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Anthocyanins Protect Against A2E Photooxidation and Membrane Permeabilization in Retinal Pigment Epithelial Cells¶

Young P. Jang[1,2], Jilin Zhou[1], Koji Nakanishi[2] and Janet R. Sparrow*[1,3]

[1]Department of Ophthalmology, Columbia University, New York, NY
[2]Department of Chemistry, Columbia University, New York, NY
[3]Department of Pathology and Cell Biology, Columbia University, New York, NY

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ABSTRACT

The pyridinium bisretinoid A2E, an autofluorescent pigment that accumulates in retinal pigment epithelial cells with age and in some retinal disorders, can mediate a detergent-like perturbation of cell membranes and light-induced damage to the cell. The photodynamic events initiated by the sensitization of A2E include the generation of singlet oxygen and the oxidation of A2E at carbon–carbon double bonds. To assess the ability of plant-derived anthocyanins to modulate adverse effects of A2E accumulation on retinal pigment epithelium (RPE) cells, these flavylium salts were isolated from extracts of bilberry. Nine anthocyanin fractions reflecting monoglycosides of delphinidin, cyanidin, petunidin and malvidin were obtained and all were shown to suppress the photooxidation of A2E at least in part by quenching singlet oxygen. The anthocyanins tested exhibited antioxidant activity of variable efficiency. The structural characteristics relevant to this variability likely included the ability to form a stable quinonoidal anhydro base at neutral pH, a conjugated diene structure in the C (pyrane) ring, the presence of hydroxyl groups on the B (benzene) ring and the relative hydrophobicity conferred by the arrangement of substituents on the B ring. Cells that had taken up anthocyanins also exhibited a resistance to the membrane permeabilization that occurs as a result of the detergent-like action of A2E.

INTRODUCTION

It is widely held that diets rich in fruits and vegetables can defend against some forms of degenerative disease. The protective effects are usually attributed to the antioxidant vitamins E and C and to carotenoids such as β-carotene. There are, however, other phytochemicals that may play a significant preventive role, one of these being anthocyanin (1). The latter polyphenolic compounds are water-soluble pigments that account, in part, for the red, purple and blue colors of fruits and flowers. Anthocyanins belong to the flavonoid family of compounds and occur in nature as glycosides (anthocyanosides). A particularly rich source of anthocyanins is the bilberry plant (*Vaccinium myrtillus*, Ericaceae), extracts of which are used as nutritional supplements (2).

Although all anthocyanins have the structure of 2-phenylchromenylium salts (Fig. 1), the nonsugar portion of the compound occurs naturally in 6 different forms, all varying in the number and arrangement of hydroxyl and methoxyl groups (3). In addition, each of these three-ringed structures can be glycosylated by different sugars, the most common being glucose, although galactose, rhamnose, arabinose and xylose residues are also found. Orally administered anthocyanins are absorbed within the gastrointestinal system of humans in their unchanged glycosylated forms (3) and have been shown to be incorporated into cultured endothelial cells when delivered in the medium (4).

Because the structure of anthocyanins is consistent with an ability to donate hydrogen atoms, it has been speculated for some time that these compounds have antioxidant capability, *in vivo*. Accordingly, anthocyanins have been shown to scavenge chemically generated superoxide (5–7) and nitric oxide radicals (5), to diminish H_2O_2–mediated cytotoxicity (4) and to reduce α-tocopheroxyl radical ($TocO^{\bullet}$) to α-tocopherol (TocOH) (8). The *in vitro* antioxidant activities of anthocyanins have even been reported to supersede that of vitamin E (9,10). On the other hand, anthocyanins do not exhibit an ability to induce NAD(P)H:quinone oxidoreductase (2), a Phase 2 enzyme that inactivates carcinogenic electrophiles and exhibits antioxidant activity (11).

Although there is as yet no evidence that bilberry extracts can protect or improve vision (12), one of the degenerative disorders for which these extracts are marketed is age-related macular degeneration (AMD). The etiology of atrophic AMD is still poorly understood, but it is generally accepted that AMD begins with the death of retinal pigment epithelial (RPE) cells, the degeneration of photoreceptor cells and the resultant loss of vision following

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*To whom correspondence should be addressed: Department of Ophthalmology, Columbia University, New York, NY 10032, USA. Fax: 212-305-9638; e-mail: jrs88@columbia.edu

Abbreviations: AMD, age-related macular degeneration; cya-ara, cyanidin 3-arabinoside; cya-gal, cyanidin 3-galactoside; cya-glc, cyanidin 3-glucoside; DAPI, 4′,6-diamidino-2-phenylindole; DPBS, Dulbecco's phosphate-buffered saline; del-ara, delphinidin 3-arabinoside; del-gal, delphinidin 3-galactoside; del-glc, delphinidin 3-glucoside; ESI, electrospray ionization; FAB-MS, fast atom bombardment ionization mass spectrometry; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*′-ethanesulfonic acid; HPLC, high-performance liquid chromatography; LCMS, liquid chromatography–mass spectrometry; mal-ara, malvidin 3-arabinoside; mal-glc, malvidin 3-glucoside; pet-glc, petunidin 3-glucoside; Q-TOF, quadrupole time-of-flight; RPE, retinal pigment epithelium; $TocO^{\bullet}$, α-tocopheroxyl radical; TocOH, α-tocopherol.

