Structural requirements for inhibition of cytokine-induced endothelial activation by unsaturated fatty acids[1]

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Abstract Dietary long-chain fatty acids (FA) may influence pathological processes involving endothelial activation, including inflammation and atherosclerosis. We have previously shown that the n–3 FA docosahexaenoate (DHA) inhibits endothelial activation in the range of nutritionally achievable plasma concentrations. The present study assessed structural determinants for this effect. Saturated, monounsaturated, and n–6 and n–3 polyunsaturated FA were incubated with cultured endothelial cells for 24–72 h alone, and then in the presence of interleukin-1, tumor necrosis factor, or bacterial lipopolysaccharide for an additional 24 h before assessing the expression of the vascular cell adhesion molecule-1 (VCAM-1) or other products of endothelial activation. No FA tested per se elicited endothelial activation. While saturated FA did not inhibit cytokine-induced expression of adhesion molecules, a progressively increasing inhibitory activity was observed, for the same chain length, with an increase in double bonds. Comparison of FA with the same length and number of unsaturation and only differing for the double bond position or for the *cis*/*trans* configuration indicated no difference in inhibitory potency, indicating no effect of the double bond position or configuration. As judged by Northern analysis, these latter FA also inhibited VCAM-1 messenger RNA steady state levels to the same extent, indicating a pre-translational site of action attributable to the single double bond. Thus the double bond is the minimum necessary and sufficient requirement for FA inhibition of endothelial activation. These properties are likely relevant to the anti-atherogenic and anti-inflammatory properties ascribed to n–3 FA, which are able to accommodate the highest number of double bonds in a fatty acid of given chain length.—De Caterina, R., W. Bernini, M. A. Carluccio, J. K. Liao, and P. Libby. **Structural requirements for inhibition of cytokine-induced endothelial activation by unsaturated fatty acids. *J. Lipid Res.* 1998.** 39: **1062–1070.**

Supplementary key words leukocyte adhesion molecules • VCAM-1 • E-selectin • cytokines • n–3 fatty acids • omega–3 fatty acids • double bonds • atherosclerosis • inflammation

The amount and type of fatty acids (FA) in the diet influence the risk of human immune and inflammatory diseases, including atherosclerosis (1, 2). For example, a dietary shift from low to high intake of n–3 (omega–3) FA may induce clinical improvement in rheumatoid arthritis, inflammatory bowel disease, psoriasis, and atherosclerosis (2). The low prevalence of such diseases in populations consuming high amounts of fish products supports this concept (3–7). However, understanding mechanisms by which FA modulate diseases remains elusive. For years, the differential action of n–3 versus n–6 FA has been attributed to their specific enzymatic metabolism to eicosanoids, yielding different bioactive products within the prostaglandin, leukotriene, lipoxin, and hydroxy-acid families (8, 9). However a number of recent observations have suggested alternative antiinflammatory actions of certain FA, including modulation of gene expression. Examples include the reduction of hepatic synthesis of very low density lipoproteins (10), and the reduction of interleukin-1, tumor necrosis factor (11) and platelet-derived growth factor B-chain expression (12) in endotoxin-activated macrophages by n–3 FA.

In keeping with these observations, we have shown that nutritionally relevant concentrations of n–3 FA (low micromolar) modulate cytokine-induced expression of endothelial leukocyte adhesion molecules and soluble pro-inflammatory proteins (13, 14). This effect involves a prostaglandin-independent modulation of steady-state messenger RNA (mRNA) for adhesion molecules and reduced white blood cell adhesion to cytokine-activated endothelium (13, 14). These newly recognized properties of n–3 FA may be a mechanism by which their consump-

Abbreviations: FA, fatty acids; DHA, docosahexaenoic acid, docosahexaenoate; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; mRNA, messenger ribonucleic acid; HSVEC, human saphenous vein endothelial cells; HUVEC, human umbilical vein endothelial cells; HEPES, N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid]; IL, interleukin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; EIA, enzyme-immunoassay; ANOVA, analysis of variance.

[1]Presented in abstract form at the 69th Scientific Session of the American Heart Association, Dallas, TX, November 1996 (*Circulation.* 1996. **94**(Suppl.I): I–102).

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tion reduces acute inflammation and atherosclerosis, processes that share increased leukocyte adhesion to vascular endothelium as an early pathogenic event (15). An important and unexpected finding in our previous study was a differential effect of the longest chain n–3 FA docosahexaenoate (22:6 n–3) relative to the shorter and less unsaturated eicosapentaenoate (20:5 n–3), and some activity of the monounsaturate oleate (18:1 n–9) on cytokine-induced endothelial activation (13, 14). To further explain the difference in activity of such compounds and to gain further insight into the mechanism of their effects, we have compared several long-chain FA, differing in chain length and number and position of unsaturation, to determine the structure–activity relationship of reduced cytokine-induced expression of endothelial leukocyte adhesion molecules. We have also tested whether FA may themselves activate endothelium, a possibility raised in some preliminary studies (16).

