

Neuroprotective effects of anthocyanins on apoptosis induced by mitochondrial oxidative stress

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Objectives: Mitochondrial oxidative stress (MOS) is a major factor in the underlying pathology of many neurodegenerative diseases. Here, we investigated the neuroprotective effects of a unique class of nutraceutical antioxidants, anthocyanins, against MOS-induced death of cultured cerebellar granule neurons (CGNs). Callistephin and kuromanin are anthocyanins derived from strawberries and black rice, respectively, whose neuroprotective properties have yet to be examined in detail.

Methods: Glutathione (GSH)-sensitive MOS and intrinsic apoptosis were induced in CGNs by incubation with the Bcl-2 inhibitor, HA14-1. The effects of anthocyanin co-incubation on CGN survival were assessed.

Results: The anthocyanins demonstrated significant protection from MOS-induced apoptosis which was equivalent to that provided by the green tea polyphenol, epigallocatechin 3-gallate; however, neither anthocyanin was as effective as GSH at rescuing CGNs. Inhibition of Bcl-2 caused a significant reduction of mitochondrial GSH which was prevented by the anthocyanins. Furthermore, the anthocyanins inhibited iron-induced lipid peroxidation in rat brain homogenates and prevented cardiolipin oxidation induced by MOS in CGNs. MOS-induced mitochondrial fragmentation and proteolytic cleavage of the optic atrophy 1 (OPA1) fusion GTPase were also attenuated by the anthocyanins. Finally, the anthocyanins significantly enhanced GSH peroxidase activity in a cell-free assay.

Discussion: These data show that anthocyanins suppress MOS-induced apoptosis by preserving mitochondrial GSH and inhibiting cardiolipin oxidation and mitochondrial fragmentation. These nutraceutical antioxidants warrant further study as potential therapeutic agents for neurodegenerative diseases caused by MOS.

Keywords: Callistephin, Cyanidin-3-O-glucoside, Glutathione, Kuromanin, Mitochondrial oxidative stress, Neuronal apoptosis, Pelargonidin-3-O-glucoside

Introduction

Anthocyanins are a unique family of natural antioxidants responsible for the deep red, blue, and purple colors found in many fruits and vegetables. Although many berries such as blueberries, cranberries, chokeberries, and lingonberries have distinct polyphenolic profiles, they all share consistently high contents of anthocyanins.¹ For example, strawberries contain four major anthocyanins of which callistephin (pelargonidin-3-O-glucoside) typically makes up 60–80% of the total anthocyanin content.² A structural comparison of antioxidant families is reviewed

by Rice-Evans *et al.*³, in which a ranking of compounds based on their trolox equivalent antioxidant activities was determined. According to this ranking, many anthocyanins and their aglycone anthocyanidin derivatives appear to have the greatest intrinsic antioxidant activity, along with other well-known natural antioxidants like epigallocatechin 3-gallate (EGCG), a potent polyphenolic antioxidant found in green tea. Thus, anthocyanins are natural compounds which possess significant intrinsic antioxidant and free radical scavenging activities.^{4,5}

Oxidative stress is commonly regarded as a key factor in the pathology of various neurodegenerative diseases and likely plays a central role in aging as well.^{6,7} Many studies have established links between

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oxidative stress and neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, amyotrophic lateral sclerosis, and multiple sclerosis, as well as acute ischemic neuronal injury such as stroke.^{8–12} In particular, *mitochondrial oxidative stress* (MOS) is extremely detrimental to neuronal survival and often underlies neurodegeneration.^{13–16} As a result, inhibition of MOS via mitochondrial-targeted antioxidants is currently a major strategy being investigated for therapeutic intervention in multiple neurodegenerative diseases.^{17–23}

The intrinsic antioxidant and free radical scavenging properties of anthocyanins suggest that they may possess significant neuroprotective activity as well. Indeed, either pure anthocyanins, anthocyanin-enriched fractions, or anthocyanin metabolites have been shown to protect PC12 cells and SH-SY5Y neuroblastoma cells from hydrogen peroxide-induced death *in vitro*.^{24–26} Similarly, anthocyanidin components of bilberries rescue RGC-5 retinal ganglion cells from peroxynitrite-induced damage and protect retinal ganglion cells from excitotoxicity *in vivo*.²⁷ Anthocyanin-enriched fractions of purple grape juice, bilberry, mulberry, and purple sweet potato color have been used to reduce oxidative stress and protect neurons in diverse paradigms of CNS injury *in vivo* such as that induced by carbon tetrachloride, psychological stress, accelerated senescence, d-galactose, middle cerebral artery occlusion, or ischemia/reperfusion.^{28–33} Collectively, these studies indicate that anthocyanins are capable of protecting neurons from an array of oxidative insults.

The effects of anthocyanins on neuronal apoptosis induced specifically by MOS have not been previously examined, nor has their neuroprotective mechanism of action been studied in detail. Here, we investigated the neuroprotective effects of two purified anthocyanins, callistephin (pelargonidin-3-*O*-glucoside) and kuromanin (cyanidin-3-*O*-glucoside), found in high concentrations in strawberries and black rice, respectively, against MOS-induced apoptosis in primary cultures of rat cerebellar granule neurons (CGNs). Our findings indicate that these anthocyanins protect CGNs from MOS-induced apoptosis by preserving mitochondrial glutathione (GSH) and preventing cardiolipin oxidation and mitochondrial fragmentation. Thus, we propose that anthocyanins, or their metabolites, protect neurons from MOS by acting locally at the level of the mitochondria to enhance the resistance of these organelles to oxidative damage.

