The Omega-3 Fatty Acid Docosahexaenoate Reduces Cytokine-Induced Expression of Proatherogenic and Proinflammatory Proteins in Human Endothelial Cells

Raffaele De Caterina, Myron I. Cybulsky, Steven K. Clinton, Michael A. Gimbrone Jr, Peter Libby

Abstract The mechanisms by which dietary fatty acids can modulate atherogenesis and inflammation are poorly understood. Induction in endothelial cells of adhesion molecules for circulating leukocytes and of inflammatory mediators by cytokines probably contributes to the early phases of atherogenesis and inflammation. We report here that incorporation into cellular lipids of docosahexaenoic acid (DHA), a specific fatty acid of the ω3 family, decreases cytokine-induced expression of endothelial leukocyte adhesion molecules, secretion of inflammatory mediators, and leukocyte adhesion to cultured endothelial cells. DHA, but not eicosapentaenoic acid, decreased in a dose- and time-dependent fashion the expression of vascular cell adhesion molecule 1 (VCAM-1) induced by interleukin (IL)-1, tumor necrosis factor (TNF), IL-4, or bacterial lipopolysaccharide, with half-maximum inhibition at <10 μmol/L. This reduction required prolonged (24- to 96-hour) exposure of endothelial cells to DHA and correlated with the degree of DHA incorporation into cellular lipids. DHA also limited cytokine-stimulated endothelial cell expression of E-selectin and intercellular adhesion molecule 1 and the secretion of IL-6 and IL-8 into the medium but not the surface expression of constitutive surface molecules. Cyclooxygenase inhibition did not block the effect of DHA on VCAM-1. In parallel with reduced surface VCAM-1 protein expression, DHA reduced VCAM-1 mRNA induction by IL-1 or TNF. DHA treatment also reduced the adhesion of human monocytes and of monocytic U937 cells to cytokine-stimulated endothelial cells. These properties of DHA may contribute to antiatherogenic and anti-inflammatory effects of ω3 fatty acids. (Arterioscler Thromb. 1994;14:1829-1836.)

Key Words • omega-3 fatty acids • n-3 fatty acids • fish oils • endothelial leukocyte adhesion molecules • atherogenesis

Dietary long-chain omega-3 polyunsaturated fatty acids (ω3 FA) modulate a wide range of cell responses.1-3 Epidemiological studies suggest an inverse relation between intake of ω3 FA and the incidence of cardiovascular disease,4-12 at least in part because of reduced atherosclerosis.13 Consumption of ω3 FA diminishes atherogenesis in nonhuman primates14,15 as well as in other animal species (reviewed in References 1 and 2). In addition to atherothrombotic diseases, epidemiological studies demonstrate an inverse relation between dietary intake of ω3 FA and some inflammatory/immune diseases such as psoriasis, bronchial asthma, and thyrotoxicosis.4

Both atheroma formation and inflammation require the adhesion of circulating leukocytes to the endothelium and their subsequent transendothelial migration.16-23 These processes depend on the endothelial expression of endothelial leukocyte adhesion molecules, such as vascular cell adhesion molecule 1 (VCAM-1), intracellular adhesion molecule 1 (ICAM-1), and E-selectin, that recognize cognate ligands on leukocytes.24-26 We hypothesized that ω3 FA modulate endothelium-dependent mechanisms in atherogenesis and inflammation such as their expression of leukocyte adhesion molecules or the production of chemoattractants. We show here that one ω3 FA, docosahexaenoic acid (DHA), reduces cytokine-stimulated expression of such molecules and concomitantly reduces monocyte adhesion to activated endothelial cells.

