in the presence of resveratrol. The samples were kept at room temperature protected from light and then run again at 4 hours. Under these conditions, HQ alone (Supplementary Fig. S5A) and BQ alone (Supplementary Fig. S5B) both still produced a peak near 3.19 minutes, but HQ had degraded to additional peaks with retention times near 2.1 and 2.9 minutes, which could not be characterized under the conditions of this experiment. The combinations of HQ + resveratrol (Supplementary Fig. S5C) and BQ + resveratrol (Supplementary Fig. S5D) at 4 hours showed similar chromatographic peaks to those present at 30 minutes. In contrast, with resveratrol alone, there was a minimally degraded stable peak at 7.9 minutes (Supplementary Fig. S5E).

Mass spectra corresponding to the four chromatographic peaks shown by HQ + resveratrol in Figure 6B are shown in Figure 7. UV peaks 1 to 4 showed products with $[M+H]^+$ at 445.1284 m/z (Fig. 7A), 337.1078 m/z (Fig. 7B), 443.1133 m/z (Fig. 7C), and 229.0865 and 777.1968 m/z (Fig. 7D), respectively. Mass spectra corresponding to the new peaks formed by BQ + resveratrol in Figure 6C are shown in

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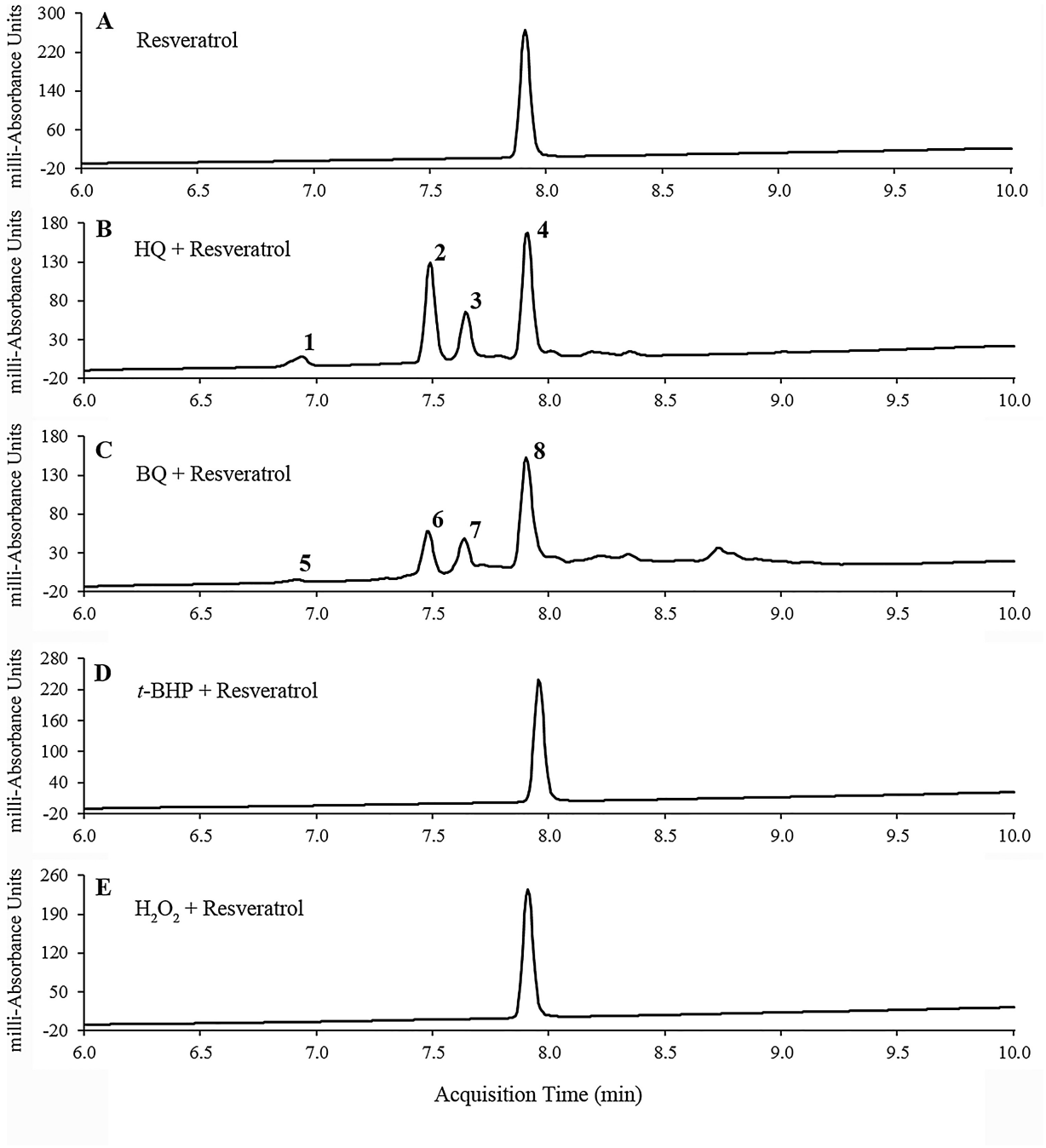

