Fatty acid modulation of endothelial activation\textsuperscript{1–3}

Raffaele De Caterina, James K Liao, and Peter Libby

ABSTRACT  Dietary balance of long-chain fatty acids may influence processes involving leukocyte-endothelial interactions, such as atherogenesis and inflammation, that involve increased endothelial expression of leukocyte adhesion molecules, or endothelial activation. We compared the ability of various saturated, monounsaturated, and polyunsaturated fatty acids to modulate endothelial activation. Consumption of the n–3 fatty acid docosahexaenoic acid (DHA; 22:6n–3) reduced endothelial expression of vascular cell adhesion molecule 1 (VCAM-1), E-selectin, intercellular adhesion molecule 1 (ICAM-1), interleukin 6 (IL-6), and IL-8 in response to IL-1, IL-4, tumor necrosis factor, or bacterial endotoxin, with a half-maximal inhibitory concentration (IC\textsubscript{50}) of 1–25 μmol, ie, in the range of nutritionally achievable plasma concentrations. The magnitude of this effect paralleled its incorporation into cellular phospholipids. DHA also reduced the adhesion of human monocytes and monocyte U937 cells to cytokine-stimulated endothelial cells. These effects were accompanied by a reduction in VCAM-1 messenger RNA, indicating a pretranslational effect. To assess structural fatty acid determinants of VCAM-1 inhibitory activity, we compared various saturated, monounsaturated, and n–6 and n–3 polyunsaturated fatty acids for their VCAM-1 inhibitory activity. Saturated fatty acids did not inhibit cytokine-induced expression of adhesion molecules. However, a progressive increase in inhibitory activity was observed with dietary intake of fatty acids with the same chain length but increasing double bonds, ie, from monounsaturated to n–6 and, further, to n–3 fatty acids. Thus, the greater number of double bonds seems critical for the greater activity of n–3 compared with n–6 fatty acids in inhibiting endothelial activation. These properties are likely to be relevant to the antiatherogenic and antiinflammatory properties of n–3 fatty acids.  \textit{Am J Clin Nutr} 2000;71(suppl):213S–23S.

KEY WORDS  Long-chain fatty acids, atherogenesis, inflammation, endothelium, leukocytes, monocytes, adhesion molecules

INTRODUCTION  Highly unsaturated fatty acids, and n–3 fatty acids in particular, are receiving increasing attention as potential antiatherogenic and antiinflammatory agents. Atherosclerosis and inflammation share similar mechanisms in their early phases, involving increased interactions between vascular endothelia and circulating leukocytes. It was logical, therefore, to investigate a role for fatty acids in the modulation of such interactions. This line of research is leading to a new understanding of the mechanism of action of these nutrients. In this article we will first summarize the biological concepts of the pathogenesis of atherosclerosis. We will then review major findings as to the role of fatty acids in such modulation. Recent findings of ours and of others have led to a new way of thinking about fatty acids and their balance in the diet and, consequently, in membrane phospholipids as modulators of cell responsiveness to cytokines. This concept has broad implications in human pathobiology, nutrition, and therapeutics, with special reference to atherosclerosis and inflammation.

EARLY PHASES OF ATHEROSCLEROSIS  Atherosclerotic lesions originate in discrete points of the arterial tree (mainly branching points, bifurcations, and the convex site of bending arteries) characterized by low or oscillatory shear stresses (1) that can favor the passive transport of arterial blood components into the vessel wall. Late, complex lesions, usually observed in adults, can assume different appearances, reflecting different stages in plaque evolution and perhaps different natural histories in plaque development (2, 3). However, most investigators now agree that arterial fatty streaks represent the earliest stage of plaque development (2, 4–10). This is the earliest detectable lesion in hypercholesterolemic animal models of atherosclerosis in different species (2, 4–10), and is present in the coronary arteries of 50% of young humans between 10 and 14 y of age, as observed in an autopsy study (2). Fatty streaks are areas of focal intimal thickening produced by the intimal accumulation of lipid-laden macrophages (foam cells) surrounded by extracellular matrix and a variable number of lymphocytes. The relation of fatty streaks to more advanced atherosclerotic lesions has long been disputed (11–13) and their full reversibility is generally accepted. However, observations in various animal models (2, 4–10) and particularly in primates (\textit{Macaca nemestrina}) with low-level hypercholesterolemia (8) have clarified that fatty streaks indeed

\textsuperscript{1}From the CNR Institute of Clinical Physiology and the Scuola Superiore Sant’ Anna, Pisa, Italy, and the Vasculature Medicine and Atherosclerosis Unit, Brigham and Women’s Hospital, Boston.

