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Fish oil increases antioxidant enzyme activities in macrophages and reduces atherosclerotic lesions in apoE-knockout mice

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Abstract

Objectives: The molecular and cellular mechanisms that fish oil (FO) exerts its physiological function are complicated. The present study brings evidence on the in vivo effect of FO on the development of atherosclerosis in apolipoprotein E knockout (apoE$^{-/-}$) mice. We also test the hypothesis that the modulation of the cellular oxidative stress and antioxidant status contributes to the anti-atherosclerotic effect of FO. **Methods and results:** ApoE$^{-/-}$ mice were fed a diet rich either in FO or corn oil (CO) for 10 weeks. Both FO and CO had a plasma triacylglycerol-raising effect in apoE$^{-/-}$ mice, whereas aortic atherosclerotic lesions were significantly reduced in the mice that had consumed a high FO diet compared to those fed a high CO diet. The levels of hepatic superoxide dismutase (SOD) and catalase (CAT) activities were remarkably higher in the mice fed the FO diet than in mice fed the CO diet and the control diet. We then investigated the effects of FO and CO on the production of superoxide anion ($O_2^{\cdot-}$) and reactive oxygen species (ROS) in cultured J774 macrophages. Antioxidant status was assessed by the determination of antioxidant enzyme activities. Both FO and CO induced high levels of $O_2^{\cdot-}$ and total ROS at a short time in macrophages. However, only the FO group restored the induction of $O_2^{\cdot-}$ and ROS to near basal levels after oil treatment for 24 h. Throughout the time course experiments, antioxidant enzyme activities in the FO group mostly displayed a greater increase than in the corresponding CO group after the same time period of oil treatment. **Conclusions:** In the present study, FO reduced the formation of atherosclerotic lesions in the aortic arteries of apoE$^{-/-}$ mice not through any lipid-lowering effect. The protective role of FO in the development of atherosclerosis may result from its antioxidative defense mechanism through the induction of antioxidant enzyme activities.

Keywords: Antioxidant enzymes; ApoE-knockout mice; Atherosclerosis; Fish oil; Macrophages

1. Introduction

In many epidemiological and experimental studies, it has been found that marine n-3 polyunsaturated fatty acids (PUFAs) in fish oil (FO) have beneficial effects with regard to the development of cardiovascular disease [1,2]. However, some studies have reported no beneficial association between fish consumption and cardiovascular disease [3,4]. Most studies to date primarily concern the causal relationship between FO and the development of coronary heart disease. More studies are thus needed to clarify the mechanisms by which dietary n-3 PUFAs exert their physiological function.

Atherosclerosis is a multi-factorial disease that can be modulated through diet and cellular oxidative stress/antioxidant status. It is known that oxidatively modified LDL plays an important role in the development of atherosclerosis [5]. Dietary PUFAs may be incorporated into lipoproteins, thereby potentially affecting the susceptibility of LDL to oxidative modification. The major n-3 PUFAs found in FO are eicosapentaenoic acid [EPA, 20:5(n-3)] and docosahexaenoic acid [DHA, 22:6(n-3)]. There are conflicting results among studies on the susceptibility of LDL oxidation due to n-3 PUFAs supplementation. In some human studies, enhanced oxidation of LDL was observed [6,7], whereas in some other studies no effect of dietary n-3 PUFAs on LDL oxidation was observed [8,9]. In animal studies, treatment with n-3 PUFAs was shown to either increase [10] or decrease [11] susceptibility to LDL oxidation. This raises the important question of whether excess supplementation

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with n-3 PUFA-enriched oil may render an animal more prone to atherosclerosis.

The accumulation of lipid-laden foam cells derived from macrophages in the aortic intima is an early event in atherogenesis [5,12]. Reactive molecules such as free radicals and lipid peroxides, which can be generated by cells of the arterial wall, may be responsible for the formation and progression of pathological state. In this study, we examined the effect of dietary FO on the atheroscrotic parameters in apolipoprotein E-knockout (apoE$^{-/-}$) mice, a genetically engineered mouse model of atherosclerosis. The apoE$^{-/-}$ mice have markedly elevated plasma cholesterol and triacylglycerol concentrations due to the accumulation of VLDL and IDL in the blood [13]. It has been found that FO is an efficient triacylglycerol-lowering agent in both humans and animal models with the exception of the apoE$^{-/-}$ mice [14,15], indicating that apoE is necessary for FO to lower plasma TG concentrations. It would seem, therefore, that the absence of apoE in apoE$^{-/-}$ mice prevents FO modulation of TG metabolism [15]. This animal model should allow us to measure the contribution of FO to atherogenesis independently of the lipid-lowering mechanism.

