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Protective Effects of Anthocyanins against Amyloid β-Peptide-Induced Damage in Neuro-2A Cells

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ABSTRACT: Alzheimer's disease is neuropathologically characterized by amyloid β-protein (Aβ) deposition, resulting in neurotoxicity. Herein, we focused on the prevention of anthocyanins from amyloid-mediated neurodysfunction. The data demonstrated that combined exposure of $A\beta_{1-40}$ and $A\beta_{25-35}$ to Neuro-2A cells resulted in reactive oxygen species (ROS) production and perturbation of calcium homeostasis. The expressions of LXRα, ApoE, ABCA1, and seladin-1 genes were significantly down-regulated upon Aβ challenge. β-Secretase, the rate-limiting enzyme that catalyzes amyloid precursor protein transform to Aβ, was up-regulated by Aβ treatment. For the duration of Aβ stimulation, malvidin (Mal) or oenin (Oen; malvidin-3-*O*-glucoside) was added, and the protective effects were observed. Mal and Oen showed protective effects against Aβ-induced neurotoxicity through blocking ROS formation, preserving Ca^{2+} homeostasis, and preventing Aβ-mediated perturbation of certain genes involved in Aβ metabolism and cellular defense. The present study implicates anthocyanin as a potential therapeutic candidate for the prevention of amyloid-mediated neurodysfunction.

KEYWORDS: Alzheimer's disease, amyloid β-protein, anthocyanin, β-secretase, neuroprotection, Neuro-2A cells

INTRODUCTION

Deposition of senile plaques composed of amyloid β-peptide (Aβ) in the brain is the neuropathological hallmark of Alzheimer's disease (AD), which is an age-related dementia affecting millions of adults and elderly individuals worldwide. Amyloid precursor protein (APP), a type I integral membrane protein, is subject to sequential proteolytic processing that results in Aβ species formation.[1] The reaction is catalyzed first by β-secretase and then by γ-secretase, which are members of the intramembrane protease superfamily. In the nonamyloidogenic pathway, APP undergoes ectodomain shedding by α-secretase—which is a member of the α-disintegrin and metalloprotease family—following the cleavage of the APP C-terminal membrane-connected residue by γ-secretase and the generation of a nontoxic peptide as well as the APP intracellular domain.[2] However, in the amyloidogenic pathway, APP is first cleaved by β-secretase (also named β-site APP cleaving enzyme, BACE) and then by γ-secretase to generate Aβ. The β-sheet content of Aβ is correlated with insolubility and associated with neurotoxicity. Amyloid plaque formation and the development of neurofibrillary tangles and gliosis also accompany this process. Oxidative stress-inducing effect of Aβ might be involved in the pathology and mechanism of neurodegeneration.[3] Aβ-mediated neurotoxicity is caused by the intracellular accumulation of reactive oxygen species (ROS), leading to lipid oxidation and GSH depletion.[4] Burst of ROS levels may result in DNA damage and activation of the DNA checkpoint signal.[5] Furthermore, calcium plays a critical role in signal transduction, survival, and axon guidance in neuronal cells, and Aβ-induced perturbation of Ca^{2+} homeostasis leads to dysfunction in both cortical and hippocampal neurons.[6]

Previously, it has been discovered that apolipoprotein E (ApoE), the major cholesterol transporter in the central nervous system, is associated with the development of AD.[7] Although the exact mechanism by which ApoE influences the risk for developing AD is still unclear, intracellular cholesterol levels can modulate the processing of APP to Aβ. Additionally, liver X receptors (LXRs), ligand-activated nuclear receptors that function as heterodimers with the retinoid X receptor (RXR), induce a variety of genes involved in lipid efflux and transport, such as ApoE and the ATP-binding cassette transporter.[8] LXRs have been suggested as critical pharmacological targets for modulating cerebral Aβ metabolism, as it both inhibits APP processing and promotes Aβ clearance.[8,9] Moreover, seladin-1, an evolutionarily highly conserved gene with homology to a family of flavin adenine dinucleotide-dependent oxidoreductases, catalyzes the production of the sterol intermediates in the cholesterol metabolic pathway[10] and shows a neuroprotective effect against AD. Thus, it is important to investigate the expression of ApoE and its associated regulators and transporters to reveal the correlation between cholesterol metabolism and AD prevention.