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\textsuperscript{3}Address reprint requests to R De Caterina, Laboratory for Thrombosis and Vascular Research, CNR Institute of Clinical Physiology, Via Savi 8, I-56126 Pisa, Italy. E-mail: rdecater@po.ifc.pi.cnr.it.

precede more advanced atherosclerotic lesions, which develop at
the same critical points of the arterial vasculature. Therefore, an
understanding of the inception of atherosclerosis requires an
understanding of the pathogenesis of fatty streaks (3, 14–17).

Contrary to previous opinions (18, 19), it is now mostly
accepted that endothelial damage, in the form of focal desqua-
mation with intimal denudation, is not required and is usually
not present at the inception of the atherosclerotic process.
Rather, endothelial dysfunction (an earlier, subtler, and more
common set of alterations that is not dependent on the physical
loss of the endothelial layer) can initiate the entire process. A
substantial role is now ascribed, in these early phases, to the
monocyte-macrophage (20).

In normal physiologic conditions, the vascular endothelium
contributes to vascular homeostasis by adaptively altering its
functional state. This happens through a continuous monitoring
by the vascular endothelium of blood borne and locally generated
stimuli and through an immediate response to changes in its envi-
ronment (14, 16, 21). Functional properties of the endothelium
include active regulation of hemostasis; control of platelet func-
tion, coagulation, and fibrinolysis (21); and control of vascular
tone, endothelial permeability, and medial smooth muscle cell
growth (22). Maladaptive changes in endothelial functions
induced by qualitatively or quantitatively abnormal stimuli can
result in localized alterations in the interactions of cellular and
macromolecular components acting at the blood vessel wall inter-
facing, such as changes in the antithrombotic properties of the
endothelium, altered control of vascular tone, altered permeabil-
ity to plasma lipoproteins, hyperadhesiveness to blood leuko-
cytes, and increased cytokine and growth factor production.
These alterations can be collectively termed endothelial dysfunc-
tion (23), a term now used by cardiologists for endothelium-
dependent alterations of vascular tone. The term endothelial activ-
ity more specifically describes the functional changes that
endothelia may undergo under the influence of various stimuli—
the best studied of which are inflammatory cytokines and bacte-
rial endotoxin—and the acquisition of new functional and
antigenic properties, most of which influence interactions with
blood leukocytes. Endothelial activation plays an important role
in the initiation, progression, and clinical emergence of athero-
sclerosis (14, 16, 23) and is a pivotal process in monocyte adhesion.

Monocyte binding to the endothelium

Leukocyte binding to the endothelium is a prominent feature of
several inflammatory and immunologic disorders. In acute inflam-
mation, polymorphonuclear leukocytes or lymphocytes, to a morphologically
normal arterial endothelium is typical of diet-induced atheroscle-
rosis in animals (24). Similarly, many features of the selective
recirculation of lymphocytes that occur in a variety of immune
reactions are explained by the preferential binding of lymphocyte
subtypes to district-specific lymphatic endothelia (25).

Leukocyte binding to cultured endothelial cells has been studied
extensively in vitro in an attempt to identify and study the
mechanisms mediating this cell-to-cell interaction. It is now clear
that activation of leukocytes, endothelial cells, or both can lead to
increased adhesion of polymorphonuclear leukocytes, monocytes,
or lymphocytes to the endothelium. Several protein families, each
with distinct functions, provide “traffic signals” for leukocytes.
These include 1) the selectin family of adhesion molecules, which
appear to recognize a sialylated carbohydrate determinant on their
cognate ligands (26, 27); 2) chemoattractants, some of which
(classical chemoattractants such as N-formyl peptides, comple-
ment components, leukotriene B₄, and platelet-activating factor)
act broadly on neutrophils, eosinophils, basophils, and monocytes,
whereas others (chemokines such as monocyte chemoattractant
protein 1 (MCP-1) and interleukin 8 (IL-8)) have selectivity for
leukocyte subsets (28, 29); and 3) the immunoglobulin superfami-
ly members on the endothelium [intercellular adhesion molecule
1 (ICAM-1), ICAM-2, ICAM-3, and vascular cell adhesion mole-
cule 1 (VCAM-1)] that recognize integrin ligands on the leukocyte
surface in a paradigm first established with ICAM-1 binding to
leukocyte function associated antigen 1 (LFA-1) (30; Figure 1).

