Bilberry (*Vaccinium myrtillus*) Anthocyanins Modulate Heme Oxygenase-1 and Glutathione *S*-Transferase-pi Expression in ARPE-19 Cells

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Purpose. To determine whether anthocyanin-enriched bilberry extracts modulate pre- or posttranslational levels of oxidative stress defense enzymes heme-oxygenase (HO)-1 and glutathione *S*-transferase-pi (GST-pi) in cultured human retinal pigment epithelial (RPE) cells.

Methods. Confluent ARPE-19 cells were preincubated with anthocyanin and nonanthocyanin phenolic fractions of a 25% enriched extract of bilberry (10^{-6}–1.0 mg/mL) and, after phenolic removal, cells were oxidatively challenged with H_2O_2. The concentration of intracellular glutathione was measured by HPLC and free radical production determined by the dichlorofluorescin diacetate assay. HO-1 and GST-pi protein and mRNA levels were determined by Western blot and RT-PCR, respectively.

Results. Preincubation with bilberry extract ameliorated the intracellular increase of H_2O_2-induced free radicals in RPE, though H_2O_2 cytotoxicity was not affected. By 4 hours, the extract had upregulated HO-1 and GST-pi protein by 2.8- and 2.5-fold, respectively, and mRNA by 5.5- and 7.1-fold, respectively, in a dose-dependent manner. Anthocyanin and nonanthocyanin phenolic fractions contributed similarly to mRNA upregulation.

Conclusions. Anthocyanins and other phenolics from bilberry upregulate the oxidative stress defense enzymes HO-1 and GST-pi in RPE, suggesting that they stimulate signal transduction pathways influencing genes controlled by the antioxidant response element. (*Invest Ophthalmol Vis Sci.* 2007;48: 2343–2349) DOI:10.1167/iovs.06-0452

Oxidative stress is implicated in the pathogenesis of age-related macular degeneration (AMD), including impairment and death of retinal pigment epithelial (RPE) cells.[1] Human studies show that dietary intake and supplementation with antioxidant nutrients, including β-carotene, vitamins C and E, and zinc, are associated with reduced risk of AMD. Anthocyanins are potent antioxidants in vitro[2] and may also affect visual function in vivo,[3] but little information is available regarding their action in the retina. The low bioavailability and cellular concentrations of anthocyanins suggest that their bioactivity may result more from modulating redox regulation than from direct quenching of reactive oxygen species (ROS).[4] Anthocyanins are absorbed and excreted both in unmetabolized glycosylated forms[5] and as glucuronidated and methylated derivatives.[6] Although little information is available regarding the form, distribution, or retention of anthocyanins in the retina, data on glycosylated anthocyanin activity in RPE cells in vitro may provide a biological plausibility for its putative role in promoting visual function. Bilberries, an anthocyanin-rich fruit, have long been used in traditional herbal therapies for the treatment of eye disorders. Recently, Fursova et al.[7] found that supplementation with bilberry extract decreased serum and retinal lipid peroxides and slowed the development of cataract and macular degeneration in OXYS rats, a hypertensive strain that presents with a shortened lifespan and early phenotypes of age-related disorders.

Upregulation of stress proteins is a universal protective response to adverse conditions, including oxidative stress.[8] Heme-oxygenase (HO)-1, a heat-shock protein (Hsp32), catalyzes the rate-limiting first step in heme catabolism to carbon monoxide (CO), free Fe^{2+}, and biliverdin that is converted to the antioxidant bilirubin by biliverdin reductase.[9] Bilirubin also modulates cell signal transduction pathways relevant to inflammation.[9] Free Fe^{2+} rapidly induces ferritin expression and the ATPase Fe^{2+}-secreting pump to decrease Fe^{2+}, thereby limiting oxidative damage created via the Fenton reaction.[10] CO, the third product of HO-1 activity, stimulates cell signaling similar to nitric oxide, but absent its radical activity. For example, CO mediates vasodilation, inhibits platelet aggregation, and suppresses cytokine production, all factors associated with the amelioration of AMD pathophysiology.[11] Thus, HO-1 induction may confer protection in the retina by increasing resistance to oxidative stress, inflammation, and apoptosis.[12]

Two HO isoforms, inducible HO-1 and constitutive HO-2, have been found in human RPE cells.[13] Exposure to light upregulates retinal HO-1 and HO-1 protein is increased in RPE by neovascular AMD.[14] Although the impact of these changes in RPE is not fully elucidated, HO-1 upregulation is generally thought to protect the cells.[15] HO-1 shares genomic regulatory mechanisms with other protective enzymes, including glutathione *S*-transferase (GST) and NAD(P)H:quinone oxidoreductase (NQO1), enzymes that detoxify byproducts of oxidative stress.[16] Inducing enzymes involved in glutathione (GSH) biosynthesis also enhances cellular antioxidant defenses.[17] Age-related declines in GSH are associated with increased risk of AMD.[18] The purpose of this study was to investigate the effects of anthocyanin and other phenolic compounds from bilberry (*Vaccinium myrtillus*) on quiescent and oxidatively stressed RPE cells in vitro.

