JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Fucoxanthin Ameliorates Inflammation and Oxidative Reponses in Microglia

Ratih Pangestuti,[†] Thanh-Sang Vo,[‡] Dai-Hung Ngo,[‡] and Se-Kwon Kim*[,†,‡]

[†]Marine Bioprocess Research Center and [‡]Marine Biochemistry Laboratory, Department of Chemistry, Pukyong National University, Busan 608-737, Republic of Korea

ABSTRACT: Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disease that slowly destroys memory and thinking skills. In the brains of AD patients, signs of neuronal degeneration are accompanied by markers of microglial activation and inflammation as well as oxidant damage. This study tested the hypothesis that fucoxanthin, which is known to exert a variety of pharmacological properties, would ameliorate oxidative stress and inflammation in amyloid-β_{42} (Aβ_{42})-induced BV2 microglia cells. It was found that fucoxanthin treatment attenuated pro-inflammatory secretion in BV2 cells as determined by ELISA analysis. Suppressive effects of fucoxanthin on the phosphorylation mitogen-activated protein kinase (MAPK) pathway were confirmed. Moreover, fucoxanthin was able to inhibit free radical-induced DNA oxidation in BV2 cells. This effect was associated with a significant reduction of intracellular reactive oxygen species (ROS) formation and recovery of antioxidative enzymes. The findings in this study suggest that fucoxanthin may serve as a negative feedback regulator of inflammation and oxidative stress in BV2 cells and thereby may protect neuronal cells from neurotoxic mediators released by microglia.

KEYWORDS: *Alzheimer's diseases, microglial, inflammation, oxidative, fucoxanthin*

INTRODUCTION

Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disease characterized by the accumulation of amyloid-β (Aβ) plaques and the formation of neurofibrillary tangles inside the neurons.[1] Aβ is implicated in the pathology through both direct toxicity to neurons and potentiating neuronal damage by microglial activation.[2] Microglia are the resident immune cells in the central nervous system (CNS); they enter the system from the blood circulation early in an organism's development and serve a role of immune surveillance.[3] As an important line of CNS defense, microglia becomes quickly activated in response to injuries or immunologic stimuli.[4] Once activated, microglia functions similarly to macrophages by undergoing phagocytosis, antigen presentation, rapid proliferation, and neurotoxic factors secretion.[5] The neurotoxic factors are nitric oxide (NO), prostaglandin E_2 (PGE_2), pro-inflammatory cytokines [tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6)], cathepsin B, cathepsin D, and reactive oxygen species (ROS). Studies have continuously demonstrated that excessive quantities of neurotoxic factors released by microglia can be deleterious to neurons. The damaging effects of neurotoxic mediators secreted by microglia have been observed in vivo as well as in vitro.[6] Moreover, activation of microglia and excessive amounts of pro-inflammatory mediators released by microglia are continuously observed during the pathogenesis of AD.[7]

In addition to microglial inflammatory response, accumulating evidence also supports the concept that oxidative imbalance and subsequent oxidative stress play an important role in the pathophysiology of AD.[8] This hypothesis of oxidative stress for the AD etiology is supported by the observation in some clinical trials that high doses of antioxidants might be beneficial to AD patients.[9] Notably, the CNS is particularly susceptible to oxidative stress as a result of high oxygen consumption, abundant lipid content, relatively low antioxidant levels, and low regenerative capacity.[10] Therefore, it is important to tightly regulate oxidative damage mediated by free radicals.[11] For those reasons, neuroprotective agents with anti-inflammatory and antioxidant properties would be more effective in AD prevention compared to other agents with anti-inflammatory or antioxidant properties only.

Carotenoids are linear polyenes that function as light-harvesting pigments and protectors against harmful ROS.[12] Carotenoids are synthesized by plants, algae, fungi, and microorganisms, but not animals; hence, animals have to obtain carotenoids from their diets. Fucoxanthin is one of the major carotenoids found in brown algae and diatoms.[13] Fucoxanthin has a unique structure including an unusual allenic bond and a 5,6-monoepoxide in its molecule.[14] Besides their role in photosynthetic and pigmentation effect, fucoxanthin has long been reported to provide health benefit effects including antiobesity,[15] anti-inflammatory,[13] and antioxidant activities.[16] Furthermore, several scientific studies have provided insight into protective effects of fucoxanthin in neuroblastoma and cortical neurons.[17] On the basis of the aforementioned consideration, this study was undertaken to investigate the potential role of fucoxanthin in the response of microglia to Aβ and oxidative stress.

MATERIAL AND METHODS

Materials. Cell culture medium [Dulbecco's modified Eagle's medium (DMEM)], penicillin/streptomycin, fetal bovine serum (FBS), and the other materials required for culturing cells were purchased from Gibco

Received: January 3, 2013
Revised: April 1, 2013
Accepted: April 3, 2013
Published: April 3, 2013

BRL, Life Technologies (Grand Island, NY, USA). Fucoxanthin (F6932), amyloid-β_{42} (Aβ_{42}), H_2O_2, Griess reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) were purchased from Sigma (St. Louis, MO, USA). TRIzol RNA extraction was provided by Invitrogen Ltd. (Paisley, UK). RT-PCR reagents were purchased from Promega (Madison, WI, USA). Specific antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and Amersham Pharmacia Biosciences (Piscataway, NJ, USA); other chemicals and reagents used in this study were of analytical grade.

Preparation of Aβ_{42} Oligomers. Oligomeric Aβ_{42} was prepared as previously described.[18] Synthetic Aβ_{42} was dissolved in HFIP to 1 mmol/L. The HFIP was then removed in a hood for 24 h and then evaporated using a Speed Vac, and the dried HFIP film was stored at −20 °C. The peptide film was suspended to make a 5 mmol/L solution in anhydrous DMSO. Aβ_{42} oligomers were prepared by diluting the solution in medium without phenol red to a concentration of 100 μM and incubated overnight at 4 °C.

Cell Culture and Viability Assay. The murine BV2 microglia was a kind gift of Prof. Il Whan Choi (Inje University, Korea). BV2 cells were grown in T-75 tissue culture cell flasks SPL Life Science (Seoul, Republic of Korea) at 5% CO_2 and 37 °C humidified atmosphere using DMEM supplemented with 5% FBS, 2 mM glutamine, and 100 μg/mL penicillin−streptomycin. For viability assay, cells were seeded into 96-well plates at a density of 2×10^4 cells/well, and incubated with serum-free medium for 24 h, followed by treatment with different concentrations of fucoxanthin. After incubation for 24 h, 100 μL of MTT (0.5 mg/mL final concentration) was added, and incubation was continued for another 4 h. The absorbance was measured at 540 nm by using an enzyme-linked immunosorbent assay (ELISA) microplate reader, Tecan GmbH (Grödig, Austria). Relative cell viability was calculated compared with the absorbance of the untreated control group.