Methods

Endothelial Cell Culture

Human saphenous vein endothelial cells (HSVEC) were isolated from outgrowths of explants from unused portions of saphenous veins harvested for coronary artery bypass surgery. HSVEC were harvested enzymatically with type II collagenase 0.1% as described27 and maintained in medium 199 (Gibco) containing HEPES (25 mmol/L), heparin (1%), ECGF (50 μg/mL), l-glutamine (1%), antibiotics, and 5% fetal calf serum (FCS). Once grown to confluence, the cells were replated on low-pyrogen fibronectin (1.5 μg/cm^2) at 20 000 cells/cm^2. HSVEC so isolated form a confluent monolayer of polygonal cells and express von Willebrand factor as determined by their content of specific mRNA and immunoreactive protein.28,29

Reagents

The following unsaturated FA, representing the most abundant unsaturated FA esterified in cell membrane phospholipids, and their properties

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ids, were used: oleic acid (18:1, o9), linoleic acid (18:2, o6), arachidonic acid (20:4, o6), eicosapentaenoic acid (EPA; 20:5, o3), and DHA (22:6, o3), all obtained >99% pure as sodium salts from Nu-Chek Prep. These FA were dissolved in water and then in tissue culture medium, divided into aliquots under nitrogen stream, and maintained at ~80°C until used. For control experiments, DHA as free acid from two other commercial sources (Calbiochem-Behring and Aldrich Chemicals) was also used. These materials were dissolved first in a small volume of 99% ethanol. Control incubations contained equal amounts of this solvent.

The following human recombinant cytokines were used: interleukin (IL)-1a (Hoffmann-La Roche, at 1 to 10 ng/mL), IL-1B (1 to 10 ng/mL), tumor necrosis factor (TNF)-a (1 to 10 ng/mL), and IL-4 (50 to 100 ng/mL) (all from Genzyme). Bacterial lipopolysaccharide (LPS) from Escherichia coli (1 to 15 μg/mL), indomethacin, aspirin, (+)-o-tocopherol acid succinate (vitamin E), and butylated hydroxytoluene (BHT) were from Sigma. Vitamin E and BHT were dissolved in 99% ethanol and then in medium. Control incubations contained equal amounts of this solvent.

Detection of Cell Surface Molecules

Assay of cell surface molecules was carried out by either cell surface enzyme immunoassays (EIA) or flow cytometry using mouse anti-human monoclonal antibodies against VCAM-1 (Ab 1/6), E-selectin (Ab H18/7), ICAM-1 (Ab HU 5/3), major histocompatibility complex class 1 (MHC-I, W6/32), or the monoclonal antibody E1/1, recognizing a constitutive and non-cytokine-inducible endothelial cell antigen.31 EIA were carried out by incubating monolayers first with saturating concentrations of specific monoclonal antibodies against the target molecule, then with biotinylated goat anti-mouse IgG (Vector), and finally with streptavidin–alkaline phosphatase (Zymed). Layers were washed three times between each incubation step, and integrity of the monolayer was monitored by phase-contrast microscopy. The surface expression of each protein was quantified spectrophotometrically, reading the optical density of the wells (410 nm) 15 to 60 minutes after the addition of a chromogenic substrate (paranitrophenylphosphate) as described.30

For flow cytometric analysis, the surface expression of adhesion molecules was assessed by incubating HSVEC, suspended in Hanks' buffered saline solution with 3 mmol/L EDTA, with the specific primary antibody for 30 minutes at 4°C, and subsequently with goat anti-mouse F(ab')2 from IgG (H+L), labeled with fluorescein-isothiocyanate (Caltag) at 4°C. After washings and fixation in 1% formaldehyde, the cell suspension was analyzed on a Becton-Dickinson FACScan analyzer. Results were plotted as intensity of fluorescence (in arbitrary units, on a logarithmic scale, on the abscissa) versus cell number (on the ordinate; total cell count, 10⁶ for each condition).

Control of FA Incorporation Into Total Cell Lipids

Relative amounts of the individual chemical species of the o3 and o6 families were quantified by integration of peaks obtained by gas-liquid chromatographic separation of FA from HSVEC monolayers. After chloroform-methanol (2:1) extraction, total cell lipids were subjected to acidic transesterification, and fatty acid methyl esters were analyzed by flame-ionization capillary gas-liquid chromatography (on a Hewlett-Packard model 5890 apparatus) using a 30 m Supelcowax 10 (2.40 μm film thickness) column. A temperature gradient from 170°C to 225°C was used at a rate of 1°C/min. The hydrogen, air, and carrier (helium) pressures were 0.8, 0.9 to 1, and 0.6 bar, respectively. Individual peaks were integrated automatically with a Hewlett-Packard integrator and identified by comparison with retention times of FA standards.

