

Anthocyanins Protect Against Ethanol-Induced Neuronal Apoptosis via GABA_{B1} Receptors Intracellular Signaling in Prenatal Rat Hippocampal Neurons

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Abstract Here, we investigated the possible involvement of gamma-aminobutyric acid B1 receptor (GABA_{B1}R) in mediating the protective effects of black soybean anthocyanins against ethanol-induced apoptosis in prenatal hippocampal neurons because GABARs are known to play an important role in the development of central nervous system. Treatments were performed on primary cultures of prenatal rat hippocampal neurons transfected with or without GABA_{B1}R small interfering RNA (siRNA). The results showed that, when ethanol treatment was followed by anthocyanins treatment, cellular levels of proapoptotic proteins such as Bax, activated caspase-3, and cleaved poly (ADP-ribose) polymerase 1 (PARP-1) were decreased, and the cellular level of the antiapoptotic protein Bcl-2 was increased compared to treatment with ethanol alone. Furthermore, the effects of ethanol on cellular levels of GABA_{B1}R and its downstream signaling molecules such as protein kinase A, calcium/calmodulin-dependent protein kinase II (CaMKII), and phosphorylated cAMP response element binding protein were diminished or reversed by anthocyanins treatment. The ability of anthocyanins to reverse the effects of ethanol on cellular levels of Bax, Bcl-2, active caspase-3, cleaved PARP-1, GABA_{B1}R, and CaMKII were abrogated in cells transfected with GABA_{B1}R siRNA. In a GABA_{B1}R-dependent manner, anthocyanins also inhibited the ability of ethanol to elevate intracellular free Ca²⁺ level and increase the proportion of cells with low mitochondrial membrane potential in the population. Cell apoptosis assay and morphological studies also confirmed the neuroprotective effect of anthocyanins against ethanol via GABA_{B1}R. Our data suggest that GABA_{B1}R plays an

important role in the neuroprotective effects of anthocyanins against ethanol.

Keywords Apoptosis · Anthocyanins · GABA_{B1} receptor · Intracellular signaling · Neuroprotection · siRNA

Introduction

Exposure to high levels of ethanol in utero results in lifelong mental and physical abnormalities that are collectively referred to as fetal alcohol syndrome (FAS) [1]. Damage to the central nervous system of fetuses due to ethanol exposure is the main cause of FAS [2, 3]. The cortex, hippocampus, and thalamus are brain structures that are particularly sensitive to alcohol exposure [4].

Ethanol affects the central nervous system in many ways. Many of the effects of ethanol such as dependence and behavior impairment are mediated by receptors for gamma-aminobutyric acid (GABA), the principal inhibitory neurotransmitter in the central nervous system [5]; GABA_B receptors (GABA_BRs) belong to the G-protein coupled receptor family and are involved in various physiological and pathological processes [6–8]. They play an important role in the development of the central nervous system because they are involved in memory and learning. They are important for synaptic plasticity and mediate behavior modifying actions of ethanol. Gamma aminobutyric acid B receptor 1 has been reported to improve behavior impaired by ethanol in *Drosophila* [9]. GABA_BR signaling activates adenylyl cyclase and protein kinase A (PKA). cAMP is generated by the action of adenylyl cyclase, and it is an activator of PKA. Phosphorylation and activation of the transcription factor cAMP response element binding protein (CREB) takes place after the activation of PKA. Transcription of cAMP inducible genes by phosphorylated

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CREB (p-CREB) is important for maintaining synaptic plasticity. Besides activation by GABA_BR, adenylyl cyclase activity is also modulated upon phosphorylation by Ca²⁺/calmodulin kinase (CaMKII) [10]. Ethanol crosses the blood–brain barrier and induces neurodegeneration through the intrinsic apoptotic pathway by activating Bax and promoting cleavage of caspase-3 [11]. Reactive oxygen species (ROS) are generated during ethanol metabolism by the brain [12, 13]. The fetal brain develops in a low oxygen environment and has low antioxidant capacity [14, 15].

Natural antioxidants extracted from fruits and vegetables are ideal candidates to ameliorate oxidative stress and scavenge ROS induced by ethanol. Anthocyanins and flavonoids are plant-derived polyphenols that differ in their oxidation states. Flavonoids have been shown to prevent ethanol-induced apoptosis in cultured fetal rhombencephalic neurons [16]. Flavonoids originating from grape seed have a neuroprotective role against ethanol in the cerebellum and hippocampus of mice [17]. In fact, it has been demonstrated that dietary flavonoids can protect brain and liver against ethanol-induced damage [18]. Electron delocalization properties and the ability to form resonance structures make anthocyanins the most potent antioxidants among flavonoids [19, 20]. In fact, anthocyanins are one of the most effective natural antioxidants. They have cardioprotective, anti-inflammatory, antidiabetic, and anticarcinogenic properties [19, 21]. Anthocyanins are the main flavonoids components of red wine and have been shown to reduce ethanol-induced lipid peroxidation, modulate redox status, and improve spatial memory in the hippocampus and cerebellum of adult rats [22–24]. Anthocyanins extracted from berries can improve cognitive brain function and reduce age-associated oxidative stress [19, 21, 25, 26]. They have been shown to prevent learning and memory loss in estrogen-deficient rats [27].

Black soybean is rich in anthocyanins and has been used for centuries as a medicinal food in China, Korea, and Japan [28]. Therefore, it is probable that black soybean anthocyanins have healthful effects. The neuroprotective effects of anthocyanins against ethanol-induced damage to prenatal brain tissues and the involvement of GABA_BR in this process have not been examined in detail. Here, we compared GABA_B1R siRNA transfected and nontransfected prenatal hippocampal neuronal cells and showed that anthocyanins from black soybeans acted via GABA_B1R to protect cultured prenatal hippocampal neurons against ethanol-induced apoptosis.

Materials and Methods

Treatment of Animals

Female Spargue–Dawley rats ($n=10$, 250 g body weight) were housed in a temperature-controlled environment (Geyongsang

National University Neurobiology Laboratory, Jinju, Korea) with lights on from 0800–2000 hours (12 h light/dark) cycle and allowed ad libitum access to food. At gestational day (GD) 17.5, pregnant rats were killed by decapitation after an intravenous injection (i.v.) of pentobarbital sodium (3 mg/100 g b.w). All the experimental procedures were approved by the animal ethics committee of the Division of Applied Life Sciences, Department of Biology, Gyeongsang National University, South Korea.

Primary Cultures of Hippocampal Neurons

The primary cultures of hippocampal neurons were prepared from brains of GD 17.5 fetuses. Hippocampal tissues were treated with 0.25 % trypsin-EDTA for 20 min, dissociated by mechanical trituration in ice-cold calcium- and magnesium-free Hank's balanced salt solution and collected by centrifugation. Cells (1×10^6 cells/ml) were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 10 % heat-inactivated fetal bovine serum, 1 mM pyruvate, 4.2 mM sodium bicarbonate, 20 mM HEPES, 0.3 g/l bovine serum albumin, 50 U/ml penicillin, and 50 mg/l streptomycin and plated in poly-lysine (0.02 g/l) coated cell culture plates or chamber slides. Cultures were maintained at 37 °C in a humidified atmosphere with 5 % CO₂. Proliferation of neuroglia cells was inhibited by adding 10 μM cytosine β-D-arabinofuranoside (Sigma, St. Louis, MO, USA) to the growth medium followed by incubation for 12 h.