Methods

Reagents

HA14-1 (2-amino-6-bromo- α -cyano-3-(ethoxycarbonyl)-4H-1-benzopyran-4-acetic acid ethyl ester)

and GSH-monoethyl-ester were obtained from Calbiochem (San Diego, CA, USA). Callistephin chloride, kuromanin chloride, thiobarbituric acid, GSH peroxidase cellular activity assay kit, GSH peroxidase, and monoclonal antibody against β -tubulin were purchased from Sigma-Aldrich (St Louis, MO, USA). Mitochondria/cytosol fractionation kits were obtained from BioVision (Mountain View, CA, USA). GSH/GSSG assay kit was purchased from Oxford Biomedical Research (Oxford, MI, USA). Acridine orange 10-nonyl bromide and mitotracker green were obtained from Invitrogen (Carlsbad, CA, USA). Trichloroacetic acid was from Fisher Scientific (Pittsburgh, PA, USA). Polyclonal antibody against β -actin was obtained from Cell Signaling (Beverly, MA, USA). Horseradish peroxidase-linked secondary antibodies and reagents for enhanced chemiluminescence detection were from Amersham Biosciences (Piscataway, NJ, USA). Fluorescein isothiocyanate (FITC)-conjugated secondary antibody for immunofluorescence was from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

CGN culture

CGNs were isolated from postnatal day 7 Sprague-Dawley rat pups of both sexes, as previously described.³⁴ CGNs were plated on poly-L-lysine coated, 35 mm-diameter plastic dishes at 4.0×10^6 cells/well in Basal Medium Eagle's containing 10% fetal bovine serum, 25 mM KCl, 2 mM L-glutamine, and penicillin/streptomycin (100 U/ml/100 μ g/ml). Cytosine arabinoside (10 μ M) was added to the culture medium 24 hours after plating to limit the growth of nonneuronal cells. This protocol produced cultures ~95% pure for granule neurons. Typically, experiments were performed after 6–7 days in culture. All animal manipulations were performed in accordance with and under approval of the University of Denver Institutional Animal Care and Use Committee.

Treatment protocol

CGNs were incubated with anthocyanins, or other antioxidants, \pm HA14-1 for 24 hours for immunocytochemistry and apoptosis induction, and 4 hours for all other experiments. All incubations were performed in plating medium containing fetal bovine serum and 25 mM KCl, unless otherwise stated.

Immunocytochemistry and apoptosis assay

CGNs were fixed in 4% paraformaldehyde for 1 hour followed by incubation in a blocking solution made up of 5% bovine serum albumin (BSA) in 0.2% triton-X100 in phosphate-buffered saline (PBS; pH = 7.4) for 1 hour. Following blocking, primary antibody to β -tubulin was prepared at a dilution of 1:200 in

2% BSA in 0.2% triton-X100 in PBS and then incubated overnight at 4°C. Secondary antibody, conjugated to FITC, was prepared at a 1:250 dilution in 2% BSA and 0.2% triton-X100 in PBS with DAPI at 10 µg/ml and incubated for 1 hour at 25°C. Cells were then washed thoroughly with PBS and five images were captured per well (triplicate wells for each condition equals one experiment) on a Zeiss Axiovert-200M epi-fluorescence microscope for apoptosis quantification. CGNs were scored as apoptotic if they had condensed and/or fragmented nuclei.

Cardiolipin oxidation assay

CGNs were treated for 4 hours and then incubated at 25°C with 200 µM acridine orange 10-nonyl bromide and Hoechst for 20 minutes, washed twice with PBS, and imaged immediately under Cy3 and DAPI filters using a 40× objective.

Mitotracker staining

CGNs were treated for 4 hours and then incubated at 37°C for 20 minutes with mitotracker green and Hoechst (1:1000) in Hank's balanced salt solution (pH 7.4), and then imaged immediately under FITC and DAPI filters with a 40× objective.

Thiobarbituric acid-reactive substances (TBARS) assay

This assay was performed and modified as previously described.^{35–38} Ferrous iron, thiobarbituric acid, and trichloroacetic acid solutions were prepared immediately before use with sterile, deionized water flushed with nitrogen gas for 30 minutes. Brains from 7-day-old rat pups were homogenized and diluted 1:10 (w:v) in Krebs's buffer (pH 7.4). Samples were immediately prepared with 500 µl of homogenate, callistephin (100, 200, and 400 µM), or kuromanin (100, 200, and 400 µM), either alone or containing ferrous iron at a final concentration of 200 µM. All samples were queued to 750 µl with Krebs's buffer. Prepared samples were incubated at 37°C while shaking for 20 minutes. Then, 300 µl of 12.5% trichloroacetic acid in 0.9 N HCl was added to each sample to stop the reaction along with 70 µl of 10% thiobarbituric acid in 0.9 N NaOH/0.9% NaCl solution. Samples were boiled for 20 minutes, cooled slightly, and centrifuged at 2000 rpm for 15 minutes. The supernatant was transferred to duplicate wells on a 96-well plate and absorbance was read once per minute for 10 minutes at 532 nm. All readings for a sample were averaged together. The sample absorbance (lacking iron) was subtracted from the corresponding sample absorbance (with iron) to account for the intrinsic absorbance of the anthocyanins.