Determination of Nitric Oxide Production. NO levels in the culture supernatants were measured by the Griess reaction. In brief, BV2 cells were preincubated overnight in 24-well plates using DMEM without phenol red at a density 2×10^5 cells/mL, followed by the treatment with fucoxanthin. After treatment with fucoxanthin, NO production was stimulated by adding Aβ_{42} and incubation for a further 24 h. Then 50 μL of culture supernatants from each sample was mixed with the same volume of the Griess reagent (1% sulfanilamide/0.1% *N*-1-naphthylethylenediamine dihydrochloride/2.5% phosphoric acid) following the incubation for 15 min. Absorbance values were read at 540 nm using an ELISA microplate reader. The values obtained were compared with those of standard concentrations of sodium nitrite dissolved in DMEM, and the concentrations of nitrite in conditioned media of treated cells were calculated.

Determination of Prostaglandin-E_2 Production. Assesment of PGE_2 synthesis was performed by enzyme immunoassay without prior extraction or purification using a commercially available PGE_2 enzyme immunometric assay kit (R&D Systems Inc., Minneapolis, MN, USA). BV2 cells were treated with different concentrations of fucoxanthin and stimulated with Aβ_{42} for 24 h. The conditioned media were collected to perform PGE_2 enzyme immunometric assay (PGE_2-EIA) according to the instructions of the manufacturer. The concentration of PGE_2 was calculated according to the equation obtained from the standard curve plot using PGE_2 standard solution in the EIA kit.

Measurement of Pro-inflammatory Cytokine Production. The inhibitory effects of fucoxanthin on the production of pro-inflammatory cytokines were determined by an ELISA. BV2 cells were treated with different concentrations of fucoxanthin and stimulated with Aβ_{42} for 24 h. The conditioned media were collected and analyzed as per the manufacturer's instruction with Quantikine mouse TNF-α, IL-6, and IL-1β immunoassay (R&D Systems Inc.). The concentrations of TNF-α, IL-6, and IL-1β were calculated according to the equation obtained from the standard curve plot using TNF-α, IL-6, and IL-1β standard solution in the ELISA kit.

Measurement of Intracellular ROS by 2′,7′-Dichlorofluorescein Diacetate Assay. Intracellular formation of ROS was assessed according to a method described previously by employing oxidation sensitive dye (2′,7′-dichlorofluorescein diacetate) DCFH-DA as the substrate. BV2 cells were grown in black microtiter 96-well plates, labeled with 20 μM DCFH-DA in Hank's balanced salt solution (HBSS), and kept for 20 min in the dark. The nonfluorescent DCFH-DA dye, which freely penetrates into cells, was then hydrolyzed by intracellular esterase and trapped inside the cells. Cells were treated with different concentrations of fucoxanthin and incubated for 1 h. After the cells had been washed three times with PBS, 500 μM H_2O_2 (in HBSS) was added. The formation of fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS was read every 30 min at the excitation wavelength of 485 nm and the emission wavelength of 528 nm (GENios microplate reader, Tecan Austria GmbH, Grodig/Salzburg, Austria). Dose-dependent and time-dependent effects of treatments were plotted and compared with fluorescence intensity of the control group in which samples were not treated.

Genomic DNA Isolation. Briefly, cells cultured in 10 cm culture dishes were washed twice with PBS and scraped into 1 mL of PBS containing 10 mM EDTA. After centrifugation, cells were dissolved in RNase (0.03 mg/mL), NaOAC (0.175 M), proteinase K (0.25 mg/mL), and SDS (0.6%). The mixture was then incubated for 30 min at 37 °C and 1 h at 55 °C. Following incubation, phenol/chloroform/isoamyl alcohol was added at a 1:1 (v/v) ratio, and the mixture was centrifuged at 6000*g* for 5 min at 4 °C. Following centrifugation, the supernatant was mixed with 100% cold ethanol at a 1:1.5 (v/v) ratio and kept for 15 min at −20 °C. After centrifugation at 12000*g* for 5 min, the pellet was dissolved in 10 mM TE buffer (pH 7.5), and the purity of DNA was determined using a spectrophotometer at 260/280 nm. Furthermore, the quality of isolated DNA was evaluated using 1% agarose gel electrophoresis in 0.04 M Tris−acetate, 0.001 M EDTA buffer (pH 7.5).

Determination of Radical-Mediated DNA Damage. Hydrogen peroxide mediated DNA oxidation was determined as described previously.[19] Briefly, 40 μL of DNA reaction mixture were prepared by adding predetermined concentrations of test sample (or the same volume of distilled water as control), 100 μM final concentration of $FeSO_4$, 0.1 mM final concentration of H_2O_2, and 5 μL of isolated genomic DNA in the same order. Then the mixture was incubated at room temperature for 10 min, and the reaction was terminated by adding 10 mM final concentration of EDTA. An aliquot (20 μL) of the reaction mixture containing about 1 μg of DNA was electrophoresed on a 1% agarose gel for 40 min at 100 V. Gels were then stained with 1 mg/mL ethidium bromide and visualized under UV light using AlphaEase gel image analysis software (Alpha Innotech, San Leandro, CA, USA).

RNA Extraction and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was extracted from BV2 cells in the presence or absence of fucoxanthin using TRIzol reagent (Invitrogen, Paisley, UK), as reported in the manufacturer's manual. An equal amount of RNA (2 μg) was used for each cDNA synthesis reaction using a reverse transcription system (Promega). Single-stranded cDNA was amplified by PCR with specific primers. The following PCR conditions were applied for all amplifications: 27 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. The resulting cDNAs were separated by electrophoresis on 1% agarose gel for 15 min at 100 V, followed visualization under UV light after ethidium bromide staining. Band intensities were quantified with Multi gauge software (Fujifilm Life Science, Tokyo, Japan), and bands of specific genes were normalized using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference.

Western Blot Analysis. Standard procedures were used for Western blotting, where BV2 cells were lysed in RIPA buffer (Invitrogen). Cell debris was removed by centrifugation followed by quick freezing of the supernatants. The protein concentrations were determined according to the bicinchoninic acid (BCA) assay. Cell lysates were separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. The membranes were blocked with 5% bovine serum albumin (BSA) and incubated with different antibodies, which were used to detect respective proteins using a chemiluminescent ECL assay kit, according to the manufacturer's instructions. Western blots were visualized using a LAS3000 luminescent image analyzer (Fujifilm Life Science), and protein expression was quantified by Multi gauge V3.0 software (Fujifilm Life Science).

