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Inhibition of Oxidative Injury of Biological Membranes by Astaxanthin

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Abstract: The value of astaxanthin, a carotenoid pigment, in the treatment of oxidative injury is assessed. Astaxanthin protects the mitochondria of vitamin E-deficient rats from damage by Fe^{2+}-catalyzed lipid peroxidation both *in vivo* and *in vitro*. The inhibitory effect of astaxanthin on mitochondrial lipid peroxidation is stronger than that of α-tocopherol. Thin layer chromatographic analysis shows that the change in phospholipid components of erythrocytes from vitamin E-deficient rats induced by Fe^{2+} and Fe^{3+}-xanthine/xanthine oxidase system was significantly suppressed by astaxanthin. Carrageenan-induced inflammation of the paw is also significantly inhibited by administration of astaxanthin. These data indicate that astaxanthin functions as a potent antioxidant both in vivo and in vitro.

REACTIVE OXYGENS are produced by various enzymatic and nonenzymatic processes in living organisms. Under pathological conditions they may induce peroxidation of polyunsaturated fatty acids in biological membranes leading to functional impairment (1,2). Such oxygen toxicity has been postulated to underlie the pathogenesis of several diseases such as postischemic reflow injury (3, 4), retinopathy in premature infants (5), shock (6) and cerebral infarction (7). For example, paraquat (8) and adriamycin (9) also catalyze the formation of reactive oxygens and result in pulmonary fibrosis and cardiomyopathy, respectively. Furthermore, both activated neutrophils and macrophages produce active oxygen and significant fractions of these metabolites are released extracellularly, and induce various inflammatory responses (10, 11).

Superoxide has been assumed to be a primary source for other reactive oxygens, such as H_2O_2 and $^{\cdot}OH$ (1). Intracellular compartments are highly enriched with anti-oxidants such as superoxide dismutase, catalase, glutathion peroxidase, and glutathion. Thus, reactive oxygens are efficiently detoxicated (12). However, in extracellular space (13, 14) such as

plasma, concentrations of these enzymes and scavengers are low. High levels of extracellularly released superoxide cannot therefore be dismutated efficiently enough to protect cell membranes from oxygen toxicity.

Astaxanthin (AX) is a carotenoid pigment (Fig. 1) found in many animals and plants (15), and the compound can be purified in large quantity from yeast, *Phaffia rhodozyma Miller* (16). This compound may serve as an efficient and safe antioxidant. However, relatively little attention has been brought to the action of this compound. During the course of our studies on the protective action of various drugs against the impediment of biological membrane by reactive oxygens, we have found by using vitamin E deficient rats that astaxanthin protects biological membranes from hazardous oxygen species *in vivo* and *in vitro*.

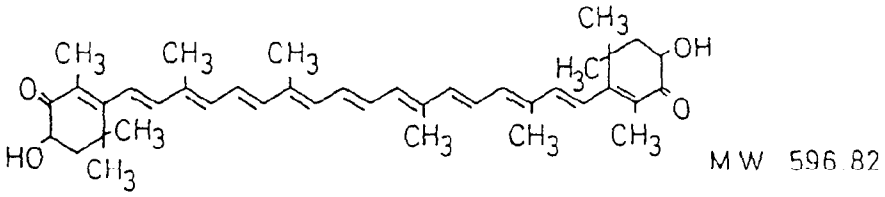

FIGURE 1. Chemical structure of Astaxanthin. Astaxanthin (AX) (3,3′-dihydroxy b,b-carotene-4,4′-dione), a carotenoid and a precursor of astacin.

Materials and Methods

Chemicals: Adenosine diphosphate (ADP), carrageenan (type IV), thiobarbituric acid (TBA), vitamin E (VE) and xanthine were purchased from Sigma Chemicals (St. Louis). Xanthine oxidase was obtained from Boehringer Mannheim Co. (West Germany). Astaxanthin was kindly donated by Suntory Co. (Osaka). Vitamin E deficient diet was obtained from Oriental Yeast Co. (Tokyo). Other chemicals were obtained from nacalai tesque Co. (Kyoto). For *in vitro* experiments, AX was dissolved in dimethyl sulfoxide.

