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Antihypertensive Potential and Mechanism of Action of Astaxanthin: II. Vascular Reactivity and Hemorheology in Spontaneously Hypertensive Rats

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The current study was designed to determine the effects of a dietary astaxanthin (ASX-O) on vascular reactivity in spontaneously hypertensive rats (SHR), in order to verify its antihypertensive action mechanism. We evaluated contractions induced by phenylephrine (Phe), angiotensin II (Ang II) and the xanthine/xanthine oxidase (Xan/XOD) system, and relaxations induced by sodium nitroprusside (SNP) as well as endothelium-dependent relaxations mediated by acetylcholine (ACh) in thoracic aorta of the SHR, with and without ASX-O intervention. We also investigated the effects of ASX-O on blood rheology using a microchannel array system. In this study, ASX-O showed a significant modulatory effect on nitric oxide (NO)-induced vasorelaxation by the NO-donor SNP ($p < 0.05$). However, it did not show significant effects in restoring the impaired endothelium-dependent relaxation to ACh in the SHR. On the other hand, the constrictive effects by Phe, Ang II and Xan/XOD were ameliorated by ASX-O ($p < 0.05$). ASX-O also demonstrated significant hemorheological effect by decreasing the microchannel transit time of whole blood. In conclusion, the results suggest that ASX-O may act in modulating the blood fluidity in hypertension, and that the antihypertensive effects of ASX-O may be exerted through mechanisms including normalization of the sensitivity of the adrenoceptor sympathetic pathway, particularly [alpha]-adrenoceptors, and by restoration of the vascular tone through attenuation of the Ang II- and reactive oxygen species (ROS)-induced vasoconstriction.

Key words astaxanthin; hypertension; mechanism; vasorelaxation; blood rheology

Diseases such as hypertension, atherosclerosis, hyperlipidemia, and diabetes are associated with vascular, functional and structural changes including endothelial dysfunction, altered contractility and vascular remodeling. The role of vascular endothelium in modulating vascular tone and maintaining cardiovascular homeostasis in blood vessels, through the production of an array of both relaxant (e.g., nitric oxide [NO], prostacyclin) and constrictor factors (such as thromboxane, endothelin), has been widely reported.^{1–4)}

The development of hypertension is accompanied by changes in the rheological properties of blood, particularly by an increased red blood cell (RBC) aggregation, leading to further pathological complications. Plasma viscosity contributes to the cardiovascular risks and may be of special importance in cases of reduced blood flow, as commonly occurs in patients with advanced atherosclerosis,⁵⁾ and it is also related to the extension of coronary heart disease.^{6,7)} Effects of rheological properties of blood on thrombogenesis and atherosclerosis has been cited in several reports.^{8–10)}

We previously investigated blood pressure (BP) lowering-effects of the dietary astaxanthin (ASX-O) in spontaneously hypertensive rats (SHR).¹¹⁾ The current study was designed to investigate the antihypertensive mechanisms of action of ASX-O by investigating its effects on vascular reactivity in the SHR. Contractile and relaxant responses of the vascular system were studied on aortic rings from ASX-O-treated SHR. Moreover, the effects of ASX-O on blood fluidity and rheology were evaluated by microchannel transit time analysis.

MATERIALS AND METHODS

General Procedures Male SHR (7 weeks old) were used in this study. The animals were obtained from colonies of specific pathogen-free rats maintained by Japan Shizuoka Laboratory Company (Japan SLC, Shizuoka, Japan). Housing conditions were thermostatically maintained at $24 \pm 1^\circ\text{C}$ with constant humidity (60%) and lighting (12 h light/dark cycle, light on: 07:30–19:30). The animals were housed for at least 1 week before the experiments and fed a normal diet (Lab MR, NOSAN, Yokohama, Japan) and water given *ad libitum*. Body weights were measured daily during the experimental period. ASX-O, composed of 5.5% astaxanthin (ASX) in edible oil base, was obtained from Fuji Chemical (Fuji Chemical Industry Co., Ltd., Toyama, Japan) and dissolved and diluted in olive oil (Wako Pure Chemicals, Osaka, Japan). Administered doses were calculated as ASX in the dietary ASX-O. The animals were divided into two groups (8 rats/group) and were treated daily for 7–9 weeks. One group was administered ASX (5 mg/kg/d, *p.o.*) and the other one was similarly treated with olive oil (1 ml/kg/d).

