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Effect of Docosahexaenoic Acid and Ascorbate on Peroxidation of Retinal Membranes of ODS Rats

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Mutant male osteogenic disorder Shionogi (ODS) rats, unable to synthesize ascorbic acid, were fed diets containing a high content of docosahexaenoic acid (DHA) and different amounts of ascorbic acid, to study the effect of DHA on peroxidative susceptibility of the retina and possible antioxidant action of ascorbic acid. ODS rats were fed from 7 weeks of age with diets containing high DHA (6.4% of total energy). A control group received a diet high in linoleic acid. The diets also contained varying amounts of ascorbic acid. Fatty acid compositions and phospholipid hydroperoxides in rod outer segment (ROS) membranes, and retinal ascorbic acid were analyzed. DHA in ROS membranes was significantly increased in rats fed high DHA, compared with the linoleic acid diet. Levels of phospholipid hydroperoxides in the DHA-fed rats were significantly higher than the linoleic acid-fed rats. Ascorbic acid supplementation did not suppress the phospholipid hydroperoxide levels after a high DHA diet, even when the supplement increased the content of retinal ascorbic acid. In conclusion, high DHA feeding induced a marked increase of phospholipid hydroperoxides in ROS membranes of ODS rats. Supplementation of ascorbic acid did not reverse this increase.

Keywords: DHA; Ascorbic acid; ODS rats; Rod outer segments; Fatty acids; Lipid peroxidation

Abbreviations: DHA, docosahexaenoic acid; ROS, rod outer segment; CL-HPLC, chemiluminescence-high performance liquid chromatography; ODS rat, osteogenic disorder Shionogi rat; PCOOH, phosphatidylcholine hydroperoxide; ECD, electrochemical detector

INTRODUCTION

Docosahexaenoic acid (DHA, 22:6n3) is a major fatty acid in the retina of mammals.[1–3] Although the physiological function of DHA in vision is not very clear, a previous study has shown that DHA may influence neural function through activation of a retinoid X receptor signaling pathway.[4] Increasing acyl chain unsaturation from 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphocholine to 1-stearoyl-2-docosahexaenoyl-*sn*-glycero-phosphocholine results in a 3-fold increase in *Ka*, which means there is stronger binding of metarhodopsin and transducin.[5,6] Another study showed that dietary DHA can partially counteract kainate neurotoxicity in the rat retina.[7] It seems to be necessary to supplement DHA to maintain the neural function of mammals, especially in their developing stages. However, the high content of DHA indicates the potential for harm, since DHA would be very sensitive to oxidative stress, especially in an organ such as retina, which is always exposed to external light and oxygen.

Depletion of rat retinal DHA reduces the susceptibility of the ROS to acute light damage; the relationship between retinal light damage and ROS lipids does not depend on the total unsaturated fatty acid content of ROS. Rather, the damage appears to

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be related to the relative levels of DHA and 22:5n-6.[8–10] These studies were carried out by exposing rats to approximately over 10 times luminous intensity over the control. Some studies have been done in chronic and normal light conditions, in which accumulation of lipid peroxides in the retina has been demonstrated.[11,12] With Sprague–Dawley rats, our previous study indicated that although the DHA content in ROS membranes was significantly increased in the rats fed high DHA (9.69% total energy), there was no corresponding increase in the hydroperoxide levels of ROS membranes under normal light conditions.[13] Possibly, antioxidants present in the retina may have contributed to the result. One of these may be ascorbic acid.

