Neuroprotective effect of astaxanthin on H2O2-induced neurotoxicity in vitro and on focal cerebral ischemia in vivo

Ya-Peng Lu, Si-Yuan Liu, Hua Sun, Xiao-Mei Wu, Jie-Jia Li, Li Zhu

Institute of Nautical Medicine, Nantong University, Nantong 226001, China
School of Life Sciences, Nantong University, Nantong 226007, China

ARTICLE INFO

Article history:
Accepted 5 September 2010
Available online 21 September 2010

Keywords:
Astaxanthin
Neuroprotection
Cerebral ischemia–reperfusion
Oxidative stress

ABSTRACT

Astaxanthin (AST) is a powerful antioxidant that occurs naturally in a wide variety of living organisms. Much experimental evidence has proved that AST has the function of eliminating oxygen free radicals and can protect organisms from oxidative damage. The present study was carried out to further investigate the neuroprotective effect of AST on oxidative stress induced toxicity in primary culture of cortical neurons and on focal cerebral ischemia–reperfusion induced brain damage in rats. AST, over a concentration range of 250–1000 nM, attenuated 50 μM H2O2-induced cell viability loss. 500 nM AST pretreatment significantly inhibited H2O2-induced apoptosis measured by Hoechst 33342 staining and restored the mitochondrial membrane potential (MMP) measured by a fluorescent dye, Rhodamine 123. In vivo, AST prevented cerebral ischemic injury induced by 2 h middle cerebral artery occlusion (MCAO) and 24 h reperfusion in rats. Pretreatment of AST intragastrically twice at 5 h and 1 h prior to ischemia dramatically diminished infarct volume and improved neurological deficit in a dose-dependent manner. Nissl staining showed that the neuronal injury was significantly improved by pretreatment of AST at 80 mg/kg. Taken together, these results suggest that pretreatment with AST exhibits noticeable neuroprotection against brain damage induced by ischemia–reperfusion and the antioxidant activity of AST maybe partly responsible for it.

1. Introduction

Ischemic stroke is a leading cause of death and disability across the world. In spite of substantial research and development efforts, no sufficient therapy for this obstinate illness is available. A variety of mechanisms are involved in ischemic brain injury (Cheng et al., 2004). Ischemia induces an imbalance of endogenous oxidants and antioxidants and overproduction of toxic free radicals (Chan, 2001; Mattson et al., 2001; Traystman et al., 1991). Reperfusion also comes with massive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that potentiate initial brain damage (Kuroda and Siesjo, 1997; Traystman et al., 1991). Accumulations of toxic free radicals, therefore, not only increase the susceptibility of brain tissues to oxidative damage but also trigger various cascades of ischemic injury, leading to...
either direct injury via membranous lipid peroxidation and protein and DNA oxidation or indirect damage via inflammation and apoptosis (Chan, 2001; Kuroda and Siesjo, 1997; Traystman et al., 1991). For these reasons, antioxidant agents have been the focus of studies for developing neuroprotective drugs to prevent and treat stroke.

AST is widely distributed in nature, and it is the principal pigment in crustaceans, salmonoids, and many other organisms (Liu et al., 2009). A number of studies on AST have in vitro and in vivo demonstrated its antioxidant effect (Liu et al., 2009; Palezza and Krinsky, 1992b). In addition to these, several other biologic activities of AST, including anticancer, antiinflammatory, antidiabetic, immunomodulatory activities, also have been reported (Hussein et al., 2006). In recent years, the protective effect of AST against ischemia–reperfusion injury has been paid more attention. AST and its derivative have shown to reduce injury of hepatocyte and cardiocyte following ischemia–reperfusion (Curek et al., 2010; Gross and Lockwood, 2005). The mechanisms of action may include antioxidant and antiinflammatory activity. Of note, AST can cross the blood brain barrier in mammals (Ikedo et al., 2008), for which it has attracted considerable attention as a potential neuroprotective agent. As noted earlier, AST shows significant neuroprotective effect by preventing the ischemia-induced impairment of spatial memory in mice induced by bilateral common carotid occlusion, 2 vessels occlusion (Hussein et al., 2005).