All experimental procedures were performed in accordance with the standards established by the 'Guide for the Care and Use of Laboratory Animals at Toyama Medical and Pharmaceutical University'.

Measurement of BP and Heart Rate (HR) in Conscious Rats Arterial BP and HR were determined by a tail cuff system. The rats were lightly supported in a holder made of cloth mesh and maintained at $37 \pm 1^\circ\text{C}$ (Model THC-1 Digital Thermo, Softron, Tokyo, Japan). BP from the tail artery

was indirectly measured using a tail-cuff apparatus (BP-98, Softron) which was controlled with a personal computer. Values are presented as the average of three separate measurements.

Measurement of Blood Cell Counts and Plasma Fibrinogen Blood from the heart of each sacrificed SHR was separately collected into heparinized syringes containing 5% heparin and 2% sodium citrate, and was instantly and gently mixed. A small portion of the blood was used for the cell counts which were determined by a cell counter (Celltac α , Nihon Kohden, Tokyo). Plasma was immediately separated from the other portion of the blood by centrifugation at 3000 *g* for 15 min (Kubota 8700, Kubota, Tokyo). The measurement of plasma fibrinogen was performed at the Special Reference Laboratories (SRL) (Tokyo).

Vascular Reactivity Experiments The experiments were conducted as previously described by Goto *et al.*¹²⁾ Briefly, ASX-O- and olive oil-treated SHR (δ , 14–16 weeks age, 254–320 g body weight) were anesthetized (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 (Niko Bioscience T-634, 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 6 × 10⁻² M potassium chloride (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.

Contractile Responses In a series of experiments, each of the rings was pretreated with 10⁻⁴ M N^G-nitro-L-arginine methyl ester (L-NAME) for 60 min and then tested agonists were added, separately. In one experiment, 10⁻⁴ M xanthine was added for 5 min followed by 10 mU/ml xanthine oxidase (XOD). In two other sets, angiotensin II (Ang II) was added at 10⁻⁷ M and 10⁻⁶ M, separately. The contraction was calculated as a percentage of the corresponding contraction induced by 6 × 10⁻² M KCl.

Relaxant Responses In another series of experiments, each of the rings was contracted by treatment with 5 × 10⁻⁷ M phenylephrine (Phe). When the contraction reached a plateau, ACh was added at concentrations ranging from 10⁻⁹ to 10⁻⁴ M, cumulatively. In another set, similar cumulative concentrations of sodium nitroprusside (SNP) were added. The relaxation was expressed as a percentage of the decrease in the maximal tension contracted with 5 × 10⁻⁷ M Phe.

Microchannel Array Flow Analysis The transit time of a whole blood through the microchannel array was measured and used as a marker index of blood fluidity. The detailed procedures and apparatus of the microchannel analysis (Microchannel Flow Analyzer [MC-FAN] type KH-2, Hitachi Haramachi Electronics Co., Ltd., Hitachi, Tokyo) were described previously.^{13,14)} Briefly, microgrooves formed on the surface of a single crystal silicon substrate were converted to

leak-proof microchannels by covering them tightly with an optical flat glass plate. The microgrooves in the silicon microchannel chip (Bloody-6-5, 8736 channels; width, 5 μ m; depth, 4.5 μ m; length, 20 μ m—Hitachi Haramachi Electronics Co., Ltd.) were pre-filled with saline. The heparinized whole blood samples were forced to flow through the microchannels under a pressure difference of 20 cm H₂O. To assess the filterability of the whole blood, the transit time for each 20, 40, 60, 80 and 100 μ l of blood was determined. These measurements were performed immediately after blood sampling at a room temperature between 20 and 25 °C. The MC-FAN was calibrated with the saline before each new measurement. The blood passage through an individual channel was observed and recorded using a video microscope system (WAT-231S, WATEC, Tokyo).

Statistical Analysis Statistical significance was determined by the Student's *t*-test for unpaired observations and Mann-Whitney Rank Sum Test. One-way Analysis of Variance (ANOVA) was performed for multiple comparisons between the groups. Differences with *p* < 0.05 were considered statistically significant.