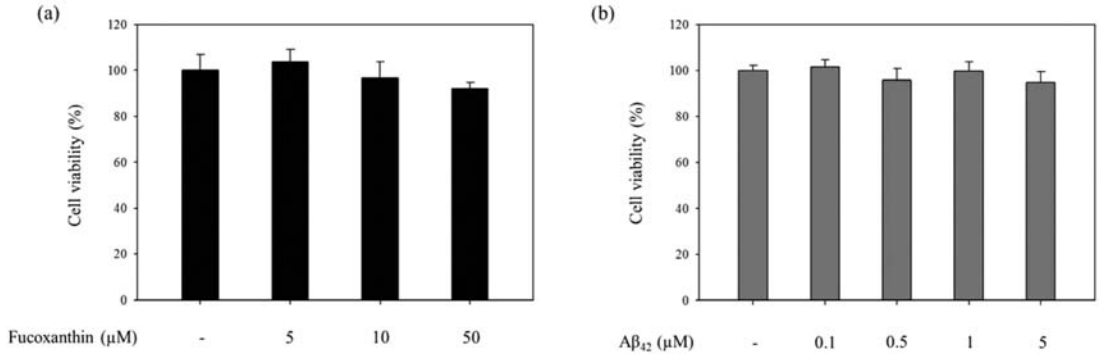

Figure 1. Cell viability values of BV2 and PC12 cells. The cytotoxicity of fucoxanthin in BV2 cells was assessed by MTT assay (a). The viability of BV2 cells following treatment with different concentration of Aβ_{42} for 24 and 48 h was assessed by MTT assay (b). Values correspond to means of three independent experiments and are shown as a percentage of viable cells compared with the viability of untreated cells.

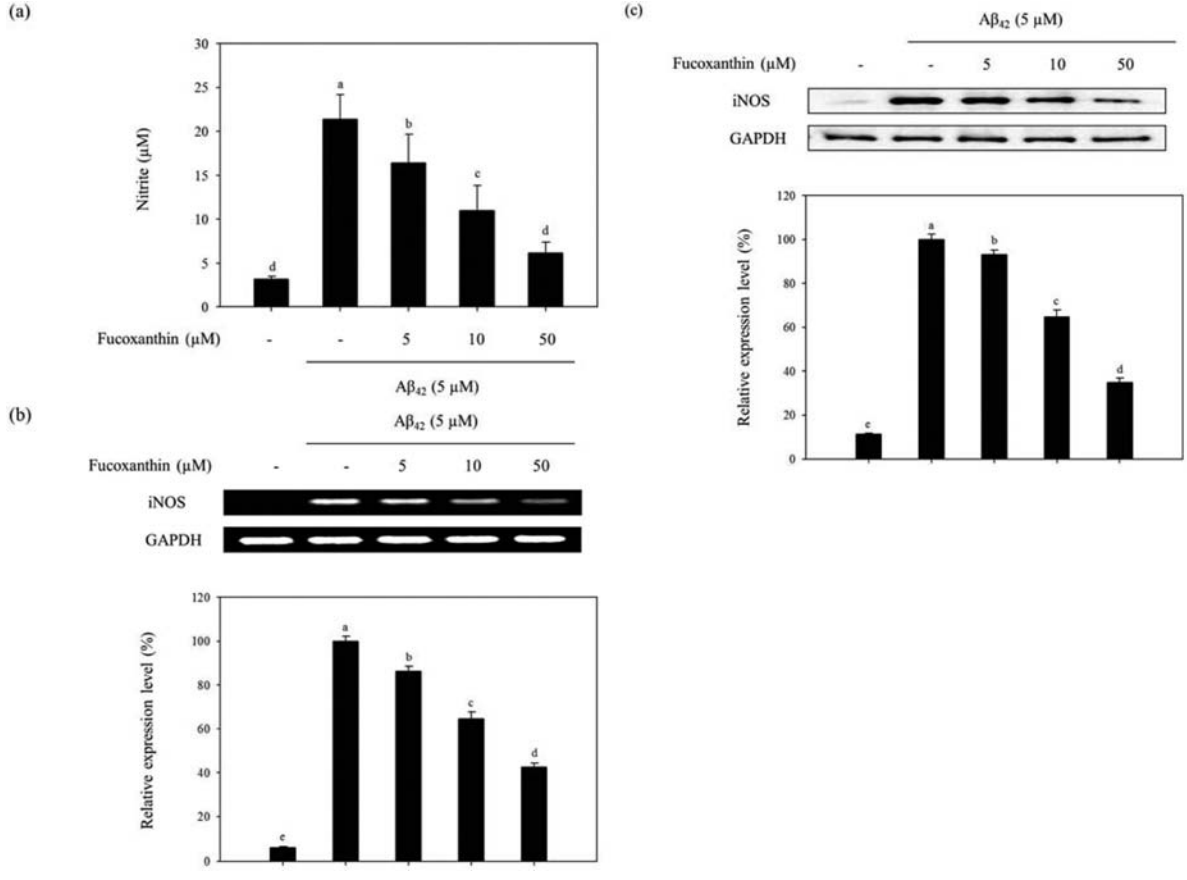

Figure 2. Inhibitory effects of fucoxanthin on NO production and iNOS expression in BV2 cells. Following fucoxanthin pretreatment for 4 h, BV2 cells were stimulated with Aβ_{42} (5 μM) and incubated for 24 h. The NO amounts were determined using Griess reagent (a). In parallel experiments, the expressions of iNOS mRNA were measured by RT-PCR (b); and the expressions of iNOS protein were measured by Western blot analysis (c). GAPDH was used as an internal control for PCR and Western blot analysis. Values correspond to the mean ± SD of three independent experiments. Different letters a−e indicate significant difference ($p < 0.05$) by Duncan's multiple-range test.

Statistical Analysis. The data were presented as the mean ± SD ($n = 3$). Differences between the means of the individual groups were assessed by one-way ANOVA with Duncan's multiple-range tests. Differences were considered to be significant at $p < 0.05$. The statistical software package SPSS v.16 (SPSS Inc., Chicago, IL, USA) was used for the analysis.

RESULTS

Effect of Fucoxanthin on Cell Proliferations. Cellular compatibility of fucoxanthin in BV2 cells was tested at three different concentrations (5, 10, and 50 μM) using the MTT cell viability assay. As shown in Figure 1a, fucoxanthin did not show any toxic effect to BV2 cells at the tested concentrations ($p < 0.05$). These data indicated that fucoxanthin could be used safely in further experiments as it does not have a negative effect on BV2 cell proliferation. Furthermore, because we will use Aβ_{42} to induce microglial inflammatory response, it is necessary to ascertain the nontoxic concentration of Aβ_{42} in BV2 cells. Treatments of Aβ_{42} up to the concentration of 5 μM did not exert any toxic effect in BV2 cells ($p < 0.05$) (Figure 1b). Therefore,