siRNA Transfection and Drug Treatment

GABA_B1R complementary DNA (cDNA) plasmid (Novartis Pharma, Basel, Switzerland) was digested with *Xba*I and *Eco*RI to release the insert from pCI vector. The PCR primers used for amplification of GABA_B1R cDNA were 5'CGGTAATACGACTCACTATAGGGAGACGCTACCATCCAACAGACCA-3' and 5' GCGTAATACGACTCACTATAGGGAGATCCTGTGAGCTCATGTTGGAA-3' and contained T7 promoter sequences (underlined) at their 5' ends. The PCR reaction amplified a 420-bp fragment of GABA_B1R cDNA 1,096–1,516 bp from that is known to have the highest silencing activity. The PCR product was used as template for double-stranded RNA (dsRNAs) synthesis using the MEGA script[®] RNAi kit (Ambion, Austin, TX, USA). Subsequent processing into short fragments for transfection was performed using a ShortCut RNAi Kit (New England Biolabs, Buckinghamshire, UK). Liposome solution (DMEM containing Lipofectamine2000TM, Invitrogen, Carlsbad, CA, USA) and an equal volume of dsRNAs (21 bp) solution were incubated separately for 5 min at room temperature and then combined and incubated for 20 min. The mixture was added to cells (1×10^6 cells/ml) that had been starved by 24 h incubation in DMEM without antibiotics and serum at a final

concentration of 40 nM GABA_{B1}R siRNA, and negative control siRNA was purchased from (Qiagen, Inc). After 48 h, cells were transferred to the normal growth medium with or without drug supplements. The seven treatment groups included in this study were the following: (1) control; (2) ethanol, 100 mM; (3) baclofen, 50 μM; (4) anthocyanins, 0.1 mg/ml; (5) ethanol, 100 mM plus anthocyanins, 0.1 mg/mL; (6) ethanol, 100 mM plus GABA_{B1}R siRNA, 40 nM; (7) and ethanol, 100 mM plus GABA_{B1}R siRNA 40 nM, plus anthocyanins, 0.1 mg/mL. After 20 min of drug treatment, the cells were harvested for analyses. In all experiments, ethanol treatment was followed by anthocyanins treatment for additional 20 min.

Extraction of Anthocyanins

Anthocyanins were extracted from Korean black soybeans provided by the agriculture research facility of GNU. Anthocyanins were extracted from whole beans a total weight (1,500 g) three times by stirring with 1,500 mL of 95 % methanol/1 % HCl for 72 h at room temperature in the dark. The methanol extract was concentrated using a rotary evaporator, made up to 150 ml and loaded on a XAD-7 column. The column was washed with distilled water until the elute was a soft red color. The column was then washed with ethyl acetate until the color on the entire column changed to purple except for a 1-cm layer at the very bottom of the column. Anthocyanins were then eluted by passing 95 % methanol/1 % HCl through the column until the color on the entire column changed to red. The elute containing anthocyanins was concentrated to 100 ml using a rotary evaporator, and fines were removed by passing through a 0.45-μm filter. The anthocyanins concentrate was loaded onto a Sephadex column and eluted using a 50 % methanol/50 % distilled water/1 % HCl solution until between 800 and 1,000 mL of a red colored elute was collected. The red colored anthocyanin elute was evaporated to dryness using a rotary evaporator. The resulting anthocyanins powder was stored at -20 °C.

Western Blotting

Cultured hippocampal cells were homogenized in cell lysis buffer (Cell Signaling no. 9803, Danvers, MA, USA). Cell lysates were cleared by two rounds of centrifugation at 12,000 rpm for 10 min at 4 °C, and the supernatant saved for analyses. Protein concentration was determined using the Bio-Rad protein assay kit. Thirty micrograms of total proteins were resolved on duplicate 12 % sodium dodecyl sulfate polyacrylamide gels and transferred to a polyvinylidene fluoride membrane for immunoblot analysis as we described previously [11]. The following primary antibodies were used for the immunoreactions: guinea pig antirat GABA_{B1}R (1:500 dilution,

Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit antirat PKA-α (1:500 dilution, Santa Cruz Biotechnology), rabbit antirat CaMKII (1:500 dilution, Cell Signaling), rabbit antirat p-CREB (1:500 dilution, Cell Signaling), Bax and Bcl-2 rabbit antirat (1:500 dilution, Santa Cruz Biotechnology), PARP-1 (1:500 dilution, Santa Cruz Biotechnology), rabbit antirat caspase-3 (1:500 dilution, Cell Signaling), or antiactin (1:1,000 dilution, Sigma-Aldrich, Jerusalem, Israel; used as loading control). Incubation with primary antibody was performed for 24 h at 4 °C. After rinsing the blots, horseradish peroxidase-conjugated goat antimouse, mouse antigoat, or goat antirabbit IgG-HRPs (Santa Cruz Biotech 1:1,000) were incubated with the blots for 2 h at room temperature, and the antigens were detected by enhanced chemiluminescence (Western blotting detection reagents, Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to manufacturer's instructions. In some cases, blots were stripped and reprobed as follows. After washing in Tris-buffered saline containing 0.1 % (v/v) Tween-20 (TBST) the membrane was stripped using ReBlot Plus Strong Antibody stripping solution (Millipore, Temecula, CA, USA) according to manufacturer's instructions. The stripped membrane was washed with TBST (4 washes of 5 min) and then used for reprobing. Western blots were analyzed by densitometry using the computer-based Sigma Gel system (SPSS Inc., Chicago, IL, USA). Density values were expressed as mean±SEM.

Intracellular Free Ca²⁺ Measurement

The intracellular Ca²⁺ concentration was measured with the fluorescent Ca²⁺ indicator fura-2 acetoxymethyl ester (Fura-2AM). Drug treatments were performed as described under "siRNA transfection and drug treatment" in triplicate plates; each containing 1×10⁶ cells. After treatment, cells were washed twice with Krebs buffer and then incubated for 60 min in DMEM media containing 5 μM Fura-2AM at 37 °C in a humidified incubator with 5 % CO₂. Cells were washed twice with Locke's solution (pH 7.8), and Fura-2AM fluorescence signals of Ca²⁺ were measured using a luminescence spectrophotometer (LS50B, Perkin Elmer, Boston, MA, USA) with excitation wavelengths of 340 and 380 nm and emission measurements at 510 nm. The 340 nm/380 nm fluorescence ratio, averaged over a period of 2 s, was monitored. Fluorescence signals were acquired stored and analyzed using a computer with universal imaging software or a MicroVAX II computer with origin 7 software. Intracellular calcium was determined from the Grynkiewicz equation [29]:

$$[Ca] = K_d \times \frac{(R - R_{\min})}{(R_{\max} - R)} \times \frac{Sf_2}{Sb_2}$$

where K_d is the dissociation constant of the Fura-2AM Ca²⁺ interaction was taken to be 225 nM in the cytosolic environment;

R is the 340 nm/380 nm fluorescence ratio; R_{\min} is ratio with zero Ca^{2+} ; R_{\max} is the ratio with saturating Ca^{2+} (using calcium chloride); Sf_2 is the fluorescence at 380 nm with zero Ca^{2+} ; and Sb_2 is the fluorescence at 380 nm with saturating Ca^{2+} .

Flouro-Jade B and Propidium Iodide Staining

Flouro-Jade B (FJB) staining was performed as previously described [30]. Primary cultures of hippocampal neurons from the GABA_{B1}R siRNA transfected and nontransfected groups were plated 48 h after transfection in poly-D-lysine coated chambers. After 4 days growth, cultures were treated as indicated in the figure legends for 12 h at 37 °C. Then, cells were fixed in 4 % paraformaldehyde in phosphate-buffered saline (PBS) for 5 min and stored at -70 °C. Slides were air-dried for 3 h and then subjected, in order, to the following treatments: 10 min in 0.06 % potassium permanganate solution, distilled water rinse, 20 min in 0.1 % acetic acid containing 0.0004 % FJB (Calbiochem, San Diego, CA, USA) and three washes in distilled water. The slides were allowed to dry at 55 °C for 10 min and viewed under a confocal microscope using an FITC filter with (Olympus Fluoview FV1000, Japan). For propidium iodide (PI) staining, slides were immersed with gentle mixing in PI solution (1 µg/ml in PBS) for 20 min at room temperature and then washed twice with PBS for 10 min. Glass cover slips were mounted on the slides with mounting medium prior to viewing.