Drugs and Chemicals All the reagents used in these experiments were of analytical grades. Xanthine, XOD, L-NAME, ACh, Phe, SNP, KCl and Krebs solution reagents were obtained from Wako (Wako Pure Chemicals, Tokyo). Other materials were purchased as follows: pentobarbital sodium from Tokyo Chemical Industry (TCI) (Tokyo Kasei, Tokyo), heparin from Mochida (Novo-Heparin, Mochida, Tokyo), sodium citrate from Fuso (Fuso, Osaka) and Ang II from Calbiochem (Calbiochem, CA, U.S.A.).

RESULTS

Effects of ASX-O on the BP In this study, ASX-O showed significant BP-lowering effects on the arterial BP, as represented by the effect on the mean blood pressure (MBP) (*p* < 0.05) (Fig. 1) and summarized in Table 1. Throughout the 7 weeks treatment period, the daily administered ASX-O showed no significant or consistent effect on the HR, however, a decrease in the HR was exhibited on week 7. The body weight (BW) of ASX-O-treated SHR was not changed compared to the control group.

Blood Cell Counts and Plasma Fibrinogen ASX (5 mg/kg) neither changed the blood cell count indices nor did it cause a significant change in the plasma fibrinogen level compared to the control (Table 2).

Table 1. Effects of ASX-O on BP, HR and BW in SHR in the 7th Week of the Treatment

| | Control-SHR | ASX-SHR |
|--------------|-------------|----------------------|
| SBP, mmHg | 197 ± 3 | 173 ± 1 [†] |
| DBP, mmHg | 163 ± 2 | 135 ± 1* |
| MBP, mmHg | 174 ± 2 | 148 ± 1* |
| HR, beat/min | 362 ± 12 | 280 ± 6* |
| BW, g | 266 ± 3 | 260 ± 4 |

Control- and ASX-SHR were treated with olive oil (1 ml/kg/d) and ASX (5 mg/kg/d), respectively. SBP=systolic blood pressure, DBP=diastolic BP, MBP=mean BP, HR=heart rate and BW=body weight. The values represent the mean ± S.E.M. (*n* = 8 rats per group). **p* < 0.05 vs. the control group (*t*-test), [†]*p* < 0.05 vs. the control group (Mann-Whitney Rank Sum Test).

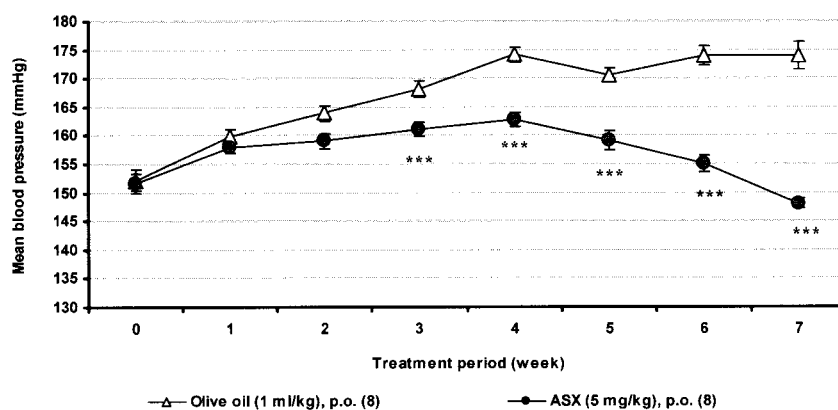


Fig. 1. Effects of Oral Administration of ASX-O on MBP in SHR

Each data point represents the mean \pm S.E.M. of 8 rats per group. *** $p < 0.001$ vs. the vehicle control group (t -test).