Rats: Vitamin E deficient rats were prepared according to the method of Machilin *et al.* (17). Three groups (10 rats for each group) of male Wistar rats (6 to 7 weeks of age) were fed for 2–4 months. Each group had a different diet, normal (group 1), vitamin E-free (group 2) and vitamin E-free plus astaxanthin (containing 1 mg astaxanthin/100 g) (group 3).

Mitochondria and erythrocyte ghosts: Liver mitochondria were isolated from each group of animals according to a modification of Hogeboom and Schneider (18), and erythrocyte ghosts were prepared by the method of Dodge *et al.* (19).

Mitochondrial functions: Oxidative phosphorylation and respiratory control index (the ratio of phosphorylative accelerated respiration expressed by state 3 to the substrate level respiration expressed by state 4) were measured by a Clark-type oxygen electrode (20). Mitochondria were incubated in a medium containing 0.15 M KC1-10 mM Tris-HC1 buffer (pH 7.4) to eliminate the inhibitory effect of sucrose on lipid peroxidation by Fe^{2+} and thiobarbituric acid reaction (21, 22).

Lipid peroxidation and lipid analysis: Erythrocyte ghosts were prepared according to the method of Blight and Dyer (22). Fe^{2+}-induced lipid peroxidation of the ghosts was assayed by thiobarbituric acid reaction (21). Lipid peroxidation *in vitro* was induced by Fe^{2+}

and xanthine/xanthine oxidase system. Membrane phospholipids were analyzed by thin layer chromatography.

Protein content: Membrane protein was determined according to Lowry's method using bovine serum albnumin as a standard (23).

