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A fish oil-rich diet reduces vascular oxidative stress in apoE$^{-/-}$ mice

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Abstract

Oxidative stress contributes to lipid peroxidation and decreases nitric oxide (NO) bioavailability in atherosclerosis. While long-chain (n-3) polyunsaturated fatty acids (PUFA) are easily oxidized *in vitro*, they improve endothelial function. Hence, this study postulates that long-chain (n-3) PUFA decrease atherogenic oxidative stress *in vivo*. To test this, apoE$^{-/-}$ mice were fed a corn oil- or a fish oil (FO)-rich diet for 8, 14 or 20 weeks and parameters related to NO and superoxide ($O_2^{\cdot -}$) plus markers of lipid peroxidation and protein oxidative damage in the aortic root were evaluated. The FO-rich diet increased NO production and endothelial NO synthase (NOS) expression and lowered inducible NOS, p22^{phox} expression and $O_2^{\cdot -}$production after 14 and 20 weeks of diet. Protein lipoxidative damage (including 4-hydroxynonenal) was decreased after a long-term FO-diet. This supports the hypothesis that a FO-rich diet could counteract atherogenic oxidative stress, showing beneficial effects of long-chain (n-3) PUFA.

Keywords: *Atherosclerosis, 4-hydroxynonenal, polyunsaturated fatty acids, nitric oxide, superoxide anion, aortic root*

Introduction

Atherosclerosis is a multi-factorial disease. It involves oxidative stress and endothelial dysfunction and can be regulated through diet. Under physiological conditions, endogenous antioxidant defences maintain what seems to be a tentative balance between nitric oxide (NO) and superoxide anion ($O_2^{\cdot -}$). The endothelial dysfunction is due to increased $O_2^{\cdot -}$ production, which inactivates NO by forming peroxynitrite ($ONOO^-$), an adduct with oxidizing capacity. Thus, a decline in NO bioavailability is observed [1].

It has been reported that the restoration of NO levels regresses atherosclerosis [2], whereas chronic inhibition of NO production accelerates the disease [3]. Moreover, NO generated by endothelial NO synthase (eNOS) is believed to have an anti-inflammatory effect [4] and to prevent endothelial dysfunction [5], whereas NO produced by inducible NO synthase (iNOS) contributes to tissue injury in atherosclerosis [6]. NAD(P)H oxidases are an enzymatic source of $O_2^{\cdot -}$ [7,8] that have been identified in adventitial fibroblasts, endothelial cells, vascular smooth muscle cells and macrophages present in the vascular wall.

Dietary constituents have been pointed out both as causative effects in oxidative stress and as protective agents. Epidemiological studies show an inverse correlation between the intake of long-chain (n-3) polyunsaturated fatty acids (PUFA) and the incidence of cardiovascular disease [9] and fish and fish oil (FO) are the main sources of these fatty acids. They improve vascular dilation through endothelium-dependent responses in porcine coronary arteries [10], in the aorta of healthy rats [11,12] and in healthy volunteers [13]. The long-chain (n-3) PUFA reduce the

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atherosclerotic lesions by several mechanisms [14–16]. However, there is no direct evidence of the effects of FO on NO, $O_2^{\cdot -}$ and $ONOO^-$ generation in apoE$^{-/-}$ mice, which are hypercholesterolemic and, thus, spontaneously develop atherosclerosis. Their atherosclerotic lesion composition is similar to those of humans and it is accepted as the only model that develops extensive atherosclerotic lesions on a chow diet [17].

In the present study we examine the *ex vivo* impact of feeding apoE$^{-/-}$ mice with corn oil (CO)- or FO-rich diets for different periods of time on parameters related to NO and $O_2^{\cdot -}$ generation during the development of atherogenesis in the aortic root. Also, protein oxidative modifications related to PUFA peroxidation were evaluated to contribute to explaining the beneficial effects of FO on atherosclerosis. 4-hydroxynonenal (HNE) is not only the major bioactive marker of lipid peroxidation and the reactive aldehyde that modifies the protein component of low density lipoproteins, but is also a growth regulating factor and signalling molecule involved in cell proliferation, differentiation and apoptosis interacting with cytokines [18]. Therefore, the beneficial effects of a FO-rich diet on the appearance of HNE or other lipid peroxidation effects in aortic tissue affected by atherosclerosis might have particular value for better understanding of the pathophysiology of atherosclerosis and oxidative stress.

Materials and methods

Animals

Three groups of 10 male apoE$^{-/-}$ mice for each diet (Charles River, L'Arbresle, France) were fed from the age of 4 weeks for 8, 14 or 20 weeks on semi-purified diets prepared in our laboratory containing 0.15% cholesterol and 5% lipids (Table I). The lipids were either CO as the control diet, rich in linoleic acid (18:2n-6, 55.1 mol%), or menhaden oil as the FO diet, rich in eicosapentanoic acid (EPA, 20:5n-3, 14.0 mol%) and docosahexanoic acid (DHA, 22:6n-3, 9.2 mol%) [14]. All dietary components were purchased from Sigma-Aldrich Co (St. Louis, MO) with the exception of mineral and vitamin mixes, which were from ICN Pharmaceuticals (Costa Mesa, CA).