Assessment of Total Protein Synthesis

HSVEC were cultured in 12-well plates in the presence/absence of DHA for 24 to 96 hours and subsequently stimulated with IL-1a for periods up to 24 hours. At the time of IL-1 addition, [3H]leucine (New England Nuclear) was also added at 0.2 μCi/10⁶ cells, and total protein synthesis was then evaluated by counting radioactivity after cold trichloroacetic acid precipitation of supernatants and of total cell extracts at various time points, as described.22

Assays for Prostacyclin, IL-6, and IL-8

These assays were carried out by commercially available EIA for the stable hydrolytic product of prostacyclin, 6-keto-PGF₁α (Cayman Chemical), or for IL-6 and IL-8 (R & D), assayed directly in HSVEC supernatants.

Isolation of RNA and Northern Analysis

Cultured HSVEC were harvested by trypsin/EDTA and lysed in guanidinium isothiocyanate by use of a syringe and 21 gauge needle. RNA was purified by ultracentrifugation through CsCl.33 RNA concentration and purity were determined from the A260 and A260/A280 ratio, respectively. RNA quality was confirmed by gel electrophoresis before Northern analysis. For this, 20 μg of cellular RNA was applied to each lane, separated on a 1.2% agarose/formaldehyde (2.2 mol/L) gel, transferred to a nylon membrane (Hybond-N, Amersham), and immobilized by shortwave UV illumination. The membranes were prehybridized for at least 4 hours before hybridization with 32P-labeled DNA probe for VCAM-1 or o-actin labeled by random hexanucleotide priming (Pharmacia Inc) to specific activities >108 cpm/μg DNA.

Preparation of Human Monocytes and U937 Cells and Adhesion Assays

Human peripheral blood monocytes were obtained from the white blood cell–rich fraction (leukopheresis packs) that are a by-product of the platelet pheresis program of the Dana Farber Cancer Institute. Cells were obtained by centrifugation on Ficoll-Hypaque density gradient at 15°C (LMS, Organon Teknika) followed by counterflow centrifugation elutriation in a Beckman 12-21 M/E centrifuge using a JE-6 elutration rotor and a 6-mL Sanderson chamber (Beckman). A modified Doherty method34 was used;35 the elutration buffer was Dulbecco's modified phosphate-buffered saline (DPBS, Gibco), supplemented with 3 mmol/L EDTA and 0.25% human serum albumin (HSA, Sigma). The mononuclear cell fraction isolated by Ficoll-Hypaque density gradient at the Ficol-plasma interface was loaded at 14 mL/min onto the elutriator centrifuge rotor head (2500 rpm at 10°C), and fractions of elutriated cells were collected. Monocytes were eluted at 21.5 mL/min. Monocyte suspensions with this technique are 89±4% pure, with 8% lymphocytes, <2% granulocytes, and essentially no free platelet contamination as determined by light scatter (FACScan, Becton Dickinson) and cell surface antigen analysis with monoclonal antibodies directed to CD14. Monocytes were resuspended in cold DPBS containing 0.75 mmol/L Ca²⁺, 0.75 mmol/L Mg²⁺, and 0.25% HSA. U937 cells were obtained through American Tissue Culture Collection and grown in RPMI medium 1640 (Gibco) containing 10% FCS. Both monocytes and U937 cells were concentrated by centrifugation at 1×10⁶ cells/mL. For the adhesion assays, HSVEC were grown to confluence in the presence or absence of DHA (10 μmol/L) in six-well tissue culture plates for 96 hours, after which IL-4 (50 ng/mL) or IL-1α (10 ng/mL) was added for an additional 24 hours to induce the expression of VCAM-1. As a control, some monolayers were treated with a mouse anti-human monoclonal antibody (E1/6) against VCAM-1. The adhesion assay was performed by adding 1 mL of the concentrated U937 cell suspension to each monolayer under rotating conditions (63 rpm) at 21°C.36,37 After 10
minutes, nonadhering cells were removed by gentle washing with medium 199, and monolayers were fixed with 1% paraformaldehyde. The number of adherent cells was determined by counting six different fields by use of an ocular grid and a 20x objective (0.16 mm²/field). Fields for counting adherent leukocytes were randomly selected at half-radius distance from the center of the monolayers; counts were performed by two independent blinded observers, with interobserver variability <8%.