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Table 1. Heme-oxygenase and Glutathione *S*-transferase-pi Lux Primer Definitions and Sequences

Gene	Primer	Strand	3′ Loc	Sequence
HO-1	X06985.1_633RL	Reverse	633	cgcatATCTCCAGGGAGTTCATGcG
HO-1	X06985.1_633RL/611FU	Forward	611	ACATTGCCAGTGCCACCAAG
GST-pi	NM_000852.2_135FL	Forward	135	cggtcGAAGGAGGAGGTGGTGACcG
GST-pi	NM_000852.2_135FL/155RU	Reverse	155	TAGGCAGGAGGCTTTGAGTGAG
GAPDH	NM_002046			Certified LUX Primer Set*
β-Actin	NM_001101			Certified LUX Primer Set*

* Invitrogen, Carlsbad, CA.

Materials and Methods

RPE Cell Culture

ARPE-19 cells were obtained from the American Type Culture Collection (Manassas, VA) and propagated according to methods described by Dunn et al.[19] Cells were maintained in Dulbecco's modified Eagle's medium and Ham's F12 medium, supplemented as described with the exception that, after confluence, fetal bovine serum was decreased to 5% to promote differentiation.

Bilberry Extract

An extract of bilberry (25% anthocyanin enriched) used commercially in dietary supplements was a gift from Artemis International Inc. (Fort Wayne, IN). Concentrations of the extract are expressed as dry weight per milliliter of medium.

Total Phenols Assay and Total Anthocyanins Assay

Total phenols were determined colorimetrically by the Folin-Ciocalteu assay.[20] Total anthocyanins were estimated by the pH differential absorbance method.[21] Absorbance was measured in a spectrophotometer (UV-1601; Shimadzu, Columbia, MD) and results expressed as grams cyanidin-3-glucoside equivalents (CGE) per 100 grams dry weight.

Anthocyanin Chromatography

To determine whether the observed effects of bilberry were due to anthocyanins or to other phenolic constituents of the bilberry, the extract was fractionated by semipreparative methanolic gradient elution from a reversed-phase C18, silica bonded, low-pressure chromatography column (15 × 300 mm). Fractions were analyzed and combined into two pools, one containing only anthocyanins and the other containing all the remaining phenolic compounds from the extract. Anthocyanin components were analyzed by HPLC with electrochemical detection (ECD; ESA Inc., Chelmsford, MA) using anthocyanin standards as described by Milbury[22] with identities verified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an HPLC system (model 1100; Agilent, Palo Alto, CA) fitted with a photodiode array (PDA; UV G1315A; Agilent) and a Bruker ion trap MS/MS detector and electrospray interface (Esquire; Brucker Daltonics, Billerica, MA).

Cell Treatment and Viability Assays

The 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay, an assays of early mitochondrial dysfunction, was used to determine cell viability.[23,24] MTT was obtained from Invitrogen-Molecular Probes, Inc. (Eugene, OR).

Briefly, cells grown in 96-well plates were washed twice with PBS before treatment. Cells were exposed to serum-free medium with or without bilberry extract for 4 hours, rinsed free of bilberry medium, and then challenged with either control media or H_2O_2 for 2 hours. Stock solutions of H_2O_2 (30%; Fisher Scientific, Pittsburgh, PA) or bilberry extracts were made in water and final dilutions prepared in serum-free medium (without phenol red) immediately before the experiments. After treatment, cells were allowed to recover in phenol red–free preconditioned medium for 1 hour. MTT was added during the entire recovery period. When viability was assessed 24 hours after treatment, MTT was incorporated for the last hour. Absorbance and fluorescence measurements were performed on a plate reader (FluoStar Optima; BMG Labtech GmbH, Offenburg, Germany). Once preliminary studies demonstrated clear protein upregulation by 24 hours after treatment and detectable upregulation by 4 hours, cells were harvested at 4 hours after treatment for both protein and mRNA determinations. Butylated hydroxytoluene (BHT) and β-naphthoflavone (BNF) were obtained from Sigma-Aldrich (St. Louis, MO).

