Fucoxanthin, a Marine Carotenoid, Attenuates β-Amyloid Oligomer-Induced Neurotoxicity Possibly via Regulating the PI3K/Akt and the ERK Pathways in SH-SY5Y Cells

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1. Introduction

Alzheimer’s disease (AD), one of the most common neurodegenerative disorders affecting aging populations, is characterized by neurofibrillary tangles, synaptic impairments, and loss of neurons. Oligomers of β-amyloid (Aβ) are widely accepted as the main neurotoxins to induce oxidative stress and neuronal loss in AD. In this study, we discovered that fucoxanthin, a marine carotenoid with antioxidative stress properties, concentration dependently prevented Aβ oligomer-induced increase of neuronal apoptosis and intracellular reactive oxygen species in SH-SY5Y cells. Aβ oligomers inhibited the prosurvival phosphoinositide 3-kinase (PI3K)/Akt cascade and activated the proapoptotic extracellular signal-regulated kinase (ERK) pathway. Moreover, inhibitors of glycogen synthase kinase 3β (GSK3β) and mitogen-activated protein kinase (MEK) synergistically prevented Aβ oligomer-induced neuronal death, suggesting that the PI3K/Akt and ERK pathways might be involved in Aβ oligomer-induced neurotoxicity. Pretreatment with fucoxanthin significantly prevented Aβ oligomer-induced alteration of the PI3K/Akt and ERK pathways. Furthermore, LY294002 and wortmannin, two PI3K inhibitors, abolished the neuroprotective effects of fucoxanthin against Aβ oligomer-induced neurotoxicity. These results suggested that fucoxanthin might prevent Aβ oligomer-induced neuronal loss and oxidative stress via the activation of the PI3K/Akt cascade as well as inhibition of the ERK pathway, indicating that further studies of fucoxanthin and related compounds might lead to a useful treatment of AD.
pathway, leading to neuronal apoptosis [5]. Therefore, molecules which could concurrently regulate oxidative stress and signaling pathways might produce neuroprotective effects against Aβ oligomers.

Fucoxanthin, a marine carotenoid mainly extracted from edible brown seaweeds, was reported to possess beneficial biological effects, including antioxidative stress and anti-inflammation activities [7, 8]. We have previously reported that fucoxanthin can inhibit acetylcholinesterase in vitro and attenuate scopolamine-induced cognitive impairments in mice, therefore suggesting that fucoxanthin might be useful against Aβ oligomer-induced neurotoxicity. Aβ monomer-induced cell death in microglia and cortical neurons [10, 11]. Moreover, fucoxanthin was reported to inhibit Aβ precursor protein-cleaving enzyme 1 (BACE-1), an enzyme that cleaves the Aβ precursor protein into Aβ monomers [12]. Taken together, these reports suggest that fucoxanthin might inhibit Aβ-mediated neurotoxicity. However, it remains unknown whether fucoxanthin can prevent Aβ oligomer-induced neurotoxicity, and moreover, how fucoxanthin is able to produce neuroprotective effects in vitro.

In this study, we have shown for the first time that fucoxanthin significantly attenuates Aβ oligomer-induced neuronal apoptosis as well as the increase of intracellular ROS in SH-SY5Y cells. We have also demonstrated that fucoxanthin concurrently produced neuroprotective effects possibly via regulating prosurvival PI3K/Akt and proapoptotic ERK pathways.

2. Materials and Methods

2.1. Chemicals and Reagents. Fucoxanthin was purified from Sargassum hornoeri according to our published procedures [9]. Briefly, a fucoxanthin-rich solution was first obtained by extraction with ethanol (ethanol-to-sample ratio 1:4) at 30°C for 2 h. The solution was concentrated at 25°C. Wastes including lipid and chlorophylls were precipitated when the ethanol reached approximately 63%. Fucoxanthin was obtained by precipitation when the ethanol concentration reached near 40%. The purity of fucoxanthin was over 90% as examined by high-performance liquid chromatography.