Recently, flavonoids have been revealed to show inhibitory effects on β-secretase and have contributed to the development of naturally occurring β-secretase inhibitors for AD prevention.[11] It has also been suggested that consumption of foods rich in polyphenols, particularly anthocyanins, is associated with health improvement, but the mechanisms contributing to these advantageous effects remain to be completely investigated.[12]

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Anthocyanins, members of a subfamily of flavonoids, possess multifunctional benefits including antioxidative and anti-inflammatory properties, cardioprotection, and chemoprevention for cancer.[13] An animal study revealed that daily consumption of berries high in anthocyanin has beneficial effects on cognitive performance and prevented memory deficits,[14] Notably, our group observed that anthocyanin-rich extracts derived from mulberry (*Morus atropurpurea* L.) showed a cognition promotion effect on senescence-accelerated mice.[15] Furthermore, malvidin (Mal), one of the major anthocyanidins that is enriched in mulberries, berries, and grapes, has been identified that shows potential inhibitory efficacy against Aβ fibril formation.[16] However, the protective effects of Mal and its analogue against Aβ-induced neurodysfunction have not yet been revealed. Herein, we demonstrated that Aβ treatment caused an increase in oxidative stress and the intracellular Ca^{2+} concentration and that the LXR-regulated genes involved in cholesterol metabolism were down-regulated upon Aβ stimulation. The stimulation also promoted gene and protein expression of β-secretase. Importantly, both Mal and oenin (Oen; malvidin-3-*O*-glucoside) displayed protective effects against Aβ-induced perturbation in neuronal cells.

MATERIALS AND METHODS

Materials. Anthocyanins Mal and Oen were purchased from Extrasynthese Corp. (Genay Cedex, France). 2′,7′-Dichlorofluorescein diacetate (DCFH-DA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and synthetic amyloid β-protein (Aβ) (Aβ_{1-40} and Aβ_{25-35}) were all obtained from Sigma Aldrich Co. (St. Louis, MO). The Trizol RNA extraction reagent was obtained from MDBio Co. (Frederick, MD). Other high grade reagents were purchased from commercial companies.

Aβ Preparation. Aβ1−40 and Aβ25−35 were, respectively, dissolved in sterile deionized water at a concentration of 0.1 mM as stock solutions and stored at −20 °C before the experiment. The stocks were diluted to the desired final concentrations in treatment medium prior to use. The combination of Aβ1−40 and Aβ25−35 was also prepared beforehand.

Cell Culture. Mouse neuroblastoma Neuro-2A cell line (BCRC no. 60026) was purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2 in α-minimum essential medium (Eagle) with 7% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin antibiotics. Cells were, respectively, treated with different concentrations of Aβ_{1-40} (0−10 μM), Aβ_{25-35} (0−1 μM), or a combination (0.5 μM Aβ_{25-35} and 5 μM Aβ_{1-40}) for the indicated period of time.

Cell Viability Assay. Neuro-2A cells were preincubated with various concentrations of Aβ or anthocyanins for a total period of 48 h. The cells were then washed with PBS and incubated with MTT (0.5 mg/mL) for 3 h in a conditioned incubator. The blue formazan crystals that formed were dissolved using dimethyl sulfoxide (100 μL) and were subsequently quantified spectrophotometrically by measuring the absorbance at 570 nm with a spectrophotometer (Hitachi, Tokyo, Japan).

Cell Cycle Evaluation. Neuro-2A cells were preincubated with Aβ for 24 h and then incubated with or without 50 μM Mal or Oen for a total period of 48 h. Cells were harvested and then incubated with a 40 μg/mL PI solution for 15 min. After they were washed with cold PBS, the cells were resuspended in 300 μL of PBS and then analyzed by flow cytometry. Data were collected from 10000 cells and evaluated by CELL QuestTM software.

ROS Formation. The method was followed by Eruslanov and Kusmartsev[17] with slight modification. Neuro-2A cells were preincubated with Aβ for 24 h and then incubated with or without 50 μM Mal or Oen for an additional 12 h. Cells were harvested and then incubated with a DCFH-DA/PBS solution at a reaction concentration of 20 μM for 30 min. After the cells were washed with cold PBS, the ROS level was analyzed by flow cytometry (FACS Calibur, Becton-Dickinson Immunocytometry System, Franklin Lakes, NJ). Data were collected from 10000 cells and evaluated by CELL QuestTM software.

Intracellular Calcium Concentration. Neuro-2A cells were preincubated with Aβ for 24 h and were then incubated with or without 50 μM Mal or Oen for an additional 12 h. Cells were harvested and incubated with Fluo 3− acetoxymethyl ester (Sigma Aldrich, St. Louis, MO) at the reaction concentration of 20 μM for 30 min. After the cells were washed with cold PBS, the intracellular calcium level was analyzed by flow cytometry (FACS Calibur, Becton-Dickinson Immunocytometry System). Data were collected from 10000 cells and evaluated by CELL QuestTM software.