Latest research shows that AST may ameliorate cerebral ischemia–reperfusion injury in rats by intraventricular injection (Shen et al., 2009).

The effectiveness of AST pretreatment by oral administration in protecting against cerebral ischemia injury has not been evaluated. The present study aims to investigate whether AST can reduce brain infarct volume and neurological dysfunction that follow cerebral ischemia–reperfusion (Curek et al., 2010; Gross and Lockwood, 2005). For these reasons, antioxidant agents have been the focus of studies for developing neuroprotective drugs to prevent and treat stroke. The effectiveness of AST pretreatment by oral administration in protecting against cerebral ischemia injury has not been evaluated. The present study aims to investigate whether AST can reduce brain infarct volume and neurological dysfunction that follow cerebral ischemia–reperfusion injury in rats by oral administration, as well as the effect of AST on H2O2-induced neuronal damage using cultured cortical neurons to elucidate the neuroprotective mechanism.

2. Results

2.1. Neuroprotective effect of AST against cerebral ischemia–reperfusion insult

The neuroprotective efficacy of AST to ischemia–reperfusion insult was evaluated by comparing the infarct volume (Figs. 1A, B) and the neurological deficit (Fig. 1C) in rat model of MCAO. The changes of infarct volume were assessed using the vital dye 2,3,5-triphenyltetrazolium chloride (TTC). Infarct volume in vehicle-treated MCAO group was 36.5±4.2%. While in 50 μg/kg and 80 μg/kg AST-pretreated groups, infarct volume significantly reduced to 14.6±5.4% and 11.4±4.9%. Nimodipine (NIM) as a positive control at a dose of 10 mg/kg also reduced infarct volume to 24.6±5.7%. The results of Zea Longa test showed that the neurological deficit score was obviously increased to 3.5±0.5 in vehicle-treated rats after ischemia–reperfusion. In 50 μg/kg and 80 μg/kg AST-pretreated rats, the neurological deficit were mild and the scores decreased to 1.5±0.7 and 0.9±0.8, respectively. After pretreated with NIM, the neurological deficit score also decreased to 2.3±0.7.

2.2. Effect of AST on histopathology after cerebral ischemia–reperfusion

On Nissl-stained sections, in the ipsilateral brain, many atrophic neurons with shrunken cytoplasm and damaged nuclei were observed in vehicle group while no apparent morphological changes in sham-operated group (Fig. 2A). As illustrated in Fig. 2B, in sham-operated group, the number of cells was 128.3±10.1, which was significantly reduced to 40.3±8.0 in vehicle-treated group after MCAO. Pretreated with AST (80 mg/kg, intragastrically) twice at 5 h and 1 h prior to MCAO, the number of cells in the same brain area was elevated to 99.5±12.6.

2.3. Protective effect of AST against H2O2-induced neuronal cell death

Cortical neurons were exposed to H2O2 (25–400 μM) for 18 h and cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. As shown in Fig. 3A, H2O2 decreased the cell viability in a dose dependent manner. Exposure of 25, 50, 100 and 400 μM of H2O2 reduced cell viability to 87.1±3.8%, 63.9±3.1%, 51.1±2.1%, 40.9±0.6%, and 38.3±1.7% of control. The concentration of 50 μM was used for the determination of H2O2-induced neuronal cell damage in the present experiments. AST (250, 500, and 1000 nM) containing 0.5% Dimethyl Sulphoxide (DMSO), which showed no toxic effect on the cells (Fig. 3B), significantly increased the cell viability to 73.9±5.1%, 89.7±2.4%, 80.4±5.7% of control respectively.

2.4. AST protects neuronal cells against H2O2-induced apoptosis

Chromatin condensation and nuclear fragmentation were observed in neurons treated with 50 μM H2O2, whereas the control culture showed round blue nuclei of viable neurons in Hoechst 33342 staining (Fig. 4A). Treatment with 50 μM H2O2 produced 45.4±3.9% apoptotic cells of cultured cortical neurons, compared with 9.1±0.9% apoptotic cells in control cultures. The addition of AST (500 nM) significantly decreased H2O2-induced apoptotic cells to 27.3±3.5% (Fig. 4B).