Ascorbic acid is an essential nutrient for humans and certain animals that cannot synthesize this vitamin. Because intense light exposure decreases the level of retinal ascorbic acid, it is believed to be a potent antioxidant.[14] Like man, Wistar Shionogi rats of the (od/od) substrain with an osteogenic disorder are unable to synthesize L-ascorbic acid. Thus, osteogenic disorder Shionogi (ODS) rats provide a useful model for elucidating the physiological role of ascorbic acid, especially its antioxidative function. In this study, young male ODS rats were treated with diets of preformed DHA (6.4% of total energy) with a constant amount of linoleic acid (18:2n6) and various amount of ascorbic acid, to assess the effects on fatty acid composition of the ROS membrane during development. The peroxidative extent of ROS membranes with different levels of DHA was evaluated by comparison of their phospholipid hydroperoxide levels using a highly sensitive analytical technique, chemiluminescence-high performance liquid chromatography (CL-HPLC). Varying the ascorbic acid in the diet allowed us to determine the efficacy of this antioxidant in preventing oxidative stress under normal light conditions.

MATERIALS AND METHODS

Animals

ODS od/od rats (male, Clea Japan Inc., Tokyo, Japan) were supplemented with a vitamin C deficient standard diet and ascorbic acid in drinking water until 6 weeks. The amount of ascorbic acid taken from drinking water by rats per day was almost equivalent to those fed with solid diet (300 mg ascorbic acid/kg diet). At the age of 7 weeks, ODS od/od rats were first fed the basal diet containing 5% (w/w) high-oleic safflower oil and 300 mg ascorbic acid/kg diet for six days, then were randomly assigned to five diet treatments containing 10% (w/w) fat for 32 or 33 days. Groups 1–4 were fed diets with high DHA (92% DHA ethyl ester in purity, 6.4% total energy) and linoleic acid (2.3% total energy) and ascorbic acid of 100 mg (G1), 300 mg (G2), 600 mg (G3), 3000 mg/kg diet (G4), Group 5 was fed a diet with high linoleic acid (8.6% total energy), and 300 mg/kg diet of ascorbic acid. As seen in Table II, the PUFA% in Group 5 was almost the same as Groups 1–4 (Tables I and II). So Group 5 was served as the control group. Group 1 (100 mg ascorbic acid diet) was fed the diet for 33 days and the other groups were for 32 days. For each group, six rats were used. Animals were housed individually in a controlled temperature of 22 ± 1°C and humidity of 50–60% with a 12-h light/dark cycle

TABLE I Composition of experimental diets

Ingredient	g/kg diet G1–G4	g/kg diet G5
Casein	200	
DL-methionine	3	
Glucose	225	
Cellulose	50	
α-Cornstarch	150	
Sucrose	225	
RRR-α-tocopherol equivalent (mg/kg of diet)	700	
Vitamin mix	10	
Choline bitartrate	2	
Mineral mix	35	
Test lipids	100	
Safflower oil	0	40
High-oleic safflower oil	65	60
DHA ethyl ester*	35	0

* The purity was 92%.

TABLE II Fatty acid composition of dietary lipids (for 7-weeks rats)

Group	1	2	3	4	5
LA level (en%)*	2.4	2.4	2.4	2.4	8.7
DHA level (en%)	6.6	6.6	6.6	6.6	0
Ascorbic acid (mg/kg diet)	100	300	600	3000	300
Fatty acid					
16:0	3.4	3.4	3.4	3.4	5.5
16:1(n-7)	0.1	0.1	0.1	0.1	0.1
18:0	1.5	1.5	1.5	1.5	2.3
18:1(n-9)	50.3	50.3	50.3	50.3	50.0
18:2(n-6)	10.9	10.9	10.9	10.9	40.3
18:3(n-3)	0.2	0.2	0.2	0.2	0.5
20:4(n-6)	0.6	0.6	0.6	0.6	0
20:4(n-3)	0.2	0.2	0.2	0.2	0
20:5(n-3)	0.9	0.9	0.9	0.9	0
22:5(n-6)	0.3	0.3	0.3	0.3	0
22:5(n-3)	0.6	0.6	0.6	0.6	0
22:6(n-3)	30.5	30.5	30.5	30.5	0
Others	0.5	0.5	0.5	0.5	1.3
PUFA (%)	44.0	44.0	44.0	44.0	40.8
MUFA (%)	50.4	50.6	50.6	50.6	49.5
P/S ratio	29.5	29.5	29.5	29.5	5.2
n-6 (%)	11.9	11.9	11.9	11.9	40.0
n-3 (%)	32.2	32.2	32.2	32.2	0.3
n-6/n-3 ratio	0.4	0.4	0.4	0.4	133.3

* en%, % total energy.