Real-Time Polymerase Chain Reaction (PCR) Analysis. Neuro-2A cells were preincubated with Aβ for 24 h and then incubated with or without 50 μM Mal or Oen for a total period of 48 h. The total cellular RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's protocols and was quantified by an absorbance at 260 nm. The RNA purity was determined using A260/A280 ratio (average ≥1.7). The total RNA from each specimen was first reverse-transcribed into cDNA using a SYBR GreenER Two-Step qRT-PCR kit (Invitrogen), and then, the PCR amplification was performed using the ABI7300 Real-Time PCR System as follows: 1 cycle at 50 °C for 2 min and denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A linear regression was performed, and the slope, relating the Ct to log ng RNA, was calculated. The Ct value of the target gene was normalized to the housekeeping gene, GAPDH. The BACE-1 gene was amplified using SuperScript One-Step RT-PCR with the Platinum *Taq* System (Invitrogen). Specimens were subjected to 30 cycles of amplification (cDNA synthesis at 50 °C for 30 min followed by predenaturation at 94 °C for 2 min, and the PCR amplification was performed with denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and the final extension at 72 °C for 10 min). PCR products were then electrophoresed on a 1.8% agarose gel and visualized by ethidium bromide staining. The results were then digitized using the UVP BioDoc-It System. Table 1 shows the primer sequences used for the real-time quantitative PCR.

Western Blot Assay. Neuro-2A cells were preincubated with Aβ for 24 h and then incubated with or without 50 μM Mal or Oen for a total period of 48 h. Cells were harvested, and protein concentrations were quantified using a Bio-Rad Protein Assay Kit (Bio-Rad) according to the manufacturer's instructions. Protein extracts were diluted in 5 × sodium dodecyl sulfate (SDS) sample buffer (8% SDS; 0.04% Coomassie blue R-250; 40% glycerol; 200 mM Tris, pH 6.8; and 10% 2-mercaptoethanol) and were subsequently boiled for 10 min. Samples were electrophoresed in a 10% SDS−polyacrylamide gel electrophoresis mini-gel and then transferred onto polyvinylidenedifluoride membranes (Millipore Corp., Bedford, MA) with transfer buffer (48 mM Tris, 39 mM glycine, 0.0037% SDS, and 20% methanol) at 350 mA for 60 min. The membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween-20 (PBST) for 1 h. The membranes were immunoblotted with a rabbit antimouse β-secretase primary antibody (Abcam Inc., Cambridge, MA) in a PBST solution containing 5% bovine serum albumin at 4 °C overnight. After consecutive 30 min washes in PBST, the membranes were incubated with a horseradish peroxidase-labeled secondary antibody for 60 min at room temperature and then washed with PBST for 30 min. The final detection was performed using enhanced chemiluminescence (ECL kit) Western blotting reagents (Amersham Pharmacia Biotech, NJ).

Statistical Analysis. All data are presented as the mean ± standard deviation (SD). Statistically significant differences were based on

Table 1. Sequences of Primers Used for Real-Time Quantitative PCR

gene name[a]	Gene Bank ID	forward primer	reverse primer
LXRα	NM_013839	GGCTGCAGGTGGAGTTCATC	AATGAGCAGAGCAAACTCAGCAT
LXRβ	NM_009473	GATCCTCCTCCAGGCTCTGAA	TGCGCTCAGGCTCATCCT
ApoE	NM_009696	CCTGAACCGCTTCTGGGAT	GCTCTTCCTGGACCTGGTCA
ABCA1	NM_013454	GAAGCCAGTTGTGACAAAACTAAATT	GCAACACTGTGGTGGCTTCA
Bcl-2	NM_009741	TCGCAGAGATGTCCAGTCAG	CCTGAAGAGTTCCTCCACCA
Seladin-1	NM_053272	CATCGTCCCACAAGTATG	CTCTACGTCGTCCGTCA
GAPDH	NM_008084	TCAACGGCACAGTCAAGG	ACTCCACGACATACTCAGC

[a] ABCA1, ATP-binding cassette transporter A1.

comparison with the untreated groups and were calculated by a one- or two-way analysis of variance. Statistically significant individual group means were then compared using Student's *t* test.

RESULTS

Protective Effects of Anthocyanins on Aβ-Induced Neurotoxicity. The effects of different forms of Aβ-mediated neurotoxicity in Neuro-2A cells are shown in Figure 1A. Cells were treated with various concentrations Aβ for 48 and 72 h, respectively, and the results indicated that Aβ_{25-35} (Figure 1A, upper panel) was associated with a higher toxic effect on cells than Aβ_{1-40} (Figure 1A, bottom panel). We further investigated the combination effect of Aβ (Aβ_{25-35} and Aβ_{1-40}) at a concentration that was significantly toxic to Neuro-2A cells. As shown in Figure 1B, Neuro-2A cells were treated with Aβ (0.5 μM Aβ_{25-35} and 5 μM Aβ_{1-40}) for 48 h, and the cell viability was significantly ($p < 0.05$) decreased as determined by the MTT assay. To investigate the protective effect of anthocyanin against Aβ-induced cytotoxicity, anthocyanin (50 μM) was directly added into the medium of the Aβ-treated cells after 24 h stimulation, and the cells were then incubated for an additional 24 h. The data showed that cotreatment with anthocyanin led to a significant increase in cell viability as compared with Aβ-treated alone group ($p < 0.05$; Figure 1B).

