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Anthocyanin Extracts from Black Soybean (*Glycine max* L.) Protect Human Glial Cells Against Oxygen-Glucose Deprivation by Promoting Autophagy

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Abstract

Anthocyanins have received growing attention as dietary antioxidants for the prevention of oxidative damage. Astrocytes, which are specialized glial cells, exert numerous essential, complex functions in both healthy and diseased central nervous system (CNS) through a process known as reactive astrogilosis. Therefore, the maintenance of glial cell viability may be important because of its role as a key modulator of neuropathological events. The aim of this study was to investigate the effect of anthocyanin on the survival of glial cells exposed to oxidative stress. Our results demonstrated that anthocyanin extracts from black soybean increased survival of U87 glioma cells in a dose dependent manner upon oxygen-glucose deprivation (OGD), accompanied by decrease levels of reactive oxygen species (ROS). While treatment cells with anthocyanin extracts or OGD stress individually activated autophagy induction, the effect was significantly augmented by pretreatment cells with anthocyanin extracts prior to OGD. The contribution of autophagy induction to the protective effects of anthocyanin was verified by the observation that silencing the Atg5 expression, an essential regulator of autophagy induction, reversed the cytoprotective effect of anthocyanin extracts against OGD stress. Treatment of U87 cells with rapamycin, an autophagy inducer, increased cell survival upon OGD stress comparable to anthocyanin, indicating that autophagy functions as a survival mechanism against oxidative stress-induced cytotoxicity in glial cells. Our results, therefore, provide a rationale for the use of anthocyanin as a preventive agent for brain dysfunction caused by oxidative damage, such as a stroke.

Key Words: Anthocyanin, Oxygen-glucose deprivation, Glial cells

INTRODUCTION

Anthocyanins are water-soluble pigments that belong to the large class of polyphenols and are responsible for the reddish-blue color in a variety of plant tissues (Clifford, 2004). Although the absorption and metabolism-based pharmacokinetics of anthocyanin in serum, subsequent to their bioavailability, are dependent on their nature of chemical structure (Prior and Wu, 2006; McGhie and Walton, 2007), an increasing number of studies provide evidences for health-benefits of anthocyanin including anti-atherogenic activity, vision improvement, anticancer and anti-inflammatory activities (Kamei *et al.*, 1995; Wang *et al.*, 1999; Matsumoto *et al.*, 2003; Xia *et al.*, 2006). These physiological functions of anthocyanin are largely based on their anti-oxidant function as a free radical scavenger but recent studies have revealed that anthocyanins regulate the expression of several genes related to atherosclerosis, and induce apoptosis or autophagy (Longo *et al.*, 2008; Lee *et al.*, 2009b; Mauray *et al.*, 2010; Paixão *et al.*, 2011).These observations suggest that anthocyanin may play a role in the modulation of signal pathways involved in cell death and inflammation upon exposure to oxidative stress.

In the brain, supplementation of blueberries in the diets of mouse or rats resulted in enhanced short-term memory and improvements in motor behavior (Casadesus *et al.*, 2004; Papandreou *et al.*, 2009). Furthermore, diatery supplements of blueberry fed for 8 weeks to 19-month-old rats were shown to be effective in reversing the course of neuronal and behavioral aging (Joseph *et al.*, 1999; Ramirez *et al.*, 2005). As a possible mechanism for this neuroprotective effect, it was suggested

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that the induction of hippocampal heat shock protein (Hsp70) in response to lipopolysaccharide (LPS) challenge was restored in the blueberry-fed old rats, to comparable response levels as those observed in young rats (Galli *et al.*, 2006). The neuroprotective effect of anthocyanin was demonstrated in vitro by showing that cyanidin-3-glucoside (C3G) extracted from mulberry has cytoprotective effects on PC12 cells exposed to oxidative stress such as oxygen-glucose deprivation (OGD) or hydrogen peroxide. In addition, these extracts were also effective in the decrease of infarction volume observed in an in vivo mouse model of ischemia with transient middle cerebral artery occlusion (Kang *et al.*, 2006).

