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 astrogliosis. 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, dietary 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...
that the induction of hippocampal heat shock protein (Hsp70) in response to lipopolysaccharide (LPS) challenge was re-
stored 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 vi-
tro 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 effect-
tive 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 ho-
meostasis throughout the normal CNS by secretion of vari-
ous 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, in-
cluding 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 neuro-
supportive effects of astrocytes in normal CNS, reactive as-

trocytes 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 glo-


coside 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 Cul-
ture Collection (Manassas, VA, USA) were maintained in mini-

umum essential medium (MEM) supplemented with 10% heat
inactivated fetal bovine serum and antibiotics solution (penicil-
in G 100 unit/ml and streptomycin 100 mg/ml) at 37°C in a
humidified incubator with 5% CO₂. Before exposure to OGD,
U87 cells were seeded onto 35 mm culture dish (Iwaki, Tokyo,
Japan) at the density of 5×10⁶ cells /ml and incubated over-
night. 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) N₂, 10% (v/v)
H₂ and 5% (v/v) CO₂ (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 en-
zymes using 2-(4, 5-dimethyltriazol-2-yl)-2, 5-diphenyl tetra-
zolium bromide (MTT, Duchefa, Haarlem, The Netherlands)
dyes. After OGD stress in the absence or presence of antho-
cyanin 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 us-
ing 2’, 7’-dichlorofluorescein diacetate (DCF-DA, Molecular
probes, Eugene, OR, USA). DCF-DA is hydrolyzed by intracel-
lular 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. Quan-
tification 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, pH 7.5) 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, Rock-
ford, IL, USA). Equal amount of proteins was separated on
12% SDS-PAGE and transferred to polyvinylidene difluoride
membrane (Millipore, Bedford, MA, USA). After incubation

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 Sci-
cence (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
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