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Docosahexaenoic acid attenuates VCAM-1 expression and NF-κB activation in TNF-α-treated human aortic endothelial cells☆

Tzu-Ming Wang[a], Chun-Jung Chen[b], Tzong-Shyuan Lee[c], Han-Yi Chao[a], Wen-Huey Wu[d], Shu-Chen Hsieh[e], Huey-Herng Sheu[f], An-Na Chiang[a,*]

[a]Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei 112, Taiwan
[b]Department of Education and Research, Taichung Veterans General Hospital, Taichung 407, Taiwan
[c]Institute of Physiology, National Yang-Ming University, Taipei 112, Taiwan
[d]Department of Human Development and Family Studies, National Taiwan Normal University, Taipei 106, Taiwan
[e]Institute of Food Science and Technology, National Taiwan University, Taipei 112, Taiwan
[f]Department of Internal Medicine, Taichung Veterans General Hospital, Taichung 407, Taiwan

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Abstract

This study was conducted to test the hypothesis that n-3 polyunsaturated fatty acids are able to down-regulate expression of adhesion molecules and nuclear factor-κB (NF-κB) activation in vascular endothelial cells, in addition to reducing atherosclerotic lesions in vivo. We report here that docosahexaenoic acid (DHA) reduces atherosclerotic lesions in the aortic arteries of apolipoprotein E knockout (apoE$^{-/-}$) mice. Consistent with the observation in animal study, DHA inhibited THP-1 cell adhesion to tumor necrosis factor α (TNF-α)-activated human aortic endothelial cells (HAECs). Expression of vascular cell adhesion molecule 1 (VCAM-1) and intercellular adhesion molecule 1 (ICAM-1) on the cell surface of HAECs was determined by cell-surface enzyme-linked immunosorbent assay. DHA and eicosapentaenoic acid decreased VCAM-1 expression in a dose-dependent manner in TNF-α treated HAECs, while *cis*-linoleic acid and arachidonic acid did not have any significant effect on either VCAM-1 or ICAM-1 expression. Moreover, DHA significantly reduced VCAM-1 protein expression in the cell lysates of TNF-α-treated HAECs, as determined by Western blot analysis. In line with NF-κB signaling pathway, DHA suppressed the TNF-α-activated IκBα phosphorylation and degradation as well as IκB kinase-β phosphorylation. Subsequently, translocation of the NF-κB (p50/p65) and AP-1 (c-Fos/c-Jun) subunits was down-regulated by DHA in the nucleus of HAECs. These results suggest that DHA negatively regulates TNF-α-induced VCAM-1 expression through attenuation of NF-κB signaling pathway and AP-1 activation. This study provides evidence that DHA may contribute to the prevention of atherosclerosis and inflammatory diseases in vivo.

Keywords: Docosahexaenoic acid; Polyunsaturated fatty acids; apoE knockout mice; VCAM-1; ICAM-1; Human aortic endothelial cells

1. Introduction

Many lines of evidence suggest a beneficial effect of fish oil-supplement diets on atherothrombotic disorders [1–3]. We previously reported the protective role of fish oil in the development of atherosclerosis in apolipoprotein E knockout (apoE$^{-/-}$) mice [4]. It is widely accepted that n-3 polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA, C22:6n-3) and eicosapentaenoic acid (EPA, C20:5n-3), are active components of fish oil and have diverse biological functions in human health and disease. Studies have shown that consumption of n-3 PUFAs reduces the risk of hyperlipidemia, inflammation, atherosclerosis, neurological disorders as well as cancer [5–10].

Inflammation is an essential factor for acute and chronic human diseases and is characterized by activation of inflammatory cytokines or inflammatory mediators [11]. Vascular endothelial cells are the primary target of immunological attack in inflammatory vascular diseases, and their injury can lead to the pathogenesis of atherosclerosis [12]. During atherosclerotic progression, monocyte recruitment to the endothelium is enhanced by adhesion molecules on the surface of endothelial cells, including vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and E-selectin [13]. The cytokine-induced expression of cell adhesion molecules is regulated predominantly by activation of nuclear factor-κB (NF-κB) [14]. This finding has led to our interest in determining the beneficial effects of n-3 PUFAs on the regulation of monocytic adherence to endothelial cells, adhesion molecule expression and modulation of NF-κB signaling pathway.

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* Corresponding author. Tel.: +886 2 28267122; fax: +886 2 28264843.
E-mail address: anchia@ym.edu.tw (A.-N. Chiang).

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NF-κB is a transcription factor that plays a key role in cell adhesion, cellular growth, differentiation, inflammation and survival [14–19]. The NF-κB family comprises five members, relA (p65), relB, c-Rel, p50/p105 (NF-κB1) and p52/p100 (NF-κB2). In mammals, NF-κB dimers residing in the cytoplasm are maintained in an inactive state by the inhibitory IκB proteins (IκBα, IκBβ and IκBε) and are activated rapidly by cytokines or pro-inflammatory stimuli, such as tumor necrosis factor α (TNF-α) and interleukin 1. Activated NF-κB has been observed in human and experimental atherosclerotic lesions [20,21]. Phosphorylation of IκBα is a key regulatory step in the canonical or classical pathway of NF-κB activation [17,22]. Following cytokine stimulation, IκB kinase (IKK), an activator of the signaling cascade, phosphorylates the IκBα protein, resulting in the release and translocation of NF-κB to the nucleus. As NF-κB subunit levels increase in the nucleus, the NF-κB subunit can bind to NF-κB sites in the promoter of the inflammatory target genes [14,17,18,22]. Dysregulation of NF-κB activation plays an important role in a number of diseases including atherosclerosis, cancers and rheumatoid arthritis [16,20,23].