Diets

Diets were prepared weekly and stored at −20°C to prevent oxidation and food was provided and removed daily. The peroxide content of the FO diet was <10 meq/kg (iodometric determination from IUPAC). Food was restricted to 5–6 g per day to avoid differences in cholesterol ingestion and body weights were recorded weekly. Mice were fasted overnight at the end of the experiment. An alternating 12-h light–dark cycle was maintained.

Table I. Composition of semi-purified diets.

Components	Amount, g/kg diet
Casein	224.5
Cornstarch	445
Sucrose	223
Cellulose	31
DL-Methionine	1
Mineral mix[a]	14
Vitamin mix[b]	10
Cholesterol	1.5
Oil[c]	50
Water	350

[a]AIN-93 M-MX;
[b]AIN-93-VX;
[c]Corn oil or menhaden oil.

Preparation of samples

At 12, 18 or 24 weeks of age, mice were anaesthetized with isofluorane (Abbott Laboratories, Madrid, Spain) and bled by cardiac puncture using heparin as an anti-coagulant. The procedures and the care of the mice complied with the European Union guidelines.

The hearts from seven apoE$^{-/-}$ mice for each age and dietary group were perfused with cold phosphate-buffered saline (PBS) pH 7.4 and placed in 0.9% saline for at least 1 h. This allowed the heart muscle to relax, facilitating the isolation of the aortic root. Strips or homogenized strips of the aortic root were used for the assays. The limited amount of samples available from each mouse prevented the performance of all assays on each sample.

Hearts from the other three mice in each group were also perfused with cold PBS and fixed with 4% paraformaldehyde, washed in 10 mM PBS plus 20 mM glycine, blocked with 1% BSA in 10 mM PBS and cryoprotected overnight with 35% sucrose at 4°C under mild shaking. They were then frozen on a cryostat mount with OCT (Tissue-Tek, Zoeterwoude, The Netherlands) and stored at −80°C; cross-sections were used for $p22^{phox}$ and HNE evaluations.

NO production and eNOS and iNOS expression

NO was detected by electron spin resonance [12,19]. Aortic root strips (3–5 mg) were pre-incubated at 37°C for 15 min in 0.90 ml PBS, pH 7.4 and exposed to the spin trapping agents diethyldithiocarbamic acid (5 mM final concentration) and $FeSO_4.7H_2O$ (50 μM final concentration), for 30 min. Aortic root strips were then stored at −80°C for later electron spin resonance analysis. The electron spin resonance detectable paramagnetic complex was measured in a Bruker 300E spectrometer (Bruker Instruments Company, Billerica, MA). The signal was evaluated at 2.04 g. Results are expressed as arbitrary intensity units per milligram of tissue.

To study eNOS and iNOS protein expression, the aortic root strips (4–6 mg) were pulverized in liquid

N_2 and transferred for 30 min to a buffer (100 µl/mg tissue) containing Tris-HCl 62.5 mM (pH 6.8), 2% SDS, 10% glycerol and a mammalian protease inhibitor cocktail (Sigma). After maintaining it for 30 min on ice, the homogenate was sonicated for 10 s, boiled for 3 min and centrifuged at 13 000xg for 10 min at 4°C. The supernatant was collected and total protein concentration was measured using the Bio-Rad protein assay (Hercules, CA). For Western blotting of eNOS and iNOS, the proteins were separated on 7.5% SDS-polyacrylamide gel under reducing conditions. Purified bovine eNOS and iNOS (2 µg) (Cayman, Huissen, The Netherlands) were also loaded as positive controls. After electrophoresis, the protein was transferred to a nitrocellulose membrane. Blots were then incubated in a blocking buffer (Tris-buffered saline, containing 0.05% Tween-20 or TBST) and 5% non-fat dry milk for 2 h at room temperature and incubated overnight at 4°C with a mouse monoclonal eNOS antibody (Calbiochem, Darmstadt, Germany) or rabbit polyclonal iNOS antibody (Chemicon Immunostains, Chandler's Ford, UK). After several washes in TBST the membranes were incubated for 1 h at room temperature with the corresponding secondary antibodies (Bio-Rad). The membranes were washed again with TBST and incubated with the chemiluminescence reagent (Bio-Rad). Individual eNOS and iNOS bands were quantified by densitometric scanning using Phoretix (Nonlinear Dynamics, Newcastle-upon-Tyne, UK) and normalized with a polyclonal antibody against γ-tubulin (Sigma) by calculating NOS/γ-tubulin ratio. Results are expressed as arbitrary densitometric units.