Flow Cytometric Analysis of Mitochondrial Membrane Potential ($\Delta\Psi_m$)

Mitochondrial membrane potential ($\Delta\Psi_m$) was monitored using the JC-1 mitochondrial membrane potential detection kit (Biotium Inc., Hayward, CA, USA) according to the manufacturer's protocol. Primary cultures of hippocampal neurons from GD 17.5 fetal brain were transfected or not with siGABA_{B1}R RNA and treated with test compounds as described under "siRNA transfection and drug treatment." After drug treatment, cells were harvested, stained with JC-1 reagents at 37 °C for 15 min, washed twice in 1× assay buffer, and resuspended in 0.5 ml PBS for FACS analysis (FACSCalibur Flow Cytometer; Becton Dickinson, San Jose, CA, USA). Analyses were performed on triplicate samples. JC-1 aggregates in healthy polarized mitochondria and emits a red fluorescence at 590 nm. JC-1 monomers that leak from stressed depolarized mitochondria emit green fluorescence at 530 nm. The red and green fluorescence were measured in the green (FL-1) and red (FL-2) channels of the flow cytometer, respectively. The cells were then immediately observed under a fluorescence microscope using a "dual-band pass" filter designed to simultaneously detect fluorescein and rhodamine dyes.

Flow Cytometric Analysis for Apoptosis Using Fluorescence-Activated Cell Sorting Assay

Fragmentation of genomic DNA to sub-G1 DNA was assessed with the aid of fluorescence-activated cell sorting (Becton Dickinson, Franklin Lakes, NJ, USA) analysis as described [31]. GD 17.5 hippocampal cells were cultured in DMEM medium same to that used for primary culture until 80 % confluence. Total cells were collected and resuspended in 100 µl PBS, fixed with 1 ml 70 % ethanol (-20 °C), and washed with PBS. Each pellet was then resuspended in 250 µl PBS containing 1 mg/ml RNase and incubated on ice for 30 min. After the incubation with 250 µl propidium iodide solution (0.5 mg/ml in PBS; Sigma) for 30 min at room temperature then cells were subjected to fluorescence-activated cell sorting assay.

Statistical Analysis

All the bands from Western blot were scanned and analyzed by densitometry using the Sigma Gel System (SPSS Inc., Chicago, IL, USA). All the density values were expressed as the mean±SE. Statistical analysis of the results were carried out by one-way ANOVA, followed by the Duncan's least significant difference post hoc test to determine group differences (F values). Differences with a $p < 0.05$ were considered statistically significant.

Results

Anthocyanins Abrogate the Negative Effects of Ethanol on Cellular Levels of Some Apoptosis Marker Proteins Through GABA_{B1}R

We measured cellular levels of a few apoptosis marker proteins to ascertain whether anthocyanins can protect against ethanol-induced apoptosis in primary cultures of prenatal rat hippocampal neurons. The cellular level of the proapoptotic protein Bax increased upon exposure to ethanol for 20 min, whereas the cellular level of the antiapoptotic protein Bcl-2 was reduced significantly increasing Bax/Bcl-2 ratio [$F(1, 8)=8.7, p < 0.05$; Fig. 1] as expected. Furthermore, we measured the expressions levels of both activated caspase-3 and cleaved PARP-1 as they are important executioners of apoptosis. Activated caspase-3 cleaves PARP-1 and ultimately results in apoptotic and necrotic cell death [32]. Significant increases in active caspase-3 [$F(1, 10)=16.8, p < 0.05$] level and cleaved PARP-1 [$F(1, 8)=11.6, p < 0.05$] level were observed in ethanol-treated cells (Fig. 1). Therefore, ethanol treatment increases levels of some but not all proapoptosis markers.

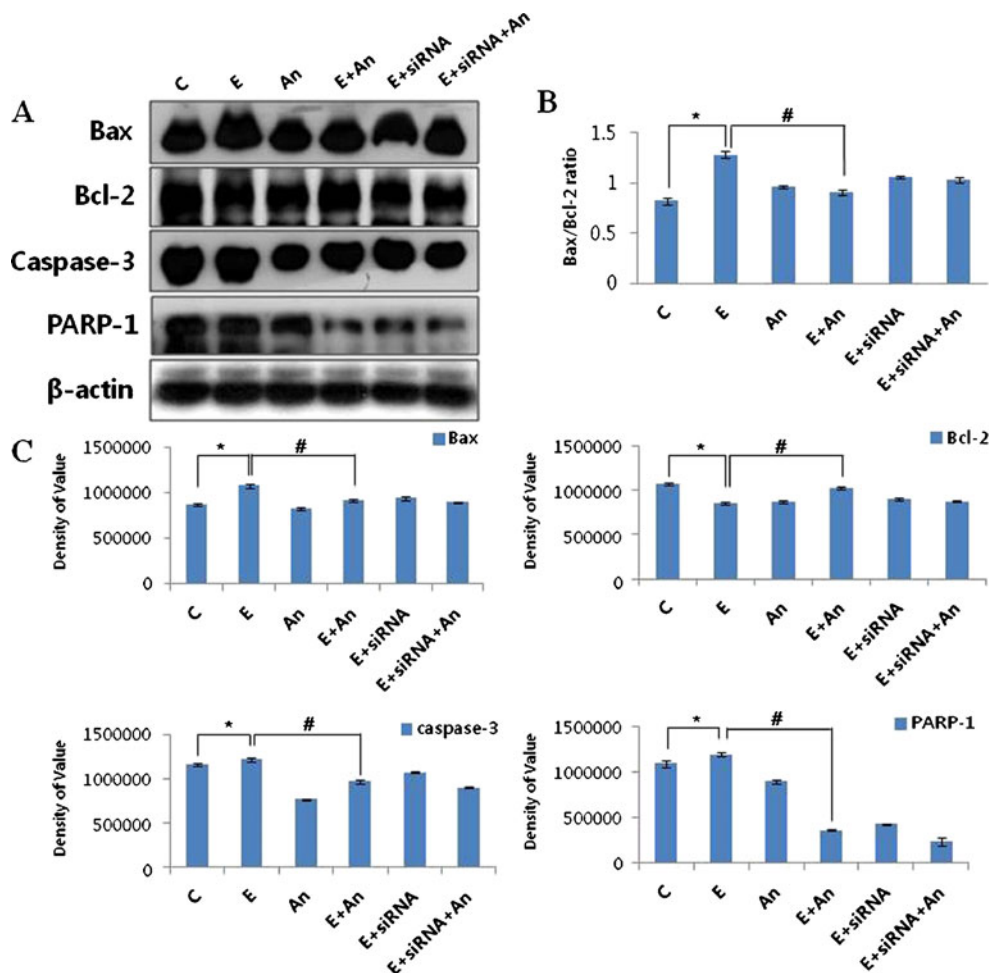


Fig. 1 Treatment of anthocyanins following ethanol treatment abrogates the negative effects of ethanol on cellular levels of some apoptosis marker proteins through GABA_{B1R}. Shown are the results of Western blot analysis of apoptosis marker proteins in protein extracts of cultured prenatal rat hippocampal cells. The various groups that were analyzed are as follows: control (C); 100 mM ethanol (E), 0.1 mg/ml anthocyanins (An), 100 mM ethanol+0.1 mg/ml anthocyanins (E+An), 100 mM ethanol added to cells transfected with 40 nM GABA_{B1R} siRNA (E+siRNA), and 100 mM ethanol+0.1 mg/ml anthocyanins added to cells transfected with 40 nM GABA_{B1R} siRNA (E+siRNA+An). The groups that did not receive siRNA (C, E, An, E+An) received the vehicle that