Dichlorofluorescein Assay

Intracellular free radical production was determined using the dichlorofluorescein (DCF) assay described by Sohn et al.[25] DCFH-DA (2′,7′-dichlorofluorescin-diacetate) was acquired from Invitrogen-Molecular Probes, Inc. (Eugene, OR). After fluorescence measurements were completed, excess H_2O_2 (500 mM) was added to assess maximum assay fluorescence and assure equivalent DCFH-DA loading.

Protein Analysis by Western Blot

HO-1, GST-pi, and β-actin protein levels were determined by Western blot analysis. Extraction reagent (CytoBuster Protein; Novagen-Calbiochem, La Jolla, CA) containing protease inhibitors (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA) was used to extract protein and samples were stored at −80°C until electrophoresis.

Protein concentration was determined by the bicinchoninic (BCA) protein assay (Pierce Biotechnology Inc., Rockford, IL). After resolution, proteins were electrotransferred onto polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA). Primary anti-human HO-1 and GST-pi IgG1 mouse isotypes were obtained from BD Bioscience (San Jose, CA) and housekeeping gene β-actin mouse monoclonal antibody was obtained from Novus Biologicals (Littleton, CO). Visualization was accomplished using horseradish peroxidase-conjugated sheep anti-mouse IgG (GE Healthcare, Arlington Heights, IL) and chemiluminescent substrates (SuperSignal West; Pierce). Exposed x-ray film was quantified by densitometry analysis (Quantity One software; Bio-Rad Laboratories, Hercules, CA).

RNA Extraction and RT-PCR Analysis

Cell mRNA was extracted (RNeasy Kits; Qiagen Inc., Valencia, CA) and stored at −80°C until quantitative RT-PCR analysis. A reverse transcription kit (Script III; Invitrogen Corp., Carlsbad, CA) was used to synthesize cDNA for amplification. RT-PCR was conducted in a PCR system (Prism 7000; Applied Biosystems, Foster City, CA) using certified housekeeping fluorogenic primers (GAPDH and β-actin) and custom gene primers (D-LUX, Invitrogen) described in Table 1. Data analysis was performed using the $2^{-\Delta\Delta CT}$ method[26] normalized to GAPDH mRNA and expressed relative to the control subjects.

Data Analysis and Statistics

Data are expressed as a percentage of control data or as increases (*x*-fold) over values obtained under control conditions and are presented as mean ± SD of results in three or more independent exper-

iments, each performed using triplicate cell culture plates with a minimum of eight wells per condition. Statistical analysis was performed using ANOVA followed by the Dunnett or Bonferroni tests. $P \leq 0.05$ was considered to show statistic significance.

Results

The bilberry extract contained 28.1% CGE as determined by pH differential spectroscopic analysis of anthocyanins. HPLC-UV (photodiode array) and HPLC-ECD analysis indicated total anthocyanin content as 27.7% and 27.9% CGE, respectively. The total phenols content was 61.8 g catechin equivalents/100 g. Fifteen anthocyanins were identified by HPLC-ECD and by LC-MS/MS, using authentic standards of delphinidin 3-galactoside, delphinidin 3-glucoside, cyanidin 3-galactoside, delphinidin 3-arabinoside, cyanidin 3-glucoside, petunidin 3-galactoside, cyanidin 3-arabinoside, petunidin 3-glucoside, peonidin 3-galactoside, petunidin 3-arabinoside, peonidin 3-glucoside, malvidin 3-galactoside, peonidin 3-arabinoside, malvidin 3-glucoside, and malvidin 3-arabinoside. Using low-pressure, semipreparative chromatography, we made sample pool from fractions 55 to 70 that contained polyphenols and phenolic acids but was free of anthocyanins. The constituent phenolics included *trans*-resveratrol, ferulic acid, *p*-coumaric acid, quercetin, and caffeic acid; chlorogenic acid, which was the most abundant phenolic acid, was present at approximately 60 μg/g in the original extract. A sample pool created from fractions 70 to 75 included all anthocyanins present in the bilberry extract.

After growing for 10 days, confluent ARPE-19 cells displayed the cobblestone morphology described by Dunn et al.[19] and exhibited a robust defense against H_2O_2 with a 50% lethal dose (LD_{50}) of ~500 μM H_2O_2. Treatment of ARPE-19 cells grown for <10 days with 500 μM H_2O_2 resulted in >70% cell death as illustrated in 9-day-old cultures (Fig. 1). At 3 days in culture, although cells appeared confluent microscopically, the LD_{50} for H_2O_2 by the MTT assay was 41±14 μM.