For neutrophil and, probably, lymphocyte adhesion, selectins
mediate the initial tethering of the circulating leukocyte
over the endothelium, allowing it to roll over the endo-
thelium, slowing down its speed considerably. Antagonists of
L-selectin and E-selectin inhibit neutrophil and monocyte influx
in response to inflammatory agents (31, 32). Selective targeted
disruption of the gene for another such molecule, P-selectin,
which is contained preformed in endothelial Weibel-Palade bod-
ies, can also affect leukocyte rolling (33). The slowing down of
a leukocyte effected by interactions between selectins and carbo-
hydrates allows the leukocyte to sense the presence of chemotac-
tic gradients and elicit a chemoattractant-receptor–mediated
event, ie, the activation of some integrin-type leukocyte ligand
exhibiting new activation epitopes (30, 34, 35). Final firm attach-
ment of leukocytes to the endothelium requires the interaction
of integrin ligands on the leukocyte surface with immunoglobulin
superfamily members, ie, ICAM-1, ICAM-2 and VCAM-1,
expressed on the endothelium (30, 36) (Figures 1 and 2).

The possible sequential interactions between selectins and carbo-
hydrates, chemoattractants and receptors, and immunoglobu-
lin and integrins [for neutrophils and possibly also for lympho-
cyte homing (37, 38)] and the multiple molecular choices
available for each of these ligand-to-ligand interactions provide
great combinatorial diversity in signals. This diversity allows
the selective responses of different leukocyte classes to inflamma-
tory agents, the preferential recirculation patterns of lymphocyte
subpopulations, or the selective binding of monocytes to arterial
endothelium during early phases of atherogenesis.

Because monocyte recruitment into the intima of large arteries
is specific to atherosclerosis but not to other forms of leukocyte-to-
endothelium interactions, it was hypothesized that these localized
monocyte-to-endothelium interactions reflect specific molecular
changes in the adhesive properties of the endothelial surface, lead-
ing to expression of “athero-ELAMs,” ie, endothelium-leukocyte
adhesion molecules (ELAMs) on the endothelial surface in the
early phases of atherosclerosis. The first such protein, originally
identified in the hypercholesterolemic rabbit model, is VCAM-1
(Figure 2), a member of the immunoglobulin superfam-
ily, expressed on human vascular endothelium in 2 molecular
forms (118 and 98 kDa) arising from alternative splicing of unprocessed
messenger RNA (mRNA) (39, 40). Both forms are able to bind a
heterodimeric integrin receptor, very-late-antigen 4 (VLA₄), with
leukocyte selectivity of expression on monocytes and lymphocytes
but not on neutrophils. This explains the selective pattern of inhibi-
tion of monocyte but not neutrophil adhesion by antibodies
directed against VCAM-1 and the selectivity of monocyte recruit-
ment in early atherogenesis (41). Endothelial cells express
VCAM-1 early during cholesterol feeding in rabbits, before the
appearance of macrophages and foam cells in the intima of a developing fatty streak, in a temporal pattern consistent with its pathogenetic role in lesion development (42).

Interaction between VCAM-1 and VLA-4 is only one of the possible ligand-to-ligand interactions involved in monocyte recruitment in early atherogenesis. The interactions between the regulatable (and, to a large extent, constitutive) endothelial molecule ICAM-1 and the integrin ligands LFA-1 and CD11b/CD18 (Mac-1) (30), between endothelial E-selectin and monocytic sialylated Lewis x carbohydrate (43), and between monocytic L-selectin and an as yet incompletely characterized inducible endothelial ligand (44) likely contribute to monocyte binding to an activated endothelium. In addition, endothelial monocyte-specific soluble products, which are also inducible by cytokines and endotoxin such as MCP-1, M-CSF, IL-6, and IL-8, are augmented several-fold in response to bacterial endotoxin and cytokines such as IL-1 and tumor necrosis factor (TNF). Resting, unactivated endothelial cells express negligible or low amounts of these molecules, with the notable exception of ICAM-1. After endotoxin and cytokines interact with their specific cell surface receptors, a cascade of intracellular events occurs, ultimately leading to the surface appearance or secretion of these products of endothelial activation. Because most adhesion molecules are not expressed in basal conditions, cytokine activation requires initiation of transcription (51). Also, different adhesion molecules, which are products of separate genes, are expressed simultaneously and in conjunction with increased gene expression of other endothelial products such as MCP-1, M-CSF, IL-6, and IL-8, and tissue factor. This leads to the hypothesis that activation of one or several transcription factors, including the early-response genes (c-Jun and c-Fos) and the nuclear factor-κB (NF-κB) system, leads to concerted activation of genes. The NF-κB system in particular has received increasing attention over the past several years as a common denominator of endothelial activation and is possibly causally linked with adhesion molecule expression (52).

