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Antiatherogenic effects of n-3 fatty acids - evidence and mechanisms

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ABSTRACT: N-3 (omega-3) (polyunsaturated) fatty acids are thought to display a variety of beneficial effects for human health. Clues to the occurrence of cardiovascular protective effects have been, however, the spur for the first biomedical interest in these compounds, and are the best documented. Historically, the epidemiologic association between dietary consumption of n-3 fatty acids and cardiovascular protection was first suggested by Bang and Dyerberg, who identified the high consumption of fish, and therefore, of fish oil-derived n-3 fatty acids, as the likely explanation for the strikingly low rate of coronary heart disease events reported in the Inuit population. Since their initial reports, research has proceeded in parallel to provide further evidence for their cardioprotection and to understand underlying mechanisms. Decreased atherogenesis is currently thought to be a part of the cardiovascular protection by n-3 fatty acids. This article summarizes the evidence for such a claim and the mechanisms putatively involved. (Heart International 2006; 3-4: 141-54)

KEY WORDS: Coronary heart disease, Fish, Fish oil, Omega-3 fatty acids, n-3 fatty acids, Cardioprotection, Nutrigenomics

INTRODUCTION

N-3 (omega-3) (polyunsaturated) fatty acids, mainly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), feature a variety of beneficial effects for human health, ranging from fetal development to cancer prevention (1). However, cardiovascular protective effects have been the spur for the first biomedical interest in these compounds, and are the best documented. The epidemiologic association between dietary consumption of n-3 fatty acids and cardiovascular protection was first suggested by Bang and Dyerberg (2, 3). The authors identified the high consumption of fish, and therefore, of fish oil-derived n-3 fatty acids, as the likely explanation for the strikingly low rate of coronary heart disease events reported in the Inuit population (2, 3). Since their initial reports, research has proceeded in parallel to provide further evidence for this cardioprotection and to understand underlying mechanisms. Decreased atherogenesis is currently thought to be a part of the cardiovascular protection by n-3 fatty acids, and is likely to occur in addition to effects on arrhythmias and sudden cardiac death (4, 5), as recently reviewed (6). This article summarizes the evidence for such a claim and the mechanisms putatively involved.

EVIDENCE FOR ANTIATHEROGENIC EFFECTS OF N-3 FATTY ACIDS

Effects of n-3 fatty acid in animal models of atherosclerosis

In animal models, the hypotriglyceridemic action of n-3 fatty acids, which is very well documented in humans, is not consistently observed, while a reduction in high density lipoprotein (HDL)-cholesterol has been consistently reported (7). Putative antiatherogenic ef-

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fects of n-3 fatty acids are, therefore, not attributable to the favorable effects on serum lipids that occur in humans (see below). N-3 fatty acids have been reported to lessen the development of atherosclerotic lesions in several animal models. However, results have not been unequivocal due to differences between species chosen and variations in study design. N-3 fatty acids were usually supplemented as fish oils. Studies aimed at determining their effect on atherogenesis or on lesion regression have been performed in non-human primates, pigs, rabbits and mice. The design of most of these studies has been criticized for a variety of reasons. One is that the supplementation with fish oils reached extremely high levels, between 10 and 40% of total energy intake as n-3 fatty acids, in most studies, while realistic values should be 1-2% (7). Another reason is the frequent lack of an appropriate control. In some cases, fish oils were simply substituted for saturated fats; in others, the control oil had a different polyunsaturated/saturated (P/S) ratio, a variable that might *per se* have an influence on lesion development (8, 9). In addition, the choice of the endpoint parameters and relative methods in the evaluation of atherosclerosis has been quite variable. The choice of the experimental model is also relevant in explaining the different outcome: in some cases atherogenesis has been induced by drastic dietary changes, quite different from the pathophysiological conditions occurring in humans. Ideally, animal models showing similarities with the inception and progression of human atherosclerosis should be used, and control oil should be carefully chosen (7). The majority of experimental studies available in the literature do not meet these criteria.