Anthocyanins Prevent Aβ-Induced Cell Cycle Arrest. As shown in Table 2, the cell cycle of Aβ-treated cells was arrested at S (17%) and G2/M (19%) phases, which was a significant difference as compared to the control group (8 and 9%, respectively). Both Mal and Oen cotreatment with Aβ-stimulated cells significantly ($p < 0.05$) inhibited the disruption in cell cycle mediated by Aβ.

Anthocyanins Prevent Intracellular ROS Formation upon Aβ Stimulation. The mechanism underlying Aβ-induced neural cell degeneration could potentially involve free radical production. The data revealed that an increase in ROS formation was observed in cells treated with Aβ (0.5 μM Aβ_{25-35} and 5 μM Aβ_{1-40}) (Table 3). However, cells treated to anthocyanins and Aβ showed lower ROS content as compared with Aβ-treated alone group ($p < 0.05$).

Inhibitory Effects of Anthocyanins on the Aβ-Induced Disturbance of Ca^{2+} Homeostasis. Next, we performed experiments to investigate the effect of Aβ on intracellular Ca^{2+} homeostasis. The basal intracellular calcium content determined by flow cytometry was significantly higher in Aβ-treated cells (155 ± 7.9%) as compared with the control (100 ± 2.4%). The increase in $[Ca^{2+}]$ induced by Aβ treatment (0.5 μM Aβ_{25-35} and 5 μM Aβ_{1-40}) was significantly diminished by Mal and Oen treatment (decrease to 119 ± 9.3 and 126 ± 7.3% of control value, respectively) (Table 3).

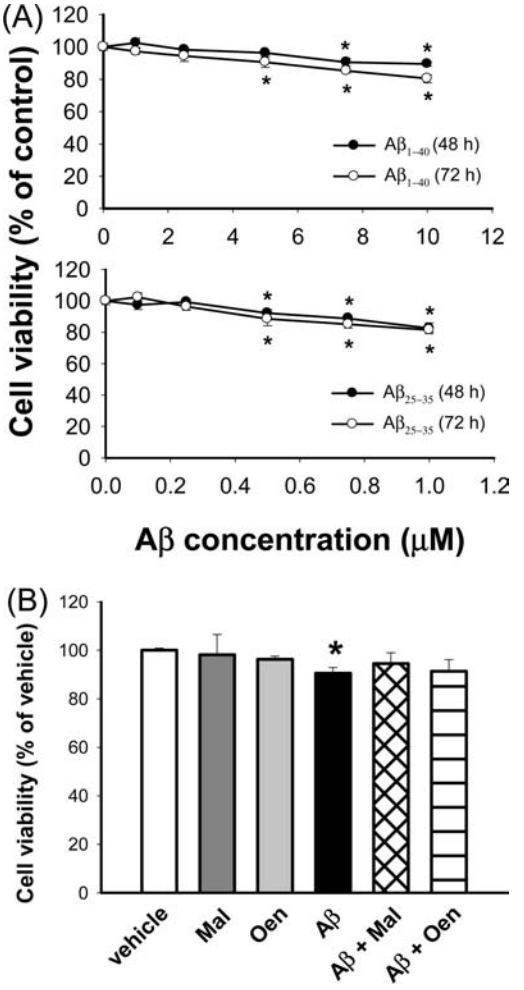

Figure 1. Effects of anthocyanins against Aβ-induced neurotoxicity in Neuro-2A cells. Cells were treated with the indicated concentrations of Aβ_{1-40} and Aβ_{25-35} (A) for 48 and 72 h, respectively, and the neuroprotective effects of anthocyanins (B) were assessed using MTT assay. Neuroprotective effects of anthrocyanins were evaluated by cells treated with or without Mal or oenin (Oen) at a final concentration of 50 μM in the presence of Aβ (combination of 0.5 μM Aβ_{25-35} and 5 μM Aβ_{1-40}) for 48 h, and the cell viability was assessed using MTT assay. Data are from three independent experiments and are expressed as the mean ± SD. * represents significant differences from the control group ($p < 0.05$).