$O_2^{\cdot-}$ production and p22^{phox} expression

The $O_2^{\cdot-}$ production was measured by chemiluminescence, following Tarpey et al. [20]. Aortic root strips (2–3 mg) were incubated in 50 mM potassium phosphate buffer pH 7.4 containing EDTA (100 µM) and 2,3-dimethoxy-1,4-naphthoquinone (100 µM) for 3 h at 37°C. This was followed by addition of coelenterazine (10 µM). One strip from the same mice was evaluated in the same conditions but in the presence of 60 U/ml Cu-Zn superoxide dismutase, an $O_2^{\cdot-}$ scavenger. The signal was measured with a luminometer (TD-20/20, Turner Designs, CA) and results are expressed as arbitrary chemiluminescence units per milligram of tissue.

The expression of p22^{phox} was evaluated by immunofluorescence in seven cross serial sections (five for positive studies and two for negative controls; 10 µm) of the aortic root from three mice in each group. The images were captured by an Olympus confocal laser IX-70 Fluoview 500 (Hamburg, Germany). We used anti-mouse p22^{phox} (Santa Cruz Laboratories, CA) as the primary polyclonal antibody. The sections were visualized after incubation with a corresponding secondary antibody (Molecular Probes, Paisley, UK). The fluorescence was quantified in lesioned areas using Metamorph analyser software (Universal Imaging Corporation, Toronto, Canada). Results are expressed as arbitrary fluorescence units.

Protein oxidative adducts

Protein oxidative damage was evaluated using Western-blot analyses according to previously published protocols [21]. The aortic roots from mice of 12 and 24 week of age were homogenized under antioxidant conditions as previously described [21]. Immunodetection was performed using as primary antibodies a polyclonal anti-dinitrophenylhydrazine reactive carbonyls (DNP) (Sigma), a polyclonal anti-malondialdehyde-lysine (MDAL) (Academy Bio-Medical Company, Dallas, TX) and a polyclonal anti-neuroketals (Chemicon). The monoclonal antibody to β-actin (Sigma) was used to control protein loading. Control experiments showed that omission of primary or secondary antibody addition produced blots with no detectable signal. For detection of protein carbonyls, and prior to electrophoresis, samples were derivatized with DNP as previously described [21]. Protein bands were visualized with the chemiluminescence ECL method (Millipore Corporation, Billerica, MA). The density of the immunoreactive bands was determined by densitometric analysis using a GS-800 Calibrated Densitometer (Bio-Rad).

HNE-histidine adduct was evaluated by immunohistochemical positive staining using three cross-serial sections from three mice in each group of 18 weeks of age, which were mounted on a slide coated with 3-amino-propyl-triethoxy silane. Immunohistochemistry was performed in a three-step procedure (LSAB kit, Dako, Copenhagen, Denmark). The first step was incubation with anti-mouse HNE monoclonal antibody against HNE-histidine adduct [22]. The second step consisted of incubation with biotinylated secondary anti-mouse and anti-rabbit immunoglobulins (AB2) for 30 min. The final step was to incubate with streptavidin horseradish peroxidase for 30 min. The reaction was visualized by 3,3'-diaminobenzidine tetrahydrochloride in organic solvent (DAB) positivity after 10 min of incubation. Negative controls from each mouse were processed without primary antibodies. Intensity and distribution of HNE immunostaining in the blood vessels were analysed qualitatively with an Olympus BX light microscope.

Statistical analysis

All data are expressed as means ± standard error (SE). The differences between the two treatment groups were analysed using Student's *t*-test for unpaired observations. The effect of age and diet on all variables

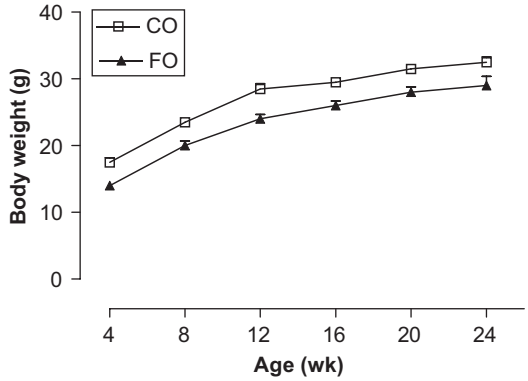

Figure 1. Body weight of apoE$^{-/-}$ mice fed CO and FO-rich diet at 12, 18 and 24 weeks of age. Values are means±SE, $n=10$ mice/diet.

was assessed by 2-way ANOVA, allowing us to study their interaction. Only the effects and interactions with a significant difference of at least $p < 0.05$ are presented. Statistical analysis was performed using SPSS, version 12.0.