Several dietary and therapeutic approaches to anti-inflammation are based on the inhibition of NF-κB activation [24–27]. Growing evidence from animal and human studies indicates that n-3 PUFAs have an immunomodulatory effect [6,28]. This report addresses the impact of n-3 PUFAs on the modulation of atherogenesis in apoE$^{-/-}$ mice and the molecular mechanisms of adhesion molecule regulation as well as the NF-κB signaling pathway in human aortic endothelial cells (HAECs) when exposed to TNF-α. The present study brings evidence on the effect of PUFAs on the development of atherosclerotic lesions. The cellular model allows us to clarify the possible contribution of DHA to atherosclerotic prevention.

2. Materials and methods

2.1. Animals and diets

Male apoE$^{-/-}$ mice were obtained from Jackson laboratory (Bar Harbor, ME, USA) and bred in the animal center at National Yang-Ming University. All animal experiments and care were approved by the Animal Care and Use Committee of National Yang-Ming University. At 12 weeks of age, 40 mice were randomly divided into five groups (n=8). The control group was given the normal chow diet, and 1.1% ethanol in phosphate-buffered saline (PBS: 150 mM NaCl, and 20 mM sodium phosphate, pH 7.4) every day by gavage. The other four groups were fed normal chow ad libitum and 200 mg/kg (~1.8 kcal/kg) of DHA, EPA, *cis*-linoleic acid (LA) or arachidonic acid (AA) in 1.1% ethanol in PBS (pH 7.4) by gavage every day. After 10 weeks on these diets, the mice were deprived of food overnight and weighed before the end of experiment. The mice were anaesthetized with CO_2 and blood samples were collected by heart puncture. The blood was centrifuged at 12,000×*g* for 15 min, and the plasma supernatant was stored at −35°C until analysis.

2.2. Assessment of atherosclerotic lesions in apoE$^{-/-}$ mice

The severity of atherosclerotic lesions in the aortic sections from the apoE$^{-/-}$ mice was assessed as described previously [4]. Briefly, the aortic vessels were removed and fixed with formal-sucrose solution (4% paraformaldehyde, 5% sucrose, 20 μM butylated hydroxytoluene, and 2 μM EDTA, pH 7.4) at room temperature overnight. The section of the ascending aorta was embedded in O.C.T. compound (Sakura Finetechnical, Tokyo, Japan) and frozen. Serial sections (20 μm) were transversely cut and fixed on polylysine-coated slides, then stained with Oil Red O. The dyed specimens were photographed using a digital camera (Nikon, Tokyo, Japan). Images were transferred to personal computer and sizes of aortic sinus lesions were determined by using an Olympus Cue-2 image analysis system with the morphometry software WIPLab 3.0. The area of the lesions was quantified and found to cover a span of 1 mm of the aorta starting from the aortic valve sinus. Units of measurement of atherosclerotic lesion area are expressed in mm^2×1000.

2.3. Measurement of plasma lipid and fatty acid levels

Plasma derived from the hyperlipidemic apoE$^{-/-}$ mice was diluted with 150 mM NaCl and 1 mM EDTA (pH 7.4), so that the optical density (OD) measurement and lipid concentrations were brought into the normal range. The plasma total cholesterol, high-density lipoprotein (HDL)-cholesterol and triglyceride concentrations were measured enzymatically using commercial kits (Wako Chemicals, Richmond, VA, USA). The concentration of non-HDL-cholesterol was calculated as the difference between total cholesterol and HDL-cholesterol. Fatty acid composition of the plasma in apoE$^{-/-}$ mice was determined according to the method of Miles et al. [29]. Briefly, total lipid was extracted from plasma with chloroform/methanol (2:1, v/v). Plasma fatty acids were prepared by saponification at 70°C in methanolic 0.5 M KOH and neutralized using concentrated sulfuric acid. Fatty acids were then extracted into chloroform and washed twice in 0.88% KCl. After evaporation to dryness, fatty acid methyl esters were prepared by reaction with excess of diazomethane in ether. Fatty acid methyl esters were extracted with hexane and were analyzed using a Hewlett-Packard 6890 gas chromatograph equipped with a flame ionization detector and a 0.32 mm×25 m BPX70 capillary column. Helium at 2.0 ml/min was used as the carrier gas. The column temperature was maintained at 170°C for 10 min after sample injection and was programmed to increase from 170°C to 220°C at 5°C/min before being maintained at 220°C for 15 min. Fatty acid methyl esters were identified by comparison of their retention times with those of the selected standards.

2.4. Cell culture

Human aortic endothelial cells (HAECs, Cascade Biologics, Portland, OR, USA) were grown in Medium 200 (Cascade Biologics) supplemented with low serum growth supplement (complete medium) containing 2% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1 μg/ml hydrocortisone, 10 ng/ml human epidermal growth factor, 3 ng/ml human fibroblast growth factor, 10 μg/ml heparin and 1% antibiotic mixture. Bovine serum albumin (BSA)-bound fatty acid was made by adding fatty acid solution to 150 mM NaCl containing 12% (w/v) fatty acid-free BSA (Sigma, St. Louis, MO, USA) dropwise with gentle stirring and sterilized by 0.45-μm filter. The stock solution of BSA-bound fatty acid was added to each culture medium and adjusted to the desired concentration of PUFA. The cells were incubated with PUFA in a 5% CO_2 humidified atmosphere at 37°C for 24 h before activation by TNF-α.

2.5. Monocyte adhesion assay

HAECs were cultured in 24-well plates at a density of 6×10^4 cells/well. After incubation for 24 h, HAECs were treated with 80 μM of PUFAs for another 24 h, followed by stimulation with 10 ng/ml of TNF-α for 3 h. The THP-1 monocytes (5×10^5 cells/well) were labeled with 2′, 7′-bis (2-carboxyethyl)-5(6)-carboxy- fluorescein acetoxy-methyl ester (BCECF/AM, 10 μM, Boehringer-Mannheim) and allowed to interact with HAECs for 30 min at 37°C. Unbound cells were removed by gently washing twice with PBS, and the adherent cells were photographed by an inverted fluorescent microscope (Eclipse TS-100, Nikon). Fluorescence was measured with an automated fluorometer (Multiskan Spectrum, Thermo Fisher Scientific, Vantaa, Finland) at an excitation wavelength of 485 nm and emission at 530 nm.