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(lights on 7 A.M. and off at 7 P.M.). The illuminance level measured at the front of the cages was 70–100 lux of cool, white fluorescent light (Toshiba FLR40S, W/M). To prevent the autoxidation of DHA in the diet, the diet was prepared beforehand without adding DHA and stored at −20°C. DHA stored at −75°C was mixed with the diet every day immediately before feeding. Water was available *ad libitum*. The experimental diet was made available to the rats in the evening and removed the next morning, and there was no significant difference in growth rates for various diet groups.

Rats were killed at 81 days (Group 1) and 80 days (Groups 2–5). Rat retinas were extruded through a slit made across the entire cornea,[15] quickly frozen in dry ice, and stored at −75°C until used.

Preparation of the ROS Membrane

All isolations were performed at 4°C. Two retinas were pooled for separation of the ROS. A 50 μl of the retina homogenate (2 retinas in 1.8 ml buffer) was used for ascorbic acid analysis as described below, before the membrane isolation. ROS was isolated by discontinuous sucrose gradient centrifugation as described by Stinson *et al*.[16].

Fatty Acid Analysis

Total lipids were extracted and methylated as described before.[13] Separation of fatty acid methyl esters was performed by gas–liquid chromatography utilizing fused silica BPX70 capillary column (25 m × 0.22 mm I.D.; SGE, Australia) in a splitless mode (Shimadzu GC 14A). Helium was used as the carrier gas. The initial oven temperature was 50°C, held for 1 min, then increased to 160°C at 20°C/min, followed by an increase in temperature to 300°C at 3°C/min and maintained for 5 min. The injector and detector temperatures were 250°C. With these conditions, fatty acid methyl esters from the membrane of one hemi-retina could be detected.

Peroxidation Assay

Six samples for each group were used for direct assay of phospholipid hydroperoxides by CL-HPLC.[17,18] Phospholipids were extracted with chloroform–methanol (2:1, v/v). The chloroform layer was collected, dried under argon and re-dissolved in chloroform–methanol (2:1 by volume), then injected into a CL-HPLC unit for phospholipid hydroperoxide analysis. The CL-HPLC system included a JASCO Finepak SIL column (5 μm, 250 × 4.6 mm; Japan Spectroscopic Co., Tokyo, Japan) which was placed in an oven at 35°C, with a mobile phase of isopropanol/methanol/water (13.5:4.5:2, v/v/v) at a flow rate of 1.8 ml/min by a JASCO 980-PU pump. In the post-column detection system, phospholipid absorption at 210 nm was monitored with a JASCO 970-UV detector and hydroperoxide specific chemiluminescence was measured with a CLD-110LH detector (Tohoku Electronic Ind. Co., Sendai, Japan). The CL reagent was a mixture of 10 mg/l of cytochrome c and 2 mg/l of luminol in 50 mM borate buffer at pH 10.0.

Quantification of Ascorbic Acid

Using HPLC with an electrochemical detector (ECD), both ascorbic acid and dehydroascorbic acid in one eighteenth of a retina could be quantified efficiently. Briefly, 50 μl of methaphosphoric acid (10% w/v) was added to 50 μl of the retinal homogenate, centrifuged at 10,000*g* for 10 min at 4°C. The supernatant (2 μl) was applied to HPLC. Reducing treatment of dehydroascorbic acid was carried out by adding 50 μl of 10 mM dithiothreitol in 1 M K_2HPO_4 to 100 μl of above supernatant, letting it stand at room temperature for 5 min, and then adding 50 μl of methaphosphoric acid (25% w/v). A 2 μl of the mixture was injected into HPLC-ECD for total ascorbic acid quantification.[19] Conditions for HPLC analysis were the same as described previously.[20]

Statistical Methods

Statistical analysis were performed first between DHA and linoleic acid groups with same ascorbate levels (Group 2 and Group 5), then between Groups 1–4 with same DHA levels. Data were assessed by multiple comparison tests. Significant effects of treatment were defined utilizing Scheffe's method as *post-hoc* test. All data were presented as the mean ± standard deviation (SD).

