Anthocyanins Protect against Kainic Acid-induced Excitotoxicity and Apoptosis via ROS-activated AMPK Pathway in Hippocampal Neurons

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SUMMARY

Introduction: Excitotoxicity is an important mechanism involved in neurodegeneration. Kainic acid (KA)-induced excitotoxicity results an unfavorable stress, and we investigated the signaling pathways activated in such conditions. **Aims:** Here, we sought to determine the cellular and biochemical benefits of anthocyanins extracted from Korean black bean against KA-induced excitotoxicity and neuronal cell death. **Methods and results:** Mouse hippocampal cell line (HT22) and primary prenatal rat hippocampal neurons were treated with KA to induce excitotoxicity. Incubation of the cells with KA alone significantly decreased cell viability, elevated intracellular Ca^{2+} level, increased generation of reactive oxygen species (ROS) and loss of mitochondrial membrane potential ($\Delta\psi_{\text{M}}$). These events were accompanied by sustained phosphorylation and activation of AMP-activated protein kinase (AMPK). Kainic acid induced upregulation of Bax, decrease in Bcl-2, release of cytochrome-c, and activation of caspase-3 in both cell types. Anthocyanins attenuated KA-induced dysregulation of Ca^{2+} , ROS accumulation, activation of AMPK, and increase in percentage of apoptotic cells. Pretreatment of the cells with compound C, an inhibitor of AMPK, diminished the KA-induced activation of AMPK and caspase-3. The activation of AMPK through elevation of cellular ROS and Ca^{2+} levels is required for KA-induced apoptosis in hippocampal neurons. **Conclusions:** In summary, our data suggest that although anthocyanins have diverse activities, at least part of their beneficial effects against KA-induced hippocampal degeneration can be attributed to their well-recognized antioxidant properties.

Introduction

The exact mechanisms involved in onset and progression of neurodegenerative diseases including Alzheimer's, Parkinson's, and Huntington's diseases are still poorly defined, but excitotoxicity is recognized as one of the mechanisms involved [1,2]. Aberrant excitation involving the excitatory glutamate receptors is recognized to have an important role in the pathology of neurodegenerative disorders including Alzheimer's, Parkinson's, and Huntington's diseases [3–6]. Agonist-induced excitotoxicity resulting from overstimulation of the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors subclass of glutamate receptors has been widely used in brain injury models [7–9]. Kainic acid (KA) is a nondegradable analog of glutamate and is an agonist for AMPA/kainate receptors that exerts its effects on different parts of the brain [10–12]. Effects of KA are mediated via activation of these glutamate receptors and include the induction of inflammatory responses, production of cytokines and neuronal death.

The exact molecular mechanisms by which KA induces excitotoxicity and cell death remain unclear; however, oxidative stresses and the activation of proinflammatory cytokines are major contributors [13]. Additionally, KA increases neuronal excitability, production of reactive oxygen species (ROS), and lipid peroxidation [14–16]. Both *in vitro* and *in vivo* studies demonstrate that KA induces cell death via accumulation of intracellular calcium, which stimulates ROS production and mitochondrial dysfunction, thereby leading to neuronal cell death [13,17]. There is evidence for activation of calpain- and caspases-induced neural apoptosis following KA exposure [18]. Besides oxidative stress and intracellular calcium overload, KA can activate the glutamate receptors leading to depletion of neuronal energy stores and thereby activating pathways of energy metabolism [19–21].

A body of work indicates that KA excitotoxicity is associated with energy depletion. AMP-activated protein kinase (AMPK), the multifunctional metabolic and energy sensor in the brain, is activated by conditions of cellular energy depletion [22,23]. Oxidative stress, glucose deprivation, heat shock, hypoxia, and

ischemia are some of the stresses that induce activation of AMPK [23,24]. AMP-activated protein kinase is activated when it is phosphorylated at Thr172 on its catalytic α subunit, and activated AMPK further phosphorylates key enzymes of lipid metabolism [25,26]. A modest activation of AMPK-induced neurogenesis and improved cognition in animals, but sustained AMPK activation reduced cognition and induced apoptosis in a number of *in vitro* and *in vivo* studies [27,28]. Therefore, we investigated the role of AMPK in KA-induced neurotoxicity. We report here that KA-induced activation of AMPK is associated with induction of apoptotic cell death in immortalized mouse hippocampal cells (HT22) and in primary cultures of prenatal rat hippocampal neurons.