was used to deliver siRNA at the same time as the other two groups received siRNA (E+siRNA, E+siRNA+An). All treatments were performed 48 h after transfection. The duration of treatment was for 20 min of ethanol followed by additional 20 min of anthocyanins at 37 °C. **a** Immunoblot of Bax, Bcl-2, Caspase-3, PARP-1, and β-actin. β-actin was used as loading control. **b** Histogram showing the ratio of proapoptotic protein Bax and antiapoptotic protein Bcl-2 quantification of signals on the Western blots in protein extracts of cultured prenatal rat hippocampal neuronal cells. **c** Quantification of signals on the Western blots. Bars represent mean±SEM (n=3). **p*<0.05, significantly different from control; #*p*<0.05 significantly different from ethanol

Treatment of anthocyanins for 20 min following ethanol treatment protected against the ethanol-induced increase in cellular Bax level and decrease in Bcl-2 level. It also significantly [*F*(1, 4)=12.6, *p*<0.05] reduced their ratio while simultaneously decreased cellular levels of active caspase-3 [*F*(1, 10)=21.4, *p*<0.05] and cleaved PARP-1 [*F*(1, 8)=17.7, *p*<0.05] (Fig. 1). These results indicated that anthocyanins protect against ethanol-induced apoptosis. In cells transfected with GABA_{B1R} siRNA, the ability of anthocyanins to reverse the effect of ethanol on Bax/Bcl-2 ratio, active caspase-3 and cleaved PARP-1 levels was abrogated or diminished, suggesting that GABA_{B1R} was required for the protective action of anthocyanins.

Anthocyanins Modulate the Effect of Ethanol on Cellular Levels of GABA_{B1R} and its Downstream Signaling Molecules

To extend our study to sort out the mechanism involved in ethanol induced apoptosis in both hippocampal neuronal cells, we measured the expressions levels of GABA_{B1R} and its downstream molecules. GABA_{B1R} was transfected with targeted siRNA in prenatal hippocampal neuronal cells. This was confirmed through Western blot analysis using agonist and antagonist of GABA_{B1R} (Fig. 2a). Ethanol treatment significantly increased the levels of GABA_{B1R} and its downstream signaling molecules, CaMKII and p-CREB. It

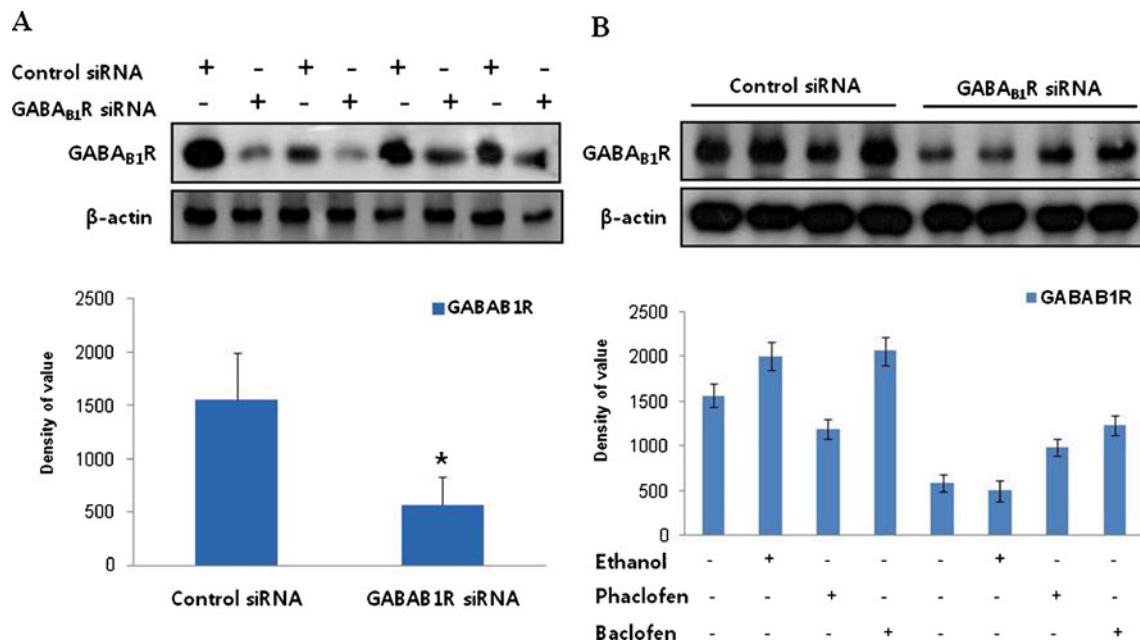


Fig. 2 **a** The expression levels of control and targeted siRNA GABA_{B1}R protein along with or without drug treatment in prenatal hippocampal neuronal cells. **a** Shown are the results and quantification signals of Western blot analysis of nontargeted and targeted siRNA GABA_{B1}R protein levels in cultured prenatal rat hippocampal neuronal cells. Prenatal hippocampal neuronal cells were transfected with targeted GABA_{B1}R siRNA and then incubated in DMEM media for 48 h. **b** Shown are the results and quantification signals of Western blot analysis of non-targeted and targeted siRNA with or without drug treated GABA_{B1}R protein levels in cultured prenatal rat hippocampal neuronal cells. The various groups that were analyzed are as follows: control (C); 100 mM ethanol (E); 50 μM Baclofen (Ba); and 100 μM Phaclofen. All treatments were performed 48 h after transfection. The duration of treatment was for 20 min at 37 °C. Bars represent mean ± SEM (n=3). *p<0.05. **b** Treatment of anthocyanins following ethanol treatment abrogates the negative effects of ethanol on cellular levels of GABA_{B1}R and some GABA_{B1}R downstream signaling molecules. Shown are the results of Western blot analysis of GABA_{B1}R and its

downstream signaling molecules in protein extracts of cultured fetal rat hippocampal cells. The various groups that were analyzed are as follows: control (C); 100 mM ethanol (E); 50 μM Baclofen (Ba); 0.1 mg/mL anthocyanins (An); 100 mM ethanol+0.1 mg/mL anthocyanins (E+An); 100 mM ethanol added to cells transfected with 40 nM GABA_{B1}R siRNA (E+siRNA); and 100 mM ethanol+0.1 mg/ml anthocyanins added to cells transfected with 40 nM GABA_{B1}R siRNA (E+siRNA+An). The groups that did not receive siRNA (C, E, Ba, An, and E+An) received the vehicle that was used to deliver siRNA at the same time as the other two groups received siRNA (E+siRNA, E+siRNA+An). All treatments were performed 48 h after transfection. The duration of treatment was 20 min of ethanol followed by additional 20 min of anthocyanins at 37 °C. **a** Immunoblot of GABA_{B1}R, PKA-α, CaMKII, p-CREB, and β-actin. β-Actin was used as loading control (**b**). Quantification of signals on the Western blots. Bars represent mean ± SEM (n=3). *p<0.05, significantly different from control; #p<0.05, significantly different from ethanol

decreased the expressions level of PKA-α in no siRNA transfection groups but not in the transfection group (Fig. 2b). Compared to the ethanol-treated group, GABA_{B1}R, CaMKII, and p-CREB levels were lower, but PKA-α level is high in the ethanol plus anthocyanin-treated group, suggesting that anthocyanins interfere with ethanol signaling. Baclofen, a specific agonist of GABA_{B1}R, was used for comparison.