Aβ1-42 peptide was purchased from GL Biochem (Shanghai, China). 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) was obtained from Sigma Chemicals (St. Louis, MO, USA). Soluble Aβ oligomers were prepared as previously described [13, 14]. Briefly, Aβ1-42 peptide was dissolved in HFIP to form Aβ monomers. After thoroughly vortexing, 1 mM Aβ monomer solution was aliquoted in 100 μl stock, and stored at −20°C. Milli-Q water (900 μl) was added to 100 μl Aβ1-42 solution before the experiments. Aβ solution was further spin-vacuumed and incubated at room temperature for 20 min. HFIP was completely evaporated to obtain the solution of 50 μM Aβ. The Aβ solution was kept at room temperature under constant stirring for 48 h and centrifuged at 14000g for 15 min at 4°C. The supernatant (about 900 μl) which contained mainly soluble Aβ oligomers was collected.

SB415286 was obtained from Sigma Chemicals. U0126, wortmannin, and LY294002 were received from LC Laboratories (Woburn, MA, USA). Antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA).

2.2. SH-SY5Y Cells Culture. SH-SY5Y cells were maintained in high glucose modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and penicillin (100 U/ml)/streptomycin (100 μg/ml) at 37°C with 5% CO2. The medium was refreshed every two days. Before experiments, SH-SY5Y cells were seeded in DMEM with 1% fetal bovine serum for 24 h.

2.3. Cell Viability Measurements. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay based on our previous protocol [15, 16]. Briefly, 10 μl MTT (5 mg/ml) was added to each well in 96-well (100 μl medium/well) plates. Then, plates were incubated at 37°C for 4 h, and 100 μl solvate (0.01 N HCl in 10% SDS) was added. After 16–20 h, the absorbance of samples was measured at a wavelength of 570 nm with 655 nm as the reference wavelength.

2.4. Fluorescein Diacetate (FDA)/Propidium Iodide (PI) Double Staining. FDA/PI double staining was performed according to our previous publication [17]. Viable cells were visualized by the fluorescein formed from FDA by esterase activity in the cytoplasm. Nonviable cells were visualized by PI, which only penetrates the membranes of dead cells. Cells were examined after incubation with 10 μg/ml FDA and 5 μg/ml PI at 37°C for 15 min. Images were obtained by UV light microscopy and compared with those taken under a phase-contrast microscopy (Nikon, Tokyo, Japan). To quantitatively evaluate cell viability, images of each well were taken from five randomly selected fields, and the number of FDA-positive and PI-positive cells was counted. The percentage of cell viability was analyzed using the equation % of cell viability = [number of DFA-cell positive cells / (number of PI-positive cells + number of DFA-positive cells)] × 100%.

2.5. Hoechst Staining. Chromatin condensation was measured by staining the cell nuclei with Hoechst 33342 as previously described [18, 19]. Cells in 6-well (2 ml medium/well) plates were washed with ice-cold phosphate-buffered saline, fixed, and membrane permeabilized with 4% formaldehyde in 0.1% Triton X-100 for 15 min. Cells were then stained with Hoechst 33342 (5 μg/ml, Thermo Fisher Scientific, Shanghai, China) at 4°C for 5 min. Images were obtained by a fluorescence microscope at 100x magnification (Nikon). To determine the proportion of apoptotic nuclei in each group, images of each well were taken from five randomly selected fields, and the number of pyknotic nuclei and total nuclei was counted. The percentage of pyknotic nuclei was then analyzed using the equation % of pyknotic nuclei = number of pyknotic nuclei / number of total nuclei × 100%.

2.6. Intracellular ROS Measurements. The level of intracellular ROS was measured by 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-H2DCF-DA, Sigma) as reported in our previous publication [20]. Briefly, cells were washed once with ice-cold phosphate-buffered saline and incubated with 10 μM carboxy-H2DCF-DA at 37°C for 10 min. Cells were then washed once with ice-cold phosphate-buffered saline and scanned with a plate reader...
at 485 nm excitation and 520 nm emission. Images were acquired by a fluorescence microscope (Nikon). Unless otherwise indicated, the fluorescence intensity in SH-SY5Y cells without treatment is expressed as a percentage of the control.