RESULTS

Fatty Acids in ROS Membranes After Dietary Manipulation

As shown in Table II, linoleic acid was maintained at the same level (about 11%) in all groups that received a high dose of DHA (30.5%) in their diets. As expected, significant increases in DHA levels in ROS membranes were produced in all high DHA feeding groups examined (Group 2 and Group 4), compared with the control group, Group 5, which was fed high linoleic acid (Table III). The dietary manipulation produced DHA levels ranging from 47% (Group 2) down to 37% (Group 5). Changes in 22:5n3 level showed a similar tendency to DHA, i.e. 22:5n3 was significantly increased in the high DHA-fed groups

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TABLE III Fatty acid composition of lipid from 80-day-old ODS rat ROS membrances ($n = 5$)

Group	2	4	5
Fatty acid			
16:0	16.55 ± 0.78^{a}	19.40 ± 1.79^{b}	19.24 ± 2.24^{b}
18:0	23.87 ± 0.92^{a}	22.23 ± 0.41^{b}	25.07 ± 1.83^{a}
18:1n9	5.24 ± 2.22	6.77 ± 1.33	6.80 ± 1.43
18:1n7	1.67 ± 0.33	1.68 ± 0.30	1.99 ± 0.29
18:2n6	2.02 ± 1.06	2.44 ± 1.19	1.68 ± 1.65
20:4n6	2.83 ± 0.84^{a}	2.89 ± 0.53^{a}	4.21 ± 0.62^{b}
22:4n6	0.11 ± 0.15^{a}	0.11 ± 0.15^{a}	0.65 ± 0.37^{b}
22:5n6	0.23 ± 0.18^{a}	0.48 ± 0.34^{a}	3.81 ± 0.23^{b}
22:5n3	0.43 ± 0.12^{a}	0.55 ± 0.08^{a}	nd^{b}
22:6n3	47.04 ± 4.64^{a}	43.44 ± 3.90^{a}	36.55 ± 3.99^{b}

Values given are means ± SD. For each measurement, 2 retinas were pooled. Data were assessed between Group 2 and Group 5 which had same ascorbic acid levels, then between Group 2 and Group 4 which had same DHA levels, using Student's *t*-test. Means within the same row not followed by a common letter are significantly different ($p < 0.05$). nd, Not detected.

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(Group 2 and Group 4) compared with the control group (Group 5). On the other hand, addition of dietary DHA was associated with a significant decline in n-6 fatty acids, 20:4n6, 22:4n6 and 22:5n6. The lower arachidonic acid level was obtained in Group 2 and Group 4, compared with the high arachidonic acid level in Group 5. Another n-6 fatty acid, 22:5n6, was especially reduced from 3.8% in Group 5 to 0.2% in Group 2. However, 18:2n6 was inversely increased from 1.7% (Group 5) to 2.4% (Group 4), but the change was not significant. All these results were consistent with our previous study.[13]

Ascorbic Acid Concentrations in Retinas of ODS Rats Fed with Different Amount of Ascorbic Acid

We found elevations of both reduced ascorbic acid and total ascorbic acid concentrations, corresponding to increasing amounts of ascorbic acid supplementation (Table IV). Groups fed 300 mg ascorbic acid supplemented-diets (Group 2 and Group 5) were considered to be the control groups. There was no significant difference in the growth rates between these groups. Group 1 was the ascorbic acid-deficient group; the retinal ascorbic acid concentration was significantly lower than almost all other groups.