Table 2. Blood Cell Counts and Plasma Fibrinogen Level in SHR in Week 7 of the Treatment with ASX-O and Olive Oil

| | WBC ($\times 100/\mu\text{l}$) | RBC ($\times 10000/\mu\text{l}$) | HGB (g/dl) | HCT (%) | MCV (fl) | MCH (pg) | MCHC (g/dl) | PLT ($\times 10000/\mu\text{l}$) | Fibrinogen (mg/dl) |
|---------|-------------------------------------|---------------------------------------|----------------|----------------|----------------|----------------|----------------|---------------------------------------|-----------------------|
| Control | 43.5 \pm 4.1 | 858.2 \pm 5.1 | 14.7 \pm 0.1 | 45.3 \pm 0.3 | 52.7 \pm 0.2 | 17.1 \pm 0.1 | 32.5 \pm 0.2 | 60.7 \pm 3.4 | 285.5 \pm 7.4 |
| ASX | 40.4 \pm 3.5 | 833.3 \pm 7.1 | 14.2 \pm 0.1 | 44.2 \pm 0.4 | 53.0 \pm 0.3 | 17.0 \pm 0.2 | 32.1 \pm 0.2 | 55.1 \pm 4.4 | 276.6 \pm 1.8 |

Blood cell counts of SHR treated with the control olive oil (1 ml/kg/d) and ASX (5 mg/kg/d) for 7 weeks. WBC=white blood cell count, RBC=red BC, HGB=hemoglobin, HCT=hematocrit, MCV=mean cell volume, MCH=MC hemoglobin, MCHC=MCH concentration and PLT=platelet count. Data represents the mean \pm S.E.M. ($n=6-8$ rats).

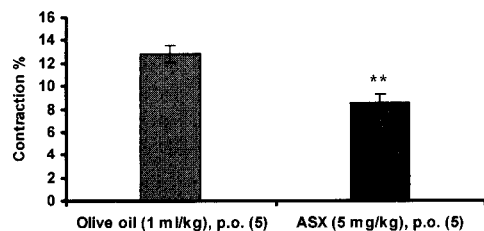


Fig. 2. Effects on Xan/XOD-Induced Contraction in SHR Group Treated with ASX

Data represent the mean \pm S.E.M. ($n=5$). ** $p < 0.01$ vs. the control (t -test).

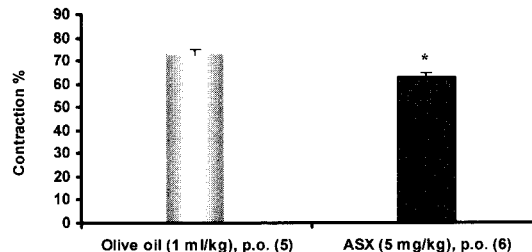


Fig. 3. Contractile Response to Phe in ASX-O-Treated SHR

Contraction was induced by 5×10^{-7} M Phe, and calculated as a percentage of the corresponding contraction induced by 6×10^{-2} M KCl. Data are the mean \pm S.E.M. ($n=5-6$). * $p < 0.05$ vs. the control (t -test).

Vascular Reactivity of ASX-O on Rat Aorta, ex Vivo

1) Effects on Contraction Induced by the Xanthine/XOD System: The xanthine/XOD-induced contraction of the aortic preparations was significantly reduced in the ASX-O-treated SHR compared to the olive oil-treated ones ($p < 0.05$) (Fig. 2).

2) Contractile Responses to Phe and Ang II: ASX-O demonstrated a significant reduction of the contractile responses of the aortic preparations to Phe (at 5×10^{-7} M) ($p < 0.05$) (Fig. 3) and the responses to Ang II (10^{-7} M) ($p < 0.001$) (Fig. 4), compared to the vehicle control.

3) Vasorelaxant Responses to SNP and ACh: The vasorelaxant response to SNP (at 10^{-9} – 10^{-8} M) was significant in the ASX-O-treated group compared to the control group (Fig. 5). However, the response to ACh was not significant (Fig. 6).

Microchannel Array Flow Analysis The effects of ASX-O on the blood fluidity were evaluated by the microchannel transit time of the whole blood, measured by the MC-FAN. The transit time of the blood was significantly decreased in the ASX-O-treated SHR compared to the control ($p < 0.05$) (Fig. 7).

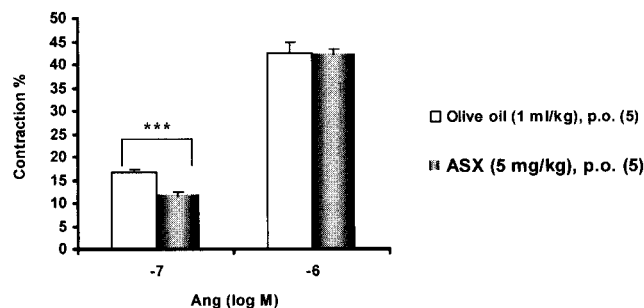


Fig. 4. Contractile Response to Ang II in SHR Treated with ASX-O

Contraction was calculated as a percentage of the corresponding contraction induced by 6×10^{-2} M KCl. Data represent the mean \pm S.E.M. ($n=5$). *** $p < 0.001$ vs. the vehicle control (t -test).