Fig. 6. HPLC-UV chromatograms of resveratrol with HQ, BQ, *t*-BHP, and H_2O_2. Resveratrol was prepared by itself as a control in water (**A**). Binary mixtures of resveratrol were prepared in water with HQ (**B**), BQ (**C**), *t*-BHP (**D**), and H_2O_2 (**E**). The solutions were kept at room temperature for 30 minutes, and then analyzed using an LCMS method with UV detection. The UV chromatograms at 254 nm are shown with the time axis expanded near the region where resveratrol and resveratrol-related reaction products eluted from the column. Numbered peaks 1 to 4 on the HQ + resveratrol chromatogram (**B**) and peaks 5 to 8 on the BQ + resveratrol chromatogram (**C**) were identified by LCMS analysis and are characterized in Figure 7 and Supplementary Figure S6, respectively.

Supplementary Figure S6. UV peaks 5 to 8 showed products with $[M+H]^+$ at 445.1277 and 463.1385 m/z (Supplementary Fig. S6A), 337.1074 m/z (Supplementary Fig. S6B), 443.1129 and 551.1337 m/z (Supplementary Fig. S6C), and 229.0866 and 777.1971 m/z (Supplementary Fig. S6D), respectively. Unreacted resveratrol was present in both HQ + resveratrol (peak 4) and BQ + resveratrol (peak 8) at $[M+H]^+$ 229.0865 and 229.0866 m/z, respectively.

Discussion

In this study, we have shown that resveratrol can protect human donor RPE cells against oxidative stress induced by HQ, an important component of cigarette smoke. This effect is associated with improved mitochondrial function, stimulation of cellular antioxidant systems, and activation of downstream ER stress proteins. Resveratrol also preserved