First discovered in lymphocytes, where it has a role in controlling the activation of genes encoding for the immunoglobulin κ chains (53), the NF-κB system is now known as a much more gen

Endothelial activation as a transducer of atherogenic risk factors

In view of this evidence for the participation of leukocyte adhesion molecules, chemoattractants, and cytokines in early atherogenesis, we must consider the signals that regulate this expression. The gene expression of VCAM-1, as well as that of other adhesion molecules such as ICAM-1, E-selectin, and of inducible soluble endothelial products such as MCP-1, M-CSF, IL-6, and IL-8, is augmented several-fold in response to bacterial endotoxin and cytokines such as IL-1 and tumor necrosis factor (TNF). Resting, unactivated endothelial cells express negligible or low amounts of these molecules, with the notable exception of ICAM-1. After endotoxin and cytokines interact with their specific cell surface receptors, a cascade of intracellular events occurs, ultimately leading to the surface appearance or secretion of these products of endothelial activation. Because most adhesion molecules are not expressed in basal conditions, cytokine activation requires initiation of transcription (51). Also, different adhesion molecules, which are products of separate genes, are expressed simultaneously and in conjunction with increased gene expression of other endothelial products such as MCP-1, M-CSF, IL-6, and IL-8, and tissue factor. This leads to the hypothesis that activation of one or several transcription factors, including the early-response genes (c-Jun and c-Fos) and the nuclear factor-κB (NF-κB) system, leads to concerted activation of genes. The NF-κB system in particular has received increasing attention over the past several years as a common denominator of endothelial activation and is possibly causally linked with adhesion molecule expression (52).

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activating subunits are separated from I-κB at the beginning of mRNA transcription (52). Nucleotide sequences of several genes that provide a signal necessary for the translocation of activating subunits to the nucleus. There the activating subunits are bound to an inhibiting protein, I-κB, which allows the attachment of a ubiquitin molecule to the κB that allows the attachment of a ubiquitin molecule to theκB. After a phosphorylation of I-κB, allowing the activat-
ing subunits to be translocated to the nucleus. Then the activating subunits can bind specific “consensus” sequences in the promoter region of several genes that provide a signal necessary for the beginning of mRNA transcription (52). Nucleotide sequences able to bind specifically to NF-κB–like factors (NF-κB elements) have been identified in many human genes, including those for the inducible endothelial leukocyte adhesion molecules and secretable cytokines (52). The NF-κB system provides a potential common link to coordinate the expression of the variety of endothelial genes involved in endothelial activation. Stimuli able to activate the NF-κB system appear also to induce oxidant stress (55, 56) in the form of reactive oxygen species, i.e., the superoxide anion and hydrogen peroxide. Antioxidants can inhibit such activation (55, 56), thus giving an important molecular rationale for the therapeutic use of such substances in vascular disease initiation and progression.

A model for other endothelial leukocyte adhesion molecules and cytokine-induced endothelial products, IL-1, TNF, and IL-4 can induce VCAM-1 expression in vitro. These cytokines can be produced by monocyte-macrophages—and, to some extent, by T lymphocytes—inferring developing lesions (57). Therefore, such stimuli might provide a paracrine mechanism to amplify the local reaction at the site of a fatty streak, enhancing local monocyte recruitment. The question remains, however, of what initiates the entire atherogenic process. Some hints may come from the notion that cholesterol-induced atherosclerosis in animals is invariably accompanied by both endothelial activation and the focal expression of VCAM-1 (42) and the focal accumulation of LDL in the arterial intima (58). LDL or some of their biotransformation products may stimulate monocyte recruitment. Indeed, several lines of evidence suggest that the critical process that heightens the atherogenicity of LDL is the oxidative modification of LDL in the arterial intima, a microenvironment protected from circulating antioxidants (59–62). Indeed, minimally oxidized LDL or β-VLDL can heighten monocyte adhesiveness to endothelial cells (63), and also increase endothelial production of MCP-1 and M-CSF in vitro (64, 65). As to the exact component of oxidized LDL able to confer such a property, Kume et al (66) reported that a lysophospholipid associated with oxidized LDL particles, lysophosphatidylcholine (alone or in combination with cytokines), can stimulate the expression of some endothelial leukocyte adhesion molecules, including VCAM-1 and ICAM-1, in cultured endothelial cells under certain conditions. Conversely, the protective effect of HDL on atherosclerosis may result in part from inhibition of LDL oxidation (67–69). Other circulating products or metabolites might act by similar mechanisms in conditions associated with enhanced atherosclerotic risk independent of the lipid status. Such factors could include the advanced glycosylation end products associated with diabetes, lipoprotein(a) (a modified LDL particle that appears to be an independent risk factor for atherosclerosis), or homocysteine, as occurs in homocysteinuria and possibly in subtler forms of congenital or acquired enzyme defects in the homocysteine biosynthetic pathway (cystathionine β-synthase or tetrahydrofolate reductase), partly due to vitamin (eg, folate) deficiency. In addition to these humoral stimuli, endothelial gene expression also responds to hemodynamic forces (70, 71), potentially explaining the localization of atherosclerosis at particular points of the arterial vasculature. All these issues are currently under investigation.