METHODS

Endothelial cell culture

Human saphenous vein endothelial cells (HSVEC) were harvested from unused portions of saphenous veins obtained for coronary bypass surgery, by enzymatic digestion with 0.1% type II collagenase, as previously described (17), and maintained in medium 199 (Gibco BRL, Life Technologies Inc. Grand Island, NY) containing N-[2-hydroxyethyl]piperazine-N′-[2-ethanesulfonic acid] (HEPES, 25 mol/L), heparin, endothelial cell growth factor (50 μg/mL), l-glutamine, antibiotics, and 5% fetal calf serum. Cells were plated on low-pyrogen fibronectin (1.5 $\mu g/cm^2$) or gelatin at 20,000 $cells/cm^2$. HSVEC so isolated form a confluent monolayer of polygonal cells and were characterized by morphologic criteria, by their constitutive expression of von Willebrand factor immunoreactive protein, and by their cytokine-induced expression of E-selectin (17). Cell density at the beginning of incubation experiments was chosen in order to reach confluency 72 h later, i.e., at the time of assessment of adhesion molecule expression. Control experiments were also performed on human umbilical vein endothelial cells (HUVEC)(obtained through Dr. Michael A. Gimbrone, Jr., Vascular Research Division, Brigham and Women's Hospital, Boston, MA). Cells were grown in 96-, 12-, or 6-well flat-bottom cluster plates for the assessment of adhesion molecule expression by enzyme-immunoassays, or flow cytometry, or for control of FA toxicity, respectively.

Reagents

FA used in this study included all the most common saturated, mono- and polyunsaturated FA present in cell membranes, as well as some rarer FA used to clarify structure–activity relationships. They were all obtained as >99% pure sodium salts (soaps) from Nu Chek Prep, Inc., Elysian, MN. Eicosapentaenoic acid (EPA) obtained as free acid from Sigma and docosahexaenoic acid from Aldrich Chemicals and Calbiochem-Behring yielded identical results. Saturated fatty acids included C16:0 (palmitic), C18:0 (stearic) and C20:0 (arachidic). Monounsaturated FA included C16:1 Δ9*cis* (palmitoleic), C18:1 Δ9*cis* (oleic), C18:1 Δ9*trans* (elaidic) and C18:1 Δ9*cis*-12-OH (ricinoleic). n–6 Polyunsaturated comprised C18:2 Δ9*cis*-12*cis* (linoleic), C18:3 Δ6*cis*-9*cis*-12*cis* (gamma-linolenic), C20:3 Δ8*cis*-11*cis*-14*cis* (homo-gamma-linolenic), and C20:4 Δ5*cis*-8*cis*-11*cis*-14*cis* (arachidonic). n–3 Polyunsaturated fatty acids comprised C18:3 Δ9*cis*-12*cis*-15*cis* (alpha-linolenic), C20:5 Δ5*cis*-8*cis*-11*cis*-14*cis*-17*cis* (eicosapentaenoic, EPA), C22:5 Δ7,10,13,16,19 all *cis* (docosapentaenoic, DPA), and C22:6 Δ4,7,10,13,16,19 all *cis* (docosahexaenoic acid, DHA). Sodium salts were dissolved in water and then in tissue culture medium, divided into aliquots under a nitrogen stream, and maintained at −80°C until used. Free acids were dissolved first in a small volume of 99% ethanol and then in medium. In this case, control incubations contained equal amounts of the solvent. Tissue culture medium in which FA were dissolved always contained 5% serum.

The following human recombinant cytokines were used: interleukin (IL) −1α (a gift from Hoffmann-La Roche, Nutley, NJ, at 0.1–10 ng/mL, and IL-1β (1–10 ng/mL), tumor-necrosis factor (TNF)-α (1–10 ng/mL), from Genzyme. Bacterial lipopolysaccharide (LPS) from *Escherichia coli* (0.01–10 μg/mL) was purchased from Sigma.

Controls for toxicity

To evaluate possible cytotoxic effects of treatments, we monitored cell count, Trypan blue exclusion, and [^{3}H]leucine incorporation into total cell-associated and released proteins. For this last assessment, we performed trichloroacetic acid precipitation of total cell extracts and cell supernates to assess protein synthesis, as previously described (18).

Detection of cell surface molecules

Assay of cell surface molecules used cell surface enzyme immunoassay (EIA) with mouse anti-human monoclonal antibodies (as hybridoma supernatants) against VCAM-1 (Ab E1/6) and E-selectin (Ab H18/7). Monolayers were first incubated 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). Cell layers were washed thrice between each incubation step, and their integrity was monitored by phase-contrast microscopy. The surface expression of each protein was quantified spectrophotometrically (at 410 nm wavelength) 15–60 min after the addition of the chromogenic substrate *p*-nitro-phenylphosphate (Sigma) in the presence of levamisole as an inhibitor of cellular phosphatases, as previously described (13, 19). Characterization of the effect of DHA on the surface expression of adhesion molecules was also performed by flow cytometry, as previously described (13, 20).

Isolation of RNA and Northern analysis

Total cellular RNA was isolated by a single extraction using an acid guanidinium thiocyanate–phenol–chloroform method. RNA concentration and quality were determined from the A260 and A260/A280 ratios, respectively. RNA quality was confirmed by gel electrophoresis prior to Northern analysis. For this, 20 μg of cellular RNA was applied to each lane, separated on a 1% agarose–formaldehyde gel, transferred to a nylon membrane (Amersham Hybond-N, Arlington Heights, IL) and immobilized by shortwave UV illumination. The membranes were prehybridized for at least 2 h, before hybridization, with a ^{32}P-labeled DNA probe for VCAM-1, labeled by random hexanucleotide priming (Pharmacia, Piscataway, NJ) to specific activities $>10^8$ cpm/μg DNA, and autoradiographed. Details of these methods have been already described (13).