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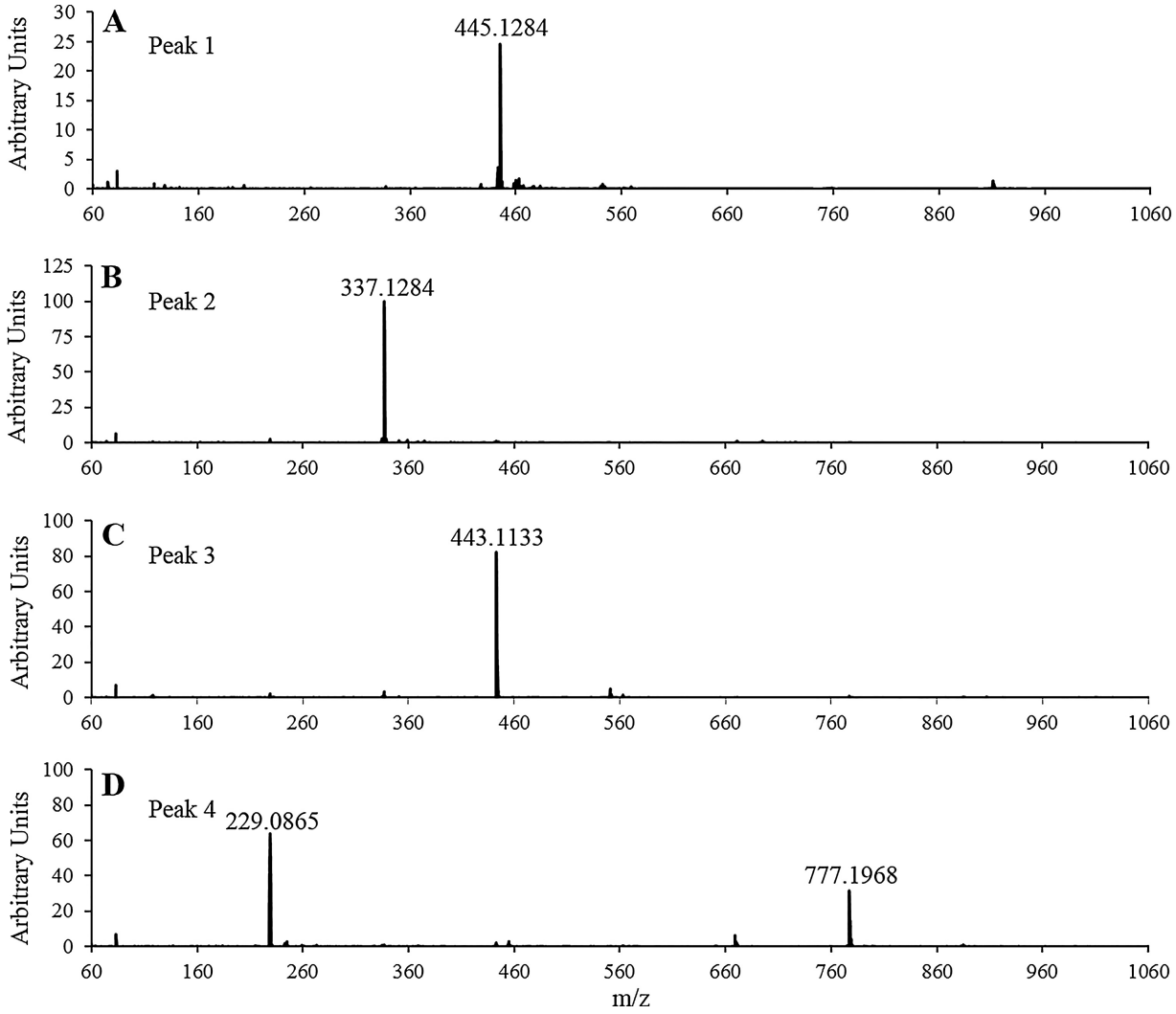

Fig. 7. Mass spectra of products from reaction of HQ with resveratrol. Mass spectra corresponding to the four UV peaks (peaks 1–4) from HQ + resveratrol (Fig. 6B) were identified with LCMS analysis and used to characterize additional reaction products (**A–D**). Positive-ion mass spectral data were acquired in full-scan mode over the range of 60 to 1700 m/z with reference ion 922.009798 m/z.

cell viability in the presence of other oxidants, BQ, *t*-BHP, and H_2O_2, but to different degrees. HQ interacts with resveratrol in solution, which resulted in the formation of novel species as detected by LCMS. A similar interaction was observed between BQ and resveratrol but not between *t*-BHP and resveratrol or H_2O_2 and resveratrol.

Mitochondrial function is critical to cell survival, and HQ is known to target this organelle in RPE cells specifically through decreases in membrane potential.[26,27] Our work confirmed that HQ can virtually eliminate RPE mitochondrial respiration. To our knowledge, we are the first to demonstrate the specific effects of HQ on primary human RPE cells with the XFe24 Flux Analyzer. Strikingly, in the presence of resveratrol, the deleterious effects of HQ on mitochondrial function were prevented, which implies that the structural integrity and function of the electron transport system were protected. In contrast, aged primary human RPE cells treated with H_2O_2 did not have altered mitochondrial respiration or reactive oxygen species (ROS) levels compared with baseline.[28] In ARPE-19 cells, pretreatment with resveratrol exerts a direct protective effect against mitochondrial dysfunction induced by acrolein, another toxin in cigarette smoke, through increases in basal respiration, ATP turnover, and respiratory capacity compared with cells that were not pretreated.[29] In that study, resveratrol treatment alone increased basal respiration and spare capacity compared with untreated cells; in contrast, we did not observe any differences in function compared with untreated cells, potentially due to our shorter incubation times with resveratrol alone.[29] Our results suggest that resveratrol can preserve mitochondrial function in RPE under HQ-induced oxidative stress, and it is likely that these effects directly or indirectly contributed to improved cell viability. Although it is beyond the scope of the current manuscript, it would be of interest to understand whether resveratrol similarly improves mitochondrial function and cell viability in vivo.