CGN subcellular fractionation

Following treatment, CGNs were placed on ice and washed once with ice cold 1X PBS. Then, 200 µl of cytosolic buffer were added to each well for 20 minutes. Wells were scraped and duplicate wells were combined and transferred to microcentrifuge tubes. CGNs were then fractionated into mitochondrial and cytosolic fractions as per the manufacturer's protocol from Biovision.

GSH assay

GSH assay kit from Oxford Biomedical Research was adapted from the manufacturer's instructions for cuvettes to be used in a 96-well plate format by proportionally reducing the volumes of reagents and sample from 1 ml to 300 µl.

Cell lysis and immunoblotting

After treatment, whole-cell lysates of CGNs for Western blotting were prepared as described previously.³⁹ Equal amounts of cell protein, as determined by a BCA protein assay kit (Pierce Chemical Co., Rockford, IL, USA), were run on polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes for immunoblotting. Membranes were blocked in PBS with 0.1% triton-X100 (PBS-T) containing 1% BSA and 0.01% sodium azide for 1 hour at 25°C. Primary antibodies were prepared in blocking solution and incubated with the membranes for 1 hour. Membranes were next washed five times over 30 minutes with PBS-T. Secondary antibodies prepared in PBS-T were incubated with the membranes for 1 hour and then membranes were washed again for five times over 30 minutes. Finally, immunoreactive proteins were detected by enhanced chemiluminescence.

GSH peroxidase activity assay

GSH peroxidase activity was assayed as per the GSH peroxidase cellular activity assay manufacturer's instructions for the kit (Sigma-Aldrich) with one adjustment. The volume was corrected to allow the assay to be performed in a 96-well plate in place of cuvettes.

Data analysis

In general, experiments were performed using duplicate or triplicate wells for each treatment condition. Each experiment was generally performed at least three separate times. Data shown represent the means ± SEM for the number (*n*) of independent experiments performed. Statistical analysis was typically performed using one-way ANOVA with a *post hoc* Tukey's test and a *P* value <0.05 was considered statistically significant.

Results

Anthocyanins protect CGNs from MOS-induced apoptosis

As a means of inducing MOS in CGNs, we utilized the Bcl-2 homology-3 domain (BH3) mimetic, HA14-1. This compound was initially identified by anti-cancer researchers using a structure-based, drug design strategy to find small organic molecules predicted to fit into the hydrophobic surface groove (i.e., the BH3 groove) of the pro-survival Bcl-2 protein.⁴⁰ We have previously shown that HA14-1 induces the intrinsic apoptosis of CGNs principally via a MOS-dependent mechanism which is completely suppressed by GSH.^{41,42} There are a number of distinct compounds in the anthocyanin family and they all possess significant intrinsic antioxidant activity. In the current study, we chose to focus on callistephin and kuromanin because they are commercially available in their pure forms. In contrast, many studies using anthocyanin-enriched extracts or juices are confounded by the fact that additional compounds with unknown activities are also present in these preparations.

An initial screen of several natural antioxidant compounds demonstrated that 100–200 μ M concentrations of callistephin or kuromanin provided significant protection to CGNs against MOS-induced

apoptosis. The protective effects of these anthocyanins were similar to those provided by green tea EGCG, as we have previously reported.⁴³ On the other hand, natural antioxidants such as curcumin, quercetin, and resveratrol failed to offer any significant protection in this model of MOS-induced neuronal death despite the fact that each of these compounds has demonstrated beneficial effects in other models of neuronal injury.⁴⁴

To more precisely assess the neuronal injury induced by HA14-1 in CGNs, we initially examined the integrity of the microtubule network and nuclear morphology. MOS induced by the Bcl-2 inhibitor, HA14-1, caused a complete disassembly of the microtubule network in CGNs as shown by immunocytochemistry for beta-tubulin (Fig. 1, compare Con to HA14). The anthocyanins, callistephin (200 μ M) and kuromanin (200 μ M), each preserved the microtubule cytoskeleton when administered in combination with HA14-1. GSH monoethylester is shown as a positive control for neuroprotection in this model and completely preserved the microtubule network in CGNs exposed to the Bcl-2 inhibitor. Nuclei were scored as apoptotic if fragmented or condensed using de-colorized DAPI images like the ones shown in Fig. 1. HA14-1 induced marked nuclear condensation and