Effects of Aβ on the Expression of Genes Associated with ApoE Synthesis and Antiapoptotic Defense. We further examined the effect of Aβ on the LXR-regulated ApoE level. Aβ (0.5 μM Aβ_{25-35} and 5 μM Aβ_{1-40}) significantly reduced the

Table 2. Cell Cycle Profiles of Neuro-2A Cells Treated with Aβ and Anthocyanins[a]

treatment[b]	G0/G1 phase	S phase	G2/M phase
(A) control	83 ± 2.4	7.6 ± 2.1	8.7 ± 1.1
(B) Aβ	62 ± 3.1#	17 ± 1.5#	19 ± 1.6#
(C) Aβ + Mal	76 ± 3.2*	12 ± 1.2*	12 ± 1.4*
(D) Aβ + Oen	75 ± 4.1*	13 ± 1.2*	11 ± 1.2*

[a] Cells were cotreated with Aβ (combination of 0.5 μM $Aβ_{25-35}$ and 5 μM $Aβ_{1-40}$) and anthocyanins (50 μM) for 48 h. Cells were harvested and stained with PI and analyzed by flow cytometry. Data were evaluated using ModFit LT software. Ten thousand cells were collected, and defined events were summed as 100% [b] (A) Control, (B) Aβ (combination of 0.5 μM $Aβ_{25-35}$ and 5 μM $Aβ_{1-40}$), (C) Aβ + Mal (50 μM), and (D) Aβ + Oen (50 μM). Data are presented as the percentage of total cells. # and * mean significant differences from the control group, and the group of Aβ-treated alone at $p < 0.05$, respectively.

Table 3. Effects of Anthocyanins on Intracellular ROS Formation and $[Ca^{2+}]i$ Homeostasis in Neuro-2A Cells upon Aβ Stimulation[a]

treatment[b]	ROS formation	$[Ca^{2+}]i$ (% of vehicle)
(A) control	100 ± 11.2	100 ± 2.4
(B) Aβ	190 ± 20.5#	155 ± 7.9#
(C) Aβ + Mal	108 ± 18.0*	119 ± 9.3*
(D) Aβ + Oen	123 ± 5.6*	126 ± 7.3*

[a] Cells were cotreated with Aβ for 24 h and then treated with or without anthocyanins (50 μM) for an additional 12 h. For ROS and $[Ca^{2+}]i$ determination, cells were stained with DCFH-DA and Fluo3-AM fluorescein, respectively, for 30 min and subsequently analyzed by flow cytometry. Data were evaluated using ModFit LT software. Ten thousand cells were collected, and defined events were summed as 100%. [b] (A) Control, (B) Aβ (combination of 0.5 μM $Aβ_{25-35}$ and 5 μM $Aβ_{1-40}$), (C) Aβ + Mal (50 μM), and (D) Aβ + Oen (50 μM). # and * mean significant differences from the control group and the group of Aβ-treated alone at $p < 0.05$, respectively.

level of LXRα but not that of LXRβ, and ApoE along with its transporter ABCA1 were also decreased upon Aβ stimulation (Figure 2). Seladin-1, which catalyzes cholesterol synthesis, was suppressed by Aβ treatment. Furthermore, the antiapoptotic gene, Bcl-2, was also inhibited. However, anthocyanin treatment significantly improved the Aβ-induced down-regulation of these genes.

Anthocyanin-Induced Inhibition of β-Secretase Expression in Neuronal Cells. We investigated the gene and protein expression of β-secretase, which is the key enzyme involved in amyloidogenesis. Neuro-2A cells were treated with Aβ (0.5 μM $Aβ_{25-35}$ and 5 μM $Aβ_{1-40}$) for 24 h and were then incubated with or without anthocyanins (50 μM) for an additional 24 h. The expression of β-secretase gene (Figure 3A) and protein (Figure 3B) was significantly ($p < 0.05$) increased upon Aβ stimulation by 1.7- and 1.8-fold, respectively, as compared with the blank. The Aβ-induced increase in β-secretase expression was significantly ($p < 0.05$) inhibited by Mal or Oen treatment.

DISCUSSION

It has been postulated that the amyloidogenic processing of APP to Aβ, particularly the aggregation and deposition-prone Aβ senile plaque, plays a critical role in the pathogenesis of AD. A corollary to the amyloid cascade hypothesis is that amelioration or inhibition of Aβ-mediated neurotoxicity is likely to be one of the future treatments for AD.[18] Incidentally, Neuro-2A cells, which have been widely used as a cell model to investigate the role of phytochemicals in protection against AD-related neurotoxicity, serve as a reliable and convenient cell line for Aβ-relevant research.[4,19] Furthermore, amyloidogenic residues $Aβ_{25-35}$ and $Aβ_{1-40}$ have been implicated in neurotoxicity and have been discussed in various studies both in vitro and in vivo.[20,21] However, it is evident from the literature that there has been less focus on investigating the combination effects of different amyloids. Herein, we have described a protective effect by which naturally occurring anthocyanins ameliorate Aβ-induced neurotoxicity in Neuro-2A cells.