Anthocyanins are natural plant pigments and occur in edible plant materials such as fruits, vegetables, and beans [29]. It has been suggested that consumption of foods rich in polyphenols, particularly anthocyanins, is associated with health improvement [30]. Anthocyanins constitute a subfamily of flavonoids that possess antioxidative, antiinflammatory, and antineurodegenerative properties [31–33]. Antioxidants have been extensively studied as potential therapeutic agents for various neurodegenerative disorders. It has been reported that the anthocyanin, cyanidin-3-O-glucoside, protects against cerebral ischemia and mitochondrial damage induced by β -amyloid [34,35]. Therefore, we examined the effect of anthocyanins extracted from Korean black bean on KA-induced neurodegeneration. We report here that KA treatment induced apoptosis in HT22 mouse hippocampal cells and in primary cultures of prenatal rat hippocampal neurons. Pretreatment with anthocyanins protected against KA-induced apoptosis in both these cell types. Kainic acid treatment elevated intracellular calcium, increased oxidative stress, activated AMPK, and activated the mitochondrial pathway of apoptosis, whereas pretreatment with anthocyanins reversed these trends.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA). Cytosine β -D-arabinofuranoside, kainic acid and other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless indicated otherwise. Anthocyanins were extracted from Korean black bean (*Glycine max* (L.) Merr.) seed coat by a well-established method [36,37]. Anthocyanins extracted by this procedure have been shown to contain delphinidin-3-glucoside, cyanidin-3-glucoside, and petunidin-3-glucoside. Rabbit-derived anti-Bcl-2 (Sc-492), anti-Bax (Sc-493), and goat-derived polyclonal anticytochrome-c (Sc-8385) (dilution used, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit-derived anticaspase-3 (#9662), anti-AMPK α (#2603), and antiphospho-AMPK α (Thr-172) (#2535) (dilution used, 1:500; Cell Signaling Technology Danvers, MA, USA), anti- β -actin (A5060, dilution used, 1:2000; Sigma, St. Louis, MO, USA), horseradish peroxidase-conjugated anti-rabbit IgG (Sc-2004, dilution used, 1:1000; Santa Cruz Biotechnology), and horseradish peroxidase conjugated anti-goat IgG (Sc-2768, dilution used, 1:1000; Santa Cruz Biotechnology) were used in immunoblotting experiments.

Animal Treatment

Female Sprague-Dawley rats weighing 220–250 g ($n = 20$) were housed in a temperature-controlled environment under a 12:12 light/dark cycle and fed *ad libitum*. Pregnancy was timed from the day of insemination, which was designated as gestational day (GD) 0.5. At GD 17.5, pregnant mothers were killed by decapitation immediately after sedation by an i.v. injection of pentobarbital sodium (3 mg/100 g b.w.). All the experimental procedures were approved by the animal ethics committee (IACUC) of the Division of Applied Life Sciences, Department of Biology, Gyeongsang National University, South Korea.

In Vitro Culture of Hippocampal Cells

Cultures of rat embryonic prenatal hippocampal cells were prepared as we previously described [38]. Mouse hippocampal cells (HT22) were cultured in high-glucose DMEM supplemented with 10% FBS and 1% antibiotics at 37°C in humidified air containing 5% CO₂.

Drug Treatment

HT22 cells and primary hippocampal neuronal cells were grown for 5 days and then divided into groups for the treatments. The treatment groups were as follows: (1) Control: incubated in DMEM medium for 12 h; (2) KA treatment: incubated in DMEM containing KA (100 μ M) for 12 h; (3) anthocyanins treatment: incubated in DMEM containing anthocyanins (0.2 mg/mL) for 12 h; (4) Anthocyanins + KA treatment: incubated in DMEM containing anthocyanins (0.1 mg/mL) for 12 h and then postincubated in DMEM containing KA (100 μ M) for 12 h; (5) Anthocyanins + KA treatment: incubated in DMEM containing anthocyanins (0.2 mg/mL) for 12 h and then incubated in DMEM containing KA (100 μ M) for 12 h. Treatment for Groups 4 and 5 (Anthocyanins + KA) was carried out at day 5 while the treatment to the first three groups was applied at 5.5 day. All the cells were harvested at day 6 and used for the desired analyses.

Cell Viability Assay

The colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay was employed to measure cell viability. Cells were seeded into 96-well plates (1×10^5 cells/well) in 200 μ L of DMEM media. After 5 days growth, anthocyanins and KA treatments were performed as described under "Drug treatment". At end of the treatments, MTT (5 mg/mL in phosphate buffer saline, PBS) was added to each well, and the plates were incubated for 4 h at 37°C. Formazan dissolved in dimethyl sulfoxide (DMSO) added to the wells, and plates were agitated for 10 to 20 min on a shaker. Absorbance was then measured at 550–570 nm (L1) and 620–650 nm (L2) in a scanning microplate reader. The L2 absorbance measures cell debris and well imperfections. The corrected absorbance ($A = L1 - L2$) of each well was used to calculate the percentage of cell survival as 100 X absorbance of treated wells/absorbance of control wells.