Quantitation of densities of autoradiographic bands for Northern hybridization was performed with the aid of the National Institutes of Health "Image" software on a MacIntosh Quadra 800 computer (Apple, Cupertino, CA).

Experimental designs

To test for a possible stimulatory effect of FA on adhesion molecule expression, FA were incubated for 24–72 h with HSVEC or

HUVEC. Positive controls for adhesion molecule expression included a 12–24 h stimulation with IL-1α, IL-1β, TNFα, or LPS. Based on our previous results, showing the time-dependent increase of the inhibitory effect of DHA on cytokine-induced adhesion molecule expression, the experimental design in this set of experiments included incubation of FA with HSVEC or HUVEC for 48 h before addition of cytokine or LPS. After an additional 16–24 h, surface expression of adhesion molecules was assessed.

Statistics

Each EIA included 8–24 replicates for each condition, and each experiment was replicated a minimum of 3 times. Multiple comparisons were performed by one-way analysis of variance (ANOVA) and individual differences were tested by the Fisher's protected least significant difference test after demonstration of significant intergroup differences by ANOVA. Such analyses were performed with the aid of the Statview II statistical package (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Toxicity studies

None of the FA tested exhibited significant toxicity (cell count and Trypan blue exclusion) when used at concentrations up to 25 μm for periods as long as 72 h before cytokine stimulation. More detailed toxicity studies were performed for oleic, ricinoleic, arachidonic, eicosapentaenoic, or docosahexaenoic acids, showing similar toxicity becoming apparent (>15% decline in cell count and protein synthesis) at concentrations >100 μm.

Unsaturation appears required for inhibition of cytokine-stimulated adhesion molecule expression by FA

None of the long-chain saturated FA studied here inhibited cytokine-induced endothelial adhesion molecule expression. Unsaturated FA did inhibit adhesion molecule expression. This was already apparent with monounsaturates (oleic, ricinoleic, palmitoleic acids) with minimum effective concentrations of 25–50 μm (**Fig. 1**).

DHA inhibits cytokine-stimulated adhesion molecule expression to a greater extent than other FA tested

Under all experimental conditions tested (different incubation times, different concentrations, different types and concentrations of the stimulating cytokines), DHA, the longest and the most unsaturated FA used in our comparison, inhibited endothelial activation most potently (**Fig. 2**, upper panel). Optimum conditions for producing this effect, assessed by EIA and flow cytometry, included a long pre-incubation time (48–72 h before the addition of the stimulating cytokine) and the use of sub-maximal concentrations of cytokines. Under these circumstances, DHA exerted inhibition at concentrations as low as 1–5 μm, and for all cytokines tested, as well as for LPS (Fig. 2, lower panel). DHA also inhibited cytokine-stimulated E-selectin surface expression (by cell surface EIA) to an extent paralleling that of VCAM-1 under all circumstances tested (different incubation times and cytokines) to an extent averaging 20% less than for VCAM-1 (not shown). Pre-incubation of fatty acids with endothelial cells was always required for the effects described. No effect was apparent when FA were co-administered with the stimulating cytokines or LPS. For DHA (13) or oleic acid, for which extensive incorporation data were available, the effect increased in parallel with increased incorporation, suggesting incorporation as a requirement for FA action. To support this hypothesis, experiments were also performed by performing cytokine stimulation after the FA pre-incubation in the absence of FA, removed by washing with serum-containing medium at the time of cytokine administration. Under these circumstances, in which increased incorporation continued to be documented, FA still inhibited endothelial activation, ruling out the necessity of the actual pres-

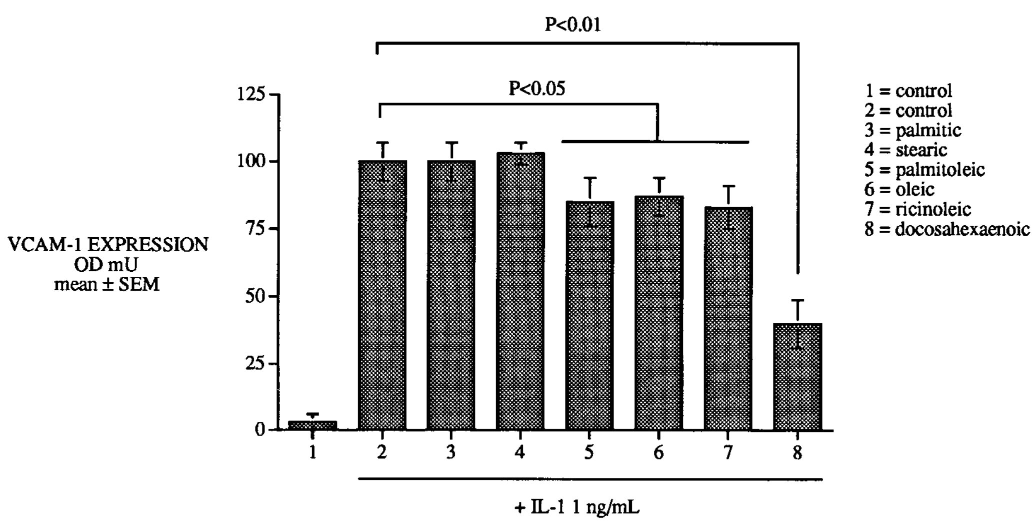

Fig. 1. Various unsaturated, but not saturated FA, modulate IL-1α-stimulated VCAM-1 expression by HSVEC. FA (sodium salts) were incubated at 25 μm for 72 h with HSVEC, followed by 16 h stimulation with IL-1α, in the continous presence of FA. At the end of this incubation, VCAM-1 expression was assessed by a cell surface EIA. Saturated FA (palmitate and stearate) did not affect stimulated VCAM-1 expression. Monounsaturated FA produced significant, although modest, inhibition. DHA inhibited VCAM-1 expression most potently. The graph shows one of six similar experiments, yielding similar results. For each column, n = 8.

