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Astaxanthin attenuates glutamate-induced apoptosis via inhibition of calcium influx and endoplasmic reticulum stress

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ABSTRACT

Astaxanthin (AST) is a carotenoid that has been shown to have neuroprotective effects. In this study, it was found that AST significantly inhibited glutamate-induced loss of cell viability and apoptosis. AST pretreatment attenuated glutamate-induced activation of caspase-3, reduction of anti-apoptotic protein Bcl-2, and increase of pro-apoptotic protein Bak. In addition, AST pretreatment suppressed the production of intracellular reactive oxygen species. AST treatment also prevented glutamate-induced increase of the level of activated p38 mitogen-activated protein kinase (MAPK), which has been shown to promote apoptotic events. Furthermore, AST treatment greatly reduced the elevation of intracellular calcium level induced by glutamate and inhibited the activity of calpain, a calcium-dependent protease that plays an important role in mediating apoptosis stimulated by calcium overload in cytoplasm. Both oxidative stress and calcium overload can lead to endoplasmic reticulum (ER) stress. C/EBP-homologous protein (CHOP) is a bZIP transcription factor that can be activated by ER stress and promotes apoptosis. Here we found that AST attenuated glutamate-induced elevation of CHOP and ER chaperone glucose-regulated protein (GRP78). Overall, these results suggested that AST might protect cells against glutamate-induced apoptosis through maintaining redox balance and inhibiting glutamate-induced calcium influx and ER stress.

1. Introduction

Glutamate is an excitatory neurotransmitter important for neuronal processes including learning and memory. However, excessive accumulation of extracellular glutamate in the brain causes aberration in neuronal excitation processes (Sheldon and Robinson, 2007). The resulted excitotoxicity has been shown to induce damage and death of neuronal cells and is linked with the pathogenesis of neurological disorders such as cerebral ischemia, Alzheimer's disease (AD), Parkinson disease (PD) and amyotrophic lateral sclerosis (ALS) (Choi, 1992; Lancelot and Beal, 1998; Olney, 1969). In addition to the excitotoxicity initiated by the overactivation of glutamate receptor, nonreceptor-mediated toxicity caused by the overproduction of reactive oxygen species also contributes to glutamate-induced neuronal cell death (May et al., 2006; Murphy et al., 1989; Sun et al., 2010). A rapid calcium (Ca^{2+}) influx can be induced by glutamate treatment, leading to an increase of intracellular Ca^{2+} concentration (Gao et al., 2008; Yang et al., 2013). Proteases such as calpains, which can be activated by high levels of intracellular Ca^{2+}, have been reported to mediate apoptosis induce by excessive glutamate (Araújo et al., 2010; Rami et al., 2000; Ray et al., 2000; Zhang and Bhavnani, 2006). Calpain targets calcium regulatory proteins such as sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (French et al., 2006) and calcium channel proteins (Pedrozo et al., 2010), and causes Ca^{2+} release from endoplasmic reticulum (ER). Prolonged Ca^{2+} depletion in ER impairs protein processing and promotes ER stress (Brostrom and Brostrom, 2003; Thuerauf et al., 2001). It has been shown that glutamate can induce ER stress in neuronal cells (Mei et al., 2014; Pan et al., 2012). Severely impaired ER function triggers apoptotic signaling that leads to cell death and has been implicated in the development of neurodegenerative diseases including AD, PD and ALS (Doyle et al., 2011; Kanekura et al., 2009).

Astaxanthin (AST, 3, 3′-dihydroxy-β, β,-carotene-4, 4′-dione) belongs to the family of xanthophylls, the oxygenated derivatives of carotenoids. It is widely distributed in nature and is the principal pigment in many organisms such as crustaceans, salmonids and red yeast (Higuera-Ciapara et al., 2006). As a strong antioxidant, AST effectively scanvenges oxygen free radicals and reduces oxidative stress in both cell and animal models (Campoio et al., 2011; Marin et al., 2011; Wolf et al., 2010). Numerous health-promoting benefits have been associated with AST supplementation, including immunomodulation, prevention and treatment of cardiovascular diseases and cancer

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(Chew et al., 1999; Macedo et al., 2010; Pashkow et al., 2008). Evidence also suggests that AST has neuroprotective effects. In animal model of brain ischemic injury, AST treatment has been shown to reduce cerebral infarction caused by cerebral artery occlusion via inhibiting ischemia-mediated oxidative stress and apoptosis (Shen et al., 2009). In addition, AST has been found to inhibit cytotoxicity and apoptosis induced by beta-amyloid peptide or 6-hydroxydopamine in neuronal cells (Chang et al., 2010; Ikeda et al., 2008; Wang et al., 2010).

In the present study, we studied the effects of AST on glutamate-induced cytotoxicity in SH-SY5Y cells. The results showed that AST significantly inhibited glutamate-induced loss of cell viability and apoptosis by ameliorating the effect of glutamate on the expression of Bcl-2 family proteins, preventing the reduction of anti-apoptotic Bcl-2 and the increase of pro-apoptotic Bak. Further analyses indicated that AST inhibited glutamate-induced apoptotic signaling via preventing glutamate-induced elevation of intracellular Ca^{2+} concentration and production of reactive oxygen species. In addition, AST attenuated glutamate-induced increase of the level of CCAAT/Enhancer-binding protein (C/EBP)-homologous protein (CHOP) and ER chaperone glucose-regulated protein 78 (GRP78). Therefore, the prevention of ER stress may be an important mechanism by which AST exerts protective effects against glutamate-induced cytotoxicity.

