Neuropharmacology 84 (2014) 13–18

Contents lists available at ScienceDirect

Neuropharmacology

journal homepage: www.elsevier.com/locate/neuropharm

The protective effect of astaxanthin on fetal alcohol spectrum disorder in mice

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ARTICLE INFO

Article history:
Received 9 December 2013
Received in revised form
16 March 2014
Accepted 16 April 2014
Available online 26 April 2014

Keywords:
Ethanol
Embryo
Astaxanthin

ABSTRACT

Astaxanthin is a strong antioxidant with the ability of reducing the markers of inflammation. To explore the protective effect of astaxanthin on maternal ethanol induced embryonic deficiency, and to investigate the underlying mechanisms, we detected the morphology, expression of neural marker genes, oxidative stress indexes, and inflammatory factors in mice model of fetal alcohol spectrum disorder with or without astaxanthin pretreatment. Our results showed that astaxanthin blocked maternal ethanol induced retardation of embryonic growth, and the down-regulation of neural marker genes, Otx1 and Sox2. Moreover, astaxanthin also reversed the increases of malondialdehyde (MDA), hydrogen peroxide (H_2O_2), and the decrease of glutathione peroxidase (GPx) in fetal alcohol spectrum disorder. In addition, maternal ethanol induced up-regulation of toll-like receptor 4 (TLR4), and the down-streaming myeloid differentiation factor 88 (MyD88), NF-κB, TNF-α, and IL-1β in embryos, and this was inhibited by astaxanthin pretreatment. These results demonstrated a protective effect of astaxanthin on fetal alcohol spectrum disorder, and suggested that oxidative stress and TLR4 signaling associated inflammatory reaction are involved in this process.

1. Introduction

Fetal alcohol syndrome (FAS), a consequence of prenatal alcohol exposure, is diagnosed based on (i) prenatal and/or postnatal growth retardation; (ii) craniofacial abnormalities, including microcephaly, short palpebral fissures, and a deficient philtrum (Wattendorf and Muenke, 2005); and (iii) central nervous system dysfunction. FAS is the severe end of the spectrum of congenital abnormalities that alcohol can cause; the entire range of problems is referred to as fetal alcohol spectrum disorder (FASD). FASD has been estimated to affect 8–10 of 1000 newborns per year (Sampson et al., 1997). Thus, it is a major public health concern. Although scientists have performed many studies on FASD, the mechanisms causing the damage are not yet elucidated. Furthermore, there is no known effective strategy for prevention (other than ethanol abstention) or treatment.

Oxidative stress has been linked to the development of FASD (Henderson et al., 1995). Studies demonstrated that reactive oxygen species (ROS) accumulated in the fetuses of ethanol-consuming mothers and in neonatal animals fed with ethanol (Heaton et al., 2002; Henderson et al., 1995). Our previous studies showed that the generation of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) played a critical role in ethanol-induced brain damage (Peng et al., 2005; Long et al., 2010). Astaxanthin (AST), known as a carotenoid pigment, is a strong antioxidant which protected membranous phospholipids and other lipids against peroxidation (Palozza and Krinsky, 1992). Evidence showed that AST had up to several-fold stronger free radical antioxidant activity than vitamin E and carotene (Kurashige et al., 1990; Guerin et al., 2003). In double-blind, randomized controlled trials, AST was found to lower oxidative stress in several human health conditions (Kidd, 2011). Moreover, it is known that ROS up-regulate proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-1 (IL-1), and IL-6. High levels of these cytokines are associated with neurotoxicity. Whereas, AST has been found to reduce makers of

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http://dx.doi.org/10.1016/j.neuropharm.2014.04.013
0028-3908/

inflammation, e.g., TNF-α. Thus, AST has been deemed to be safe and has potential as a therapeutic antioxidant and anti-inflammation agent for further testing in human diseases (Fassett and Coombes, 2009). In this study, we explored the protective effect of astaxanthin on maternal ethanol induced embryonic deficiency, and investigated the underlying mechanisms.