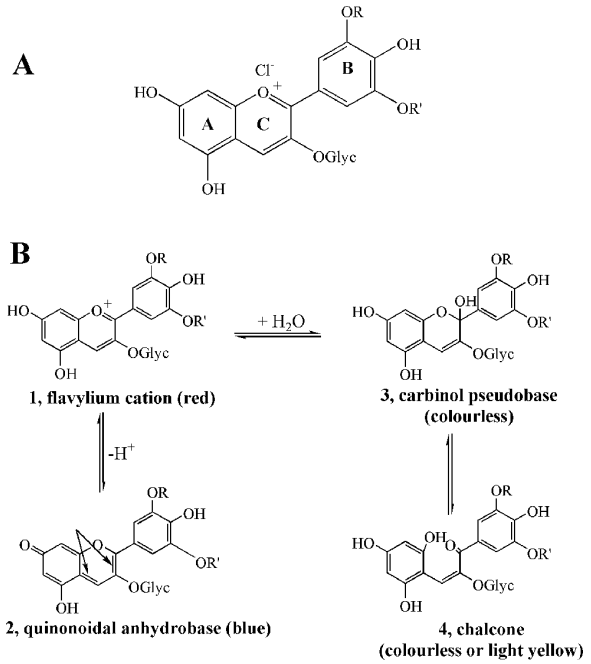

Figure 1. A: Anthocyanins, a subclass of flavonoid, are defined by a three-ring ($C_6C_3C_6$) structure consisting of two benzene rings (A and B) flanking the oxygen-containing pyran ring (C). Individual differences result from variation in the number and arrangement of the hydroxyl groups as well as the extent of alkylation and/or glycosylation of these groups. **B**: Anthocyanins undergo reversible structural changes with four structural types existing in equilibrium: flavylium cation **1**, quinonoidal anhydro base **2**, colorless carbinol bases **3** and pale yellow chalcones **4**. The relative concentrations of these species at equilibrium are dependent on acid–base proton transfer, hydration of the pyrilium nucleus and ring-chain tautomeric reactions. Acid–base equilibrium between the flavylium cation and the quinonoidal anhydro base is pH dependent. The diene structure (arrows) on the quinonoidal anhydro base **2** is an excellent substrate for singlet oxygen.

thereafter. Aging of the RPE cell is accompanied by the progressive accumulation of fluorescent pigments that constitute lipofuscin deposits in the cell. There is a growing body of evidence linking RPE lipofuscin with AMD. For instance, histological analyses of human donor eyes (13,14), in addition to noninvasive fundus spectrophotometry (15,16) and confocal ophthalmoscopy (17), have shown that RPE cells overlying the macula exhibit the most pronounced age-related accumulation of lipofuscin. The monitoring of RPE lipofuscin through the detection of fundus autofluorescence has also revealed that areas of RPE atrophy develop at sites of previously increased fluorescence (17–19). Studies concerned with examining associations between RPE lipofuscin and RPE cell death have shown that a constituent of RPE lipofuscin, the bisretinoid fluorophore A2E and its photoisomer iso-A2E (20), can perturb cellular membranes (21,22) and can mediate blue-light damage to RPE (23,24). The photosensitization of A2E leads to the generation of singlet oxygen and reaction of the latter at carbon–carbon double bonds leads to A2E photooxidation (25,26). Ultimately, the photochemical events provoked by the irradiation of A2E in RPE cells initiates cell death (23) by way of a pathway that involves the participation of cysteine-dependent proteases (caspases) to cleave cellular substrates, and that is modulated by the mitochondrial protein Bcl-2 (27).

Extracts of bilberry are widely used in the United States as phytoceuticals although little work has been done to elucidate their therapeutic effectiveness. This is cause for some concern because preparations of bilberry, like most other plant medicinals, are not regulated by the U.S. Food and Drug Administration. If bilberry extract has therapeutic properties, it would likely be because of its content of anthocyanins. In this study, anthocyanins were isolated from bilberry extracts and analyzed for the ability to modulate adverse effects of A2E on RPE cells.

MATERIALS AND METHODS

Reagents. Bilberry extract was from Nature's Resource Premium Herb (Mission Hills, CA); ethanolamine, trifluoroacetic acid and vitamin E were purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI); *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) was obtained from Sigma Chemical Company (St. Louis, MO); acetonitrile was purchased from Fisher Scientific Company (Fair Lawn, NJ) and Dulbecco's phosphate-buffered saline (DPBS) from GIBCO-BRL (now Invitrogen, Carlsbad, CA). HP-20 resin was purchased from Supelco (Bellefonte, PA). All other chemicals were from Sigma. A2E was synthesized as previously described (20).

Cell culture, treatment and illumination. Human adult RPE cells (ARPE-19; American Type Culture Collection, Manassas VA) lacking endogenous A2E (21) were grown as previously described (21,23). Confluent cultures were allowed to accumulate A2E from a 20 μ*M* concentration in media, an extracellular concentration that does not induce membrane damage, and cultures were illuminated at 430 nm (± 30 nm; 8 mW/cm^2) under conditions that have been previously described (23,25,27). Alternatively, cells were incubated in 100 μ*M* A2E to test for membrane permeabilization (21). When applicable, cultures were treated with anthocyanins (100 μ*M*) dissolved in PBS buffer.

Evaluation of the singlet oxygen quenching activity of anthocyanins. To measure the singlet oxygen quenching activity of anthocyanins, A2E (100 μ*M*) and anthocyanins (100 μ*M*) were incubated together with a single oxygen generator: the endoperoxide of 1,4-dimethylnaphthalene (10 m*M* in 100 μL of methanol) (25,26). The mixture was stirred overnight in the dark at room temperature; following which, A2E was quantified by high-performance liquid chromatography (HPLC) using A2E as the external standard.

Mass spectrometry. Fast atom bombardment ionization mass spectrometry (FAB-MS) for oxidized A2E was performed on a JMS-HX110A/110A tandem mass spectrometer (JEOL, Akishima, Tokyo, Japan), using 10 kV acceleration voltage and fitted with a Xe beam FAB gun (6 kV) on the MS-1 ion source. 3-Nitrobenzyl alcohol was used as matrix. Liquid chromatography–mass spectrometry (LCMS) analysis of the anthocyanins was performed on a quadrupole time-of-flight (Q-TOF) detector (Micromass, Manchester, UK) with an electrospray ionization (ESI) source. ESI capillary voltage was 3 kV. The sample was introduced with a flow-injection mode using 80% acetonitrile and 0.1% TFA in water as a solvent. Flow rate was 0.2 mL/min.