Results

Body weight

Both groups of mice had similar body weight gains. They had the highest rate of body weight gain during the first 4 weeks of the study and they reached the maximal weight at 24 weeks of age (Figure 1).

NO production and eNOS and iNOS expression

After receiving a FO-rich diet until 18 and 24 weeks of age, NO generation by the aortic root of apoE$^{-/-}$ mice was ~30% higher than for the CO group ($p < 0.01$ and $p < 0.05$, respectively) (Figure 2). Both age ($p < 0.001$) and diet ($p < 0.001$) affected NO production, though no interaction between the two was detected.

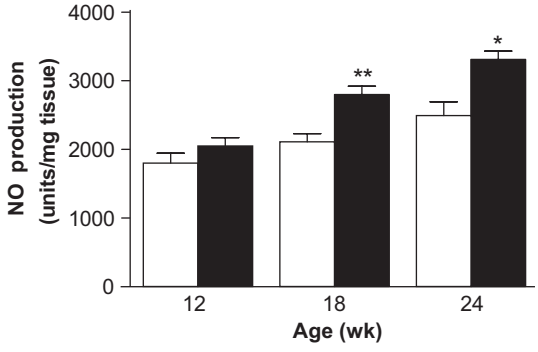

Figure 2. NO production by the aortic root strips from apoE$^{-/-}$ mice fed CO (□) or FO (■) at 12, 18 and 24 weeks of age, measured by electron spin resonance exposed to diethyldithiocarbamic acid and $FeSO_4.7H_2O$. Values are means±SE, $n=7$ mice/diet. The differences between the two groups were analysed using Student's *t*-test. $^{*}p < 0.05$; $^{**}p < 0.01$.

To determine the involvement of eNOS and iNOS in the increased NO production by blood vessels in mice treated with FO, we measured the expression of both proteins in the aortic root by immunoblotting. Representative Western blots conducted on eNOS and iNOS proteins and the accompanying histograms illustrate the mean of these protein levels for each group. The eNOS expression (Figure 3A) increased at 18 ($p < 0.01$) and 24 ($p < 0.05$) weeks of age in mice receiving the FO-rich diet, and only an independent effect of diet ($p < 0.01$) was observed. As opposed to this, iNOS expression decreased at 18 ($p < 0.05$) and 24 ($p < 0.05$) weeks of age (Figure 3B). An independent effect of diet ($p < 0.01$) was detected for iNOS.

$O_2^{\cdot -}$ production and p22^{phox} expression

To evaluate whether NAD(P)H oxidases-dependent $O_2^{\cdot -}$ formation could decrease NO bioavailability by forming $ONOO^-$, we measured the production of $O_2^{\cdot -}$ (Figure 4) and the expression of p22^{phox} (Figure 5) in the aortic root. At 12 weeks of age there was no significant differences in $O_2^{\cdot -}$ production for the mice receiving the FO-rich diet, but at 18 and 24 weeks it decreased ($p < 0.05$) (Figure 4). Independent effects of age ($p < 0.001$) and diet ($p < 0.05$) were observed for $O_2^{\cdot -}$ production. The FO-rich diet reduced p22^{phox} expression (Figure 5) by 37% ($p < 0.01$) at 18 weeks of age and by 21% ($p < 0.05$) at 24 weeks (Figures 5A–C) and an independent dietary effect ($p < 0.001$) was observed.

Protein oxidative adducts

After quantitative analysis of different types of protein oxidative damage in aortic root lysates, the results (Figure 6) reveal that although DNP-reactive carbonyls and NKTL—derived from (n-3) PUFA peroxidation—and MDAL content was significantly higher in FO than CO at 12 weeks of age, later on these differences disappeared. The effect of age in DNP and NKTL content was significant for the FO group ($p < 0.01$), suggesting that effects of FO may show a biphasic response in terms of protein oxidative modification.

Subintimal accumulation of macrophages and fibroblast proliferation and no immunohistochemical signal for HNE can be observed in the negative control of the aortic root (Figure 7A); only cholesterol clefts, tissue surface and artefacts showed some DAB positivity. In sections from mice fed the CO-rich diet (Figure 7B) there was subintimal accumulation of foamy macrophages and fibroblasts that showed a strong HNE immunohistochemical signal. This signal was stronger than for negative controls. Some stage of atherosclerosis and immunopositivity was present

