

Fatty acid modulation of endothelial activation¹⁻³

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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 (IC₅₀) 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 monocytic 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. *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 (*Macaca nemestrina*) with low-level hypercholesterolemia (8) have clarified that fatty streaks indeed

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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 desquamation 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 environment (14, 16, 21). Functional properties of the endothelium include active regulation of hemostasis; control of platelet function, 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 interface, such as changes in the antihemostatic properties of the endothelium, altered control of vascular tone, altered permeability to plasma lipoproteins, hyperadhesiveness to blood leukocytes, and increased cytokine and growth factor production. These alterations can be collectively termed *endothelial dysfunction* (23), a term now used by cardiologists for endothelium-dependent alterations of vascular tone. The term *endothelial activation* 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 bacterial 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 atherosclerosis (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 inflammation, polymorphonuclear leukocytes bind to the endothelium in postcapillary venules. Adhesion of monocytes, but not of polymorphonuclear leukocytes or lymphocytes, to a morphologically normal arterial endothelium is typical of diet-induced atherosclerosis 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

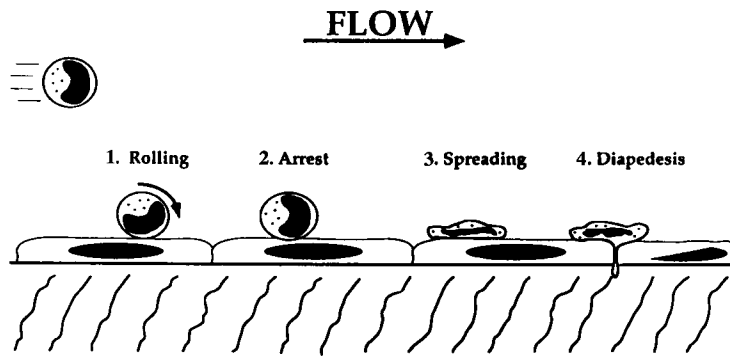
appear to recognize a sialylated carbohydrate determinant on their cognate ligands (26, 27); 2) chemoattractants, some of which (classical chemoattractants such as *N*-formyl peptides, complement components, leukotriene B₄, and platelet-activating factor) act broadly on neutrophils, eosinophils, basophils, 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 superfamily members on the endothelium [intercellular adhesion molecule 1 (ICAM-1), ICAM-2, ICAM-3, and vascular cell adhesion molecule 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 endothelium, 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 bodies, can also affect leukocyte rolling (33). The slowing down of a leukocyte effected by interactions between selectins and carbohydrates allows the leukocyte to sense the presence of chemotactic 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 attachment 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 carbohydrates, chemoattractants and receptors, and immunoglobulins and integrins [for neutrophils and possibly also for lymphocyte 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 inflammatory 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, leading 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 superfamily, 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 inhibition of monocyte but not neutrophil adhesion by antibodies directed against VCAM-1 and the selectivity of monocyte recruitment in early atherogenesis (41). Endothelial cells express VCAM-1 early during cholesterol feeding in rabbits, before the





	Rolling	Activation ----->	Firm adhesion	Transendothelial migration
Leukocyte	sLe ^x and other sialylated, fucosylated structures L-selectin	Cytokine, chemokine, and chemoattractant receptors	β -1, β -2, and β -3 Integrins	PECAM-1 and β -1 and β -2 Integrins
Endothelial	P-selectin L-selectin ligand E-selectin CD34 MAdCAM-1	Chemokines (eg, IL-8, MCP-1, MIP-1 β) PAF PECAM-1 E-selectin	ICAM-1, ICAM-2, VCAM-1, MAdCAM-1	PECAM-1 ICAM-1 VCAM-1

FIGURE 1. Adhesive interactions during leukocyte (monocyte) emigration. Upper panel: series of events as identified by intravital microscopic studies under flow. The adhesive interactions involved in leukocyte emigration involve several distinct phases: 1) initial transient adhesion (rolling), 2) activation, 3) firm adhesion (arrest and spreading), and 4) transendothelial migration (diapedesis). Lower panel: molecules involved in these steps. Recent *in vitro* and *in vivo* studies indicate that rolling is mediated by multiple low-affinity interactions between selectin receptors and their cognate carbohydrate ligands. Firm adhesion and diapedesis are largely dependent on integrin and immunoglobulin-like proteins. CD34, cluster of differentiation 34; PECAM, platelet-endothelial cell adhesion molecule; ICAM, intercellular adhesion molecule; IL, interleukin; MAdCAM, mucosal adhesion cell adhesion molecule 1; MCP, monocyte chemoattractant protein; MIP, macrophage inhibitory protein; PAF, platelet activity factor; sLe^x, sialyl Lewis^x; VCAM, vascular cell adhesion molecule.

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 VLA₄ 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 [a monocyte-selective chemoattractant (45)], and macrophage colony-stimulating factor (M-CSF) [able to promote activation and maturation of monocytes and macrophages (46)], are likely to be involved in monocyte recruitment in atherogenesis and have all been detected in atherosclerotic lesions in experimental animals or humans (46–50). So far, VCAM-1 is the only endothelial leukocyte adhesion molecule that appears to be selective for monocytes but not neutrophils.