Studies in non-human primates

Monkeys have been used to determine the effects of n-3 fatty acids on lipoprotein cholesterol and triglyceride plasma levels (10-13), and on atherosclerosis development (14-16). Almost all these studies are difficult to compare with one another due to metabolic differences between species and to different study designs. Usually, n-3 fatty acids were substituted for fat in high amounts, so that the experimental results can be either attributed to n-3 fatty acid or to the removal of hyperlipidemic (saturated) fatty acids. A consistent finding in monkey studies has been the reduction in plasma HDL-cholesterol, an effect often observed in other species (7). The development of atherosclerosis in general appears to be retarded or diminished (14, 15). These results were obtained with the concomitant removal of saturated fats from the diet, replaced by high amounts of fish oil without balancing the saturated/unsaturated ratio. Studies aimed at demonstrating the effect of alpha-linolenic acid (ALA) on atherogenesis also support a protective effect for this specific n-3 fatty acid (17, 18). In one regression study, dietary supplementation with a relatively low dose of fish oil (2.5% of total energy intake) in addition to an atherogenic and to a therapeutic diet, using sunflower oil as control for both, caused an increase in cholesterol and phospholipid content in the aortic intima and no changes in atherosclerosis (16). In an *in vivo* baboon model, high doses of fish oil - against olive oil supplementation - prevented platelet deposition on a plastic vascular shunt and vascular lesion formation in response to mechanical vascular injury in endarterectomized carotids as well as in uninjured aortas (19). Recently, in a macaque model, the effect of fish oil on the long-term occlusive tendency of aortocoronary vein bypass grafts was evaluated and found ineffective against olive oil (20).

Studies in swine

Pigs have been widely used in studies of lipoprotein metabolism, which appears to be similar to humans, and for the study of effects on atherosclerosis, since spontaneous atherogenesis occurs in this species with an early spontaneous beginning. However, native lesion development in the pig is slow, and needs to be accelerated by a high-fat diet, cholesterol and bile acids supplementation. Fish oils have usually decreased triglycerides and HDL-cholesterol (7). The effects on atherosclerosis are, again, conflicting. A significant dose-dependent reduction in the extent of aortic and coronary luminal encroachment has been sometimes observed when the vessel was mechanically abraded, while atherosclerosis progression was prevented in non-abraded arteries (21-24). However, aortic lesion area was not reduced in one set of studies (21-24). In addition, the question of lesion regression was specifically addressed. A favorable effect of fish oil supplementation after a period of atherosclerosis induction by an atherogenic diet was observed in one set of experi-

ments, and regression appeared to be more evident with low plasma cholesterol levels. In these studies, however, there was no control oil or a control oil with a P/S fatty acid ratio different from that of fish oil (25, 26). When a control oil with a matching P/S fatty acid ratio was used in one lesion-regression study, fish oil had no effect on lesion regression (27). This was accompanied by an unfavorable increase in low-density lipoproteins (LDL), decrease in HDL, and increased susceptibility of LDL to oxidation (28).

Studies in rabbits

Rabbits provide a convenient model for atherosclerosis, since atherosclerosis can be easily and quickly induced in this species. This is usually achieved by feeding either a cholesterol-rich or a casein-based, fat-free diet. In addition, Watanabe heritable hyperlipidemic rabbits (WHHL), lacking functional native LDL receptors and thereby promoting atherosclerosis, are a well-established atherosclerosis model, resembling one type of human inheritable type of atherosclerosis. However, lipoprotein metabolism is different in rabbits and humans. In rabbits, an increase in dietary cholesterol does not result in LDL, but on β-very-low density lipoproteins (β-VLDL) elevation, so that any change in lipoprotein levels is not necessarily pertinent to most situations occurring in humans. Studies aimed at verifying the effect of n-3 fatty acids in rabbit atherosclerosis models are inconsistent both with regard to lipoproteins and triglyceride/cholesterol concentrations (7). Moreover, results of n-3 fatty acid supplementation on atherosclerosis are conflicting in this species, and most studies lack an adequate control. Fish oils were reported to inhibit atherosclerosis development in cholesterol fed rabbits (29-31), to enhance lesion formation (32, 33), or to have no effect (34, 35). However, fish oils reduced intimal proliferation in arteries after ballon injury (36, 37). This effect was inversely related to serum cholesterol values, in agreement with data obtained in porcine models (26, 38). The efficacy of fish oil was also apparently enhanced by vitamin E supplementation (31).

In WHHL rabbits, n-3 fatty acids were initially reported to have either no effect on plasma lipids and aortic lesion size (39), or to lower triglycerides, total lipoproteins and cholesterol in female rabbits, but to be ineffective on lesion size of treated vs. untreated controls (40). With a similar experimental protocol, but with different criteria for lesion evaluation, fish oil reduced triglyceride and cholesterol levels and aortic lesions (41). Recently, a direct comparison of fish oil against olive oil treatment confirmed the occurrence of a hypolipidemic effect of fish oil in this species, and these findings were associated with a retardation of atherosclerosis development in young WHHL rabbits (42).

