ANTIOXIDANTS & REDOX SIGNALING
Volume 5, Number 1, 2003

Rapid Letter

Astaxanthin Limits Exercise-Induced Skeletal and Cardiac Muscle Damage in Mice

WATARU AOI,[1,2] YUJI NAITO,[1] KUNIHIRO SAKUMA,[3] MASASHI KUCHIDE,[4] HARUKUNI TOKUDA,[4] TAKASHI MAOKA,[5] SHINYA TOYOKUNI,[6] SHIGENORI OKA,[7] MASAHIRO YASUHARA,[3] and TOSHIKAZU YOSHIKAWA[1]

ABSTRACT

Dietary antioxidants may attenuate oxidative damage from strenuous exercise in various tissues. Beneficial effects of the antioxidant astaxanthin have been demonstrated *in vitro,* but not yet *in vivo.* We investigated the effect of dietary supplementation with astaxanthin on oxidative damage induced by strenuous exercise in mouse gastrocnemius and heart. C57BL/6 mice (7 weeks old) were divided into groups: rested control, intense exercise, and exercise with astaxanthin supplementation. After 3 weeks of exercise acclimation, both exercise groups ran on a treadmill at 28 m/min until exhaustion. Exercise-increased 4-hydroxy-2-nonenal-modified protein and 8-hydroxy-2′-deoxyguanosine in gastrocnemius and heart were blunted in the astaxanthin group. Increases in plasma creatine kinase activity, and in myeloperoxidase activity in gastrocnemius and heart, also were lessened by astaxanthin. Astaxanthin showed accumulation in gastrocnemius and heart from the 3 week supplementation. Astaxanthin can attenuate exercise-induced damage in mouse skeletal muscle and heart, including an associated neutrophil infiltration that induces further damage. *Antioxid. Redox Signal.* 5, 139–144.

INTRODUCTION

INTENSE EXERCISE leads to production of reactive oxygen species (ROS), mainly via the mitochondrial electron transport chain, xanthin oxidase, and phagocytes (1, 12). Exercise-induced ROS oxidize several targets such as proteins, lipids, and DNA in these tissues, causing oxidative damage in skeletal muscle, heart, and liver (1, 12). Previous studies have demonstrated that dietary supplementation with antioxidants such as vitamin E, vitamin C, and carotenoids can decrease oxidative damage induced by intense exercise (6, 12).

Astaxanthin, a carotenoid pigment found in algae, fish, and birds, shows highly potent antioxidative (8, 14, 16), immunomodulatory (4), antineoplastic (13), and antiinflammatory (3) effects. Astaxanthin esters biosynthesized by a unicellular alga, *Haematococcus pluvialis,* are obtained from cultures (14) and can be used for studies in animals. We examined immunohistochemically and biochemically whether dietary astaxanthin could attenuate delayed-onset oxidative damage induced by intense exercise in skeletal muscle and heart in mice.

Recent experiments in several models indicated that an inflammatory response is induced by intracellular production of ROS, which increaseactivity of certain redox-sensitive transcription factors. Nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) are typical transcription factors controlled by ROS that regulate gene expression for many chemokines, inflammatory cytokines, and adhesion molecules (18, 22). In response to these mediators, phagocytes infiltrate into tissues expressing them, where these cells induce proteolysis, ultrastructural derangement, and further oxidative damage. ROS, then, are not only a direct cause of oxidative damage; they also act as a second messenger initiating delayed-onset inflammatory damage. In previous studies, the

Departments of [1]Medicine, [2]Physiology, [3]Legal Medicine, and [4]Biochemistry, Kyoto Prefectural University of Medicine, Kyoto, 602-0841; [5]Sun Biolex Co., Kyoto, 606-0805; [6]Department of Pathology and Biology of Diseases, Kyoto University, Kyoto, 606-8501; and [7]Nagase Co., Kobe, 651-2241, Japan.

inflammatory cascade was shown to be accelerated in skeletal and cardiac muscles by oxidative stress (19, 23). Thus, delayed-onset muscle damage after exercise, including oxidative injury, is induced by the inflammatory cascade; damage and neutrophil infiltration occur together after a delay, not immediately after exercise (5). Antioxidants may inactivate transcription factors, decreasing expression of inflammatory mediators and ultimately neutrophil infiltration. We therefore also studied whether dietary astaxanthin decreased neutrophil infiltration in skeletal muscle and heart induced by strenuous exercise.