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 atheroge-

nesis, 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, 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- κ B (NF- κ B) system, leads to concerted activation of genes. The NF- κ B 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- κ B system is now known as a much more gen-

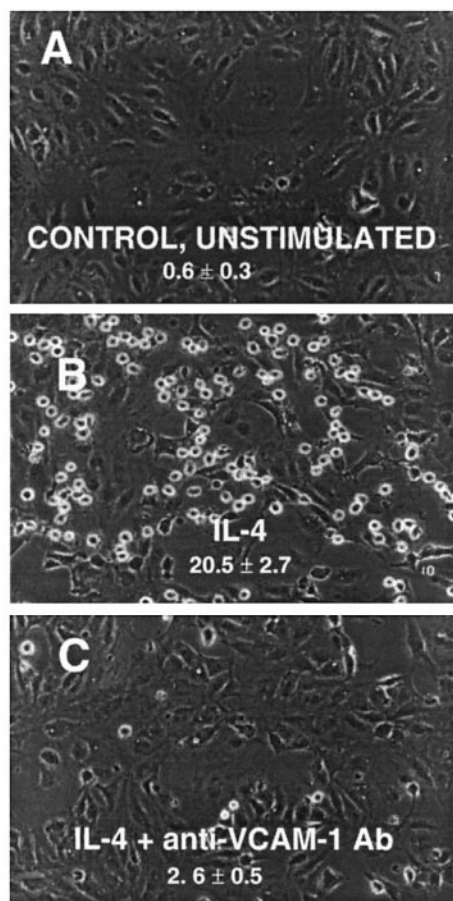


FIGURE 2. The role of vascular cell adhesion molecule (VCAM)-1 in cytokine-induced mononuclear cell adhesion in a typical experiment performed under rotation (63 rpm), mimicking flow conditions somewhat. For these experiments, monocytoid U937 cells were grown in RPMI medium (GIBCO BRL, Grand Island, NY) with 10% fetal calf serum and concentrated by rotation at room temperature and 1×10^9 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 $\mu\text{g/L}$ 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). $\bar{x} \pm \text{SEM}$. In this system, a control nonrelevant antibody (HU 8/4) is completely ineffective (not shown).

eral control mechanism of cell function than originally thought (54). The system consists of a family of transcription factors present in the cytoplasm as dimeric proteins. Normally, these subunits are bound to an inhibiting protein, I- κ B. After a phosphorylation of I- κ B that allows the attachment of a ubiquitin molecule to the inhibitor and renders it susceptible to proteolytic degradation, the activating subunits are separated from I- κ B, allowing the activating subunits to be translocated to the nucleus. There 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- κ B-like factors (NF- κ B elements) have been identified in many human genes, including those for the inducible endothelial leukocyte adhesion molecules and secretable cytokines (52). The NF- κ B 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- κ B system appear also to induce oxidant stress (55, 56) in the form of reactive oxygen species, ie, 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—infiltrating 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.

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 endothelial 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 stabilization of I- κ B, the inhibitor of the transcription factor NF- κ B (74). Because NF- κ B 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 synthase 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 streaks. 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 have a longer-term counterpart in allowing endothelial expression of leukocyte adhesion molecules and chemoattractants. In more general terms, however, the notion of a negative regulation of endothelial activation, as shown by the effects of nitric oxide, identifies a novel, previously unknown mechanism of action for potentially protective factors. Thus, one may speculate that nutritional interventions such as dietary enrichment with L-arginine or administration of novel, long-acting nitric oxide donors may put this novel mechanism to use to counteract the effects of proatherogenic stimuli [such as cytokines, but also, potentially, oxidized LDL, homocysteine, advanced glycation end products of diabetes, and lipoprotein(a)] at the level of the arterial endothelium. By the same token, other known protective factors (ie, estrogens) may act—aside from and beyond influencing plasma lipids—at the level of endothelial activation by influencing leukocyte-endothelial interactions and the start of the response of vessel walls to proatherogenic factors. This view advances our understanding of the initial phases of atherosclerosis, linking biological observations with epidemiologic data and with preventive and, possibly, new therapeutic approaches to this disease.

MODULATION OF ENDOTHELIAL-LEUKOCYTE INTERACTIONS BY n-3 FATTY ACIDS

The epidemiologic evidence of an association between dietary n-3 polyunsaturated fatty acid (PUFA) intake and protection from cardiovascular disease (75-82) is explained, at least in part, by the decreased incidence of atherosclerosis. Apart from numerous animal studies showing decreased atherosclerosis in animals treated with n-3 PUFAs (reviewed in 83), recent evidence has been obtained from human autopsy studies about such effects in Alaskan natives (who consumed high amounts of fish-derived products) and nonnatives (who consume mostly Western-type diets). In the study of Newman et al (84), which reported a

decreased percentage of intima covered with fatty streaks and raised lesions in Alaskan natives with high dietary intakes of n-3 PUFAs (80) compared with nonnatives, the magnitude of difference in fatty streak development appeared to be larger in younger age groups (84), suggesting that diet affects mainly the early events leading to fully developed atherosclerotic lesions. A recent study of n-3 PUFA supplementation after coronary bypass surgery indicated that such treatment significantly reduces vein graft stenosis (85), a process that can be regarded as an accelerated form 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 expression of endothelial leukocyte adhesion molecules and then characterized 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 significant incorporation of this fatty acid in cell membrane phospholipids) 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 inhibition could be shown with IL-1 α , IL-1 β , TNF, IL-4, and bacterial lipopolysaccharide (**Figure 3**). Inhibition of adhesion molecule expression 1) occurred in a range of DHA concentrations compatible with nutritional supplementation of this fatty acid to individuals consuming a normal Western diet (**Figure 3A**), 2) occurred at any time after the appearance of a cytokine effect, modifying the specific kinetics of surface expression of adhesion 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 incorporation of n-3 PUFAs and was inversely related to the amount of n-6 fatty acids (**Figure 4**). Experiments assessing the incorporation of [14 C]DHA into cell phospholipids showed a significant incorporation 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 destination 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 molecules involved in leukocyte recruitment. The effect was also seen for other cytokine-activated products, ie, 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, ie, reduced monocyte or monocytoïd 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