Reactive oxygen species (ROS) are produced during normal cellular function. ROS include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$). Their high chemical reactivity leads to the oxidation of proteins, DNA or lipids. Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) are primary antioxidant enzymes that protect against molecular and cellular damage caused by ROS. Emerging evidence indicates that ROS are important risk factors in the pathogenesis of many diseases if the antioxidant system is impaired. Compared with the considerable literature describing the effects of FO on cardiovascular disease, relatively little information is available about any association between FO and ROS generation or between FO and antioxidant status. The present study brings together evidence on the effect of FO on ROS production and antioxidant enzyme activities in J774 macrophages.

The aim of this study was to examine the in vivo effect of FO supplementation to apoE$^{-/-}$ mice on the development of atherosclerotic lesions in the aortic wall. In addition, alterations in the oxidative stress and antioxidant status that are related to the etiology of atherogenesis were also investigated using J774 macrophages as the cellular model.

2. Methods

2.1. Mice and diets

The apoE$^{-/-}$ mice were purchased from Jackson laboratory (Bar Harbor, ME). At 10 weeks of age, 24 male apoE$^{-/-}$ mice were assigned randomly to three groups of eight. The mouse diets were based on the AIN-93G formulation recommended by the American Institute of Nutrition for rodent feeding studies [16]. The control diet consisted of the following ingredients per kg: 140 g casein, 715 g corn flour, 50 g solkafloc, 40 g corn oil (CO), 50 g vitamin and mineral mixture, 2 g choline bitartrate, 2 g L-cystine, 1 g DL-methionine. In the two high fat diets the 200 g/kg corn flour in the basal diet was replaced with 200 g/kg of CO or FO (purchased from ICN Biomedicals, Inc). Mice were allowed free access to the diets for 10 weeks during the experiment and were deprived of food overnight before the end of experiment. The investigation conformed with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

The mice were anesthetized with CO_2 and blood samples were collected for determination of lipid concentrations. The heart and entire aorta were rapidly removed for histopathological analyses of aortic atherosclerotic lesions. The liver was also removed and frozen in liquid nitrogen, then stored at −80 °C for antioxidant enzyme analyses. Before the analyses, the liver tissue was homogenized in 10 volumes of 50 mmol/l phosphate buffer (pH 7.4) on ice for 30 s using a polytron homogenizer. The homogenate was transferred into centrifuge tubes and centrifuged at 9000 × *g* at 4 °C for 20 min. The resultant supernatant was used to measure antioxidant enzyme activity and the amount of protein present.

The fatty acids were extracted from dietary oils with chloroform/methanol (2:1 v/v), saponified at 70 °C in methanolic 0.5 M KOH, and derivatized with excess diazomethane in ether. Fatty acid methyl esters (dissolved in hexane) were separated by gas chromatography (Hewlett–Packard 6890 gas chromatograph) equipped with a flame ionization detector and a 25 m × 0.32 mm BP 70 capillary column. Fatty acid methyl esters were identified by a comparison of their retention times with those of standards. The fatty acid composition of the diets is shown in Table 1. Saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and PUFAs were calculated by summing the four SFAs, two MUFAs and four PUFAs, respectively, as denoted in Table 1.

Table 1
Fatty acid composition of dietary oils

	CO	FO
g/100 g total fatty acids		
12:0	4.2	ND
14:0	4.6	9.3
16:0	13.5	18.4
16:1	ND	13.6
18:0	3.1	4.8
18:1 (n-9)	21.6	10.5
18:2 (n-6)	52.8	9.6
18:3 (n-3)	ND	5.7
20:5 (n-3)	ND	14.0
22:6 (n-3)	ND	12.8
P:M:S	52.8:21.6:25.4	42.1:24.1:32.5

P:M:S, PUFA:MUFA:SFA; ND, not detectable.

