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Astaxanthin Protects Neuronal Cells against Oxidative Damage and Is a Potent Candidate for Brain Food

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Abstract

Astaxanthin (AST) is a powerful antioxidant that occurs naturally in a wide variety of living organisms. Based on the report claiming that AST could cross the brain-blood barrier, the aim of this study was to investigate the neuroprotective effect of AST by using an oxidative stress-induced neuronal cell damage system. The treatment with DHA hydroperoxide (DHA-OOH) or 6-hydroxydopamine (6-OHDA), either of which is a reactive oxygen species (ROS)-inducing neurotoxin, led to a significant decrease in viable dopaminergic SH-SY5Y cells by the MTT assay, whereas a significant protection was shown when the cells were pretreated with AST. Moreover, 100 nM AST pretreatment significantly inhibited intracellular ROS generation that occurred in either DHA-OOH- or 6-OHDA-treated cells. The neuroprotective effect of AST is suggested to be dependent upon its antioxidant potential and mitochondria protection; therefore, it is strongly suggested that treatment with AST may be effective for oxidative stress-associated neurodegeneration and a potential candidate for natural brain food.

Astaxanthin (AST; fig. 1), a red-orange carotenoid pigment, occurs naturally in many well-known aquatic animals such as shrimp, crab and salmon. As it belongs to the xanthopyll class of carotenoids, AST is closely related to β-carotene, lutein, and zeaxanthin, sharing with them many of the general metabolic and physiological activities attributed to carotenoids. On the other hand, AST has unique chemical properties based on its molecular structure. The presence of the hydroxyl (OH) and keto (C = O) moieties on each ionone ring explains some of its unique features, i.e. a high antioxidant activity. In recent years, a number of in vitro and in vivo studies on AST have demonstrated its antioxidant effect, for example the quenching effect on singlet oxygen, a strong scavenging effect on superoxide, hydrogen peroxide, and hydroxyl radicals and an inhibitory effect on lipid peroxidation [1–3]. In addition to these, several other biologic activities of AST, including anticancer, anti-inflammatory, antidiabetic, immunomodulatory activities and a neuroprotective effect, have also been reported [4].

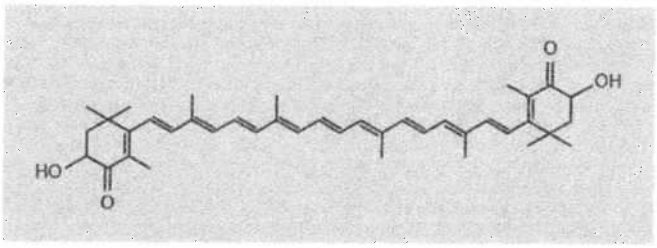

Fig. 1. Chemical structure of AST.

Parkinson's disease (PD) is a neurodegenerative disorder characterized by a preferential loss of the dopaminergic neurons. The mechanism responsible for degeneration of dopaminergic neurons is incompletely understood; however, an increasing body of evidence suggests that oxidative stress, mitochondrial inhibition and impairment of the ubiquitin-proteasome system may be largely involved as major biochemical processes in the degenerative cascade [5]. In our previous study, we demonstrated that reactive oxygen species (ROS)- and mitochondrial dysfunction-mediated apoptotic signaling increased within a few hours after treatment with DHA hydroperoxides (DHA-OOH) and resulted in dopaminergic SH-SY5Y cell death [6]. In addition, the in vitro experimental model using neurotoxic compounds such as 6-hydroxydopamine (6-OHDA) has also revealed that the neuronal cell death was regulated by ROS generation, mitochondrial inhibition and other oxidative stress-related signaling molecules [7–9].

Currently, most efforts to prevent and treat neurodegenerative disorders focus on diet, lifestyle modification and drugs that target the disease processes, and among them, several natural antioxidant food factors have been focused on in recent years, which are also named brain foods. AST is a powerful antioxidant, and Hussein et al. [10] recently reported that AST prevented the ischemia-induced impairment of spatial memory in mice. Although these facts suggested that AST might be a potent candidate for a natural neuroprotective agent, further basic evidence to demonstrate the neuroprotective effect of AST is needed.

The human dopaminergic neuronal cell line SH-SY5Y processes many types of substantia nigra neurons [11]. Therefore, in this study we investigated whether AST would prevent DHA-OOH- or 6-OHDA-induced cytotoxicity in SH-SY5Y cells. We examined the effects of AST on cell viability in DHA-OOH- or 6-OHDA-treated cells. The possible mechanisms of AST protection were investigated by measuring intracellular ROS generation and accumulation of AST in the cells.