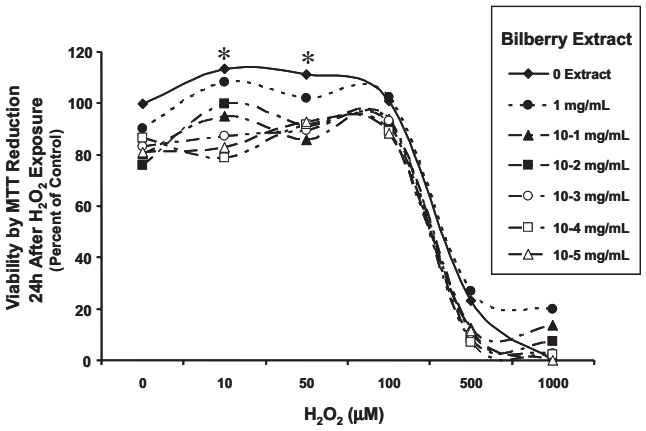

Figure 1. H_2O_2-induced cell death in RPE cells grown 9 days in culture is not prevented by preincubation with bilberry extract. RPE cells were either untreated or preincubated for 4 hours with bilberry extract (10^{-5}–1.0 mg/mL) in serum-free medium. The cells were challenged with H_2O_2 for 2 hours before being rinsed with serum-free medium and left to recover in medium containing 5% serum for 24 hours. During the last hour of the recovery period, MTT was added to the media to assess cell viability. Cell tolerance to H_2O_2 varied in this assay, depending on the time in culture of the RPE cells. Cells cultured for 3, 9, 10, or 35 days exhibited H_2O_2 LD_{50}s of 41 ± 14, 342 ± 15, 566 ± 14, and 583 ± 14 μM, respectively. Low levels of H_2O_2 demonstrated mild proliferative stimulation in 9-day cultures that was not significant in cultures grown 10 days or more. $^*P < 0.05$ versus control (no H_2O_2). All data points at 500 and 1000 μM H_2O_2 are decreased ($P < 0.05$) versus all control cultures. Data represent the mean, $n = 8$ cultures for each condition. Standard deviation bars are omitted for clarity.

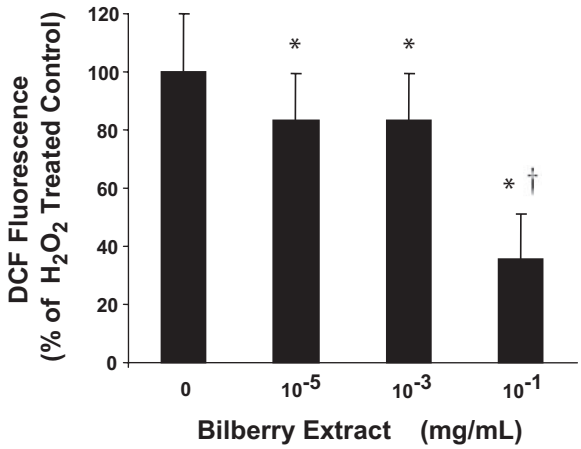

Figure 2. Intracellular radical quenching in 10-day cultured RPE cells treated with bilberry extract assessed by the DCF assay. RPE cells were either untreated or were preincubated for 4 hours with 0, 10^{-5}, 10^{-3} or 10^{-1} mg/mL bilberry extract in serum-free medium. The cells were then challenged with 500 μM H_2O_2 for 2 hours before being rinsed with PBS and loaded with DCF for 30 minutes before the fluorescence was read. Cells not treated with H_2O_2 showed a basal intracellular DCF fluorescence level that was approximately 30% of the level measured in cells treated with 500 μM H_2O_2 and not significantly different from the H_2O_2-treated cells that were preincubated with 10^{-1} mg/mL bilberry extract. $^*P < 0.05$ versus control cells (no bilberry preincubation and treated with H_2O_2). †$P < 0.05$ versus cells preincubated with 10^{-5} and 10^{-3} mg/mL bilberry extract and then treated with H_2O_2. Data represent the mean ± SD of results in three independent experiments each performed with triplicate wells.

The effect of bilberry preincubation on H_2O_2-induced RPE cell cytotoxicity is illustrated in Figure 1. In 9-day cultures, lower dose challenges of H_2O_2 showed stimulation of additional proliferation that was inhibited by bilberry extract. Bilberry extract preincubation with doses between 10^{-5} and 1.0 mg/mL did not shift viability curves to the right (i.e., increase resistance to H_2O_2). Similar experiments performed at 10 days in culture also revealed no shift in viability curves, suggesting no increase in ARPE19 cell resistance to H_2O_2-induced cytotoxicity (data not shown).