Studies in mouse models

Studies in mouse models have been scarce. Reiner et al evaluated the effect of n-3 fatty acids on the development of atherosclerosis and on the secretory activity of peritoneal macrophages in the atherosclerosis-susceptible strain C57BL/6J (43). The authors compared a saturated fatty acid- and a fish oil-supplemented diet; fish oils diminished the lesion size. Macrophages displayed a decreased ability to produce basal tumor necrosis factor (TNF)-α and lipopolysaccharide (LPS)-elicited TNF-α and interleukin (IL)-1β production, a reduction of lipoprotein lipase expression, and an enhancement of nitrate synthesis, used as a nitric oxide production index. In a recent study on murine macrophages, fish oil reduced the expression of intercellular adhesion molecule (ICAM)-1 and of scavenger receptor A type I and II (44). These findings suggest an effect of n-3 fatty acids on macrophage phenotypes and on their role in lesion formation.

Recently, transgenic mouse models of atherosclerosis have been introduced (45). The LDL receptor-deficient (LDLR-/-) mouse develops atherosclerosis when put on a Western-type, high-fat diet (46). In this model, LDL is not efficiently cleared from plasma, and hypercholesterolemia and atherogenesis proceed with a pattern similar to the human situation.

The apolipoprotein (apo) E-deficient mouse is another atherogenesis model (47, 48). In this model, there is no need to modify the diet, since atherosclerosis proceeds spontaneously and very rapidly. The serum lipid profile is quite different from the human condition, however, since apo E is a constituent of all lipoproteins except LDL, and serves as a ligand for receptors involved in the clearance of chilomicrons and VLDL remnants. The only human counterpart of this situation is the genetic defect of apo E, a rare clinical condition that leads to severe atherosclerosis.

In both LDL-receptor knockout and apo E knockout models, atherosclerotic lesions begin to spread from the thoracic aorta to the whole aorta and its main branches, predominantly at bifurcation sites. The lesion formation pattern is similar in both models (49). Although gender influence is controversial, actively produced estrogens seem protective.

While the effect of n-3 fatty acids in the LDL receptor model had not been the object of any published work until recently, studies of n-3 fatty acids in apo E-/- mice have been done. Calleja et al fed apo E-/- mice diets enriched with different oils commonly used in human nutrition, without adding cholesterol (50). Following evaluation of the lesion area, male animals appeared to respond to sunflower oil, rich in n-6 polyunsaturated fatty acids, while females responded to palm oil and elevated concentrations of olive oil, rich in monounsaturated fatty acids. Adan et al recently fed 7-week-old apo E-deficient mice an atherogenic diet in the presence or absence of DHA supplementation (1% final concentration) for 8 weeks (51), and observed no effect of DHA on atherosclerosis: the size and extension of lesions in the aortic arch and the thoracic and abdominal aorta were similar in both experimental groups. Cholesterol and cholate in the diet might have influenced these results. We recently evaluated the effects of n-3 (in the form of fish oil) compared with n-6 fatty acids (in the form of corn oil) in both the apo E- and the LDL receptor-knockout models. Both n-3 and n-6 fatty acid supplementation retarded atherosclerosis development in LDL receptor-/- mice, with a stronger effect seen with n-3 fatty acids, especially in the regions (such as the aortic arch) more susceptible to lesion development. There was an important strain-dependence of the effect, with no protection against atherosclerosis in apo E-/- mice (52).

In summary, animal experimental studies with n-3 fatty acids are hampered by differences in study design and species. No definite claim on the existence of true antiatherogenic effects of n-3 fatty acids can be done to date on this basis.

Studies in humans

Nutritional intake of n-3 fatty acids is highly likely to lead to cardioprotection. This has been recently confirmed by a 30-yr follow-up in men who were free of overt cardiovascular disease at baseline and who consumed up to 35 g fish per day (53); and recently, baseline blood values of long-chain n-3 fatty acids have been associated with the reduced risk of sudden death in a nested-case study conducted on healthy subjects from the Physicians' Health Study cohort (54). Recently, the American Heart Association recommended that all adults eat fish twice a week as a means of coronary heart disease prevention (55). However, because of the multifactorial nature of ischemic heart disease, of which atherosclerosis is one, albeit important, component, evidence about the occurrence of a true antiatherogenic effect of n-3 fatty acids in humans is not easy to gather. Autopsy studies in Alaskan natives (consuming high amounts of fish-derived products) and non-natives, mostly consuming Western-type diets provide circumstantial evidence about a lesser extent of atherosclerosis in populations exposed to a high nutritional intake of n-3 fatty acids. Newman et al (57) reported reduced percent of intima covering with fatty streaks and raised lesion in Alaskan natives, with a high n-3 fatty acid dietary intake (56), vs. non-natives (57). In their study, the magnitude of difference in fatty streak development appears larger in younger age groups (57) (Fig. 1), suggesting an effect of diet mainly in the early events leading to fully developed atherosclerotic lesions. Prospective studies in humans are few, but mostly pointing to the true occurrence of such effects. A study of high-dose n-3 fatty acid supplementation on coronary artery disease regression, evaluated by angiography, was negative (58), but a subsequent well-controlled study (the study on prevention of coronary atherosclerosis by intervention with marine omega-3 fatty acids (SCIMO)) showed a slower progression in subjects supplemented with lower doses (1.65 g/day of EPA + DHA) (59). Interestingly, the same authors recently reported no effect of the treatment on carotid intima-media thickness evaluated by carotid ultrasound in the very same subjects (60), indicating some district-specificity for the fish oil effect. It has been speculated that 0.5-2.0 g n-3 fatty acids per day are effective in reducing clinical endpoints (61). By contrast, higher doses would yield no effect (58). However, this contention is based on very few studies examining the effects of these substances on true atherogenesis and not on a mixed endpoint. One study after coronary bypass surgery indicated that n-3 fatty acids significantly reduced vein graft stenosis (62), a process