2. Materials and methods

2.1. Animals

Wild type C57BL/6 (WT) mice were obtained from the Experimental Animal Center of Sun Yat-sen University, Guangzhou, China. And they were maintained in the Division of Laboratory Animal Resources, a facility accredited by the Association of the Assessment and Accreditation of Laboratory Animal Care International. All mice were housed in specific pathogen-free environment on a 12-h light/dark cycle (8:00–20:00) with access to chow and water *ad libitum*. Female mice selected for the experiment weighted from 18 to 22 g. All aspects of the animal care and experimental protocols were approved by the Animal Research Committee protocols of Sun Yat-sen University. Pregnancy was confirmed by the presence of vaginal plug and this was considered as gestational day 0 (E0).

To evaluate the effect of ethanol on embryonic development, successfully mated female mice were randomly divided into 4 ethanol (EtOH) groups and one control group. There were 6 mice in each group (3 for morphology assessment and 3 for RT-PCR and western blotting). Mice in the ethanol groups were given intraperitoneal ethanol (25%, v/v, dissolved in lactate Ringer's injection [LR]) at 10:30 on G8, at a dose of 0.02 ml/g body weight. Mice in the control group were injected with 0.02 ml/g of LR.

2.2. Experimental treatment

We obtained astaxanthin from Sigma–Aldrich Chemical Co. (St Louis, MO, USA). Along with intraperitoneal EtOH (0.02 ml/g, on G8), pregnant mice were given intraperitoneal AST (10 mg/ml, dissolved in normal saline buffer [NS]) at 10:00 on G7 and G8, at a dosage of 1.0, 10, 50, or 100 mg/kg body weight per day. Mice in the control group were given intraperitoneal NS (0.02 ml/g on G7 and G8) and intraperitoneal LR (0.02 ml/g on G8). There were 6 mice in each group (3 for morphology assessment and 3 for RT-PCR and western blotting). The embryo numbers for each group were: 64 in control group, 43 in EtOH group, 44 in EtOH + 1.0 mg/kg d AST group, 50 in EtOH+10 mg/kg·d AST group, 52 in EtOH + 50 mg/kg d AST group, 52 in EtOH + 100 mg/kg d AST group.

2.3. Morphology assessment

On G10.25, 3 pregnant mice in each group (randomly selected) were sacrificed, and the embryos were explanted using an anatomical microscope with the removal of the maternal decidua in phosphate buffer solution (PBS). Photos of whole embryos were taken by a digital camera under an anatomical microscope at a fixed magnification, and head length (HL, distance between the anterior end of the telencephalon and the posterior end of the midbrain), head width (HW, distance between the 2 sides of the telencephalon), and crown rump length (CRL) were measured. We photographed a transparent ruler at the same magnification, and all the measurements (HL, HW, and CRL) were calculated by comparison with the ruler image by use of Adobe PhotoShop CS3 extended (Adobe Systems, Inc., Mountain View, CA).

2.4. Real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

Embryos were dissected from pregnant mice on G9.25, and brains were collected and stored at −80 °C until use. Total RNA was extracted from brain tissues with the Trizol reagent (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized from 1 μg of total RNA using a Reaction Ready™ first-strand cDNA synthesis kit (SuperArray Bioscience Corporation). After incubation at 70 °C for 3 min and cooling down to 37 °C for 10 min, RT cocktail was added to the annealing mixture and further incubated at 37 °C for 60 min. Two microliters of 1:2 diluted cDNA was subjected to real-time quantitative PCR using Bio-Rad iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad). PCR was performed in a 25 μl volume using SYBR GreenER qPCR Super Mix for iCycler (Invitrogen). All primers were purchased from SuperArray Bioscience Corporation. All PCR assays were performed in triplicate. The reaction conditions were: 50 °C for 2 min, 95 °C for 3 min, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s, and 72 °C for 15 s. Beta-actin was amplified from all samples on each plate as housekeeping genes. The reaction mixtures without template cDNA were used as negative controls.