MATERIALS AND METHODS

Animals and experimental design

Twenty-seven female C57BL/6 mice (7 weeks old; Clea Japan, Osaka, Japan) were acclimated for 1 week in an air-conditioned room (22 ± 2°C) with a 12-h light/12-h dark cycle (lights on, 7:30 a.m. to 7:30 p.m.). Mice were divided into three groups of nine animals each: rested controls (R), intense exercise (E), and exercise plus dietary astaxanthin supplementation (EA). All mice were acclimated to running on a motor-driven treadmill designed for mice, beginning at 5 m/min for 10 min/day, 3 days/week. Over a 3-week period, intensity was gradually increased to 28 m/min while a 10-min duration was maintained. On the day of the experiment, E and EA mice performed a bout of treadmill running to exhaustion at the above speeds. Exhaustion was defined as the inability of a mouse to right itself when laid on its side. The diet for the astaxanthin supplementation group was prepared by mixing CE-2 powder with astaxanthin (Fuji Chemical Industry, Toyama, Japan) at 0.02% (wt/wt). The food intake was recorded daily for 3 weeks before the final day of the experiment. Mice were weighed every 3 days. Significant differences were not observed between groups in weight gain or daily food intake. At 24 h after exhaustive exercise, mice were decapitated and then exsanguinated. The left gastrocnemius and heart were quickly removed.

In an additional experiment, 12 mice were divided into a control diet group ($n = 6$) and an astaxanthin supplementation group ($n = 6$). Quantitative analysis was performed for astaxanthin concentration in gastrocnemius and heart after mice had consumed the respective diet for 3 weeks.

Immunohistochemistry for 4-hydroxy-2-nonenal (4-HNE)-modified proteins

Serial 8-μm transverse sections cut with a cryostat (5030 Microtome, Bright Instrument Co. Ltd., Huntingdon, Cambridgeshire, U.K.) were affixed to silanized slides (Dako Japan, Tokyo, Japan). All subsequent steps were carried out as described previously (21). In brief, the sections were incubated overnight at 4°C with a primary antibody diluted in phosphate-buffered saline (PBS). Sections then were rinsed well with PBS and incubated with biotinylated anti-mouse IgG (1:300 dilution; Dako) for 30 min at room temperature. After extensive rinsing, sections were incubated for 30 min with peroxidase-streptavidin conjugate (Vector Laboratories, Burlingame, CA, U.S.A.), and visualized with diaminobenzidine and hydrogen peroxide (H_2O_2). Sections then were mounted in a glycerol-based medium containing *p*-phenylenediamine. A negative control with omission of the primary antibody was included in the immunostaining procedures in each instance.

Production of 8-hydroxy-2'-deoxyguanosine (8-OHdG)

DNA was extracted from heart and gastrocnemius using a DNeasy kit (Qiagen, Valencia, CA, U.S.A.). Extracted DNA was digested by DNase I, alkaline phosphatase (type III), and phosphodiesterase, and then analyzed by HPLC with electrochemical detection as previously described (11).

Myeloperoxidase (MPO) activity

MPO activity was determined by the method of Smith *et al.* (24). Tissue samples were homogenized in 30 m*M* potassium phosphate buffer (pH 7.4) using a Polytron tissue homogenizer. The homogenate was centrifuged at 12,000 rpm for 15 min at 4°C. The resulting pellet was homogenized again in 50 m*M* potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide. MPO activity was assessed by continuously monitoring H_2O_2-dependent oxidation of *o*-dianisidine dihydrochloride at 480 nm and 37°C. One unit of activity was defined as a change in absorbance of 1.0 at an optical density of 480 nm. Activity is stated as units per gram of wet tissue.