Assay of cell viability. Apoptotic cells and membrane-permeabilized cells were labeled with the membrane-impermeant dye Dead Red (Molecular Probes, Eugene, OR) and the nuclei of all cells with 4′,6-diamidine-2-phenylindole (DAPI), as previously described (25). Counting was performed from digital images; nonviable cells were expressed as a proportion of the total number of cells in an illuminated field.

Activity-guided fractionation of bilberry and isolation of anthocyanins. Bilberry extract (10 g) was dissolved in 250 mL of deionized water and fractionated between the organic and aqueous phases by extracting an aqueous solution of bilberry with 250 mL of ethyl acetate three times. HP-20 dianion resin was used to divide aqueous layer into 10 subfractions (H_2O:MeOH = 100:0 → 0:100). The fractions were assayed for their ability to inhibit A2E-oxidation by exposing preparations of A2E (20 μ*M* in DPBS diluted from stock solution of A2E in DMSO) with and without each fraction (250 μg/mL), to 430 nm light for 10 min (0.36 mW/mm^2). Anthocyanins were isolated by preparative HPLC of the aqueous layer of bilberry extract.

HPLC. An HP 1100 Series HPLC equipped with a photodiode array detector was used with a reverse-phase C18 column (150 mm × 4.6 mm, Cosmosil 5C18, Nacalai Tesque, Japan) for quantitation studies and a Vydac C18 (250 mm × 22 mm, 10 μm, Grace Vydac, Hesperia, CA) for anthocyanin separation. Detection was at 520 nm. A2E was quantitated with the following gradient of acetonitrile in water (containing 0.1% TFA): 85–95% (vol/vol, 10 min), 95–100% (5 min), 100% (5 min) and a flow rate of 0.8 mL/min with monitoring at 430 nm. In some experiments, A2E was

extracted from RPE cells with $CHCl_3$ after homogenizing with a glass tissue homogenizer. A2E was used as external standard for quantitation, and A2E levels were normalized to cellular protein concentration determined by the Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA). Anthocyanins were quantitated with the following gradient of 5% formic acid in acetonitrile: 95–90% (vol/vol, 5 min), 90–85% (10 min) and a flow rate of 0.8 mL/min at 520 nm. To isolate anthocyanins by preparative HPLC, a solvent system of 5% formic acid in acetonitrile was used as the mobile phase with the following gradient: 93% (vol/vol, 30 min), 93–90% (10 min), 90–87% (30 min), 87–77% (20 min), 77–75% (10 min), 75–30% (20 min) and a flow rate of 4.0 mL/min at 520 nm; injection volume was 100 μL (40 mg/mL).

Hydrolysis of anthocyanins. To remove sugar residues from the anthocyanins, the pigments were boiled in 2 M HCl for 1 hour to generate aglycones of cyanidin, petunidin and malvidin. Each aglycone was purified by HPLC.

Statistical analysis. Data were analyzed by one-way ANOVA, and the Newman Keul Multiple Comparison test (Prism, GraphPad Software, San Diego, CA).

RESULTS

Anthocyanins isolated from bilberry

We began this work with a reexamination of the ability of bilberry extracts to inhibit A2E photooxidation. From the FAB-MS spectra obtained after samples of A2E were irradiated at 430 nm, it was clear that bilberry extracts reduced A2E photooxidation. As shown in Fig. 2, in the absence of the antioxidant, we detected oxidation at as many as 6 double bonds along the side arms of A2E (MHz 608, 624, 640, 656, 672 and 688). Conversely, with the addition of bilberry extract, only three higher molecular weight species (MHz 608, 624 and 640) were detected.

Anthocyanins were subsequently isolated from the total bilberry extract. Separation of the organic soluble phase from the water-soluble fraction in ethyl acetate demonstrated that the antioxidant activity resided primarily in the aqueous phase. The latter fraction was then concentrated and chromatographed on a Dianion HP-20 column using a water-methanol gradient. Each of the nine fractions that were resolved were coincubated with A2E before 430 nm irradiation; all demonstrated an ability to inhibit A2E photooxidation.

With the application of reverse-phase HPLC and gradient elution, nine individual anthocyanins, all monoglycosylated, were resolved in the water-soluble active fractions (Fig. 3); the identity of the peaks was determined on the basis of previously described elution orders and mass spectra (2,28) obtained by LCMS. The sequence of elution of the compounds and corresponding mass-to-charge ratio (MHz) as determined by mass spectroscopy was delphinidin 3-galactoside (del-gal), MHz 465; delphinidin 3-glucoside (del-glc), MHz 465; cyanidin 3-galactoside (cya-gal), MHz 449; delphinidin 3-arabinoside (del-ara), MHz 435; cyanidin 3-glucoside (cya-glc), MHz 449; cyanidin 3-arabinoside (cya-ara), MHz 419; petunidin 3-glucoside (pet-glc), MHz 479; malvidin 3-glucoside (mal-glc), MHz 493 and malvidin 3-arabinoside (mal-ara), MHz 463. This elution order is consistent with their known hydrophobicities (29,30). Of the nine anthocyanins isolated from the extract, the predominant compounds (~58%) were del-gal (~9%), del-glc (~13%), cya-gal (~24%) and cya-glc (~12%).