Methods

Materials

AST was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DHA (purity = 98%) was purchased from the Cayman Chemical Co. (Ann Arbor, Mich., USA). 6-OHDA hydrochloride was obtained from Wako Pure Chemical Industries, Ltd.

DHA-Hydroperoxide Preparation

The DHA-OOH were prepared by the reaction of lipoxidase (from soybeans, Wako Pure Chemical Industries, Ltd.) with docosahexaenoic acid, as previously described [6]. The reaction mixture containing 83.6 mg docosahexaenoic acid, 16 mg lipoxidase and 220 ml borate buffer (200 mm, pH 9.0) was used, and the reaction was carried out in a flask filled with oxygen at room temperature. After incubation for 10 min, to terminate the reaction, HCl was added to the mixture until the solution pH was below 4.0. The formed hydroperoxides were extracted twice with an equal amount of chloroform/methanol (1:1), and the collected chloroform layer was then evaporated. The obtained DHA-OOH was dissolved in ethanol. The identification was performed by high-performance liquid chromatography (HPLC) analysis monitored at A_{234}, and the concentration was quantified using a lipid hydroperoxide kit (Cayman) and compared with a standard curve prepared using authentic 13-HPODE.

Cell Culture and Cell Viability

Human dopaminergic neuroblastoma SH-SY5Y cells were grown in Cosmedium-001 (Cosmo-Bio, Tokyo, Japan) containing 5% fetal bovine serum. The cells were seeded on plates coated with polylysine and cultured at 37°C. The cell viability was quantified by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, different concentrations of AST were added to the cells for 4 h. Following the removal of excess AST agent, the cells were washed three times with FBS-free DMEM prior to the addition of 10 μM DHA-OOH or 100 μM 6-OHDA for 24 h, followed by further incubation with 0.5% MTT solution (5 mg/ml) for 4 h. The cells were then lysed with 0.04 N HCl in isopropyl alcohol, and the absorbance was read at wavelengths of 550 nm (peak) and 630 nm (bottom).

Analysis of Reactive Oxygen Species Production

The intracellular ROS level was detected by flow cytometry using DCHF-DA that is oxidized by hydrogen peroxide or low-molecular-weight peroxides to produce the fluorescent compound 2,7-dichlorofluorescein (DCF). In this study, the SH-SY5Y cells (which had reached approximately 80% confluence) seeded on six-well plates were washed twice with serum-free DMEM and thereafter incubated for 4 h in serum-free DMEM in the presence of 100 nM AST. After washing with serum-free DMEM, the cells were loaded with carboxy-H2DCFDA for 30 min, prior to exposure to 10 μM DHA-OOH or 100 μM 6-OHDA for 30 min. Followed by treatment with 6-OHDA, the cells were washed once with PBS+ and PBS–, respectively, and then collected into vials. The fluorescence of DCF in the supernatant was measured by an EPICS Elite Flow Cytometer (Beckman Coulter, Inc., Fullerton, Calif., USA).

Analysis of Astaxanthin Concentration in Different Fractions of the Cell

AST contents in cell membrane, mitochondrial and cytosolic fractionation and media were quantified by HPLC. Five dishes (9-cm cell culture dishes) of confluent SH-SY5Y cells were incubated with 100 nM AST for 4 h. The media were collected. The cells were washed with PBS three times, and then were fractionated into cell membrane, mitochondria and cytosol as described by Pallotti [12]. The media and fractionations were dissolved in 200 ml of acetone and filtered through a