It has long been assumed that glial cells including astrocytes serve merely as structural supports for neurons in the central nervous system (CNS). However, accumulating evidence has proposed that astrocytes provide microenvironments for homeostasis throughout the normal CNS by secretion of various neurotrophic factors, cytokines, and neurotransmitters, in response to various signals via specific receptors (Markiewicz and Lukomska, 2006). In pathological conditions, astrocytes become reactive in response to most forms of CNS injury, including infection, trauma, ischemia, and neurodegenerative diseases. The basic process of reactive astrocytes involves cellular hypertrophy, changes in gene expression profile, and induction of astrocyte proliferation. In contrast to neurosupportive effects of astrocytes in normal CNS, reactive astrocytes exhibit both beneficial as well as harmful effects on neuronal survival and function (Hamby and Sofroniew, 2010; Sofroniew and Vinters, 2010). Therefore, astrocyte survival could be considered a key determinant for CNS outcome, neuronal degeneration or repair of neuronal activity. While the protective effect of anthocyanin in response to oxidative stress have been demonstrated in a variety of cells, its effect on glial cells, which are prone to oxidative stress exposure, has not been sufficiently investigated. Several tumor cell lines of glioma origin are used for model of astrocytes in vitro (Jung *et al.*, 2010; Poulletier *et al.*, 2011). Although glioma cell lines and normal astrocytes exhibit different responses in survival rate to deregulation of proliferative signals (Senger *et al.*, 2002; Sick *et al.*, 2011), but show similar susceptibilities to oxidative stress-induced apoptosis or autophagy (Bonini, *et al.*, 2004; Hwang *et al.*, 2010). In this study, therefore, we examined the effect of anthocyanin extracts from black soybeans on the survival of U87 glioma cells under OGD, which mimics the ischemic condition in vivo. We found that anthocyanin exhibited a protective effect on the survival of U87 cells in response to oxidative stress, which is associated with an increase in autophagy induction under conditions of hypoxic stress.

MATERIALS AND METHODS

Extraction and purification of anthocyanins

Black seed coated soybean (*Glycine max* L.) cultivar Cheongja 3 developed by the National Institute of Crop Science (NICS) was selected for the source of anthocyanin in this study. The extraction of anthocyanin contents was performed as in the previous studies (Ha *et al.*, 2009; Lee *et al.*, 2009a). Anthocyanin contents were determined by means of high performance liquid chromatography using a Dionex Ultimate 3000 series (Dionex Softron GmbH, Germering, Germany). Among the anthocyanins in Cheongja 3, cyanidin-3-*O*-glucoside was the major anthocyanin constituent, representing 68.3% of anthocyanin, followed by delphinidin-3-*O*-glucoside (25.2%), and petunidin-3-*O*-glucoside (6.5%).

Cells culture and OGD treatment

Human glioblastoma cells (U87) from American Tissue Culture Collection (Manassas, VA, USA) were maintained in minimum essential medium (MEM) supplemented with 10% heat inactivated fetal bovine serum and antibiotics solution (penicillin G 100 unit/ml and streptomycin 100 mg/ml) at 37°C in a humidified incubator with 5% CO_2. Before exposure to OGD, U87 cells were seeded onto 35 mm culture dish (Iwaki, Tokyo, Japan) at the density of 5×10^5 cells /ml and incubated overnight. OGD treatment was performed as previously described (Jung *et al.*, 2010). Briefly, the cells were washed twice with degassed DMEM without glucose and serum and immediately treated with various concentration of anthocyanin extracts from black soybeans or rapamycin (Sigma-Aldrich, St. Louis, MO, USA) as indicated. Afterwards, the cells were incubated in an anaerobic chamber containing 85% (v/v) N_2, 10% (v/v) H_2 and 5% (v/v) CO_2 (Thermo Forma, Marietta, Oh, USA) at 37°C for 5 h.