Anthocyanins protected against A2E-oxidation in a cell-free system

Given that anthocyanins are purported to have antioxidant properties (1,8,31), we sought to establish whether they could inhibit light-induced photooxidation of A2E. For these experiments, A2E in aqueous media was exposed to 430 nm illumination in the presence and absence of anthocyanins. To test correlations between chemical structure and antioxidant activity, we probed with anthocyanins varying in terms of the hydroxyl and methoxyl substituents on the B ring: delphinidin, cyanidin and petunidin and malvidin. We also compared these anthocyanins with and without (aglycones) the sugar residues galactose, glucose and arabinose bound to their structures at the 3-O position. The mixtures were subsequently analyzed by quantifying the consumption of A2E as A2E-photooxidation occurred. Blue-light irradiation resulted in an HPLC profile that revealed a decrease in the absorbance of the A2E peak, reflecting a loss of A2E as compared with control non-illuminated samples (data not shown). This photooxidation-associated consumption in A2E was attenuated, however, when the irradiated mixture contained the anthocyanins or anthocyanidins

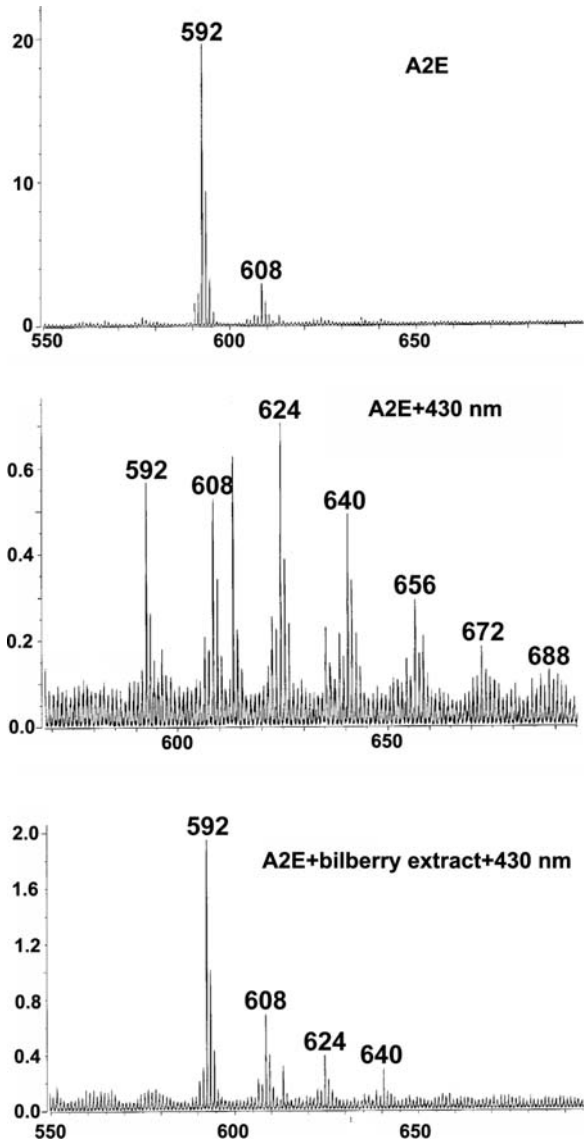

Figure 2. Bilberry extract reduced A2E photooxidation. FAB-MS of A2E (200 μ*M* in PBS), A2E irradiated at 430 nm (blue light) and A2E irradiated in the presence of bilberry extract. The molecular ion peak at MHz 592 corresponds to the molecular mass of A2E. The photooxidation of A2E is indicated by the presence of additional peaks that differ by mass 16. Illumination in the presence of bilberry extract reduces the intensity of these peaks and eliminates MHz 656, 672 and 688.

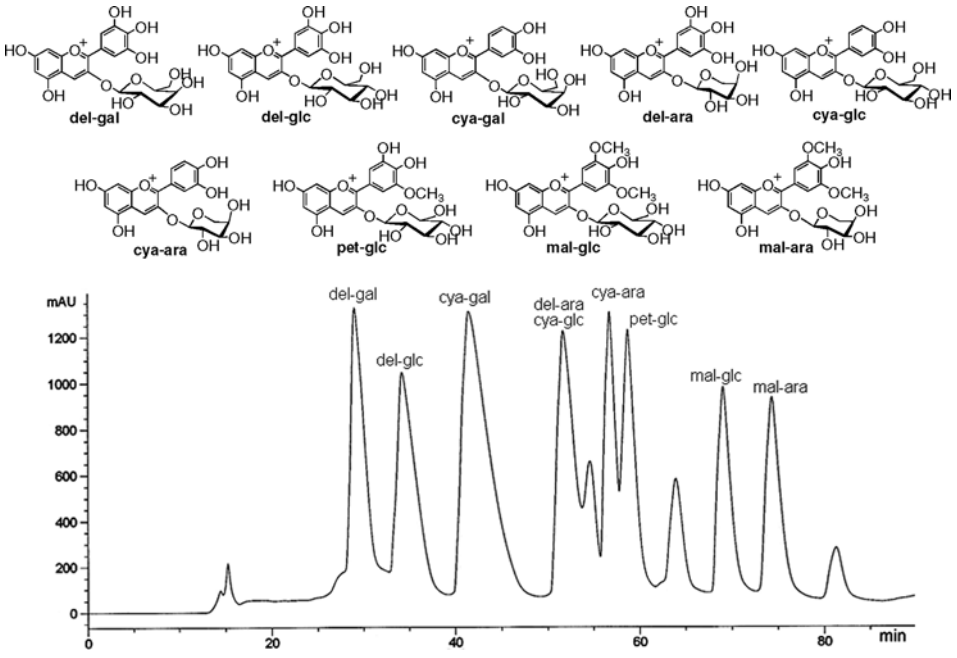

Figure 3. Reverse-phase HPLC profile of anthocyanins isolated from water-soluble extracts of bilberry. Monitoring was performed at 520 nm. Nine species were detected: delphinidin 3-galactoside (del-gal), delphinidin 3-glucoside (del-glc), cyanidin 3-galactoside (cya-gal), delphinidin 3-arabinoside (del-ara), cyanidin 3-glucoside (cya-glc), cyanidin 3-arabinoside (cya-ara), petunidin 3-glucoside (pet-glc), malvidin 3-glucoside (mal-glc) and malvidin 3-arabinoside (mal-ara). The structures of these compounds are presented above.