2.6. Cell-surface enzyme-linked immunosorbent assay (ELISA)

To examine whether polyunsaturated fatty acids could regulate the expression of VCAM-1 and ICAM-1 on HAEC surface, cell-surface ELISA was conducted. Briefly, at 95% confluence of HAECs in 96-well microplates, 20, 40, 80 or 160 μM of PUFAs were added to HAECs for 24 h before treatment with 10 ng/ml of TNF-α for 6 h. The cells were washed and then incubated with goat anti-human VCAM-1 or ICAM-1 monoclonal antibody (Abcam, Cambridge, UK) diluted 1:2000 in PBS containing 1% BSA and 0.05% Tween-20. After incubation of the cells at room temperature for 1 h, the plates were washed three times with PBS and then treated with peroxidase-conjugated rabbit anti-goat IgG (1:2000 dilution in PBS containing 1% BSA and 0.05% Tween-20). After 1 h of incubation at room temperature, the plates were washed three times with PBS and treated with 200 μl freshly made 4% *o*-phenylenediamine and 0.04% H_2O_2 in a mixture of 50 mM citrate buffer and 100 mM phosphate buffer (pH 7.4). After incubation in a dark place for 15 min, 50 μl/well of 2 M H_2SO_4 was added, and spectrophotometric readings were made at 492 nm using a microplate reader.

2.7. Western blot analysis

The effect of PUFAs on the cellular expression of VCAM-1 and ICAM-1 was also determined in TNF-α-challenged HAECs by Western blot analysis. Moreover, the expression of phospho-IκBα, IκBα degradation, phospho-IKK-β and nuclear subunits of p50/p65 as well as c-Fos/c-Jun was analyzed to determine whether the regulation of adhesion molecule expression by PUFAs was associated with the changes in the NF-κB signaling pathway. Briefly, HAECs were pretreated with 80 μM PUFAs for 24 h in six-well plates and treated with 10 ng/ml TNF-α for another 6 h. Cells were then washed with cold PBS, centrifuged at 1200×*g* for 10 min at 4°C and finally lysed with lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA and 1 mM phenylmethylsulfonyl fluoride for 1 h. Next, the cell debris was removed by centrifugation at 4000×*g* for 30 min at 4°C, followed by quick freezing of the supernatants. The protein concentration was determined using the Bradford assay (Bio-Rad, Hercules, CA, USA) with BSA as the standard. Nuclear extracts of HAECs were prepared according to the method of You et al. [30]. Equal amounts of cellular proteins were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electophoresis and transferred onto polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The immunoblots were then placed in Tris-buffered saline blocking solution (10 mM

Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20, and 5% nonfat milk) for 1 h. Expression of phospho-IκBα, total IκBα, phospho-IKK-β and total IKK-β was analyzed using the proteins from the cell lysates, while expression of NF-κB (p50/p65) and AP-1 (c-Fos/c-Jun) subunits was determined from the nuclear proteins. The transferred blots were incubated with the primary antibody for 1 h, then washed extensively with 1× Tris-buffered saline and 0.05% Tween 20. Following incubation with horseradish peroxidase-conjugated secondary antibody incubation and washing, bound IgG was visualized using an enhanced chemiluminescence detection kit system (GE Healthcare, Piscataway, NJ, USA). The blot was then stripped for further probing with actin or B23 antibody (Santa Cruz, CA, USA) as the internal controls for the cellular or nuclear proteins, respectively.

2.8. Statistical analysis

Data are given as mean±S.E.M. The results were analyzed by one-way analysis of variance. All statistical analyses were performed using SPSS version 12.0. Differences between the mean values were considered significant if $P<.05$.

3. Results

3.1. Plasma lipid and fatty acid levels in apoE$^{-/-}$ mice

To address the effects that might be exerted by n-3 PUFA in vivo, certain biological parameters were estimated in apoE$^{-/-}$ mice at the end of the experimental period. By 10 weeks of the experiment, the body weight of the five groups of mice did not differ (data not shown). The levels of plasma triglyceride, total cholesterol, and HDL-cholesterol among the mice in the PUFA-treated groups were similar to those of mice fed the control diet (Table 1). The changes in fatty acid composition of the plasma in apoE$^{-/-}$ mice are compared in Table 2. It shows that the plasma fatty acid composition was positively correlated with the PUFA-treated by gavage. Mice treated DHA increased the proportion of DHA by 2.9-fold as compared with the levels in control mice. Significant increases in the composition of other corresponding fatty acids were also found in the plasma of apoE$^{-/-}$ mice treated with EPA, LA, and AA.

3.2. Effect of PUFAs on atherosclerotic lesions in apoE$^{-/-}$ mice

Representative histological sections from the control and PUFA-treated mice are presented in Fig. 1A–E. At the age of 22 weeks, all examined apoE$^{-/-}$ mice showed the presence of atherosclerotic lesions in their aortas. The mean atheromatous area through the region of the aortic valve in DHA and EPA groups was 53% and 71% of the controls, respectively. However, the atherosclerotic lesion area of mice in the LA and AA groups was 1.67- and 1.42-fold greater than the lesions in the control group, respectively. Morphometric analyses showed that the area of aortic atherosclerotic lesions was significantly reduced in the DHA-treated mice (Fig. 1F). Among the four PUFA-treated groups, the mice in the LA group had the largest aortic lesions.

