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Effect of docosahexaenoic acid intake on lipid peroxidation in diabetic rat retina under oxidative stress

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Abstract

Docosahexaenoic acid (DHA) plays an important role in visual function but has a highly oxidation-prone chemical structure. Therefore, we investigated how dietary DHA affects the generation of lipid peroxides in rat retina under oxidative stress in diabetes with/without vitamin E (VE) deficiency. Streptozotocin-induced (50 mg i.p./kg B.W.) diabetic Sprague–Dawley (SD) rats were assigned to four groups: (i) control/VE(+), (ii) DHA/VE(+), (iii) control/VE(−) and (iv) DHA/VE(−), and raised for 28 days. We then measured lipid peroxide levels in the retina, serum and liver. With a normal intake of VE, dietary DHA increased only the retinal level of thiobarbituric acid-reactive substances (TBARS) slightly. In contrast, in rats with VE deficiency, dietary DHA increased serum and liver lipid peroxide levels but not in the retina. These results suggest that dietary DHA does not necessarily promote lipid peroxidation in the retina even under high oxidative stress.

Keywords: *Docosahexaenoic acid, oxidative stress, lipid peroxidation, vitamin E, retina, diabetes*

Introduction

Docosahexaenoic acid (22:6$n-3$; DHA) and eicosapentaenoic acid (EPA) are both $n-3$ polyunsaturated fatty acids (PUFAs) predominant in fish oils. DHA is very prone to lipid peroxidation because of its unstable chemical structure with six double bonds. Intake of fish oil rich in DHA and EPA enhances sensitivity to lipid peroxidation and promotes the requirement of vitamin E (VE) in the liver [1]. Song et al. [2] have reported that DHA intake increases the levels of phospholipid hydroperoxides and thiobarbituric acid-reactive substances (TBARS) in the serum, liver and kidney of rats. On the other hand, dietary DHA does not promote lipid peroxidation in rat tissues, particularly liver, to the extent expected from the peroxidizability index of the lipids [3–5]. This phenomenon is particularly prominent in the case of DHA intake, is less prominent in the case of EPA intake, and absent in the case of α-linolenic acid intake [5]. In addition, other reports have indicated that tissue TBARS levels do not increase, or are even decreased, by intake of fish oil [6,7]. Hence, the results obtained so far appear to be difficult to interpret. However, our previous results obtained in studies of tissue lipid peroxidation suggest that the oxidative stability of DHA *in vivo* is not as low as would be expected from its unstable chemical structure with six double bonds [3–5].

DHA is distributed at high levels in the neural system, especially the retina and brain. Within the

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retina, DHA is present at high proportions in the phospholipids (PLs) of photoreceptor cells, particularly in the rhodopsin-containing rod outer segment (ROS) membranes [8–12]. The retina is thought to be highly sensitive to photooxidation because of its high proportion of DHA [13]. It is reported that in the retina, light induces the generation of lipid peroxides and reactive oxygen species [14], and also decreases DHA in the ROS membranes [15], which is considered to be a cause of retinal light damage [16,17]. In contrast, rats fed a diet containing fish oil rich in DHA show partial protection against acute light-induced lesions in the ROS, whereas retinal susceptibility to lipid peroxidation is increased but the levels of VE and DHA are unchanged relative to the control [18]. We have measured the levels of phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) in the retina of rats fed high levels of DHA [19]. In spite of an increase in the proportion of DHA in the ROS membrane lipids, there was no increase in the level of hydroperoxide in the retinal membranes. Therefore, we were interested in clarifying whether DHA clearly promotes the generation of lipid peroxides in the retina, especially under severe oxidative stress.

It is well known that diabetes induces oxidative stress [13,20,21]. Therefore, in the present study, we investigated whether dietary DHA promotes the generation of lipid peroxides and free radicals in the rat retina, as well as the serum and liver, under the oxidative stress of diabetes. Furthermore, to obtain more conclusive evidence, we conducted experiments under more severe conditions induced by diabetes with VE deficiency.

Materials and methods

Animals and diets

The experiments were done in accordance with the guidelines of the Animal Committee of the Incorporated Administrative Agency, National Institute of Health and Nutrition (Tokyo, Japan).