Intracellular Free Ca^{2+} Measurement

The intracellular Ca^{2+} concentration was measured with Fura-2 acetoxymethyl ester using a previously described fluorescence-based method [39]. Treatments were performed as described under “Drug treatment” on cells grown in triplicate plates, each containing 1×10^6 cells/mL. At the end of treatment, cells were washed twice with Locke’s solution (154 mM NaCl, 25 mM KCl, 2.3 mM CaCl_2 , 3.6 mM NaHCO_3 , 8.6 mM HEPES, and 5.6 mM glucose) (pH 7.8) and then incubated for 1 h at 37°C in a humidified incubator in DMEM media containing $5 \mu\text{M}$ Fura-2. Cells were then collected, washed twice with Locke’s solution, and fluorescence signals were measured with a luminescence spectrophotometer (LS50B, Perkin Elmer, Boston, MA, USA). Excitation wavelength was alternated between 340 and 380 nm, and the fluorescence emission at 510 nm was measured. The $340 \text{ nm}_{\text{Ex}}/380 \text{ nm}_{\text{Ex}}$ fluorescence ratio, averaged over a period of 2 s, was measured. Fluorescence signals were acquired, stored, and analyzed using a computer with universal imaging software or a MicroVax II computer with image 7 software. Intracellular calcium was determined using the Grynkiewicz equation [40].

$$[\text{Ca}] = K_d \times \frac{(R - R_{\min})}{(R_{\max} - R)} \times \frac{\text{Sf2}}{\text{Sb2}}$$

where K_d , the dissociation constant of the Fura-2 Ca^{2+} interaction was taken to be 225 nM in the cytosolic environment; R is the $340 \text{ nm}_{\text{Ex}}/380 \text{ nm}_{\text{Ex}}$ fluorescence ratio; $R_{\min}$ is the ratio with zero Ca^{2+} ; $R_{\max}$ is the ratio with saturating Ca^{2+} (using calcium chloride); Sf2 is fluorescence at 380 nm with zero Ca^{2+} ; Sb2 is fluorescence at 380 nm with saturating Ca^{2+} .

Measurement of Reactive Oxygen Species (ROS)

Intracellular ROS content in cells was visualized using 2,7-dihydrodichlorofluorescein diacetate (DCFH-DA) essentially as described [41]. Cells were grown to 80% confluence on glass chamber slides (Lab-Tek Chamber slide, USA), washed with PBS, incubated with anthocyanins 0.1 and 0.2 mg/mL for 12 h, and then exposed to KA for 12 h. Cells were then washed with PBS, $50 \mu\text{M}$ DCFH-DA in DMSO was added, and the cultures were incubated for 30 min at 37°C in the dark. After incubation, cells were washed with Locke’s solution (pH 7.4) and analyzed under a confocal laser scanning microscope (Fluoview FV 1000, Olympus, Japan) with excitation and emission wavelengths of 485 and 530 nm, respectively. Fluorescence intensity was measured using image J software and expressed as “fold of control,” which is the ratio of the fluorescence intensity of a sample to the fluorescence intensity of the control cells.

Measurement of Mitochondrial Membrane Potential

Mitochondrial membrane potential ($\Delta\psi_M$) was measured using JC-1 $\Delta\psi_M$ detection kit (Biotium Inc., Hayward, CA, USA), according to the manufacturer’s protocol. JC-1 emits either green or red fluorescence, depending on the $\Delta\psi_M$. A green

signal indicates depolarized mitochondria, while a red signal indicates polarized mitochondria. Thus, the shift from red to green fluorescence indicates a drop in $\Delta\psi_M$. All treatments were performed on triplicate samples. At the end of treatment, cells were washed two times with PBS, harvested, and stained with JC-1 reagents at 37°C for 15 min. Cells were washed and then resuspended in 1X assay buffer. Red and green fluorescence were measured in the green (FL-1) and red (FL-2) channels of a FACS Caliber flow cytometer (Becton Dickinson, San Jose, CA, USA). In total, 10,000 cells were acquired for analysis by Cell Quest software, version 3.0 (Becton Dickinson), and the percent of cells with low $\Delta\psi_M$ in the total cell population was calculated.

Western Blot Analysis

Western blot was carried out on protein extracts of cells that were prepared as previously described [38]. The protein concentration was measured using Bio-Rad protein assay solution. Equivalent amounts of protein ($30 \mu\text{g}$ per sample) were electrophoresed on 7.5–15% SDS-PAGE gels under reducing conditions and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Millipore Corporation, Billerica, MA, USA). Prestained protein markers, broad range (7–240 kDa, multicolor, Elpis Biotech, Daejeon, Korea) were run in parallel for estimation of the molecular weights of the proteins. The membrane was blocked with 5% (w/v) skimmed milk and incubated overnight at 4°C with primary antibody. Membranes were washed, probed with horseradish peroxidase-conjugated secondary antibody, and immunocomplexes were visualized using enhanced chemiluminescence ECL-detecting reagent spray (West-one™, Western blotting detection system, Intron Biotechnology, Scottsdale, AZ, USA). The X-ray films were scanned, and the optical densities of the protein bands were analyzed by densitometry using Sigma Gel, version 1.0 (SPSS, Chicago, IL, USA).