In its protective capacity, resveratrol is thought to function as an antioxidant, so we explored different antioxidant genes that may be affected by this treatment. Expression of HO-1 is highly inducible in retinal cells in response to stress and can produce large fold-changes in mRNA relative to control.[23,30] GCLC is also upregulated in response to

oxidative stress in many tissues, including the retina.[25,31] In preliminary studies, we showed that *HO-1* and *GCLC* mRNA expression is upregulated by HQ in donor RPE cells (Toimil BA, et al. *IOVS* 2012;53:ARVO E-Abstract 3195). Additional upregulation of these genes by resveratrol in the presence of HQ indicates that this treatment stimulates endogenous RPE antioxidant systems above the normal protective response to an oxidative threat.

Although our PCR results were robust and reproducible, there was a more variable additive effect of resveratrol on HQ enhanced HO-1 protein levels, and it did not cause additional increases in GCLC protein levels. GCLC protein was highly expressed at basal levels, and the fold change of *GCLC* mRNA was comparatively smaller than that of *HO-1* mRNA, which may account for this observed lack of change in GCLC protein levels. Although there is evidence that HO-1 function is important to RPE cell survival under oxidative stress, our results indicate some variability in HO-1 expression that could not be attributed to differences in experimental conditions or apparent effectiveness of the resveratrol treatment based on observations of cell morphology.[30,32] Pretreatment with other antioxidants has also been found to upregulate *HO-1* gene expression and protein levels in ARPE-19 cells, but perhaps the protection conferred by resveratrol's effects on antioxidant genes reported here is secondary to other protective pathways.[33,34] A limitation of this research is that we did not investigate if there were factors that could explain the differences between antioxidant mRNA and protein expression and the functional impact of these proteins on viability, which may represent avenues for future study.

The ER stress response is a complex system that involves signaling intended to protect cells or, if this stress cannot be resolved, promote apoptosis. In some cells, HQ upregulates CHOP, a protein commonly associated with the activation of apoptotic pathways.[11] Thus the lack of attenuation of CHOP by resveratrol reported here may initially appear discrepant with other survival-promoting effects. However, consistent with our results, in ARPE-19 cells CHOP serves a cytoprotective role under stress induced by cigarette smoke extract (CSE), and CHOP knockdown by siRNA decreases ARPE-19 cell viability under oxidative threat.[35,36]

XBP1s is a transcription factor and downstream element of the inositol-requiring enzyme 1 arm of the UPR. It has been hypothesized that XBP1s help cells adapt under ER stress conditions and restores homeostasis through the promotion of protein folding and degradation of misfolded proteins. Indeed, knockout or silencing models of XBP1s in RPE cells result in increased susceptibility to oxidative stress and decreased expression of antioxidant genes.[11,37] Furthermore, treatment with an adenovirus expressing XBP1s prior to CSE exposure decreased the number of apoptotic RPE cells.[35] In our system, we observed that resveratrol alone upregulated XBP1s. These data are all consistent with a hypothesis that resveratrol upregulates XBP1s as a protective measure to help cells manage a subsequent oxidative threat.

We found that HQ alone induced high XBP1s levels and caused decreased cell viability. Similarly CSE upregulates RPE cell XBP1s expression and is associated with ER stress and decreased cell viability.[35,38] ER stress inducers also upregulate spliced XBP1 mRNA in a dose-dependent manner in three additional cell types.[39] Resveratrol attenuated the increase in XBP1s induced by HQ, an effect that was associated with improved cell viability. Furthermore, in a model of ischemic-reperfusion injury to retinal vascular cells, resveratrol pretreatment attenuated the ratio of spliced XBP1 mRNA to total XBP1 mRNA compared with cells without pretreatment and significantly decreased degeneration.[40] Taken together, we speculate that the observed high levels of XBP1s induced by oxidants in our system and others is an adaptive response to oxidative stress, and that resveratrol attenuated the oxidant stress, as reflected by lower XBP1s levels.