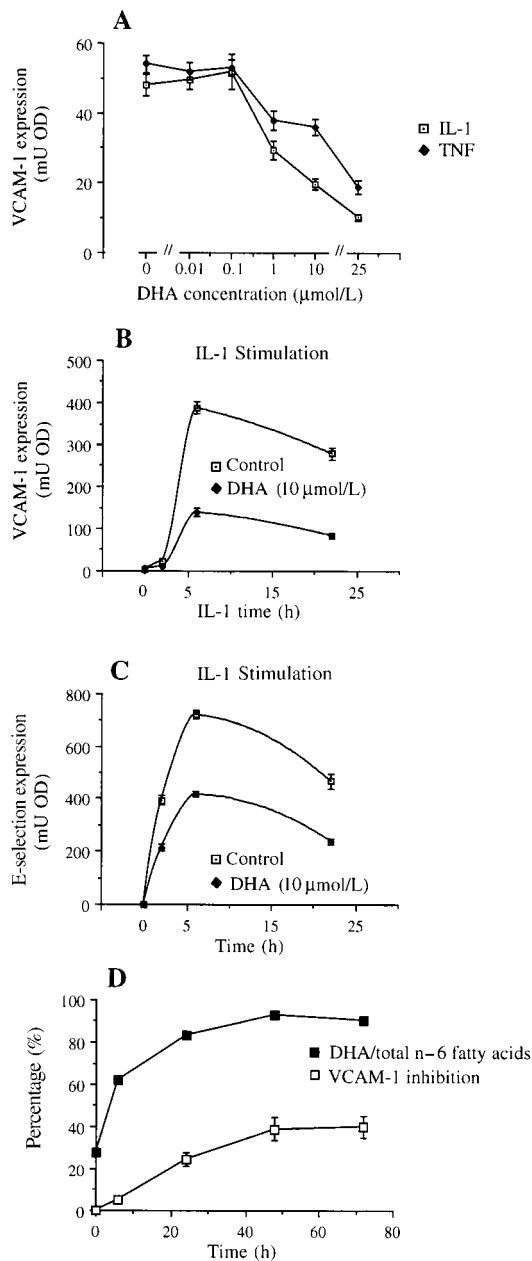


FIGURE 3. Mean (\pm SEM) inhibition of cytokine-induced expression of VCAM-1 and E-selectin in human saphenous vein endothelial cells by docosahexaenoic acid (DHA). DHA, as sodium salt, was dissolved in medium 199 (Mascia Brunelli, Milan, Italy) containing 5% fetal calf serum and incubated for 96 h with human adult saphenous vein endothelial cell monolayers in 96-well plates, after which cytokines were added for a further 24 h (panel A) or different times (panels B and C) to induce surface expression of endothelial leukocyte adhesion molecules, assessed by cell-surface enzyme immunoassays as described previously (86). A: Dose-response curves of VCAM-1 expression as a function of DHA concentration after stimulation with interleukin (IL)-1 α or tumor necrosis factor (TNF)- α , both at 10 μ g/L. B and C: Time course of VCAM-1 and E-selectin expression, respectively, after the addition of IL-1 α (at 10 μ g/L) in the absence or presence of DHA. OD, optical density. D: Parallelism between the inhibition of VCAM-1 expression by DHA and the relative abundance of DHA incorporation in cell membrane lipids, as assessed by gas-liquid chromatographic analysis of total cell-associated lipid extracts. Reproduced, slightly modified, from reference 86 with permission from the American Heart Association.

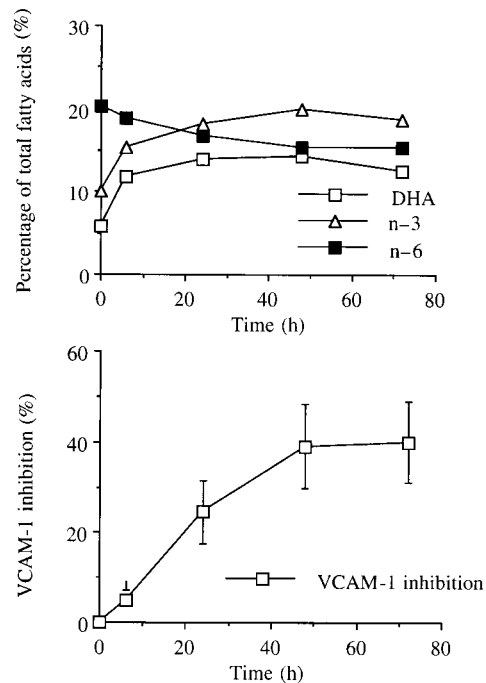


FIGURE 4. Changes in fatty acid incorporation into total cellular lipids as a function of time after incubation of human adult saphenous vein endothelial cells with docosahexaenoic acid (DHA; 10 μ mol/L) are shown in the top panel. The time course of the inhibition of surface VCAM-1 expression after stimulation with IL-1 under the same experimental conditions is shown in the bottom panel. Note the parallel increase in DHA and total n-3 fatty acids, and the decrease in total n-6 fatty acids. These changes parallel the progressive increase of VCAM-1 inhibition after IL-1 stimulation.

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- κ B 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 \leq 10 μ mol/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

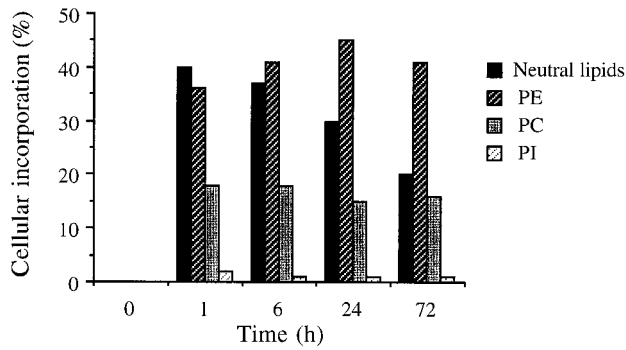


FIGURE 5. Lipid class distribution of [^{14}C]docosahexaenoic acid (DHA) as a function of time in human adult saphenous vein endothelial cells. Major neutral lipids and phospholipid classes [phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI)] were resolved by 2-dimensional thin-layer chromatography, and quantified by counting the radioactivity after scraping the silica plate in areas corresponding to migration of authentic phospholipid standards. DHA accumulated preferentially in neutral lipids at the beginning, then was redistributed into phospholipid classes, with the greatest accumulation in the PE pool.