2.2. Macrophage culture

Macrophage-like J774 cells were obtained from the American Type Culture Collection (Rockville) and cultured in RPMI-1640 containing 100 units of penicillin/streptomycin, and 10% fetal calf serum. FO or CO was mixed with 150 mmol/l NaCl containing 12% (w/v) fatty acid-free BSA and added to each culture medium with a final concentration of 3 mg/ml [17]. The culture was maintained at 37 °C in 5% CO_2 and harvested in duplicate after 1, 3 and 24 h to assess the generation of $O_2^{\cdot -}$ or ROS and to assay activities of antioxidant enzymes.

2.3. Plasma lipid analysis

Plasma derived from the hyperlipidemia apoE$^{-/-}$ mice was diluted with 150 mmol/l NaCl, 1 mmol/l EDTA (pH 7.4), so that the OD measurement and lipid concentrations were brought into the normal range. Plasma concentrations of total cholesterol (TC) and triacylglycerol (TG) were assayed enzymatically using commercial kits (Merck). The levels of HDL-cholesterol (HDL-C) and LDL-cholesterol (LDL-C) were determined by precipitation with phosphotungstic acid/magnesium chloride or with heparin/sodium citrate, respectively, using reagents supplied by Merck. The amount of cholesterol in VLDL and IDL (VLDL + IDL-C) was determined by subtracting HDL-C and LDL-C from TC.

2.4. LDL preparation and TBARS analysis

Blood was collected in the presence of 1 mmol/l EDTA in order to prevent oxidation during lipoprotein separation. Plasma was isolated by centrifugation at 1500 × *g* for 15 min and LDL was isolated from pooled plasma of two to three mice by sequential density gradient ultracentrifugation [18]. To avoid LDL aggregation during ultracentrifugation, plasma derived from the hyperlipidemia E$^{-/-}$ mice was diluted with 150 mmol/l NaCl and 1 mmol/l EDTA (pH 7.4) as above. The isolated LDL was dialyzed against EDTA-free PBS (150 mmol/l NaCl, 20 mmol/l sodium phosphate, pH 7.4). The protein content of the LDL fraction was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA) with BSA as the standard. The products of lipid peroxidation in LDL were measured as thiobarbituric acid reactive substances (TBARS) according to Yagi [19]. Plasma LDL (200 μg/ml) was incubated with 10 μM $CuSO_4$ and oxidized at 37 °C under air. The reaction was stopped by the addition of 0.04% butylated hydroxy toluene (BHT), 10% (w/v) trichoroacetic acid (TCA), and 2-thiobarbituric acid (TBA) in succession. After removal of the precipitated protein by centrifugation, TBARS were determined fluorometrically with excitation at 535 nm and emission at 553 nm. The concentrations of TBARS were calculated using tetraethoxypropane (Sigma, Tokyo, Japan) as a reference standard.

2.5. Antioxidant enzyme assays

CAT activity in the extract of macrophages or the liver homogenates of apoE$^{-/-}$ mice was assayed using the method of Aebi [20]. In this method, the decomposition of H_2O_2 due to CAT activity was assayed by the decrease in the absorbance of H_2O_2 at 240 nm. One unit (U) of CAT activity is defined as the amount of enzyme catalyzing 1 μmol of H_2O_2 per min at 25 °C. SOD activity in the extract of macrophages or the liver homogenates of apoE$^{-/-}$ mice was assayed using the hydroxylamine reduction assay of Oyanatui [21]. In this method, the reduction of hydroxylamine by $O_2^{\cdot -}$ is monitored at 550 nm utilizing the hypoxanthine/xanthine oxidase system as the source for $O_2^{\cdot -}$. One U of SOD activity is defined as the amount of enzyme necessary to decrease the reduction of hydroxylamine by 50%. GPx activity in the extract of macrophages or the liver homogenates of apoE$^{-/-}$ mice was quantified by a coupled enzyme (GPx and glutathione reductase) procedure [22]. This method follows the decrease in absorbance at 340 nm as NADPH is converted to NADP. One unit of GPx activity is defined as the amount of enzyme oxidizing 1 μmol of NADPH per min. The specific activities of the various enzymes in the mouse liver were expressed in U/mg of protein with the protein content determined as stated above. Antioxidant enzyme activities in macrophages are expressed in mU/10^6 cells.