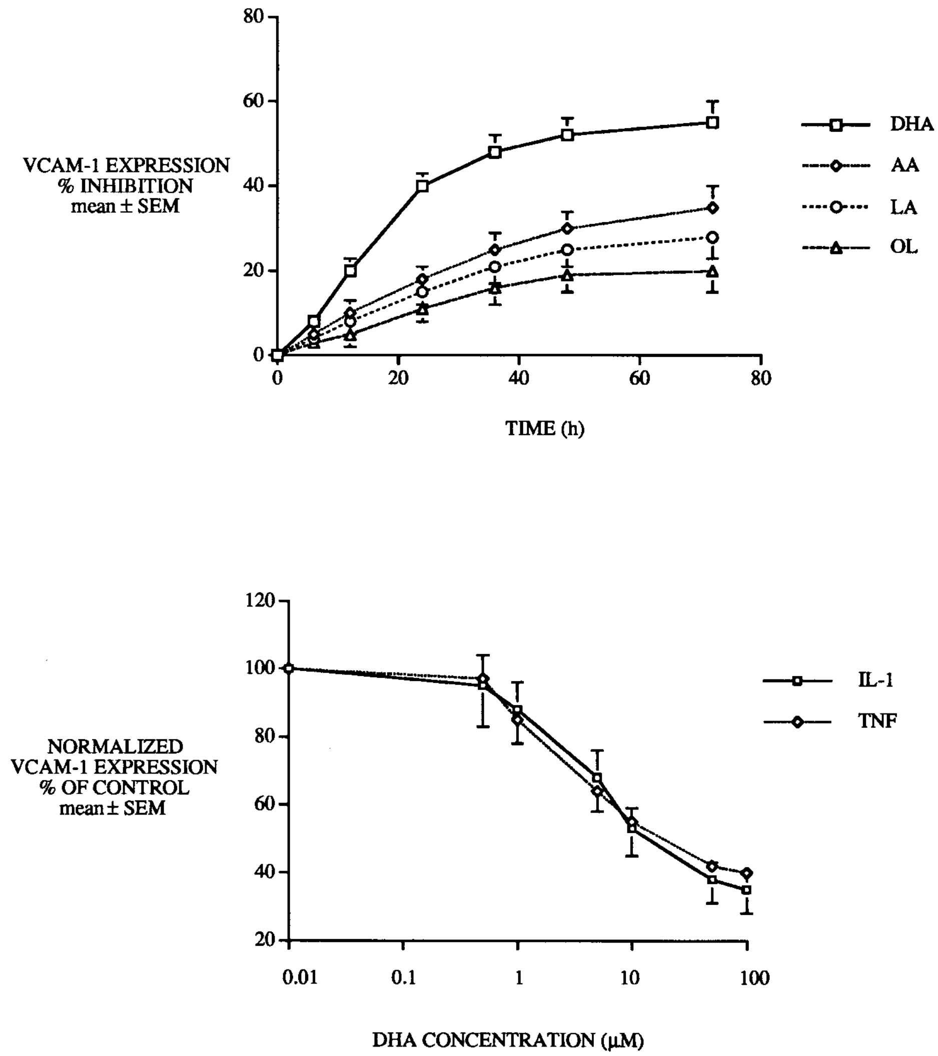

Fig. 2. Time-dependence and stimulus-independence of the inhibition of stimulated VCAM-1 expression in HSVEC by FA. The upper panel shows the time-dependency of the effect of unsaturated FA on VCAM-1 expression. FA (DHA, docosahexaenoate, AA, arachidonate, LA, linoleate, and OL, oleate) were all used at 50 μm for different incubation times (on the abscissa). At the end of the incubation time, IL-1α (1 ng/mL) was added, still in the presence of the FA, for a further 12 h. The lower panel shows concentration–response curves of VCAM-1 expression in HSVEC, normalized as % or response to stimulus alone (on the ordinate), as a function of DHA concentration, added for 72 h (on the abscissa, in logarithmic scale) in response to IL-1α or TNFα, both at 1 ng/mL. Note the similarity of the two concentration–response curves. Experiments using IL-1β, IL-4, or LPS as a stimulus yielded like results and similar degrees of inhibition at different pre-incubation times, indicating that inhibition does not depend, at any time, on the cytokine used to induce endothelial activation. Also, a qualitatively similar pattern of inhibition was seen with regard to another inducible adhesion molecule, E-selectin (data not shown).

ence of FA at the time of adhesion molecule expression for the inhibition of their expression.