(aglycones of anthocyanins) (Fig. 4). Expressed as percent protection, the reduction in the loss of A2E varied between 30 and 70% and was most pronounced for malvidin. In experiments performed to compare the antioxidant capability of vitamin E (100 μM) under identical conditions, 25% protection was observed (data not shown). The antioxidant protection afforded by mal-glc was significantly greater ($P < 0.001$) than the 3-glucosides of cyanidin, petunidin and delphinidin. On the other hand, the latter three anthocyanin species were not significantly different from one another ($P > 0.05$). Substituting arabinose for glucose at the 3-O position reduced the antioxidant efficiency in the case of malvidin ($P < 0.05$) but had no effect on delphinidin and cyanidin ($P > 0.05$). Galactose at the 3-O position also had no effect on the antioxidant activity of cyanidin ($P > 0.05$) although it may have increased the protectiveness of delphinidin ($P < 0.05$). The effect of removal of the sugar also varied with anthocyanin structure, there being a reduction when cya-glc was compared with its aglycone ($P < 0.05$) but no effect in the case of the 3-glucosides of petunidin and malvidin.

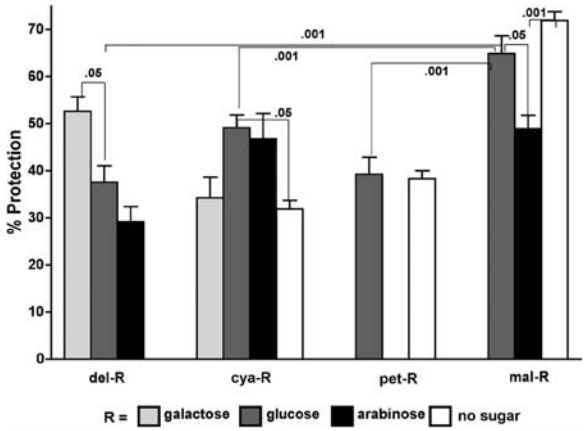

Figure 4. Protective effects of anthocyanin pretreatment on blue light–induced A2E oxidation. The loss of A2E that accompanies A2E oxidation was quantified by HPLC after A2E was irradiated at 430 nm in the presence and absence of glycosidic (galactoside, glucoside and arabinoside) and non-glycosidic (no sugar) forms of delphinidin, cyanidin, petunidin and malvidin as indicated. Anthocyanins were used at a concentration of 100 mM. Data are expressed as percent protection ([A2E + anthocyanins + blue light] − [A2E + blue light]) ÷ ([A2E] − [A2E + blue light]) × 100; mean ± SEM of three experiments. *P* values are given when significant differences apply.

Anthocyanins quench singlet oxygen

Because we have previously shown that singlet oxygen can mediate the photooxidation of A2E, we sought to determine whether the anthocyanins inhibit A2E oxidation by quenching singlet oxygen. To this end, we generated singlet oxygen from the endoperoxide of 1,4-dimethylnaphthalene, which decomposes to release singlet oxygen. We previously demonstrated that for the oxidation of A2E, the endoperoxide can substitute for blue-light irradiation (26). Accordingly, mixtures of A2E and 1,4-dimethyl naphthalene endoperoxide with and without anthocyanins were incubated for 14–15 h at room temperature. As shown in Fig. 5, quantification of A2E by HPLC revealed a 96% decrease in the level of A2E following its incubation with the singlet oxygen generator but without anthocyanins. This loss of A2E is indicative of A2E oxidation. Addition of anthocyanin (100 μM) to the A2E–endoperoxide mixture shielded A2E so that its loss was reduced to 62% (delphinidin 3-galactose) and 63% (cyanidin 3-galactose) of control A2E levels not incubated with endoperoxide ($P < 0.001$). These results demonstrate that anthocyanins can protect against A2E oxidation by quenching singlet oxygen.

Anthocyanins protect A2E-laden RPE cells from blue light–induced damage

To study anthocyanins in a cell-based system, we first incubated ARPE-19 cells with an unfractionated anthocyanin mixture, with delphinidin 3-galactose and cyanidin 3-galactose (100 μM for 2–5 days). Incorporation of anthocyanins into the cells was confirmed by light microscopy (Fig. 6). When incubated with anthocyanins for up to 5 days, the ARPE-19 cells showed no evidence of cell toxicity

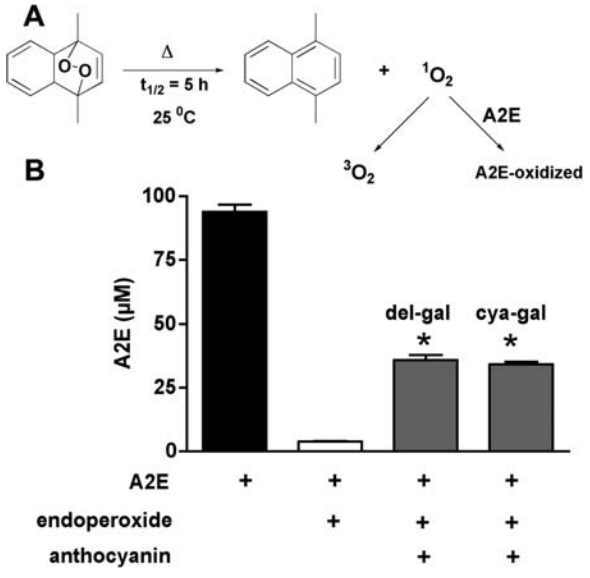

Figure 5. Anthocyanins inhibit A2E-oxidation by quenching singlet oxygen. A: A2E was oxidized (A2E-oxidized) by singlet oxygen generated from the endoperoxide of 1,4-dimethylnaphthalene (25°C in CD_3OD). B: A2E oxidation with and without the addition of delphinidin galactoside (del-gal, 100 m*M*) or cyanidin galactoside (cya-gal, 100 m*M*) was quantified by HPLC as the loss in A2E. The symbols (+) and (–) indicate the presence or absence, respectively, of a compound; $*P < 0.001$ when compared with a mixture of A2E and endoperoxide in the absence of anthocyanin. Data are the mean ± SEM of three experiments.