2.5. AST restored the MMP

In order to elucidate the protective effect of AST to the mitochondria, we monitored the changes in MMP in neurons by measuring the fluorescence intensity of Rhodamine 123. As shown in Fig. 5A, control (untreated) neurons incorporated and retained the dye, exhibiting a bright fluorescence, and H2O2 exposure resulted in a marked decrease of 51.8±6.9% in fluorescence intensity as compared with control cells (Fig. 5B). Pretreatment with AST (500 nM) restored the fluorescence intensity up to 74.3±7.3% of control cells.

3. Discussion

Results from multiple studies support the antioxidant and antiinflammatory properties of AST, establishing it as an
appropriate candidate for development as a therapeutic agent for cardiovascular oxidative stress and inflammation (Pashkow et al., 2008). To evaluate pharmacological neuroprotection of AST against the deleterious effect of cerebral ischemia–reperfusion injury, we used a model of transient focal cerebral ischemia in rats induced by 2 h MCAO and 24 h reperfusion which mimics many features of stroke in humans (Laing et al., 1993; Longa et al., 1989a). By using TTC staining, we proved that pretreatment of AST by oral administration before MCAO could significantly decrease cerebral infarct volume. Meanwhile, neurological deficit score was reduced. Moreover, according to the observation of the dose dependent effect, we found the dose of 80 mg/kg AST could produce the best neuroprotective effect in rats. Under this dosage, experiments on Nissl staining revealed that AST could reduce neuronal loss remarkably. These results suggested that AST could significantly protect brain against damage induced by ischemia–reperfusion. Some studies have also proved that AST possesses neuroprotective activity (Ikeda et al., 2008; Hussein et al., 2005; Shen et al., 2009). But so far there is no direct evidence that AST can cross the blood brain barrier under normal conditions. Additional research examining the transport across the blood brain barrier and brain distribution of AST after oral administration is needed. In addition, based on the existing studies (Rosenberg et al., 1998), we hypothesized that opening of the blood brain barrier caused by cerebral ischemia–reperfusion may provide a window for AST to reach brain.

Oxidative stress has been extensively studied in relation to pathophysiology of stroke which is suggested to play a critical role (Clemens, 2000; Gilgun-Sherki et al., 2002; Janardhan and Qureshi, 2004). It leading to ischemic cell death involves the formation of ROS/RNS through multiple injury mechanisms, such as mitochondrial inhibition, Ca++ overload, reperfusion injury, and inflammation (Coyle and Puttfarcken, 1993). The results of a growing number of studies suggest that post-ischemic oxidative stress contributes to brain injury and to expansion of ischemic lesion (Lakhan et al., 2009). Whether the neuroprotective effect of AST on focal cerebral ischemia in vivo is based on its antioxidant activity needs to evaluate.

H₂O₂, which has been found to be overproduced during ischemia–reperfusion of neural tissues (Chan, 2001; Li and Jackson, 2002; Sugawara and Chan, 2003), has been extensively used as an inducer of oxidative stress in vitro model (Lee et al., 2007; Satoh et al., 1996). The exposure of cultured cells to H₂O₂ results in an imbalance in energy metabolism and the deleterious effects of hydroxyl radicals on membrane lipids and proteins. H₂O₂ can cause neuronal cell death by inducing a delayed accumulation of extracellular glutamate and NMDA receptor stimulation with both effects being mediated by hydroxyl radicals (Halliwell, 1992; Mailly et al., 1999). Our present studies confirmed that treating cortical neurons with H₂O₂ resulted in a dose dependent viability loss. However, pretreatment with different concentrations of AST (250-
1000 nM), greatly decreased the cell viability loss. These results indicated that AST did significantly protect neuron cells from H2O2 induced cytotoxicity. H2O2-treated cells stained with fluorescent DNA binding dye, Hoechst 33342, displayed typical morphological features of apoptosis with sickle shaped-nuclei and AST pretreatment significantly reduced the apoptotic cells. As we know, mitochondrial structures are very susceptible to oxidative stress as evidenced by massive information on lipid peroxidation, protein oxidation, and mitochondrial DNA mutations. Mitochondrial have a central role in oxidative stress induced apoptosis, since cytochrome c release in the cytoplasm and opening of the permeability transition pore are important events in the apoptotic cascade (Lenaz, 1998). The loss of the MMP has been identified as the first steps in the apoptotic process (Zamzami et al., 1995). In this paper, our results were consistent with previous study that H2O2 can disrupt the MMP (Zhang et al., 2007), caused a drop in Rhodamine 123 fluorescence. When pretreatment with AST, the cells retained the dye and appeared brighter. Accordingly, we suggest that AST can protect neuron cells from H2O2 induced cytoxicity through antioxidant activity and the mitochondrial pathway.