Plasma creatine kinase (CK) activity

Plasma samples were refrigerated until assay and total CK activity was measured by using a kit (CPK 45-5; Sigma Chemical Co., St. Louis, MO, U.S.A.), with an interassay reliability of ±2%.

Quantitative analysis of astaxanthin

Astaxanthin content in each tissue was quantified by HPLC. Tissues were homogenized with acetone. After centrifugation (3,000 rpm; 10 min), supernatants were evaporated to dryness. The residue was dissolved in 200 μl of acetone and filtered through a 0.45-μm polytetrafluoroethylene membrane filter; then 20 μl of solution was subjected to HPLC on a Shimadzu LC-6A instrument equipped with a Shimadzu SPD-6AV spectrometer (Shimadzu, Kyoto, Japan) set at 460 nm. The column used was a Wakosil $5C_{18}$ (ODS) column (250 mm length × 4.6 mm internal diameter) with a mobile phase of methyl alcohol. A low flow rate was used (1.0 ml/min). Astaxanthin was quantified relative to calibration with a standard sample (F. Hoffman–La Roche, CA, U.S.A.).

Statistical analysis

Data were analyzed by using a two-way ANOVA. If the ANOVA indicated a significant difference, Tukey's HSD was performed to determine the significance between means. A value of $p < 0.05$ was considered to indicate statistical significance.

RESULTS

Immunohistochemistry for 4-HNE-modified proteins

According to immunohistochemistry, 4-HNE-modified proteins were minimal or undetected in sarcolemma of most muscle cells in the R group (Fig. 1). Expression of 4-HNE-modified proteins was noted at the surfaces of muscle cells in the E group. This immunoreactivity was remarkably decreased in mice treated with astaxanthin. Labeling of stained muscle was absent after antibody preabsorption with the 4-HNE peptide. Observations were similar between gastrocnemius and heart.

Production of 8-OHdG

At 24 h after intense exercise, 8-OHdG/10^5 × dG tended to be higher in both gastrocnemius and heart in the E group than in the R group (Fig. 2). Increases in 8-OHdG/10^5 × dG were significantly inhibited by treatment with astaxanthin ($p < 0.05$).