Male Sprague–Dawley rats (CLEA Japan, Tokyo, Japan) aged 7 weeks and weighing 200–220 g were housed individually at a controlled temperature of 22 ± 1°C and humidity of 50–60% with a 12 h light:dark cycle. They were first fed a control/VE(−) diet (Table I) formulated on the basis of the AIN-93G diet for rodents [22], and had access to water *ad libitum* for 7 days. Since we intended to focus on the events occurring under severe oxidative stress, all the rats were intraperitoneally injected with streptozotocin (Wako, Osaka, Japan, 50 mg/kg body weight) in 10 mM citrate buffer, pH 5.5, to induce diabetes on day 3 in a preliminary feeding period of 7 days. Blood samples for measurement of plasma glucose (Glucose B-Test Wako, Wako, Osaka, Japan) were taken from the tail vein 48 h after the streptozotocin injection. The rats were then randomly assigned by body weight and blood glucose level to four diet groups: (i) control/VE(+) group, (ii) DHA/VE(+) group, (iii) control/VE(−) group, and (iv) DHA/VE(−) group, and raised for 28 days.

The compositions of the experimental diets and the fatty acid compositions (g/100 g) of the dietary lipids are shown in Table I. The dietary lipids were prepared by combining stripped corn oil, olive oil and DHA ethyl esters to provide almost the same level of total PUFAs. The purity of the DHA concentrate (ethyl ester form) was 93.58%. RRR-α-tocopheryl acetate was used to adjust the dietary VE level to 6 mg/kg diet in the VE-deficient (VE(−)) groups and 100 mg/kg diet in the VE-sufficient (VE(+)) groups. To prevent autoxidation of DHA in the diet, the diet was initially prepared without added DHA and stored at −20°C. DHA stored at −80°C was mixed with the diet every day immediately before feeding. Each diet was made available to the rats in the evening and removed the following morning. Food and water were available *ad libitum*.

At the end of the study period, the rats were anesthetized with Nembutal by intraperitoneal injection and killed by cardiac puncture. Their livers were promptly removed, washed with isotonic saline and weighed. The retinas were excised and quickly frozen in liquid nitrogen. Serum was separated by centrifugation at 2700*g* at 4°C for 15 min. The tissues and sera were stored at −80°C until use.

Experimental procedures

The right retina was homogenized in 1.5 ml of ice-cold 0.1 M sodium phosphate buffer, pH 6.5, and this homogenate was used for analyses of TBARS, VE levels and fatty acid composition. The left retina was homogenized in the same buffer, and part of this homogenate was used to analyze PCOOH and PEOOH. The rest of the homogenate was centrifuged at 3000*g* at 4°C for 10 min, and the supernatant was used for measurement of free malondialdehyde plus 4-hydroxy-2-alkenals (MDA + 4HAE) and aldose reductase (AR) activity.

Measurement of PCOOH and PEOOH levels

PLs were extracted by the Bligh–Dyer method [23]. The chloroform layer was collected, dried by evaporation, redissolved in chloroform–methanol, and then injected into a chemiluminescence (CL)–HPLC unit for phospholipid hydroperoxide analysis [24]. The CL–HPLC system included a Finepac SIL-NH_2 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 (6.75:2.25:1, v/v/v) at a flow rate of 1.0 ml/min by a Shimadzu LC-10AD vp pump

Table I. Composition of experimental diets (g/kg diet) ⋆ and fatty acid composition (g/100 g) of dietary lipids given to rats.

Group	Control/VE(+)	DHA/VE(+)	Control/VE(−)	DHA/VE(−)
		%Total energy		
Linoleic acid	7.3	3.7	7.3	3.7
DHA	0	3.8	0	3.8
		g/kg Diet		
Basic components†	930	930	930	930
Test lipids‡	70	70	70	70
Stripped corn oil	58	27.7	58	27.7
Olive oil	12	25.6	12	25.6
DHA concentrate¶	0	16.7	0	16.7
		Fatty acid (g/100 g diet)		
16:0	11.0	8.6	11.0	8.6
16:1($n-7$)	0.1	0.3	0.1	0.3
18:0	2.3	2.0	2.3	2.0
18:1($n-9$)	38.9	40.6	38.9	40.6
18:2($n-6$)	45.9	23.5	45.9	23.5
18:3($n-3$)	0.8	0.5	0.8	0.5
20:4($n-6$)	–	0.3	–	0.3
20:5($n-3$) EPA	–	0.2	–	0.2
22:6($n-3$) DHA	–	22.4	–	−22.4
other	1.0	1.6	1.0	1.6
ΣSFA	13.3	10.6	13.3	10.6
ΣMUFA	39.0	40.9	39.0	40.9
ΣPUFA	46.7	46.9	46.7	46.9
P/S ratio	3.5	4.4	3.5	4.4
Σ$n-3$	0.8	23.1	0.8	23.1
Σ$n-6$	45.9	23.8	45.9	23.8
$n-6/n-3$ ratio	57.4	1.0	57.4	1.0
		mg/kg Diet		
RRR-α-tocopherol equivalent	100	100	6	6