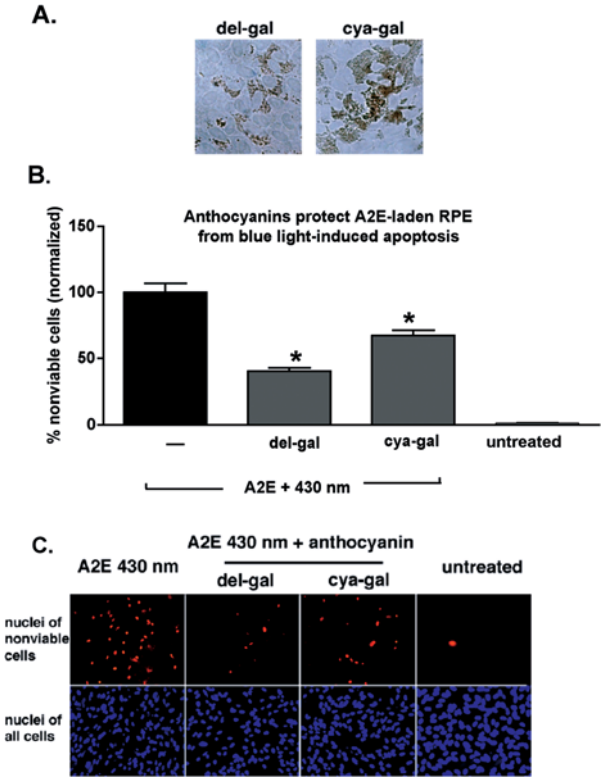

Figure 6. Anthocyanins protect A2E-laden RPE from blue light–induced death. A: Light microscopic detection of delphinidin galactoside and cyanidin galactoside incorporated by ARPE-19 cells. Phase-contrast images. B: ARPE-19 cells that had accumulated A2E were incubated without anthocyanin (–) or with delphinidin galactoside (del-gal, 100 m*M*) or cyanidin galactoside (cya-gal, 100 m*M*) for 3 days and were then irradiated at 430 nm. Values are mean ± SEM of three experiments; $*P < 0.001$ when compared with the absence of anthocyanin. C: Fluorescence detection of nuclei of nonviable cells (red) and nuclei of all cells (blue); images representative of experiments in B.

(data not shown). With the anthocyanin mixture approximately 1.1 mmol of anthocyanin was incorporated per mg protein.

We then sought to test the antioxidant capacity of cell-associated anthocyanins by testing their capacity to confer a resistance to A2E-mediated blue light damage. In these experiments, ARPE-19 cells that had accumulated A2E were subsequently incubated with and without delphinidin 3-galactose or cyanidin 3-galactose for 3 days and were then illuminated with blue light. The capacity for anthocyanins to confer a cellular resistance to blue-light damage was evident when ARPE-19 cells that had accumulated A2E were incubated. Pretreatment with delphinidin 3-galactose or cyanidin 3-galactose reduced cell death by 60% and 33%, respectively ($P < 0.001$) (Fig. 6).

Anthocyanins reduced cellular incorporation of A2E

In the foregoing experiments, cells were allowed to accumulate A2E before they were incubated with anthocyanins. However, in other experiments in which we incubated the cultured RPE cells with anthocyanins before A2E accumulation, we consistently observed by microscopic examination that the amount of A2E amassed was reduced when compared with cells that had not been pretreated with anthocyanins. To confirm these observations by quantitative assay, the amounts of A2E and iso-A2E, the latter being a less abundant photoisomer, were quantified by HPLC to determine the cellular incorporation of these fluorophores. Consistent with our previous observation that A2E and iso-A2E reach photoequilibrium at a ratio of 4:1 (A2E:iso-A2E) (20), A2E was present at a 3.8-fold greater abundance than iso-A2E (Fig. 7). Under conditions in which the cells were pretreated with anthocyanins, cellular incorporation of both A2E and iso-A2E was reduced. The decrease from delphinidin 3-galactose was 29% for both A2E and iso-A2E whereas cyanidin 3-galactose reduced the accumulation of A2E and iso-A2E by 46% and 44%, respectively (Fig. 7). Despite the reduced incorporation of A2E/isoA2E, the ratio of these photoisomers remained constant.

Anthocyanins reduced A2E-mediated membrane permeabilization

Because we have shown, using the plasma membrane as a model bilayer, that at certain extracellular concentrations A2E can induce membrane damage, we next sought to test whether anthocyanins can protect against this detergent-like activity. In these experiments, ARPE-19 cells were incubated with del-gal or cya-gal for 3 days and were incubated with A2E (100 μ*M*) for 18 hours. Membrane integrity was subsequently evaluated according to the ability of a membrane-impermeable dye to penetrate into the cell; the latter was indicated by nuclear labeling. Without anthocyanin pretreatment, nuclei were labeled in one third of the cells exposed to 100 μ*M* A2E. However, in the presence of del-gal, the number of permeabilized cells was decreased by 90% whereas with cya-gal the decrease was 72% (Fig. 8).

DISCUSSION

In aqueous environments, common anthocyanins such as those studied in the present work, exist as a mixture of several structures

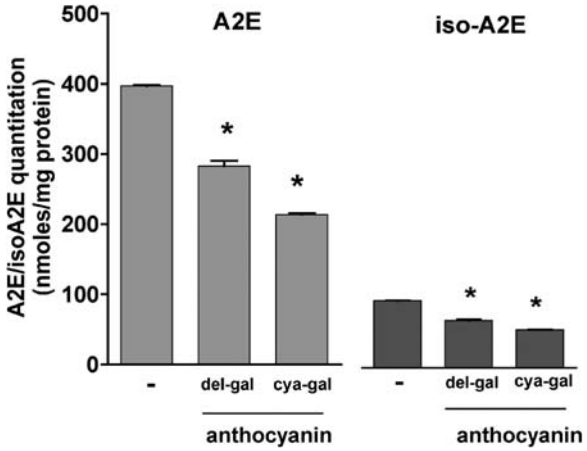

Figure 7. In cultured ARPE-19 cells that have incorporated anthocyanins, the accumulation of A2E is retarded. Cells were incubated with 100 μ*M* delphinidin 3-galactoside (del-gal) or cyanidin 3-galactoside (cya-gal) for 2 days or were not incubated (–). The cells were then allowed to accumulate A2E (20 μ*M* for 10 days) after which cell-associated A2E and iso-A2E (photoisomer of A2E) was quantified by HPLC and normalized to cellular protein levels. Percent incorporation of A2E/isoA2E into RPE cells was 71.4/70.5% for del-gal and 54.0/56.9% for cya-gal, respectively. Values are mean ± SEM of three experiments, $*P < 0.001$.