2.7. Western Blot Analysis. Western blotting was performed using a well-established protocol [21]. Cell lysates were separated on SDS-polyacrylamide gels and transferred onto polyvinylidifluoride membranes (Pall Corporation, New York, USA). After membrane blocking, proteins were detected by primary antibodies. After incubation at 4°C overnight, signals were obtained after incubation with HRP-conjugated secondary antibodies. Subsequently, blots were developed using the enhanced chemiluminescence plus kit (Beyotime, Hangzhou, China) and signals were exposed.

2.8. Statistical Analysis. Results were expressed as mean ± SEM. Differences among groups were compared by analysis of variance (ANOVA) followed by Dunnett’s or Tukey’s test. p < 0.05 was considered as statistically significant.

3. Results

3.1. Fucoxanthin Effectively Attenuates Aβ Oligomer-Induced Neuronal Apoptosis in SH-SY5Y Cells. We first evaluated the neurotoxicity of Aβ oligomers in SH-SY5Y cells. It was demonstrated that 24 h treatment of Aβ oligomers at concentrations of 1–1.5 μM significantly induced neuronal death in SH-SY5Y cells (Figure 1(a)). Therefore, we used 1 μM Aβ oligomer to induce neurotoxicity in the following study.

To investigate the neuroprotective effects of fucoxanthin, SH-SY5Y cells were pretreated with 0.3–3 μM fucoxanthin for 2 h before adding Aβ oligomers. After 24 h, cell viability was analyzed. Fucoxanthin concentration-dependently attenuated Aβ oligomer-induced reduction of cell viability (Figure 1(b)). Moreover, treatment of 3 μM fucoxanthin alone for 26 h did not induce cell proliferation or cell death (data not shown).

As shown in Figure 2, Aβ oligomers could substantially increase the number of PI-positive nonviable cells and decrease the number of FDA-positive viable cells when compared to the control group. This neurotoxicity of Aβ oligomers was largely prevented by fucoxanthin (Figure 2). Moreover, Aβ oligomers significantly increased the percentage of pyknotic nuclei in SH-SY5Y cells, suggesting that Aβ oligomers caused neuronal loss mainly via inducing cell apoptosis (Figure 3). Furthermore, fucoxanthin significantly decreased Aβ oligomer-induced neuronal apoptosis, as demonstrated by the decrease in the percentage of pyknotic nuclei in the fucoxanthin plus Aβ oligomer group as compared to that in the Aβ oligomer group (Figure 3).

3.2. Fucoxanthin Effectively Attenuates Aβ Oligomer-Induced Increase of ROS in SH-SY5Y Cells. Aβ oligomers significantly increased the level of intracellular ROS in SH-SY5Y cells (Figure 4). This increase in ROS was significantly attenuated by treatment with 3.0 μM fucoxanthin. This finding provides additional support for the neuroprotective effects of fucoxanthin against Aβ oligomer-induced toxicity in SH-SY5Y cells (Figure 4).

3.3. Concurrent Activation of the PI3K/Akt Pathway and Inhibition of the ERK Pathway Attenuate Aβ Oligomer-Induced Neuronal Loss in SH-SY5Y Cells. We further investigated if signaling pathways were involved in Aβ oligomer-induced neuronal loss in our model. We first...
Figure 2: Fucoxanthin attenuates Aβ oligomer-induced neuronal loss as evidenced by FDA/PI double staining. SH-SY5Y cells were treated with 3 μM fucoxanthin. After 2 h, 1 μM Aβ oligomer was added. FDA/PI double staining was used to demonstrate FDA-positive viable cells and PI-positive nonviable cells at 24 h after the treatment of Aβ oligomers.