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 $\mu\text{g/L}$ 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 accommodate 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- κB 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,

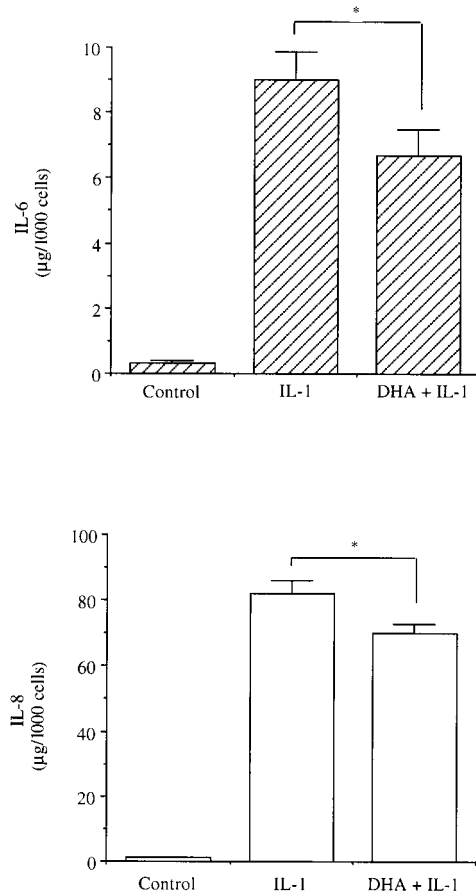


FIGURE 6. Mean (\pm SEM) production of interleukin (IL)-6 (upper panel) and IL-8 (lower panel) in the absence of endothelial activation (control), after 24-h stimulation with 10 μg IL-1 α /L and after docosahexaenoic acid (DHA) (10 $\mu\text{mol/L}$ for 48 h) in conjunction with IL-1 α (for a further 24 h still in the presence of DHA). IL-6 and IL-8 were measured in the supernate of endothelial cell cultures. Note the significant decrease in the stimulated production of both IL-6 and IL-8 after preincubation with DHA.

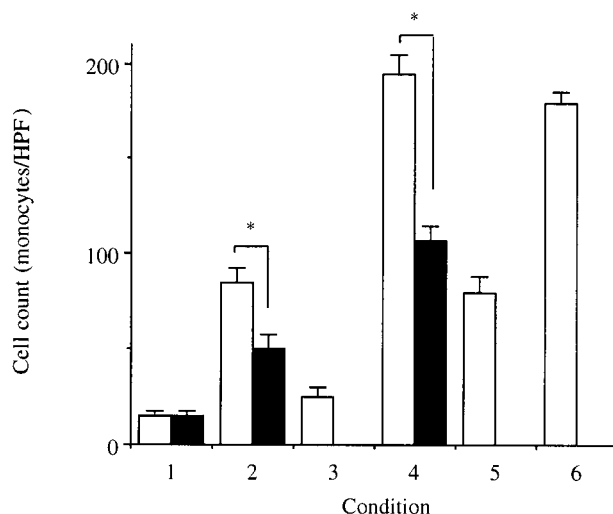


FIGURE 7. Effect of docosahexaenoic acid (DHA) on adhesion of human adult saphenous vein endothelial cells after stimulation with interleukin (IL)-4 or IL-1, in a typical experiment performed under rotation (63 rpm). Conditions: 1, unstimulated; 2, IL-4 (59 $\mu\text{g/L}$); 3, IL-4 in conjunction with antibody (Ab) E1/6; 4, IL-1 (10 $\mu\text{g/L}$); 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. $\bar{x} \pm \text{SEM}$.

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- κB activation, possibly through enhanced transcription or stabilization of the inhibitor I- κB (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

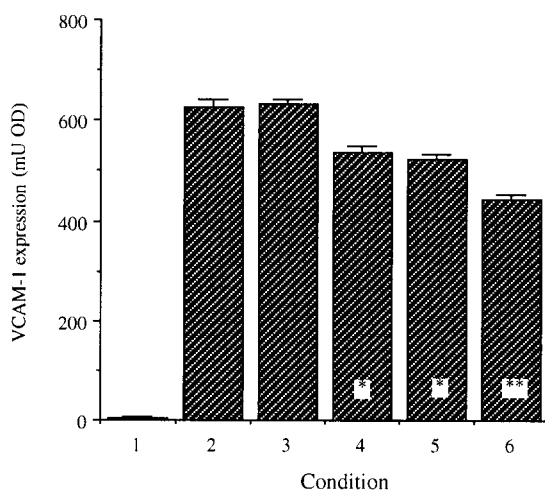


FIGURE 8. Mean ($\pm \text{SEM}$) effect of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in combination compared with either agent alone on interleukin-1 (IL-1)-stimulated vascular cell adhesion molecule 1 (VCAM-1) expression. The experimental design was similar to that described in Figure 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 $\mu\text{mol/L}$) plus IL-1 ($n = 20$); 4, DHA (10 $\mu\text{mol/L}$) plus IL-1 ($n = 20$); 5, EPA plus DHA (both 5 $\mu\text{mol/L}$) and IL-1 ($n = 29$); 6, EPA plus DHA (both 10 $\mu\text{mol/L}$) and IL-1 ($n = 30$). Although EPA was ineffective at 10 $\mu\text{mol/L}$, a synergism between the 2 fatty acids was apparent when they were used in combination. ***Significantly different from the control plus IL-1 group, * $P < 0.05$, ** $P < 0.01$ (Scheffe's test after ANOVA).