3.3. Effect of PUFAs on THP-1 monocyte adhesion to TNF-α-activated HAECs

Monocyte adhesion to endothelial cells is an essential event in the initiation of atherosclerosis development [12]. Therefore, we examined the effect of PUFAs on THP-1 monocyte adhesion to TNF-α-activated HAECs to link the antiatherosclerotic actions by DHA in vivo and in vitro. The binding of THP-1 cells to HAECs was at the basal level in the control group, while THP-1 cell adhesion was remarkably increased when HAECs were treated with TNF-α (10 ng/ml) (Fig. 2). The adhesion of THP-1 monocytes to TNF-α-stimulated HAECs was significantly suppressed by DHA, but not by EPA, LA and AA.

Table 1
Plasma lipid (mmol/L) in apoE$^{-/-}$ mice treated with n-3 (DHA, EPA) and n-6 (LA, AA) polyunsaturated fatty acids

Variable	Control	DHA	EPA	LA	AA
Triglyceride	2.2±0.15	2.5±0.20	2.3±0.23	2.7±0.22	2.6±0.24
Total cholesterol	22.1±1.5	23.2±1.7	24.7±2.0	27.1±2.0	26.4±2.2
HDL-cholesterol	2.3±0.17	2.9±0.29	2.7±0.18	2.6±0.21	2.8±0.27
Non-HDL-cholesterol	19.8±1.6	20.3±1.9	22.0±1.9	25.2±2.0	23.6±2.1

Values are means±S.E.M.

Table 2
Fatty acid composition of the plasma in apoE$^{-/-}$ mice treated with n-3 and n-6 polyunsaturated fatty acids

Fatty acid	Control	DHA	EPA	LA	AA
mol/100 mol					
16:0	18.3 ± 0.3^{a}	17.4 ± 0.3^{a}	16.8 ± 0.6^{a}	12.5 ± 0.3^{c}	14.5 ± 0.6^{b}
16:1	2.8±0.2	3.1±0.2	2.5±0.3	2.4±0.3	2.9±0.4
18:0	6.4±0.6	5.6±0.3	6.0±0.5	6.2±0.4	6.8±0.3
18:1	19.1 ± 0.9^{a}	18.4 ± 0.5^{a}	18.7 ± 0.5^{a}	14.6 ± 0.8^{b}	18.1 ± 1.0^{a}
18:2(n-6)	41.5 ± 2.1^{b}	38.9 ± 3.3^{b}	39.5 ± 2.8^{b}	54.8 ± 2.7^{a}	40.6 ± 2.1^{b}
18:3(n-6)	1.2 ± 0.1^{abc}	1.5 ± 0.1^{abc}	1.7 ± 0.2^{a}	1.1 ± 0.1^{c}	1.3 ± 0.1^{abc}
20:4(n-6)	8.4 ± 0.6^{b}	7.8 ± 0.5^{bc}	7.2 ± 0.5^{bc}	6.2 ± 0.3^{c}	12.5 ± 0.3^{a}
20:5(n-3)	0 ± 0^{c}	0.6 ± 0.1^{b}	3.2 ± 0.2^{a}	0 ± 0^{c}	0.8 ± 0.1^{b}
22:5(n-3)	0.6 ± 0.04^{b}	1.5 ± 0.1^{a}	1.8 ± 0.2^{a}	0.7 ± 0.1^{b}	0.5 ± 0.1^{b}
22:6(n-3)	1.8 ± 0.1^{bc}	5.2 ± 0.3^{a}	2.5 ± 0.1^{b}	1.4 ± 0.1^{c}	2.1 ± 0.1^{bc}

Values are means±S.E.M., $n=4$. Means in a row with different superscripts differ, $P<.05$.

3.4. Effect of PUFAs on VCAM-1 and ICAM-1 expression in TNF-α-treated HAECs

To examine the effect of PUFAs on adhesion molecule expression, we measured the expression of VCAM-1 and ICAM-1 on TNF-α-activated HAECs by cell-surface ELISA. As shown in Fig. 3A, DHA suppressed cell-surface VCAM-1 expression in a dose-dependent manner at concentrations ranging from 40 to 160 μM, while EPA suppressed VCAM-1 expression only at higher doses (80 and 160 μM). However, no significant change was seen in the LA and AA-treated groups. A similar assay was conducted for ICAM-1 expression. DHA suppressed ICAM-1 expression only at 160 μM, whereas EPA, LA and AA did not reveal significant effect on ICAM-1 expression over the range of concentrations used.

The effect of PUFAs on adhesion molecule expression in TNF-α-treated HAECs was also examined by Western blot analysis as shown in Fig. 3B. TNF-α evoked a 26.5-fold increase in VCAM-1 expression compared with the control group, and this induction was effectively inhibited by DHA. However, DHA had no significant suppressive effect on ICAM-1 expression. Moreover, EPA, LA, and AA did not have any significant effect on either VCAM-1 or ICAM-1 expression in TNF-α-treated HAECs.

3.5. Effect of PUFAs on NF-κB signaling pathway in TNF-α-stimulated HAECs

The NF-κB signaling pathway has been reported to play an essential role in the regulation of adhesion molecule expression [14]. We therefore investigated whether NF-κB activation was involved in the regulation of VCAM-1 expression by DHA in TNF-α-stimulated HAECs. It is well known that NF-κB activation is regulated by the phosphorylation of IκBα and its subsequent degradation [17,22]. Western blot analyses were performed to examine the expression of phospho-IκBα and total IκBα in TNF-α-activated HAECs. We found that expression of phospho-IκBα was significantly enhanced in TNF-α-treated cells, while DHA-pretreated cells efficiently suppressed the enhancement (Fig. 4A). In contrast, the other PUFA-treated cells did not show down-regulation of the TNF-α-enhanced phosphorylation of IκBα. Consistent with this result, expression of total IκBα was significantly decreased in the cell lysates of TNF-α-treated HAECs,