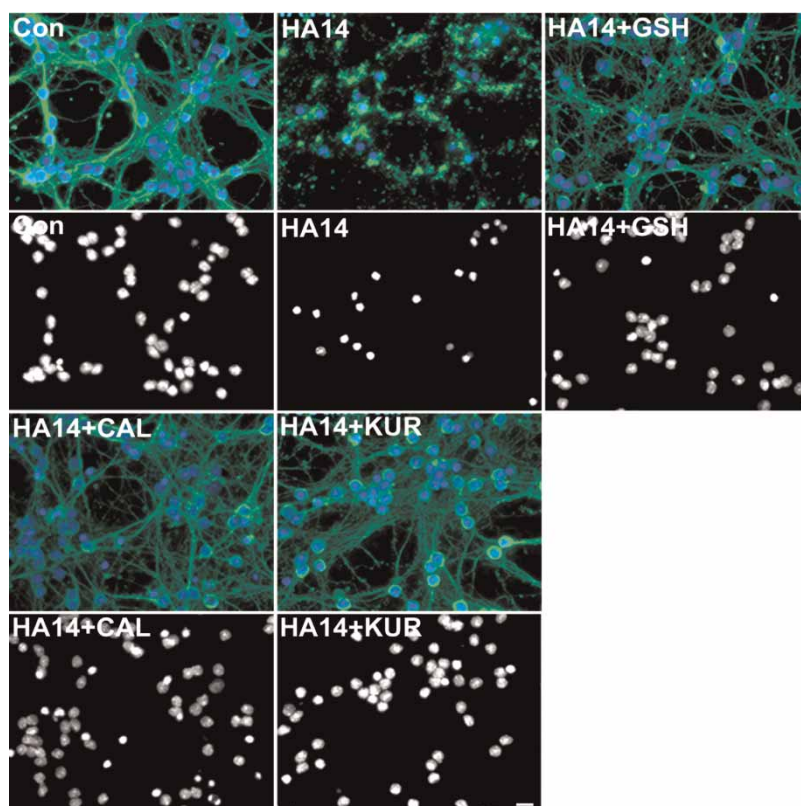


Figure 1 Anthocyanins significantly protect CGNs from MOS-induced apoptosis. CGNs were co-incubated with HA14-1 (15 μ M) and the antioxidants shown at the following concentrations: GSH monoethyl ester (2 mM), callistephin (CAL; 200 μ M), and kuromanin (KUR; 200 μ M). Some cells were incubated with HA14-1 alone or no treatment (control; Con) for 24 hours. Immunocytochemistry shows beta-tubulin (green) and DAPI (blue). Scale bar = 10 μ m. Black and white images show decolorized DAPI fluorescence to highlight nuclear morphology.

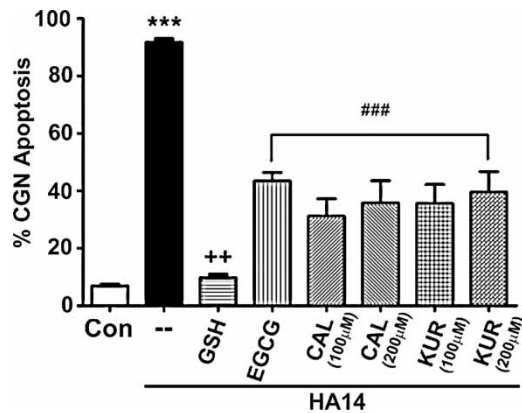


Figure 2 Quantification of CGN apoptosis. Percent apoptosis of four combined independent experiments as conducted in Fig. 1. CGNs were considered apoptotic if that displayed condensed or fragmented nuclei. Results are shown as mean \pm SEM, $n = 4$. Abbreviations used: Con, control; HA14, HA14-1 or Bcl-2 inhibitor; GSH, glutathione monoethyl ester; EGCG, epigallocatechin 3-gallate; Cal, callistephin; Kur, kuromanin. *** Indicates $P < 0.001$ compared to control, ### indicates $P < 0.001$ compared to HA14 treatment, and ++ indicates $P < 0.01$ compared to other antioxidant treatments. All determined by one-way ANOVA with *post hoc* Tukey's test.

some fragmentation which were both significantly diminished by co-incubation with either of the anthocyanins or GSH. The data from four independent experiments were combined and are shown in Fig. 2 as the (means \pm SEM) percentage of CGNs undergoing apoptosis for each treatment group. Callistephin and kuromanin each significantly reduced MOS-induced CGN apoptosis to levels comparable to those observed for the green tea polyphenol, EGCG. However, neither the anthocyanins nor EGCG were as effective as GSH at protecting CGNs from MOS-induced death.

Anthocyanins preserve mitochondrial GSH in CGNs exposed to the Bcl-2 inhibitor HA14-1

After establishing that the anthocyanins, callistephin, and kuromanin significantly protected CGNs from MOS-induced apoptosis, we next investigated the mechanism of this neuroprotection. First, given that exogenous GSH protected the CGNs from the Bcl-2 inhibitor, we analyzed the effects of MOS and anthocyanins on mitochondrial GSH content. GSH is an essential intrinsic antioxidant and maintenance of the mitochondrial GSH pool in particular is critical for cell survival.⁴⁵ As shown in Fig. 3, incubation with the Bcl-2 inhibitor, HA14-1, significantly reduced the mitochondrial GSH content of CGNs and predictably, co-incubation with GSH monoethyl ester prevented this depletion. Interestingly, all of the polyphenols tested preserved mitochondrial GSH levels in the presence of HA14-1 with callistephin and kuromanin being slightly more effective than