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; P/S, ΣPUFA/ΣSFA

⋆ The energy density of all diets was 16.8 MJ/kg diet (4020 kcal/kg), using Atwater energy factors for energy calculation. † The basic components of the diet given to all the groups were as follows (g/kg): corn starch, 399.986 g; casein, 200.0 g; glucose, 142.0 g; sucrose, 90.0 g; cellulose powder, 50.0 g; AIN-93G mineral mixture, 35.0 g; AIN-93 vitamin mixture (VE free), 10.0 g; L-cystine, 3.0 g; *tert*-butylhydroquinone, 0.014 g. ‡ Fat energy percentage is 15.9% of total energy. ¶ DHA ethyl esters prepared from the ethyl esters of the orbital fat of tuna were used (Maruha Corporation, Tsukuba, Japan). The purity was 93.58%.

(Shimadzu Co., Kyoto, Japan). In the post column detection system, PL absorption at 210 nm was monitored with a Shimadzu SPD 10AV vp detector (Shimadzu Co., Kyoto, Japan) and hydroperoxide-specific chemiluminescence was measured with a CLA-FS1LHe detector (Tohoku Electronic Ind. Co., Sendai, Japan). The CL reagent was a mixture of 10 mg/l cytochrome *c* and 2 mg/l luminol in 50 mM borate buffer at pH 10.0, which was introduced into the reactor at a rate of 1.8 ml/min. The PCOOH and PEOOH produced from photooxidation of β-oleoyl-γ-palmitoyl-L-α-phosphatidylcholine and β-oleoyl-γ-palmitoyl-L-α-phosphatidylethanolamine were used as standards for preparing calibration curves for PCOOH and PEOOH. The concentrations of authentic PCOOH and PEOOH were determined by the ferrous oxidation-xylenol orange assay [25].

TBA value

TBA value was measured according to the method of Ohkawa et al. [26] for the retina and liver, or that of Yagi [27] for the serum, with a minor modification: butylated hydroxytoluene (BHT) was added to the reaction mixture at a final concentration of 0.45 mM.

Measurement of free MDA + 4HAE

Measurement of free MDA + 4HAE was performed using a commercial kit (LPO-586, OXIS International Inc., Portland, OR, USA).

Measurement of α-tocopherol concentration

Concentration of α-tocopherol was analyzed using HPLC as described by Ueda et al. [28].

Measurement of AR activity

AR activity was measured according to the method of He et al. [29].

Retinal fatty acid analysis

Fatty acid composition of retinal lipids was analyzed as described by Wang et al. [19] and Sekine et al. [30].

Briefly, total lipids were extracted using the Folch method [31]. Chloroform–methanol (2:1, v/v) containing 2.25 mg/l BHT as an antioxidant was used. Separation of fatty acid methyl esters was carried out by gas–liquid chromatography utilizing a capillary column (ULBON HR-SS-10, 2.5 mm i.d. × 25 m; Shinwa Chemical Industries, Kyoto, Japan) in splitless mode (Shimadzu GC 2010).

Measurement of other parameters

The levels of serum glucose, total protein, triacylglycerol (TG), PLs, total cholesterol (T-CHO) and HDL-cholesterol (HDL-CHO) were determined using commercially available clinical assay kits.

Statistical analysis

The results are expressed as means ± standard deviation (SD). Data were subjected to two-way ANOVA. When overall significance ($P < 0.05$) was attained, individual comparisons between groups were made using Student's *t*-test.

Results

Growth, food intake, liver weight, and serum glucose levels

There were no significant differences in body weight gain, food intake and serum glucose levels among the four groups (Table II). Relative liver weights were significantly different due to dietary oil, but no significant differences were recognized between the control/VE(+) group and DHA/VE(+) group, or between the control/VE(−) group and DHA/VE(−) group using Student's *t*-test.