Figure 3: Fucoxanthin attenuates Aβ oligomer-induced neuronal apoptosis as evidenced by Hoechst staining. SH-SY5Y cells were treated with 3 μM fucoxanthin. After 2 h, 1 μM Aβ oligomer was added. Hoechst staining was used to measure the number of pyknotic nuclei with condensed chromatin at 24 h after the treatment of Aβ oligomers. Data were the mean±SEM of three separate experiments; **p < 0.01 versus the control group; ***p < 0.01 versus the Aβ oligomer group (ANOVA and Tukey’s test).
used Western blotting analysis to explore the alterations of signaling pathways induced by Aβ oligomers. Aβ oligomers were added to SH-SY5Y cells for various durations, and the cellular proteins were extracted. It was demonstrated that Aβ oligomers time-dependently decreased the expressions of pSer473-Akt and pSer9-GSK3β, suggesting that Aβ oligomers inhibited the PI3K/Akt pathway (Figures 5(a) and 5(b)). Furthermore, Aβ oligomers also increased the expression of pERK in a time-dependent manner in SH-SY5Y cells, indicating that Aβ oligomers might activate the ERK pathway in our model (Figure 5(c)).

The PI3K/Akt pathway is widely accepted as a prosurvival signaling pathway, while the ERK pathway is generally considered as a proapoptotic pathway [4, 5]. To explore if the regulation of these signaling pathways could attenuate Aβ oligomer-induced neuronal loss, two inhibitors were used. SB415286 is a GSK3β-specific inhibitor, while U0126 is a MEK-specific inhibitor. Both SB415286 and U0126 concentrations could dependently attenuated Aβ oligomer-induced neuronal loss in SH-SY5Y cells (Figure 6).

Moreover, the combination of SB415286 and U0126 synergistically prevented neurotoxicity induced by Aβ oligomers, suggesting that concurrently activation of the PI3K/Akt pathway and inhibition of the ERK pathway could attenuate Aβ oligomer-induced neuronal loss in SH-SY5Y cells (Figure 6).

3.4. Fucoxanthin Prevents the Alterations of the PI3K/Akt and the ERK Pathways Induced by Aβ Oligomers in SH-SY5Y Cells. To study whether fucoxanthin attenuated Aβ oligomer-induced neuronal loss via regulating the PI3K/Akt and the ERK pathways, we used Western blotting analysis. SH-SY5Y cells were pretreated with 3 μM fucoxanthin for 2h before adding Aβ oligomers. After 6h, proteins were extracted. As demonstrated in Figures 7(a) and 7(b), fucoxanthin prevented Aβ oligomer-induced decrease of pSer473-Akt and pSer9-GSK3β in a concentration-dependent manner, suggesting that fucoxanthin could attenuate the inhibition of the PI3K/Akt pathway induced by Aβ oligomers. Moreover, fucoxanthin concentration...
dependently prevented Aβ oligomer-induced increase of pERK, indicating that fucoxanthin also attenuated the activation of the ERK pathway induced by Aβ oligomers (Figure 7(c)). The treatment of fucoxanthin alone did not significantly change the expressions of pSer473-Akt, pSer9-GSK3β and pERK (Figure 7).

In addition, LY294002 and wortmannin, two PI3K inhibitors were used to confirm if fucoxanthin attenuated Aβ oligomer-induced neuronal loss via regulating the PI3K/Akt pathway. As shown in Figure 8, both PI3K inhibitors significantly abolished the neuroprotective effects of fucoxanthin against neuronal loss induced by Aβ oligomers, providing a support that fucoxanthin produced neuroprotective effects via the activation of PI3K/Akt pathway.

4. Discussion

We have reported, for the first time, that fucoxanthin significantly attenuated Aβ oligomer-induced neurotoxicity in SH-SY5Y cells. We further demonstrated that the neuroprotective effects of fucoxanthin against Aβ oligomer-induced neuronal loss and oxidative stress possibly via activating the prosurvival PI3K/Akt pathway and inhibiting the proapoptotic ERK pathway, concurrently.