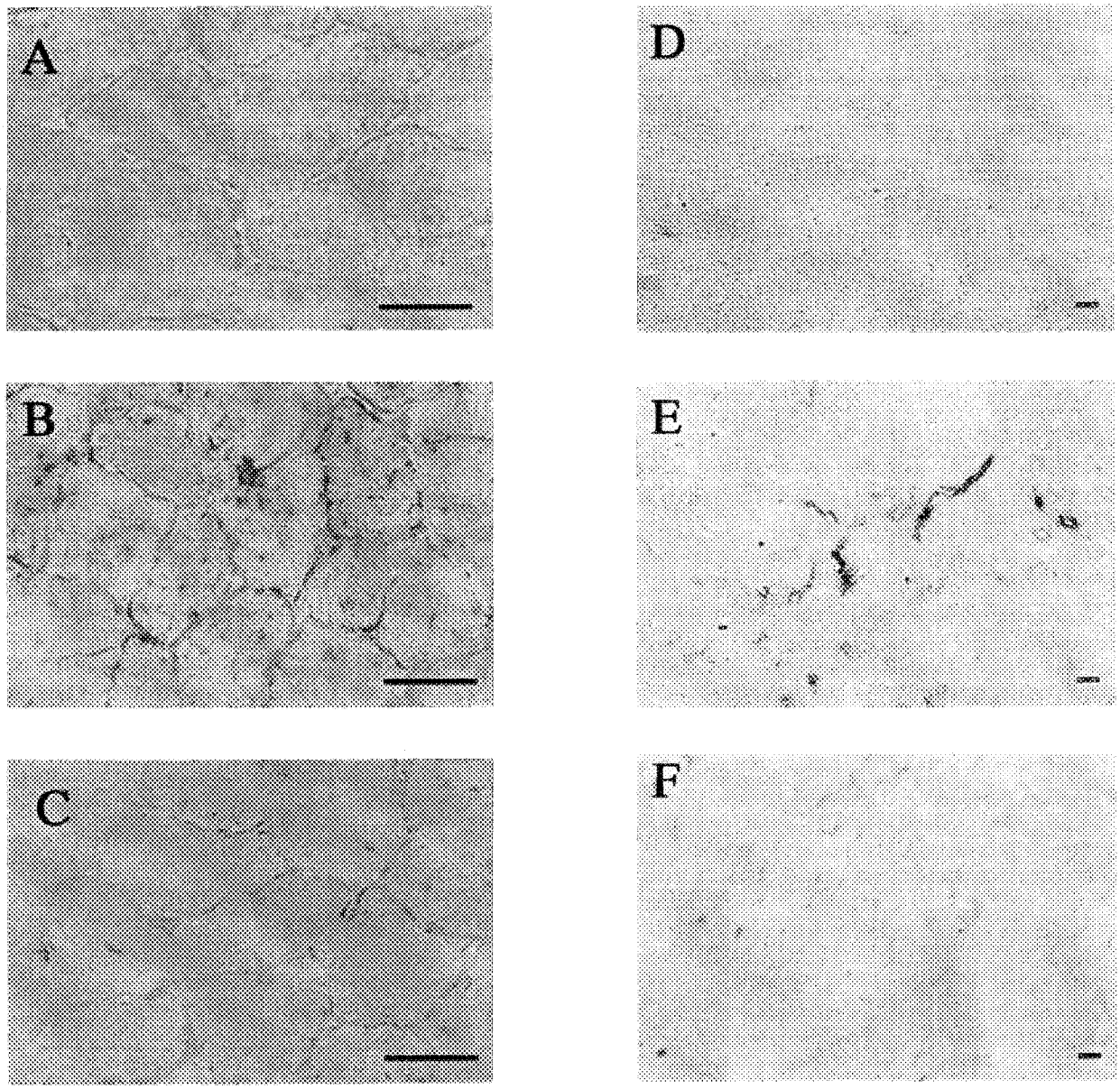

FIG. 1. Immunohistochemical findings for 4-HNE-modified proteins in gastrocnemius (A–C) and heart (D–F) after intense exercise. (A and D) Tissues from rested control mice. (B and E) Tissues from exercised mice. (C and F) Tissues from acute exercised mice receiving astaxanthin. Scale bar = 50 μm.

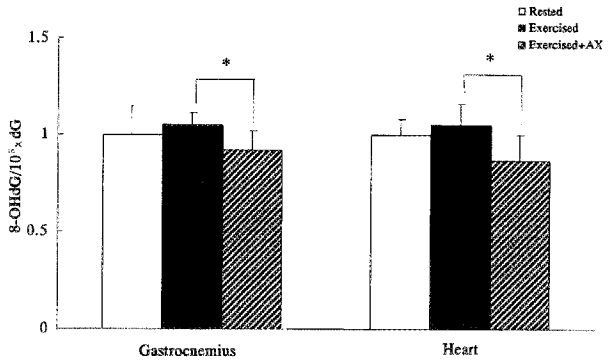

FIG. 2. Production of 8-OHdG in gastrocnemius and heart. Data are presented as means + SE for four mice. *$p <$ 0.05 (Tukey's HSD). AX, astaxanthin.

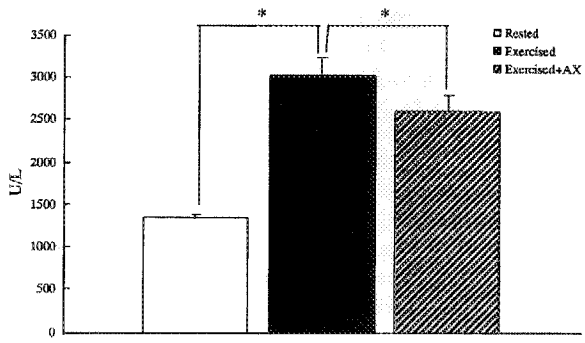

FIG. 4. CK activity in plasma. Data are presented as means + SE for eight mice. *$p < 0.05$ (Tukey's HSD). AX, astaxanthin.