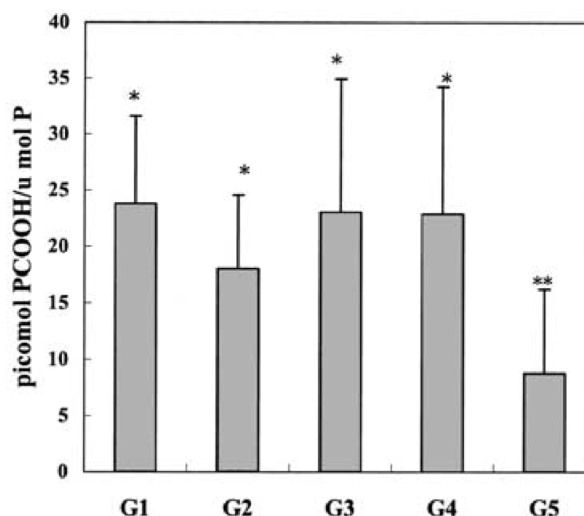

FIGURE 1 Phosphatidylcholine hydroperoxide in retinal ROS membranes of ODS rats fed with different amounts of DHA and ascorbic acid ($n = 6$). Values are mean ± SD. * and **, significantly different at $p < 0.05$ (Student's *t*-test for Group 2 and Group 5, Scheffe's method for Groups 1–4).

On the other hand, the retinal ascorbic acid concentration in Group 4 was significantly higher, due to the very high supplementation of ascorbic acid. We also determined the concentration of dehydroascorbic acid by reducing it with dithiothreitol to quantify the total amount of vitamin C by HPLC-ECD. But dehydroascorbic acid concentrations were similar and low in all groups. The mean values were below 1.0 nmol/retina. There was no significant difference in concentration in either ascorbic acid or its oxidized form between Group 2 and Group 5, although the level of phospholipid hydroperoxide in Group 2 was higher than that in Group 5 (see below).

Phospholipid Hydroperoxides in ROS Membranes with Different Amount of DHA and Ascorbic Acid

Phospholipid hydroperoxides were quantified by CL-HPLC, which can detect as little as one femtomol of phospholipid hydroperoxide. Figure 1 shows that significantly high amounts of phosphatidylcholine hydroperoxide (PCOOH) were detected in ROS

TABLE IV Ascorbic acid concentration (nmol/retina) in the retina of ODS rats fed different amounts of ascorbic acid ($n = 6$)

Group	1	2	3	4	5
Ascorbic acid (mg/kg diet)	100	300	600	3000	300
Ascorbic acid	4.26 ± 0.76^{a}	6.74 ± 1.73^{ab}	8.26 ± 2.06^{b}	11.45 ± 2.00^{c}	8.58 ± 2.26^{b}
Total ascorbic acid	4.08 ± 0.97^{a}	7.64 ± 1.63^{b}	8.88 ± 1.86^{b}	12.17 ± 1.68^{c}	8.82 ± 1.83^{b}

Values given are means ± SD. For each measurement, 2 retinas were pooled. Data were assessed first between Group 2 and Group 5 using Student's *t*-test, which had same ascorbic acid levels, then by multiple comparison tests between Groups 1–4 with same DHA levels. Significant effects of treatment were defined utilizing Scheffe's method. Means within the same row not followed by a common letter are significantly different ($p < 0.05$).

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membranes of all DHA fed groups (Groups 1–4) compared with the control group (Group 5). All groups fed with the same dose of DHA had similar levels of PCOOH, without respect to the supplemental amount of ascorbic acid. The difference in DHA content of retinas between high DHA groups and the high linoleic acid group was about 10% of total fatty acid composition. An equivalent value of PCOOH in ROS membranes of ODS rat was below 50 picomol PCOOH/μmol phospholipids, which was near to the value reported in plasma from healthy adults (40–80 pmol/μmol phospholipids).[18] Phosphatidylethanolamine hydroperoxide levels were much less than PCOOH, which were detected in only seven of 30 samples (data not shown).