Inhibition of cytokine-stimulated adhesion molecule expression progressively increases with the number of unsaturations and does not depend on chain length

Our present findings, as well as previous ones of ours, establish differences in potency between FA, even within the categories of n–3 and n–6 FA, with regard to the inhibition of cytokine-induced endothelial activation. We therefore investigated structural determinants for this difference in potency by comparing FA of the same chain length but different degree of unsaturation, as well as, conversely, FA with the same number of double bonds, but different chain lengths. The degree of unsaturation, but not chain length per se, appeared to influence inhibition of cytokine-induced endothelial activation (**Fig. 3**). The position (n–3 vs. n–6) of the last double bond relative to the methyl end of the FA carbon chain also did not change FA potency when variables such as chain length and number of unsaturation were kept equal (**Fig. 4**).

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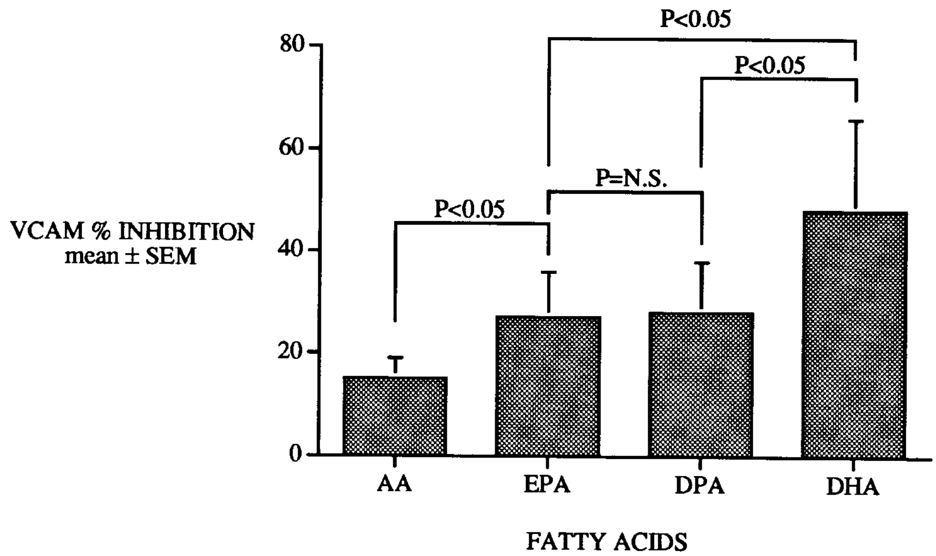

Fig. 3. A comparison of the inhibitory effects of arachidonic (AA), eicosapentaenoic (EPA), docosapentaenoic (DPA), and docosahexaenoic (DHA) acids (all at 25 μm) on LPS-induced (100 ng/mL, 16 h) VCAM-1 activation in HSVEC. AA and EPA have identical chain lengths and differ by the presence of one additional double bond in the n–3 position in the case of EPA. DPA, the elongation product of EPA, has the same number of double bonds (5) as EPA, but 2 extra carbon atoms. DHA has an additional double bond. Note the significantly greater activity of EPA versus AA and of DHA versus DPA, but the equal potency of EPA and DPA, suggesting a progressive increase in potency with the increase in number of double bonds. Each column represents ≥16 replicates; data are representative of three similar experiments yielding similar results. Similar results were also obtained using IL-1α as a stimulus.

The *cis/trans* configuration of the double bond does not influence FA potency in inhibiting cytokine-induced endothelial activation

A comparison of two monounsaturates only differing for the *cis/trans* configuration of the double bond, oleic and elaidic acids, is shown in **Fig. 5**. The two FA exhibited identical potency in inhibiting cytokine-induced expression of VCAM-1.

A single double bond, independently from the *cis/trans* configuration, is able to inhibit VCAM-1 mRNA steady state concentrations

A comparison of the autoradiographic bands for VCAM-1 mRNA levels obtained at Northern analysis from endothelial cells pre-treated with either oleic acid or elaidic acid and then stimulated with LPS is shown in **Fig. 6**. Treatment with these monounsaturates was accompa-

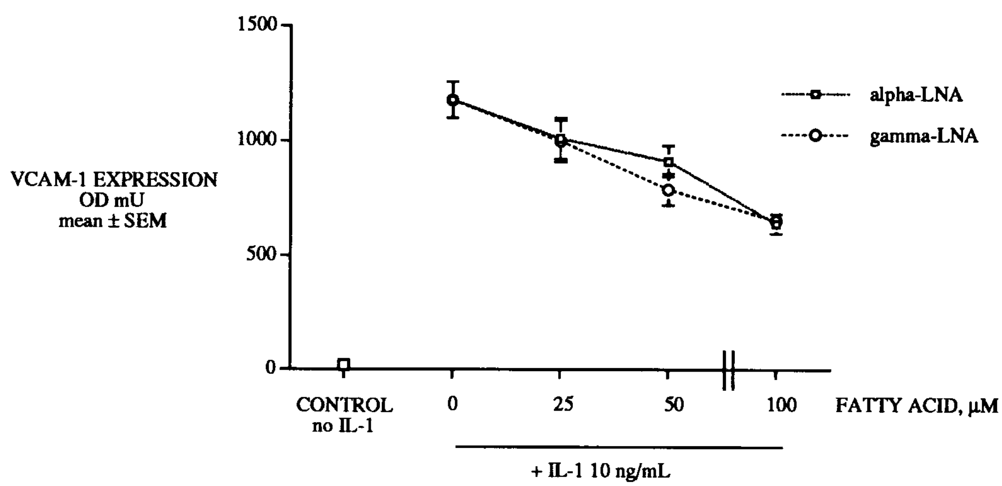

Fig. 4. The n–3/n–6 position of the last double bond does not affect endothelial activation. The figure shows a comparison of concentration–response curves of VCAM-1 expression (on the ordinate) as a function of increasing concentrations of the polyunsaturated FA alpha-linolenic and gamma-linolenic acids (on the abscissa), with a 72-h pre-incubation time. These two FA share the same chain length (18 carbon atoms) and number of unsaturations (3), but only differ in the position of the double bonds, the former being an n–3 FA, the latter being an n–6 FA. Note the virtual superimposition of the two curves indicating equal potency in this assay.