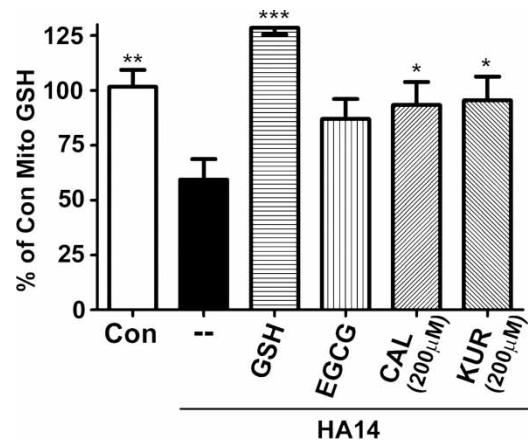


Figure 3 Anthocyanins preserve mitochondrial GSH in MOS-challenged CGNs. CGNs were co-incubated with HA14-1 (15 μ M) and the antioxidants shown at the following concentrations: GSH monoethyl ester (2 mM), EGCG (25 μ M), callistephin (CAL; 200 μ M), and kuromanin (KUR; 200 μ M). Some cells were incubated with HA14-1 alone or no treatment (control; Con) for 4 hours. Cells were then fractionated using differential centrifugation into mitochondrial and cytosolic fractions, and GSH was measured in mitochondrial fractions as described in Methods. Data shown on the Y-axis represent the mitochondrial GSH content as a percentage of that in control CGNs, mean \pm SEM, $n = 8$, * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$ when compared to HA14 by a two-tailed *t*-test. Abbreviations used are the same as in Fig. 1.

EGCG. Thus, anthocyanins preserved the essential pool of mitochondrial GSH in CGNs subjected to MOS.

Anthocyanins inhibit lipid peroxidation in vitro

Hydrogen peroxide is formed in relatively large quantities within mitochondria downstream of the action of superoxide dismutase on superoxide radicals which leak from the electron transport chain. Hydrogen peroxide on its own is not extremely reactive; however, its reaction with ferrous iron (Fe^{2+}) yields the highly reactive hydroxyl radical via the Fenton reaction. Hydroxyl radicals can oxidize membrane lipids to initiate a lipid peroxidation chain reaction resulting in the formation of damaging lipid hydroperoxides. Based on the data presented above, anthocyanins would be predicted to mitigate lipid peroxidation *indirectly* by maintaining mitochondrial GSH levels during MOS. Additionally, anthocyanins possess intrinsic free radical scavenging activity. Therefore, we measured the capacity of anthocyanins to *directly* inhibit lipid peroxidation *in vitro* in a cell-free assay using rat brain homogenate incubated with ferrous iron. The TBARS assay is a classic way to measure lipid peroxidation in biological systems. TBARS generated during the assay are measured by absorbance at 532 nm. Anthocyanins are highly pigmented compounds which made it necessary for us

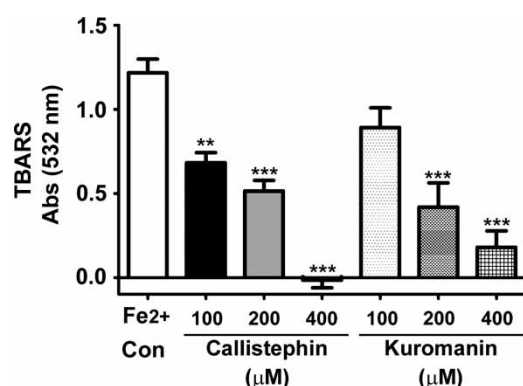


Figure 4 Anthocyanins inhibit lipid peroxidation *in vitro*. Whole P7 rat brains were homogenized and treated with callistephin or kuromanin (at the concentrations indicated), or no treatment (control). Lipid peroxidation was induced using 200 μ M ferrous iron and assayed for TBARS by absorbance at 532 nM. Higher absorbance indicates increased levels of lipid peroxidation. Data shown represent the mean \pm SEM, $n = 4$. Experiments performed in duplicate ** = $P < 0.01$, *** = $P < 0.001$, versus iron control (one-way ANOVA with *post hoc* Tukey's test).

to correct the assay for their interfering absorbances. Samples of rat brain homogenate were prepared in duplicate; one was reacted with iron and one without iron as a control for the intrinsic anthocyanin absorbance at each concentration. The anthocyanin absorbance at each concentration (measured in the absence of iron) was then subtracted from the corresponding absorbance of the paired sample that was reacted with iron. Using this method, both callistephin and kuromanin dose-dependently inhibited lipid peroxidation induced by iron with the 400 μ M dose of each anthocyanin reducing TBARS to essentially non-detectable levels (Fig. 4).

Cardiolipin oxidation induced by MOS in CGNs is reduced by callistephin

The TBARS assay demonstrated that anthocyanins are capable of inhibiting lipid peroxidation directly *in vitro* in rat brain homogenates. In CGNs incubated with the Bcl-2 inhibitor, HA14-1, mitochondria are specifically targeted and therefore, an assay to specifically evaluate the oxidation of mitochondrial lipids would yield the most relevant information. Cardiolipin is a lipid found exclusively in mitochondrial membranes and it plays a critical role in tethering cytochrome C to the inner mitochondrial membrane and preventing its release from mitochondria. Indeed, cytochrome C release during intrinsic apoptosis is linked to cardiolipin peroxidation.^{46–48} Peroxidation of cardiolipin can be assessed by assaying the binding of the fluorescent dye, acridine orange 10-nonyl bromide (AO), to this membrane lipid.⁴⁹ AO binds quite strongly to reduced cardiolipin and the dye is released when cardiolipin is oxidized making it

easy to visualize. Untreated (control) CGNs have a very bright and punctate AO fluorescence consistent with it staining reduced cardiolipin within mitochondria (Fig. 5(A)). In comparison, CGNs incubated with HA14-1 and therefore, undergoing MOS, fluoresce very dimly using the identical exposure conditions. This marked reduction in fluorescence indicates that most of the cardiolipin has been oxidized. However, co-incubation of CGNs with HA14-1 and callistephin significantly inhibited the oxidation of cardiolipin and maintained the bright and punctate AO staining consistent with protection from MOS-