2.5. Western blot analysis

Embryos were dissected from pregnant mice on G9.25, and brains were collected and stored at −80 °C until use. Tissue was lysed in RIPA Lysis Buffer. The lysates were separated by 10% SDS–PAGE then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was then incubated at room temperature in 5% milk blocking buffer solution for 1 h, followed with the blocking solution containing first antibody overnight at 4 °C. Otx1, Sox2, toll-like receptor 4 (TLR4), myeloid differentiation factor 88 (MyD88), nuclear factor kappa B (NF-κB) p65, or β-actin was detected with specific primary antibodies (anti-Otx1 antibody at 1:1000 dilution, Chemicon; anti-Sox2 antibody at 1:1000 dilution, Chemicon; anti-TLR4 antibody at 1:1000 dilution, Chemicon; anti-MyD88 antibody at 1:1000 dilution, Chemicon; anti-NF-κB antibody at 1:1000 dilution, Chemicon; anti-β-actin antibody at 1:1000 dilution, Chemicon;). After washing three times with Tris buffer solution (TBS) for 5 min, the blot was incubated with a second antibody at 1:2000 dilution (Chemicon). The blot was again washed three times with TBS before being exposed to the SuperSignal West Dura Extended Duration substrate (Pierce).

2.6. Determination of oxidative stress indexes and inflammatory reaction indexes

On G9.25, embryo brains were collected and homogenized in NS and tested for H_2O_2 content with the BIOXYTECH H_2O_2-560 kit (Oxis International Inc., Portland, OR), for MDA with the BIOXYTECH MDA-586 kit (Oxis International Inc., Portland, OR), and for glutathione peroxidase (GPx) with the BIOXYTECH GPX-340 kit (Oxis International Inc., Portland, OR), according to the protocols provided by the manufacturer. Total protein was extracted and protein concentration was determined. Also, the tissue lysates were detected for TNF-α and IL-1β levels with ELISA kits (ASSAYPRO EMT 2010-5, EMI2200-1, MO, USA).

2.7. Statistical analysis

Descriptive results were expressed as mean ± SEM. Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) version 12.0. One-way ANOVA was used for multiple comparisons. Post hoc analyses, when appropriate, were performed using LSD. Pearson's correlation analysis was used to analyze the correlation of two variants. A p value less than 0.05 was considered statistically significant.

3. Results

3.1. Retardation of embryonic growth and down-regulation of neural marker genes were induced by ethanol, and were antagonized with AST

To investigate the underlying mechanisms and to further explore antagonists, we treated pregnant mice with ethanol and AST, and examined the embryonic growth indexes and the neural marker gene expression. A maternal treatment of 0.02 ml/g ethanol caused significant decreases of HL, HW, and CRL in embryos (Fig. 1). An intraperitoneal treatment of 50 or 100 g/kg/d AST antagonized ethanol-induced embryonic abnormalities. No significant differences were shown in embryo growth with the treatment of 0.02 ml/g ethanol and 100 g/kg/d AST as compared with the controls.

Otx1 and Sox2 are neural marker genes that increase in expression during neural development (Papanayotou et al., 2008; Simeone et al., 1993). We detected Otx1 and Sox2 expression by RT-PCR and western blotting in embryos with a maternal treatment of 0.02 ml/g ethanol, and found significant down-regulations (Fig. 2). An intraperitoneal treatment of AST antagonized reduced ethanol-induced down-regulation of Otx1 and Sox2 expression. Our results indicated that AST as an antagonist, protected against ethanol-induced embryonic damage.

3.2. Maternal administration of AST inhibited ethanol-induced embryonic oxidative stress

We detected the levels of H_2O_2, MDA and GPx in ethanol-administrated embryos with or without AST pretreatment. Maternal ethanol at a dose of 0.02 ml/g on G8 caused a significant increase in H_2O_2 and MDA, and a significant decrease in GPx, as compared with the control embryos (Fig. 3). These changes of oxidative stress indexes were reversed by a pretreatment of AST on G7 and G8 with a dose–response relation. This data confirmed that FASD was mediated by oxidative stress, and suggested that AST protected the embryos from ethanol-induced neurotoxicity via ROS inhibition.