Resveratrol and HQ and resveratrol and BQ interacted to form new peaks on LCMS. In contrast, no new peaks were identified when resveratrol was incubated with *t*-BHP or H_2O_2. Given the similar retention time of the main peak for HQ and BQ at 30 minutes, as well as the fact that the reaction products of resveratrol with either HQ or BQ are similar in retention time and mass, we hypothesize that HQ rapidly oxidizes to BQ, and BQ then reacts with resveratrol through an addition reaction. The earlier retention times of these peaks on a reverse-phase HPLC indicate that these compounds are more polar than resveratrol alone, which is consistent with the addition of the more polar HQ or BQ to resveratrol. This reaction may occur through two previously reported mechanisms, both of which would produce compounds that match our reported molecular weights.[41,42] Based on our LCMS results and the conditions of the experiment, which included a mobile phase with 0.3% formic acid to break up ionic interactions and a nonpolar organic solvent to disrupt hydrophobic interactions, we are confident that these detected chromatographic peaks are new covalently bonded compounds, and not molecules formed simply by ionic or hydrophobic interactions.

The scavenging properties of resveratrol have been well established, and resveratrol forms an adduct in the presence of acrolein.[43] However, to our knowledge, resveratrol-oxidant adducts, such as those formed by resveratrol and HQ and resveratrol and BQ, have not been previously tested in a biologic system. Resveratrol protected against decreases in RPE cell viability induced by multiple different oxidants. However, the degree of protection varied by oxidant, and was greatest for the resveratrol-HQ and resveratrol-BQ combinations. Thus it is tempting to speculate that the greater protective effects against HQ and BQ may be related to the formation of new covalently bonded resveratrol-HQ and resveratrol-BQ compounds. Although it is beyond the scope of the current report, it would be of interest to isolate and characterize these compounds formed by HQ and resveratrol to confirm their structure (which we have inferred in Supplementary Figure S7) and determine their biological function, both individually and in combination with each other. These experiments are currently underway in our laboratory.

The effects on various biologic targets that we observed by cotreatment of resveratrol in the presence of HQ, such as the additive effects on HO-1 and GCLC mRNA expression, and the lack of effect of resveratrol on HQ-induced CHOP protein levels, are distinct from the effect of resveratrol to attenuate XBP1s protein levels and improve cell viability. Accordingly, the effectiveness of resveratrol is likely not exclusively a result of a simple neutralization of HQ, and, as described earlier, the formed compounds may be biologically active. Regardless, because resveratrol can also protect against *t*-BHP and H_2O_2 but does not form new compounds with either oxidant, we hypothesize that a direct interaction between resveratrol and HQ or BQ is not the sole mechanism of resveratrol protection against these oxidants.

In preliminary experiments, resveratrol given prior to HQ provided inconsistent protective effects, as indicated earlier, therefore resveratrol was coincubated with HQ in subsequent experiments. These variable results may be attributed to the anti- and pro-oxidant abilities of resveratrol.[44] Additionally, in a study that examined resveratrol cytotoxicity in GRX cells, 24 hours pretreatment with resveratrol (50 µM) significantly decreased cell viability, and increased ROS production at 24 hours with 1, 10, and 50 µM resveratrol doses.[45] Thus if resveratrol were to be used therapeutically to protect against HQ-induced oxidative damage it may be necessary to reduce the time that RPE cells are exposed to resveratrol, as we have demonstrated in this study.

Conclusions

Resveratrol protects RPE cells under oxidative stress caused by HQ that is associated with preserved mitochondrial function, upregulation of antioxidant mRNA and proteins, and activation of the ER stress response. Resveratrol can also protect RPE cells from other oxidants, but the degree of protection varies, which may be related to the reactivity of resveratrol with certain oxidants to form novel compounds. The resveratrol-oxidant byproducts may provide new avenues of study into therapeutic compounds to treat RPE cell dysfunction. We continue to investigate this direct interaction and believe that this work will contribute to an understanding of how resveratrol can preserve RPE function in response to environmental oxidative stressors, which could potentially reveal new therapeutic pathways to target, or may lead to the use of resveratrol as a treatment for AMD.

Acknowledgments

Supported in part by the Unrestricted Research to Prevent Blindness grant; the National Institutes of Health Core Grant P30EY005722; and the National Science Foundation Award no. CHE-0923097.

Disclosure: **S.E. Neal**, None; **K.L. Buehne**, None; **N.A. Besley**, None; **P. Yang**, None; **P. Silinski**, None; **J. Hong**, None; **I.T. Ryde**, None; **J.N. Meyer**, None; **G.J. Jaffe**, None

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