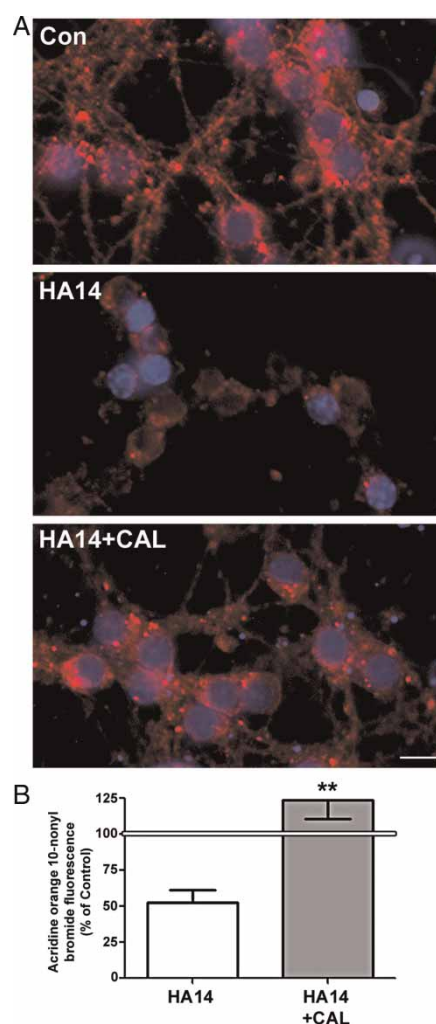


Figure 5 Callistephin suppresses cardiolipin oxidation in MOS-challenged CGNs. (A) CGNs were co-treated with 200 μ M callistephin and 15 μ M HA14-1, HA14-1 alone, or no treatment (control) for 4 hours. After this, cells were stained with acridine orange 10-nonyl bromide (AO). AO binds exclusively to reduced cardiolipin and is released when cardiolipin is oxidized. AO fluorescence is shown in red and Hoechst nuclear staining is shown in blue. Images are representative of similar results obtained in three independent experiments. Scale bar = 10 μ m. (B) Quantification of AO fluorescence from ~ 150 CGNs per treatment group that were incubated as described in (A). Data are expressed as a percentage of the AO fluorescence observed in control CGNs. ** = $P < 0.01$ when compared to HA14 by a two-tailed *t*-test.

induced apoptosis. Quantification of AO fluorescence revealed that incubation with HA14-1 induced an approximately 50% decrease in reduced cardiolipin and this effect was essentially blocked by co-incubation with callistephin (Fig. 5(B)). These data indicate that MOS-induced CGN death is associated with pronounced cardiolipin oxidation and this effect is significantly mitigated by the anthocyanin callistephin.

Mitochondrial fragmentation induced by MOS in CGNs is prevented by callistephin

In addition to increased oxidation of cardiolipin, MOS induced by the Bcl-2 inhibitor, HA14-1, is also associated with significant mitochondrial fragmentation.⁴² Mitotracker staining of live control CGNs revealed an intricate network of tubular mitochondria (Fig. 6(A)). In marked contrast, CGNs incubated for 4 hours with HA14-1 displayed fragmented

mitochondria that appeared round and punctate. This mitochondrial fragmentation induced by MOS was remarkably diminished by co-incubation with callistephin. We have previously reported that cleavage of the mitochondrial fusion GTPase, optic atrophy 1 (OPA1), occurs coincident with mitochondrial fragmentation in CGNs exposed to apoptotic inducers like HA14-1.⁵⁰ Co-incubation with either callistephin or kuromanin reduced OPA1 cleavage in CGNs exposed to the Bcl-2 inhibitor (Fig. 6(B)). Thus, the anthocyanins attenuated both mitochondrial fragmentation and OPA1 cleavage induced by MOS in CGNs.

GSH peroxidase activity is potentiated by anthocyanins

Because the anthocyanins preserved mitochondrial GSH content in CGNs subjected to MOS, we evaluated the effects of these compounds on GSH peroxidase (GSHpx) activity *in vitro* in a cell free assay. This assay measures the activity of GSHpx indirectly by coupling the reaction to GSH reductase activity. Briefly, GSHpx utilizes GSH as an electron donor to reduce an organic hydroperoxide and in the process generates GSSG. The GSSG is then reduced by the action of GSH reductase via the oxidation of NADPH to NADP⁺, resulting in a corresponding decrease in absorbance at 340 nm. A small amount of GSHpx was used to generate a low basal rate of GSHpx activity (Fig. 7; Con). Interestingly, inclusion of either callistephin or kuromanin to the assay dramatically enhanced the activity of GSHpx (Fig. 7). These results suggest that, in addition to their intrinsic free radical scavenging activity, anthocyanins may have an additional effect of potentiating GSH peroxidase activity. At present, the mechanism underlying this effect is unclear but it may involve an