2.6. Assessment of atherosclerotic lesions

For each mouse, the heart and entire aorta with its main branches were dissected entirely to the iliac bifurcation; the fat and connective tissue adhering to the adventitia were cleaned from the aorta as much as possible. A modified method of Paigen et al. was used to quantitate the areas of aortic atherosclerosis for each mouse [23]. The vessels were removed and fixed with formal-sucrose (4% paraformaldehyde, 5% sucrose, 20 μmol/l butylated hydroxytoluene, 2 μmol/l EDTA, pH 7.4) at room temperature overnight. The upper section of the ascending aorta was embedded in O.C.T. compound (Sakura Finetechnical Co., Ltd., Tokyo) and frozen. Serial sections (20 μm) were transversely cut and fixed on polylysine-coated slides, then stained with Oil Red O and counterstained with hematoxylin. The samples were randomized and examined by an operator "blind" to the origin of the samples. The area of the lesions was quantitated and this covered a span of 1 mm of the aorta starting from the aortic valve sinus. Quantification of the atherosclerotic lesions was carried out using an Olympus Cue-2 image analysis system with the morphometry software WIPLab 3.0.

2.7. ROS levels in macrophages

The ROS levels in macrophages were measured using the dye 2′ ,7′ -dichlorodihydrofluorescein diacetate (DCFH-

Table 2
Plasma lipid profiles and levels of LDL-TBARS in apoE-knockout mice fed control, CO, or FO diets

	Control	CO	FO
TG[a] (mmol/l)	2.0 ± 0.13	2.8 ± 0.33*	3.6 ± 0.48**
TC[b] (mmol/l)	21.6 ± 2.9	22.8 ± 3.1	23.3 ± 2.5
HDL-C[b] (mmol/l)	2.0 ± 0.1	2.2 ± 0.2	2.1 ± 0.3
LDL-C[b] (mmol/l)	4.4 ± 0.6	4.9 ± 0.9	4.1 ± 0.7
VLDL + IDL-C[b] (mmol/l)	15.2 ± 2.7	15.7 ± 3.3	17.2 ± 2.9
TBARS (nmol/mg LDL)	22.3 ± 3.8	23.7 ± 2.9	24.1 ± 2.5

Values are mean ± S.D. ($n = 8$ for each group except that $n = 3$ for TBARS analysis). TBARS, thiobarbituric reactive substance.
*$P < 0.001$ vs. control.
**$P < 0.01$ vs. CO.
[a] For TG, 1 mmol/l equals 88.0 g/dl.
[b] For cholesterol (C), 1 mmol/l is 25.91 g/dl.

DA) [24]. This reduced dye was added to cells (1×10^6 cells/ml) at a final concentration of 10 μM. The fluorescence of the oxidized dichlorofluorescein was monitored by Flow Cytometry with an excitation wavelength of 488 nm and an emission wavelength of 525 nm. Similar measurements were made with 10 μM hydroethidine (HE) which has been reported to be more specifically oxidized by $O_2^{\cdot-}$ [25]. In this case, the fluorescence was monitored by Flow Cytometry with excitation at 495 nm and emission at 637 nm. The results were expressed as the relative fluorescence intensity. $O_2^{\cdot-}$ and ROS levels in macrophages without oil treatment were used as the control.

2.8. Statistical analyses

Data are given as mean ± S.D. Results were analyzed by one-way analysis of variance (ANOVA) using the SAS program (version 6.12, SAS Institute, Cary, NC). Differences between mean values were evaluated by the post-hoc test and were considered significant if $P < 0.05$.

3. Results

The body weight of the eight mice in each group before study entry (10 weeks of age) was 22 ± 2 g and at the end of the study at 20 weeks of age was 28 ± 3 g, 29 ± 3 g and 27 ± 2 g in control, high CO, and high FO diet, respectively. The differences in body weights between the three groups were not significant.