**Experimental Designs**

Cultured HSVEC were preincubated for 0 to 96 hours with various unsaturated FA and then stimulated with the cytokines (IL-1α, IL-1β, TNF, or IL-4) or LPS for an additional 0 to 24 hours in the continued presence of FA or in control conditions. Subsequently the expression of endothelial surface molecules (cell surface EIA and/or flow cytometry), of endothelial secreted products (EIA), of adhesion molecule mRNA (Northern analysis), or of parameters of cell viability (morphology, number, total protein synthesis) was assessed or the adhesion was measured.

**Statistics**

Multiple comparisons were performed by one-way ANOVA and individual differences tested by Fisher’s protected least significance difference test after the demonstration of significant intergroup differences by ANOVA. Student’s t test for unpaired data was used to compare the IL-1α-stimulated production of IL-6 and IL-8 with/without DHA preincubation. Comparisons of distribution of fluorescent intensities at flow cytometry were performed by the Kolgomorov-Smirnov statistics. Significance level was set at P<.05. Results are expressed as mean±SEM unless otherwise indicated.

**Results**

**Specific FA Reduce the Surface Expression of VCAM-1 in Response to Cytokines**

The surface expression of VCAM-1 induced by IL-1α or TNF was used to screen the effects of various unsaturated FA. Preincubation of HSVEC for ≤24 hours with DHA and, to a lesser extent, with oleic acid, but not of other FA tested (including, notably, EPA), significantly decreased the expression of VCAM-1 induced by IL-1α or TNF as assayed by cell surface EIA (Fig 1). Since DHA produced the greatest inhibition, further experiments focused on this FA.

**DHA Inhibits VCAM-1 and E-Selectin Expression by HSVEC in a Time- and Dose-Dependent Fashion**

DHA inhibited cytokine-induced expression of all three adhesion molecules assayed (VCAM-1, E-selectin, and ICAM-1) in a concentration-dependent manner (half-maximal inhibition at <10 μmol/L). Dose-response curves for VCAM-1 after both IL-1α and TNF are shown in Fig 2A. DHA reduced cytokine-stimulated expression of VCAM-1 more than that of E-selectin (see Fig 2B and 2C). Inhibition of ICAM-1 was the least pronounced: with 72 hours of preincubation with DHA and after a further 24 hours of stimulation with IL-1α 10 ng/mL, ICAM-1 inhibition was 23% compared with 53% inhibition for VCAM-1 in the same experiment (P<.01 for both, P<.05 in the comparison of percent inhibition for these two adhesion molecules). Inhibition of adhesion molecule expression was observed at all time points examined during cytokine stimulation (Fig 2B and 2C). DHA inhibited VCAM-1 expression with all stimuli tested, including IL-1α (1 to 10 ng/mL), IL-1β (1 to 10 ng/mL), TNF-α (1 to 10 ng/mL), IL-4 (50 to 100 ng/mL), or LPS (1 to 10 μg/mL). When tested in the same experimental conditions, all stimuli used, although different in their potency and selectivity in eliciting adhesion molecule expression, did not affect the degree of DHA effect: for instance, with 72 hours of preincubation with DHA followed by 24 hours of stimulation with either IL-1α 10 ng/mL or IL-4 50 ng/mL, VCAM-1 expression declined 53% and 56%, respectively (P=NS) (see also the similar dose-response curves for IL-1α and TNF in Fig 2A). When tested comparatively after IL-1α stimulation, DHA from the three different commercial sources used were all effective and equipotent in inhibiting VCAM-1 expression (P<.05 for each DHA source relative to control, P=NS for comparisons between pairs of different DHA sources).

