Antihypertensive and Neuroprotective Effects of Astaxanthin in Experimental Animals

Ghazi HUSSEIN,*a,b Masami NAKAMURA,b Qi ZHAO,b Tomomi IGUCHI,b Hirozo GOTO,c Ushio SANKAWA,a and Hiroshi WATANABEB

*a International Research Center for Traditional Medicine, Toyama Prefecture; Toyama 939–8224, Japan; b Division of Pharmacology, and c Department of Kampo Diagnostics, Institute of Natural Medicine, Toyama Medical & Pharmaceutical University; Toyama 930–0194, Japan.

Received May 18, 2004; accepted October 5, 2004

Astaxanthin is a natural antioxidant carotenoid that occurs in a wide variety of living organisms. We investigated, for the first time, antihypertensive effects of astaxanthin (ASX-O) in spontaneously hypertensive rats (SHR). Oral administration of ASX-O for 14 d induced a significant reduction in the arterial blood pressure (BP) in SHR but not in normotensive Wistar Kyoto (WKY) strain. The long-term administration of ASX-O (50 mg/kg) for 5 weeks in stroke prone SHR (SHR-SP) induced a significant reduction in the BP. It also delayed the incidence of stroke in the SHR-SP. To investigate the action mechanism of ASX-O, the effects on PGF2α-induced contractions of rat aorta treated with Nω-nitro-l-arginine methyl ester (L-NAME) were studied in vitro. ASX-O (1 to 10 μg) induced vasorelaxation mediated by nitric oxide (NO). The results suggest that the antihypertensive effect of ASX-O may be due to a NO-related mechanism. ASX-O also showed significant neuroprotective effects in ischemic mice, presumably due to its antioxidant potential. Pretreatment of the mice with ASX-O significantly shortened the latency of escaping onto the platform in the Morris water maze learning performance test. In conclusion, these results indicate that astaxanthin can exert beneficial effects in protection against hypertension and stroke and in improving memory in vascular dementia.

Key words astaxanthin; hypertension; spontaneously hypertensive rat (SHR); carotenoid; nitric oxide (NO)

Astaxanthin (ASX), a red carotenoid pigment, is a biological antioxidant that occurs naturally in a wide variety of living organisms. ASX has shown some pharmacological activities, including anti-oxidative,1—4) anti-tumor and anti-cancer,5,6) anti-inflammatory,7,8) anti-diabetic9) and immunomodulatory activities.10,11) It was reported to exhibit free radical scavenging activity and to protect cells against lipid peroxidation and the oxidative damage of low-density lipoproteins (LDL)-cholesterol and of the cell membrane.12) The role of the oxidation of unsaturated lipids in LDL and its complex sequela in the pathogenesis of a number of cardiovascular diseases (CVD) has been widely investigated.13,14) Epidemiological and clinical data have indicated that dietary antioxidants, such as carotenoids, might protect against CVD15,16) and help to prevent several human diseases such as prostate cancer and inflammatory disorders.12)

In this study, we first investigated the effects of ASX on hypertension in rats in vivo. A dietary product of astaxanthin (ASX-O) was used in this experiment. The present study was also designed to investigate mechanisms of action and effects of ASX-O on vascular reactivity in rat aortic preparations. We evaluated the effects of ASX-O on the aorta in relation to the endothelium and nitric oxide (NO). Moreover, the effects of ASX-O administration on behavior were studied in mice.

To the best of our knowledge, this is the first report on the antihypertensive and neuroprotective effects of ASX in experimental animals; however a mixture composition for low-density lipoprotein and the oxidative damage of low-density radical scavenging activity and to protect cells against lipid peroxidation. Further, the present study was conducted as by Goto et al.18) Briefly, Wistar rats (♂, 20—40 weeks) weighing 350—450 g were anesthetized

MATERIALS AND METHODS

General Procedures Male rats of Wistar Kyoto (WKY) (7 weeks), Wistar standard breed (20—40 weeks), spontaneously hypertensive rat (SHR) (7 weeks) and stroke-prone SHR-SP strains (10 weeks) were used in this study. The neuropsychological tests were performed in accordance with the guidelines established by the Guide for the Care and Use of Laboratory Animals at Toyama Medical and Pharmaceutical University.