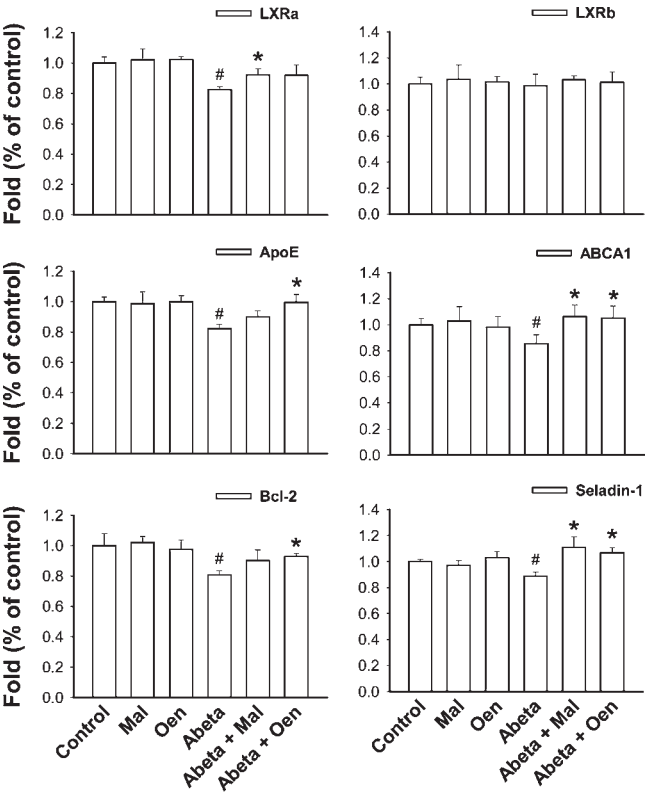

Figure 2. Effects of anthocyanins on target genes expression in Neuro-2A cells upon Aβ stimulation. Cells were challenged with Aβ for 24 h and then treated with or without anthocyanins (50 μM) for an additional 12 h. Cells were harvested, and total RNA was prepared for reverse transcription. cDNA was then amplified, and target genes were examined by real-time PCR. Aβ, combination of 0.5 μM $Aβ_{25-35}$ and 5 μM $Aβ_{1-40}$. # and * represent significant differences from the control group and the group of Aβ-treated alone at $p < 0.05$, respectively.

Anthocyanin is a member of the flavonoid family that shows multiphysiological functions, especially in AD prevention.[22] Anthocyanin-rich fruits slow deleterious effects of aging on neuronal communication and behavior.[23] Furthermore, the inhibition of $Aβ_{25-35}$ fibril formation by Mal and Oen is dramatic.[16] It has been reported that daily consumption of berries rich in anthocyanins (blueberries and bilberries) showed salutary benefits in the prevention of memory deficits and promoted cognitive performance of mice using step-down inhibitory avoidance, open field, elevated plus-maze, and radial maze tasks.[14] In our previous findings, we observed that supplementation of anthocyanin-rich mulberries (*M. atropurpurea* L.) significantly improved the cognitive performance of senescence-accelerated