TUNEL Staining

Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using DeadEnd™ Fluorometric TUNEL System (Promega, MA, USA), according to the supplier recommendations and protocol. Cells were grown in chamber slides and treated as described under “Drug treatment”. At the end of treatment, cells were fixed with 4% paraformaldehyde, washed with 1X PBS, and permeabilized with 1% TritonX-100. After TUNEL staining, the slides were treated with a freshly prepared $1 \mu\text{g}/\text{mL}$ solution of propidium iodide (PI) in PBS for 15 min at room temperature and then rinsed with distilled water. Glass cover slips were mounted on the slides with mounting medium. A PI filter was used to detect PI staining (red color), and an FITC filter was used to detect TUNEL staining (green color). Images were acquired under a confocal laser scanning microscope (Fluoview FV 1000). All samples were analyzed with at least two biological replicates, and three images from each replicate were taken using a $60\times$ objective for manually counting the TUNEL-positive (green) and PI-positive (red) cells. Percent TUNEL-positive cells were calculated as $100 \times \text{TUNEL-positive cells} / \text{Total numbers of cells}$.

Data Analysis and Statistics

Bands from Western blots were scanned and analyzed by densitometry using the Sigma Gel System (SPSS Inc.). The density values were expressed as the mean $\pm$ SEM. Group differences were analyzed using a one-way analysis of variance (ANOVA) followed by Student's *t*-test. Differences with a *P*-value less than 0.05 were considered as statistically significant.

Results

Anthocyanins Protect Against KA-Induced Cytotoxicity and Disturbance of Ca^{2+} Homeostasis

Kainic acid exposure is known to activate caspases and induce neuronal apoptosis [18]. To establish conditions for KA-induced apoptosis in HT22 cells and primary cultures of hippocampal neurons, we treated both types of cells with KA (100 μM) for various time periods and monitored the cellular level of the major apoptotic inducer, caspase-3, by Western blot analysis. A significant increase in the activation of caspase-3 was observed after 2, 12, and 24 h of KA treatment in both types of cells (Figure 1A). The cell viability was significantly decreased by KA treatment in both HT22 and primary hippocampal neuronal cells (Figure 1B,C). To investigate whether anthocyanins protect against KA-induced damage, the cells were pretreated with anthocyanins for 12 h followed by KA (100 μM) posttreatment for 12 h. Kainic acid induced loss of cell viability was significantly attenuated by pretreatment with both 0.1 and 0.2 mg/mL anthocyanins (Figure 1B,C). Calcium homeostasis regulates neuronal activity, neural development and governs neurotransmitter release. Disturbance in Ca^{2+} homeostasis or its dysregulation results in neuronal cell death. Measurement of intracellular free Ca^{2+} with the fluorescent Ca^{2+} indicator, Fura-2, showed that KA treatment increased intracellular free Ca^{2+} peak in both type of cells compared with control (Figure 1D,E). Both HT22 cells and primary hippocampal cells pretreated with anthocyanins (0.1 and 0.2 mg/mL, respectively) decreased intracellular Ca^{2+} . As pretreatment with anthocyanins effectively inhibits the KA-induced elevation of cytosolic Ca^{2+} levels, these results support our hypothesis that anthocyanins effectively protect against KA-induced excitotoxicity.

Anthocyanins Inhibit KA-Induced Increased in ROS Production and $\Delta\psi_{\text{M}}$ Loss

Anthocyanins are natural antioxidants and are known to inhibit ROS accumulation in neuronal cells. Therefore, we performed experiments to ascertain whether anthocyanins could affect KA-induced oxidative cell death in HT22 cells. Intracellular ROS level was examined using DCFH-DA, a cell-permeable probe. When nonpolar and nonfluorescent DCFH-DA dye is added to cells, it diffuses across the cell membrane and is hydrolyzed by intracellular esterases to liberate nonfluorescent 2,7-dichlorodihydrofluorescein (DCFH), which upon reaction with ROS forms highly fluorescent dichlorofluorescein (DCF). Dichlorofluorescein fluorescence was assayed by confocal microscopy. Compared with

control cells, the ROS content of KA-treated HT22 cells was almost sixfold higher (Figure 2A,B). Pretreatment with anthocyanins significantly impaired the ability of KA to induce accumulation of ROS (Figure 2A,B).

To clarify the antiapoptotic effect of anthocyanins, we evaluated the loss of mitochondrial membrane potential ($\Delta\psi_{\text{M}}$), an early event in apoptosis. As shown in Figure 2C,D, the percentage of cells with low $\Delta\psi_{\text{M}}$ was $28.66 \pm 6.2\%$ in control HT22 cells and was increased to $65.32 \pm 4.1\%$ in KA-treated cells. When the KA treatment was preceded by treatment with 0.1 mg/mL or 0.2 mg/mL anthocyanins, the percentage of cells with low $\Delta\psi_{\text{M}}$ was reduced to $44.2 \pm 3.45\%$ and $35.56 \pm 8.5\%$, respectively, indicating that the anthocyanins had a protective effect against KA-induced collapse of mitochondrial potential.