Previous studies showed that AST protects against lipid peroxidation and oxidative damage to cell membranes, cells, and tissues through free radical scavenging activity (Lim et al., 1992). AST has a molecular structure similar to that of β-carotene. However, in contrast to 11 in β-carotene, it has 13 conjugated double bonds which gives it significantly greater antioxidant capacity (Shibata et al., 2001). AST stabilizes free radicals by adding them to its long double-bond chain rather than donating an atom or electron to the radical. Consequently, it can resist chain reactions that occur when a fatty acid is oxidized, thus allowing it to scavenge or quench longer than antioxidants that can’t stop this chain reaction (Kurashige et al., 1990). Moreover, AST has hydroxyl groups in the 3 and 3′ positions, making the molecule highly polar and dramatically enhancing its membrane function to protect against oxidative stress (Shibata et al., 2001). Because of its polar end groups, AST spans the cell membrane bilayer allowing it to sit near the lipid–water interface, where free radical attack first occurs and contributes to cell membrane mechanical strength (Palozza and Krinsky, 1992a). We considered that the free radical scavenging activity and the ability of enhancing membrane function may have contributed to the protective effect against H2O2-induced cytotoxicity and depolarization of MMP in this study. Our results also indicate that AST alone at 2000 nM showed significant cell death. The cytotoxic effect of AST which is peer reviewed (Liu et al., 2009) maybe due to its prooxygenic agent activity, just like other carotenoids. The nature of the biological environment in which AST resides will influence its behaviour (El-Agamey et al., 2004).

As discussed earlier, the NF-κB inflammatory pathway has been shown to be at least partially regulated by ROS and has been implicated in stroke (Wang et al., 2007). AST has been
shown to inhibit inflammation induced nitric oxide production and inflammatory gene expression by suppressing I-κB kinase-dependent NF-κB activation (Lee et al., 2003). A separate study demonstrated the ability of AST to suppress the development of inflammation in an endotoxin-induced uveitis model showed that in vivo AST (100 mg/kg) was as strong an antiinflammatory as prednisolone (10 mg/kg) (Ohgami et al., 2003). Accordingly, we hypothesized that the significant neuroprotective effect showed by AST at relatively high dose are not only due to its significant antioxidant property but also due to its antiinflammatory activity.

In recent times, of the more than 100 neuroprotective agents that reached randomized clinical trials in focal ischemic stroke, none has proven unequivocally efficacious (O’Collins et al., 2006). In this paper, our findings have verified the neuroprotective effect of AST against ischemia–reperfusion induced rat brain damage and the antioxidant activity of AST maybe partly responsible for it. These results, in conclusion, suggest that AST might be a promising neuroprotective agent which has potential clinical value.

4. Experimental procedures

4.1. Materials

AST, TTC, MTT, Hoechst 33342 and Rhodamine 123 were purchased from Sigma-Aldrich Corporation (Saint Louis, USA). H2O2 (purity=30%) was obtained from AMRESCO Corporation (Solon, USA). Culture mediums were purchased from Invitrogen Corporation (Carlsbad, USA). Common inorganic salts were purchased in China.