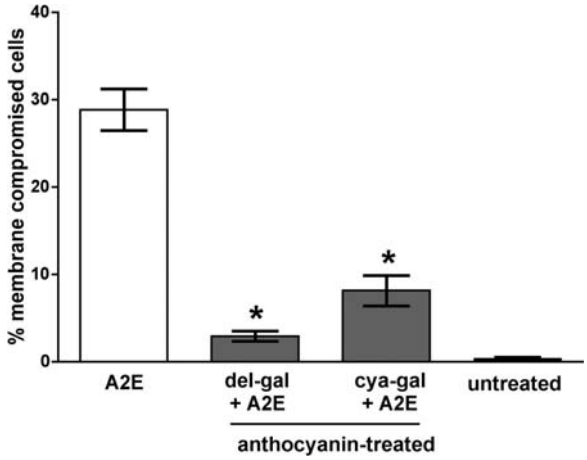

Figure 8. Anthocyanins protect against A2E-induced membrane damage. ARPE-19 cells incorporated delphinidin galactoside (del-gal) and cyanidin galactoside (cya-gal) from a 100 μ*M* concentration for 3 days before being incubated with 100 μ*M* A2E for 18 h. Nuclei of membrane-compromised cells were labeled with a membrane-impermeable dye and counted. Values are mean ± SEM of three experiments; $*P < 0.001$ when compared with A2E without prior incorporation of anthocyanin.

in chemical equilibrium: flavylium cation, a red pigment; quinonoidal anhydrobase (blue); carbinol pseudobase (colorless) and chalcone (colorless or light yellow) (2,32). The latter is quite unstable and easily decomposes to other compounds. At acidic pH, anthocyanins exist chiefly in the form of the flavylium cation. As the pH increases, deprotonation occurs so that at neutral pH, anionic forms of the quinonoidal base predominate (33). In our experiments carried out at neutral pH, the anthocyanins exhibited a blue color, indicating that the quinonoidal anhydrobase likely dominated. This form is the most efficient for the quenching of nonradical singlet oxygen because its unsaturated diene conjugation (double-single-double bond arrangement) allows for the addition of singlet oxygen and the formation of endoperoxide at these electron-rich double bonds. Given that A2E is photooxidized by the singlet oxygen generated upon photosensitization of A2E, quenching of singlet oxygen by the quinonoidal anhydrobase form is likely to be at least partially responsible for the anthocyanin-mediated antioxidant effects we observed. Indeed, although A2E is itself an efficient quencher of singlet oxygen (26), anthocyanins can intercede.

The availability of hydroxyl groups on the B (benzene) ring of anthocyanins is also fundamental to the antioxidant activity of these compounds because these hydroxyl moieties can donate hydrogens to scavenge radicals (1,31,34,35). For instance, the antioxidant protection provided by glycosides of cyanidin are in large part attributable to the presence of hydroxyl groups at ortho positions on the B ring. The donation of a hydrogen by anthocyanins serves to scavenge oxygen radicals such as superoxide ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) and by contributing a hydrogen to the peroxyl radical can also break the chain reactions involved in lipid peroxidation (35). The extent to which these moieties contributed to the antioxidant activity we observed is not yet clear. It is relevant that in addition to singlet oxygen, superoxide is generated by the photosensitization of A2E (36) (Kim, S. R. and Sparrow, J. R., unpublished); however, we have not yet determined whether superoxide contributes to the photooxidation of A2E. Nevertheless, superoxide that directly contributes to blue light/A2E–mediated cellular damage may have been quenched by anthocyanins. It is also noteworthy that the protection afforded by anthocyanins was less pronounced in experiments that assayed the ability of anthocyanins to protect A2E from endoperoxide-derived singlet oxygen (Fig. 5) than in the acellular assays of A2E photooxidation (Fig. 4) and in cell viability assays (Fig. 6). This difference may indicate that under the latter two conditions, superoxide scavenging by the anthocyanins contributed to the protective effects. The absence of differences among the anthocyanins that are dihydroxylated or trihydroxyated on the B ring (cya-glc and pet-glc versus del-glc) is not necessarily instructive on the issue of superoxide scavenging because two hydroxyl groups (cyanidin and petunidin) can confer greater antioxidant activity than one, but three hydroxyl groups do not provide further antioxidant activity (1). Under the experimental conditions involving blue-light illumination, we cannot exclude the possibility that some of the protection observed was a result of anthocyanin-mediated screening of blue light. However, given the evidence presented above that anthocyanins quench endoperoxide-derived singlet oxygen, these pigments clearly also play an antioxidant role.