Ethanol-Induced Disturbance of Ca²⁺ Homeostasis is Inhibited by Anthocyanins Through GABA_{B1}R

Ca²⁺ homeostasis has an important role in the release of neurotransmitters and in the development of neurons and its disturbance causes neuronal cell death. Ethanol-induced neurotoxicity is mainly linked to the dysregulation of intracellular Ca²⁺ concentrations [33]. Therefore, cytosolic free Ca²⁺ concentrations without and with drug treatments were

compared using the fluorescent Ca²⁺ indicator, fura-2AM (Fig. 3). Compared with the control group, ethanol treatment for 20 min significantly increased peak Ca²⁺ concentration in the nontransfected group than transfected group. In the nontransfected group, the ethanol-induced increase in peak Ca²⁺ concentration was inhibited almost completely by anthocyanins treatment for 20 min following ethanol treatment. This protective effect of anthocyanins against the ethanol-induced increase in peak Ca²⁺ concentration was not evident in the GABA_{B1}R siRNA transfected group, suggesting that the protective effect of anthocyanins requires the GABA_{B1}R. The GABA_{B1}R agonist baclofen also decreased the concentration of intracellular Ca²⁺. These results showed that ethanol elevates intracellular Ca²⁺ concentrations, while anthocyanins played a role in reversing this increase via GABA_{B1}R, suggesting that anthocyanins may protect against ethanol-induced cell death.

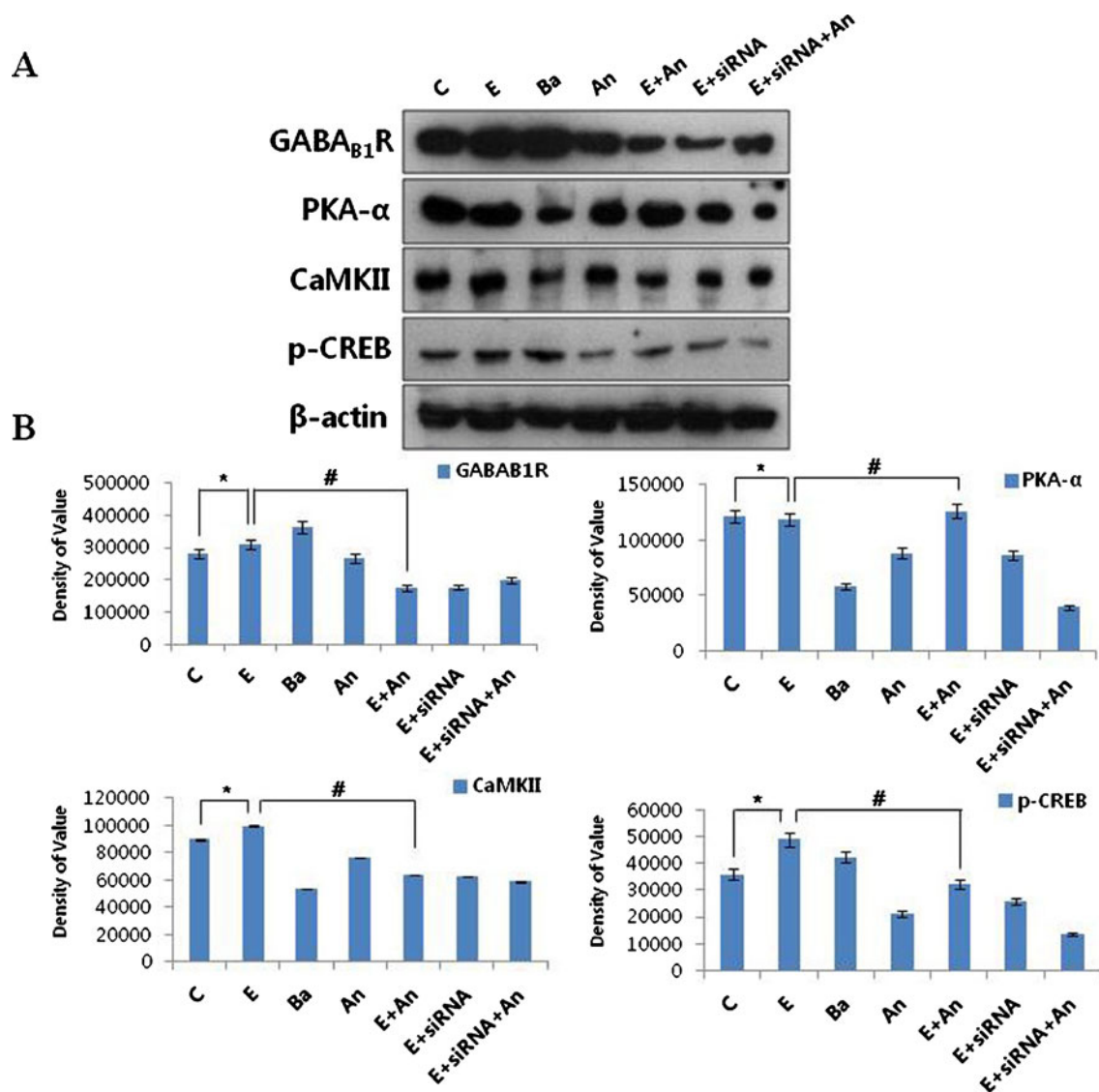


Fig. 2 (continued)

Ethanol-Induced Collapse of Mitochondrial Membrane Potential ($\Delta\Psi$ M) is Inhibited by Anthocyanins Through GABA_{B1}R

The abnormal increase in intracellular Ca²⁺ level induced by ethanol leads to collapse of the mitochondrial membrane potential ($\Delta\Psi$ M), and this collapse along with release of cytochrome-c can activate the apoptotic pathway [34]. FACS analysis showed that the ethanol-treated nontransfected population had a significantly higher percentage of cells with lower mitochondrial potential (24.04 %) compared with control (11.56 %). The group treated with anthocyanins for 20 min following ethanol exposure had a lower percentage of cell population with lower mitochondrial potential (15.86 %) compared to the ethanol-treated group, indicating that anthocyanins inhibit ethanol toxicity. However, in cells transfected with GABA_{B1}R siRNA, anthocyanin was unable

to reverse the adverse effect of ethanol on mitochondrial membrane potential (Fig. 4).