Cell viability

Cell viability was determined by the colorimetric assay which measures the reducing activity of mitochondrial enzymes using 2-(4, 5-dimethyltriazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT, Duchefa, Haarlem, The Netherlands) dyes. After OGD stress in the absence or presence of anthocyanin or rapamycin, cells were incubated with MTT (0.5 mg/ml) and incubated for 2 h at 37°C. After removing the medium, the formazan crystals were dissolved by acid isopropyl alcohol and subsequently distilled water. The extent of reduction of MTT was quantified by measuring the absorbance at a 570 nm using a Victor 3 spectrophotometer (PerkinElmer, Turku, Finland). The relative viability was expressed as a percentage of control cells.

Flow cytometric analysis of ROS

ROS generation was determined by flow cytometry using 2', 7'-dichlorofluorescein diacetate (DCF-DA, Molecular probes, Eugene, OR, USA). DCF-DA is hydrolyzed by intracellular esterase to yield a reduced, non-fluorescent compound, DCFH. The ROS produced by cells oxidized the DCFH to highly fluorescent DCF. After exposure to OGD stress, cells were incubated with 10 μM DCF-DA for 30 min at 37°C and then washed twice with ice-phosphate buffered saline. Quantification of ROS levels from each sample was measured using a FACS Calibur™ (Becton Dickinson, San Jose, CA, USA) with excitation at 488 nm and emission at 525 nm.

Western blotting

Whole cell lysates were prepared using RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH7.5) with protease inhibitors (Roche, Mannheim, Germany) and immediately sonicated three times for 15 sec each on ice followed by centrifugation at 13,200 rpm at 4°C for 20 min. The concentration of protein from each sample was measured by the BCA assay kit (Pierce, Rockford, IL, USA). Equal amount of proteins was separated on 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After incubation

with 0.1% TTBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20) containing 5% non-fat dried milk for 30 min, the membranes were incubated with the antibodies against LC3 (Sigma-Aldrich) (1:10,000), ATG5 (Epitomics, Burlingame, CA, USA) (1:1,000) or beta-actin (Sigma-Aldrich) (1:15,000). Following washing and incubation with G-horseradish peroxidase-conjugated secondary antibodies (Millipore) (1:2,000), the immunoreactive bands were visualized by enhanced chemiluminescence kit from Pierce. Quantification of intensities for each band was determined by multi gauge 2.2 software (Fuji Photo Film Co, Tokyo, Japan).

Small interfering RNA (siRNA) transfection

To inhibit autophagy induction, cells were transfected with a total 100 nM of ATG5 siRNA (Genolution, Seoul, Korea) using the Neon™ Transfection system kit (Invitrogene, Paisley, UK) at 1,300 V, 30 ms in antibiotic-free medium, according to the manufacturer's instructions. The targeted sequences of ATG5 were GGAAUAUCCUGCAGAAGAA and sequences of negative control were CCUACGCCACCAAUUUCGU. After 48 h of transfection, cells were subjected to expose to OGD stress.

Statistical analysis

The data are presented as means ± SD obtained from at least three independent experiments. The significant differences between groups were determined using unpaired Student's t-test. Values with $p<0.05$ was considered significant.

RESULTS

Protective effect of anthocyanin extracts from black soybeans on OGD susceptibility

As shown in Fig. 1A, the dose of anthocyanin extracts we used in this study did not affect on the survival or growth rate of U87 glioma cells, up to 100 μg/ml, as determined by MTT assay. To examine the effect of anthocyanin on the survival of glioma cells upon oxidative stress, U87 cells were exposed to OGD stress in the presence or absence of anthocyanin extracts. As shown in Fig. 1B, the pretreatment of anthocyanin extracts prior to OGD exposure increased the viability of U87 cells in dose-dependent manner. Cell viability determined after oxidative stress was 13.4% in control cells, but were 29.4%