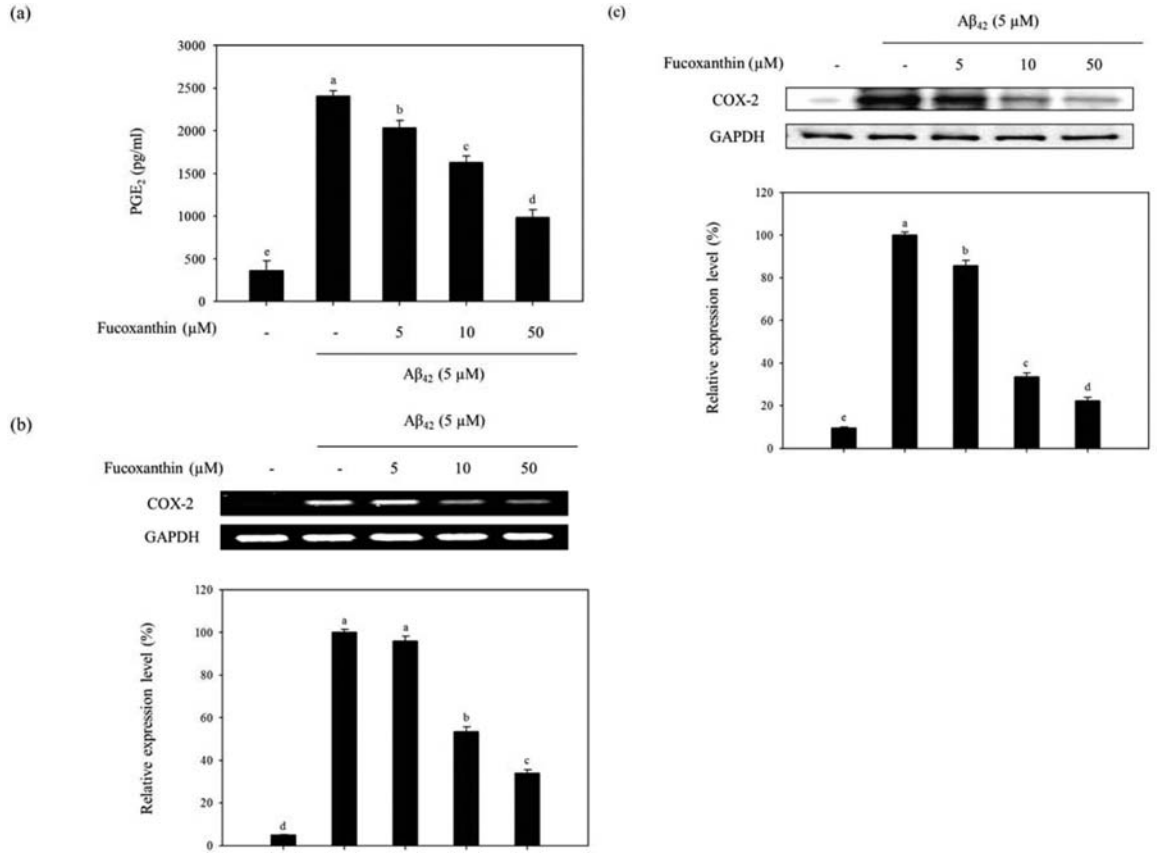

Figure 3. Inhibitory effects of fucoxanthin on PGE_2 production and COX-2 expression in BV2 cells. Following fucoxanthin pretreatment for 4 h, BV2 cells were stimulated with Aβ_{42} (5 μM) and incubated for 24 h. The PGE_2 amounts were determined using ELISA (a). In parallel experiments, the expressions of COX-2 mRNA were measured by RT-PCR (b) and the expressions of COX-2 protein were measured by Western blot analysis (c). GAPDH was used as an internal control for PCR and Western blot analysis. Values correspond to the mean ± SD of three independent experiments. Different letters a−e indicate significant difference ($p < 0.05$) by Duncan's multiple-range test.

Table 1. Effects of Fucoxanthin on the Pro-inflammatory Cytokine Production in Aβ_{42}-Stimulated BV2 Cells[a]

	pro-inflammatory cytokine concentration (pg/mL)		
	TNF-α	IL-6	IL-1β
blank	52.56 ± 6.45e	128.83 ± 21.92e	105.38 ± 10.96 d
control	1257.44 ± 86a	1719.17 ± 59.16a	482.13 ± 21.74a
fucoxanthin, 5 μM	1020.19 ± 45.52b	1386 ± 75.9b	396.05 ± 8.77b
fucoxanthin, 10 μM	710.12 ± 6.72c	914.5 ± 69.06c	359.69 ± 15.82b
fucoxanthin, 50 μM	384.69 ± 30.14d	401.67 ± 50.91d	305.19 ± 212.99c

[a]Values correspond to the mean ± SD from three independent experiments. Different letters a−e indicate significant difference ($p < 0.05$) by Duncan's multiple-range test. Control, + Aβ42 − fucoxanthin; blank, − Aβ42 − fucoxanthin.

5 μM of Aβ_{42} as a nontoxic concentration was selected for further analysis.

Effect of Fucoxanthin on the Production of Nitric Oxide and Prostaglandin-E_2 Production. NO is a gaseous free radical, which is generated through conversion of L-arginine to L-citruline by enzymes of the nitric oxide synthase (NOS). To determine the effect of fucoxanthin on NO production levels, BV2 cells were pretreated with various concentrations of fucoxanthin for 4 h and then stimulated with Aβ_{42} for another 24 h. The cell culture medium was then harvested to determine the nitrite content by Griess reaction. Results showed that Aβ_{42} increased the production of NO up to 5-fold compared with untreated cells (Figure 2a). Furthermore, inducible NOS (iNOS) gene and protein levels were detected using PCR and Western blot analyses. As shown in Figure 2b,c, fucoxanthin effectively inhibited Aβ_{42}-induced iNOS expression in a concentration-dependent manner.

In the next step, to investigate the inhibitory effect of fucoxanthin on microglial activation, PGE_2 levels were measured using PGE_2-ELISA. Incubation with Aβ_{42} (5 μM) for 24 h significantly increased the production of PGE_2 compared to nonstimulated cells ($p < 0.05$) (Figure 3a). Treatment with fucoxanthin (5, 10, and 50 μM) 4 h before the addition of Aβ_{42} attenuated PGE_2 production with a minimum effective inhibition by 5 μM concentration (2034.2 ± 89.09 pg/mL). Additionally, preincubation of BV2 cells with fucoxanthin down-regulated cyclooxygenase-2 (COX-2) expression (Figure 3b,c). These results indicated that fucoxanthin suppressed Aβ_{42}-induced PGE_2 production through down-regulation of COX-2 gene and protein expressions.

dx.doi.org/10.1021/jf400015k | J. Agric. Food Chem. 2013, 61, 3876−3883

Effect of Fucoxanthin on the Production of Pro-inflammatory Cytokines. It has been shown previously that exposure of microglia to Aβ_{42} resulted in the production and secretion of various pro-inflammatory cytokines, including TNF-α, IL-6, and IL-1β.[14] In this study, we tested whether fucoxanthin could reduce the generation of pro-inflammatory cytokines elicited by Aβ_{42} stimulation. Fucoxanthin treatment dramatically inhibited pro-inflammatory cytokine production (Table 1). We further analyzed pro-inflammatory cytokine mRNA expressions induced by PCR analysis. As shown in Figure 4, fucoxanthin treatments remarkably down-regulated the gene expression of TNF-α, IL-6, and IL-1β.