TABLE 1

Effects of n-6 and n-3 fatty acids, compared with saturated fatty acids, on vascular cell adhesion molecule 1 (VCAM-1) expression by human saphenous vein endothelial cells after incubation with 1 μ g interleukin 1 α


Fatty acid (25 μ mol/L)	VCAM-1 inhibition %
Arachidonic (20:4n-6)	15 \pm 4 ^a
Eicosapentaenoic (20:5n-3)	25 \pm 9 ^b
Docosapentaenoic (22:5n-3)	28 \pm 12 ^b
Docosahexaenoic (22:6n-3)	48 \pm 18 ^c

¹ $\bar{x} \pm$ 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.

mechanism prevent cell activation. Alternatively, it might be that PUFAs induce some hydrogen peroxide-degrading enzyme, eg, glutathione peroxidase (93). These hypotheses are currently being tested.

A BROADER PERSPECTIVE ON FATTY ACIDS AS MODULATORS OF CELL 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-relation analysis of the inhibitory properties on endothelial activation of fatty acids, see reference 101. 

REFERENCES

1. Ku DN, Giddens DP, Zarins CK, Glagov S. Pulsatile flow and atherosclerosis in the human carotid bifurcation: positive correlation between plaque location and low and oscillating shear stress. *Arteriosclerosis* 1985;5:293-302.
2. Strydom H. Evolution and progression of atherosclerotic lesions in coronary arteries of children and young adults. *Arteriosclerosis* 1989;88(suppl):1-32.
3. Fuster V, Badimon L, Badimon JJ, Chesebro JH. The pathogenesis of coronary artery disease and the acute coronary syndromes (first of two parts). *N Engl J Med* 1992;326:242-51.
4. Gerrity RG. The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am J Pathol* 1981;103:181-90.
5. Joris I, Znad T, Nunnari JJ, Krolkowski FJ, Majno G. Adhesion and emigration of mononuclear cells in the aorta of hypercholesterolemic rats. *Am J Pathol* 1983;113:341-58.
6. Faggiotto A, Ross R, Harker L. Studies of hypercholesterolemia in the nonhuman primate. I. Changes that lead to fatty streak formation. *Arteriosclerosis* 1984;4:323-40.
7. Rosenfeld ME, Tsukada T, Gown AM, Ross R. Fatty streak initiation in Watanabe heritable hyperlipemic and comparably hypercholesterolemic fat-fed rabbits. *Arteriosclerosis* 1987;7:9-23.
8. Masuda J, Ross R. Atherogenesis during low level hypercholesterolemia in the nonhuman primate. I. Fatty streak formation. *Arteriosclerosis* 1990;10:164-77.
9. Faggiotto A, Ross R. Studies of hypercholesterolemia in the nonhuman primate. II. Fatty streak conversion to fibrous plaque. *Arteriosclerosis* 1984;4:341-56.
10. Masuda J, Ross R. Atherogenesis during low level hypercholesterolemia in the nonhuman primate. II. Fatty streak conversion to fibrous plaque. *Arteriosclerosis* 1990;10:178-87.
11. Mitchell JRA, Schwartz CJ. *Arterial Disease*. Oxford, UK: Blackwell Scientific Publishers, 1965.
12. Smith EB, Slater RS. Lipids and low-density lipoproteins in intima in relationship to its morphological characteristics. In: Schletter D, Weizel A, eds. *Atherogenesis: initiating factors*. Ciba Foundation symposium no. 12. Amsterdam: Elsevier Publishing Co, 1973:39-51.
13. Smith EB, Slater RS, Crothers DX. Quantitative interrelationships between plasma constituents and normal and atherosclerotic human intimal tissue. In: Schletter D, Weizel A, eds. *Atherosclerosis III*. Berlin: Springer-Verlag, 1974:96-102.

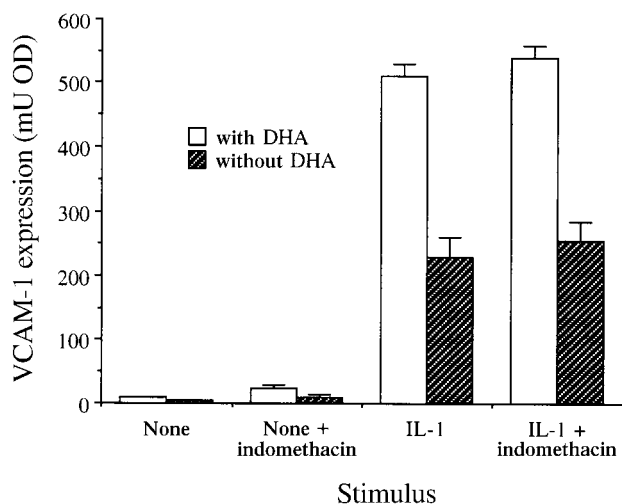


FIGURE 9. Mean (\pm SEM) inhibition of vascular cell adhesion molecule 1 (VCAM-1) expression by docosahexaenoic acid (DHA) in the absence or presence of 5 μ mol indomethacin/L. Note that DHA inhibition of VCAM-1 expression was unaffected by indomethacin.