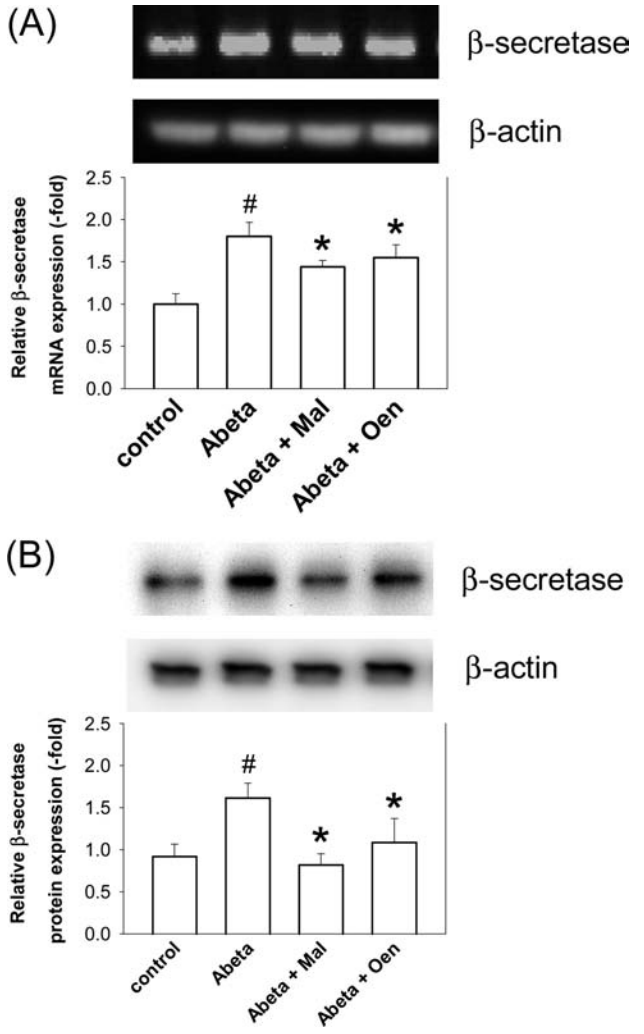

Figure 3. Effects of anthocyanins on Aβ-induced β-secretase expression in Neuro-2A cells. Cells were treated with Aβ for 24 h followed by the treatment with Mal (50 μM) or Oen (50 μM) for an additional 24 h. The expression of β-secretase gene (A) and protein (B) was evaluated. The results from triplicate independent experiments of A and B were quantified by using a LabWorks densitometer. Aβ, combination of 0.5 μM Aβ_{25-35} and 5 μM Aβ_{1-40} combination. # and * represent significant differences from the blank and the group of Aβ-treated alone at $p < 0.05$, respectively.

mice (SAMP), using passive and active avoidance tests, and this protective effect might be resulted from the reduction of Aβ deposition and promotion of antioxidant capacity.[15] By the way, Oen is the most abundant anthocyanins present in mulberry. However, the possible mechanism has not yet been revealed and discussed in an in vitro study. Aβ_{1-40} and Aβ_{25-35} seem to be attracted more attention on their neurotoxicity involved in AD. Recently, Tarozzi et al. [24] reported that anthocyanin (cyanidin 3-*O*-glycoside, Cy-3G) showed neuroprotective effect by inhibiting the aggregation of Aβ. They also found that 50 μM Cy-3G protected SH-SY5Y cells against Aβ_{1-40} oligomer-induced toxicity. Furthermore, it has been suggested that Aβ_{1-40} or Aβ_{25-35} showed neurotoxicity through mediating apoptotic cell death.[25] However, it seems to be fewer considerations paid on the combined effect of these Aβ during the neurotoxic states, even both of them involved in the process of AD. Besides, the progressive loss of synapses in the brain is the other critical characterization of AD, and that seems to implicate that the loss of neurofunction is more crucial and decisive than the death of neuron itself under the attack of Aβ.[26] The concentrations of insoluble and soluble Aβ were still so marginal even in the brain from AD patients,[27] and an animal study also revealed that mice administrated with 1 μg of Aβ_{25-35} significantly led to the impairment of behavior using avoidance and maze tests.[28] All of the evidence show that the influence of Aβ on neurofunction and cognition ability is so tricky and delicate. In the present study, we attempted to reveal the original effects of neurotoxic Aβ on neurocyte during the aggregation and deposition process. First, we examined the cytotoxic properties of Aβ_{1-40} and Aβ_{25-35}, and the data revealed that Aβ_{25-35} showed a greater influence on cell proliferation than Aβ_{1-40} (Figures 1 and 2). We also found that cells treated with Mal or Oen (a 48 h incubation with a concentration greater than 150 μM) showed cytotoxicity but not at lower dosages (data not shown). We further investigated whether the Aβ combination at a lower dosage caused neurotoxicity in Neuro-2A cells. The data suggest that either Mal or Oen shows intervention effects against the Aβ-induced inhibition of cell viability (Figure 1B). Furthermore, the cell cycle in Aβ-treated cells was arrested at S and G2/M phases, corresponding to a significant difference when compared with the control (Table 2), and Mal or Oen cotreatment with Aβ-stimulated cells significantly recovered the disruption in cell cycle mediated by Aβ. It has also been suggested that amyloid might influence cell cycle progression.[29] APP can generate a number of isoforms of amino acid residues in length and exist in the brain. However, we can only find single use of these Aβ in the present researches, but no reports reveal the effect of their combination. Herein, we showed the effect of the combination of Aβ_{1-40} and Aβ_{25-35} on neurocytes. The data suggested that physiological concentrations of Aβ (5 μM Aβ_{1-40} and 0.5 μM Aβ_{25-35}) showed significant influence on the cell viability, respectively, but the combination of Aβ_{1-40} and Aβ_{25-35} did not show more cytotoxicity. Previously, Schaeffer et al. [30] reported that both Aβ_{1-40} and Aβ_{25-35} at high doses (12 μM) showed cytotoxicity in neuroncytes. However, combination of these Aβ at the same final dose unexpectedly showed no cytotoxic effect. Interestingly, similar results did not show in our study, and it might be due to sequence-sensitive effects of Aβ in the aggregation process. The results suggest that lower concentrations of Aβ induced neurons to reenter the cell cycle, and different concentrations have differential abilities to promote neurons into various cell cycle phases or trigger their death.