2. Materials and methods

2.1. Materials

Dulbecco's modified eagle's medium (DMEM) and 0.25% trypsin-EDTA were purchased from Invitrogen (Eugene, OR, USA). Fetal bovine serum (FBS), penicillin and streptomycin were purchased from Thermo Scientific (Rockford, IL, USA). AST was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Sodium glutamate hydrate and Brandford protein assay kit were purchased from Sangon Biotech (Shanghai, China). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetra-zolium bromide (MTT) cell proliferation and cytotoxicity assay kit, antibodies for Bak, GRP78 and CHOP were purchased from Wanleibio Co., Ltd. (Shenyang, China). Dimethyl sulfoxide (DMSO), Hoechst 33258, caspase 3 activity kit, caspase 4 activity kit, fluo-3 AM, BCA protein assay kit, BeyoECL plus Western blotting detection system, donkey anti-goat IgG (H+L) and goat anti-rabbit IgG (H+L) antibodies were purchased from Beyotime Institute of Biotechnology (Haimen, China). 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) was purchased from Sigma Chemical (St. Louis, MO, USA). The calpain activity assay kit was purchased from Genmed Inc. (Shanghai, China). Antibodies for p38 mitogen-activated protein kinase (MAPK), phospho-p38 MAPK (Thr180/Tyr182), p44/p42 MAPK, phospho-p44/p42MAPK (Thr202/Tyr204) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies for Bcl-2 and calpain 1 were purchased from Boster Institute of Biotechnology (Wuhan, China). Antibody for actin was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA).

2.2. Cell culture

Human neuroblastoma SH-SY5Y cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. The cells were maintained at 37 ℃ in a humidified atmosphere with 5% CO2 and 95% air.

2.3. Cell viability

The cell viability was determined by MTT assay. SH-SY5Y cells were seeded in 96-well plates with a density of 4×10^3 and 2×10^3/well for evaluating glutamate dose response and the effect of AST on glutamate-induced cytotoxicity, respectively. Primary cultures of hippocampal neurons were prepared from the brains of newborn Sprague/Dawley rats. To examine the effect of AST on glutamate-induced cytotoxicity, hippocampal neurons were seeded with a density of 2×10^3/well in the growth neurobasal medium containing 2% B27 and 1% FBS. After treatment, MTT solution (100 μl, 0.5 g/l) was added into each well and incubated at 37 °C for 6 h. The medium was then removed and DMSO was added to each well and incubated at 37 °C for 15 min to dissolve the formazan. The colored product was then measured at 570 nm using a microplate reader.

2.4. Hoechst 33258 nuclear staining assay

The apoptotic cells were observed by Hoechst 33258 nuclear staining assay. SH-SY5Y cells pretreated with AST (50 μg/l) for 24 h were treated with 20 mM glutamate for 24 h. After the treatment, the cells were fixed for 20 min at room temperature. The fixed cells were incubated with Hoechst 33258 for 5 min at room temperature in the dark. The fluorescence was then examined using an Olympus BX53 fluorescence microscope. For quantification, cells exhibiting chromatin condensation and intense fluorescence were considered as apoptotic cells.

2.5. Measurement of caspase activities

The activities of caspase 3 and caspase 4 were determined using commercial available kits according to the manufacturer's instructions. SH-SY5Y cells pretreated with AST (50 μg/l) for 24 h were treated with 20 mM glutamate for 24 h (for caspase-3) or 18 h (for caspase-4). After treatments, cells were harvested by trypsinization and the total cell lysates were prepared. To evaluate the activity of caspase 3, cell lysates were incubated with Ac-DEVD-pNA and reaction buffer for 10 h at 37 ℃. Substrate cleavage was then measured using a spectrometer at 405 nm. To evaluate the activity of caspase 4, cell lysates were incubated with Ac-LEVD-pNA and reaction buffer for 1 h at 37 ℃. The samples were then measured using a spectrometer at 405 nm.

2.6. Measurement of intracellular reactive oxygen species level

Intracellular reactive oxygen species levels of cells were measured using fluorescent probe DCFH-DA. SH-SY5Y cells pretreated with AST (50 μg/l) for 24 h were treated with 20 mM glutamate for 2 h. The cells were washed twice with PBS and incubated with 5 μM DCFH-DA for 30 min at 37 ℃ in the dark. The cells were then washed twice with PBS and the fluorescence was examined using an Olympus BX53 fluorescence microscope.

2.7. Measurement of intracellular Ca^{2+} level

Intracellular Ca^{2+} levels were measured using fluo-3AM. SH-SY5Y cells pretreated with AST (50 μg/l) for 24 h were treated with 20 mM glutamate for 30 min. The cells were washed twice with PBS and incubated with 5 μM fluo-3AM in Krebs-Ringer buffer (D-glucose 10 mM, NaCl 120 mM, KCl 4.5 mM, Na_2HPO_4 0.7 mM, NaH_2PO_4 1.5 mM and $MgCl_2$ 0.5 mM, pH 7.4) for 40 min at 37 ℃ in the dark. The cells were then washed twice with PBS and incubated for additional 20 min at 37 ℃. The cells were collected by trypsinization and the fluorescence intensity was measured by a fluorospectrophotometer (F-2700 Techcomp (China) Ltd.).

2.8. Measurement of calpain activity

The activity of calpain was determined using a commercial available kit according to the manufacturer's instructions. The assay used a 7-amino-4-methylcoumarin (AMC) labeled calpain substrate Leu-Leu-Val-Tyr. Upon cleavage of the substrate by calpain, AMC was released and its amount was quantified using a fluorospectrophotometer (F-2700 Techcomp (China) Ltd.).