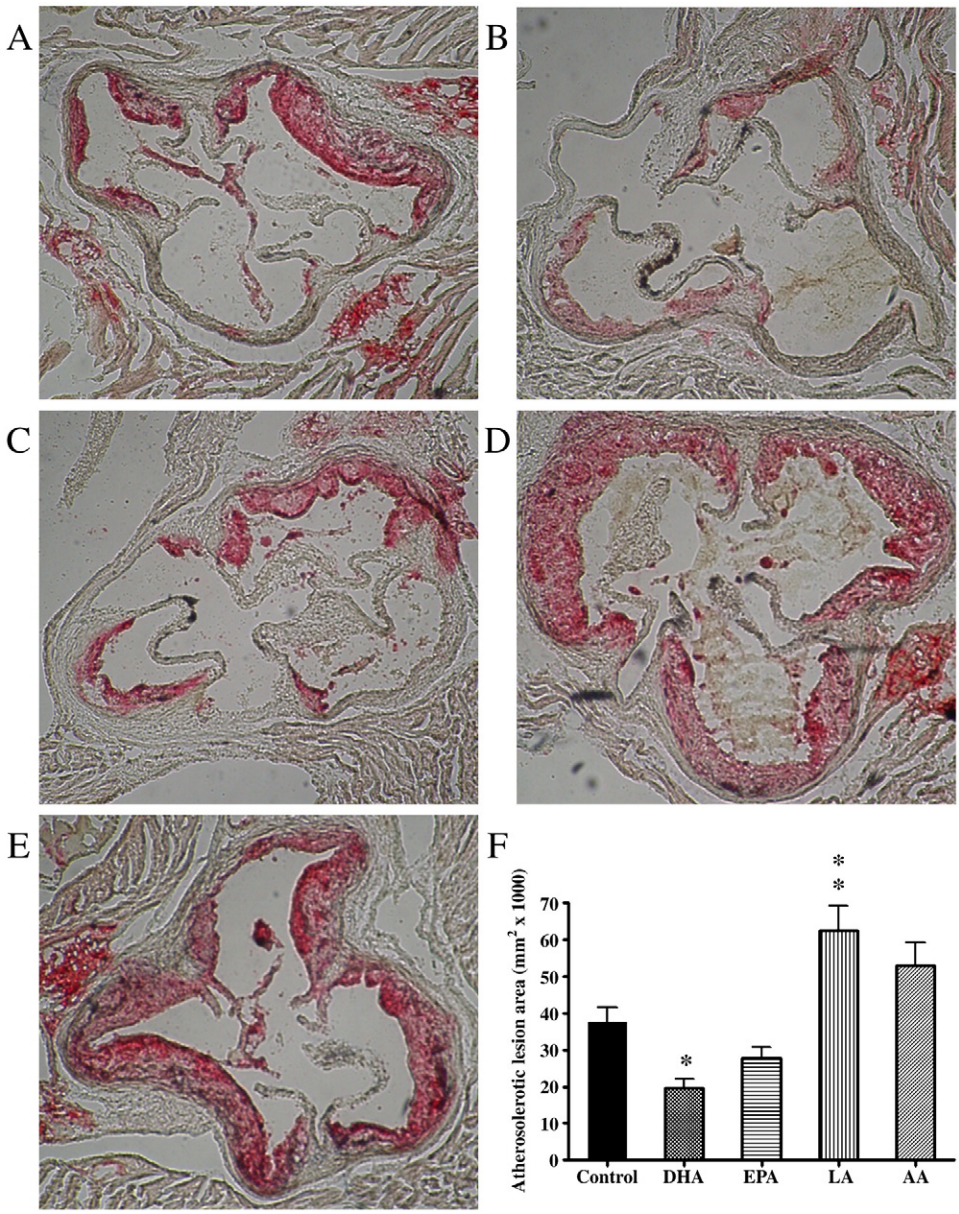

Fig. 1. Representative photomicrographs of atherosclerotic lesions in cross sections of the aortic root from apoE$^{-/-}$ mice fed a normal (A) or high PUFA diet (B–E) diet for 10 weeks. The lipids in the lesions were stained by Oil Red O (original magnification ×40). The bar graph represents the histopathological analyses (F). Values are means+S.E.M. *P<.05; **P<.01, versus control group.

while DHA inhibited the TNF-α-stimulated IκBα degradation (Fig. 4B). Treatment of HAECs with other PUFAs had no significant effect. Considering that IKKs are upstream kinases of IκB in the NF-κB signaling pathway [16], we next examined the effects of PUFAs on TNF-α-induced cytoplasmic IKK-β activation in HAECs. As shown in Fig. 4C, TNF-α up-regulated IKK-β phosphorylation, but DHA inhibited the activation. EPA, LA and AA did not show any significant effect on IKK-β phosphorylation.

Both NF-κB and AP-1 are potential therapeutic targets for atherosclerosis [23,31]. To obtain insight into the possible effect of DHA on the prevention of atherosclerosis, we next asked whether DHA might play a role in the regulation of nuclear NF-κB and AP-1 subunit translocation in the TNF-α-treated HAECs. As shown in Fig. 5, TNF-α resulted in a significant induction of nuclear NF-κB (p50/p65) and AP-1 (c-Fos/c-Jun) translocation, while DHA significantly suppressed all of these inductions. EPA only mildly suppressed the induction of nuclear c-Fos translocation in the TNF-α-stimulated HAECs. In contrast, LA and AA did not affect the nuclear translocation of NF-κB and AP-1 subunits in TNF-α-activated HAECs.