DCF assays were conducted to determine whether bilberry could be transported into RPE cells to affect intracellular radical production. Preincubation with 10^{-5} and 10^{-1} mg/mL bilberry extract decreased intracellular radical formation by 18% and 65%, respectively (Fig. 2). These results suggest that bilberry preincubation reduced intracellular radicals by radical quenching or modulation of cellular redox status to near basal levels; nevertheless, treatment with 500 μM H_2O_2 induced cellular damage sufficient to cause cell death even in the presence of intracellular anthocyanins (Fig. 1).

When assessed at 24 hours after treatment, no increase in HO-1 protein expression was observed in 10-day RPE cells exposed to 200 μM H_2O_2 for 2 hours, suggesting that antioxidant defenses were adequate. In contrast, cells exposed to 500 μM H_2O_2 had a 10-fold increase in HO-1 protein expression 24 hours after treatment (Figs. 3A, 3B), a reflection of the induction of oxidative stress. Upregulation of HO-1 protein was not prevented by a 4-hour preincubation with 1.0 mg/mL bilberry extract before H_2O_2 exposure. Increased HO-1 protein expression was observed in both control cells and those exposed to 200 μM H_2O_2 (Fig. 3B), demonstrating that bilberry alone can upregulate HO-1. HO-1 protein levels were then assessed immediately after a 4-hour exposure to bilberry extract (10^{-6}–1.0

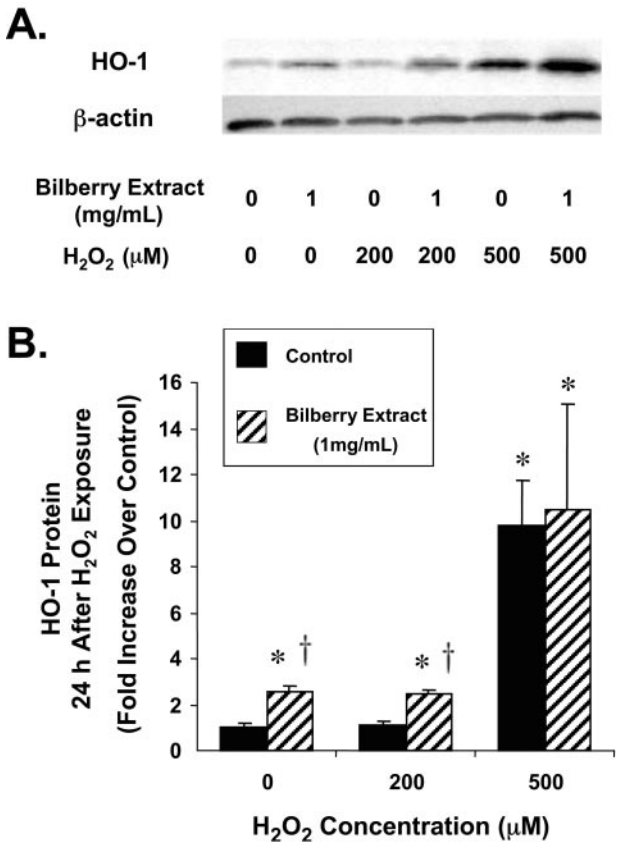

FIGURE 3. (**A**, **B**) Upregulation of HO-1 protein expression by 500 μM H_2O_2 and by 1.0 mg/mL bilberry extract. RPE cells cultured for 10 days after plating were preincubated for 4 hours with either serum-free medium along or containing 1.0 mg/mL extract. The medium containing extract was twice washed from the cells with fresh medium, and then the cells were incubated an additional 2 hours with serum- and phenol-free medium containing either 200 or 500 μM H_2O_2. The cells were then refed medium containing 2% serum for 24 hours and the protein extracts harvested. Protein was separated by SDS-PAGE followed by immunoblot analysis using anti-human HO-1 and anti-human β-actin antibodies. Quantification was performed by densitometry analysis, and the values were adjusted to the corresponding β-actin values obtained from the same gel. The results are expressed as relative units. $^*P < 0.05$ versus control cells. $\dagger P < 0.05$ versus cells treated with 200 μM H_2O_2. Values are mean ± SD of results of three independent experiments, each performed with three or more cultures.

mg/mL). Collected data from different experiments show a dose-dependent increase in HO-1 protein with bilberry treatment (Fig. 4).

BHT, an antioxidant response element (ARE) agonist, was used as a positive control at 300 and 1000 μM and upregulated HO-1 protein expression by 1.6- and 2.3-fold at 4 hours, respectively (Fig. 5). Although the increase induced by 300 μM BHT was not statistically significant in the Western blot analysis of GST-pi protein expression, bilberry extract and H_2O_2 induced significant increases (Fig. 6).