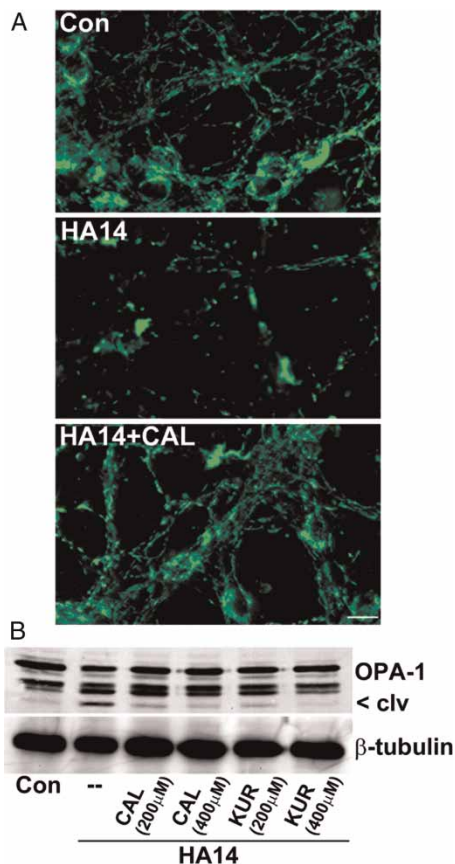


Figure 6 Anthocyanins inhibit MOS-induced mitochondrial fragmentation and cleavage of the OPA1 fusion GTPase. (A) CGNs were treated as in Fig. 5. Live cells were stained using mitotracker green fluorescence to show mitochondrial morphology. Scale bar = 10 μm. (B) CGNs were co-treated with HA14-1 and either callistephin or kuromanin (at the concentrations indicated), HA14-1 alone, or no treatment (control) for 4 hours. Following treatment, cells were lysed and western blots were performed for OPA1. Arrow indicates the presence of an OPA1 cleavage product (clv). beta-Tubulin is shown as a loading control. Results shown are indicative of three independent experiments.

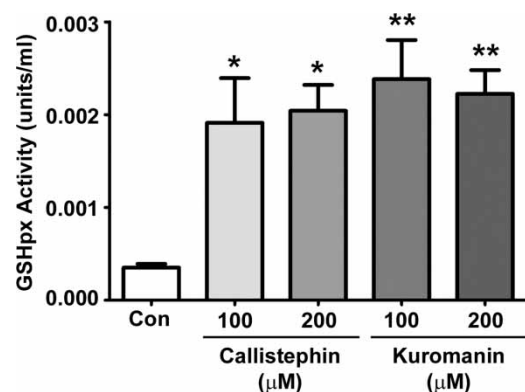


Figure 7 Anthocyanins stimulate GSHpx activity *in vitro*. Glutathione peroxidase (GSHpx) activity (control), alone or measured in the presence of either callistephin or kuromanin, is shown as the rate of enzyme activity (units/ml). * = $P < 0.05$, ** = $P < 0.01$ versus control, analyzed by one-way ANOVA with *post hoc* Tukey's test. $n = 5$ experiments performed in duplicate.

enhancement of GSH recycling in the presence of the anthocyanin.

Discussion

Anthocyanins are polyphenolic compounds that are rapidly gaining attention as possible therapeutic agents for neurodegeneration. Despite their relative hydrophilicity compared to many other antioxidants, anthocyanins and their aglycone anthocyanidin derivatives have been found to cross the blood brain barrier and enter the CNS.^{51–53} Consistent with their ability to access various regions of the brain, anthocyanins have demonstrated neuroprotective effects in multiple *in vivo* models of neuronal injury or neurodegeneration. For example, anthocyanins have been shown to protect neurons in models of cerebral ischemia-reperfusion injury.^{32,33} In addition, diets rich in anthocyanins have been suggested to slow the process of age-related cognitive decline.⁵⁴ In support of this, a recent study showed that blueberry supplementation in a small population of older adults experiencing early signs of dementia resulted in measurable cognitive improvement in tasks such as paired associative learning and word list recall.⁵⁵ Anthocyanins have also been shown to provide significant neuroprotection in animal models of Parkinson's disease. An anthocyanin-enriched extract from mulberry fruit significantly decreased dopaminergic neuronal cell loss in the substantia nigra of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice.⁵⁶ Similarly, oral administration of pelargonidin significantly protected dopamine neurons from unilateral intrastriatal 6-hydroxydopamine lesioning and attenuated the behavioral deficits observed in lesioned rats.⁵⁷ These studies indicate that anthocyanins have significant potential as therapeutic agents for neurodegenerative diseases, cognitive decline, and ischemic injury such as stroke.