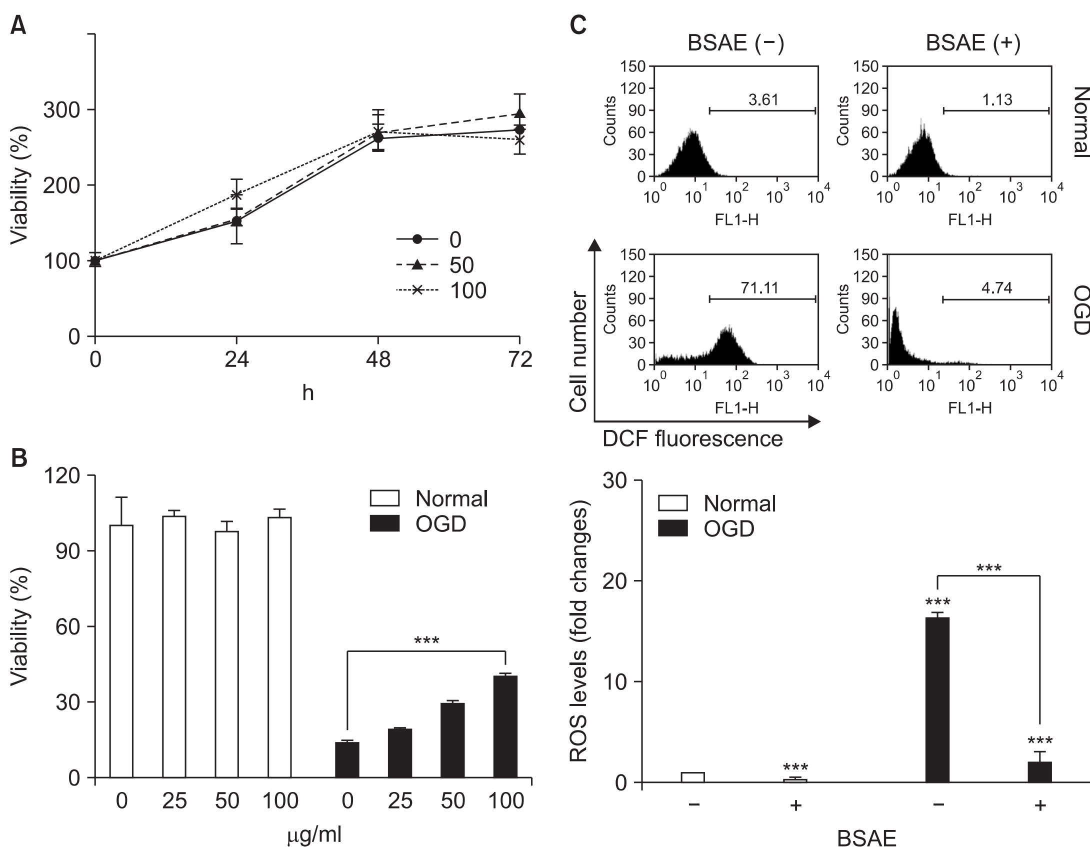

Fig. 1. Anthocyanin from black soybean increased survival of U87 cells upon OGD with decrease in ROS accumulation. (A) The effect of anthocyanin extracts on the growth rate of U87 cells was determined after incubation with 0, 50, or 100 μg/ml of black soybean anthocyanin extracts (BSAE) for 72 h. (B) The effects of anthocyanin on the survival of U87 cells were examined by pretreatment with BSAE at a concentration of 25, 50 or 100 μg/ml before exposure to OGD (B). The viability was determined by MTT assay as described in materials and methods. The value from control cells, BSAE-untreated cells (A) or OGD-untreated cells (B) was set to 100% for relative viability. Values are presented as mean ± S.D. from three independent experiments. (C) Intracellular ROS levels were determined by measuring DCF-DA fluorescence intensity using flow cytometric analysis. The representative (up) and quantitative (down) results for Intracellular ROS levels after OGD in the absence or presence of 100 μg/ml of BSAE. The fold changes in the mean intensities from three experiments were provided as mean ± S.D. (***$p<0.001$).