3.1. Plasma lipid levels and hepatic antioxidant enzyme activities in apoE$^{-/-}$ mice

The diet enriched with FO resulted in a significant increase in plasma n-3 PUFA levels (data not shown), which reflected the fatty acid compositions of the diets. Compared with mice fed the control and CO diets, the high FO treatment significantly increased plasma levels of TG (Table 2). In contrast, TC levels and plasma concentrations of HDL-, LDL-, and VLDL + IDL-cholesterol did not differ between the three groups. Moreover, there was no difference of TBARS levels in LDL between any two of the groups.

Hepatic SOD, and CAT activities were significantly elevated in mice fed with FO and CO diets relative to the control diet mice. The levels of these two antioxidant enzyme activities in the mice fed the FO diet were also remarkably higher than those of the CO diet mice. For GPx activity, the CO and FO diet mice showed a significantly higher levels than the control mice. However, the FO diet mice showed no significant difference in GPx activity compared to the CO diet mice (Table 3).

3.2. Analysis of atherosclerotic area in the aorta

The aortic arteries from all apoE$^{-/-}$ mice (controls and those fed high fat diets) at the age of 20 weeks showed the presence of atherosclerotic lesions. Intimal cells in atheromatous lesions included macrophage-derived foam cells and smooth muscle cells. Some lesions exhibited a greater development with a fibrous cap and atheroma. The spread of lipid distribution in atheromatous lesions was more extensive in the CO group (Fig. 1B) than that in the control (Fig. 1A). When the atherosclerotic lesions were viewed under low power, the mice fed the control diet showed multiple lesions covering over 50% of the circumference of the aortic wall. For the mice fed the high CO diet, lesions could be seen to involve over 90% of the circumference of the aortic wall. However, macrophage-derived foam cells in the atheromatous lesions were significantly less in the FO group (Fig. 1C). In all groups of apoE$^{-/-}$ mice, atherosclerotic plaques were predominantly found at the aortic root. The areas of aortic atherosclerotic lesions were significantly reduced ($P < 0.01$) in mice that had consumed a high FO diet for 10 weeks, compared to those fed a high CO diet (Fig. 1D).

3.3. ROS and antioxidant enzyme activities in macrophages

To evaluate the effect of dietary oils on oxidative stress, $O_2^{\cdot-}$ and ROS were determined in macrophages treated with CO or FO for different time periods. The production of $O_2^{\cdot-}$ and ROS was significantly increased in macrophages treated with 3 mg/ml oil for 1 h, but the induction in the CO group

Table 3
Hepatic antioxidant enzyme activities in apoE-knockout mice fed control, CO, or FO diets

	Control	CO	FO
U/mg protein			
SOD	46.3 ± 5.3	56.9 ± 8.4*	78.1 ± 9.3**
CAT	185 ± 13	214 ± 19*	288 ± 17**
GPx	0.41 ± 0.05	0.60 ± 0.09*	0.55 ± 0.14*

Values are mean ± S.D. ($n = 8$). The definition of enzyme unit (U) was described in Section 2.
*$P < 0.05$ vs. control.
**$P < 0.001$ vs. CO.