Anthocyanins Protect Against KA-induced Activation of the Mitochondrial Apoptotic Pathway

The KA-induced spike in intracellular free Ca^{2+} levels and collapse of mitochondrial membrane potential (Figures 1 and 2) suggested that KA should activate the mitochondrial pathway of apoptosis. Therefore, apoptotic signaling pathways were investigated in KA-treated HT22 cells and primary hippocampal neuronal cells. Bax and Bcl-2 are members of the Bcl-2 family of proteins and are the main regulators of the mitochondrial pathway of apoptosis. Commitment to apoptosis was measured by comparing the cellular level of Bax (proapoptotic protein) and Bcl-2 (antiapoptotic protein) after various treatments. Our results demonstrated that Bax content increased and Bcl-2 content decreased upon treatment of HT22 cells and primary cultures of hippocampal neurons with KA as expected (Figure 3A,B). Cells treated with anthocyanins before treatment with KA had lower levels of Bax and higher levels of Bcl-2 compared with cells treated with KA (Figure 3A,B) consistent with the protective effect of anthocyanins on KA-induced cell death and loss of mitochondrial membrane potential (Figure 1, Figure 2C,D).

To demonstrate the downstream events involved in KA-induced damage of mitochondrial membranes, we estimated changes in cytochrome-c levels in the cytosolic fraction. After 12 h of KA treatment, there was a significant increase in cytochrome-c compared with control in HT22 cells and primary cultures of hippocampal neurons (Figure 3C). Cells pretreated with anthocyanins had lower levels of cytochrome-c compared with cells treated with KA alone.

Anthocyanins Inhibit the KA-induced Activation of AMPK

We hypothesized that the KA-induced increase in intracellular free calcium and accumulation of ROS would activate AMPK by phosphorylation of its α subunit (AMPK α) at Thr172. Therefore, we evaluated the phosphorylation status of AMPK α in primary hippocampal neuronal cells after different periods of treatment with KA. The Western blot data showed that 30 min, 2 h, and 12 h of KA treatment increased the phosphorylation of AMPK α significantly without any change in total AMPK level (Figure 4A). Pretreatment with anthocyanins inhibited the KA-induced

Figure 1 Effect of anthocyanins on Kainic acid (KA)-induced neuronal cell death. **(A)** Western blot analysis of activated caspase-3 levels in HT22 cells and primary cultures of rat fetal hippocampal neurons (Neurons) treated with KA (100 μ M) for indicated time periods. Actin levels are shown as loading control. Shown are representative Western blots. Represented in the graph are the density values (mean $\pm$ SEM; $n = 3$) of the activated caspases-3 bands in the Western blots. The density values are expressed in arbitrary units (a.u). Significant difference ($P < 0.05$) from control (Con) is indicated by the asterisk. **(B, C)** MTT assay of KA-induced changes in cell viability without or with anthocyanin pretreatment. HT22 cells **(B)** and primary cultures of rat fetal hippocampal neurons **(C)** were grown for 12 h in DMEM medium without (Con) or with 100 μ M KA (KA) or 0.2 mg/mL anthocyanins (Antho) supplement. For KA treatment following anthocyanin pretreatment, cells were treated with 0.1 mg/mL anthocyanins (Antho1 + KA) and 0.2 mg/mL anthocyanin (Antho2 + KA) for 12 h followed by 100 μ M KA treatment for 12 h. Cell viability was determined by MTT assay. Data are the mean $\pm$ SEM of three independent experiments ($n = 3$), with three plates in each experiment. Significant differences ($P < 0.05$) from the KA sample are indicated by the asterisks. KA-induced elevation of intracellular free Ca^{2+} level. HT22 cells **(D)** and primary cultures of fetal rat hippocampal neurons **(E)** were grown without treatment for 12 h **(C)**, treated with 100 μ M KA (KA) for 12 h or pretreated with anthocyanins (0.1 and 0.2 mg/mL) for 12 h, and posttreated with 100 μ M KA for 12 h (Antho + KA). Fura-2 AM labeling of intracellular free Ca^{2+} was performed at the end of treatments. Spectra are representative of three independent experiments with triplicate samples.