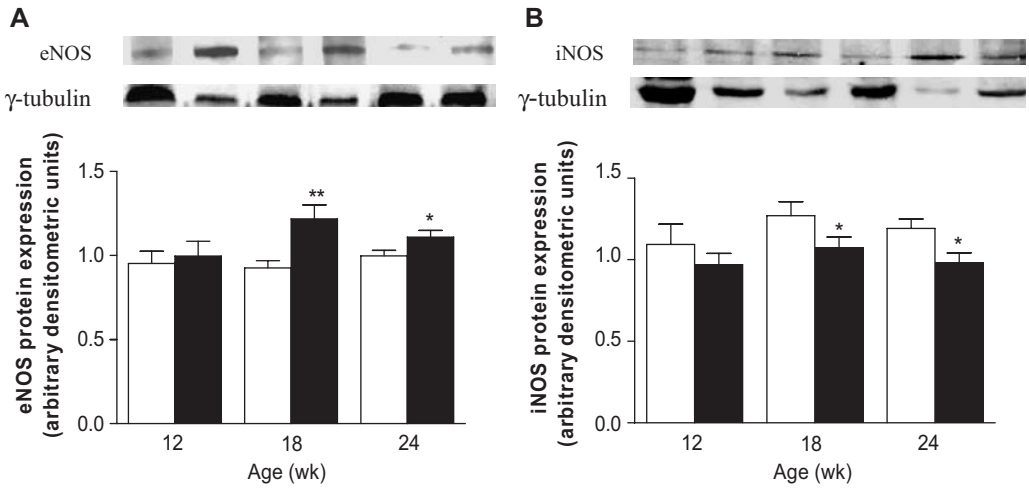

Figure 3. Immunoblot analysis of eNOS (A) and iNOS (B) in the aortic root homogenates from apoE$^{-/-}$ mice fed CO (□) or FO (■) at 12, 18 and 24 weeks of age. Data were obtained by densitometric scanning and normalized with a polyclonal antibody against γ-tubulin. Values are means ± SE, $n = 6$ mice/diet. The differences between the two groups were analysed using Student's t-test. $^{*}p < 0.05$; $^{**}p < 0.01$.

in the media. Subintimal cholesterol clefts, tissue surfaces and artefacts in frozen tissue from mice fed the CO-rich diet showed some DAB positivity. In sections from mice fed the FO-rich diet (Figure 7C) there was subintimal accumulation of foamy macrophages and fibroblasts with a positive HNE immunohistochemical signal, but less than in the CO-fed mice. Less pronounced stage of atherosclerosis and the HNE-immunopositivity was present in the aortic media. Cholesterol clefts, tissue surfaces and artefacts in frozen tissue from mice fed the FO-rich diet showed some DAB positivity.

Discussion

Atherosclerosis involves endothelial dysfunction related to an increased rate of inactivation of the NO generated by eNOS, due to an enhancement of $O_2^{\cdot -}$ generated by NAD(P)H oxidases present in vascular cells [7]. In the non-phagocytic cells, NAD(P)H oxidases are active in the resting state and the intracellular output of $O_2^{\cdot -}$ is very low. The recruitment and subsequent migration of monocytes into the atherosclerotic vessel wall introduces other cellular sources of free radicals, such as NO from iNOS and $O_2^{\cdot -}$ from the NAD(P)H oxidases [7]. Thus, there is considerable interest in increasing NO bioavailability and decreasing $O_2^{\cdot -}$ generation to slow down atherosclerosis development.

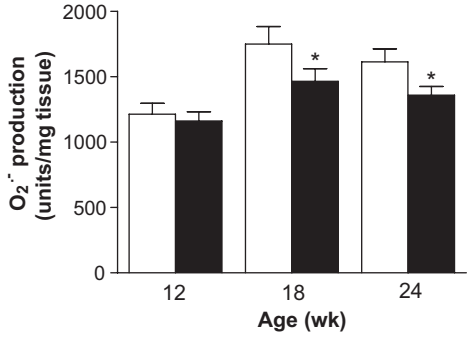

Figure 4. $O_2^{\cdot -}$ production by the aortic root strips from apoE$^{-/-}$ mice fed CO (□) or FO (■) at 12, 18 and 24 weeks of age, measured by chemiluminescence in the presence of 10 μM coelenterazine. Values are means ± SE, $n = 7$ mice/diet. The differences between the two groups were analysed using Student's t-test. $^{*}p < 0.05$.

Fish, FO or EPA and DHA all reduce the risk for atherosclerosis by lowering plasma triacylglycerol concentration, blood pressure and platelet aggregation [9,15]. The present study reports that a FO-rich diet enhances the production of NO measured in the absence of interaction with $O_2^{\cdot -}$ [23]. NO from eNOS decreases vascular tone, vascular smooth muscle cell proliferation [24], leukocyte adhesion to endothelium [25], oxidation of low-density lipoproteins [26] and platelet aggregation [27]. The NO production *ex vivo* reported in the current paper is the result of increased eNOS expression and decreased iNOS expression by the aortic root of apoE$^{-/-}$ mice and may also indicate a compensatory protective mechanism in inflammatory and vascular diseases. The specific removal of the eNOS in apoE$^{-/-}$ mice results in a marked acceleration of atherosclerotic lesion formation in the aorta, and in significant coronary atherosclerosis [28]. Studies of the vasoprotective effects of chronic eNOS overexpression in eNOS-transgenic mice have yielded conflicting results. Reduced lesion formation has been demonstrated in mice after carotid artery ligation [24]. However, the same group observed that eNOS-transgenic mice showed accelerated atherosclerosis under hypercholesterolemia [29], due to a dysfunction of eNOS because of a reduction in tetrahydrobiopterin that resulted in decreased NO production and enhanced $O_2^{\cdot -}$ production.