MPO activity

Tissue-associated MPO activity was increased from control concentrations of 0.64 ± 0.15 to 2.15 ± 0.31 in gastrocnemius and 1.18 ± 0.30 to 2.39 ± 0.38 in heart at 24 h after intense exercise (Fig. 3). These increases in MPO activity were significantly inhibited by treatment with astaxanthin ($p < 0.05$).

CK activity

Plasma CK activity was significantly greater in the E group (3,018 ± 231.7 U/L) than in the R group (1,361 ± 17.6 U/L) (Fig. 4). The increase in plasma CK activity was significantly inhibited by treatment with astaxanthin (2,608 ± 186.5 U/L) ($p < 0.05$).

Astaxanthin content in tissues

Astaxanthin had accumulated not only in liver, but also in gastrocnemius and heart at 3 weeks of feeding of a diet containing 0.02% astaxanthin (Table 1). No astaxanthin was detected in the tissues of rats fed a normal diet.

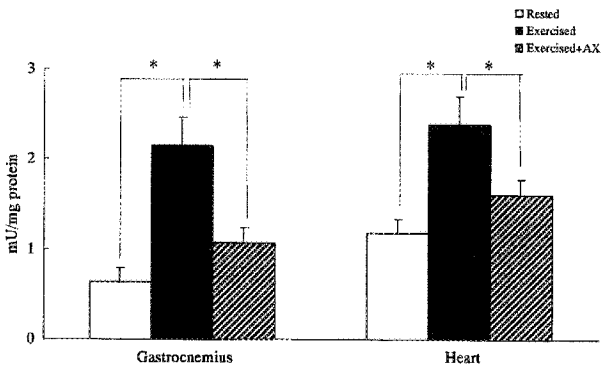

FIG. 3. MPO activity in gastrocnemius and heart. Data are presented as means + SE for eight mice. *$p < 0.05$ (Tukey's HSD). AX, astaxanthin.

DISCUSSION

The present study is the first to demonstrate that astaxanthin can attenuate exercise-induced oxidative damage in gastrocnemius and heart. We measured 4-HNE-modified proteins and 8-OHdG as markers of oxidative damage to lipids and DNA, respectively. These markers have recently been validated as quantitative indications of oxidative damage (10). Our study is also the first to immunohistochemically localize oxidative damage induced by strenuous exercise in skeletal muscle and heart using a monoclonal antibody against 4-HNE-modified protein.

Astaxanthin has been reported to be more effective than other antioxidants, such as vitamin E and β-carotene, in preventing lipid peroxidation in solutions and in various biologic membrane systems (8, 16). Goto *et al.* (9) reported that the superior antioxidant activity of astaxanthin may involve the unique structure of the terminal ring moiety, where radicals can be trapped, in addition to trapping at the conjugated polyene chain. In the terminal ring, the hydrogen atom at the C3 methine was suggested to be a radical trapping site (9). Our data documented that astaxanthin indeed is absorbed and transported into skeletal muscle and heart in mice, even though most dietary carotenoids accumulate mainly in the liver and show relatively little distribution to other peripheral

TABLE 1. ASTAXANTHIN CONTENT AND RATIO OF ITS ISOMERS IN TISSUES

	Gastrocnemius	*Heart*	*Liver*
Content (μg/g of tissue)	18.6	46.7	270.3
Isomer ratio (%) *trans*	72.2	76	68.1
cis	27.8	24	31.9

Values are means for six mice receiving an astaxanthin diet for 3 weeks.

tissues, including skeletal muscle and heart. This unique pharmacokinetic characteristic of astaxanthin makes it well suited to decreasing oxidative stress in gastrocnemius and heart.