**Inhibition of Cytokine-Induced VCAM-1 Expression by HSVEC Requires Prolonged Preincubation Times and Correlates With the Alteration of Ratios of Specific FA in Cellular Phospholipids**

Inhibition of VCAM-1 expression by DHA depended on the time of incubation of HSVEC with DHA, requiring pretreatment and increasing for up to 72 hours. The relationship of these kinetics to the reequilibration of polyunsaturated FA pools inside the cells was explored by parallel assessment of the FA composition of endothelial cells upon incubation with DHA (Fig 2D). HSVEC, at passage 3 to 4, contained 5.7% of DHA, as percent of total FA, at baseline. This content represented 57% of total ω3 FA present at baseline. Incubation of HSVEC with 10 μmol/L DHA progressively increased total DHA incorporated in cellular lipids up to a maximum of 14.3% at 48 hours, accompanied by increased total ω3 FA and a corresponding decrease of total ω6 FA. At 48 hours, DHA content represented 72% of total ω3 FA. Total ω3/ω6 FA

![Fig 1](http://atvb.ahajournals.org/)

Bar graph showing the surface expression of vascular cell adhesion molecule (VCAM-1) by human saphenous vein endothelial cells after 6 hours of stimulation with recombinant human tumor necrosis factor (TNF-α) (1 ng/mL) following preincubation for 24 hours with different fatty acids (FA) (arachidonic acid, AA; docosahexaenoic acid, DHA; eicosapentaenoic acid, EPA; omega acid, OLEATE), all at 10 μmol/L, compared with medium without FA supplementation (control). In a typical screening experiment, VCAM-1 expression is measured in milliunits of optical density (OD) by a cell-surface enzyme immunoassay. Baseline expression of VCAM-1 (in the absence of TNF-α) is 5±3 mU. Horizontal lines join groups of treatment for which significant (*)P<.05) differences are found after ANOVA. In similar screening experiments, qualitatively superimposable findings were obtained for different times of TNF incubation and using IL-1α as a stimulus.

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Fig 2. Graphs showing the inhibition of cytokine-induced expression of vascular cell adhesion molecule (VCAM)-1 and E-selectin in human saphenous vein endothelial cells (HSVEC) by docosahexaenoic acid (DHA). DHA, as sodium salt, was dissolved in medium 199 containing 5% serum and incubated for 96 hours (A through C) with HSVEC monolayers in 96-well plates, after which cytokines were added for a further 24 hours to induce surface expression of leukocyte adhesion molecules. This was assessed by cell-surface enzyme immunoassays. A, Dose-response curves of VCAM-1 expression as a function of DHA concentration are shown, after stimulation of HSVEC monolayers with Interleukin (IL)-1α or tumor necrosis factor (TNF)-α, both at 10 ng/mL. B and C, Inhibition of VCAM-1 and E-selectin, respectively, as a function of time after the addition of IL-1α (at 10 ng/mL). D, Percent inhibition of IL-1α-stimulated VCAM-1 expression (c) is shown as a function of DHA preincubation time, as obtained in cell-surface enzyme immunoassay experiments; each time point is the mean±SEM from three or more experiments, each consisting of ≥16 comparisons between DHA-treated and control cell monolayers. D also shows the corresponding time course of DHA incorporation into total cell lipids (•) from parallel HSVEC cultures, expressed as the ratio of DHA (in percent) to total n-6 fatty acid (FA) in total cellular lipid, as determined by gas-liquid chromatography.

Flow Cytometry Also Demonstrates DHA-Induced Inhibition of Cytokine-Stimulated VCAM-1 Expression in HSVEC

Flow cytometric analysis of suspended HSVEC also showed decreased cytokine-stimulated cell surface expression of VCAM-1 by DHA (Fig 3). DHA pretreatment significantly (P<.01) decreased the median intensity of fluorescence and splayed the distribution, indicating that DHA limited cytokine-induced VCAM-1 expression in a substantial population of cells.