Measurement of Blood Pressure and Heart Rate in Conscious Rats Arterial blood pressure (BP) and heart rate (HR) were determined by a tail cuff system. The rats were lightly supported in a mesh holder made of cloth and maintained at 37 ± 1°C (Model THC-1 Digital Thermo, Softron, Tokyo, Japan). BP from the tail artery was indirectly measured using a tail cuff apparatus (BP-98, Softron, Tokyo) which was controlled with a personal computer. Values are presented as the average of three separate measurements.

Relaxation Experiments Using Rat Aorta The experiments were conducted as by Goto et al.18) Briefly, Wistar rats (♂, 20—40 weeks) weighing 350—450 g were anesthetized

© 2005 Pharmaceutical Society of Japan
(50 mg/kg i.p. pentobarbital) and sacrificed by cutting the abdominal aorta. Aortic rings (3 mm) were prepared from the thoracic aorta, mounted on steel hooks in a Magnus chamber (Kishimoto UC-5TD, Kyoto, Japan) and attached to a force-displacement transducer (Kishimoto UM-203, Kyoto) and a recorder (Recti-Horiz 8K, NEC, Tokyo). Baths were filled with Krebs solution maintained at 37 °C and bubbled continuously with 5% CO₂ in O₂ at a pH of 7.4. The rings were equilibrated for 40 min at an initial resting tension of 1 g, and then contracted with 60 mM KCl to determine the optimal resting tension. When the contraction reached a steady maximal response, 10⁻⁶ M acetylcholine (ACh) was added to confirm the status of the endothelium. ACh induced relaxation in rings with intact endothelium, whereas the relaxation disappeared in the denuded ones. The Krebs solution was then replaced and the experiments were carried out.

In a series of experiments, each of the rings was contracted by treatment with 3 × 10⁻⁶ M PGF₂α and then ASX-O was added at concentrations ranging from 1 × 10⁻⁶ to 1 × 10⁻⁴ M cumulatively. Relaxation was expressed as the percentage of the decrease in maximal tension obtained by PGF₂α-induced contraction.

The effects of ASX-O on the responses induced by the xanthine/xanthine oxidase (XOD) system were studied by the addition of xanthine and XOD to ASX-O-pretreated aortic rings.

To investigate the effects of nitric oxide (NO), the preparations with the endothelium were exposed to 10⁻⁴ M N⁶-nitro-1-arginine methyl ester (L-NAME) for 60 min prior to the addition of ASX-O. In some experiments, the endothelial lining of the rings was removed by pressing the ring slightly and rolling it gently onto a filter paper few times. Removal of the endothelial lining was functionally confirmed by ACh, as described above.

**Transient Cerebral Ischemia**

ICR mice (δ, 8 weeks) were subjected to transient cerebral ischemia induced by bilateral common carotid artery occlusion, 2 vessel occlusion (2VO), 1 h after administration of the substance as described by Watanabe et al. In brief, the mice were anesthetized with pentobarbital sodium (50 mg/kg i.p.), and the arteries were exposed and carefully separated from the adjacent veins and sympathetic nerves, then occluded by artery clips (Roboz Surgical Instruments, MD, U.S.A.) for 20 min. While the arteries were clamped, 0.3 ml of blood was withdrawn from the tail vein. Then, the clips were removed and cerebral blood flow was restored. The skin incision was closed and the mice were kept in an air-conditioned room at 25 ± 1 °C. Sham-operated mice were subjected to the same procedure without carotid clamping and withdrawal of the blood.