### FIGURE 2

The role of vascular cell adhesion molecule (VCAM)-1 in cytokine-induced mononuclear cell adhesion in a typical experiment performed under rotation (63 rpm), mimicking flow conditions somewhat. For these experiments, monocytoid U937 cells were grown in RPMI medium (GIBCO BRL, Grand Island, NY) with 10% fetal calf serum and concentrated by rotation at room temperature and 1 × 10^6 cells/L. Human saphenous vein endothelial cells were grown to confluence in 6-well tissue culture plates, after which human recombinant interleukin (IL)-4 (Genzyme, Cambridge, MA) was added at 50 μg/L for 24 h. Control endothelial cells do not normally support mononuclear cell adhesion (panel A). Adhesion is dramatically increased after treatment of endothelial cells with cytokines (in this case IL-4; panel B). The increased mononuclear cell adhesion in these conditions is due to a large extent, to VCAM-1, as assessed by the inhibition obtained in the presence of the anti-VCAM-1 antibody (Ab) E 1/6 (panel C). τ ± SEM. In this system, a control nonrelevent antibody (HU 8/4) is completely ineffective (not shown).

Regulation of endothelial activation as a possible mode of action of antiatherogenic substances

Cytokine-induced endothelial activation increases the surface expression of endothelial leukocyte adhesion molecules and the secretion of soluble proatherogenic products (such as MCP-1 and
M-CSF) many-fold. Activated endothelial cells may thus provide a
target for therapeutic interventions. In a set of investigations, we
showed that several nitric oxide donors can reduce the expression of
adhesion molecules and cytokine-inducible, secretable endothe-
lial products by cytokine-activated endothelial (72) and smooth
muscle (73) cells. This finding raises the possibility that nitric
oxide acts as an endogenous antiatherogenic agent. Subsequent
work has shown that these effects occur through induction and sta-
bilization of IκB, the inhibitor of the transcription factor NF-κB
(74). Because NF-κB activation can control the coordinated
expression of a variety of adhesion molecules and chemoattractants
derived from endothelia or smooth muscle cells, these findings
account for a variety of long-term, cyclic guanosine 5’-
monophosphate (GMP)-independent actions of nitric oxide in the
arterial wall. Sources of nitric oxide in the vasculature include both
endothelial cells (mostly by means of the constitutively expressed
isoform of the enzyme nitric-oxide synthase now called NOSIII)
and other cell types (monocyte-macrophages and smooth muscle
cells, mostly by means of the cytokine-inducible nitric oxide syn-
thase called NOSII). The notion of the regulation of endothelial
activation by nitric oxide, itself a product of the vessel wall, adds
complexity to the entire scheme of the regulation of the expression
of ligands and soluble effectors in the origin of fatty stakes. One
may speculate that in a normal endothelial cell, endothelium-
derived nitric oxide contributes to maintaining an antiatherogenic
profile. Conversely, endothelial dysfunction, primarily manifested
by an alteration of endothelium-derived vasodilation, also might
show a role as a proinflammatory endothelial level. The term endo-
thelial dysfunction is often used to describe this phenomenon,
which might play a key role in the development of atherosclerosis.