DISCUSSION

The target of retinal light damage may first be polyunsaturated fatty acids, and is known to be associated with an increase in conjugated dienes and a decrease in ROS DHA levels. As a diet-induced decrease in a readily oxidizable polyunsaturated fatty acid such as DHA could impart protection against light damage, we are very interested in whether a diet-induced increase in DHA could increase the peroxidative susceptibility of ROS membranes. Actually, most light damage studies are carried out with Sprague–Dawley rats and light levels in the rearing environment are in excess of “standard light rearing conditions”.[14,21,22] Our previous study using young Sprague–Dawley rats provided data on the levels of phospholipid hydroperoxide, which showed no positive relationship between DHA content and oxidative susceptibility *in vivo* when reared under a normal light condition, although UV irradiation of ROS membranes did increase the phospholipid hydroperoxide levels with high DHA content.[13] As described in the introduction, we supposed the possible role of antioxidants such as ascorbic acid in retina.

It is well established that ascorbic acid accumulates in ocular tissues of many animal species at a concentration several times higher than the plasma level. One role of ascorbic acid in the eye might be to protect against damage that can result from exposure to sunlight.[23] To study the effect of dietary ascorbic acid on antioxidative function, an animal model that cannot synthesize ascorbic acid is necessary. As far as we know, this is the first report on ascorbic acid levels and phospholipid hydroperoxide levels of retina with ODS rats. By comparison of present and previous data, a significant difference is that the present study using ODS rats shows that supplementation of high-dose DHA enhances the retinal phospholipid hydroperoxide levels, regardless of ascorbic acid levels, whereas for Sprague–Dawley rats it does not. We do not know if the genetic background is the reason. Actually, many factors such as light conditions, could affect the result. For example, Organisciak *et al.* using albino Sprague–Dawley rats found that supplementation of ascorbic acid or its derivatives, by intraperitoneal or intraocular injections prior to light exposure, reduces the loss of rhodopsin and photoreceptor cell nuclei resulting from intense light. Ascorbic acid may act in an antioxidative fashion by inhibiting oxidation of membrane lipids during intense light.[24,25] However, in a recent study with albino rats, intraperitoneal injection of ascorbic acid failed to protect against blue light-induced retinal damage.[26]

By using ODS rats, Kimura *et al.* show that dietary ascorbic acid depresses plasma and low density lipoprotein lipid peroxidation.[27] But Cadenas *et al.* indicate that ascorbic acid at the dietary levels studied (150–900 mg/kg diet) did not affect lipid peroxidation of liver and plasma.[28] According to the result of Kimura *et al.*, although supplementing 300 mg ascorbic acid/kg diet was insufficient to maintain a hepatic concentration of ascorbic acid comparable to that in the liver of ODS +/+ rats, it keeps lipid peroxide concentration in plasma LDL and liver within the normal ranges seen in the ODS +/+ rats.[27] We can guess that the ascorbic acid level in the retina of ODS rats fed with 300 mg ascorbic acid/kg diet may be lower than that of ODS +/+ rats. Moreover, by comparing with the studies with Sprague–Dawley rats,[14,24] we noted another difference that the ascorbic acid levels in ODS rat retinas were quite low even fed with 300 mg ascorbate/kg diet, which has been considered as enough dietary requirement of ascorbic acid to maintain normal body weight. To reach normal ascorbate levels for Sprague–Dawley rats ($\geqq$ 13 nmol/retina) on standard house diets, over 3000 mg/kg diet of ascorbate should be supplied to ODS rats (Table IV, Group 4). Such lower ascorbic acid levels in retinas of ODS rats may have contributed to the result, i.e. higher DHA content in retinas seems to play a more important role in peroxidative susceptibilities.

In conclusion, high dietary doses of preformed DHA increased the DHA content of ROS significantly in young ODS rats compared with low n-3 and high linoleic acid feeding. There was a significant difference in phospholipid hydroperoxides between ROS with high and low DHA content under normal light conditions. Dietary supplementation of ascorbic acid increases the retinal ascorbic acid concentrations, but the increase did not affect the hydroperoxide levels derived from the high dosage of DHA.

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