Effect of Fucoxanthin on the Phosphorylation of MAPK. The mitogen-activated protein kinases (MAPK) signaling pathway has been implicated to play key regulatory roles in the production of pro-inflammatory mediators. Hence, in an attempt to determine whether the inhibition of pro-inflammatory mediators by fucoxanthin is mediated through the MAPK signaling pathway, we examined the effect of fucoxanthin in Aβ_{42}-induced phosphorylation of MAPK. Following stimulation with Aβ_{42} for 1 h, the phosphorylation of c-Jun N-terminal kinase (JNK), extracellular signal-regulated kinase (ERK), and p38 MAPK was dramatically increased. However, preincubation with fucoxanthin down-regulated the phosphorylation of JNK, ERK, and p38 MAPK (Figure 5), suggesting that phosphorylation of MAPK is required for the up-regulation of pro-inflammatory mediators in Aβ_{42}-stimulated BV2 cells.

Protective Effects of Fucoxanthin on Cellular DNA Oxidation. Free radicals can react with different components of DNA to produce a plethora of DNA lesions. These free radicals can modify DNA bases, induce inter- and intrastrand cross-links, promote DNA−protein cross-links, and create strand break.[20] In this study, fucoxanthin was analyzed for its protective effects on free radical-induced DNA oxidation using genomic DNA isolated from BV2 cells. The isolated DNA was subjected to oxidation by H_2O_2 generated via Fenton reaction. After 30 min of reaction, almost all DNA was degraded in the control group treated only with H_2O_2. Notably, a clear dose-dependent DNA oxidation inhibition effect could be observed when fucoxanthin was added into the reaction mixture (Figure 6). At 50 μM concentration of fucoxanthin, DNA damage was inhibited around 85%.

Intracellular ROS Scavenging Effect of Fucoxanthin. The intracellular ROS scavenging effect of fucoxanthin in BV2 cells was assessed using a fluorescence sensitive dye, DCFH-DA. As shown in Figure 7, progressive increments in DCF fluorescence, which indicated oxidation of DCFH-DA by intracellular radicals, were observed with incubation time up to 180 min. Fucoxanthin significantly reduced DCF fluorescence intensity, resulting in increased scavenging activity against intracellular ROS in a concentration-dependent manner ($p < 0.05$). The presence of fucoxanthin at concentrations of 10 and 50 μM led to a remarkable reduction in fluorescent intensity, resulting in fluorescent intensity almost similar to that of the blank group. These results confirmed that fucoxanthin could exert a substantial effect against intracellular ROS formation.

Antioxidant Enzymes. The antioxidant property of fucoxanthin in BV2 cells was determined through the gene expression levels of superoxide dismutase (SOD) and glutathione (GSH). To determine how fucoxanthin affects gene expression levels of antioxidative enzymes, BV2 cells were treated with fucoxanthin and then stimulated with 50 μM H_2O_2. The gene expression levels of antioxidative enzymes were determined by PCR analysis. The expression levels of antioxidant enzymes were up-regulated in the presence of fucoxanthin dose-dependently (Figure 8).

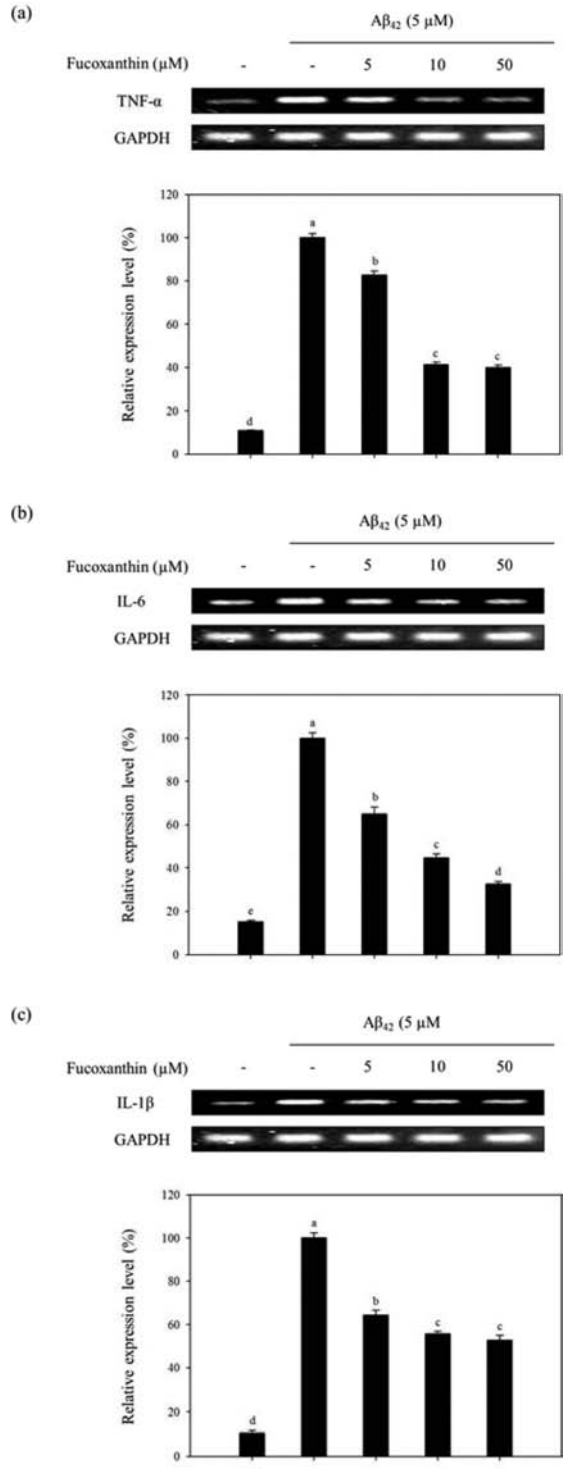

Figure 4. Inhibitory effects of fucoxanthin on TNF-α, IL-6, and IL-1β production and expression in BV2 cells. Following fucoxanthin pretreatment for 4 h, BV2 cells were stimulated with Aβ_{42} (5 μM) and incubated for 24 h. The expressions of TNF-α (a), IL-6 (b), and IL-1β (c) mRNA were measured by PCR analysis. GAPDH was used as an internal control for PCR. Values correspond to the mean ± SD of three independent experiments. Different letters a−e in each sample indicate significant difference ($p < 0.05$) by Duncan's multiple-range test.