DHA Inhibits IL-1-Induced IL-6 and IL-8 Production by HSVEC

As in the case of adhesion molecule expression on the cell surface, DHA inhibited HSVEC secretion of the...
TABLE 1. Effect of DHA (10 µmol/L, 96-h Preincubation) on the Secretion of IL-6 and IL-8 by HSVEC

<table>
<thead>
<tr>
<th>Condition</th>
<th>IL-6, ng/10⁶ Cells</th>
<th>IL-8, ng/10⁶ Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, no IL-1α</td>
<td>&lt;0.2</td>
<td>0.5±0.1</td>
</tr>
<tr>
<td>Control+IL-1α, 10 ng/mL</td>
<td>9.0±0.9</td>
<td>83.3±3.1</td>
</tr>
<tr>
<td>DHA (10 µmol/L)+IL-1α, 10 ng/mL</td>
<td>6.5±0.6*</td>
<td>69.3±1.8*</td>
</tr>
</tbody>
</table>

DHA indicates docosahexaenoic acid; IL, interleukin; and HSVEC, human saphenous vein endothelial cells. Values are mean±SEM of protein concentrations assayed in duplicate in medium from ≥4 monolayers in each control unstimulated condition and ≥8 monolayers for each stimulated condition. Experimental conditions in 12-well plates are otherwise similar to those described in Fig 2A through 2C.

**P<.01 in comparison with corresponding control, stimulated condition (Student's t test for unpaired data).

TABLE 2. Selectivity of DHA Effects on Endothelial Cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cells x 10⁶/cm²</th>
<th>Secreted proteins</th>
<th>Cell-associated proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43±2</td>
<td>92±3</td>
<td>1076±17</td>
</tr>
<tr>
<td>24 h DHA</td>
<td>54±6</td>
<td>73±4</td>
<td>1285±23</td>
</tr>
<tr>
<td>92 h DHA</td>
<td>73±4</td>
<td>85±4</td>
<td>1428±34</td>
</tr>
<tr>
<td>Viability</td>
<td>&gt;95%</td>
<td>79±3</td>
<td>1483±17</td>
</tr>
</tbody>
</table>

DHA indicates docosahexaenoic acid; Ag, antigen; MHC, major histocompatibility complex; OD, optical density; and IL, interleukin. Values are mean±SEM.

*BHBT (20 µmol/L) at maximum concentrations compatible with HSVEC viability (data not shown).

DHA Also Reduces VCAM-1 mRNA Accumulation by Cytokine-Stimulated EC

Northern analysis of VCAM-1 mRNA at various time points after the addition of IL-1α in DHA-treated and control HSVEC cultures revealed a decrease in steady-state message levels, consistent with the observed reduction of VCAM-1 protein expression (Fig 4). DHA diminished levels of mRNA encoding VCAM-1 in IL-1α-stimulated HSVEC in a time-dependent manner. The reduction was most pronounced after 24 h of incubation with DHA (Fig 4).

Vascular cell adhesion molecule (VCAM)-1 mRNA (upper panel) and ethidium bromide staining of total RNA (lower panel) at various time points after interleukin (IL)-1 stimulation in docosahexaenoic acid (DHA)-treated human saphenous vein endothelial cells (10 µmol/L, 48 hours of preincubation) and in control conditions. DHA preincubation increases VCAM-1 mRNA (by about 50% at densitometry) during the time course of mRNA induction.
The effect of docosahexaenoic acid (DHA) treatment of human saphenous vein endothelial cells (HSVEC) on the adhesion of monocyte-like cells to HSVEC after cytokine stimulation. U937 cells adhere only to a minor extent to HSVEC not exposed to cytokines (A). The adhesion is dramatically increased by treatment of the monolayer with interleukin (IL)-4 (B), a phenomenon that is almost entirely accounted for by the expression of vascular cell adhesion molecule (VCAM)-1, since it is almost completely inhibited by a treatment of the monolayer with saturating concentrations of the antibody E1/6 for 1 hour before the assay (C). DHA treatment of the monolayer also inhibits the adhesion of U937 cells to a substantial extent (D), paralleling the inhibition of VCAM-1 expression as assessed by cell-surface enzyme immunoassay and flow cytometry. IL-1α-induced U937 cell adhesion was also reduced (10% to 40% in three different experiments, data not shown). Quantitatively similar results were obtained with flow-elutriated human monocytes. Photographs show randomly selected half-radius fields; insets show (mean±SEM) cell counts per 0.16 mm² (see "Methods" for details).