We therefore hypothesized that n–3 PUFAs may modulate
atherogenesis by affecting endothelial activation. We used human
adult saphenous vein endothelial cells activated by cytokines in
an in vitro model of the early steps in atherogenesis. We first
assessed the effects of various fatty acids on the surface expres-
sion of endothelial leukocyte adhesion molecules and then char-
acterized the mechanisms and functional relevance of such
effects. One n–3 fatty acid, docosahexaenoic acid (DHA;
22:6n–3), when added to cultured endothelial cells hours or days
before stimulation with cytokines (early enough to allow a sig-
nificant incorporation of this fatty acid in cell membrane phos-
pholipids) inhibited events connected with endothelial activation
significantly, including the expression of adhesion molecules
such as VCAM-1, E-selectin, and, to a lesser extent, ICAM-1,
after stimulation with virtually any stimulus able to elicit the
coordinated expression of such genes (86, 87). Thus, this inhibi-
tion could be shown with IL-1α, IL-1β, TNF, IL-4, and bacterial
lipopolysaccharide (Figure 3). Inhibition of adhesion molecule
expression (1) occurred in a range of DHA concentrations com-
patible with nutritional supplementation of this fatty acid to indi-
viduals consuming a normal Western diet (Figure 3A), 2
curred at any time after the appearance of a cytokine effect,
modifying the specific kinetics of surface expression of adhe-
sion molecules (Figures 3, B and C), and 3) was related in its
magnitude strictly to the extent of incorporation into total cell
lipids (Figure 3D). Closer analysis of this last relation is shown in

Figure 4. The extent of the inhibitory effect of VCAM-1 paralleled
the incorporation of DHA and the overall increase in incorpora-
tion of n–3 PUFAs and was inversely related to the amount of
n–6 fatty acids (Figure 4). Experiments assessing the incorpora-
tion of [14C]DHA into cell phospholipids showed a significant incor-
poration of DHA into the phosphatidylethanolamine pool, which is
a specific and not particularly abundant phospholipid pool likely
to be found in the inner plasma membrane. Therefore, the destina-
tion of DHA is possibly a strategic position from which to alter
intracellular signal transduction pathways (88; Figure 5). This
effect was not limited to the expression of transmembrane mole-
cules involved in leukocyte recruitment. The effect was also seen
for other cytokine-activated products, i.e., the soluble proteins IL-6
and IL-8 (Figure 6) involved in either the amplification of the
inflammatory response (IL-6; 89) or specific chemoattraction for
granulocytes (IL-8; 28), and was accompanied by a functional
counterpart, i.e., reduced monocyte or monocytoid cell adhesion
to cytokine-activated endothelium (Figure 7).

One way to unravel the molecular mechanism by which n–3
PUFAs, and DHA in particular, inhibit endothelial activation
and VCAM-1 expression is to proceed backward from protein to
mRNA analysis and, further, to pathways controlling mRNA
accumulation. We first showed that DHA’s effects on VCAM-1
expression are accompanied by parallel reductions in VCAM-1
mRNA steady state concentrations, as assessed by Northern
analysis (86, 87). Similar results from experiments with a remarkably similar design were reported by Weber et al (90). These authors also showed, by using electrophoretic mobility shift assay, an inhibition by DHA of the activation of the NF-κB system of transcription factors (90). These results need to be confirmed. However, potential mechanisms for fatty acid inhibition of the activation of this system of transcription factors on cytokine stimulation can be hypothesized on the basis of comparative experiments that we performed to assess the fatty acid specificity of the effects described.

**CONTROL OF ENDOTHELIAL ACTIVATION AS A GENERAL PROPERTY OF UNSATURATED FATTY ACIDS**

In earlier experiments, with doses ≤10 μmol/L, DHA appeared to be relatively selective in decreasing cytokine-stimulated VCAM-1 expression [although a synergism with eicosapentaenoic acid (EPA) was already apparent (Figure 8)]. To understand whether there was anything specific for DHA in inhibiting cytokine-induced endothelial activation, careful dose-response studies with various fatty acids had to be performed. The availability of VCAM-1 surface enzyme immunoassays, allowing fast processing of 96-well plates of cultured endothelial cells, allowed us to compare the effects of various concentrations of a variety of fatty acids differing in chain length, number, and position of unsaturation. Saturated fatty acids (eg, 16:0, 18:0, and 20:0), monounsaturated fatty acids (eg, cis-16:1n−9 and cis-18:1n−9), n−6
PUFAs (eg, 18:3n-6 and 20:4n-6), and n-3 PUFAs [eg, 18:4n-3, 20:5 n-3, 22:5n-3 (docosapentaenoic acid; DPA) and DHA] were incubated with human saphenous vein endothelial cells alone for 24–48 h and then in the presence of IL-1 or TNF at 1–10 \( \mu \)g/L for another 24 h. No fatty acids per se elicited endothelial activation as assessed by surface enzyme immunoassay or flow cytometry, nor did saturated fatty acids inhibit cytokine-induced expression of adhesion molecules. However, a progressive increase of inhibitory activity was observed, for the same chain length, with the increase in double bonds accompanying the transition from monounsaturated fatty acids to n-6, and, further, to n-3 PUFAs (Table 1; 91). Thus, the greater number of double bonds seems critical for the greater activity of n-3 compared with n-6 fatty acids in inhibiting endothelial activation.