dx.doi.org/10.1021/jf400015k | J. Agric. Food Chem. 2013, 61, 3876−3883

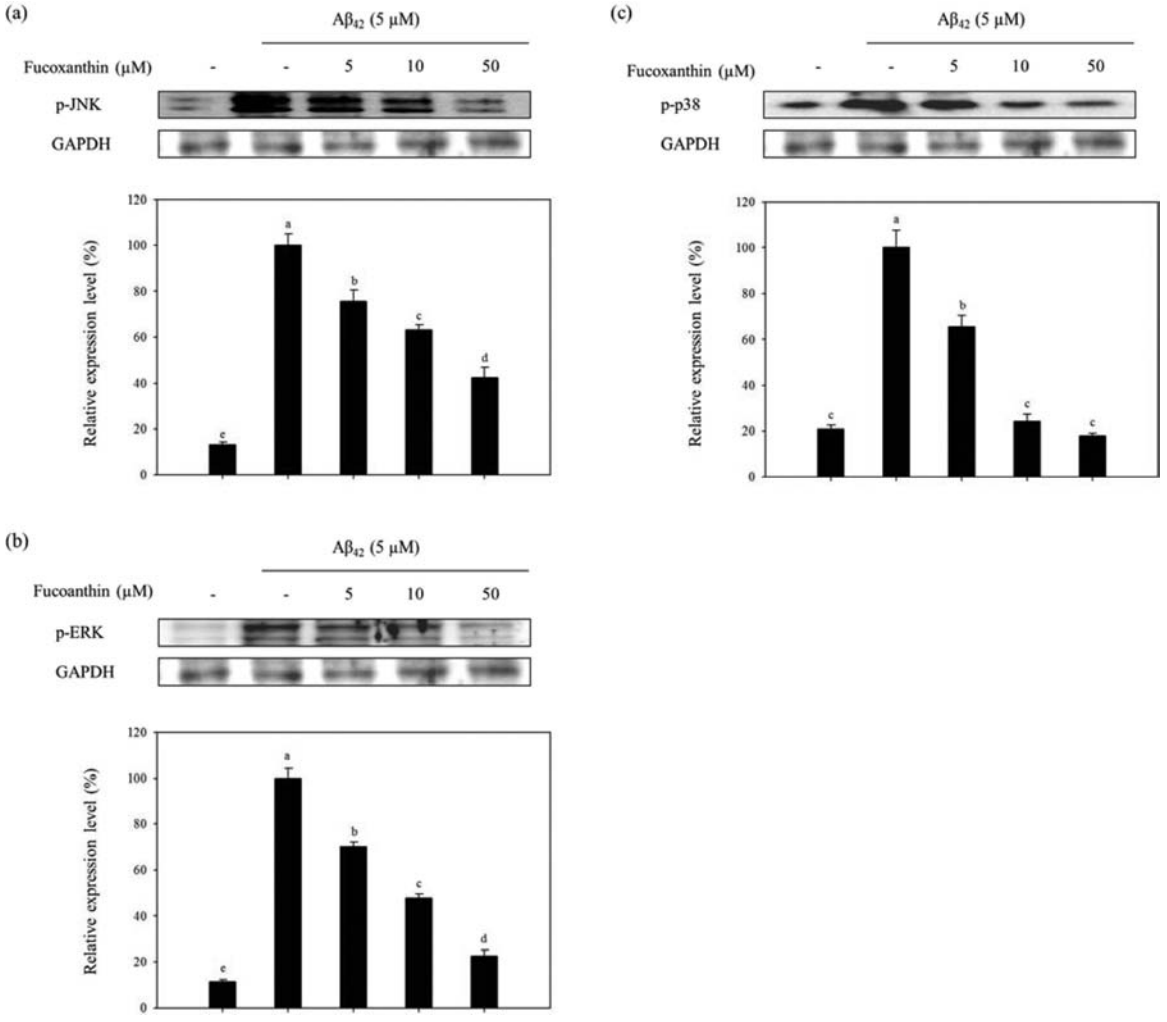

Figure 5. Inhibitory effects of fucoxanthin on phosphorylation of MAPK in BV2 cells. BV2 cells were preincubated with fucoxanthin for 4 h and then stimulated with Aβ_{42} (5 μM). The expression levels of p-JNK (a), p-ERK (b), and p-p38 (c) were measured by Western blot analysis. GAPDH was used as an internal control. Values correspond to the mean ± SD of three independent experiments. Different letters a–e indicate significant difference ($p < 0.05$) by Duncan's multiple-range test.

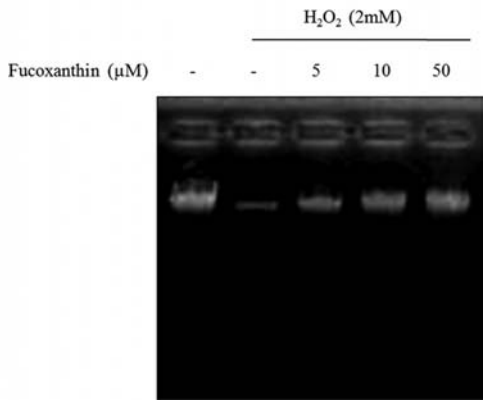

Figure 6. DNA oxidative protection by fucoxanthin in BV2 cells. Similar results were obtained in three independent experiments of identical treatments.

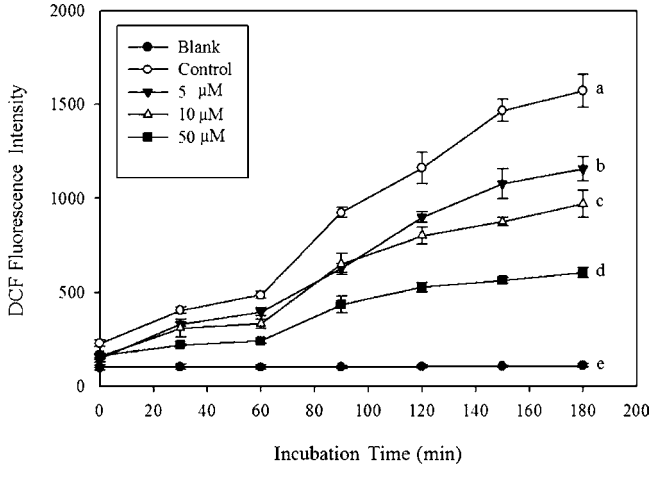

Figure 7. Radical scavenging effect of fucoxanthin in BV2 cells. Values correspond to the mean ± SD from three independent experiments. Different letters a–e indicate significant difference ($p < 0.05$) by Duncan's multiple-range test.