Figure 2 Effect of anthocyanins on Kainic acid (KA)-induced reactive oxygen species (ROS) production. HT22 cells were grown without treatment for 12 h (Con), treated with 100 μM KA (KA) for 12 h or pretreated with anthocyanins (0.1 and 0.2 mg/mL) for 12 h, and then posttreated with 100 μM KA for 12 h (Antho1 + KA and Antho2 + KA, respectively). **(A)** Shown are representative dichlorofluorescein (DCF) fluorescence and corresponding bright field images (acquired with a 60X objective) indicating intracellular ROS concentration. Scale bar = 20 μm. Clear differences of fluorescence intensity can be observed in the different groups. **(B)** DCF fluorescence intensity in the images was quantified using image J software and expressed in arbitrary units. Data are presented as "fold of control", which was calculated as the DCF fluorescence intensity in each experimental group relative to the mean DCF fluorescence intensity of the control group. Data represent mean (±SEM). Significant difference ($P < 0.05$) among the different experimental groups is indicated by the asterisks. **(C)** Flow cytometric analysis of mitochondrial membrane potential ($\Delta\psi_m$) by JC-1 staining. Collapse of $\Delta\psi_m$ is associated with high FL1 fluorescence (green) and low FL2 fluorescence (red). Results of one representative experiment are shown. The numbers indicate cell population in the quadrants indicated by the red ellipse, expressed as a percentage of the total cell population. **(D)** Graphical representation of the data in panel (C). Shown is the percentage of cell population that accounts for cells with low $\Delta\psi_m$. Data are the mean ± SEM of three independent experiments ($n = 3$). Symbols: *represents significant difference ($P < 0.05$) between the Antho+KA and Con groups; #represents significant difference ($P < 0.05$) between Con and KA.

phosphorylation of AMPK α in HT22 cells and in primary hippocampal neuronal cells (Figure 4B).

Anthocyanins Attenuate KA-induced Apoptosis in Part Via Mitigation of KA-induced AMPK Activation

TUNEL is a well-established method for the detection of apoptosis and a relatively late apoptotic marker during excitotoxicity. We studied the protective effects of anthocyanins against KA-induced apoptosis in HT22 cells and in primary cultures of hippocampal neurons using a standard TUNEL and PI staining assay. Our results showed that the percentage of TUNEL-positive apoptotic cells in the population was greater in KA-treated cells compared with untreated control in both cell types (Figure 5A,B,C and D). The percentage of apoptotic cells was significantly low in cells pretreated with anthocyanins compared with the cells treated with

KA alone. These results revealed that the dose of anthocyanins used in our experiments protects neuronal cells from KA-induced apoptosis. These results were confirmed at the molecular level by evaluating the cellular level of activated caspase-3 that has been shown to be essential for the characteristic DNA fragmentation of apoptotic cells. The content of activated caspase-3 was increased by KA, and this increase was inhibited when the KA treatment was preceded by treatment with anthocyanins (Figure 6A,B).

Next, we tested whether inhibition of AMPK α phosphorylation would have any effect on apoptotic signaling using compound C, an ATP-competitive inhibitor of AMPK. Preliminary experiments showed that pretreatment with a test dose of 20 μM compound C for different time intervals (1, 2, and 3 h) significantly reduced the KA-induced phosphorylation and activation of AMPK in primary cultures of hippocampal neurons (Figure 6C). Pretreatment with compound C before KA treatment did not significantly lower the level of activated caspase-3 compared with KA

Figure 3 Effect of anthocyanins on Kainic acid (KA)-induced modulation of cellular levels of marker proteins of the mitochondrial pathway of apoptosis. HT22 cells and primary cultures of hippocampal neurons (Neurons) were grown without treatment for 12 h (Con), treated with 100 μ M KA (KA) or 0.2 mg/mL anthocyanins (Antho) for 12 h or pretreated with anthocyanins (0.1 and 0.2 mg/mL) for 12 h, and then posttreated with 100 μ M KA for 12 h (Antho1 + KA and Antho2 + KA, respectively). Shown are Western blot analyses for Bax (A), Bcl-2 (B), and cytochrome-c (C) that were performed on protein extracts from these samples. For each data set, β -actin signals are shown as loading control. Graphs represent mean $\pm$ SEM ($n = 3$) of the density values of each protein band expressed in arbitrary units (a.u.). Symbols: [#]significant difference ($P < 0.05$) from Con; ^{*}significant difference ($P < 0.05$) from KA.

treatment alone (Figure 6D), suggesting that activation of AMPK was required for KA-induced apoptosis. Our finding suggests that KA-induced cell death in HT22 neuronal cells and primary cultures of hippocampal neurons results from both oxidative stress and overactivation of AMPK signaling. This long-term activation of AMPK α further activates the mitochondrial cell death pathway and leads to neuronal apoptosis. The failure of compound C to inhibit apoptosis through inhibition of caspase-3 may be attributed to the direct activation of AMPK by ROS.

Discussion

The present study showed that anthocyanins from Korean black bean could strongly protect both HT22 mouse hippocampal cells and primary cultures of fetal rat hippocampal neurons from KA-induced excitotoxicity. The mechanism and targets of anthocyanin-mediated neuroprotection are not fully understood. Our results demonstrate that pretreatment of cells with anthocyanins significantly protects against KA-induced excito-