4.2. Animals and treatments

Male Sprague–Dawley rats (250–280 g body wt) were offered by the Experimental Animal Center of Nantong University, Nantong, China. Animals were housed in a room with temperature of 21–25 °C, relative humidity of 50–60%, and a 12-h light/12-h dark cycle. They had free access to food and water. All procedures used in this study were in accordance with our institutional guidelines, which comply with international rules and policies. Rats were randomly divided into six groups: sham-operated group, vehicle-pretreated group, 10 mg/kg NIM pretreated group and 20, 50, 80 mg/kg AST-pretreated groups. In these doses, no-observed adverse effects of the AST was found in rats (Stewart et al., 2008). Rats in AST-pretreated groups were received oral administration of AST (20, 50, 80 mg/kg) twice at 5 h and 1 h prior to ischemia in order to achieve persistent high plasma drug levels during ischemia–reperfusion (Kurihara et al., 2002). There is already evidence that NIM, when administered during the early stage of focal ischemia, can favorably modify the outcome of stroke by reducing the Ca2+ entry and free radicals levels during both the ischemic and reperfusion periods (Uematsu et al., 1989). In this study, 10 mg/kg of NIM was treated once at 1 h prior to ischemia as a positive control. AST and NIM were suspended in a 0.5% sodium carboxymethylcellulose solution. Rats in sham-operated group and vehicle-pretreated group

Fig. 3 – Protective effect of AST on H2O2 induced decrease in cell viability measured by MTT assay. (A) Cortical neuronal cells were incubated with different concentrations of H2O2 for 18 h. (B) Cortical neuronal cells were incubated with different concentrations of AST for 22 h. (C) Cortical neuronal cells were exposed to various concentrations of AST (250, 500, 1000 and 2000 nM) for 4 h and then were treated with H2O2 (50 μM) for 18 h. Cell viability was assessed by MTT assay. Values are percentage to the control (no drugs) and are mean±SD (n=6) and were analyzed by one-way ANOVA followed by Tukey’s multiple comparison tests as a post hoc comparison. *p<0.01 versus control, †p<0.05 and ‡p<0.01 versus H2O2.
were given 0.5% sodium carboxymethylcellulose solution intragastrically.

4.3. MCAO-induced focal cerebral ischemia in rats

After pretreatment with AST, rats were anesthetized using chloral hydrate (300 mg/kg, i.p.). The MCA was occluded with a 4-0 silicone-coated nylon suture treated with Heparin sodium (6250 U/mL) by surgical operation in terms of report by Gerriets et al., 2003). Throughout the procedure, body temperature was maintained at 37±0.5 °C with a thermostatically controlled infrared lamp. The 2 h after the induction of ischemia, the filament was slowly withdrawn and the animals were then returned to their cages for a period of 24 h of reperfusion. Sham-operated animals were subjected to the same surgical procedure, but the suture was not advanced beyond the internal carotid bifurcation. After revival from anesthesia, animals were housed back with room temperature 24±1 °C.

4.4. Determination of infarct volume

Animals were killed 24 h after reperfusion and brains were removed rapidly and frozen at −20 °C for 5 min. Coronal slices were made at 2 mm from the frontal tips, and sections were immersed in 2% TTC at 37 °C for 15 min. After the end of staining, color images of these slices were captured using a digital camera (Canon PowerShot S60, Japan). All brain slices of each experimental group were analyzed for the infarct volume with the Image-J analysis software (O’Donnell et al., 2004). Percentage infarct volume was calculated using the equation: \[ \frac{(V_C - V_L)}{V_C} \times 100 \], where \( V_C \) is the volume of control hemisphere and \( V_L \) is the volume of non-infarcted tissue in the lesioned hemisphere (Swanson et al., 1990).

4.5. Neurological deficit

Neurological deficit was measured at 24 h after reperfusion according to the method of Zea Longa test (Longa et al., 1989b). Neurological evaluation were scored on a 5-point scale: 0, no neurological deficit; 1, failure to extend left forepaw fully; 2, circling to the left; 3, inability to bear weight on the left; 4, no spontaneous walking with depressed level of consciousness.

4.6. Nissl staining

After 2 h ischemia and 24 h reperfusion, rats were anesthetized with 10% chloral hydrate (400 mg/kg, intraperitoneal injection) and perfused with 200 ml saline and subsequently with 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Rat brains were then removed and post-fixed for 24 h in the same fixative. The post-fixed brains were dry-protected in 25% sucrose in PBS. The brain tissue was then sectioned coronally 20 μm in thickness from 2.3 mm posterior to the bregma with a cryostat (LAICA CM1900, Germany). Sections from 4 rats in each group were treated with Nissl staining for histological
assessment of damage using a method described previously (Zhang et al., 2008). The number of neuronal cells in the border of the infarct area were compared.