Aβ oligomers are widely accepted as the main neurotoxins to induce neuronal loss in the brain of AD patients...
However, the neurotoxicity of Aβ oligomers formed by different formation protocols varies widely in vitro. In our study, we used a protocol of Aβ oligomer formation which is derived from Roger Anwyl’s lab. We found that Aβ oligomers substantially induced neuronal apoptosis at micromolar levels in SH-SY5Y cells, which is consistent with previous publications. We also found that fucoxanthin at 3 μM could significantly attenuate Aβ oligomer-induced neuronal loss in SH-SY5Y cells, as indicated by the MTT assay, FDA/PI double staining and Hoechst staining. These results are consistent with previous publications showing that fucoxanthin can decrease oxidative stress in neurons, and therefore might be useful in the central nervous systems.

Previous studies have reported that Aβ oligomers could act on both prosurvival and proapoptotic pathways. Therefore, we studied which signaling pathways are mainly involved in Aβ oligomer-induced neuronal loss in our model. We found that (1) both the PI3K/Akt and the ERK pathways were altered by Aβ oligomers and (2) concurrent activation of the PI3K/Akt pathway and inhibition of the ERK pathway synergistically attenuated Aβ oligomer-induced neuronal loss. These results suggested that Aβ oligomers lead to neuronal loss possibly via simultaneous inhibition of the prosurvival PI3K/Akt pathway and activation of the proapoptotic ERK pathway.

We further investigated the regulation of signaling pathways by fucoxanthin. Fucoxanthin attenuated Aβ oligomer-induced decrease of pSer473-Akt and pSer9-GSK3β. Moreover, PI3K inhibitors significantly abolished groups in the ring structure of fucoxanthin that could donate electrons or hydrogen atoms, leading to free radical scavenging and the antioxidative stress properties of fucoxanthin. Therefore, our results provides evidence that fucoxanthin can decrease oxidative stress in neurons, and therefore might be useful in the central nervous systems.

Figure 7: Fucoxanthin prevents Aβ oligomer-induced alteration of the PI3K/Akt and the ERK pathways in a concentration-dependent manner in SH-SY5Y cells. SH-SY5Y cells were treated with 1 or 3 μM fucoxanthin. After 2 h, 1 μM Aβ oligomer was added. Western blotting analysis was used to determine the expression of (a) pSer473-Akt, (b) pSer9-GSK3β, and (c) pERK at 6 h after the treatment of Aβ oligomers. Data were the mean ± SEM of three separate experiments; *p < 0.05 and **p < 0.01 versus the control group; ***p < 0.01 versus the Aβ oligomer group (ANOVA and Tukey’s test).
Data, expressed as percentage of control, were the mean ± SEM of three separate experiments; *p < 0.01 versus the control group, **p < 0.01 versus the Aβ oligomer group, and $^*$p < 0.05 and $^*$*$p < 0.01 versus the fucoxanthin plus Aβ oligomer group (ANOVA and Tukey’s test).

In summary, we found that fucoxanthin attenuated Aβ oligomer-induced neurotoxicity and oxidative stress possibly via the activation of the PI3K/Akt pathway and the inhibition of the ERK pathway, concurrently. Based on these findings and the safety of fucoxanthin, we anticipated that further studies of fucoxanthin and related compounds might one day lead to a useful treatment of AD.

**Abbreviations**

AD: Alzheimer’s disease  
ANOVA: Analysis of variance  
Aβ: β-amyloid  
BACE-1: Aβ precursor protein cleaving enzyme 1  
carboxy-H2DCF-DA: 5-(and-6)-carboxy-2′,7′-dichlorodihydrofluorescein diacetate  
ERK: Extracellular signal-regulated kinase  
FDA: Fluorescein diacetate  
GSK3β: Glycogen synthase kinase 3β  
HFI1P: 1,1,1,3,3-Hexafluoro-2-propanol  
MEK: Mitogen activated protein kinase  
MTT: 3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide  
PI: Propidium iodide  
PI3K: Phosphoinositide 3-kinase  
ROS: Reactive oxygen species  
α7nAChR: α7 Nicotinic acetylcholine receptor.

**Conflicts of Interest**

The authors declare that they have no conflicts of interest.

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References