DISCUSSION

We previously reported the BP lowering effects of astaxanthin (ASX-O) in SHR. In the current study, in order to verify the antihypertensive mechanisms of action of this substance, we studied the effects of its oral administration in the SHR

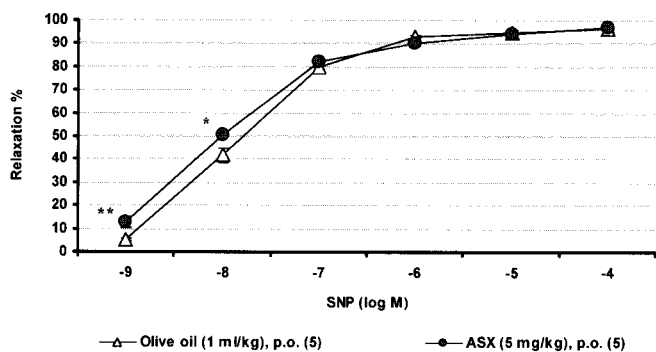


Fig. 5. Vasorelaxant Response to SNP in ASX-O-Treated SHR

Values are expressed as the percentage of decrease in maximal tension contracted with 5×10^{-7} M Phe. Data represent the mean \pm S.E.M. ($n=5$). * $p < 0.05$; ** $p < 0.01$ vs. the control (t -test).

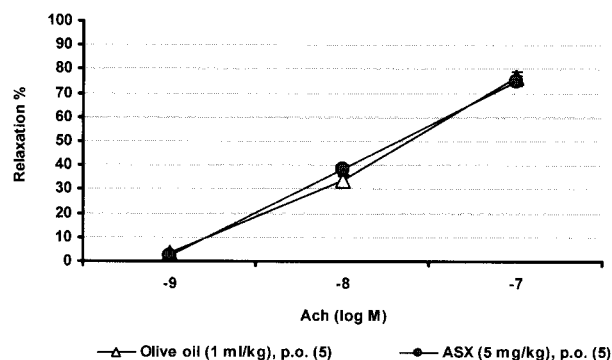


Fig. 6. Vasorelaxant Response to ACh in SHR Treated with ASX-O

Values are expressed as a percentage of the decrease in maximal tension contracted with 5×10^{-7} M Phe. Data are the mean \pm S.E.M. ($n=5$).

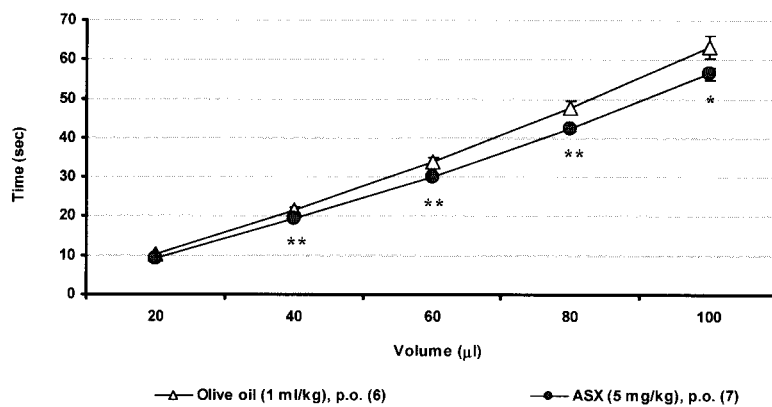


Fig. 7. Microchannel Transit Time of Whole Blood of SHR Treated with ASX-O and Olive Oil

Data represent the mean \pm S.E.M. ($n=6-7$ rats per group). * $p < 0.05$; ** $p < 0.01$ vs. the vehicle control group (t -test).