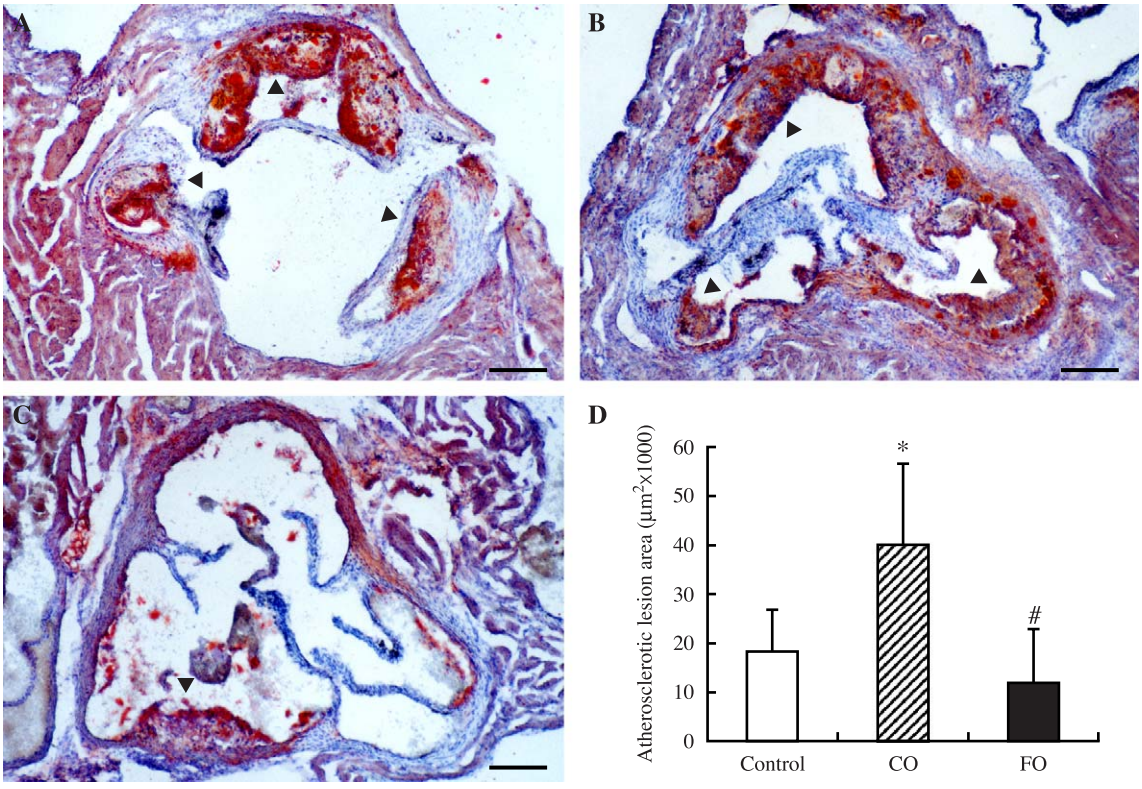

Fig. 1. Photomicrographs of atherosclerotic lesions in cross sections of the aortic root from apoE$^{-/-}$ mice fed a normal (control) or high CO or high FO diet for 10 weeks. The lipids in lesions were stained by Oil Red O and counterstained with hematoxylin. (A) ApoE$^{-/-}$ control mice ($n=8$). (B) ApoE$^{-/-}$ mice fed with CO diet ($n=7$). (C) ApoE$^{-/-}$ mice fed with FO diet ($n=8$). Bar = 0.2 mm. (D) Values are mean ± S.D. is derived from one measurement from each animal, the number of which is shown in the parentheses. *$P<0.01$ vs. control; $^{\#}P<0.01$ vs. CO.

was greater than in the FO group. The induction of $O_2^{\cdot-}$ and ROS was not sustained, but decreased to near normal upon treating the macrophages with FO for 24 h, while the CO group was still at a significantly high levels (Table 4). The changes in $O_2^{\cdot-}$ and ROS levels after oil treatment were mostly inversely related to the changes in levels of antioxidant activities. SOD and CAT activities were significantly increased after 1 h of FO treatment, and GPx activities were also increased after 24 h of FO treatment. With CO treatment, by contrast, only CAT activity was significantly elevated (Table 5). The time course changes in antioxidant enzyme activities without oil treatment did not vary significantly between the FO and CO groups (data not shown). Throughout the 24 h time course of these experiments, the antioxidant enzyme activities of the FO group mostly displayed a greater increase than in the corresponding CO group after the same time period of oil treatment (Table 5).

Table 4
The effect of FO and CO on the levels of $O_2^{\cdot-}$ and ROS in J774 macrophages

Time (h)	$O_2^{\cdot-}$		ROS	
	CO	FO	CO	FO
0	100	100	100	100
1	158 ± 15*	144 ± 16*	172 ± 18*	155 ± 16*
3	174 ± 19*	129 ± 14*	183 ± 16*	142 ± 14*
24	165 ± 16*	108 ± 13	168 ± 15*	114 ± 14

Cells were incubated with FO or CO for different time interval as shown. Values are mean ± S.D. of six determinations from three independent experiments as the percentage (%) of cells with no FO or CO treatment (control value). All of the corresponding control values (arbitrary unit) were converted as 100%.