Incidentally, these data imply that such modulatory effects of fatty acids on endothelial activation have little or nothing to do with the generation of eicosanoid mediators, which is another specific property of only some polyunsaturated fatty acids. Indeed, several lines of reasoning argue to exclude a role for eicosanoids in this phenomenon and can be summarized as follows:

1) The effect of DHA is larger than that of EPA. Because EPA is the direct precursor of the 3-series prostaglandins and of the 5-series leukotrienes, one would expect a greater effect of EPA than of DHA if eicosanoids had a role.

2) The effect is unaltered by indomethacin, a blocker of cyclooxygenase (Figure 9), which virtually rules out the participation of prostaglandins.

3) The effect is not abolished by eicosatetraenoic acid, a common blocker of all metabolism of arachidonic acid through cyclooxygenase as well as lipoxygenases (data not shown).

4) Although to a lesser extent, the effect was also observed for PUFAs that are not eicosanoid precursors and even in monounsaturated fatty acids, such as oleic acid (86); in this case, a mechanism of action of oleic acid supplementation in the medium would be seen in a relatively selective displacement and substitution of saturated fatty acids in membrane phospholipids, as we reported preliminarily (92).

Thus, the presence of at least one double bond appears to be crucial to these effects of modulation of endothelial-leukocyte interactions. The greater the number of double bonds, the greater the inhibitory effects. n-3 Fatty acids are more active than n-6 fatty acids in this regard because they accomodate more double bonds with the same chain length. One would also predict that substitution of saturated fatty acids in membrane phospholipids, even by monounsaturated fatty acids, would render endothelial cells less responsive to the stimulation of cytokines. These predictions have all been confirmed so far.

**POSSIBLE MECHANISMS OF FATTY ACID EFFECTS ON ENDOTHELIAL ACTIVATION**

The question now remains, How do fatty acids containing more double bonds in the membrane lipid bilayer lead to diminished activation of the NF-kB system in response to cytokines sufficient to reduce the subsequent start of transcription of genes encoding for endothelial leukocyte adhesion molecules? One possible explanation relates to the intracellular mediators of NF-κB activation,
namely reactive oxygen species likely formed through the activation of an NADH or NADPH oxidase after cytokine activation. The role of hydrogen peroxide appears to be crucial to this process, whereas its precursor, superoxide anion, appears to have lesser effects, as shown by the almost total abrogation of cell activation of cytokines by cell-permeable catalase (polyethylene glycol-conjugated catalase) and, conversely, the lack of action of polyethylene glycol-superoxide dismutase (72). The marginal role of the superoxide dismutase–mediated scavenging effect of O$_2^-$ could be accounted for by the spontaneous alternative dismutation of O$_2^-$ likely to occur at acidic intracellular pH. A scavenging effect of O$_2^-$ in this system in the presence of the nitric oxide radical is likely to account for the inhibition of NF-$\kappa$B activation, possibly through enhanced transcription or stabilization of the inhibitor I-$\kappa$B (74). It is conceivable that similar oxygen-scavenging reactions occur with unsaturated fatty acids. These would lead on the one hand to the initiation of fatty acid peroxidation and on the other hand the prevention of O$_2^-$ from generating hydrogen peroxide and by this

![Figure 7](image_url)

**FIGURE 7.** Effect of docosahexaenoic acid (DHA) on adhesion of human elutriated monocytes to human adult saphenous vein endothelial cells after stimulation with interleukin (IL)-4 or IL-1, in a typical experiment performed under rotation (63 rpm). Conditions: 1, unstimulated; 2, IL-4 (59 µg/L); 3, IL-4 in conjunction with antibody (Ab) E1/6; 4, IL-1 (10 µg/L); 5, IL-1 in conjunction with Ab E1/6; 6, IL-1 in conjunction with Ab HU8/4. □, Control; ■, DHA; HPF, high-power field; *$P < 0.05$. Monocyte adhesion in the presence of IL-4 was more dependent on VCAM-1 expression (assessed by the extent of inhibition obtained in the presence of the anti-VCAM-1 antibody E1/6) than in the presence of IL-1. The lack of a response to the control, nonrelevant antibody HU8/4 is also shown. DHA significantly inhibited monocyte adhesion induced by both IL-4 and IL-1. $\bar{x} \pm$ SEM.