Although some of their neuroprotective actions observed *in vivo* may be attributable to their intrinsic antioxidant capacity, several studies have demonstrated that anthocyanins also display significant anti-inflammatory effects. For instance, administration of purple sweet potato color anthocyanins to lipopolysaccharide (LPS)-treated mice significantly suppressed LPS-induced expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor- α , interleukin-1 β , and interleukin-6 in mouse brain.⁵⁸ Anthocyanins from purple sweet potato also reduced COX-2 and iNOS expression induced in rat liver by dimethylnitrosamine.⁵⁹

Yet another mechanism by which anthocyanins might protect neurons from oxidative damage is through induction of the NF-E2-related factor-2 (Nrf2)/antioxidant response element (ARE) transcriptional pathway. Nrf2 is activated during oxidative

stress and turns on the transcription of a variety of antioxidant, phase II detoxification, and cytoprotective genes, each possessing AREs within their promoter regions. Nrf2 has been established as a significant neuroprotective pathway in diverse models of neuronal injury.^{60,61} Interestingly, protocatechuic acid, which is a major metabolite of anthocyanins, has been shown to cause an Nrf2-dependent induction of GSHpx and GSH reductase in macrophages.⁶² In a similar manner, an anthocyanin fraction from purple sweet potato has been shown to induce the nuclear translocation of Nrf2 in hepatic cells.⁶³ To date, the effects of anthocyanins on Nrf2 activity in neurons has not been investigated.

A number of previous studies have investigated other potential mechanisms underlying the neuroprotective effects of anthocyanins. In the current study we show that callistephin and kuromanin protect CGNs from MOS-induced apoptosis. The mechanism of neuroprotection in our *in vitro* model involves preservation of the mitochondrial GSH pool and inhibition of cardiolipin oxidation and mitochondrial fragmentation. Two additional studies have also found a link between anthocyanins and modulation of cellular GSH status. Carlsen *et al.*⁶⁴ showed that a diet rich in berry extracts and juices significantly enhanced the promoter activity of gamma-glutamylcysteine synthetase (the rate limiting enzyme in GSH synthesis) in the brains of transgenic reporter mice. Hassimotto and Lajolo⁶⁵ recently showed that rats given a diet supplemented with anthocyanin-enriched fractions from blackberries displayed significantly increased GSH levels in the liver, kidney, and brain. Although neither of these studies provided any evidence for explicit regulation of mitochondrial GSH content, their results are consistent with our data showing that anthocyanins likely influence this key endogenous antioxidant. Moreover, our results suggest that anthocyanins may have an additional effect of enhancing GSHpx activity which would result in an increased scavenging of destructive peroxide species. Finally, it is notable that several studies have identified anthocyanins as regulators of various cell signaling pathways including an extracellular signal-regulated kinase (ERK1/2), cAMP response element-binding protein (CREB), brain-derived neurotrophic factor (BDNF) cascade, as well as a PI3K/AKT pathway.^{66,67} Thus, anthocyanins exhibit pleiotropic actions which contribute to their substantial neuroprotective capacity *in vitro* and *in vivo*.

Finally, one must interpret *in vitro* data such as ours in the context of the anthocyanin concentrations required to observe significant neuroprotective effects. In the current study, we used callistephin and kuromanin concentrations of 100 to 400 μ M. These neuroprotective concentrations are similar to those previously reported (50–100 μ M) to protect SH-

SY5Y neuroblastoma cells or PC12 cells from hydrogen peroxide or beta amyloid-induced oxidative death.^{24,26,68} However, these concentrations are also more than two orders of magnitude greater than peak plasma concentrations observed *in vivo* after an anthocyanin-enriched diet.⁶⁹ Recent studies in humans receiving oral grape juice confirm that anthocyanins are bioavailable and subject to metabolism principally by methylation or glucuronidation.⁷⁰ Interestingly, differences in the conjugated sugar moieties as well as the phenolic aglycone structure can significantly alter the metabolic profiles of anthocyanins.^{71,72} For instance, even callistephin and kuromanin which differ by a single phenolic hydroxyl group, are absorbed and metabolized differently *in vivo*.⁷³ It is somewhat difficult to reconcile the dramatically different concentrations of anthocyanins that apparently provide neuroprotective effects *in vivo* (<1 μM) versus those required *in vitro* (~100 μM). The contributions of multiple metabolites of anthocyanins that might accumulate *in vivo*, such as protocatechuic acid, cannot be dismissed. Nor should the pleiotropic effects of anthocyanins and their metabolites, particularly their noted anti-inflammatory effects, be disregarded. In addition, one must consider the distinct difference between a single *in vitro* exposure of cultured neurons to an anthocyanin which rapidly degrades at the neutral pH of culture medium versus the potential cumulative effects *in vivo* of a continuous diet rich in anthocyanins. Despite these potentially confounding issues, anthocyanins appear to be relatively nontoxic compounds that may prove to be beneficial neuroprotective therapeutics for various degenerative disorders of the nervous system.

In summary, we have investigated the neuroprotective effects of two purified anthocyanins, callistephin, and kuromanin against MOS-induced apoptosis in CGNs. We show that these anthocyanins protect CGNs from MOS-induced apoptosis by preserving mitochondrial GSH and as a result, preventing cardiolipin oxidation and mitochondrial fragmentation. In addition, these anthocyanins significantly potentiate GSHpx activity *in vitro* suggesting that they may enhance GSH recycling. We conclude that anthocyanins protect neurons from MOS by acting locally at the level of the mitochondria to increase the resistance of these critical organelles to oxidative damage. Anthocyanins represent a class of nutraceutical antioxidants that deserve further study as potential therapeutics for neurodegenerative diseases for which MOS is an underlying factor.

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