Lipid peroxide levels in the retina, serum and liver

In the retina, PCOOH and PEOOH levels were not significantly different due to dietary oil in the VE-sufficient groups (Figure 1). VE deficiency considerably lowered these hydroperoxide levels in comparison with each VE-sufficient group, whereas no significant differences were observed between the control/VE(−) group and the DHA/VE(−) group. The TBARS level in the DHA/VE(+) group was significantly higher than that in the control/VE(+) group (Figure 2). On the other hand, there was no significant difference between the control/VE(−) group and the DHA/VE(−) group. Notably, in the control diet groups, VE deficiency resulted in about a 10-fold increase in the TBARS level, whereas in the DHA diet groups, no significant increase was observed. Free MDA + 4HAE levels did not differ significantly among the treatment groups (Figure 3).

In serum, PCOOH levels did not differ significantly among the four groups, whereas the TBARS level in the DHA/VE(−) group was significantly higher than that in the control/VE(−) group or DHA/VE(+) group (Table III). In the liver, levels of PCOOH, PEOOH and free MDA + 4HAE did not differ significantly among the treatment groups. On the other hand, TBARS level in the DHA/VE(−) group was significantly higher than that in the control/VE(−) group, but not that in the DHA/VE(+) group.

α-Tocopherol levels and AR activities

VE levels in the body could greatly affect the oxidative susceptibility of DHA. Therefore, we measured the concentrations of α-tocopherol in tissues and serum (Table IV). In the retina, VE deficiency caused a significant decrease of α-tocopherol levels, but no significant differences were observed between the VE-sufficient diet groups or between the VE-deficient diet groups. In contrast, in the serum and liver, DHA intake as well as VE deficiency resulted in a further decrease in the levels of α-tocopherol.

AR can metabolize active aldehydes such as free 4HAE as well as extra glucose generated by hyperglycemia. In the retina, there were no significant differences among the treatment groups, whereas in the liver, three groups other than the control/VE(+) group had significantly higher AR activity.

Fatty acid composition of retinal lipids

We confirmed that dietary intake of DHA increased the retinal proportion of DHA (22:6) under oxidative conditions of diabetes and even with VE deficiency (Table V). In contrast, levels of $n - 6$ fatty acids, such

Table II. Effects of dietary DHA and VE deficiency on body weight gain, food intake, liver weight and serum glucose levels in diabetic rats.

	VE(+)		VE(−)		ANOVA *P*-values[¶]		
	Control	DHA	Control	DHA	Oil	VE	Oil × VE
Body weight gain (g/day)	4.88 ± 2.32	4.89 ± 1.91	4.79 ± 1.69	5.14 ± 1.67	NS	NS	NS
Food intake (g/day)	26.37 ± 6.42	25.26 ± 3.32	26.64 ± 5.87	26.70 ± 3.64	NS	NS	NS
Liver weight (g/100 g BW)	4.03 ± 0.46	3.78 ± 0.47	4.17 ± 0.50	3.71 ± 0.44	$P < 0.05$	NS	NS
Serum glucose levels (mmol/l)	20.06 ± 8.77	19.97 ± 7.25	19.18 ± 4.17	16.26 ± 1.39	NS	NS	NS

Values are means ± SD ($n = 9$). [¶]Oil, effect of dietary oil; VE, effect of dietary VE level; oil × VE, interaction between dietary oil and VE level by two-way ANOVA. NS, not significant.

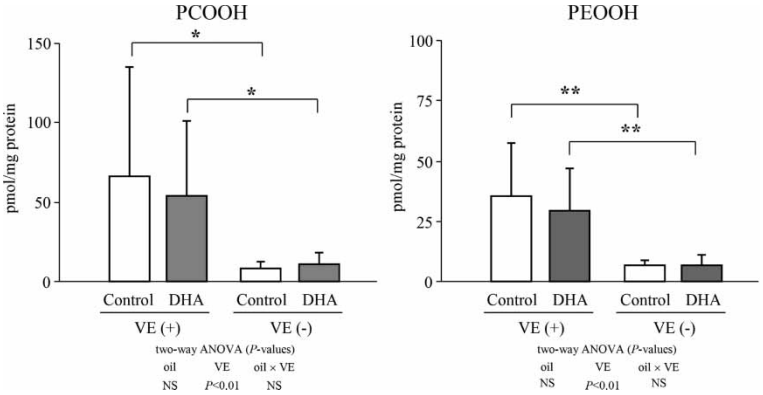

Figure 1. Effects of dietary DHA and VE deficiency on PCOOH and PEOOH levels in the retina of diabetic rats. Values are means ± SD ($n = 9$) except for the control/VE (−) group and the DHA/VE (−) group ($n = 8$). $\star P < 0.05$; $\star\star P < 0.01$. NS, not significant.

as docosatetraenoic acid (22:4) and arachidonic acid (20:4), were decreased by DHA intake.