Of the anthocyanins we tested, the most potent antioxidant protection was provided by mal-glc, a compound having methoxy groups positioned ortho to the functional hydroxyl groups in the B ring. Little difference among the other anthocyanins was observed. Certainly, the presence of a conjugated diene system in the C ring of malvidin enabled singlet oxygen quenching. An additional structural feature of malvidin, however, may be the methoxy (OCH_3) groups on the B ring. When compared with species such as cyanidin and delphinidin, which have two or three hydroxyl (OH) groups on the B ring, substitution of these hydrophilic hydroxyl (OH) groups with methoxy (OCH_3) groups likely renders malvidin more hydrophobic (37). This could be an advantage in our system. The increase in hydrophobicity may allow it to interact with the hydrophobic side arms of A2E and in so doing, to neutralize singlet oxygen or other reactive oxygen species at the site of their formation. Lipophilicity would be of added advantage to cell-associated A2E because spectroscopic studies suggest that the side arms of A2E intercalate into the nonpolar core of membranes

(21,38). Methoxy substituents can also stabilize the quinonoidal base form of malvidin (33,39). Our results showing that malvidin is more active than cyanidin and delphinidin are consistent with a study that compared these anthocyanins in an assay measuring oxidation in a liposomal system (40). Here too, the lower polarity imparted by the methoxy groups was thought to permit an attraction for the interface of the liposome. Mal-glc is also effective against lipid peroxidation (32,41).

Another feature that can influence the behavior of the compounds we tested is their glycosylation (1). Anthocyanins normally occur in fruits and vegetables with sugar residues bound to their structures, usually at the 3-position. The most common sugar is a glucose residue, but others such as the galactose and arabinose observed in the present work can also occur. Because the sugar groups are hydrophilic, glycosylation could change the antioxidant activity in our system because it may impede the ability of the compound to partition into the nonpolar domain of A2E. Not surprisingly therefore, the aglycone of malvidin provided slightly greater, although not significantly different ($P > 0.05$), protection than glucose-3-malvidin. The aglycone of malvidin has also been shown to be more efficacious than the glycosylated form in experiments evaluating antioxidant activity in a liposomal system (40). By reducing the tendency of the anthocyanin to transform to the chalcone form that can readily undergo cleavage and then degrade, sugar attachment at the 3-O position also influences the stability of the anthocyanins (39). One study suggests that the stability of bilberry anthocyanins decreases in the following order: glucosides > galactosides > arabinosides (42). Nevertheless, the results of investigations comparing the antioxidant activity of anthocyanins and their aglycones have varied widely. Some studies have reported that aglycones are more efficacious than glucosides (1,10) whereas others (1,9) found that some glucosides are better than the aglycone.

Also of importance is the stability of the aryloxyl radical ($AnO^{\bullet}$) formed from the deprotonation of the hydroxyl. A reactive secondary radical is not desirable (31). Here again lies the importance of unsaturation in the C ring of anthocyanins because the presence of the double bonds allows for electron delocalization between the A and B rings thus stabilizing the aryloxyl radical after hydrogen donation (31). Investigators have shown that ortho substitution with electron-donating alkyl or methoxy groups increases the stability of the aryloxyl radical and its antioxidant potential (1). Thus perhaps the methoxy groups ortho to the functional hydroxyl groups in the B ring of malvidin contribute to the increased antioxidant potential we observed. It is also suggested that malvidin may be a strong hydrogen donor because of the higher negative charge conferred by methoxy substituents as opposed to hydroxyl groups (40).

A good antioxidant, besides being a compound that can donate electrons or hydrogens, must also be available in tissues at sufficient concentration to be effective (32). Although anthocyanins are relatively abundant in the human diet, there has been considerable interest in their bioavailability. Initially it was thought that only aglycones are able to be absorbed through the intestinal wall. However, several anthocyanins including delphinidin and cyanidin have been detected as glycosides in human urine and plasma after their oral consumption indicating that they are absorbed in their unchanged glycoside forms (3,43,44). The mechanism by which anthocyanin glycosides are absorbed is not known, but the involvement of glucose transport receptors has been suggested (3,43). In addition, the ability of anthocyanins to competitively inhibit the transport activity of bilotranslocase, an organic anion carrier in the gastric mucosa and liver, may indicate that anthocyanins themselves are ligands for this transporter (45). Within endothelial cells, uptake into the plasma membrane and cytosol has been observed, with plasma membrane incorporation exceeding that in the cytoplasm (4).

In addition to the photooxidative damage that A2E can mediate, it can also disrupt cell membranes when presented at sufficient concentration. A2E-induced loss of membrane integrity has been witnessed as both an increase in membrane permeability and membrane blebbing (21,38). Within RPE cells of the eye, A2E-mediated disruption may be the fate of the lysosomal membrane because it is in this degradative compartment that A2E accumulates. The structural correlate for this detergent-like behavior resides in the positively charged hydrophilic head group and hydrophobic side arms of the compound. It is evident that the latter side arms associate with the hydrophobic core of the membrane bilayer because the emission maximum of cell-associated A2E corresponds most closely to that observed in nonpolar solvents (21). It is particularly interesting, therefore, that anthocyanins can provide resistance to the detergent-like perturbations mediated by A2E. Other flavonoids having structures similar to that of anthocyanins have been shown to protect membranes from detergent-induced disruption, possibly by associating with phospholipids head groups (46). These polyphenolics have also been reported to partition into the hydrophobic domain of the membrane and to decrease membrane fluidity by modifying lipid-packing order (47,48). Notably, one of the compounds bearing a methoxy substituent provoked the greatest change in membrane fluidity (47). The membrane rigidity imposed by α-tocopherol is thought to enhance its antioxidant activity (47); perhaps the methoxy moieties on malvidin, by conferring lipophilicity, serve in a similar way.

In *in vitro* experiments, we have shown that anthocyanins can serve as antioxidants that suppress photooxidative processes initiated in RPE cells by the lipofuscin fluorophore A2E. In so doing, anthocyanins reduce RPE cell damage. This work is consistent with previous reports that the anthocyanin cya-glc inhibits lipid peroxidation at neutral pH to an extent similar to (10) or considerably better than (34) that of vitamin E or its water-soluble analog Trolox. The structural criteria determining the antioxidant activity in our system appears to include the formation of stable quinonoidal anhydro base at neutral pH, the presence of a facile conjugated diene system in the C ring to allow singlet oxygen quenching, the availability of hydroxyl groups on the B ring and relative hydrophobicity. It will be important to follow this work up with *in vivo* studies aimed at evaluating anthocyanin uptake into ocular tissues.

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