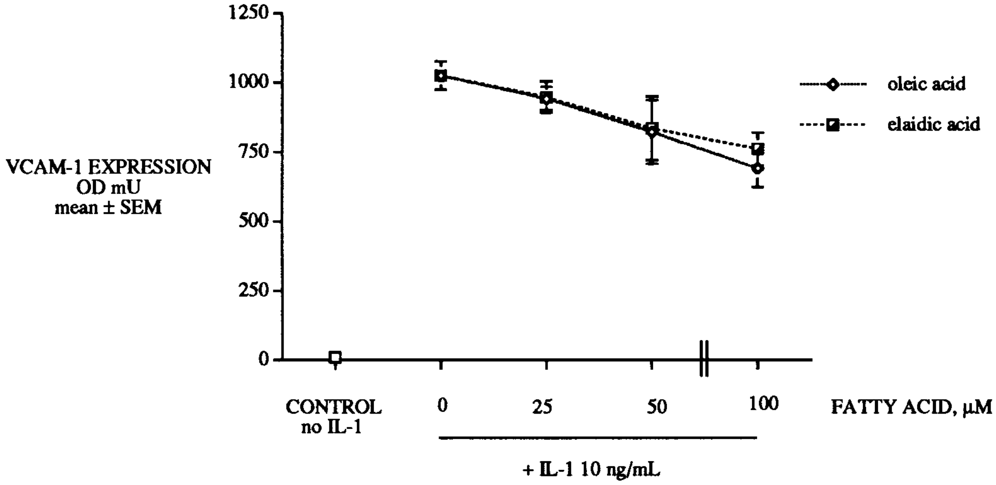

Fig. 5. The *cis/trans* configuration of the double bond does not affect endothelial activation. Concentration–response curves of VCAM-1 expression (on the ordinate) as a function of increasing concentrations of the polyunsaturated FA oleic and elaidic acids, with a 72-h pre-incubation time, (on the abscissa) are compared. These two FA share the same chain length (18 carbon atoms), number of double bonds (1), and position of the double bond (n–9), but only differ in the configuration of the double bond, oleic being *cis*, elaidic being *trans*. Note the virtual superimposition of the two curves indicating equal potency in this assay.

nied by an unequivocal reduction in VCAM-1 mRNA levels, in the experimental conditions described, indicating a pre-translational effect of the FA used and the sufficiency of a single double bond to obtain such an effect.

FA do not per se stimulate endothelial adhesion molecule expression

We tested the accessory hypothesis that FA, particularly polyunsaturated FA, may induce endothelial activation in the absence of cytokines. Experimental conditions included variations of incubation periods before detection of adhesion molecule surface expression (from 0 to 72 h) and of FA concentrations (from 10 up to 100 µm). No FA per se, under our experimental conditions, induced adhesion molecule expression, as assessed by EIA (**Table 1**).

DISCUSSION

We recently reported one mechanism by which changes in lipid membrane composition by enrichment of n–3 FA may influence atherogenesis and inflammation. In inflammation in general, as well as the special case of atherogenesis, endothelial cells express inducible leukocyte adhesion molecules (15, 21–23). We found that DHA reduces by more than 50% the endothelial responses to certain cytokines or LPS, including expression of adhesion molecules as varied as VCAM-1, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and soluble mediators of inflammation elicited after endothelial activation, such as IL-6 and IL-8 (13, 14). Surprisingly, DHA, which has a longer chain length and greater unsaturation than EPA,