4. Discussion

Accumulating evidence indicates that dietary n-3 PUFAs may help reduce atherogenesis [1,32,33]. However, the molecular mechanisms by which n-3 PUFAs prevent atherosclerosis remain unclear. In our previous study [4], we found that 200 g/kg of fish oil intake reduced the formation of atherosclerotic lesions in the aortic arteries of apoE$^{-/-}$ mice. We proposed that the protective role may result from n-3 PUFAs. In the present study, we examined the effect of n-3 PUFAs on the prevention of atherosclerosis by using a much lower level of 200 mg/kg PUFA which is in a concentration of nutritionally achievable. It clearly shows that DHA decreased atherosclerotic plaque formation in apoE$^{-/-}$ mice. Increasing evidence suggests that DHA is a unique n-3 PUFA which is sterically incompatible with

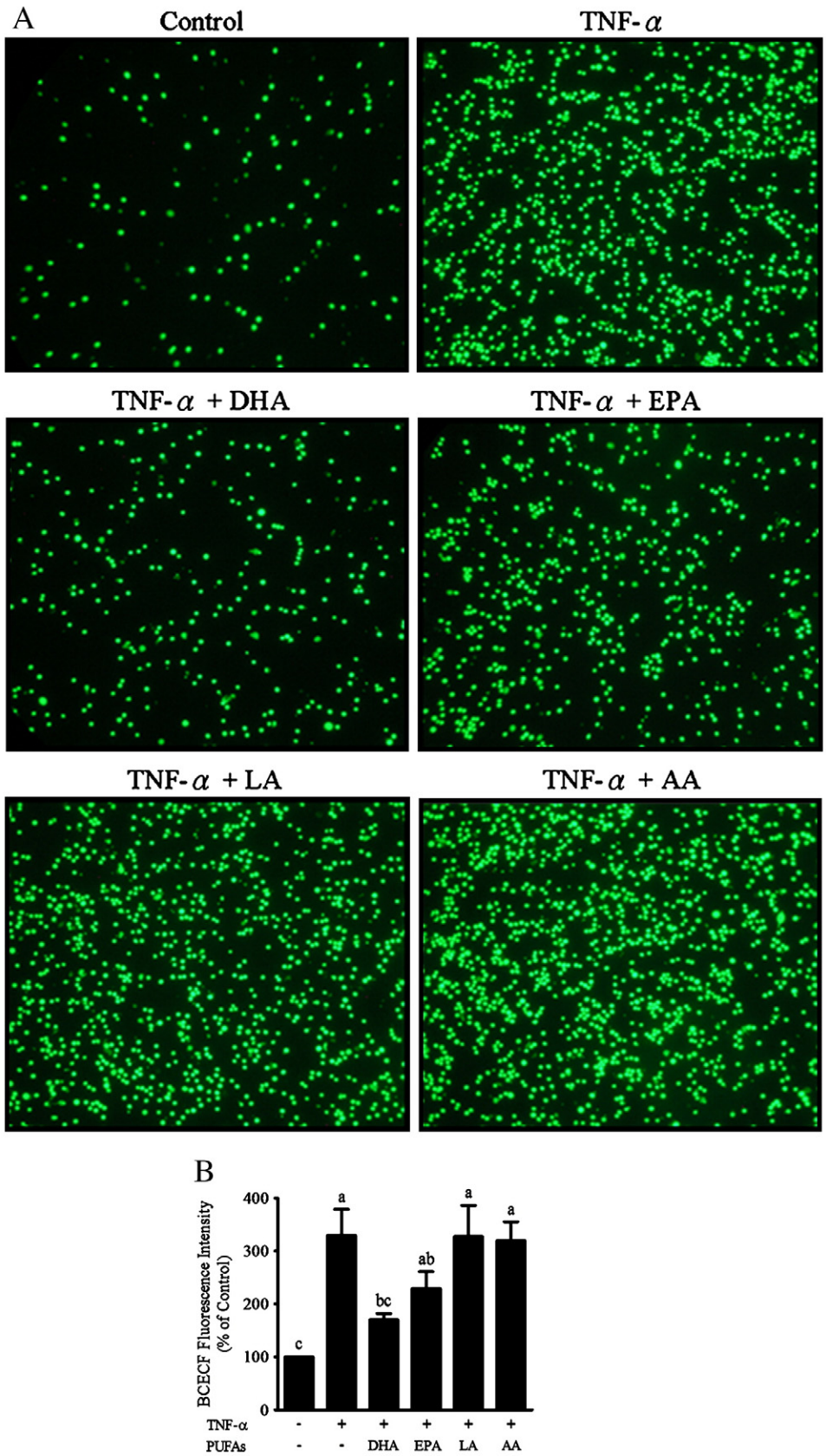

Fig. 2. Effect of PUFAs on THP-1 monocyte adhesion to HAECs stimulated with TNF-α. (A) HAECs were treated with PUFAs for 24 h and then incubated in the presence or absence of 10 ng/ml TNF-α for another 6 h. The THP-1 monocytes were labeled with BCECF/AM and allowed to interact with HAECs for 30 min. Following washing, attached THP-1 cells were visualized by an inverted fluorescent microscope. (B) The results are expressed as percentages of the controls and represent means+S.E.M. from triplicate experiments and bars with different letters (a,b,c) are significantly different ($P<.05$).