RT-PCR analysis showed upregulation of HO-1 and GST-pi mRNA by bilberry extract (Fig. 7). In addition, BNF, a more potent ARE agonist than BHT, also induced increases in HO-1 and GST-pi mRNA (Fig. 7). BHT (300 μM) significantly increased GST-pi mRNA, while an upward trend was observed for HO-1 mRNA. In other experiments, anthocyanin and polyphenolics–phenolic acid pools from the bilberry extract, as well as 300 μM BHT, induced significant increases in HO-1 mRNA. The bilberry component pools showed comparable

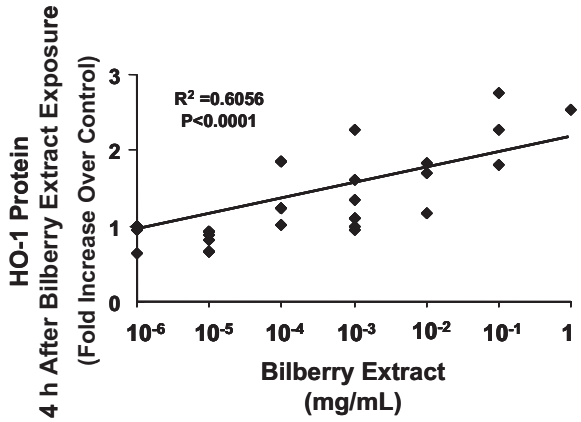

FIGURE 4. Dose–response effect of bilberry extract on HO-1 protein expression, as determined 4 hours after exposure to bilberry extract. Each depicted data point represents the mean of at least three data points (usually eight wells) for a given dose of extract, and each point is collected from independent culture experiments. The scattergram indicates a dose-dependent increase in HO-1 protein in response to bilberry treatment, determined by Western blot analysis.

potencies (Fig. 8), which appear to account for the mRNA levels observed with the intact extract (Fig. 7).

DISCUSSION

HO-1 and GST-pi are found in the retina and can be induced as a defense mechanism against oxidative stress.[8] HO-1 and GST-pi mRNA and protein levels are elevated by stressors, such as oxidant and xenobiotic compounds, and are protective of human neuronal and RPE cells in culture.[27,28] HO-1 is upregulated in rat retina exposed to visible light and is present at higher levels during daylight hours, suggesting a protective role in limiting light-induced radical damage.[29] With advanced age and in AMD, the ability to upregulate HO-1 and GST-pi diminishes and oxidative stress increases in human RPE.[14,30] Further evidence of an inverse relationship between HO-1 expression and oxidative stress is shown in Asian Indian patients with type 2 diabetes. These patients have increased oxidative damage and microangiopathy that is associated with increased NADPH oxidase expression and lower HO-1 gene expression than levels found in a comparable healthy popula-

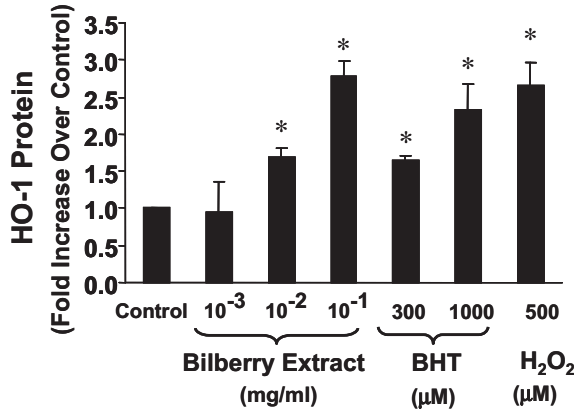

FIGURE 5. Increased HO-1 protein by Western blot analysis in response to treatment for 4 hours with media containing bilberry extract, 300 or 1000 μM BHT, or 500 μM H_2O_2. $^*P < 0.01$ versus control. Data represent results of a typical experiment (mean ± SD), with five wells used for each condition.

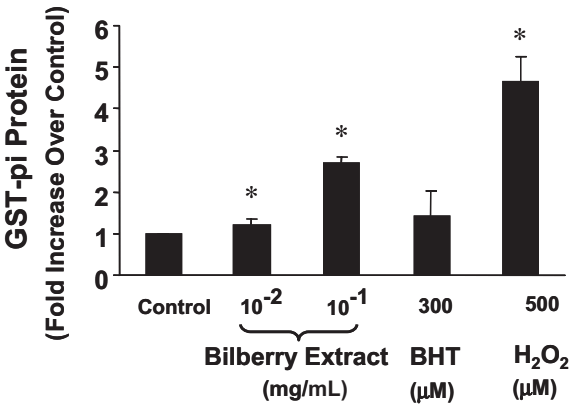

FIGURE 6. Increase in GST-pi protein by Western blot analysis in response to treatment for 4 hours with media containing bilberry extract (10^{-2} and 10^{-1} mg/mL), 300 μM BHT, or 500 μM H_2O_2. $^*P <$ 0.01 versus control. Data represent a typical experiment showing mean ± SD with four wells for each condition.