14. Gimbrone MA Jr. Endothelial dysfunction and the pathogenesis of atherosclerosis. In: Fidge N, Nestel P, eds. *Atherosclerosis VII*. New York: Elsevier Science Publishers, 1986:367–9.
15. Ross R. The pathogenesis of atherosclerosis: an update. *N Engl J Med* 1986;314:488–500.
16. Gimbrone MA Jr. Endothelial dysfunction and atherosclerosis. *J Cardiac Surg* 1989;4:180–3.
17. De Caterina R, Gimbrone MA Jr. Leukocyte-endothelial interactions and the pathogenesis of atherosclerosis. In: Kristensen SD, Schmidt EB, De Caterina R, Endres S, eds. *n-3 Fatty acids—prevention and treatment in vascular disease*. London: Springer Verlag, 1995:9–24.
18. Ross R, Glomset J. The pathogenesis of atherosclerosis. Part I. *N Engl J Med* 1976;295:369–77.
19. Ross R, Glomset J. The pathogenesis of atherosclerosis. Part II. *N Engl J Med* 1976;295:420–5.
20. Libby P. Atheroma: more than mush. *Lancet* 1996;248(suppl):S4–7.
21. Gimbrone MA Jr, ed. *Vascular endothelium in hemostasis and thrombosis*. Edinburgh: Churchill Livingstone, 1986.
22. Gimbrone MA Jr, Bevilacqua MP. Vascular endothelium: functional modulation at the blood interface. In: Simionescu N, Simionescu M, eds. *Endothelial cell biology in health and disease*. New York: Plenum Press, 1988:255–73.
23. Gimbrone MA Jr, Kume N, Cybulsky MI. Vascular endothelial dysfunction and the pathogenesis of atherosclerosis. In: Weber P, Leaf A, eds. *Atherosclerosis reviews*. Vol 25. New York: Raven Press, 1993.
24. Cybulsky MI, Gimbrone MA Jr. Endothelial-leukocyte adhesion molecules in acute inflammation and atherogenesis. In: Simionescu N, Simionescu M, eds. *Endothelial cell dysfunctions*. New York: Plenum Press, 1992:129–40.
25. Mackay C. Migration pathways and immunologic memory among T lymphocytes. *Semin Immunol* 1992;4:51–8.
26. Lasky L. Selectins: interpreters of cell-specific carbohydrate information during inflammation. *Science* 1992;258:964–9.
27. Rosen S. Cell surface lectins in the immune system. *Semin Immunol* 1993;5:237–47.
28. Oppenheim JJ, Zachariae CO, Mukaida N, Matsushima K. Properties of the novel proinflammatory supergene “intercrine” cytokine family. *Annu Rev Immunol* 1991;9:617–48.
29. Miller M, Krangel M. Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. *CRC Crit Rev Immunol* 1992;12:17–46.
30. Springer TA. Adhesion receptors of the immune system. *Nature* 1990;346:425–34.
31. Mulligan M, Varani J, Dame M, et al. Role of endothelial-leukocyte adhesion molecule 1 (ELAM-1) in neutrophil-mediated lung injury in rats. *J Clin Invest* 1991;88:1396–406.
32. Picker L, Butcher E. Physiological and molecular mechanisms of lymphocyte homing. *Annu Rev Immunol* 1992;10:561–91.
33. Mayadas T, Johnson R, Rayburn H, Hynes R, Wagner D. Leukocyte rolling and extravasation are severely compromised in P selectin-deficient mice. *Cell* 1993;74:541–54.
34. Landis R, Bennett R, Hogg N. A novel LFA-1 activation epitope maps to the I domain. *J Cell Biol* 1993;120:1519–27.
35. Diamond MS, Springer TA. A subpopulation of Mac-1 (CD11b/CD18) molecules mediates neutrophil adhesion to ICAM-1 and fibrinogen. *J Cell Biol* 1993;120:545–56.
36. Chan BMC, Elices MJ, Murphy E, Hemler ME. Adhesion to vascular cell adhesion molecule 1 and fibronectin: comparison of alpha 4 beta 1 (VLA-4) and alpha 4 beta 7 on the human B cell line JY. *J Biol Chem* 1992;267:8366–70.
37. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 1994;76:301–14.
38. Luscinskas FW, Gimbrone MA Jr. Endothelial-dependent mechanisms in chronic inflammatory leukocyte recruitment. *Annu Rev Med* 1996;47:413–21.
39. Cybulsky MI, Fries JW, Williams AJ, et al. Alternative splicing of human VCAM-1 in activated vascular endothelium. *Am J Pathol* 1991;138:815–23.
40. Cybulsky MI, Fries JW, Williams AJ, Sultan P, Gimbrone MA Jr, Collins T. Gene structure, chromosomal location, and basis for alternative mRNA splicing of the human VCAM-1 gene. *Proc Natl Acad Sci U S A* 1991;88:7859–63.
41. Bochner BS, Luscinskas FW, Gimbrone MA Jr, et al. Adhesion of human basophils, eosinophils, and neutrophils to interleukin-1-activated human vascular endothelial cells: contribution of endothelial cell adhesion molecules. *J Exp Med* 1991;173:1553–6.
42. Li H, Cybulsky MI, Gimbrone MA Jr, Libby P. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. *Arterioscler Thromb* 1993;13:197–204.
43. Erbe DV, Wolitzky BA, Presta LG, et al. Identification of an E-selectin region critical for carbohydrate recognition and cell adhesion. *J Cell Biol* 1992;119:215–27.
44. Spertini O, Luscinskas FW, Kansas GS, et al. Leukocyte adhesion molecule-1 (LAM-1, L-selectin) interacts with an inducible endothelial cell ligand to support leukocyte adhesion. *J Immunol* 1991; 147: 2565–73.
45. Rollins BJ. JE/MCP-1: an early response gene encodes a monocyte-specific cytokine. *Cancer Cells* 1991;3:517–24.
46. Clinton SK, Underwood R, Sherman ML, Kufe DK, Libby P. Macrophage-colony stimulating factor gene expression in vascular cells and in experimental and human atherosclerosis. *Am J Pathol* 1992;140:301–16.
47. Cybulsky MI, Gimbrone MA Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* 1991;251:788–91.
48. Rosenfeld M, Ylä-Herttua S, Lipton B, Ord V, Witztum J, Steinberg D. Macrophage colony-stimulating factor mRNA and protein in atherosclerotic lesions of rabbits and humans. *Am J Pathol* 1992; 140:291–300.
49. Poston R, Haskard D, Coucher J, Gall N, Johnson-Tiedy R. Expression of intercellular adhesion molecule-1 in atherosclerotic plaques. *Am J Pathol* 1992;140:665–73.
50. Nelken N, Coughlin S, Gordon D, Wilcox J. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J Clin Invest* 1991;88:1121–7.
51. Kishimoto T, Taga T, Akira S. Cytokine signal transduction. *Cell* 1994;76:253–62.
52. Collins T. Endothelial nuclear factor-kappa B and the initiation of the atherosclerotic lesion. *Lab Invest* 1993;68:499–508.
53. Bauerle PA. The inducible transcription activator NF-kappa B: regulation by distinct protein subunits. *Biochim Biophys Acta* 1991; 1072:63–80.
54. Lenardo M, Baltimore D. NF-kappa B: a pleiotropic mediator of inducible and tissue specific gene control. *Cell* 1988;58:227–9.
55. Marui N, Offermann MK, Swerlick R, et al. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest* 1993;92:1866–74.
56. Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *FASEB J* 1995;9:899–909.
57. Clinton SK, Libby P. Cytokines and growth factors in atherogenesis. *Arch Pathol Lab Med* 1992;116:1292–300.
58. Schwenke D, Carew TE. Initiation of atherosclerotic lesions in cholesterol-fed rabbits. II. Selective retention of LDL vs. selective increases in LDL permeability in susceptible sites of arteries. *Arteriosclerosis* 1989;9:908–18.
59. Witztum J. The role of monocytes and oxidized LDL in atherosclerosis. In: Leaf A, Weber P, ed. *Atherosclerosis reviews: prevention and noninvasive therapy of atherosclerosis*. Vol 21. New York: Raven Press, 1990:59–69.
60. Witztum JL, Steinberg D. Role of oxidized low-density lipoprotein in atherogenesis. *J Clin Invest* 1991;88:1785–92.
61. Witztum JL. The oxidation hypothesis of atherosclerosis. *Lancet* 1994;344:793–5.
62. Parthasarathy S. Mechanism(s) of cell mediated oxidation of low density lipoprotein. In: Nohl H, Esterbauer H, Evans CR, eds. *Free*