ROS play an important role in the pathogenesis of several human diseases, particularly disorders of the brain. The brain is particularly vulnerable and is prone to suffer from oxidative stress.[31] Increases in oxidative stress have been speculated to be involved in Aβ-mediated direct or indirect cytotoxicity.[3] In the present study, we observed that Aβ treatment significantly boosts the formation of ROS, and we also demonstrated that administration of Mal or Oen to cells in culture showed cytoprotective effects against Aβ-induced oxidative stress (Table 3). In particular, treatment of cells with the combined Aβ in the absence of anthocyanins caused a significant increase in the accumulation of intracellular ROS, while the presence of anthocyanins inhibited the Aβ-mediated radical burst.

The perturbation of Ca^{2+} homeostasis appears to be associated with dysfunction in electrical transmission and neuronal communication.[32] The amyloid peptide may disturb calcium regulation by impairing membrane ionic channel function. Furthermore, Aβ-induced increases in the intracellular calcium

concentration have been suggested as one of the mechanisms by which Aβ mediates neurotoxicity.[6] We found that the basal intracellular calcium concentration was significantly higher in cells treated with Aβ, while both Mal and Oen treatments significantly reversed this Aβ-induced increase in calcium content (Table 3).

Cholesterol is an essential component of membranes and myelin sheaths and is critical for synaptic integrity as well as proper neuronal communication.[33] However, imbalance of cholesterol absorption and metabolism potentially contributes to the development of AD. Animal studies have shown that high dietary cholesterol cause increased levels of Aβ.[34] ApoE is a major apolipoprotein in the brain that plays an important role in cholesterol metabolism and Aβ levels as well as their deposition and clearance.[7] LXRs are ligand-activated transcription factors that induce the expression of ApoE, the ATP-binding cassette transporter (ABC) and other genes involved in lipid metabolism.[35] Activation of LXRs results in a potent increase in the levels of lipidated ApoE that participates in Aβ clearance.[9] Seladin-1, another important enzyme that catalyzes the reduction of sterol intermediates in the cholesterol metabolic pathway, has been speculated to possess protective activity against Aβ-induced oxidative stress.[10] We reported that gene levels of LXR (LXRα but not LXRβ), ApoE, ABCA1, and seladin-1 were significantly decreased upon Aβ stimulation, and anthocyanin intervention reversed Aβ-induced the down-regulation of LXR-related genes (Figure 2).

Recently, it has been reported that senescence-accelerated prone mice show relatively lower cerebral Bcl-2 levels and higher Aβ deposition.[36] Bcl-2 is postulated as an antiapoptotic member that involved in the modulation of programmed cell death. Herein, we demonstrated that the Aβ-induced inhibitory effect on Bcl-2 gene expression was reversed by anthocyanin treatment.

β-Secretase is the rate-limiting enzyme for the production of Aβ, making it a particularly good drug target for the development of inhibitors that treat AD.[37] Recently, Shimmyo et al. [38] reported that naturally occurring phytochemicals, epigallocatechin-3-gallate and curcumin, suppress Aβ-induced BACE activation and neurotoxicity. In this study, we observed that exposure of $A\beta_{1-40}$ and $A\beta_{25-35}$ combination to Neuro-2A cells resulted in the up-regulation of β-secretase gene and protein levels. Mal and Oen attenuated Aβ-mediated activation of β-secretase, which plays an important role in amyloidogenesis (Figure 3).

In summary, we have demonstrated that naturally occurring anthocyanins attenuated the neurotoxicity induced by $A\beta_{25-35}$ and $A\beta_{1-40}$ combination. We have further shown that Aβ-mediated up-regulation of ROS, the perturbation of calcium homeostasis that might result in cell cycle arrest and cellular damage, and the influence on the expression of genes involved in ApoE metabolism were all blocked by anthocyanin treatment. In addition, we also revealed the inhibitory effect of anthocyanins on Aβ-induced activation of β-secretase. These findings suggest that colorful anthocyanins may have considerable potential for preventing Aβ-mediated neurodysfunction.

AUTHOR INFORMATION

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ABBREVIATIONS USED

AD, Alzheimer's disease; APP, amyloid precursor protein; Aβ, amyloid β-protein; ApoE, apolipoprotein E; ABCA1, ATP-binding cassette transporter A1; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; ADAM, β-disintegrin and metalloprotease; FBS, fetal bovine serum; LXR, liver X receptor; Mal, malvidin; Oen, malvidin-3-*O*-glucoside; ROS, reactive oxygen species; RXR, retinoid X receptor; BACE, β-site APP cleaving enzyme.

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