**Morris Water Maze Learning Performance**

One day before the start of learning, mice were given a pre-training session in which they were allowed to swim freely in a pool (70 cm diameter, with a depth of 13 cm of water maintained at 25 ± 1 °C) for 60 s without an escape platform. The pool was placed in a dimly lit test room and surrounded by visual cues. In the learning block, the pool was filled to the 13 cm depth with water, and a platform (5 cm diameter) was situated 1 cm below the surface of the water. The pool was divided into four quadrants, with the platform in a fixed position in one quadrant. Daily learning consisted of four trials in which the mouse was placed in the water from four different starting points, and the latency of escaping onto the platform was recorded. This was conducted for 5 consecutive days. A maximum of 60 s was allowed for the mouse to find the platform and climb onto it. On day 7, each mouse was subjected to a probe trial in which there was no platform present. The time of crossing the former platform quadrant and the total time of crossing all quadrants were recorded for 1 min.

**Statistical Analysis**

Statistical significance was determined by Student’s t-test for unpaired observations and by the Mann–Whitney Rank Sum Test. One-way analysis of variance (ANOVA) was performed for multiple comparisons between the groups. The significance between different groups in the Morris water maze test was analyzed by two-way repeated measures ANOVA. Differences with p < 0.05 were considered statistically significant.

**Chemicals**

Prostaglandin F₂α (PGF₂α), xanthine, xanthine oxidase (XOD), N⁶-nitro-1-arginine methyl ester (L-NAME), dimethyl sulphoxide (DMSO) and acetylcholine chloride (ACh) were obtained from Wako (Wako Pure Chemicals, Tokyo, Japan), and pentobarbital sodium was from TCI, Tokyo Kasei, Tokyo.

**RESULTS**

**Antihypertensive Effects of ASX-O**

In the first series of experiments, the effects of oral administration of ASX-O on BP in WKY rats (δ, 7 weeks) and SHR (δ, 7 weeks) were investigated. A single administration of ASX-O at doses of 5, 50 and 500 mg/kg p.o. did not affect the BP or HR in both WKY rats and SHR. Then, ASX-O was administered at 50 mg/kg p.o. to WKY rats and SHR once daily for 2 weeks. ASX-O significantly lowered the BP in SHR, while it did not produce any changes in WKY rats (Fig. 1).

Thereafter, the effect of long-term administration of ASX-O at the doses of 5 and 50 mg/kg, p.o., was examined in SHR-SP (δ, 10 weeks), which showed BP over 200 mmHg. ASX-O at the dose of 5 mg/kg produced no significant change in BP after one week of administration, although it induced a progressive reduction in BP from the second week. In contrast, at the dose of 50 mg/kg, ASX-O showed the BP lowering effect from the first week. After 5 weeks of administration, ASX-O produced a significant reduction in SBP (−6%, −4%), DBP (each −10%) and MBP (−9%, −8%) at doses of 5 mg and 50 mg/kg, respectively. On the other hand, the BP of the control group increased nearly 18 mmHg (Fig. 2). There was no significant change in the HR after ASX-O treatment. Moreover, ASX-O did not modify or show any effect on body weight in either the SHR-SP or WKY rats (data not shown).

**Time Course Effects of ASX-O on BP**

The effects on BP were measured 40 min before administration, at 0 min, and every 40 min after the administration for a 4-h period.

Compared to the control, ASX-O (50 mg/kg) administration produced significant BP lowering effects. The effects on MBP were significantly different from the control after 80 min (Fig. 3). An initial effect was shown shortly after administration, but within the first 40 min, as indicated by 0 min time in Fig. 2, suggesting an acute phase of effect by ASX-O. The HR did not show significant change during the time course (p > 0.1) (data not shown).

**Effects of ASX-O on Stroke Incidence**

In this study,
ASX-O (50 mg/kg) significantly delayed the incidence of stroke in SHR-SP. By day 4 of the post-treatment period, the stroke rate was 60% in the control and lower dose (5 mg/kg) groups, whereas the third group (50 mg/kg) did not show any sign of stroke. On day 7, the ASX-O (50 mg/kg) group showed a small incidence of stroke (20%) compared to the control that in which the incidence increased to 80% on day 14 (Fig. 4).