on vascular reactivity. We investigated these effects using a systematic approach in order to determine both contractile and relaxant responses of the aortic preparations. We found that ASX (at the dose of 5 mg/kg/d for 7 weeks) had no significant effect on ACh-induced relaxation, which is an endothelium-dependent relaxation. This result implies that the endothelial-derived NO production and/or release might not be significantly altered by ASX at the given dose in the SHR. However, in this study ASX-O showed significant enhancement of the endothelium-independent SNP-induced relaxation which is reflective of an increase in cyclic nucleotides that in turn, decrease intracellular calcium and myofilament Ca^{2+} sensitivity.¹⁵⁾

On the other hand, the constrictive effects by Phe and Ang II tend to be significantly ameliorated by ASX-O ($p < 0.05$). We focused on determining the responses to the $[\alpha]_1$ -selective agonist, Phe. ASX-O significantly ameliorated the Phe-induced contraction of the aortic rings. Hence it can be postulated that the administration of ASX-O may improve the cardiovascular functions in the SHR by normalization of the sensitivity of the adrenoceptor sympathetic pathway, particularly the $[\alpha]$ -adrenoceptors.

The vasoconstrictor Ang II had been reported to increase the nicotinamide adenine dinucleotide phosphate (NADPH)/NADPH oxidase activity and the superoxide anion (O_2^-) production in cultured vascular smooth muscle cells *in vitro*.¹⁶⁾ Those findings were consistent with the observation of the increased vascular O_2^- concentrations in rats that be-

came hypertensive as a result of chronic infusion with Ang II *in vivo*.¹⁷⁾ Interestingly, a down-regulation of the expression of the NADPH oxidase subunits and a lowering of the O_2^- level in endothelial cells by some polyphenols had been reported by Ying *et al.*¹⁸⁾ ASX is well known to have a potential antioxidant activity, and the present findings, together with those reported earlier, may urge us to consider the link between the vascular effects of ASX-O and Ang II-induced ROS-production. ASX-O, in this study, has reduced the contractile response to Ang II in the SHR aortic preparations. This effect was evident in the presence of an intact endothelium and the NO synthase inhibitor L-NAME. However, it is necessary to investigate the effect in endothelium-denuded preparations and without L-NAME. Furthermore, the vascular contraction induced by the Xan/XOD system was significantly reduced by the ASX-O treatment. This contraction is presumably induced by the oxygen-derived free radicals produced by the system. Although the inhibitory effect by the ASX-O can be explained, in part, by its scavenging activity, further investigations may be necessary.

In the microcirculation, the hemorheological factors have been implicated to play an important role in the pathogenesis of different cardiovascular diseases.¹⁹⁾ Moreover, the BP has been positively associated with plasma viscosity, for which fibrinogen is considered to be the main determinant factor.²⁰⁾ In natural medicines, some substances have been reported to have significant effects on the erythrocyte deformability and/or blood viscosity, on the fibrinogen concentration and

on blood rheology in rats.^{21,22} The microchannel (MC-FAN) has been reported as a useful tool in analyzing blood rheology in pathophysiological conditions¹⁴ and to evaluate the effects of substances on the blood flow rate and fluidity.^{13,14,23} In the current study, ASX-O significantly decreased the microchannel transit time of the whole blood compared to the vehicle control ($p < 0.05$), without affecting the blood cell counts. This suggests that ASX-O may act in modulating the blood fluidity in hypertension. This improvement of the hemorheology by ASX-O may not be related to the plasma fibrinogen level which was not significantly changed in the present experiment (Table 2). Thus this improvement is probably due to modulatory effects on the deformability or aggregation of the blood cells and/or by effects on the blood viscosity, however, further study may be needed to verify these effects.

In general, the beneficial roles of some natural antioxidants against some cardiovascular diseases have been reported,^{24,25} and several studies, both in animal models and human subjects, have reported improvements of the blood vessel function by dietary antioxidants and vitamin supplementation.^{26,27} Most recently, an ASX derivative was reported to have cardioprotection and myocardial salvage potentials in a rat infarct model.²⁸ Nevertheless, our present findings implicate ASX as a potential candidate in extending the vascular protective actions and in improving the blood fluidity in hypertension.

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). We would like to thank the grants' sponsors, and to acknowledge Fuji Chemical Industry Co., Ltd., Toyama for supplying the astaxanthin product.

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