DHA Reduces Adhesion of Mononuclear Cells to Cytokine-Stimulated EC

To study the functional consequences of DHA-induced reduction in the expression of adhesion molecules, we tested whether DHA treatment affected the adherence of human monocytes (U937 cells or flow-elutriated human monocytes) to HSVEC. Adhesion of these leukocytes to endothelial cells under nonstatic conditions partially depends on VCAM-1 expression. Indeed, monocyte/U937 cell adhesion to HSVEC was practically absent without cytokine stimulation of HSVEC and increased >30-fold after IL-4 stimulation (at 50 ng/mL) and >90-fold after IL-1α stimulation (10 ng/mL). This cytokine-stimulated adhesion was inhibited by the anti-VCAM-1 monoclonal antibody E1/6 (incubated for 1 hour before the adhesion assay) by 40±10% in the case of IL-1α and by 83±12% in the case of IL-4, a more selective inducer of VCAM-1 in HSVEC as in human umbilical vein endothelial cells. DHA pretreatment of HSVEC monolayers significantly inhibited IL-4– (Fig 5) and IL-1α–induced adhesion to a degree comparable to the inhibition of VCAM-1 surface expression as assessed by direct assays of the protein.

Discussion

This study shows that one specific ω3 FA, DHA, inhibits cytokine-stimulated expression of endothelial leukocyte adhesion molecules and of two other cytokine-induced secretable products, IL-6 and IL-8, from human endothelial cells in culture. Significant inhibition occurred with moderate, nutritionally achievable concentrations of DHA in a dose- and time-dependent fashion and was accompanied by decreased endothelial adhesivity for mononuclear cells. Our study also shows that DHA inhibition of leukocyte adhesion molecule expression does not depend on the activating stimulus or on FA metabolism through endothelial cyclooxygenase products. The effect is pretranslational, since steady-state mRNA levels decrease in parallel.

Treatment of HSVEC with DHA enriched cellular phospholipids in this FA, documenting its incorporation into biological lipid pools. Such changes closely mimic those occurring in humans during dietary supplementation with ω3 FA, which produces a sustained enrichment of DHA in cellular phospholipids. The kinetics of the effect of DHA on adhesion molecule expression paralleled its incorporation into cellular lipids (Fig 2D), indicating that this effect is not acute but rather requires incorporation into cellular phospholipids, in agreement...
with previous observations for other biological actions of ω3 FA.\(^1,2\)

Several lines of evidence point to the physiological relevance of the 30% to 60% inhibition by DHA of VCAM-1 and other cytokine-induced molecules reported here: (1) the concentrations of DHA required to inhibit cytokine-stimulated endothelial expression of leukocyte adhesion molecules lie well within the range of those achievable during in vivo administration of marine lipids with high content of ω3 FA, which increase plasma DHA concentration from the low micromolar range up to more than 100 μmol/L\(^3,4,9\) and even of the much lower plasma concentrations of DHA achievable by moderate fish intake\(^40\); (2) the observed inhibitory effect of DHA occurs independently of the stimulus used, indicating a general decrease of cell responsiveness to peptide mediators of endothelial activation; (3) DHA also blunts the production of soluble mediators produced by endothelial cells upon cytokine stimulation, providing a mechanism by which DHA interferes not only with leukocyte adhesion but also with other aspects of cytokine-induced endothelial cell inflammatory properties; and (4) DHA reduced adhesion of mononuclear cells to cytokine-stimulated endothelial cells in vitro, indicating a functional correlate of the observed reduction in adhesion molecule expression.