Serum lipid levels

Amelioration of serum lipid levels is one of the positive effects of DHA intake, as reported by our group [32,33] and others [34,35]. Even under diabetic conditions, dietary DHA also decreased the levels of TG, PLs and T-CHO (Table VI). There were no significant differences in HDL-CHO among the treatment groups.

Discussion

In this study, we investigated whether dietary DHA clearly promotes lipid peroxidation under conditions of oxidative stress in diabetes and diabetes with VE deficiency. We focused especially on the retina because it contains a very high proportion of DHA [8,11]. If DHA promotes lipid peroxidation *in vivo* as observed in the liver and kidney [2–5], the retina should readily be affected by such severe oxidative stress, leading to generation of lipid peroxides. Free radicals generated in the body attack lipids present in biomembranes, thus producing lipid hydroperoxides such as PCOOH and PEOOH at an early stage of lipid peroxidation. These hydroperoxides are then further oxidized to TBARS, and subsequently to free aldehydes, such as MDA and 4HAE, which are oxidative degradation products of $n - 6$ and $n - 3$ PUFAs. Therefore, we analyzed these products at each stage to clarify the whole process of lipid peroxidation.

First, we will discuss the events occurring in diabetes without VE deficiency. In the retina of VE-sufficient diabetic rats, dietary DHA had no effect on

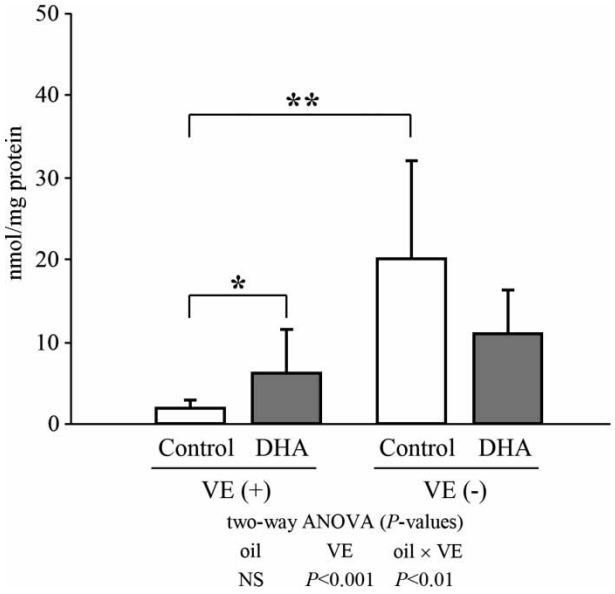

Figure 2. Effect of dietary DHA and VE deficiency on TBARS levels in the retina of diabetic rats. Values are means ± SD ($n = 9$). $\star P < 0.05$; $\star\star P < 0.01$. NS, not significant.

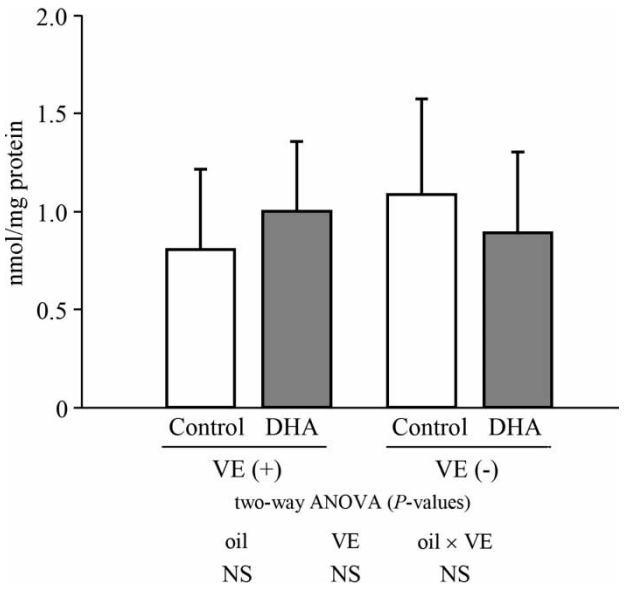

Figure 3. Effect of dietary DHA and VA deficiency on free MDA + 4HAE level in the retina of diabetic rats. Values are mean ± SD ($n = 9$). NS, not significant.

Table III. Effects of dietary DHA and VE deficiency on concentrations of lipid peroxides in serum and liver of diabetic rats.