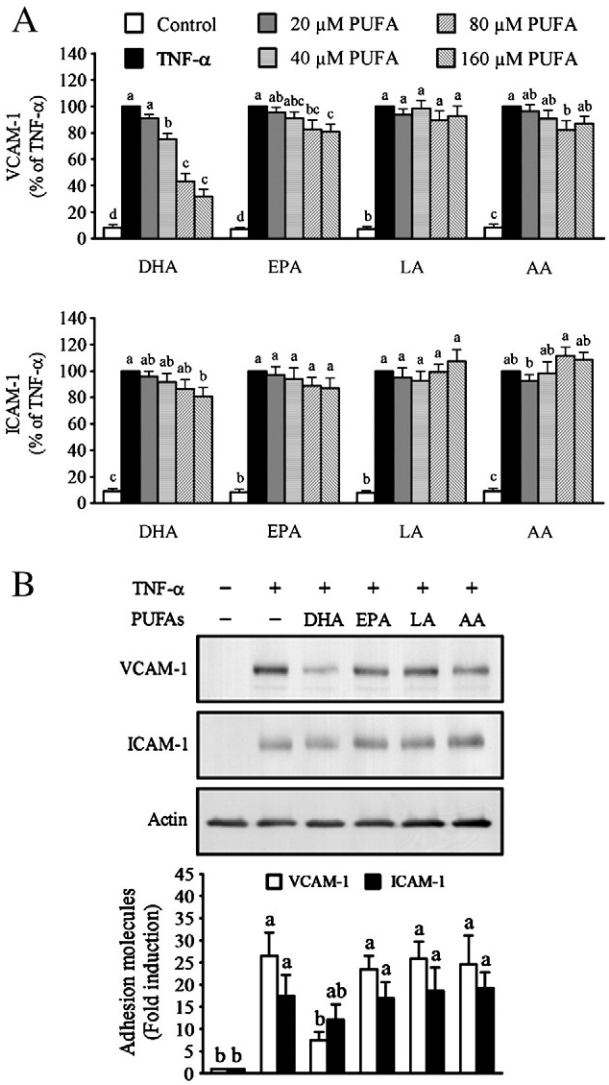

Fig. 3. Effect of PUFAs on the expression of VCAM-1 and ICAM-1 in HAECs. Cells were treated for 24 h with PUFAs prior to the addition of 10 ng/ml TNF-α. The expression of VCAM-1 and ICAM-1 was determined by cell-surface ELISA (A) or Western blot analysis (B). (A) Dose-dependent effects of PUFAs on cell-surface expression of VCAM-1 and ICAM-1 on HAECs are expressed as the percentage of TNF-α-treated group. The results represent means+S.E.M. of at least three separate experiments and bars with different letters (a,b,c) are significantly different ($P<.05$). (B) Representative gels are shown and the intensity of protein band normalized by actin was calculated as the fold of the controls and then depicted as graph bar. The results represent means+S.E.M. from triplicate experiments and bars with different letters (a,b) are significantly different ($P<.05$).

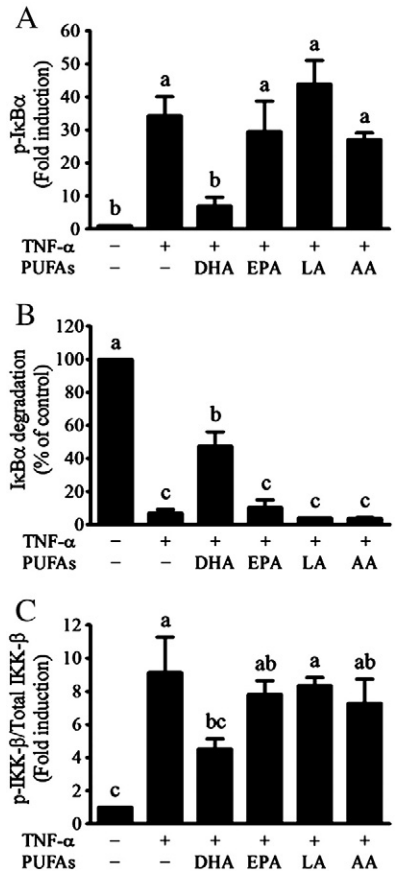

Fig. 4. Effect of PUFAs on NF-κB signaling pathway in TNF-α-stimulated HAECs. The cells were treated with 80 μM PUFAs for 24 h before the addition of 10 ng/ml TNF-α, then the cell lysates were prepared and assayed for IκBα phosphorylation (A) and degradation (B) as well as IKK-β phosphorylation (C) by Western blot analysis as described in Materials and methods. (A) The intensity of phosphor-IκBα band normalized by actin was calculated and the expression of IκBα phosphorylation was expressed as fold of the controls. (B) The intensity of IκBα band normalized by actin was calculated and IκBα degradation was depicted as the percentage of the controls. (C) The intensity of phosphor-IKK-β band normalized by total IKK-β was calculated and the expression of IKK-β phosphorylation was expressed as fold of the controls. Data represent means+S.E.M. of at least three separate experiments. Bars with different letters (a,b,c) are significantly different ($P<.05$).

cholesterol and sphingolipid and, therefore, appears to alter lipid raft behavior and protein function [34]. Moreover, the presence of DHA in membrane phospholipids can profoundly alter the lateral organization of the membrane bilayer and the consequent cellular signaling and immunosuppressive properties [35–37]. DHA appears to be the most potent inhibitor of endothelial activation, accounting for its long chain, more double bonds and special configuration.

Free fatty acids (FFA) are important components of phospholipids, triglyceride, and cholesterol ester in blood circulation, and a smaller pool of FFA bound to albumin in the plasma. From data of lipid levels (Table 1) and fatty acid composition (Table 2) in the plasma of apoE$^{-/-}$ mice, we assume that total plasma concentrations of fatty acids are in the millimolar range. The levels of albumin-bound PUFAs used (micromolar range) in the cellular study were determined in consideration with the limitation of FFA-albumin binding equilibrium in vivo [38]. The observed DHA inhibition of THP-1 monocytes to HAECs (Fig. 2) was corresponding to the anti-atherosclerotic effect of DHA in apoE$^{-/-}$ mice (Fig. 1), indicating that the concentration of albumin-bound n-3 PUFAs used in HAEC study is biologically relevant to the anti-atherogenic actions in animal study.