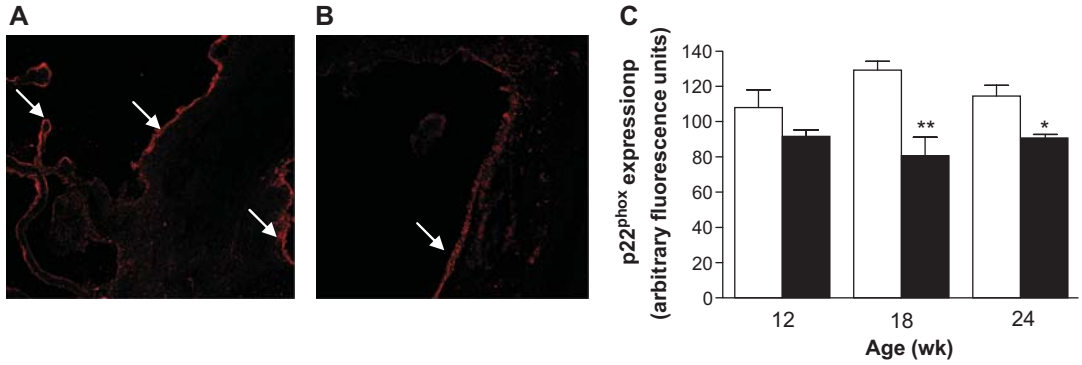

Figure 5. The p22^{phox} expression by immunofluorescence in cross-sections of aortic root isolated from apoE$^{-/-}$ mice at 12, 18 and 24 weeks of age. (A) Representative photomicrograph from an 18-week-old mouse fed CO obtained by a confocal laser. (B) Representative photomicrograph from an 18-week-old mouse fed FO obtained by a confocal laser. (C) The fluorescence was quantified in lesioned areas in mouse fed CO (□) or FO (■).The white arrows show the p22^{phox} expression. Values are means ± SE, $n = 3$ mice/diet. The differences between the two groups were analysed using Student's t-test. $^{*}p < 0.05$; $^{**}p < 0.01$. Magnification ×80.

The protection afforded by FO in the current study cannot be explained by reduction of hypercholesterolemia [30] as both groups of mice had similar cholesterol levels at all ages studied [14]. The protection is likely to be attributed to low NAD(P)H oxidases [31], which are the major source of $O_2^{\cdot -}$ in the vascular wall [7,8]. The non-phagocytic NAD(P)H oxidases are similar to neutrophil NADPH oxidase with which they share the p22^{phox} sub-unit. We observed a decrease in p22^{phox} expression and in $O_2^{\cdot -}$ generation after administering a FO-rich diet to apoE$^{-/-}$ mice. The most plausible reason for these