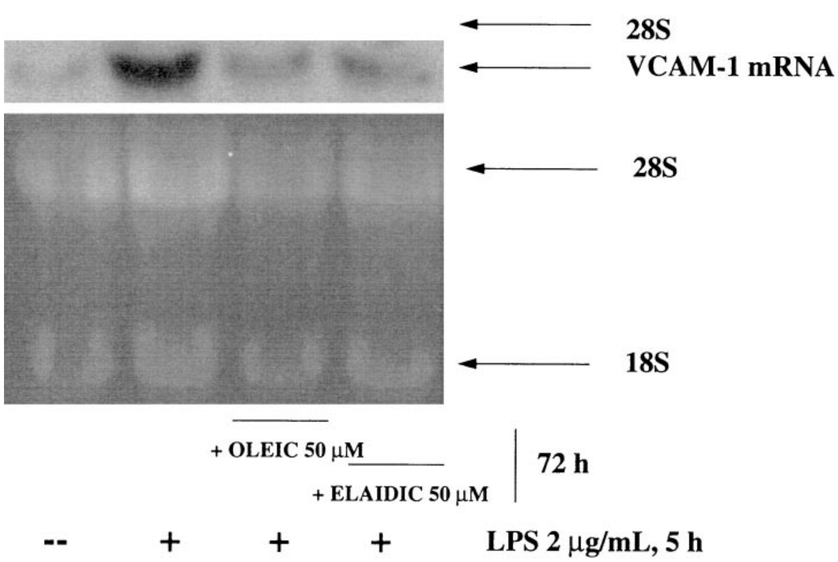

Fig. 6. The monounsaturated FA oleic and elaidic acid decrease VCAM-1 mRNA steady state levels. Northern analysis of VCAM-1 mRNA (upper panel) and ethidium bromide staining for total RNA (lower panel) at time 0 (left band), 6 h after LPS (2 µg/mL)-stimulation (middle band) and 6 h after LPS stimulation preceded by incubation with either oleic or elaidic acid (50 µm for 72 h) (right bands). Treatments with oleic and elaidic acids are associated with a quantitatively similar >50% reduction of VCAM-1 mRNA at densitometric analysis. This blot is representative of a series of three similar ones run in similar conditions and with different time points after LPS or IL-1 stimulation. Parallel experiments showed no effect of saturated FA in the same experimental conditions.

TABLE 1. The effects of FA alone (in the absence of cytokine stimulation) on VCAM-1 expression by human endothelial cells

Experimental Conditions	VCAM-1 expression
	OD mU, mean ± SEM
Control, no primary antibody	5 ± 2
Control, no stimulation	10 ± 4
Palmitate, 25 μm	12 ± 5
Stearate, 25 μm	8 ± 5
Oleate, 25 μm	9 ± 4
Palmitoleate, 25 μm	10 ± 5
Ricinoleate, 25 μm	9 ± 3
Elaidate, 25 μm	9 ± 4
Linoleate, 25 μm	13 ± 5
α-Linolenate, 25 μm	11 ± 5
γ-Linolenate, 25 μm	11 ± 5
Arachidonate, 25 μm	10 ± 6
Eicosapentaenoate, 25 μm	11 ± 3
Docosapentaenoate, 25 μm	10 ± 4
Docosahexaenoate, 25 μm	9 ± 4
IL-1α, 0.1 ng/mL	85 ± 15[a]
IL-1α, 1 ng/mL	400 ± 28[a]

FA were incubated with endothelial cell monolayers for 24 h at concentrations reported. OD mU = milliunits of optical absorbance. Results similar to FA effects were obtained with incubation times of 2, 6, 12, 48, and 72 h and with FA concentrations of 1, 5, 10, 50, and 100 μm.

[a] $P < 0.05$ vs. control.

consistently exhibited greater potency than the similar n–3 FA EPA (13, 14). Previous studies have established that such effects of DHA resulted from modulation of gene expression for endothelial leukocyte adhesion molecules, as it was accompanied by a parallel and quantitatively similar reduction of adhesion molecule messenger RNA steady state levels (13, 14). The present study therefore investigated the structural determinants of this effect. We used VCAM-1 expression as the most sensitive marker of FA effect, as inhibition of VCAM-1, under comparable conditions, exceeded that of E-selectin, IL-6, IL-8, or ICAM-1. We chose surface EIA for these experiments, because of its reproducibility (<5% coefficient of variation in multiple replicates), its sensitivity (ability to detect a 10% reduction in surface molecule expression, greater, in our hands, than that achievable by flow cytometry), and its practicability, allowing ready processing of multiple replicates. Information from Northern analysis complemented, in selected cases, information derived from the assessment of surface protein expression.

Optimal conditions to achieve inhibitory effects of FA included a relatively long incubation time (48–72 h) before the addition of the stimulating cytokine, and the use of submaximal concentrations of stimulating cytokines. The prolonged incubation time permits the incorporation of FA into cell membranes, as previously shown. Indeed we found that incorporation of DHA or AA reaches a plateau at about 72 h of incubation at 25 μm FA concentrations (13, 14). These data indicate that the effect of FA on the expression of adhesion molecule was not acute (unlike the inhibition of ion channels likely related to the antiarrhythmic potential of these substances (24–26)), but likely requires the incorporation in specific FA pools, as previously measured for DHA. For this FA we previously documented increased incorporation in total cell lipids, but also a preferential incorporation in the phospatidylethanolamine FA pool (14), from which a selective hydrolysis by phospholipase A_2 had been reported. We now confirm and extend data of time and concentration requirements to other unsaturated FA. We also describe here that the actual presence of FA at the time of cytokine or LPS stimulation is not required for the inhibition of adhesion molecule expression provided that pre-incubation with FA is performed and increased incorporation occurs. These data strongly suggest that altered FA membrane composition is required for the modulation of gene expression for adhesion molecules.

Under conditions optimized to explore FA effects on endothelial activation, we analyzed structural determinants for this action. We consistently found no effect of saturated FA on endothelial activation, while all unsaturated FA exhibited some activity. Oleic acid, the most abundant monounsaturated FA in the diet and cell membrane phospholipids, exhibited a clear, albeit quantitatively modest, inhibition. A similar degree of VCAM-1 inhibition was seen for other monounsaturates, suggesting that the inhibitory activity depends on the presence of the double bond rather than other structural features of the FA, such as chain length (palmitoleic acid), accessory substitution (such as the hydroxy-group present in ricinoleic acid), or the *trans* configuration of the double bond (elaidic acid). Other FA with higher degrees of unsaturation, belonging to the n–6 or n–3 families, produced larger effects. Parallel concentration–response curves were constructed for different FA. At the same incubation time and concentration, the FA with more double bonds (DHA) always ranked first in order of potency, accounting for the partial specificity of the effect previously attributed to DHA by us (13, 14), as well as, subsequently, by others (27). Experiments using DPA, a quantitatively rare polyunsaturate produced by chain elongation of EPA and the substrate for the final unsaturation leading to DHA, showed that even for polyunsaturates, and within the n–3 family, the presence of the extra double bond in DHA fully accounts for its greater activity. Thus, the longer carbon chain necessary to accomodate the extra double bond in DHA versus EPA is not per se responsible for any change in activity. Furthermore, the similar activity exhibited by gamma- and alpha-linolenic acids, two FA with the same chain length (18 carbon atoms) and the same number (three) of *cis* double bonds, only differing for the positions of the double bonds (the former being an n–6, the latter an n–3 FA), argues against any special property of the last (n–3) double bond in relation to the effect described.