and 40% in the cells treated with anthocyanin extracts at 50 μg/ml and 100 μg/ml, respectively. Furthermore, the DCF fluorescence intensity representing reactive oxygen species (ROS) levels was attenuated by pretreatment with anthocyanins before OGD induction over 10-fold in cells pretreated with anthocyanin extracts compared to untreated cells (Fig. 1C). Therefore, the protective effect of anthocyanin in response to OGD stress in U87 cells is associated with its free radical scavenging activity.

Induction of autophagy under hypoxic condition was enhanced in the presence of anthocyanin

It has been well known that oxidative stress induces cell death involving apoptotic pathway. However, oxidative stressors such as hydrogen peroxide and 2-methoxyestradiaol (2-ME) were shown to induce autophagic cell death in several cell lines including U87 glioma cells (Chen *et al.*, 2008; Byun *et al.*, 2009). Thus, we examined whether autophagy is induced under OGD condition in U87 cells and whether the induction is affected by the presence of anthocyanin. Autophagy is characterized by the formation of an autophagosome requiring the participation of microtubule-associated protein light chain 3 (LC3) which is normally localized to the cytoplasm (Tanida *et al.*, 2004). Thus, the conversion of the cytoplasmic form of LC3 (LC3-I; 18 kDa) to the preautophagosomal membrane-bound form of LC3 (LC3-II; 16 kDa) is used as a marker for the measurement of degree of autophagy induction. Western blotting assay for LC3 revealed an increase in the density of LC3-II bands by anthocyanin treatment alone as well as by OGD stress. The lipidation of LC3 was markedly increased by OGD in the presence of anthocyanin extracts (Fig. 2A). The mean ratio of band density of LC3-II to LC3-I was increased to 9-fold compared to control in response to OGD, and pretreat-

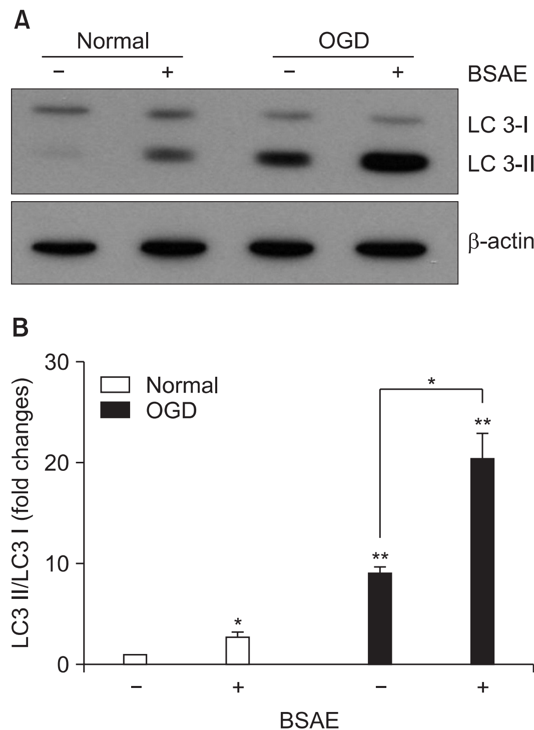

Fig. 2. Anthocyanin extracts promotes autophagy induction under OGD stress. (A) Western assay for LC3 proteins were performed with cell lysates after OGD with or without black soybean anthocyanin extracts (BSAE,100 μg/ml). Beta-actin was detected as a loading control. (B) The intensities of LC-II over LC-I was measured by densitometric analysis. The mean values from three independent experiments (± S.D) were shown after arbitrarily setting the value from control cells as 1.0. (*p<0.05, **p<0.01).