	VE(+)		VE(−)		ANOVA *P*-values[¶]		
	Control	DHA	Control	DHA	Oil	VE	Oil × VE
Serum							
PCOOH (pmol/ml)	24.72 ± 14.07	33.76 ± 35.74	34.07 ± 41.01	26.04 ± 20.20	NS	NS	NS
TBARS (nmol/ml)	4.10 ± 1.23	3.84 ± 0.87	4.51 ± 1.73	17.2 ± 11.46⋆‡	$P < 0.01$	$P < 0.01$	$P < 0.01$
Liver							
PCOOH (pmol/g tissue)	119.85 ± 35.18	112.89 ± 43.28	147.65 ± 71.97	140.16 ± 60.21	NS	NS	NS
PEOOH (pmol/g tissue)	37.13 ± 16.48	32.39 ± 8.53	48.47 ± 25.04	33.90 ± 9.47	NS	NS	NS
TBARS (nmol/g tissue)	115.20 ± 19.45	134.76 ± 26.70	111.25 ± 15.71	140.12 ± 24.15‡	$P < 0.01$	NS	NS
Free MDA + 4HAE (nmol/g tissue)	10.13 ± 1.80	9.16 ± 2.78	10.71 ± 5.63	12.45 ± 4.15	NS	NS	NS

Values are means ± SD ($n = 8 - 9$). Significantly different between VE levels in feeding the same dietary oil, using Student's *t*-test (⋆$P < 0.01$). Significantly different between oils in feeding VE(−) diet, using Student's *t*-test (‡$P < 0.01$). [¶]Oil, effect of dietary oil; VE, effect of dietary VE level; oil × VE, interaction between dietary oil and VE level by two-way ANOVA. NS, not significant.

Table IV. Effects of dietary DHA and VE deficiency on α-tocopherol concentrations in serum and tissues, and AR activities in tissues of diabetic rats.

	VE(+)		VE(−)		ANOVA *P*-values[¶]		
	Control	DHA	Control	DHA	Oil	VE	Oil × VE
Serum							
α-Tocopherol (nmol/ml)	11.42 ± 1.11	9.81 ± 0.55§§	5.50 ± 2.03⋆⋆	2.16 ± 0.28⋆⋆‡	$P < 0.001$	$P < 0.001$	$P < 0.05$
Liver							
α-Tocopherol (nmol/g tissue)	91.43 ± 19.45	68.58 ± 18.72§	15.53 ± 4.70⋆⋆	6.83 ± 0.54⋆⋆‡	$P < 0.01$	$P < 0.001$	NS
Aldose reductase (AR) (nmol NADPH/mg protein/min)	0.71 ± 0.27	1.15 ± 0.39§	1.27 ± 0.39⋆	1.17 ± 0.18	NS	$P < 0.05$	$P < 0.05$
Retina							
α-Tocopherol (nmol/mg protein)	0.46 ± 0.20	0.40 ± 0.08	0.14 ± 0.03⋆	0.13 ± 0.05⋆⋆	NS	$P < 0.001$	NS
Aldose reductase (AR) (nmol NADPH/mg protein/min)	10.3 ± 3.36	10.25 ± 2.99	10.70 ± 3.31	9.30 ± 2.59	NS	NS	NS

Values are means ± SD ($n = 8 - 9$). Significantly different between VE levels in feeding the same dietary oil, using Student's *t*-test (⋆$P < 0.01$; ⋆⋆$P < 0.001$). Significantly different between oils in feeding VE(+) diet, using Student's *t*-test (§$P < 0.05$; §§$P < 0.01$). Significantly different between oils in feeding VE(−) diet, using Student's *t*-test (‡$P < 0.01$). [¶]Oil, effect of dietary oil; VE, effect of dietary VE level; oil × VE, interaction between dietary oil and VE level by two-way ANOVA. NS, not significant.

Table V. Effects of dietary DHA and VE deficiency on fatty acid composition of retinal lipids in diabetic rats (g/100 g).