tion.[31] Similarly, RPE of control donor eyes exhibited 20-fold higher HO-1 mRNA than that of donors with diabetes, suggesting that diabetics have an impairment of endogenous defense mechanisms and a vulnerability to oxidative stress, especially in the neuroretinal cells.[32]

There is a heterogeneous mosaic pattern and an age-related decline in HO-1 protein and gene expression in the retina.[14,30] Despite general age-related declines in gene expression, translation, and transcription, the levels of most enzymes and proteins remain relatively constant.[33] This paradox has been attributed to age-associated declines in protein degradation that could result in an accumulation of damaged proteins and a decrease in the response of inducible enzymes to stimuli.[34] Potentially, interventions that stimulate upregulation of HO-1 or influence glutathione metabolism may be useful in the prevention or treatment of age-related retinopathies such as AMD.

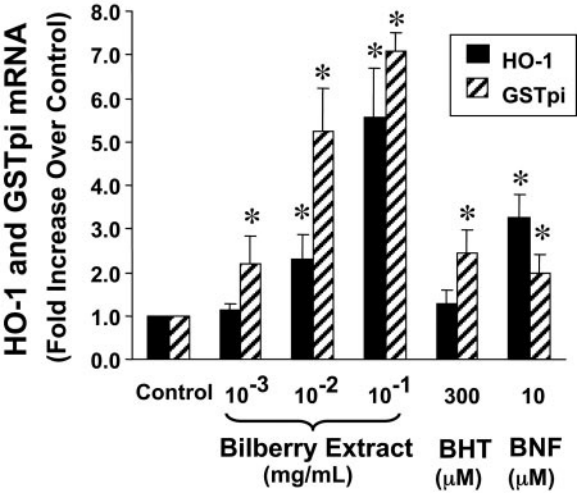

FIGURE 7. Effects of bilberry extract on HO-1 and GST-pi mRNA in RPE cultures. Cells were preincubated with increasing doses ranging from 10^{-3} to 10^{-1} mg/mL bilberry extract, 300 μM BHT, or 10 μM BNF for 4 hours. Total RNA was extracted and analyzed by RT-PCR for human HO-1 mRNA and GST-pi and then normalized to β-actin or GAPDH. Data are expressed as *x*-fold increases over untreated controls using the $\Delta\Delta_{CT}$ method. $^*P < 0.01$ compared with control. Data are the mean ± SD of results in three independent experiments, each performed in three or more cultures.

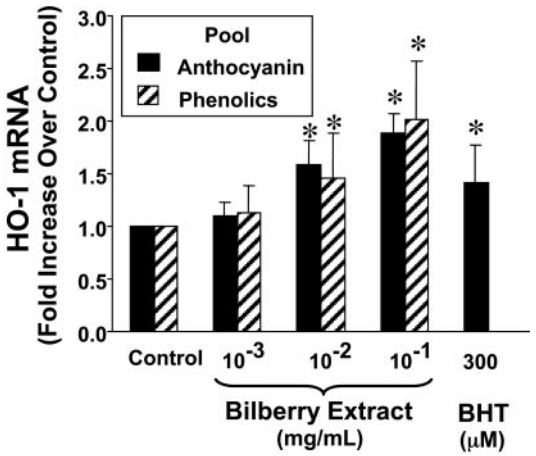

FIGURE 8. HO-1 mRNA upregulation in RPE cells in response to treatment with a pool of anthocyanin fractions (70–75) or a pool of phenolic acids and other compounds (fractions 55–70) from bilberry extract. RPE cells grown 10 days in culture were preincubated with the respective pools at concentrations of phenolics or anthocyanins representing the original amounts in 10^{-3}, 10^{-2}, and 10^{-1} mg/mL extract for 4 hours. Total RNA was extracted and RT-PCR conducted for human HO-1 mRNA and housekeeping gene GADPH mRNA. HO-1 mRNA was normalized to GAPDH and expressed relative to that of untreated control culture samples using the $\Delta\Delta_{CT}$ method. $^*P < 0.01$ compared to control. Each pool contributed approximately half of the upregulation observed with the complete mixture in Figure 7. Data are mean ± SD of results in three independent experiments, each performed in three or more cultures.