4.7. Cell culture and treatment

The primary cortical neuronal culture was prepared using a method described previously (Ho et al., 2003) with minor modifications. In brief, the cortex was aseptically removed from 13- to 15-day-old ICR mice embryo, minced with sterile surgical blades, incubated in 0.125% trypsin for 10 min and dissociated by trituration in the DNase/trypsin inhibitor solution. Dissociated cortical cells were suspended in DMEM (Dulbecco’s modified Eagle’s medium) containing 20% inactivated fetal bovine serum and penicillin (100 U) and then seeded in 96-well plates and 24-well plates precoated with poly-L-lysine at 2.0×10^6/ml. The cultures were maintained at 37 °C in a humidified environment with 5% CO₂ in a CO₂ incubator (Thermo HEPA CLASS 100, USA). Non-neuronal cell division was inhibited by an exposure to 4 μM cytosine arabinoside for 48 h. After 7 days in culture, observation through a phase contrast microscope demonstrated that the cultures were estimated to be 96% neuronal cells. All experiments were performed after the cells were in culture for 7 days. In order to produce oxidative stress, H₂O₂ was freshly prepared from 30% stock solution prior to each experiment. AST was dissolved in DMSO. The final concentration of DMSO was 0.5%, which did not affect cell viability. The cells were pretreated with various concentrations (250, 500, 1000, and 2000 nM) of AST for 4 h followed by exposure to 50 μM H₂O₂ in the presence of the same concentrations of AST for another 18 h.

4.8. MTT reduction assay for neuronal viability

Neuronal viability was assessed by using an MTT assay as described by (Jiang et al., 1996). Briefly, the medium was removed after treatment as mentioned above. The cells were washed three times with FBS-free DMEM, followed by the further incubation with MTT solution (1 mg/mL) at 37 °C for 4 h. Yellow MTT was reduced to a purple formazan by mitochondrial dehydrogenase in live cells. The assay was stopped by the addition of a 100 μL lysis buffer (20% SDS in 50% N’N-dimethylformamide, pH 4.7). Optical density was measured at 570 nm by the use of a microplate assay reader (BioTek Synergy 2™, USA).
4.9. Measurement of apoptotic neuronal death

Apoptotic neuronal death was characterized by Hoechst 33342 staining (Ban et al., 2008). After post-incubation as described above, neurons were fixed in 4% paraformaldehyde at room temperature for 20 min and then stained with 1 mg/mL Hoechst 33342 in the incubation buffer for 15 min. Apoptosis features were observed by fluorescence microscope (Leica DM IRB, Germany) and pictures were taken by camera (Canon PowerShot S60, Japan). Neurons with fragmented or condensed DNA and normal DNA were counted and the data are presented as apoptotic neurons as a percentage of total neurons. Five different fields (containing about 40–50 cells each) were counted per well in three separate experiments.

4.10. Rhodamine 123 staining

After post-incubation as described above, cells were stained with Rhodamine 123 as previously reported (Palmeira et al., 1996). In brief, cells were incubated in Hanks’ solution containing 1 mg/mL of Rhodamine 123 for 15 min at 37 °C. Unbound dye was removed by washing cells twice with prewarmed (37 °C) buffered Hanks’ solution and the cells were bathed in 100 μL of Hanks’ solution. The fluorescence was measured at an excitation wavelength of 488 nm and an emission wavelength of 510 nm using a fluorescence microscope reader (Fu et al., 1999; Li et al., 2008) (BioTek Synergy 2™, USA). Fluorescence microphotographs were taken with an inverted microscope (Leica DM IRB, Germany).

4.11. Statistical analysis

All values are presented as mean±SD. The changes in variable parameters between drug treated and vehicle groups were analyzed by one-way ANOVA followed by Tukey’s multiple comparison tests as a post hoc comparison. Differences were considered statistically significant at a level of P<0.05.

Acknowledgments

This study was supported by grants from National Program on Key Basic Research Project (Project No. 2009CB930300) and from the Administration of Science and Technology of Jiangsu (Project No. BG2007607).

REFERENCES