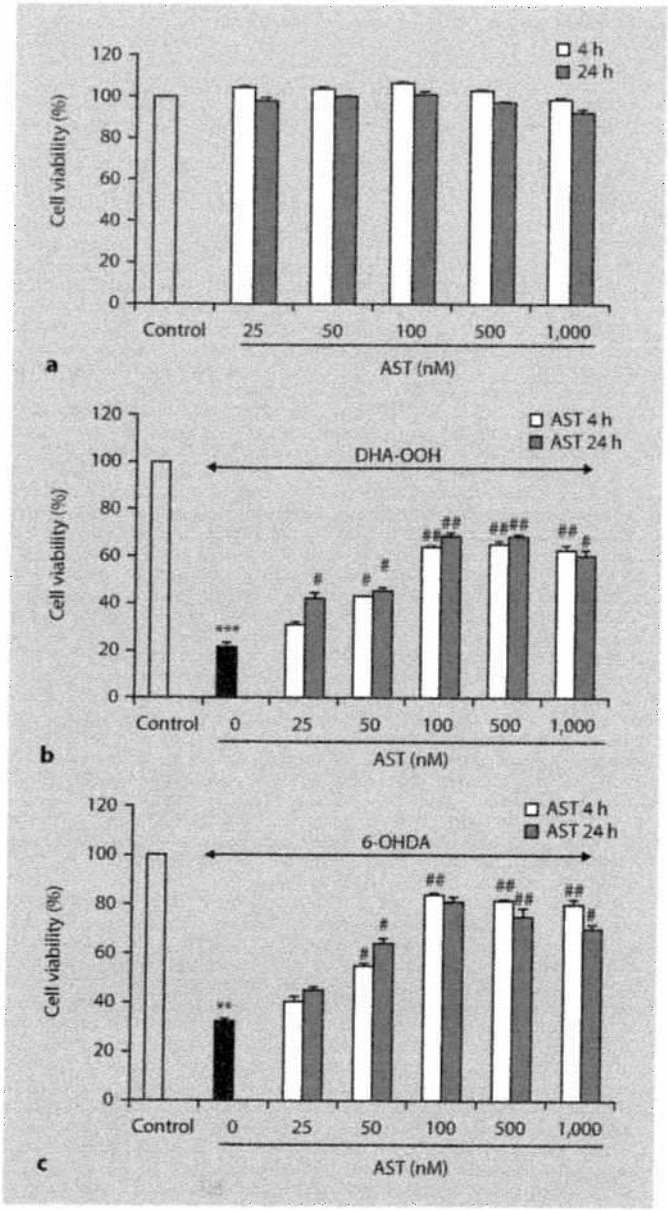

Fig. 2. Protective effect of AST on DHA-OOH- or 6-OHDA-induced decrease in cell viability measured by MTT assay. **a** SH-SY5Y cells were incubated with different concentrations of AST for 4 or 24 h, and cell viability was assessed by MTT assay. **b**, **c** Cells were incubated with or without 25–1,000 nm AST for 4 or 24 h. Then the media were removed and the cells were washed three times with FBS-free DMEM prior to the addition of 10 μM DHA-OOH or 100 μM 6-OHDA for additional 24 h. Values are percentages of the control (no drugs) of three independent experiments in triplicates and are expressed as mean ± SE ($n = 9$). ** $p < 0.01$ and *** $p < 0.001$ versus control; * $p < 0.05$ and ** $p < 0.01$ versus DHA-OOH or 6-OHDA.

0.45-mm polytetrafluoroethylene membrane filter; then 20 ml of solution was subjected to HPLC-UV using a column of Develosil ODS HG-5 (4.6 × 250 mm, Nomura Kagaku, Japan). Semi-preparative HPLC was performed at room temperature using a mobile phase consisting of methanol (95%) and water (5%) with a linear program. The flow rate was set at 0.8 ml/ml, and AST peak was collected by monitoring at 471 nm. AST was quantified relative to calibration with a standard sample. Obtained quantity of AST in media and each cellular fraction was adjusted by added total AST quantity and expressed as percent of added total AST.

Statistical Analysis

All data were analyzed using Bonferroni/Dunn's multiple comparison procedure.

Results

Effect of Astaxanthin on DHA-OOH- or 6-OHDA-Induced Cell Death

SH-SY5Y cells are widely used to study dopaminergic pathogenesis as this cell line expresses some representative dopaminergic markers such as tyrosine hydroxylase and dopamine transporter. Therefore, SH-SY5Y cells can be a suitable model system to study the role of AST against ROS-mediated dopaminergic cell death.

In this study, SH-SY5Y cells were pretreated with AST for 4 and 24 h at different concentrations, washed, and then treated with DHA-OOH or 6-OHDA for an additional 24 h. AST itself had no apparent effect on cell viability at a concentration of 25–1,000 nM even for 24 h (fig. 2a). DHA-OOH (10 μM, 24 h) and 6-OHDA (100 μM, 24 h) induced a significant decrease in cell viability by 80 and 70%. The pretreatment of SH-SY5Y cells with AST for 4 h resulted in a dose-dependent protection against DHA-OOH- or 6-OHDA-induced toxicity at a concentration ranging from 25 to 100 nM, and the most significant protection was found at a concentration of 100 nM, with respectively 65 and 84% of the control. The pretreatment with AST at a concentration of 500 and 1,000 nM caused a similar or reduced effect compared to that of 100 nM. In addition, the protective effect of AST was slightly enhanced by a longer pretreatment for 24 h the case of DHA-OOH- and 6-OHDA-treated cells (fig. 2b, c).