![Figure 8](image_url)

**FIGURE 8.** Mean ($\bar{x}$SEM) effect of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in combination compared with either agent alone on interleukin-1 (IL-1)–stimulated vascular cell adhesion molecule 1 (VCAM-1) expression. The experimental design was similar to that described in Figure 3A, with a preincubation time of 24 h before the addition of IL-1. Conditions: 1, control, unstimulated (n = 8); 2, control plus IL-1 (n = 10); 3, EPA (10 µmol/L) plus IL-1 (n = 20); 4, DHA (10 µmol/L) plus IL-1 (n = 20); 5, EPA plus DHA (both 5 µmol/L) and IL-1 (n = 29); 6, EPA plus DHA (both 10 µmol/L) and IL-1 (n = 30). Although EPA was ineffective at 10 µmol/L, a synergism between the 2 fatty acids was apparent when they were used in combination. *,$^{**}$Significantly different from the control plus IL-1 group, *$P < 0.05$, **$P < 0.01$ (Sheffe’s test after ANOVA).
A BROADER PERSPECTIVE ON FATTY ACIDS AS MODULATORS OF ENDOTHELIAL ACTIVATION

We can now formulate the broader concept that fatty acids may act as modulators of cell responsiveness to cytokines. This concept is entirely original and attractive because it can coherently explain several previously unconnected observations, specifically, 1) the reduced production of IL-1 and TNF by monocytes stimulated with bacterial lipopolysaccharides (94), 2) the reduced expression of tissue factor activity by monocytes (95), 3) the reduced accumulation of platelet-derived growth factor (PDGF) mRNA in mononuclear cells (96), and 4) the reduced in vivo adhesion of leukocytes in hamsters (97). Our theory also allows us to predict that other cytokine-induced products of endothelial cells, leukocytes, and other cytokine-responsive cells (eg, fibroblasts and smooth muscle cells) could be affected by similar mechanisms. Actually, by one of these mechanisms, fatty acids may control eicosanoid production by a mechanism different from and unrelated to substrate availability. The recent notion that cytokines such as IL-1 and phorbol esters increase the capacity of endothelial cells (and possibly other cell types) to produce prostaglandins via the induction of a recently discovered second cyclooxygenase enzyme [prostaglandin G/H synthase II, also termed cyclooxygenase 2 (98–100)], leads us to hypothesize that such synthesis may also be inhibited by DHA. The finding that DHA, but not EPA, is able to decrease endothelial surface expression of adhesion molecules could also be reverified with regard to prostaglandin G/H synthase II. If so, it might well lead to other research directions. EPA and DHA have always been referred together as n–3 PUFAs, implying similar spectra of biological and pharmacologic profiles. None of the available dietary supplements with n–3 PUFAs presently use the notion of a biologically important difference in the action of these 2 compounds. Research on ways to exploit the peculiar properties of individual fatty acids would therefore be warranted. For a complete structure-relaction analysis of the inhibitory properties on endothelial activation of fatty acids, see reference 101.

TABLE 1

<table>
<thead>
<tr>
<th>Fatty acid (25 μmol/L)</th>
<th>VCAM-1 inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic (20:4n–6)</td>
<td>15 ± 4a</td>
</tr>
<tr>
<td>Eicosapentaenoic (20:5n–3)</td>
<td>25 ± 9b</td>
</tr>
<tr>
<td>Docosapentaenoic (22:5n–3)</td>
<td>28 ± 12b</td>
</tr>
<tr>
<td>Docosahexaenoic (22:6n–3)</td>
<td>48 ± 18b</td>
</tr>
</tbody>
</table>

*4 SEM; n = 12. P < 0.01 for each condition, one-way ANOVA. Differences between arachidonic acid and the others (a), between eicosapentaenoic and docosahexaenoic acids (b), and between docosapentaenoic and docosahexaenoic acids (c) are significant, P < 0.05 (Student’s t test after Bonferroni’s correction). Data from reference 91.

REFERENCES


FIGURE 9. Mean (±SEM) inhibition of vascular cell adhesion molecule 1 (VCAM-1) expression by docosahexaenoic acid (DHA) in the absence or presence of 5 μmol indomethacin/L. Note that DHA inhibition of VCAM-1 expression was unaffected by indomethacin.
FATTY ACID MODULATION OF ENDOTHELIAL ACTIVATION


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