*$P<0.001$ vs. zero time.

Table 5
The effect of FO and CO on antioxidant enzyme activities in J774 macrophages

Time (h)	CO			FO		
	SOD	CAT	GPx	SOD	CAT	GPx
mU/10^6 cells						
0	68 ± 5	134 ± 9	53 ± 4	62 ± 5	131 ± 8	57 ± 4
1	74 ± 8	162 ± 13*	56 ± 4	108 ± 7**	152 ± 11*	58 ± 6
3	82 ± 13	177 ± 12**	57 ± 5	119 ± 12**	184 ± 13**	62 ± 5
24	76 ± 11	185 ± 14**	59 ± 6	75 ± 8***	216 ± 8**	70 ± 5**

Cells were incubated with FO or CO for different time intervals as shown. Antioxidant enzyme activities at different time points were compared with those at zero time (control). SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase. Values are mean ± S.D. of six determinations from three independent experiments. The definition of enzyme unit (mU/10^6 cells) was described in Section 2.

*$P<0.01$ vs. zero time (0 h).

**$P<0.001$ vs. zero time (0 h).

***$P<0.05$ vs. zero time (0 h).

4. Discussion

FO, which is rich in n-3 PUFAs, has been shown to be beneficial in hyperlipidemia and atherosclerosis [1,2,6,7]. On the other hand, whether dietary supplementation with n-3 PUFAs renders the LDL particles more or less susceptible to oxidative modification has not as yet been clearly shown [6–11]. Susceptibility of an organism to oxidative damage is affected by its antioxidative defense system, which in turn can be influenced by nutritional antioxidants, such as vitamin E, vitamin C, and selenium [26,27]. Endogenous antioxidant enzymes, such as SOD, CAT, and GPx, as well as antioxidant nutrients, can help to protect cells against free radical damage. Since antioxidant enzymes play an important role in controlling lipid peroxidation [28], an increase in the activities of these enzymes could delay the progression of atherosclerosis. The present study was undertaken in an attempt to assess the effects of n-3 PUFA-enriched FO on the redox status of cells in vitro and its anti-atherosclerotic effects in vivo.

ApoE$^{-/-}$ mice are excellent models for the study of atherosclerosis because they become hyperlipidemic even when they consume a low fat diet and they develop complex atherosclerotic lesions similar to those of humans [29,30]. Apolipoprotein E (apoE) is a crucial ligand for lipoprotein receptors. It allows the anchoring of TG-rich lipoproteins (TRLs) to cell surface heparan sulfate proteoglycan, facilitating their interaction with lipoprotein lipase to metabolize the TRLs [30]. Thus, apoE deficiency in mice results in major defects in TG and VLDL clearance. In this respect, our observation that the high oil treatment increased plasma TG levels may result from an impairment of TRL lipolysis in apoE$^{-/-}$ mice. Our data are in agreement with the results obtained by Asset et al. [15]. Despite having a more atherogenic serum lipid profile, it is surprising that apoE$^{-/-}$ mice fed the high FO diet had less atherosclerotic lesions than those fed the high CO diet. Moreover, the FO supplementation did not influence TBARS levels of LDL in this study. This might be due to the increased activities of hepatic antioxidant enzymes in mice fed the high FO diet. However, care must be exercised in the interpretation of the TBARS data. It is recognized that the TBARS test is not specific regarding several compounds capable of reacting with thiobarbituric acid [31]. Furthermore, TBARS analyses were performed in a small sample size ($n=3$) from the pooled plasma in the present study.