DISCUSSION

Neurodegenerative diseases are a group of progressive neurological disorders that damage or destroy the function of neurons. More than 10 million people suffer from neurodegenerative diseases worldwide annually. Although multiple factors are involved in the development of neurodegenerative diseases, dysregulation in the inflammatory network and oxidative imbalance are key components in the pathogenesis of diseases

dx.doi.org/10.1021/jf400015k | J. Agric. Food Chem. 2013, 61, 3876–3883

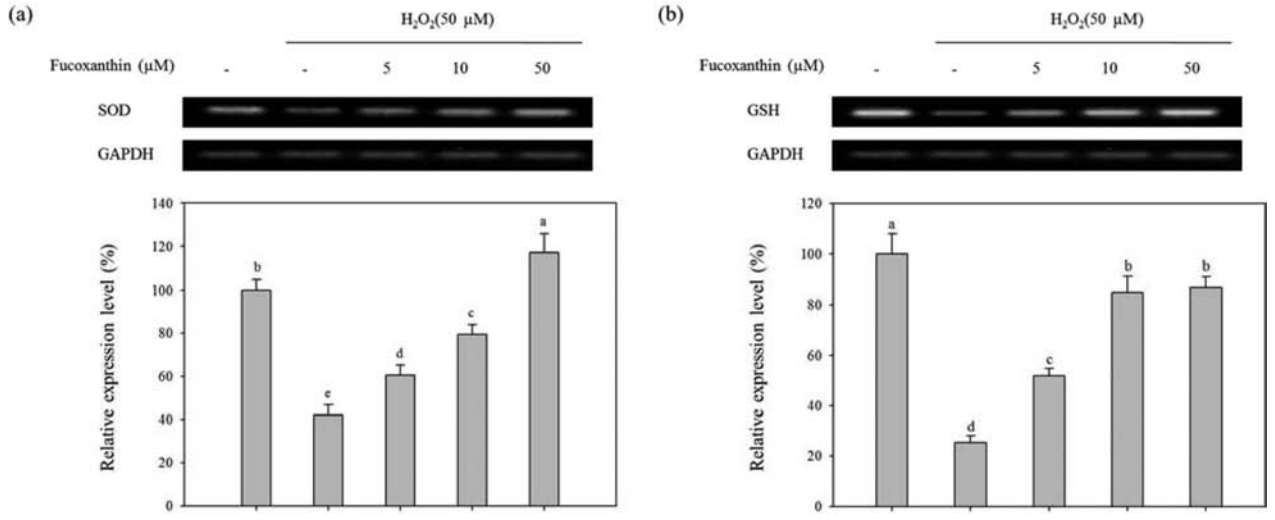

Figure 8. Effect of fucoxanthin on m-RNA expression of antioxidative enzymes in BV2 cells. BV2 cells were preincubated with fucoxanthin for 4 h and then stimulated for 24 h with H_2O_2 (50 μM). The expression levels of SOD (a) and GSH (b) mRNA were determined using RT-PCR analysis. GAPDH was used as an internal control. Values correspond to the mean ± SD of three independent experiments. Different letters a−d indicate significant difference ($p < 0.05$) by Duncan's multiple-range test.

such as AD.[21] In this study, we report that fucoxanthin suppresses indices of inflammation and oxidative damage in the BV2 cells, factors that have been implicated in AD pathogenesis. To our best knowledge, this is the first study that showed protective effects of fucoxanthin in microglial cells and identified the molecular mechanism through which fucoxanthin modulates inflammatory and oxidative responses.

In the case of pro-inflammatory mediators, there is extensive evidence to support the direct involvement of pro-inflammatory mediators in the neuronal injury that occurs in both acute and chronic neurodegenerative diseases.[8] As an example, high levels of NO that cause oxidative/nitrosative stress to neuronal cells can in turn induce apoptosis by diverse mechanisms, including induction of p53, activation of mitochondrial permeability transition to cause cytochrome *c* release, and activation of the p38 or other MAPK pathways.[22] Glial secretion of PGE_2 may impair phagocytotic clearance of Aβ by binding to the microglia EP2 receptor and enhancing microglial toxicity.[7] Pro-inflammatory cytokine TNF-α was found to potentiate glutamate neurotoxicity. TNF-α and glutamate act synergistically to induce neuronal cell death in brain slice models.[23] Fucoxanthin showed a potent anti-inflammatory activity, and part of its anti-inflammatory activity is based on the inhibition of classical nuclear factor-κB (NF-κB) and MAPK signaling pathways.[24] MAPK have been reported to play significant roles in AD progression, where Aβ_{42} activates three types of MAPK including JNK, ERK, and p38 MAPK.[25] Thus, the inhibition of MAPK may partially elucidate anti-inflammatory mechanisms of fucoxanthin, and activation of MAPK may represent the initial step in feed-forward inflammatory response in the CNS.

There is extensive literature supporting the role of oxidative damage in AD.[26] Interestingly, we found that fucoxanthin was able to protect DNA from oxidation and reduced the production of intracellular ROS. This result was in agreement with a previous finding that fucoxanthin is able to protect DNA from oxidation; this protective effect could have resulted from fucoxanthin-mediated scavenging of ROS (exogenous activity).[27] The properties underlying the scavenging activities of fucoxanthin toward free radicals relate particularly to their abilities to donate electrons or hydrogen atoms due to the presence of two hydroxyl groups in the ring structure.[28]

From the cellular view, the mechanism of ROS scavenging by fucoxanthin was understood by studying the enzymes related to ROS production. Fucoxanthin positively regulated the expression levels of antioxidative enzymes, which suggests that fucoxanthin also induces the activation of the endogenous antioxidant system and thereby protects the cells effectively against DNA oxidative damage. Cellular antioxidants are known to change their redox state and can be targeted for destruction, regulate oxidative processes involved in signal transduction, and affect gene expression and pathways of cell proliferation and death.[8] A complex natural antioxidant system exists in the biological systems, which is responsible for the prevention of damage by pro-oxidants. SOD is a metalloenzyme that removes oxygen radicals and protects against oxidative injury.[29] SOD activity can protect the brain against oxidative stress produced by oxygen radicals and represents a first line of defense against oxidative damage. Meanwhile, GSH is the major intracellular thiol and an exellent ROS scavenger.[30] An impaired endogenous antioxidant system favors accumulation of free radicals, which not only induces the process of lipid peroxidation but also plays a central role in neurodegeneration.

In summary, we have demonstrated for the first time that fucoxanthin strongly attenuated Aβ_{42}-induced microglial activation and reduced oxidative damage in BV2 cells. Fucoxanthin anti-inflammatory effects take place via attenuation of the MAPK signaling pathway. Hence, fucoxanthin can be considered as a potential anti-inflammatory and antioxidant material; however, further studies should be performed to identify its effects in animal models of neurodegenerative diseases.

AUTHOR INFORMATION

Corresponding Author

*Phone: +82-51-629-7094. Fax: +82-51-629-7099. E-mail: sknkim@pknu.ac.kr.

Funding

This research was supported by a grant from the Marine Bioprocess Research Center of the Marine Biotechnology

Program funded by the Ministry of Land, Transport and Maritime, Republic of Korea.

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

AD, Alzheimer's disease; Aβ, amyloid-β; Aβ_{42}, amyloid-β_{42}; BCA, bicinchoninic acid; CNS, central nervous system; COX-2, cyclooxygenase-2; DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; EIA, enzyme immunometric assay; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; GSH, glutathione; HBSS, Hank's balanced salt solution; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1β; IL-6, interleukin-6; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; NOS, nitric oxide synthase; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; RT-PCR, reverse transcription polymerase chain reaction; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α

■ REFERENCES

(1) Wyss-Coray, T. Inflammation in Alzheimer disease: driving force, bystander or beneficial response? *Nat. Med.* **2006**, *12*, 1005−1015.