Figure 4 Effect of anthocyanins on Kainic acid (KA)-induced activation of AMPK. (A) To optimize duration of KA treatment, primary cultures of hippocampal neurons were untreated (Con) or treated with KA (100 μ M) for 30 min, 2, 12, and 24 h, and protein extracts were subjected to Western blot analyses. Shown are representative Western blots probed with antibodies for phospho-AMPK α (Thr172) and AMPK α . The graph represents the density value in arbitrary units (a.u.) of the phospho-AMPK α bands on the blot. Data are the mean $\pm$ SEM of three independent experiments ($n = 3$). Significant differences ($P < 0.05$) from Con are indicated by the asterisk. (B) HT22 cells and primary cultures of hippocampal neurons (Neurons) were grown without treatment for 12 h (Con), treated with 100 μ M KA (KA) or 0.2 mg/mL anthocyanins (Antho) for 12 h or pretreated with anthocyanins (0.1 and 0.2 mg/mL) for 12 h, and then posttreated with 100 μ M KA for 12 h (Antho1 + KA and Antho2 + KA, respectively). Shown are Western blot analyses for phospho-AMPK α (Thr172), AMPK α , and β -actin (loading control) that were performed on protein extracts from these samples. Graph represents mean $\pm$ SEM ($n = 3$) of the density values of each protein band, expressed in arbitrary units (a.u.). Statistical difference was determined using one-way analysis of variance (ANOVA) followed by Student's t -test. Symbols: *significant difference ($P < 0.05$) from Con; #significant difference ($P < 0.05$) from KA.

toxicity and cell death. It also reduces KA-induced ROS generation, increase in intracellular free calcium, and sustained activation of AMPK which would activate the apoptotic signaling

pathway. As anthocyanins are known to be antioxidants, we suggest that the protective effect of anthocyanins in KA-induced excitotoxicity is associated with suppression of ROS, which

Figure 5 Effect of anthocyanins on Kainic acid (KA)-induced neuronal apoptosis. HT22 cells (A) and primary cultures of hippocampal neurons (B) were untreated (Con), treated with 100 μ M KA for 12 h (KA), or pretreated with 0.1 and 0.2 mg/mL anthocyanins for 12 h followed by 100 μ M KA for 12 h (Antho1 + KA and Antho2 + KA, respectively). DNA fragmentation characteristic of apoptosis and cell death were visualized with TUNEL (green) and propidium iodide (PI) (red) stains, respectively. Cells were observed by confocal microscopy with a 40 $\times$ objective field. Scale bar = 20 μ m. Arrows indicate TUNEL- and PI-stained neurons. Both concentrations of anthocyanin used effectively blocked KA-induced apoptosis, as evident from the reduced number of TUNEL-positive cells (panels c and d). (C,D) Graph shows the percentage of TUNEL-positive cells in the populations. The values represent mean $\pm$ SEM of three independent experiments. Symbols: [#]significant difference ($P < 0.05$) from Con; ^{*}significant difference ($P < 0.05$) from KA.

directly or indirectly inhibits AMPK activation and apoptotic signaling pathways.

Excitotoxicity has been described to be involved in a number of neurodegenerative disorders including Alzheimer's, amyotrophic lateral sclerosis, epilepsy, multiple sclerosis, and Huntington's disease [42]. Kainic acid is an agonist of a subtype of ionotropic glutamate receptors and has been commonly used to induce

excitotoxicity [43,44]. Substantial evidence indicates that KA activates presynaptic KA receptors located on glutamatergic terminals in the hippocampus and causes excessive release of glutamate [45–47]. Kainic acid has also been shown to be involved in the dysregulation of Ca^{+2} homeostasis [48]. Recently, it has been reported that KA induces apoptosis in cultured cerebellar granule cells through dysregulation of intracellular calcium

Figure 6 Effect of anthocyanins on Kainic acid (KA)-induced activation of caspase-3. **(A,B)** HT22 cells and primary cultures of hippocampal neurons (Neurons) were grown without treatment for 12 h (Con), treated with 100 μ M KA (KA) or 0.2 mg/mL anthocyanins (Antho) for 12 h or pretreated with anthocyanins (0.1 and 0.2 mg/mL) for 12 h, and then posttreated with 100 μ M KA for 12 h (Antho1 + KA and Antho2 + KA, respectively). Shown are Western blot analyses for activated caspase-3 that were performed on protein extracts from these samples. β -actin signals are shown as loading control **(C,D)** Effect of compound C on KA-induced activation of caspase-3. Primary cultures of hippocampal neurons were untreated (Con), treated with 100 μ M KA for 12 h, pretreated with 20 μ M compound C for the indicated periods of time, and then posttreated with 100 μ M KA for 12 h (Compound C + KA). **(C)** Shown is a representative Western blot indicating phospho-AMPK α (Thr172) and AMPK α levels. The density values are the means $\pm$ SEM ($n = 3$). Symbols: [#]significant difference ($P < 0.05$) from Con; ^{*}significant difference ($P < 0.05$) from KA. **(D)** Shown is a representative Western blot indicating activated caspase-3 and β -actin (loading control) levels. The density values are the means $\pm$ SEM ($n = 3$). Symbols: [#]significant difference ($P < 0.05$) from Con.

homeostasis, activation of caspase-3 via AMPA receptors and DNA fragmentation [49,50]. Kainic acid induced apoptosis in primary cultures of hippocampal neurons and in PC 12 cells involved the

production of ROS, induction of Bax/Bcl-2, Ca^{2+} dysregulation, and lipid peroxidation [51]. Herein, we have described a protective effect by which naturally occurring anthocyanins ameliorate

KA-induced neurotoxicity in HT22 mouse hippocampal cells and fetal rat hippocampal neuronal cells.