- radicals in the environment, medicine and toxicology. London: Richelieu Press, 1994:163–79.
63. Territo M, Berliner J, Almada L, Ramirez R, Fogelman A. Beta-VLDL pretreatment of endothelial monolayers increases monocyte but not neutrophil adhesion. *Arteriosclerosis* 1989;9:824–8.
 64. Cushing SD, Berliner JA, Valente AJ, et al. Minimally modified LDL induces monocyte chemotactic protein 1 in human endothelial and smooth muscle cells. *Proc Natl Acad Sci U S A* 1990;87:5134–8.
 65. Rajavashisth TB, Andalibi A, Territo MC, et al. Induction of endothelial expression of granulocyte and macrophage colony-stimulating factors by modified low density lipoproteins. *Nature* 1990;344:254–7.
 66. Kume N, Cybulsky M, Gimbrone MA Jr. Lysophosphatidyl-choline, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured endothelial cells. *J Clin Invest* 1992;90:1138–44.
 67. Hessler JR, Robertson AJ, Chisolm GM. LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle and endothelial cells in culture. *Atherosclerosis* 1979;32:213–29.
 68. Parthasarathy S, Barnett J, Fong LG. High density lipoprotein inhibits the oxidative modification of low-density lipoprotein. *Biochim Biophys Acta* 1990;1044:275–83.
 69. Stafforini DM, Zimmerman GA, McIntyre TM, Prescott SM. The platelet activating factor acetylhydrolase from human plasma prevents oxidative modification of low density lipoprotein. *Trans Am Assoc Physicians* 1993;106:44–63.
 70. Resnick N, Collins T, Atkinson WJ, Bonthron DT, Dewey CFJ, Gimbrone MA Jr. Platelet-derived growth factor B chain promoter contains a *cis*-acting fluid shear-stress-responsive element. *Proc Natl Acad Sci U S A* 1993;90:4591–5.
 71. Nagel T, Resnick N, Atkinson WJ, Dewey CFJ, Gimbrone MA Jr. Shear stress selectively upregulates intercellular adhesion molecule-1 expression in cultured human vascular endothelial cells. *J Clin Invest* 1994;94:885–91.
 72. De Caterina R, Libby P, Peng H-B, et al. Nitric oxide decreases cytokine-induced endothelial activation. Nitric oxide selectively reduces endothelial expression of adhesion molecules and proinflammatory cytokines. *J Clin Invest* 1995;96:60–8.
 73. Shin W, Hong Y, Peng H, De Caterina R, Libby P, Liao JK. Nitric oxide attenuates vascular smooth muscle cell activation by interferon- γ . The role of constitutive NF- κ B activity. *J Biol Chem* 1996;271:11317–24.
 74. Peng H-B, Libby P, Liao JK. Induction and stabilization of I kappa B alpha by nitric oxide mediates inhibition of NF-kappa B. *J Biol Chem* 1995;270:14214–9.
 75. Kromann N, Green A. Epidemiological studies in the Upernavik district, Greenland. *Acta Med Scand* 1980;208:401–6.
 76. Bang HO, Dyerberg J, Hjorne N. The composition of food consumed by Greenland Eskimos. *Acta Med Scand* 1976;200:69–73.
 77. Kagawa Y, Nishizawa M, Suzuki M. Eicosapolyenoic acid of serum lipids of Japanese islanders with low incidence of cardiovascular diseases. *J Nutr Sci Vitaminol (Tokyo)* 1982;28:441–53.
 78. Hirai A, Hamazaki T, Terano T. Eicosapentaenoic acid and platelet function in Japanese. *Lancet* 1980;2:1132–3.
 79. Arthaud B. Cause of death in 339 Alaskan natives as determined by autopsy. *Arch Pathol* 1970;90:433–8.
 80. Middaugh JP. Cardiovascular deaths among Alaskan natives 1980–1986. *Am J Public Health* 1990;80:282–5.
 81. Kromhout D, Bosschieter EB, De Lezenne Coulander C. The inverse relation between fish consumption and 20-year mortality from coronary heart disease. *N Engl J Med* 1985;312:1205–9.
 82. Shekelle R, Missel L, Paul O, MacMillan-Schryock A, Stamler J. Fish consumption and mortality from coronary heart disease. *N Engl J Med* 1985;313:820 (letter).