Endothelial cells, macrophages, and smooth muscle cells are able to oxidize LDL by cell-derived oxidants like $O_2^{\cdot-}$and H_2O_2 [32], resulting in the pathogenesis of atherosclerosis. These modified LDL are scavenged by macrophages and contribute to foam cell formation [5,12]. n-3 PUFAs such as EPA and DHA are highly unsaturated and exhibit hypersensitivity to lipid peroxidation [6,7]. This might be expected to lead to increased plasma atherogenic particles, which could counteract the beneficial effects of such fatty acids on cardiovascular disease. However, anti-oxidative status is another important factor involved in the pathogenesis of atherosclerotic disease [26–28]. In view of the potential importance of PUFAs in the modulation of redox status in vascular cells, we examined the generation of $O_2^{\cdot-}$ and ROS in J774 macrophages. From our data, the production of $O_2^{\cdot-}$ and ROS was significantly increased by CO or FO treatment. However, metabolism of these ROS was presumably controlled to some extent by the induction of antioxidant enzyme activities. Dismutation of $O_2^{\cdot-}$ by SOD produces the more stable species H_2O_2, which in turn is converted to water by CAT and GPx. In this study, the relatively lower levels of $O_2^{\cdot-}$ and ROS in macrophages after FO treatment correlated with, and might be due to, the higher activities of SOD, CAT, and GPx in the FO group. This result is in concordance with the studies of Frenoux et al. [33], who reported an antioxidant effect of n-3 PUFAs in spontaneously hypertensive rats. CO supplementation did not induce antioxidant enzyme activities to the same extent as FO treatment. This is considered to be the main reason why CO treatment caused higher levels of $O_2^{\cdot-}$ and ROS. The balance between oxidative stress and antioxidant status of the cell can thus minimize the oxidative perturbations caused by FO challenge. The severe atherosclerotic lesions in the aorta of animals fed high CO diet might result from the greater imbalance in redox status within vascular cells.

It has recently become apparent that PUFAs can have quite specific effects on gene expression by regulating the activity or abundance of transcription factors [34,35]. Our observation that FO results in an induction of antioxidant enzyme expression may, therefore, be mediated through an effect on transcription factors. Recent studies have shown that dietary PUFAs regulate the activity or abundance of four families of transcription factors: peroxisome proliferator activator receptors (PPARs) (α, β and γ), liver X receptor (LXRs) (α and β), hepatic nuclear factor-4 (HNF-4) α, and sterol regulatory element binding proteins (SREBPs) 1 and 2 [34]. Among these transcription factors, PPARs act as general lipid sensors for a broad spectrum of ligand-dependent transcriptional regulation. Clinical and experimental evidence suggest that PPAR activation decreases the incidence of cardiovascular disease not only by correcting metabolic disorders, but also through direct actions at the level of the vascular wall [36]. Modulation of PPARs in the cardiovascular system may have a large therapeutic potential. The presence of a PPAR-response element (PPRE) in the 5′ -flanking of Cu/Zn SOD1, the key enzyme in the metabolism of oxygen free radicals, renders it sensitive to PPAR activation [37]. Preliminary data from our laboratory, using plasmids containing PPRE in front of the luciferase reporter gene, show that n-3 PUFAs have a profound influence on PPAR-mediated gene transcription (data not shown). We thus propose that n-3 PUFAs in FO have their role in anti-atheroslerosis through gene regulation.

Atherosclerosis is a disease associated with multiple risk factors. An imbalance in the generation of oxidants and

antioxidants seems to have a vital role in the pathophysiology of atherosclerosis. The animal study has demonstrated an induction of hepatic antioxidant enzyme activities in apoE$^{-/-}$ mice following dietary supplementation with FO. These findings are in agreement with the previous data about the correlation existing between the induction of free radicals and changes in antioxidant enzyme activities in J774 macrophages under FO treatment. Decreases in oxidative perturbations may contribute to a reduced cellular cholesterol accumulation and foam cell formation in vascular cells, the hallmark of early atherosclerosis [5,12]. Nevertheless, we cannot rule out the possibility that FO may also have the function of reducing the production of inflammatory mediators, which have a role in the development of atherosclerotic lesions [38]. Moreover, n-3 PUFAs may exert other cardiovascular protective effects in that they are precursors of the 3-series prostaglandins, which are antiaggregators and vasodilators [39].

On the basis of the data presented in this study, we are in a position to propose that dietary supplementation with FO may benefit humans with atherosclerosis not through any lipid-lowering effect but through its effects on antioxidative defense mechanisms. These observations point to a new direction for understanding the physiological function of FO.

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