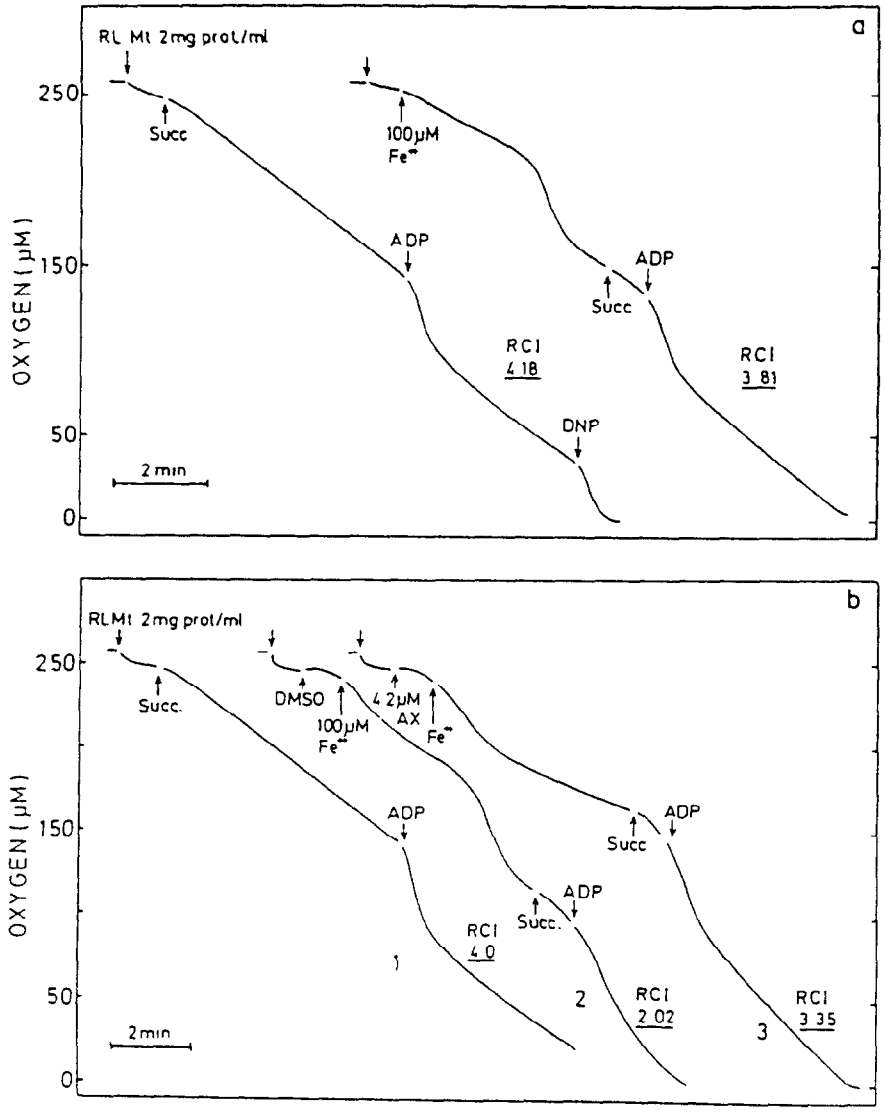

FIGURE 2. Protection by astaxanthin against Fe^{2+}-induced dysfunction of rat liver mitochondria. Liver mitochondria were isolated from normal rats and animals fed a vitamin E-deficient diet for two months. The isolated samples were washed twice with a medium containing 0.15 M KCl and 10 mM Tris-HCl buffer (pH 7.4). Respiratory activity of mitochondria was assayed with a Clark type oxygen electrode in a medium containing 0.15 M KCl, 3 mM $MgCl_2$ and 5 mM potassium phosphate buffer (pH 7.4) at 25°C. Respiratory control index of untreated rat liver mitochondria was 4.18 (trace 1 in a), and that of vitamin E-deficient rat liver mitochondria was 4.0 under succinate as respiratory substrate (trace 1 in b). Dimethyl sulfoxide, a carrier for astaxanthin was added after adding 4.2 μM astaxanthin, and Fe^{2+}-induced changes were observed (trace 3 in b). RL Mt, rat liver mitochondria; RCI, respiratory control index; DMSO, 5 μl/ml dimethyl sulfoxide; Succ, 1 mM sodium succinate; DNP, 25 μM dinitrophenol; ADP, 150 μM ADP; Fe^{2+}, 100 μM Fe^{2+}.

Carrageenan-induced inflammation: Saline solutions (1 ml) containing either vitamin E or astaxanthin were injected intraperitoneally 30 min before carrageenan treatment. Effect of astaxanthin on carrageenan-induced edema was examined by measuring the volume changes of rat paws after subcutaneous injection of carrageenan (24, 25). Anesthetized rats were given subcutaneous injections of 0.2 ml saline solution into the left paw and the same volume of saline solution containing 10 mg carrageenan into the right paw. Change in paw volume was measured hourly three times after the treatment.

Results and Discussion

Effect of astaxanthin on Fe^{2+}-induced changes in mitochondrial functions of vitamin E deficient rats. Figure 2a shows the effect of Fe^{2+} on the respiratory activity of mitochondria from intact rats. Addition of 10–100 μM Fe^{2+} to the reaction medium enhanced oxygen consumption of mitochondria and slightly decreased respiratory control index (RCI; state 3/ state 4). The mitochondria from vitamin E deficient rats (group 2) revealed RCI of 4.0 and ADP/O ratio 2.0; these values are within normal levels with succinate as a respiratory substrate (trace 1 in Fig. 2b). However, when Fe^{2+} was added to the medium, the rate of oxygen consumption increased and respiratory control index (RCI) decreased markedly (trace 2 in Fig. 2b). When astaxanthin was added prior to the treatment, the Fe^{2+}-induced increase in oxygen consumption and the decrease in respiratory

Table I

Effect of Fe^{2+} and astaxanthin on the oxidative phosphorylation of mitochondria from vitamin E deficient rats.