Anthocyanins Protect Prenatal Rat Hippocampal Neuronal Cells Against Ethanol-Induced Apoptosis

Next, we performed an apoptosis assay to determine whether anthocyanins protected against ethanol-induced apoptosis. Two groups of hippocampal neuronal cells were analyzed; one transfected with GABA_{B1}R siRNA and the other treated exactly the same way except for the addition of siRNA. As expected [35], the percentage of apoptotic cells in the ethanol treated samples was significantly higher in nontransfected population than in control and transfected group (Fig. 5). Treatment with anthocyanins for 20 min following ethanol exposure significantly reduced the percentage of apoptotic cells in the nontransfected population but not in the GABA_{B1}R siRNA

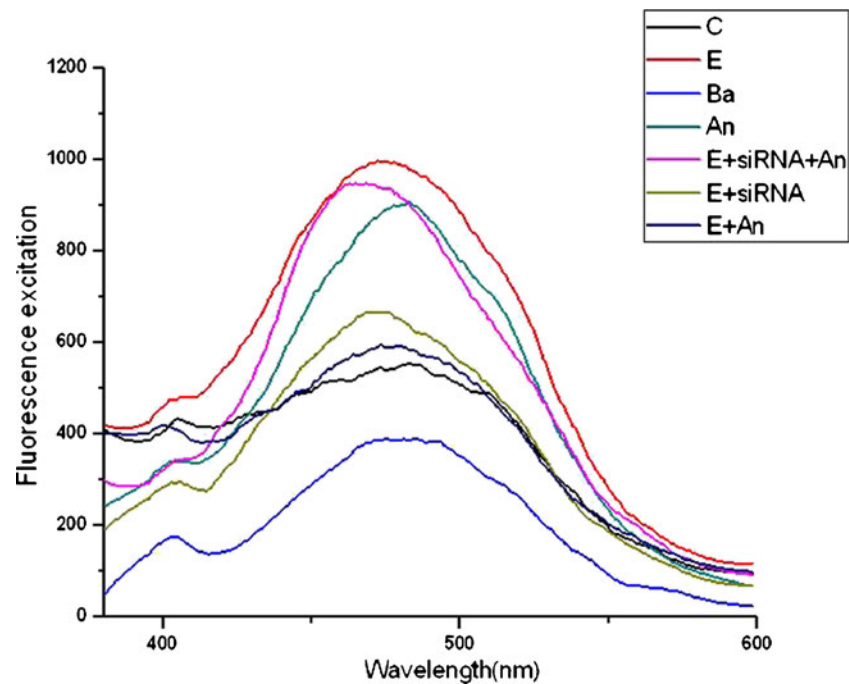


Fig. 3 Treatment of anthocyanins following ethanol treatment inhibits ethanol-induced disturbance of Ca^{2+} homeostasis. Shown are the Fura-2AM fluorescence spectra indicating peaks of intracellular $[\text{Ca}^{2+}]$ in cultured hippocampal cells subjected to the following treatments. The various groups that were analyzed are as follows: control (C); 100 mM ethanol (E); 50 μM Baclofen (Ba); 0.1 mg/ml anthocyanins (An); 100 mM ethanol+0.1 mg/ml anthocyanins added to cells transfected with 40 nM $\text{GABA}_{\text{B}1\text{R}}$ siRNA (E+siRNA+An); 100 mM ethanol added to cells transfected with 40 nM $\text{GABA}_{\text{B}1\text{R}}$ siRNA (E+siRNA);

and 100 mM ethanol+0.1 mg/mL anthocyanins (E+An). The groups that did not receive siRNA (C, E, Ba, An, and E+An) received the vehicle that was used to deliver siRNA at the same time as the other two groups received siRNA (E+siRNA, E+siRNA+An). All treatments were performed 48 h after transfection. The duration of treatment was 20 min of ethanol followed by additional 20 min of anthocyanins at 37 °C. The results are representative of three time experiments with similar results

transfected population, again indicating that the protective effect of anthocyanins required $\text{GABA}_{\text{B}1\text{R}}$.

Morphological Analysis of the Protective Effect of Anthocyanins Against Ethanol Induced Neurodegeneration

For the morphological assessment of cell death, we performed FJB and PI staining (Fig. 6). In cultured nontransfected hippocampal neurons, ethanol-treated cells showed a higher number of dead cells than control. Cells that received anthocyanins for 20 min after ethanol treatment displayed a significant reduction of cell death compared to the ethanol treatment alone indicating that anthocyanins inhibit ethanol-induced apoptosis. In contrast, no significant reduction of cell death was observed in the $\text{GABA}_{\text{B}1\text{R}}$ siRNA transfected group treated with ethanol plus anthocyanins compared to ethanol-treated nontransfected cells.

Discussion

The major finding of our in vitro study is that anthocyanins extracted from black soybeans protected against ethanol-induced degeneration of prenatal rat hippocampal neurons.

The protective effect of anthocyanins was attenuated upon transfection of cells with $\text{GABA}_{\text{B}1\text{R}}$ siRNA, indicating that the neuroprotective effect of anthocyanins involves $\text{GABA}_{\text{B}1\text{R}}$. In this study, we compared the effects of ethanol and/or anthocyanins treatment in two groups of prenatal rat hippocampal neuronal cells. One group (transfected) received $\text{GABA}_{\text{B}1\text{R}}$ siRNA, while the other group (nontransfected) did not receive any RNA during the transfection process. Baclofen, an agonist of $\text{GABA}_{\text{B}1}$ receptor, was used as a control to monitor events arising from stimulation of $\text{GABA}_{\text{B}1\text{R}}$ signaling.

Ethanol-induced apoptosis and neurodegeneration in the prenatal rat hippocampal neuronal cells (Figs. 1, 5, and 6). We have demonstrated that ethanol not only increased cellular levels of the proapoptotic protein Bax and reduced the cellular level of the antiapoptotic protein Bcl-2 but also increased their ratio in nontransfected cells. In addition to measuring levels of these apoptosis markers, we demonstrated that ethanol induces apoptosis in nontransfected cells by apoptosis assay and histochemical analyses. When ethanol treatment of nontransfected cells was followed by black soybean anthocyanins treatment, levels of apoptosis markers (such as Bax/Bcl-2 ratio, active caspase-3, and cleaved PARP-1) as well as the percentage of apoptotic cells in the population were reduced compared to ethanol-treated cells.