The main sources of ROS generation as a result of exercise are the mitochondrial electron transport chain, xanthine oxidase, and phagocytes (6, 12). Phagocytes are a particularly important source, considering that oxidative damage and phagocytic infiltration occur at the same time point after a delay following exercise, "delayed-onset damage" (5, 17, 24). In the present study, MPO, an enzyme unique to neutrophils (2, 24), was increased in gastrocnemius and heart at 24 h after exercise. In addition, plasma CK activity also was increased at 24 h after exercise. CK is present mainly in muscles, and measurement of plasma concentrations after exercise is a frequently used indicator of muscle damage (7, 15). Many studies, including the present one, have demonstrated increased plasma CK at a delayed time point after exercise (7, 15), indicating that this increase is closely related to the inflammatory response.

Dietary astaxanthin attenuated not only oxidative metabolites, but also MPO activity in muscle and CK activity in plasma compared with the exercise-only E group. Neutrophils infiltrate into tissues where cells express chemokines, cytokines, and adhesion molecules that mostly are regulated by NF-κB and AP-1 (18, 20, 22). Ordinarily, these redox-sensitive transcription factors can be localized to the cytoplasm; stimulated by stresses such as ROS, they move into nuclei and attach to binding sites on DNA (19, 23). We therefore believe that astaxanthin suppressed activity of these transcription factors by scavenging the ROS that would activate them; this decreased the expression of inflammatory mediators, attracting fewer neutrophils and lessening delayed-onset damage, including further oxidative damage.

In summary, we studied the effect of astaxanthin dietary supplementation on delayed-onset damage following intense exercise, especially oxidative damage, in gastrocnemius and heart. Dietary astaxanthin attenuated oxidative damage to lipids and DNA in these tissues, and also leakage of CK into plasma. In addition, this treatment inhibited neutrophil infiltration into the tissues. Thus, astaxanthin attenuates exercise-induced damage by directly scavenging ROS and also by down-regulating the inflammatory response.

ACKNOWLEDGMENTS

The astaxanthin diet was a gift from Fuji Chemical (Toyama, Japan).

ABBREVIATIONS

AP-1, activator protein-1; CK, creatine kinase; E, intense exercise; EA, exercise plus dietary astaxanthin supplementation; 4-HNE, 4-hydroxy-2-nonenal; H_2O_2, hydrogen peroxide; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PBS, phosphate-buffered saline; R, rested controls; ROS, reactive oxygen species.

REFERENCES

1. Bejma J and Ji LL. Aging and acute exercise enhance free radical generation in rat skeletal muscle. *J Appl Physiol* 87: 465–470, 1999.
2. Belcastro AN, Arthur GD, Albisser TA, and Raji DA. Heart, liver, and skeletal muscle myeloperoxidase activity during exercise. *J Appl Physiol* 80: 1331–1335, 1996.
3. Bennedsen M, Wang X, Willen R, Wadstroem T, and Andersen LP. Treatment of *H. pylori* infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulated cytokine release by splenocytes. *Immunol Lett* 70: 185–189, 1999.
4. Chew BP, Park JS, Wong MW, and Wong TS. A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice in vivo. *Anticancer Res* 19: 1849–1853, 1999.
5. Duarte JA, Calvalho F, Bastos ML, Soares JMC, and Appell HJ. Do invading leucocytes contribute to decreases in glutathione concentrations indicating oxidative stress in exercised muscle, or are they important for its recovery? *Eur J Appl Physiol* 68: 48–53, 1994.
6. Evans WJ. Vitamin E, vitamin C, and exercise. *Am J Clin Nutr* 72: 647S–652S, 2000.
7. Frabkiewicz-Jozko A, Faff J, and Sieradzan-Gabelska B. Changes in concentrations of tissue free-radical marker and serum creatine kinase during the post-exercise period in rats. *Eur J Appl Physiol* 74: 470–474, 1996.
8. Fukuhara K, Inokami Y, Tokumura A, Terao J, and Suzuki A. Rate constants for quenching singlet oxygen and activities for inhibiting lipid peroxidation of carotenoids and alpha-tocopherol in liposomes. *Lipids* 33: 751–756, 1998.
9. Goto S, Kogure K, Abe K, Kimata Y, Kitahama K, Yamashita E, and Terada H. Efficient radical trapping at the surface and inside the phospholipid membrane is responsible for highly potent antiperoxidative activity of the carotenoid astaxanthin. *Biochim Biophys Acta* 1512: 251–258, 2001.
10. Hattori Y, Nishigori C, Tanaka T, Uchida K, Nikaido O, Osawa T, Hiai H, Imamura S, and Toyokuni S. 8-Hydroxy-2′-deoxyguanosine is increased in epidermal cells of hairless mice after chronic ultraviolet B exposure. *J Invest Dermatol* 107: 733–737, 1997.
11. Hattori-Nakakuki Y, Nishigori C, Okamoto K, Imamura S, Hiai H, and Toyokuni S. Formation of 8-hydroxy-2′-deoxyguanosine in epidermis of hairless mice exposed to near-UV. *Biochem Biophys Res Commun* 201: 1132–1139, 1994.
12. Ji LL. Antioxidants and oxidative stress in exercise. *Proc Soc Exp Biol Med* 222: 283–292, 1999.
13. Jyonouchi H, Zhang L, Gross M, and Tomita Y. Immunomodulating actions of carotenoids: enhancement of in vivo and in vitro antibody production to T-dependent antigens. *Nutr Cancer* 21: 47–58, 1994.
14. Kobayashi M. In vivo antioxidant role of astaxanthin under oxidative stress in the green alga *Haematococcus pluvialis*. *Appl Microbiol Biotechnol* 54: 550–555, 2000.
15. Maughan RJ, Donnelly AE, Gleeson M, Whiting PH, Walker KA, and Clough PJ. Delayed-onset muscle damage