We addressed possible mechanisms by which DHA inhibits the endothelial expression of cytokine-induced molecules, using VCAM-1 expression as an index. It is unlikely that DHA acts by retroconversion to EPA, the direct precursors of eicosanoids of the ω3 series. Despite some retroconversion of DHA to EPA in DHA-treated HSVEC (upon incubation with 10 μmol/L DHA, EPA content increased from undetectable levels up to 2.1% at 72 hours), exogenous EPA did not limit cytokine-induced VCAM-1 expression. In addition, treatment of HSVEC with the cyclooxygenase inhibitors aspirin (100 μmol/L) or indomethacin (5 μmol/L), at concentrations that completely suppressed IL-1-induced production of prostacyclin, did not alter the effect of DHA. Therefore, contrary to the traditional explanation for many effects of ω3 FA,\(^41,42\) the action of DHA on endothelial cells, which do not possess a 5-lipoxygenase pathway, does not appear to depend on conversion to eicosanoids.

Oxidative products of DHA might mediate the effects described here. To address this issue we used cotreatment with two lipid-soluble antioxidants, vitamin E and BHT, in a strategy similar to the one previously used by Fox and DiCorleto\(^43\) to assess ω3 FA effects on endothelial production of platelet-derived growth factor (PDGF)-like molecules. Contrary to their findings for PDGF, we did not find a suppression of VCAM-1 expression upon treatment with antioxidants under similar experimental conditions. Furthermore, oleic acid also inhibited VCAM-1 expression (although to a lesser extent than DHA), despite its relative resistance to oxidation. Neither line of evidence, however, excludes the possibility that FA-specific oxidation products may contribute to the reported effects. Lack of direct measurement of the efficacy of these antioxidants under the experimental conditions used limits the pharmacological approach.

DHA may regulate the protein expression of cytokine-induced molecules at different levels. Interference with cytokine-receptor binding or the initial events of signal transduction seems unlikely, since DHA limited the effects of structurally unrelated agonists (various cytokines and LPS), which bind to distinct receptors and use different signal transduction pathways. In addition, the effect of DHA was quantitatively different for the various cytokine-induced molecules assayed, the effect on VCAM and E-selectin exceeding that on ICAM-1, IL-6, or IL-8. This suggests a differential regulation by DHA on the biosynthetic pathways of various proteins, which would be difficult to explain by a "membrane effect" or a general interference with cytokine signal transduction pathways. The decrease in mRNA levels specific for VCAM-1, consistent with data from measurements of protein expression, indicates a pretranslational DHA level of interference. Whether the observed effect is a decrease in mRNA stability or a decrease in transcription still needs to be addressed. Transcriptional regulation is, however, the only type of regulation for endothelial leukocyte adhesion molecules reported so far.\(^44\) An effect of DHA on cytokine-induced nuclear translocation of specific transcription factors (eg, nuclear factor-κB\(^44\)) is a likely possibility.

It is unclear why DHA but not EPA reduced cytokine-induced leukocyte adhesion molecule expression in endothelial cells. EPA alone (up to 25 μmol/L) did not modify IL-1-induced VCAM-1 expression, although this FA did modestly potentiate the effect of DHA (data not shown). EPA reduced IL-8 production stimulated by IL-1, although to a lesser extent than DHA. Several recent reports have described properties of DHA not shared by EPA,\(^45-51\) including a preferential (DHA versus EPA) release by phospholipase A\(_2\) from phosphatidylethanolamine, where most DHA usually accumulates.\(^52\) C-DHA radiolabeling of HSVEC, followed by thin-layer chromatographic separation of the various phospholipid classes, confirmed, in our hands, a preferential accumulation of DHA in phosphatidylethanolamine (data not shown).

Taken together, our results thus provide a novel mechanism by which quantitatively minor dietary lipid components may influence cell physiology and pathogenesis of certain inflammatory diseases, including atherosclerosis.

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The omega-3 fatty acid docosahexaenoate reduces cytokine-induced expression of proatherogenic and proinflammatory proteins in human endothelial cells.
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