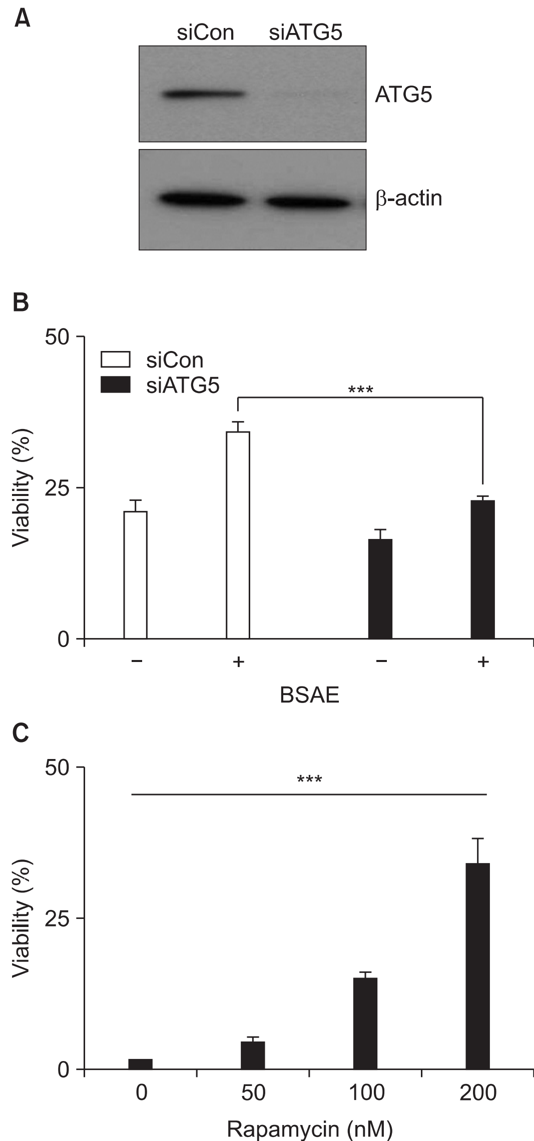

Fig. 3. Inhibition of autophagy reverses the protective effect of anthocyanin extracts on the OGD-induced cell death. (A) U87 cells were transfected with control siRNA (siCon) or Atg5 siRNA (siAtg5) for 48 h and the expression levels of endogenous Atg5 were examined by western blot analysis. (B) The relative survival of U87 cells transfected with control siRNA or Atg5 siRNA with or without BSAE under OGD stress was determined by MTT assay. The value from control cells treated with control siRNA not exposed to OGD was set as 100%. (C) The effect of rapamycin on the survival of U87 cells upon OGD was examined. Prior to exposure to OGD, indicated dose of rapamycin was added to U87 cells for 1 h. After 5 h of OGD stress, the viability was determined by MTT assay. Bars represent means ± S.D from three independent experiments. (***p<0.001).

ment with anthocyainin extracts significantly further increased the ratio to 2.3-fold of OGD, which is almost 20-fold greater than control (Fig. 2B). It should be noted that LC3 conversion by anthocyanin treatment in the absence of OGD stress, was about 3-fold greater than control.

Inhibition of autophagy reverses the protective effect of anthocyanin

Although induction of autophagy was potentiated by anthocyanin extracts under OGD condition, it is uncertain whether the autophagy induction by anthocyanin contributes the protective effect of anthocyanin or not. To clarify this point, we investigated the effect of inhibition of autophagy on cell viability after OGD stress. The expression of Atg5, an essential protein for autophagosome formation (Klionsky, 2007; Mizushima, 2007), was suppressed by siRNA transfection prior to exposure to OGD (Fig. 3A). Silencing of Atg5 expression resulted in the reduction of cell viability after OGD in the absence of anthocyanin from 20.9% to 16.3%. In addition, the increase in the viability provided by treatment anthocyanin extracts was significantly decreased by Atg5 silencing, from 34.2% to 22.7% (Fig. 3B). This result suggests that enhancement of autophagy by anthocyanin confer a survival advantage against cell death by oxidative stress. To confirm the protective effect of autophagy upon oxidative stress-induced cell death, we pretreated U87 cells with rapamycin, an autophagy inducer (Ravikumar *et al.*, 2006), before the application of OGD stress. Fig. 3C shows that pretreatment with rapamycin increased cell viability dose dependently, which was comparable to anthocyanin. Thus, autophagy induction during oxidative stress might confer an advantage to cells to sustain their viability during unfavorable environmental conditions.