Vascular Reactivity of ASX-O on Rat Aorta, in Vitro. Effects on Contraction Induced by the Xanthine/Xanthine Oxidase (XOD) System In the first relaxation experiment, the $1 \times 10^{-4} \text{M}$ L-NAME-pretreated preparations were treated with ASX-O ($1 \times 10^{-3} \text{M}$ and $1 \times 10^{-2} \text{M}$), separately. After 10 min of treatment, xanthine ($1 \times 10^{-4} \text{M}$) was added, followed by XOD (10 mU/ml) 5 min later. A transient contraction was thereby induced. The xanthine–XOD system produced 10.8% contraction, whereas ASX-O ($1 \times 10^{-3} \text{M}$) produced 8% and ASX-O ($1 \times 10^{-5} \text{M}$) 6.9% in the system-treated aorta (Fig. 5).

Effects of ASX-O and Nitric Oxide (NO) on Contraction Induced by Prostaglandin PGF$	ext{2}_{\alpha}$ After washing the preparations and equilibrating them with Krebs solution,
some preparations were contracted by treatment with PGF2α (1×10^{-6} M). When the PGF-induced contraction reached a plateau, ASX-O was added separately at concentrations ranging from 1×10^{-7} to 1×10^{-4} M.

A similar experiment was carried out simultaneously, in which the preparations were pretreated with L-NAME (1×10^{-5} M) for 60 min before the addition of PG. L-NAME is known to inhibit NO synthesis. In this experiment, ASX-O showed relaxation effects on the preparations with (+) or without (−) L-NAME-pretreatment. Relaxation was greater [percentage: 64.2, (−); 45, (+), and 63.4, (−); 14.6, (+)] at the concentrations of 1×10^{-4} M (100 μM) and 3×10^{-5} M (30 μM), respectively, while the lesser concentrations, i.e. 1×10^{-6}−1×10^{-5} M (1−10 μM), showed smaller effects (≤11.2%) (Fig. 6A). Dimethyl sulphoxide (DMSO), used to dissolve ASX-O, showed no significant effect on the preparations at concentrations ≤1%.

**Endothelium-Dependent and Endothelium-Independent Relaxation** Endothelium-dependent (ED) and endothelium-independent (EIND) relaxation experiments were carried separately on aortic preparations with intact and denuded endothelia, respectively. ASX-O produced both ED and EIND relaxations when added at cumulative concentrations of ASX-O (1×10^{-8} to 1×10^{-5} M). The intensity of the ED relaxation produced by ASX-O was greater than the EIND relaxation, 95.4% and 70.9%, respectively (Fig. 6B). Moreover, the onset and cumulative effects of the relaxation were faster and more intense in the ED relaxation than in the EIND one.

**Neuroprotective Effects of ASX-O in Mice, in Vivo**

The effects of ASX-O on the transient ischemia-induced impairment of Morris water maze performance in mice were studied in ICR mice. Transient cerebral ischemia was induced by bilateral common carotid occlusion, 2 vessels occlusion (2VO). ASX-O was given orally to the animals 1 h before the ischemia. Two days after the ischemia, the trial was performed at a rate of four trials/block/d for 5 d. The time course of change in the latency of escaping to the pool platform was recorded. The swimming time in the platform quadrant was recorded at the probe trial for 1 min after the platform was removed on day 7 of the test. These experiments showed that pretreatment of mice with ASX-O, (55, 550 mg/kg) significantly shortened the latency of escaping onto the platform, and increased the time of crossing the former platform quadrant in the probe trial (Fig. 7).

**DISCUSSION**

The role of ASX, as well as of many other antioxidant carotenoids (e.g. β-carotene, lycopene), in reducing atherogenesis and lowering the risk of some coronary heart diseases has been previously studied.20—23 However there is no previous report on the antihypertensive action of ASX. In the present study, dietary ASX-O showed a significant antihypertensive effect by lowering the BP in SHR compared to the control. It produced this effect in SHR but not in WKY. It also delayed the incidence of stroke in SHR-SP. The administration of ASX for 5 weeks produced a significant reduction in the MBP (−9, −8%), as well as in the SBP (−6, −4%) and DBP (each −10%) (data not shown) at the doses of 5 mg and 50 mg/kg, respectively, in SHR-SP. The time course effect of ASX-O suggests that ASX-O may have an initial acute effect on BP, probably due to a sympatholytic effect; however, further studies are needed to confirm this. The present results suggest that ASX-O can attenuate the development of hypertension and may help to protect the brain against stroke and ischemic insults.