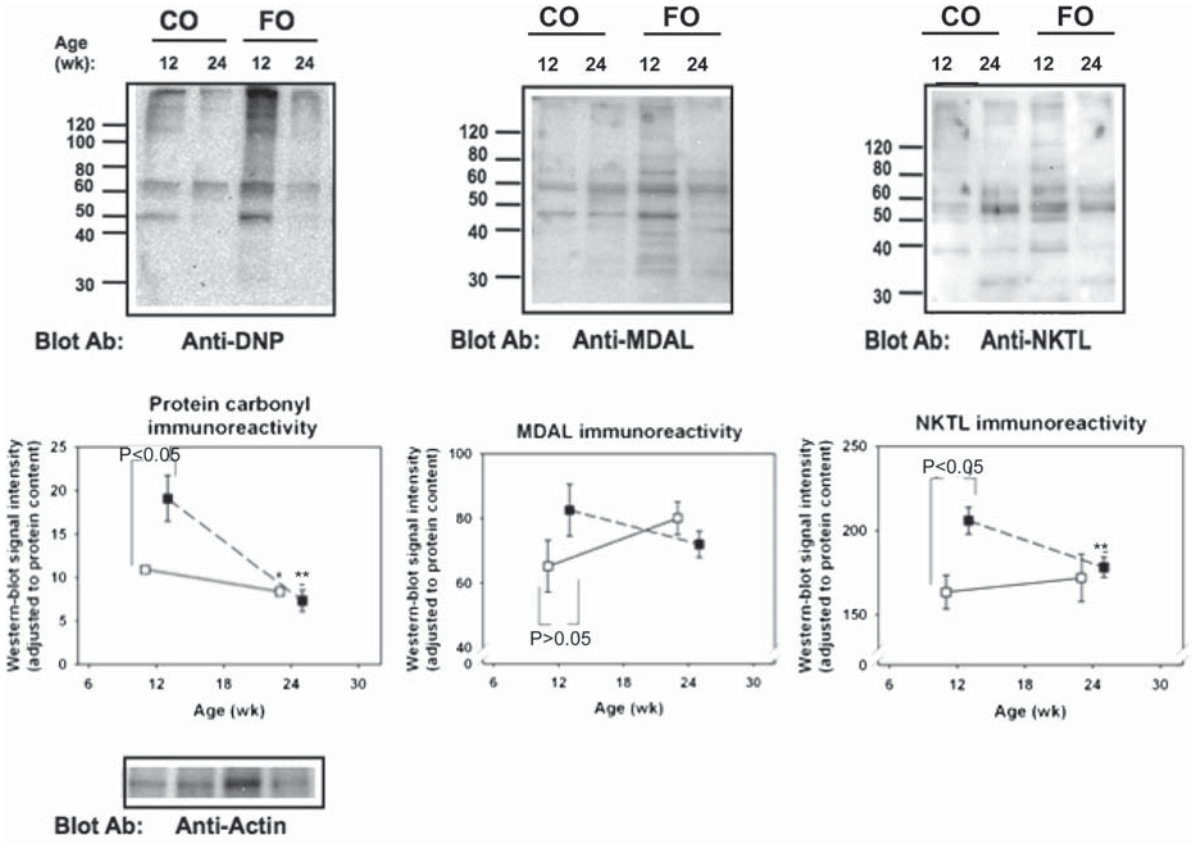

Figure 6. Immunoblot analysis of protein oxidative markers in the aortic root homogenates from apoE$^{-/-}$ mice fed CO (□) or FO (■) at 12 and 24 weeks of age. Data were obtained by densitometric scanning and values are expressed as means ± SE, $n = 7$ mice/diet. The differences between the two treatments and age groups were analysed using Student's t-test. $^{**}p < 0.01$ with reference to values obtained at 12 weeks of age for mice fed FO. Molecular weight markers are shown at the left of western-blot, representative of three different experiments. DNP: 2,4-dinitro-phenyl-hydrazine; MDAL: malondialdehyde-lysine and NKTL: neuroketals.

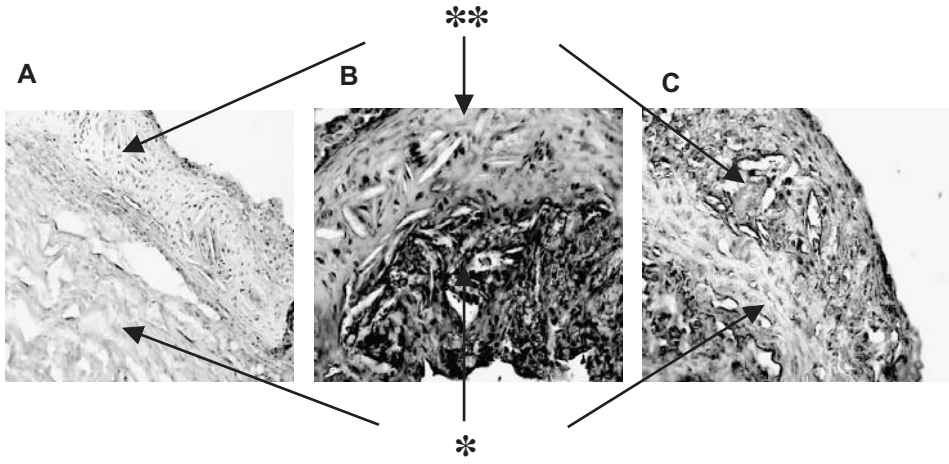

Figure 7. HNE measurement in cross-sections of aortic root isolated from apoE$^{-/-}$ mice at 18 weeks of age by immunohistochemistry. Representative micrographs from $n=3$ mice/diet. (A) Negative control as primary antibody. (B) Mouse fed CO. (C) Mouse fed FO. Magnification ×400. Subintimal accumulation of foamy macrophages (indicated by *) and fibroblasts show a strong HNE-immunohistochemical signal on B. Subintimal cholesterol clefts on B (indicated by **) also showed some DAB positivity. In sections from mice fed the FO (C) the respective HNE-immunohistochemistry showed a less pronounced reaction than that observed for B (CO).