(2) Block, M. L.; Zecca, L.; Hong, J.-S. Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat. Rev. Neurosci.* **2007**, *8*, 57−69.

(3) Lull, M. E.; Block, M. L. Microglial activation and chronic neurodegeneration. *Neurotherapeutics* **2010**, *7*, 354−365.

(4) Ransohoff, R. M.; Perry, V. H. Microglial physiology: unique stimuli, specialized responses. *Annu. Rev. Immunol.* **2009**, *27*, 119−145.

(5) Gao, H. M.; Hong, J. S. Why neurodegenerative diseases are progressive: uncontrolled inflammation drives disease progression. *Trends Immunol.* **2008**, *29*, 357−365.

(6) McGeer, P.; McGeer, E. Inflammation and the degenerative diseases of aging. *Ann. N.Y. Acad. Sci.* **2004**, *1035*, 104−116.

(7) Heneka, M. T.; O'Banion, M. K.; Terwel, D.; Kummer, M. P. Neuroinflammatory processes in Alzheimer's disease. *J. Neural Transm.* **2010**, *117*, 919−947.

(8) Butterfield, D. A.; Perluigi, M.; Sultana, R. Oxidative stress in Alzheimer's disease brain: new insights from redox proteomics. *Eur. J. Pharmacol.* **2006**, *545*, 39−50.

(9) Frank, B.; Gupta, S. A review of antioxidants and Alzheimer's disease. *Ann. Clin. Psychiatry* **2005**, *17*, 269−286.

(10) Barnham, K. J.; Masters, C. L.; Bush, A. I. Neurodegenerative diseases and oxidative stress. *Nat. Rev. Drug Discov.* **2004**, *3*, 205−214.

(11) Behl, C. Oxidative stress in Alzheimer's disease: implications for prevention and therapy. *Alzheimer's Dis.* **2005**, *38*, 65−78.

(12) Ioannou, E.; Roussis, V. Natural products from seaweeds. In *Plant-Derived Natural Products−Synthesis, Function, and Application*; Springer: Dordrecht, The Netherlands, 2009; pp 51−81.

(13) Heo, S. J.; Yoon, W. J.; Kim, K. N.; Ahn, G. N.; Kang, S. M.; Kang, D. H.; Oh, C.; Jung, W. K.; Jeon, Y. J. Evaluation of anti-inflammatory effect of fucoxanthin isolated from brown algae in lipopolysaccharide-stimulated RAW 264.7 macrophages. *Food Chem. Toxicol.* **2010**, *48*, 2045−2051.

(14) Pangestuti, R.; Kim, S. K. Biological activities and health benefit effects of natural pigments derived from marine algae. *J. Funct. Foods* **2011**, *3*, 255−266.

(15) Maeda, H.; Hosokawa, M.; Sashima, T.; Murakami-Funayama, K.; Miyashita, K. Anti-obesity and anti-diabetic effects of fucoxanthin on diet-induced obesity conditions in a murine model. *Mol. Med. Rep.* **2009**, *2* (6), 897.

(16) Sachindra, N. M.; Sato, E.; Maeda, H.; Hosokawa, M.; Niwano, Y.; Kohno, M.; Miyashita, K. Radical scavenging and singlet oxygen quenching activity of marine carotenoid fucoxanthin and its metabolites. *J. Agric. Food Chem.* **2007**, *55*, 8516−8522.

(17) Ikeda, K.; Kitamura, A.; Machida, H.; Watanabe, M.; Negishi, H.; Hiraoka, J.; Nakano, T. Effect of *Undaria pinnatifida* (Wakame) on the development of cerebrovascular diseases in stroke-prone spontaneously hypertensive rats. *Clin. Exp. Pharmacol. Physiol.* **2003**, *30*, 44−48.

(18) Resende, R.; Ferreiro, E.; Pereira, C.; Resende de Oliveira, C. Neurotoxic effect of oligomeric and fibrillar species of amyloid-β peptide 1−42: involvement of endoplasmic reticulum calcium release in oligomer-induced cell death. *Neuroscience* **2008**, *155* (3), 725−737.

(19) Milne, L.; Nicotera, P.; Orrenius, S.; Burkitt, M. Effects of glutathione and chelating agents on copper-mediated DNA oxidation: pro-oxidant and antioxidant properties of glutathione. *Arch. Biochem. Biophys.* **1993**, *304*, 102−109.

(20) Jena, N. DNA damage by reactive species: mechanisms, mutation and repair. *J. Biosci.* **2012**, *37*, 503−517.

(21) Lim, G. P.; Chu, T.; Yang, F.; Beech, W.; Frautschy, S. A.; Cole, G. M. The curry spice curcumin reduces oxidative damage and amyloid pathology in an Alzheimer transgenic mouse. *J. Neurosci.* **2001**, *21*, 8370−8377.

(22) Brown, G. C. Nitric oxide and neuronal death. *Nitric Oxide* **2010**, *23*, 153−165.

(23) Tobinick, E.; Gross, H.; Weinberger, A.; Cohen, H. TNF-α modulation for treatment of Alzheimer's disease: a 6-month pilot study. *Med. Gen. Med.* **2006**, *8*, 25.

(24) Zhu, X.; Lee, H.-G.; Raina, A. K.; Perry, G.; Smith, M. A. The role of mitogen-activated protein kinase pathways in Alzheimer's disease. *Neurosignals* **2002**, *11*, 270−281.

(25) Kim, E. K.; Choi, E. J. Pathological roles of MAPK signaling pathways in human diseases. *Biochim. Biophys. Acta: Mol. Basis Dis.* **2010**, *1802*, 396−405.

(26) Roy, A.; Jana, A.; Yatish, K.; Freidt, M. B.; Fung, Y. K.; Martinson, J. A.; Pahan, K. Reactive oxygen species up-regulate CD11b in microglia via nitric oxide: Implications for neurodegenerative diseases. *Free Radical Biol. Med.* **2008**, *45*, 686−699.

(27) Heo, S. J.; Jeon, Y. J. Protective effect of fucoxanthin isolated from *Sargassum siliquastrum* on UV-B induced cell damage. *J. Photochem. Photobiol. B: Biol.* **2009**, *95*, 101−107.

(28) Pangestuti, R.; Kim, S. K. Neuroprotective effects of marine algae. *Mar. Drugs* **2011**, *9*, 803−818.

(29) Garrel, C.; Fowler, P. A.; Al-Gubory, K. H. Developmental changes in antioxidant enzymatic defences against oxidative stress in sheep placentomes. *J. Endocrinol.* **2010**, *205*, 107−116.

(30) Gabbita, S. P.; Lovell, M. A.; Markesbery, W. R. Increased nuclear DNA oxidation in the brain in Alzheimer's disease. *J. Neurochem.* **1998**, *71*, 2034−2040.