	VE(+)		VE(−)	
	Control	DHA	Control	DHA
14:0	0.49 ± 0.12	0.48 ± 0.09	0.53 ± 0.09	0.48 ± 0.07
16:0	21.86 ± 1.25	22.76 ± 0.93	23.88 ± 1.30**	23.46 ± 0.95
16:1($n-7$)	0.53 ± 0.13	0.46 ± 0.06	0.51 ± 0.07	0.48 ± 0.07
18:0	26.61 ± 1.68	27.50 ± 0.60	28.94 ± 1.40**	28.50 ± 0.93*
18:1($n-9$)	9.80 ± 0.83	10.10 ± 0.73	10.11 ± 0.88	10.61 ± 0.70
18:2($n-6$)	1.13 ± 0.27	1.15 ± 0.17	1.10 ± 0.33	1.22 ± 0.15
20:4($n-6$)	6.63 ± 0.58	5.48 ± 0.40§§	7.20 ± 0.38*	5.82 ± 0.32‡‡
22:4($n-6$)	1.21 ± 0.11	0.64 ± 0.07§§	1.36 ± 0.14*	0.67 ± 0.05‡‡
22:6($n-3$)	17.77 ± 2.61	23.37 ± 2.15§§	16.57 ± 2.47	21.15 ± 2.73‡
Others	14.04 ± 4.24	8.16 ± 1.52	9.85 ± 1.50	7.81 ± 1.17

Values are means ± SD ($n = 8 - 9$). Significantly different between VE levels in feeding the same dietary oil, using Student's *t*-test (*$P < 0.05$; **$P < 0.01$). Significantly different between oils in feeding VE(+) diet, using Student's *t*-test ($^{\S\S}P < 0.001$). Significantly different between oils in feeding VE(−) diet, using Student's *t*-test ($^{\ddagger}P < 0.01$; $^{\ddagger\ddagger}P < 0.001$).

the levels of PCOOH and PEOOH, increased the levels of TBARS slightly but significantly, and had no effect on free MDA + 4HAE levels (Figures 1–3). On the other hand, in the serum and liver, no significant differences were observed under diabetic oxidative stress between the control and DHA diet groups in terms of PCOOH and PEOOH levels, TBARS levels, and free MDA + 4HAE levels (Table III). We have previously found that, in normal rats, intake of a high level of DHA did not increase PCOOH and PEOOH levels in the retinal ROS membranes [19], or free MDA + 4HAE in the retina [36]. In addition, Miyazawa et al. [37] reported that dietary fish oil, rich in DHA, did not significantly affect PCOOH and PEOOH levels in the normal rat brain, which contains a high level of DHA similar to the retina. We also found that a high level of dietary DHA did not increase the level of TBARS in the normal rat brain [3]. These findings suggest that dietary DHA does not promote lipid peroxidation in nervous tissues, including the retina, in normal rats. Our present results showed that dietary DHA slightly but significantly increased only the retinal TBARS level during the process of lipid peroxidation in rats with STZ-induced diabetes. This increase might be due to the influence of diabetic oxidative stress. However, this does not necessarily mean that daily intake of DHA would harm diabetic patients because the level of DHA used in this study was rather high. Therefore, the effect of daily DHA intake on the levels of various lipid peroxides, including TBARS, in diabetic patients needs to be ascertained.

We will now focus on the events occurring under more serious oxidative stress in diabetes with VE deficiency. Levels of PCOOH and PEOOH, which are early-stage products of lipid peroxidation, were decreased in the retina to the same degree in both the control/VE(−) and DHA/VE(−) groups. These results contradict the concept that VE deficiency promotes more intense oxidative stress, and thus should increase the levels of these hydroperoxides. One possible explanation for this contradiction is that, under diabetic and VE-deficient conditions, any lipid hydroperoxyl radicals that are generated may be readily degraded to further oxidized products. In the serum and liver, on the other hand, the levels of lipid hydroperoxides tended to be higher, but did not change significantly even in VE deficiency in both the control and DHA groups (Table III). TBARS, products of the next stage of oxidation, in the retina did not differ between the control/VE(−) and DHA/VE(−) groups (Figure 2). Notably, VE deficiency increased the level of TBARS significantly in the control diet groups, but not in the DHA diet

Table VI. Effects of dietary DHA and VE deficiency on concentrations of TG, PLs, T-CHO and HDL-CHO in serum of diabetic rats.

	VE(+)		VE(−)		ANOVA *P*-values[¶]		
	Control	DHA	Control	DHA	Oil	VE	Oil × VE
Triacylglycerol (TG) (mmol/l)	1.45 ± 0.82	0.79 ± 0.27§	1.18 ± 0.71	0.74 ± 0.24	$P < 0.01$	NS	NS
Phospholipids (PLs) (mmol/l)	2.06 ± 0.60	1.37 ± 0.12§§	1.70 ± 0.31	1.42 ± 0.17‡	$P < 0.001$	NS	NS
Total-cholesterol (T-CHO) (mmol/l)	1.77 ± 0.54	1.25 ± 0.15§	1.54 ± 0.19	1.34 ± 0.23	$P < 0.01$	NS	NS
HDL-cholesterol (HDL-CHO) (mmol/l)	0.70 ± 0.17	0.69 ± 0.14	0.57 ± 0.12	0.64 ± 0.11	NS	NS	NS