The 12th-century German herbalist Hildegarde von Bingen (1098–1179 CE) indicated the use of bilberry for the treatment of eye disorders.[35] Although bilberry has not been included as an ingredient in clinical trials of AMD, bilberry extracts have shown some efficacy in preclinical studies as an antioxidant, anti-inflammatory, vasoprotectant, hypoglycemic, and lipid-lowering agent—actions relevant to risk factors for AMD. Of note, using rodent models, Joseph et al.[36] found that diets rich in blueberries, an anthocyanin-rich *Vaccinium* berry related to bilberries, significantly reversed age-related declines in neuronal signal transduction, cognition, and motor behavioral deficits. While investigations of anthocyanins in human studies are limited, approximately 30 trials published during the past 45 years suggest that they possess activity regarding visual adjustments to light.[37] Although night vision improvements were found in eight studies, four randomized controlled trials yielded null outcomes. Nonetheless, the effect of bilberry on visual function in subjects with impaired night vision or eye disease has yet to be investigated.

Our results show that 3-day, undifferentiated, confluent RPE cells can be stimulated to divide further with a modest prooxidant challenge. These cells are less resistant to higher concentrations of H_2O_2 than is more developed RPE cells. These findings are consistent with Bailey et al.[38] and Wada et al.[39] who also found increased resistance to oxidative stress with increasing age and differentiation in ARPE-19 cells. We found little additional antioxidant defense benefit by extending cultures beyond 10 to 12 days to several weeks in culture. These observations underscore the importance of culture conditions for RPE cells to defining results that are dependent on the state of differentiation.

Alizadeh et al.[40] demonstrated that the oxidants *tert*-butyl hydroperoxide (tBH) and H_2O_2 upregulates HO-1 and fibroblast growth factor receptor (FGFR1) mRNA in differentiated ARPE-19 cells with concurrent downregulation of specific genes, including cellular retinaldehyde-binding protein (CRALBP)

and the RPE-specific 65-kDa protein (RPE65). Similarly, in our study, low doses of H_2O_2 induced a proliferative response in ARPE-19 cultures where nonquiescent cells may remain (9 days), whereas higher doses induced cell death. It is well established that markedly different responses to oxidative stress by proliferating and nonproliferating mammalian cells are dependent on the degree of cellular differentiation and on the magnitude of the stress stimulus.[41,42] In this regard, although the threshold of viability to the H_2O_2 challenge in fully differentiated RPE cells in our study was relatively high, the slope of the viability curves were quite steep. These experiments show that bilberry anthocyanins can be preloaded into cells and quench intracellular radicals, albeit at supraphysiologic levels; however, this radical quenching activity was insufficient to prevent cell death caused by a 500 μM H_2O_2 challenge. High levels of H_2O_2 may render catastrophic oxidant damage to surface proteins and receptors representing an insult disproportionate to in vivo conditions. Lethal cellular injury mediated by acute H_2O_2-induced oxidative stress involves an elevation of intracellular cytosolic Ca^{2+} associated with collapse of the mitochondrial membrane potential. The viability assay chosen for these experiments is an indicator of mitochondrial dysfunction leading to failure of reductive potential in the cell. Sequestration of Ca^{2+} in the mitochondrion depends on NADPH and GSH, both of which are consumed in the metabolism of H_2O_2. GSH was depleted rapidly by 500 μM H_2O_2 in our 10-day RPE cultures (data not shown). Catastrophic oxidative defense failure, observed in in vitro experiments where it is necessary to use high levels of H_2O_2, may not adequately mimic in vivo oxidative stress associated with aging. These experiments should be repeated in an optimal in vivo model over a longer time period with lower levels of oxidative stress to test the impact of long-term nutrient intakes on upregulation of protective enzymes and potential reduction in risk of chronic diseases like AMD.

Hanneken et al.[43] showed that, unlike other flavonoids, cyanidin, malvidin, and peonidin (all at 50 μM) were ineffective in protecting 1-day ARPE-19 cells from 250 μM H_2O_2. They also found that other flavonoids, including quercetin, fisetin, and galangin, protect retinal ganglion and RPE cells by stimulation of ARE responsive genes.[43,44] Neither anthocyanindins nor anthocyanins were tested for the ability to upregulate HO-1 or GST-pi. Delphinidin and cyanidin do influence expression of HO-1 in human vascular endothelial cells in vitro,[45] but it is unlikely that these aglycones (anthocyanidins) are a predominant form in vivo.[6] To our knowledge, this is the first study showing modulation of oxidative stress defense enzymes HO-1 and GST-pi by bilberry anthocyanins in RPE in vitro, suggesting the need for confirmatory studies in situ and in vivo.

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