There is a number of reports indicating that ASX is a more powerful antioxidant than other carotenoids and vitamin E, and that it may confer numerous health benefits.7,24—26 The antioxidative properties of ASX had been demonstrated in a number of different biological membranes.7,27—29 In the present study, when ASX-O was incorporated with the xanthine/XOD system, it produced relaxation of the transient contraction of the aortic rings. This transient contraction was produced by oxygen-derived free radicals that were gener-
In this study, ASX-O also showed vascular effects on the aortic preparations by inducing ED and EID vasorelaxation effects. The ED effect might result from antioxidative properties, presumably the superoxide scavenging effects of ASX in preventing superoxide-induced NO degradation, which would thus prolong its half-life and consequently its vasorelaxation effect. Therefore, the restoration of NO-induced vasorelaxation may explain, partially, the antihypertensive effects of ASX-O. In the current experiments, the effective vasorelaxant concentrations of ASX-O (30—100 μM) produced relaxation on aortic responses. This relaxation is NO-dependent at the lower dose of ASX-O (30 μM), and NO-independent at the higher dose (100 μM), as depicted in the charts and graph of Fig. 6A. However, more intensive investigations are necessary to verify the mechanisms of these effects.

Fig. 6. Vascular Reactivity of ASX-O on Rat Aorta
(A) Nitric oxide (NO)-related vasorelaxation induced by ASX-O on the aortic rings of Wistar rat, pretreated with 1×10⁻⁴ M L-NAME (+) and without pretreatment (−). All the rings had intact endothelium. ASX-O at 3×10⁻⁵ M (30 μM) produced higher relaxation (−) without L-NAME, indicating NO-dependent relaxation (6A-upper charts). At 1×10⁻⁴ M (100 μM), ASX-O showed greater relaxation (+) with L-NAME, suggesting NO-independent relaxation (6A-lower charts). ∗p<0.05 vs. the corresponding L-NAME (+) group (t-test). (B) Endothelium-dependent (ED) and endothelium-independent (EIND) relaxation induced by ASX-O. ASX-O at cumulative concentrations (1×10⁻⁸—1×10⁻⁵ M) showed ED relaxation (6B-upper chart) and EIND ones (6B-lower chart). Values are expressed as a percentage of decrease in the maximal tension contracted with 1×10⁻⁵ M PGF₂α. Data represent the mean±S.E.M. (n=4).
In addition, ASX-O showed significant neuroprotective effects at relatively high doses by preventing the ischemia-induced free radicals and their consequent pathological cerebral and neural effects. The current result indicates that ASX-O may have beneficial effects in improving memory in vascular dementia.

Some clinical studies have revealed that healthy adults can safely consume 6 mg of ASX per day from a *Haematococcus pluvialis* algal extract. In human blood, ASX is carried by the lipoproteins VLDL, LDL and HDL. ASX was reported to reduce LDL oxidizability in *vitro* and *ex vivo*, and a daily dosage as low as 3.6 mg of ASX for two consecutive weeks demonstrated that ASX could protect LDL-cholesterol against induced-oxidation in an *in vitro* test. A number of ASX-containing health products are under development based on these findings. Nevertheless, prospective clinical studies addressing the protective effects of ASX in essential hypertensive patients are crucial and recommended.

Acknowledgements This work was supported by a grant from the Regional R and D Consortium Project sponsored by the Ministry of Economy, Trade and Industry, Japan. It was also supported, in part, by a grant from the Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER). Ms. Masami Nakamura was supported by a grant from Hong Kong University. We would like to thank the grants’ sponsors, and to acknowledge Fuji Chemical Industry Co., Ltd., Toyama for supplying the astaxanthin product.

REFERENCES