Effect of Astaxanthin on DHA-OOH- or 6-OHDA-Induced ROS Generation

ROS generation has been demonstrated to be a common feature occurring in DHA-OOH- or 6-OHDA-treated cells and is also proposed as one of the initial triggers leading to activation of apoptotic signaling. In this study, we examined the effect of AST on ROS generation in SH-SY5Y cells exposed to DHA-OOH or 6-OHDA. Intracellular ROS levels were determined with DCF fluorescence by flow cytometry. As shown in figure 3, exposure of SH-SY5Y cells to DHA-OOH and 6-OHDA led to a 3.5- and 1.8-fold increases, respectively, in DCF signal compared with the control group, whereas AST pretreatment significantly inhibited the increase in DCF fluorescence in the cells treated by both toxins. In addition, we investigated the accumulation of AST in the cells. By HPLC analysis, AST was detected at 0, 9.42, 7.9, and 72.56% of the total administration levels in the cytosolic, mitochondrial, membrane fraction of the cells and the culture medium, respectively (data not shown), suggesting that AST accumulating in the membrane fraction may contribute directly to the protection against ROS-associated cell death by its potent antioxidant property.

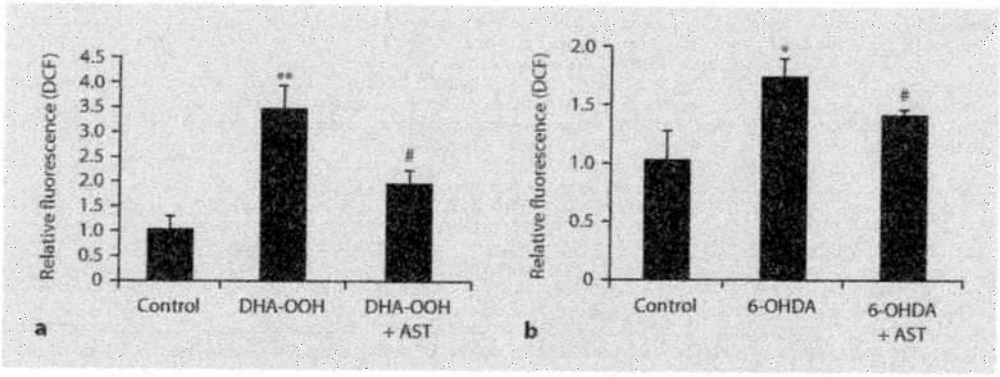

Fig. 3. Protective effects of AST on DHA-OOH- or 6-OHDA-induced ROS generation. DCF fluorescence determination was used to assess intracellular ROS generation in DHA-OOH-treated (**a**) and 6-OHDA-treated (**b**) cells. * $p < 0.05$ and ** $p < 0.01$ versus control; # $p < 0.05$ versus DHA-OOH or 6-OHDA.

Conclusion

Advances in understanding the neurodegenerative pathologies are creating new opportunities for the development of neuroprotective therapies. In this work, we have demonstrated that AST, a natural carotenoid and an abundant component in aquatic animals, significantly protected DHA-OOH- or 6-OHDA-induced cellular toxicity in human neuroblastoma dopaminergic SH-SY5Y cells.

PD is characterized by a profound loss of dopaminergic neurons in the substantia nigra. Even though the cause of PD remains largely unknown, several lines of evidence strongly suggest the involvement of oxidative stress [13]. Evidence exists that some antioxidants have a neuroprotective effect in the in vitro models of PD. However, the lack of efficacy to penetrate the blood-brain barrier has led to the failure of antioxidants to exhibit the in vivo effect. Tso et al. [14] detected AST in the brain of rats fed with natural AST, suggesting that AST could cross the blood-brain barrier in mammals.

The neuroprotective effect of AST in our study is very significant and quite powerful at the nM levels. In addition, Aoi et al. [15] have reported that AST inhibited the formation of linoleic acid-derived hexanoyl lysine adduct in mice skeletal muscle, suggesting that AST might suppress lipid peroxidation occurring in the brain due to the presence of a high concentration of polyunsaturated fatty acids. Together, it is suggested that AST may be used as a brain nutrient to protect the brain content from oxidative stress, neuronal apoptosis, and even brain aging.

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