Fe^{++} (μM)	A (nM)	RCI	(% Decrease)	ADP/O Ratio	(%)
0	0	4.00	(0)	2.00	(100)
10	0	3.37	(21)	2.04	(102)
40	0	2.92	(36)	2.02	(101)
100	0	2.44	(52)	1.98	(99)
100	45	2.64	(45)	2.04	(102)
100	420	3.21	(26)	1.85	(93)
100	4200	3.35	(22)	1.77	(89)

Experimental conditions were the same as in Figure 2. Activities of mitochondrial oxidative-phosphorylation were expressed in two characteristics, respiratory control index (RCI) and ADP/0 ratio. Numbers in parentheses represent % of the control values obtained from mitochondria which were not treated with Fe^{2+} and astaxanthin.

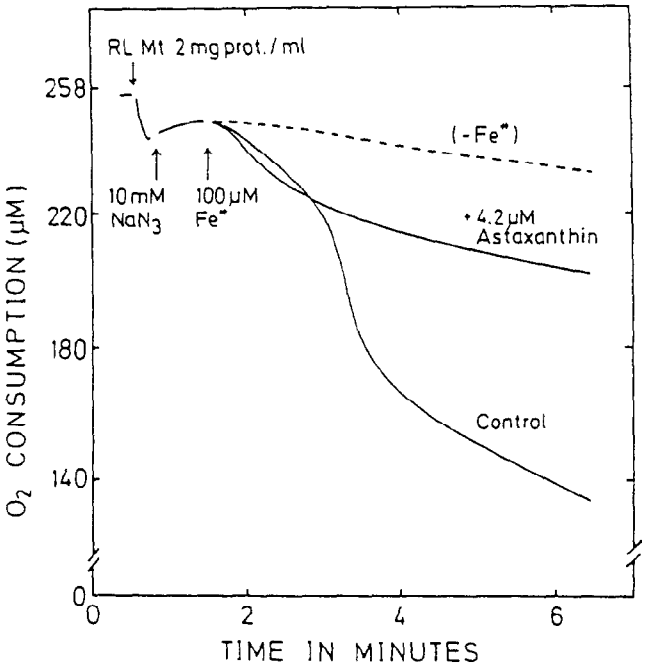

FIGURE 3. Enhancement of NaN_3-insensitive oxygen consumption by Fe^{2+} and its suppression by astaxanthin. Mitochondria were isolated from normal rat liver (group 1). Astaxanthin (4.2 μM) was added prior to each experiment. Other conditions were the same as in Figure 2.

control index were inhibited significantly (Fig. 2b, trace 3). Both the hazardous effect of Fe^{2+} and the protective effect of astaxanthin depended on their concentrations (Table I).

Figure 3 represents the enhancement of the rate of NaN_3-independent oxygen consumption by Fe^{2+}. Since NaN_3 inhibits electron transport of mitochondria, the Fe^{2+}-induced increase in oxygen consumption would have occurred independently from the mitochondrial electron transport system.

Since mitochondrial membranes are enriched with polyunsaturated fatty acids, the increased oxygen consumption might reflect the Fe^{2+}-induced lipid peroxidation. Consistent with this notion is the fact that the Fe^{2+}-induced increase in oxygen consumption was inhibited by astaxanthin that has a polyunsaturated carbon chain.

Mitochondrial function of the astaxanthin-administered vitamin E deficient rats. To test whether astaxanthin also protects mitochondrial function *in vivo*, the mitochondrial Fe^{2+}-induced lipid peroxidation was investigated in vitamin E deficient rats with or without astaxanthin administration. Figure 4 shows the effect of 100 μM Fe^{2+} on the RCI values of mitochondria isolated from different groups of rats.

The respiratory control index of mitochondria from group 1 rats was slightly inhibited by Fe^{2+}-induced lipid peroxidation; no remarkable disorders of mitochondrial function, such as oxygen consumption, were induced. Thus the defense mechanism against free radicals seems to function in control group (group 1). On the other hand, mitochondria from vitamin E deficient rats (group 2) showed a remarkable decrease in respiratory control index by Fe^{2+}. The deterioration in respiratory control index was inhibited significantly by treating animals with

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