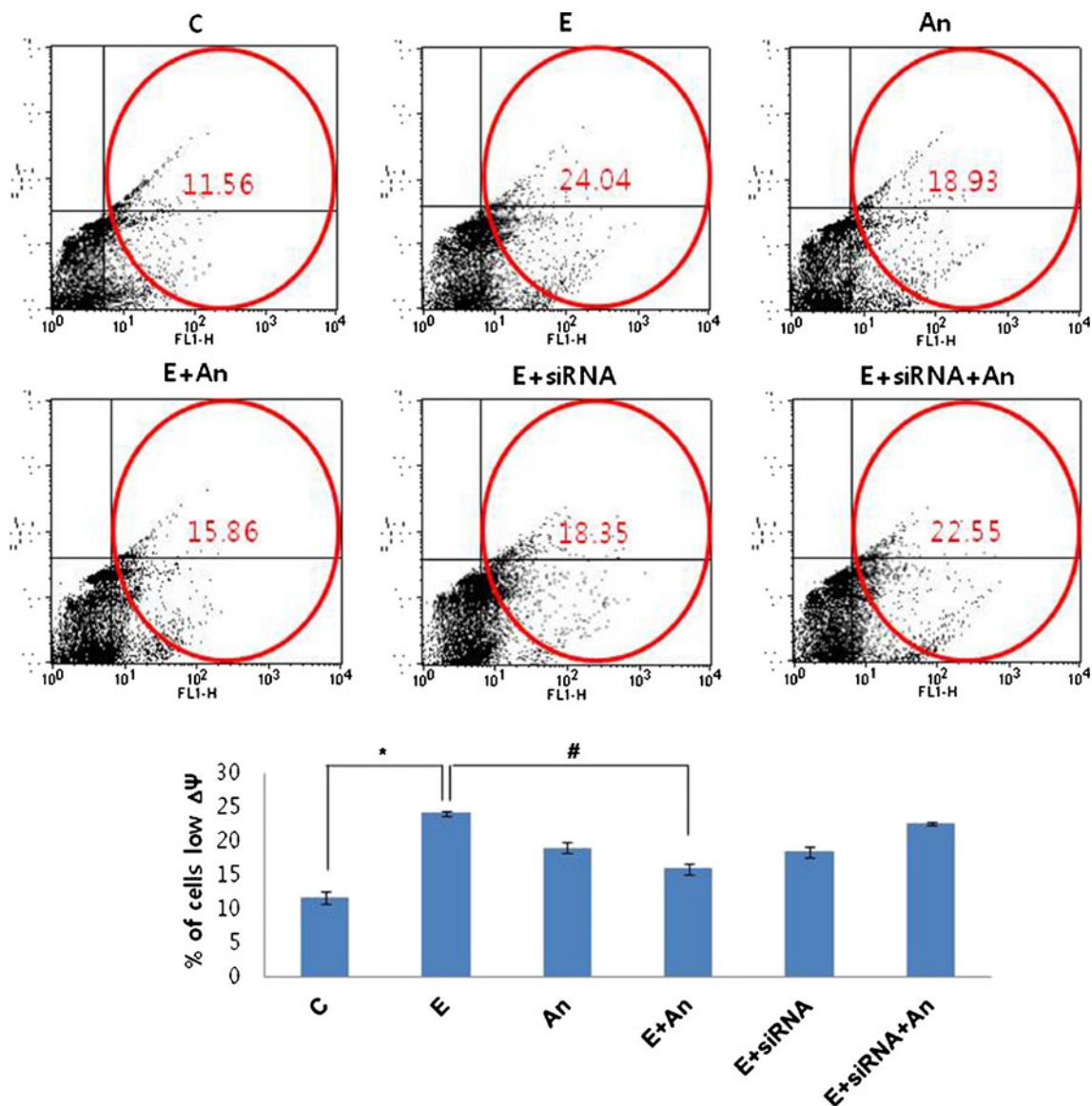


Fig. 4 Treatment of anthocyanins following ethanol treatment inhibits ethanol-induced collapse of mitochondrial membrane potential ($\Delta\Psi$ M). Shown are the results of FACS analysis of JC-1 fluorescence. The various groups that were analyzed are as follows: control (C); 100 mM ethanol (E); 0.1 mg/ml anthocyanins (An); 100 mM ethanol added to cells transfected with 40 nM GABA_{B1}R siRNA (E+siRNA); 100 mM ethanol+0.1 mg/ml anthocyanins (E+An); and 100 mM ethanol+0.1 mg/mL anthocyanins added to cells transfected with 40 nM GABA_{B1}R siRNA (E+siRNA+An); the groups that did not receive

siRNA (C, E, An, and E+An) received the vehicle that was used to deliver siRNA at the same time as the other two groups received siRNA (E+siRNA, E+siRNA+An). All treatments were performed 48 h after transfection. The duration of treatment was 20 min of ethanol followed by additional 20 min of anthocyanins at 37 °C. Data are the mean of three independent experiments ($n=3$) with three plates in each experiment. * $p<0.05$, significantly different from control; # $p<0.05$, significantly different from ethanol

All of these protective effects of anthocyanins against ethanol-induced neuronal apoptosis were more intense in the nontransfected group compared to GABA_{B1}R siRNA transfected group, indicating that GABA_{B1}R had a role in mediating the protective effects of anthocyanins.

Ethanol is known to induce neuronal apoptosis in many ways. Among them, oxidative stress is widely recognized as a major pathway by which ethanol induces neuronal

apoptosis [12, 13, 36–44]. Since ethanol is known to increase oxidative stress and the antioxidative properties of anthocyanins are well recognized, it is possible to speculate that the protective effect of anthocyanins against ethanol-induced apoptosis could be attributed at least in part to their ability to scavenge the ROS induced by ethanol treatment. It is widely accepted that neuronal degeneration after ethanol administration is associated with increase in intracellular