DISCUSSION

Anthocyanins have been reported to possess potent antioxidant activity and subsequent protective effect from oxidative damage *in vitro* and *in vivo.* However, it has not yet been clearly determined whether anthocyanins exhibit a protective role on the survival of glial cells which determines neuronal fate and subsequent clinical outcome upon oxidative stress. In our study, we showed that pretreatment with anthocyanins extracted from black soybeans prior to OGD stress in U87 glioma cells significantly increased cell viability (Fig. 1B), which is related to greater ROS clearance (Fig. 1C). Consistent with our results, it has been previously reported that pretreatment with proanthocyanidin extracts from grape seeds results in the elevation of the hydrogen peroxide tolerance in primary glial cells as measured by lactate dehydrogenase release assay (Roychowdhury *et al.*, 2001). Although different oxidative stressors were employed in our study and previous study, the protective effect of anthocyanin on glial cells may be related by the inhibition of ROS generation as demonstrated by in the decrease of DCF fluorescence signal in both studies. The DCF signals in our study increased by 15-fold and 2-fold in the absence and presence of anthocyanin, respectively, compared with control (Fig. 1C). While ROS levels were suppressed close to normal levels, the recovery of viability by anthocyanin extracts was insufficient to be explained by ROS levels, from 13% to 40. Thus, the protective effect of anthocyanin on the survival of glial cells upon OGD may be mediated by the complex mechanisms that are not directly correlated to ROS levels.

Oxidative stress has been known to induce cell death through ROS generation, which involves caspase-dependent apoptotic pathways. Recent studies, however, have suggested that ROS can induce autophagic cell death in glioma cells, which is independent of apoptotic cell death (Chen *et al.*, 2008; Byun *et al.*, 2009). In the present study, we also demonstrated that autophagy was induced in response to OGD stress, which is a metabolic as well as oxidative stress condition, as shown by activation of the autophagosomal marker LC3 (Fig. 2). Moreover, the level of autophagosome-associated LC3-II was significantly further increased by anthocyanin pretreatment in response to OGD stress. The physiological role of autophagy is essentially to remove long-lived protein and damaged organelles, thus autophagy can rescue cells in distressed conditions such as anticancer treatment or nutrient deprivation by providing molecules necessary for metabolism to sustain cell survival (Meijer and Codogno, 2009). Accordingly, OGD-mediated autophagy or its promotion by anthocyanin may either promote or impair cell death. Considering the restoration of viability by anthocyanin upon OGD stress, the enhancement of autophagy may contribute to the protective ability of anthocyanin upon OGD stress. In support of this hypothesis, the siRNA-mediated inhibition of Atg5 expression led to a decrease in the survival of U87 cells provided by pretreatment of anthocyanin extracts under OGD condition (Fig. 3A, B). Therefore, the promotion of autophagy induction by anthocyanin confers protection of U87 cells exposed to OGD stress. The importance of promotion of autophagy in the protective role of anthocyanin was confirmed by showing that viability upon OGD stress was recovered by the treatment with rapamycin, an autophagy inducer (Fig. 3C). The Silencing Atg5 also reduced the viability of U87 cells against OGD stress in the absence of anthocyanin, although to a lesser degree than in the presence of anthocyanin, suggesting that activation of autophagy by OGD stress itself may not participate to the induction of oxidative stress-mediated cell death (Fig. 3B).