Values are means ± SD ($n = 9$). Significantly different between oils in feeding VE(+) diet, using Student's *t*-test ($^{\S}P < 0.05$; $^{\S\S}P < 0.01$). Significantly different between oils in feeding VE(−) diet, using Student's *t*-test ($^{\ddagger}P < 0.01$). [¶]Oil, effect of dietary oil; VE, effect of dietary VE level; oil × VE, interaction between dietary oil and vitamin E level by two-way ANOVA. NS, not significant.

groups. In contrast, in the serum and liver, VE deficiency elevated the level of TBARS in the DHA groups, but not in the control groups (Table III). Free MDA + 4HAE, which are products of further oxidation, in the retina and liver were not affected by VE deficiency in either the control groups or the DHA groups. These results, especially with regard to TBARS levels, were contrary to our expectation that the retina, rich in readily oxidizable DHA, is more susceptible to oxidative stress than the serum and liver. The data in Table V suggest that these results were not due to loss of DHA under VE-deficient conditions, where the proportion of DHA in the DHA/VE(−) group was higher than that in the Control/VE(−) group. Therefore, we propose that some mechanisms may exist to prevent DHA-derived oxidation and formation of lipid peroxides, especially in the retina, and that this may be supported by our previous observations [19,36]. As DHA is known to be essential for normal functional development of the retina [38,39], we believe our proposal is plausible.

The effects of dietary oils and VE deficiency on α-tocopherol levels and AR activities in the retina differed from those in the serum and liver. Intake of DHA decreased the levels of α-tocopherol in the serum and liver, but not in the retina (Table IV). Previous reports from our group [32,40] and other investigators [1] have suggested that easily oxidizable DHA naturally causes a loss of α-tocopherol, as was observed in the serum and liver. However, in the retina, a different result was obtained, as observed previously [36]. There are two possible explanations for this: first, DHA in retinal biomembranes is not readily oxidized, and does not generate lipid peroxides which would decrease the α-tocopherol level; second, consumed α-tocopherol was vigorously and efficiently resupplied and/or reductively recycled. Regarding AR activities, both VE deficiency and DHA intake increased the activities of AR in the liver, but not in the retina (Table IV). In the liver, intake of DHA, as well as VE deficiency, may promote oxidative stress to induce AR. On the other hand, in the retina, AR activities were around 10 times higher than those in the liver. Therefore, oxidative stress induced by DHA intake and VE deficiency may not further induce AR. High levels of AR might efficiently remove active aldehydes generated by diabetes from the retina in the long run.

Many studies have suggested the participation of increased reactive oxygen species in the pathogenesis and/or exacerbation of diabetic complications [41,42]. Kesavulu et al. [43] have suggested that a high level of plasma lipid peroxides in patients with type 2 diabetes causes microvascular complications, including diabetic retinopathy. However, the role of readily oxidizable DHA in the pathogenesis of retinal degeneration is still controversial, as both a negative [44,45] and a positive [46–49] role have been reported elsewhere. Our present results give positive insight into the apparent paradox that DHA can have potentially beneficial actions but also adverse effects as a substrate in lipid peroxidation, which might cause retinal impairment. Recently, Rotstein et al. [50] have reported a protective effect of DHA against oxidative stress-induced apoptosis of retinal photoreceptors. The roles of DHA in the diabetic retina need to be clarified further from both positive and negative aspects.

This study investigated the role of DHA from a negative viewpoint under conditions of oxidative stress. However, we obtained data to suggest a positive effect of DHA under these conditions. Table VI shows that dietary intake of DHA decreased the serum levels of TG, PLs and T-CHO under diabetic conditions, suggesting that DHA ameliorates serum lipid levels even under oxidative stress, as well as in normal conditions [32,33].

In conclusion, under the oxidative stress of diabetes, dietary DHA was shown to increase only the TBARS level slightly in the retina, but not in the serum and liver. On the other hand, under more serious oxidative stress in diabetes with VE deficiency, lipid peroxide levels in the serum and liver generally increased in the DHA/VE(−) group, but those in the retina showed no change. These data suggest that dietary intake of DHA does not promote marked generation of retinal lipid peroxides, despite its high content in the retina, even under increased oxidative stress.

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