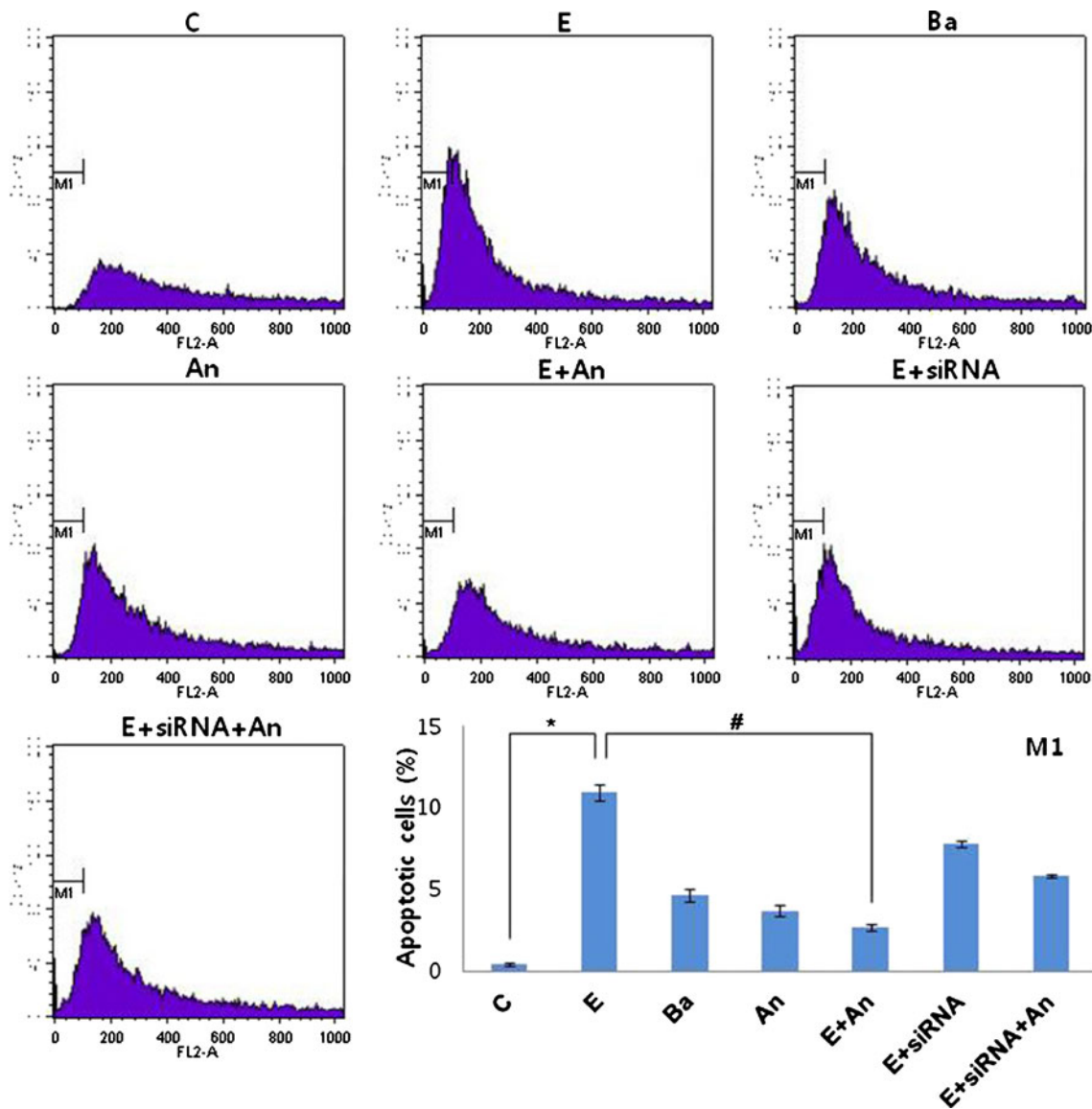


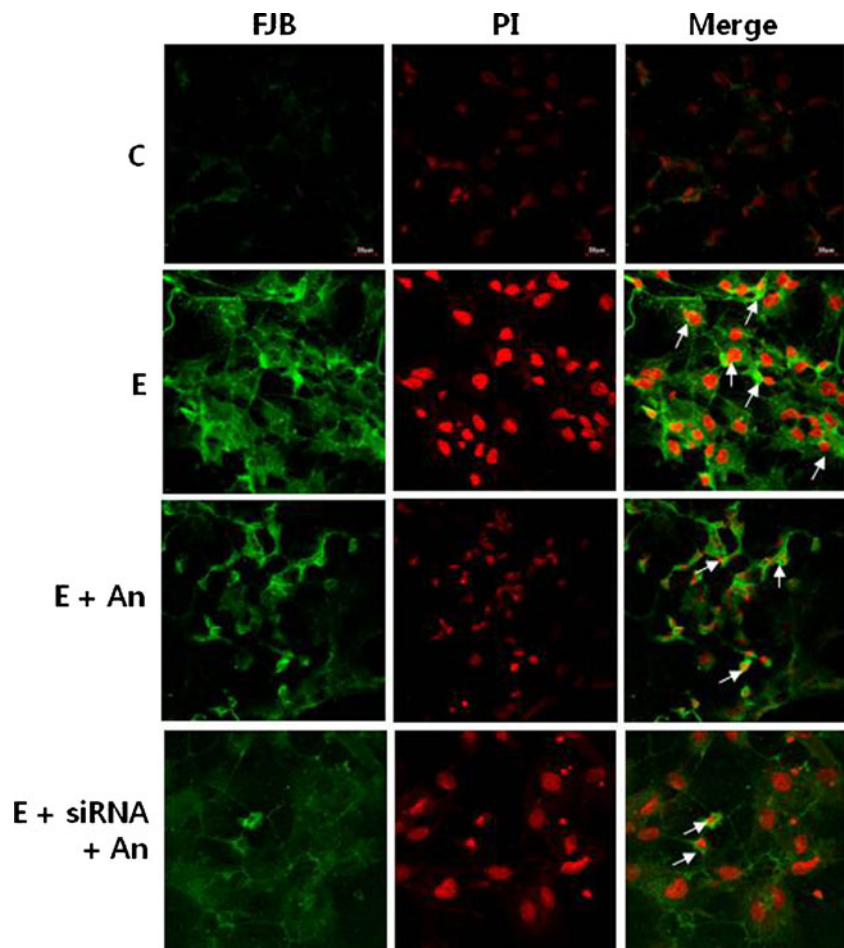
Fig. 5 Treatment of anthocyanins following ethanol treatment inhibits ethanol-induced apoptosis. Shown are the results of an apoptosis assay performed on cultured prenatal rat hippocampal neurons. The various groups that were analyzed by FACS are as follows: control (C); 100 mM ethanol (E); 50 μ M baclofen (Ba); 0.1 mg/ml anthocyanins (An); 100 mM ethanol+0.1 mg/ml anthocyanins (E+An); 100 mM ethanol added to cells transfected with 40 nM GABA_{B1}R siRNA (E+siRNA) and 100 mM ethanol+0.1 mg/ml anthocyanins added to cells transfected with 40 nM GABA_{B1}R siRNA (E+siRNA+An). The groups that did not receive siRNA (C, E, Ba, An, and E+An) received

the vehicle that was used to deliver siRNA at the same time as the other two groups received siRNA (E+siRNA, E+siRNA+An). All treatments were performed 48 h after transfection. The duration of treatment was 20 min of ethanol followed by additional 20 min of anthocyanins at 37 °C. The bar graph shows the percentage of apoptotic cells in each population. Data are the mean of three independent experiments ($n=3$) with three plates in each experiment. M1 indicates cell cycle arrest phase in mitosis. * $p<0.05$, significantly different from control; # $p<0.05$, significantly different from ethanol

Ca²⁺ concentrations. Dysregulation of calcium homeostasis and collapse of mitochondrial membrane potential activate apoptosis. Ethanol significantly increased intracellular Ca²⁺ level and increased the proportion of cells with low mitochondrial membrane potential in nontransfected prenatal rat hippocampal neuronal cells. These effects of ethanol were less marked if the neurons had been transfected with GABA_{B1}R siRNA prior to ethanol treatment [33] (Figs. 3 and 4). Similarly,

anthocyanins inhibited the ethanol-induced increase in intracellular Ca²⁺ level and loss of mitochondrial membrane potential to a greater extent in nontransfected prenatal rat hippocampal neurons than in GABA_{B1}R siRNA transfected neurons (Figs. 3 and 4). Thus, the neuroprotective effects of anthocyanins mediated by GABA_{B1}R include reversal of the detrimental effects of ethanol on Ca²⁺ homeostasis and mitochondrial membrane potential. Stimulation of GABA_BRs has been shown to inhibit

Fig. 6 Treatment of anthocyanins following ethanol treatment inhibits ethanol-induced neurodegeneration. Analysis of neurodegeneration in primary cultures of prenatal rat hippocampal neurons was examined by Flouro Jade B (FJB) and propidium iodide (PI) staining. The various groups that were analyzed are as follows: control (C); 100 mM ethanol (E); 100 mM ethanol+0.1 mg/ml anthocyanins (E+An); and 100 mM ethanol+0.1 mg/ml anthocyanins added to cells transfected with 40 nM GABA_{B1}R siRNA (E+siRNA+An). The groups that did not receive siRNA (C, E, and E+An) instead received the vehicle that was used to deliver siRNA. Cultures were exposed to test compounds for 12 h before staining with FJB (green) and PI (red). Shown images were obtained under a confocal microscope. White arrows indicate dead neuronal nuclei. Magnification with $\times 40$ objective field, scale bar= 20 μ m



calcium influx in neurons [45] and to protect against neuronal apoptosis in a cytosolic Ca²⁺-dependent manner [46].

These observations agree with our results and suggest that anthocyanins directly or indirectly activate GABA_{B1}R. The possibility that they stimulate downstream components of the GABA_{B1}R signaling pathway cannot be excluded. Ethanol and GABA_BR activation have a major effect on the cyclic AMP signaling pathway in the brain [47]. Ethanol and GABA_BR activation also affect other signaling molecules related to cAMP, such as G proteins and PKA, and may cause changes in the functions of CREB. Previous study conducted in vitro has reported that ethanol increased the cellular levels of cAMP through activated adenosine A 2 receptors, which results in the phosphorylation of CREB [48]. Several lines of evidences show that CREB phosphorylation (p-CREB) is a crucial event in the early gene expression [49–51]. Our data (Fig. 1) show that ethanol toxicity was accompanied by increase in the levels of GABA_{B1}R and its downstream signaling molecules such as PKA- α CaMKII and p-CREB (Fig. 2b). Black bean anthocyanins reversed the effect of ethanol on cellular levels of GABA_{B1}R and its downstream signaling molecules to a greater extent in nontransfected cells than in GABA_{B1}R

siRNA transfected cells. Thus, black soybean anthocyanins modulate the effects of ethanol on GABA_{B1}R levels and also modulate the effects of ethanol on the levels of downstream components of the GABA_{B1}R signaling pathway through GABA_{B1}R.

Conclusion

In conclusion, anthocyanins reverse the adverse effects of ethanol in cultured prenatal rat hippocampal neurons. They inhibit ethanol-induced processes such as apoptosis, increase in cytosolic Ca²⁺ level, mitochondrial membrane dysfunction, and neurodegeneration via GABA_{B1}R signaling. Thus, anthocyanins have considerable potential as therapeutic interventions for mitigating the deleterious effects of ethanol on prenatal brain. Realization of this potential requires further research to examine their effects in vivo.

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