Results obtained at Northern analysis with the simplest unsaturated FA used, oleic and elaidic acids, both containing only one double bond and only differing for the *cis*/*trans* configuration, parallel results obtained with the assessment of surface protein and indicate that the effect of the double bond occurs before the translation of the mRNA into protein. These results are noteworthy because they rule out a possible post-translational effect of FA, as there could be, for instance, in the transport of the protein from the sarcoplasmic reticulum to plasma membrane, where the protein can exert functions on monocyte adhesion and be detected by functional or immunological assays.

We finally show here no intrinsic stimulatory activity of VCAM-1 expression by any FA tested over a variety of incubation times. Under these experimental conditions, we did not observe the stimulation of VCAM-1 expression in endothelial cells by linoleate, as recently reported (16). Some spontaneous or enzymatic biotransformation product of linoleate could account for the results obtained by others (16).

This study focuses attention on double bonds in FA as a key structural feature for modulation of endothelial activation in response to cytokines and indicates a pre-translational site of action, but still does not clarify the molecular mechanism by which the double bond in unsaturated fatty acids exerts such an effect. Current research is therefore focusing on further identifying the site of action by which these compounds eventually modulate gene expression for cytokine-induced adhesion molecules. Further restriction of potential sites of action will require the assessment of transcriptional versus post-transcriptional regulation of VCAM-1 gene-expression and, in the case of transcriptional regulation, the most likely for genes involved in endothelial activation (27), a scrutiny of potential sites of action of FA in the cytokine signal cascade. The notion that the effects here described are independent of the cytokine used for endothelial activation supports the idea that such effects are independent from interference with specific receptors. An interference of the double bond containing FA with some potentially reactive intermediate in the cytokine signal transduction cascade, such as superoxide anion or hydrogen peroxide, appears likely, but awaits verification by specifically designed experiments. The idea that these effects, although mediated through changes in membrane fatty acid composition, are not due to general alterations of physico-chemical properties of plasma membrane such as membrane fluidity is supported by the notion that these last, at variance from the presently described effects, are almost exclusively produced by changes in *cis* polyunsaturated fatty acids (28).

The present structure–activity studies provide a framework for understanding a variety of biological effects of FA. From the perspective of atherosclerosis, diets rich in monounsaturated FA have been associated with protection from coronary artery disease (29, 30). An increase in the relative amounts of monounsaturated FA (i.e. oleate) could inhibit atherosclerosis by taking the place of saturated FA that do not limit endothelial activation. Polyunsaturated FA have a greater effect, but the net balance of their increased proportion in cell membrane phospholipids could result both from their direct effects on endothelial activation and their generation of bioactive eicosanoids, usually regarded as pro-inflammatory (8, 9). N–3 FA have a larger effect for a given chain length, as they can accommodate more double bonds, and also are poor substrates for cycloxygenases and lipoxygenases (31). DHA, among the various polyunsaturated FA, appears to be the most potent inhibitor of endothelial activation. The effects of polyunsaturated FA described above might also contribute to their putative anti-inflammatory effects (2). Finally, focusing the attention on the double bonds as the key structural requirement for the effect of unsaturated FA on endothelial activation will probably broaden our understanding of the complex relationship between lipid peroxidation and its biological effect. Lipid peroxidation, which is a function of the double bonds present in a FA, is usually regarded at present as a detrimental feature in the context of inflammation, atherosclerosis, and aging (32–34). However, the presence of double bonds may confer some protection from more direct adverse effects of free reactive oxygen species. Current ongoing research is expected to further clarify these issues.

The present work was accomplished through an ongoing collaboration between the Laboratory of Thrombosis and Vascular Research at C.N.R. Institute of Clinical Physiology in Pisa and the Vascular Medicine and Atherosclerosis Unit, Brigham and Women's Hospital, Boston, MA, fostered through travel grants by the Italian National Research Council (C.N.R.) (to Dr. De Caterina). This work was also possible thanks to contributions from NIH grants HL-48743 (to Dr. Libby) and HL-52233 (to Dr. Liao). The authors thank Dr. Michael A. Gimbrone, Jr. and Mr. William F. Atkinson, at the Vascular Research Division, Brigham and Women's Hospital in Boston, for providing the hybridomas for the experiments described, to Prof. Uberto Bortolotti, at the Division of Cardiac Surgery at Pisa University Hospital for collaboration in the setting up of tissue culture conditions in Pisa, to Mr. Guido Lazzerini, Dr. Giuseppina Basta and Mr. Claudio Lami, at the Laboratory for Thrombosis and Vascular Research-CNR Institute of Clinical Physiology in Pisa for invaluable technical help.

Manuscript received 2 June 1997 and in revised form 3 November 1997.

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