effects is a shortage of arachidonic acid [32,33] as a FO-rich diet replaces arachidonic acid by EPA and DHA in very-low-density lipoproteins plus low-density lipoproteins of rats treated in similar conditions [34] as well as in the phospholipid membranes of rat macrophages [32]. A decrease in $O_2^{\cdot -}$ production was also detected in aortic segments from healthy rats fed with FO [12], in macrophages from apoE$^{-/-}$ mice incubated in the presence of FO [16] and in human aortic endothelial cells incubated in the presence of EPA and DHA [35]. The low levels of $O_2^{\cdot -}$ are probably responsible for the decreased expression of nuclear factor–κB. This in turn was related to the reduced expression of endothelial adhesion molecules and to the reduced development of atherosclerosis observed by our group after feeding apoE$^{-/-}$ mice with a FO-rich diet [14]. All these changes explain the decreased monocyte/macrophage infiltration into the arterial wall observed by immunohistochemistry in the present study and, thus, the reduced iNOS protein expression. In this sense, iNOS deficiency significantly slows down atherosclerosis in apoE$^{-/-}$ mice by decreasing chemotaxis [36].

As $O_2^{\cdot -}$ reacts with NO mol-to-mol at an almost diffusion-controlled rate, leading to the highly oxidant $ONOO^-$, the bioactivity of NO is dependent on vascular levels of $O_2^{\cdot -}$, i.e. on the balance between $O_2^{\cdot -}$ production and $O_2^{\cdot -}$ degradation by antioxidant enzymes. The low levels of $O_2^{\cdot -}$ production observed in this paper can be responsible of the decreased $ONOO^-$ previously detected by our group [14], which in turn will favour eNOS coupling by increasing tetrahydrobiopterin [31,37]. $ONOO^-$ also has the potential to injure tissue and to generate nitrated tyrosines, a post-translational modification detected in many pathological states, such as human atherosclerotic lesions [38]. However, as we have previously observed [14], this compound was not modified in the aortic root of 12- and 18-week-old apoE$^{-/-}$ mice, and it was even slightly reduced in 24-week-old mice fed the FO-rich diet. These results should be related to the changes reported in NO and $O_2^{\cdot -}$ generation in the current paper.

Either FO or EPA and DHA, because of their high peroxidizability index [14], could be prone to peroxidation that would result in the production of highly reactive aldehydes such as malondialdehyde (MDA) and HNE. The bioactivities of HNE, which is physiologically present in various cells and tissues in particular fibroblasts and inflammatory cells [39] could also be involved. There are already indications that this aldehyde plays an important role in cellular adaptation to physiological oxidative stress as an essential component of oxidative homeostasis that affects the most fundamental antioxidant enzymatic systems such as catalase [40]. While in the short-term (12 weeks) high peroxidizability of n-3 long chain PUFA would induce increased protein oxidative damage (anti-DNP, anti-MDAL and anti-NKTL), longer-term treatments would lead to the reduced protein oxidation (HNE-histidine at 18 weeks of age) and normalization of other oxidation markers (anti-DNP and anti-NKTL at 24 weeks of age) observed in the present study. Indeed, in a very simple cellular model, our previous data [40] reveal a similar biphasic response: yeast expressing desaturase genes, thus increasing PUFA content initially shows higher sensitivity to oxidative stress, even increasing 4-HNE levels. Later on, sustained exposure to those PUFA or their oxidative byproducts induced an increase in catalase activity and finally a decreased sensitivity to hydrogen peroxide, suggesting adaptation to increased oxidative stress. The results reported here on protein oxidative modifications at 12 weeks may be the consequence of

sub-lethal doses of lipid peroxidation products, which could induce cellular adaptation and enhanced tolerance against subsequent oxidative stress by upregulating defensive systems. The messengers for this adaptive response include, among others, reactive alpha, beta-unsaturated carbonyl compounds, which may give rise to increased protein modification when evaluated by carbonyl analyses [41]. To sum up, a sustained dietary change is needed for adaptation of protein oxidative damage to potentially increased lipid peroxidation. This effect may be related to the enhancement of antioxidant capacity [42], to NO antioxidant activity [26] due to the increase of NO generation, to the reduction of reactive species such as $O_2^{\cdot -}$ and $ONOO^-$ and/or increased proteasomal turnover [43,44]. Decreased oxidative stress at the vascular level induced by a FO-rich diet in healthy rats has also been described by our group [34]. The current study suggests that the FO-rich diet reduces lipid peroxidation in the vascular wall in $apoE^{-/-}$ mice when compared to the CO-rich diet, and it helps to understand the prevention of atherosclerotic lesions observed by us under the same conditions [14] and also by other authors [16,45] .

In general, the beneficial effects of long-chain (n-3) PUFA in atherosclerosis are mostly based on their lipid-lowering effect. However, as the free radicals are also involved in atherosclerosis, an initial increase in eNOS and a decrease in iNOS expressions that will probably involve activation by kinases would probably in turn reduce oxidative stress. These findings provide important insights into the mechanisms involved in the beneficial effect of long-chain (n-3) PUFA in conditions that favour the development of atherosclerosis and support the epidemiological reports.

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