 83. Soei L, Lamers J, Sassen L, et al. Fish oil: a modulator of experimental atherosclerosis in animals. In: Kristensen S, Endres S, De Caterina R, Schmidt E, eds. *n-3 Fatty acids: prevention and treatment in vascular disease*. London: Springer, 1995:77–84.
 84. Newman WP, Middaugh JP, Propst MT, Rogers DR. Atherosclerosis in Alaska natives and non-natives. *Lancet* 1993;341:1056–7.
 85. Eritsland J, Arnesen H, Grønseth K, Field NB, Abdelnoor M. Effect of supplementation with n-3 fatty acids on graft patency in patients undergoing coronary artery bypass operation. Results from the SHOT study. *Eur Heart J* 1994;15:29 (abstr).
 86. De Caterina R, Cybulsky MI, Clinton SK, Gimbrone MA Jr, Libby P. The omega-3 fatty acid docosahexaenoate reduces cytokine-induced expression of pro-atherogenic and pro-inflammatory proteins in human endothelial cells. *Arterioscler Thromb* 1994;14:1829–36.
 87. De Caterina R, Cybulsky MA, Clinton SK, Gimbrone MA Jr, Libby P. Omega-3 fatty acids and endothelial leukocyte adhesion molecules. *Prostaglandins Leukot Essent Fatty Acids* 1995;52:191–5.
 88. Baker RR, Chang H-Y. The hydrolysis of natural phosphatidylethanolamines by phospholipase A2 from rat serum: a degree of selectivity is shown for docosahexaenoate release. *Biochim Biophys Acta* 1992;1125:56–61.
 89. Van Snick J. Interleukin-6: an overview. *Annu Rev Immunol* 1990;8:253–78.
 90. Weber C, Erl W, Pietsch A, Danesch U, Weber P. Docosahexaenoic acid selectively attenuates induction of vascular cell adhesion molecule-1 and subsequent monocytic cell adhesion to human endothelial cells stimulated by tumor necrosis factor-alpha. *Arterioscler Thromb Vasc Biol* 1995;15:622–8.
 91. De Caterina R, Bernini W, Carluccio MA, Liao JK, Libby P. Structural requirements for inhibition of cytokine-induced endothelial activation by unsaturated fatty acids. *J Lipid Res* 1998;39:1062–70.
 92. Carluccio MA, Massaro M, Bonfrate C, et al. Oleic acid inhibits endothelial activation: a direct vascular antiatherogenic mechanism of a nutritional component in the Mediterranean diet. *Arterioscler Thromb Vasc Biol* 1999;19:220–8.
 93. Meskini N, Nemoz G, Chapuy P, et al. Glutathione peroxidase activity and metabolism of arachidonic acid in peripheral blood mononuclear cells from elderly subjects. *Clin Sci* 1993;85:203–11.
 94. Endres S, Ghorbani R, Kelley VE, et al. The effect of dietary supplementation with n-3 polyunsaturated fatty acids on the synthesis of interleukin-1 and tumor necrosis factor by mononuclear cells. *N Engl J Med* 1989;320:265–71.
 95. Hansen JB, Olsen JO, Wilsgard L, Østerud B. Effects of dietary supplementation with cod liver oil on monocyte thromboplastin synthesis, coagulation and fibrinolysis. *J Intern Med Suppl* 1989;225:133–9.
 96. Kaminski WE, Jendraschak E, Kiefl R, von Schacky C. Dietary omega-3 fatty acids lower levels of platelet-derived growth factor mRNA in human mononuclear cells. *Blood* 1993;81:1871–9.
 97. Lehr A-H, Hübner C, Finckh B, et al. Dietary fish oil reduces leukocyte/endothelium interaction following systemic administration of oxidatively modified low density lipoproteins. *Circulation* 1991;84:1725–31.
 98. Maier JA, Hla T, Maciag T. Cyclooxygenase is an immediate-early gene induced by interleukin-1 in human endothelial cells. *J Biol Chem* 1990;265:10805–8.
 99. Habib A, Creminon C, Frobert Y, Grassi J, Pradelles P, Maclouf J. Demonstration of an inducible cyclooxygenase in human endothelial cells using antibodies raised against the carboxyl-terminal region of the cyclooxygenase-2. *J Biol Chem* 1993;268:23448–54.
 100. Hla T, Neilson K. Human cyclooxygenase-2 cDNA. *Proc Natl Acad Sci U S A* 1992;89:7384–8.
 101. De Caterina R, Bernini W, Carluccio MA, Liao JK, Libby P. Structural requirements for inhibition of cytokine-induced endothelial activation by unsaturated fatty acids. *J Lipid Res* 1998;39:1062–70.