and lipid peroxidation in man after a downhill run. *Muscle Nerve* 12: 332–336, 1989.
16. Naguib YM. Antioxidant activities of astaxanthin and related carotenoids. *J Agric Food Chem* 48: 1150–1154, 2000.
17. Niess AM, Dickhuth HH, Northoff H, and Fehrenbach E. Free radicals and oxidative stress in exercise-immunological aspects. *Exerc Immunol Rev* 5: 22–56, 1999.
18. Ozolins TR and Hales BF. Oxidative stress regulates the expression and activity of transcription factor activator protein-1 in rat conceptus. *J Pharmacol Exp Ther* 280: 1085–1093, 1997.
19. Peng M, Huang L, Xie ZJ, Huang WH, and Askeri A. Oxidant-induced activations of nuclear factor-kappa B and activator protein-1 in cardiac myocytes. *Cell Mol Biol Res* 41: 189–197, 1995.
20. Roebuck KA. Oxidant stress regulation of IL-8 and ICAM-1 gene expression: differential activation and binding of the transcription factors AP-1 and NF-kappaB. *Int J Mol Med* 4: 23–30, 1999.
21. Sakuma K, Watanabe K, Sano M, Sakamoto K, Uramoto I, and Totsuka T. The adaptive response of transforming growth factor-β2 and -βRII in the overloaded, regenerating and denervated muscles of rat. *Acta Neuropathol* 99: 177–185, 2000.
22. Schoonbroodt S, Legrand-Poels S, Best-Belpomme M, and Piette J. Activation of the NF-kappaB transcription factor in a T-lymphocytic cell line by hypochlorous acid. *Biochem J* 321: 777–785, 1997.
23. Sen CK, Khanna S, Reznick AZ, Roy S, and Packer L. Glutathione regulation of tumor necrosis factor-alpha-induced NF-kappa B activation in skeletal muscle-derived L6 cells. *Biochem Biophys Res Commun* 237: 645–659, 1997.
24. Smith JK, Grisham MB, Granger DN, and Korthuis RJ. Free radical defense mechanisms and neutrophilic infiltration in postischemic skeletal muscle. *Am J Physiol Heart Circ Physiol* 25: H789–H793, 1989.

Address reprint requests to:
Dr. Wataru Aoi
First Department of Medicine
Kyoto Prefectural University of Medicine
Kamikyo-ku
Kyoto 602-8566, Japan

*E-mail:*waoi@basic.kpu-m.ac.jp

Received for publication November 1, 2002; accepted November 7, 2002.