Atherosclerosis is known as a chronic inflammatory disease [12,39]. Increased expression of adhesion molecules by vascular endothelial cells has emerged as the most important mediators in vascular inflammation [40] and is responsible for further recruitment of circulating monocytes to the atherosclerotic sites [41,42]. VCAM-1 and ICAM-1 are adhesion molecules that are both expressed in vascular endothelial cells and increased under pro-inflammatory stimuli. The present study shows that DHA can significantly reduce TNF-α-induced VCAM-1 expression on HAECs using both cell-surface ELISA and Western blot analysis. These results are consistent with the previous report by De Caterina et al. [43], who demonstrated that DHA reduces the expression of adhesion molecules and inflammatory mediators in human saphenous vein endothelial cells. Furthermore, Chen et al. [44] revealed that DHA attenuates the expression of both VCAM-1 and ICAM-1 induced by lipopolysaccharide (LPS) in human retinal vascular endothelial cells. Yamada et al. [45] reported that EPA inhibits LPS-induced monocyte adhesion to the endothelial surface

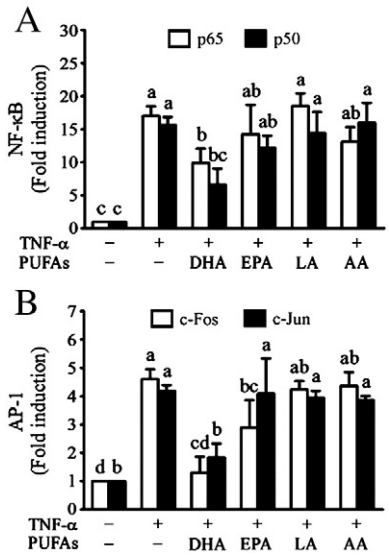

Fig. 5. Effect of PUFAs on the nuclear translocation of NF-κB (A) and AP-1 (B) subunits in TNF-α-stimulated HAECs. Cells pretreated for 24 h with 80 μM PUFAs were stimulated for another 6 h with 10 ng/ml TNF-α, then expression of nuclear NF-κB (p50/p65) and AP-1 (c-Fos/c-Jun) subunits was detected by Western blot analysis as described in Materials and methods. The results are expressed as a fold increase over the control group. Means+S.E.M. of at least three separate experiments are shown. Bars with different letters (a,b,c) are significantly different (P<.05).

surrounding the orifice of intercostal arteries of thoracic aorta in C57BL/6J mice. These findings highlight the potentially beneficial effects of n-3 PUFAs on cardiovascular disease, specifically illustrated by their ability to reduce expression of adhesion molecules. However, the influence on the regulation of adhesion molecular expression by n-3 PUFAs appears to be dependent on the type of pro-inflammatory stimulator, the cell type being treated, and the concentrations of PUFAs. Based on our observations, we suggest that DHA is more effective than EPA on reducing adhesion molecular expression in TNF-α-treated HAECs as well as suppressing THP-1 cell adhesion to TNF-α-stimulated HAECs. To further explore the possible mechanisms underlying this inhibition, we examined the effect of DHA on the NF-κB signaling pathway in the present study.

NF-κB is a redox-sensitive transcription factor that activates inflammation through regulating the gene expression of a large number of cytokines and adhesion molecules [14,16]. Disruption of NF-κB activation has been shown to delay or prevent atherogenesis [46]. Numerous natural components and therapeutic agents have been shown to inhibit NF-κB activation by preventing IκBα phosphorylation and degradation [47–51]. IKKs are the upstream kinases of IκBs in the NF-κB signaling pathway. The phosphorylation of IκB by specific IKK, followed by polyubiquitination and proteosomal degradation; this releases NF-κB, which then translocates to the nucleus and binds to the NF-κB sites of specific gene promoters thereby regulating transcription of the target genes [14,22]. Our data indicate that DHA not only inhibited the upstream IKK-β and IκBα phosphorylation as well as IκBα degradation, but also attenuated the nuclear translocation of NF-κB (p50/p65) and AP-1 (c-Fos/c-Jun) subunits. This provides evidence that DHA can attenuate NF-κB signaling pathway and AP-1 activation in TNF-α-activated HAECs. The reduction of ICAM-1 protein expression by DHA was less than the reduction of VCAM-1 expression, which reflected that the regulation of ICAM-1 expression might be less dependent on the NF-κB signaling pathway or AP-1 activation in TNF-α-treated HAECs. It has been reported that the *cis*-elements located at the promoter region of the ICAM-1 and VCAM-1 genes are different [52,53]. The human ICAM-1 promoter contains binding sites for a number of transcription factors including signal transducer and activator of transcription (STAT), NF-κB, AP-1 and antioxidant response elements (ARE) [52]. However, human VCAM-1 promoter includes binding sites for NF-κB, AP-1, GATA and thyroid hormone-responsive elements (TRE) [53]. The differential regulation of ICAM-1 and VCAM-1 expression by DHA in TNF-α-treated HAECs indicates that other complicated factors may be involved in the regulation of their gene expression.

Data from other reports indicate that DHA is a unique PUFA, because it significantly exhibits greater potency to alter the basic properties of cell membranes, including membrane fluidity, ion permeability, rapid flip-flop, lipid rafts and resident protein function [54–58]. Sun et al. [59] have reported that D-series resolvins and protectins derived from hydroxylated DHA metabolites are bioactive mediators that exert anti-inflammatory activity. Additional mechanisms have been proposed to explain the beneficial effects of DHA, including activation of peroxisome proliferator-activated receptor-gamma (PPAR-γ) [60], alteration of redox signaling [56], and inhibition of phosphatidylinositol 3-kinase/AKT [61], modulation of lipid rafts [57] and inhibition of NADPH oxidase and protein kinase C [58,59]. These studies lead us to speculate that DHA is a bioactive modulator of different intracellular signaling pathways. The precise mechanisms of fatty acids in regulating cellular signaling require further elucidation, particularly with regard to the activated mediators in different cells. It is likely that DHA is involved in many diverse biological activities and cellular functions.

In summary, the present study provides mechanisms by which DHA may exert a beneficial effect on the prevention of atherosclerosis. We have demonstrated that DHA treatment is capable of reducing atherosclerotic lesions in the aorta of apoE$^{-/-}$ mice. Our results also show that DHA can lead to the attenuation of VCAM-1 expression and NF-κB signaling pathway in TNF-α-challenged HAECs. The data reported herein provide evidence that DHA may have a potential application in the prevention of atherosclerosis and inflammatory disorders.

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