It has been reported previously groups that anthocyanin-rich extract from *P. lentiscus* berry or delphinidin, an anthocyanidin, possess the ability to induce autophagy in hepatoma cells (Longo *et al.*, 2008; Feng *et al.*, 2010). We also observed that only the treatment of anthocyanin extracts without OGD stress resulted in the activation of autophagy in U87 glioma cells as evidenced by increase in LC3 lipidation (Fig. 2A). Therefore, autophagy induction of anthocyanin is not a unique feature limited in hepatoma cells. Previous groups show that autophagy was induced under conditions in which an anthocyanin mixture or delphinidin exhibit cytotoxic effect or growth retardation effect on hepatoma cells. The present study demonstrated the activation of autophagy by anthocyanin could occur without affecting growth rate or viability of U87 cells (Fig. 1A, Fig. 2). The discrepancy for the autophagy induction and cytotoxic effect of anthocyanin in this study and previous studies may be attributed to the dose or relative composition of anthocyanin extract. The previous group showed that autophagy was induced with 200 μg/ml of anthocyanin extracts from berries (*P. Lentiscus* L.), at which survival rate of hepatoma cells were about 60% (Longo *et al.*, 2008), while 100 μg/ml anthocyanin extracts from black soybean were used in the present study. Furthermore, the relative composition of two major anthocyanins, cyanidine 3-*O*-glucoside and delphinidin 3-*O*-glucoside, was 4:1 in the extracts from berries in the pre-

vious study whereas the ration was 2.7:1 in the extracts from black soybean in the present study. Depending the chemical natures of anthocyanins, different patterns in absorption and degradation as well as distinct biological responses were observed in vivo in vitro (Prior and Wu, 2006). Thus, difference in the relative composition of these two anthocyanins and their degradation products may affect on the cell survival and autophagy induction. It has been shown that, in the presence of autophagy inhibitors, delphinidin or anthocyanin mixture was shown to induce necrosis or enhance induction of apoptosis. In the present study, Atg5 silencing did not affect the anthocyanin-treated cells but did reverse the protective effect of anthocyanin on OGD stress (data not shown and Fig. 3B). Taken together, anthocyanin-induced macroautophagy, which is observed in response to the cytotoxic dose of anthocyanin or under hypoxic condition, was required for the survival of cells in unfavorable environment conditions. It has not been determined however, whether the same mechanism of autophagy induction by anthocyanin is employed under the cytotoxic conditions in addition to viable conditions, as well as in the presence or absence of oxidative stress.

Upon ischemic injury, astrocytes are subjected to undergo swelling and the astrocyte gap junction may remain unfastened, leading to deterioration in the balance of uptake and release of glutamate, and the diffusion of proapoptotic factors, which may contribute to acceleration of cell death in surrounding neurons (Budd and Lipton, 1998; Lin *et al.*, 1998). However, considering that astrocytes are a reservoir for a high amount of antioxidants and that astrocytes release essential neurotrophic factors (Dringen *et al.*, 1999; Chen *et al.*, 2001), they may also play a protective role in the survival of neurons against oxidative stress. Supporting this presumption, failure of astrocyte functions contributed to neuronal degeneration and disruption of the astroglial scar is associated with the spread of inflammation and infarction volume after stroke in mouse models (Rossi *et al.*, 2007; Li *et al.*, 2008; Takano *et al.*, 2009). Therefore, the preservation of the viability of glial cells including astrocytes in response to oxidative stress may be crucial for the reduction of neuronal death, thereby limiting the infarction area. Based on our results, autophagy induction could be developed as a beneficial approach to promote glial cell survival in response to oxidative stress. In the present study, anthocyanin is a potential candidate to be an effective adjuvant for the prevention or reduction of glial cell death, and subsequent neuronal death during conditions of hypoxic status, such as stroke.

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