In this study, we demonstrated that anthocyanins protected HT22 cells and primary fetal hippocampal neurons from KA-induced oxidative stress and apoptosis. Anthocyanins constitute a subfamily of flavonoids that have beneficial antioxidative, antiinflammatory, and antineurodegenerative effects [31–33]. The beneficial effects of anthocyanin have been attributed to their antioxidative properties by the work of many researchers. A number of studies showed that cyanidin-3-O-glucoside has a strong neuroprotective effect in cerebral ischemia and β -amyloid-induced mitochondrial damage [34,35]. Anthocyanins containing cyanidin-3-O-glucoside have been used both *in vitro* and *in vivo* for protection against focal cerebral ischemia and exert a strong neuroprotective effect by blocking the release of apoptosis-inducing factor (AIF) [52]. While cyanidin-3-O-glucoside exerted cytoprotective effects in neuronal cells [34], blueberry constituents reduced neuronal loss induced by excitotoxic KA [53] and IH636 grape seed proanthocyanidin extract (GSPE) partially protected rodent neuronal tissue against *O*-ethyl-S,S-dipropyl phosphorodithioate (MOCAP)-induced neurotoxicity [54].

It is widely accepted that neuronal degeneration after KA administration is associated with a depletion of ATP and accumulation of Ca^{2+} in neurons. The increase in intracellular Ca^{2+} can trigger Ca^{2+} -activated formation of free radicals [55]. We found that the intracellular calcium concentration and intracellular concentration of ROS were significantly higher in cells treated with KA (Figures 1 and 2). These results are in agreement with the previous findings that are mostly focused on ROS and Ca^{2+} signaling [55–57]. Our data showing that pretreatment with anthocyanins significantly reversed the KA-induced increases in cytosolic Ca^{2+} concentration and intracellular ROS concentration are therefore consistent with the proposed role of anthocyanins in neuronal protection. The antioxidant and free radical scavenger activity of anthocyanins have recently established in hyperglycemia-induced hepatic oxidative damage consistent with our current findings [58].

Many studies have demonstrated that AMPK acts as a multifunctional metabolic sensor in the brain [23–26]. There is evidence that AMPK has a dual function in the regulation of cell death and survival that is dependent upon the type of stress, type of cells, and duration of exposure. Activation of AMPK promotes cell death *in vitro* and *in vivo* in a number of different cells including neurons [21,59,60], and inhibition of AMPK is protective in different models exposed to a variety of stressors. In this study, we observed a dramatic increase in the phosphorylation status of AMPK α in HT22 cells and primary cultures of hippocampal neurons upon exposure to a cell death-inducing dose of KA with-

out an accompanying increase in the total AMPK level (Figure 4). Pretreatment with anthocyanins inhibited the KA-induced phosphorylation of AMPK α in both types of cells. Importantly, concentrations of compound C that inhibited KA-induced AMPK α phosphorylation also prevented the KA-induced increase in cellular content of activated caspase-3 (Figure 6C,D). Our results are consistent with the notion that the long-term and overactivation of AMPK promotes cell death in HT22 cells and primary cultures of rat fetal hippocampal neurons. Our results also suggest that inhibition of KA-induced AMPK activation by anthocyanins is likely to be an important component of their neuroprotective mechanism.

Administration of KA led to an increase in Bax content and decrease in Bcl-2 content in both HT22 and primary cultures of hippocampal neurons thereby favoring apoptotic neuronal death. On the other hand, pretreatment with anthocyanins before KA treatment significantly reduced the KA-induced increase in Bax content and decrease in Bcl-2 content, thus reducing the apoptotic effect of KA (Figure 3A,B). Our present *in vitro* results indicate that the neuroprotective action of anthocyanins against KA can be at least partially attributed to the reduction in apoptosis, as anthocyanins decreased the KA-induced activation of caspase-3 (Figure 6A).

In summary, we have demonstrated that Korean black bean anthocyanins attenuated the neurotoxicity induced by KA *in vitro*. Anthocyanins are naturally occurring antioxidants. Our results show that their neuroprotective effects against KA-induced excitotoxicity can be attributed to attenuation of numerous KA-induced processes such as ROS accumulation, increase in AMPK activation, perturbation of Ca^{2+} homeostasis, loss of mitochondrial integrity, inhibition of Bax accumulation, inhibition of the release of mitochondrial cytochrome-c into the cytoplasm, and reduction in cellular content of activated caspase-3. These findings suggest that colorful anthocyanins may have considerable potential for preventing KA-mediated excitotoxicity and neurodegeneration.

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Conflict of Interest

There is no conflict of interest.

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