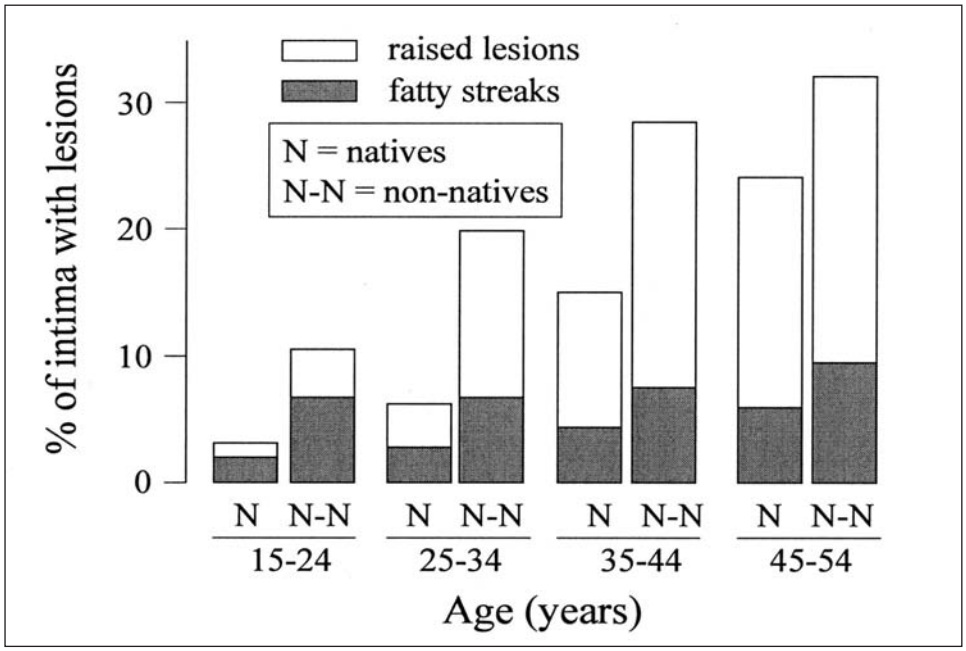

Fig. 1 - *Percent of coverage of the aorta with fatty streaks (open areas) and raised plaques (grey areas) in Alaskan natives vs. non-natives, divided by age. Notice the larger difference, attributable to the prevalence of fatty streaks, in younger age groups. Redrawn, modified, from (57).*

which may be regarded as an accelerated form of atherosclerosis. Studies on restenosis after percutaneous coronary angioplasty have been contradictory and, in the end, largely inconclusive (63-74), although issues of study design still leave open the possibility that n-3 fatty acids can have some efficacy on restenosis (75). Restenosis after percutaneous interventions is, however, the result of a mechanical injury to an already diseased vessel wall, and its relevance to native atherosclerosis is controversial.

Very recently, the results of a large Japanese study (the Japan EPA Lipid Intervention Study, JELIS) in over 20,000 Japanese subjects (overall with a high baseline intake of n-3 fatty acids compared to Western populations) were presented. Subjects were treated with either a relatively high dose of EPA (1800 mg/day) or nothing, against the background of optimal medical therapy. The study examined whether EPA administration, in both a primary and a secondary prevention setting, could reduce major coronary events, including sudden cardiac death, fatal and non-fatal myocardial infarction, unstable angina, the number of cases of angioplasty/stenting or coronary bypass grafting, in a prospective, double-blind open-label study with blinded adjudications of endpoints (76). In an average follow-up of 4.6 yrs, the study found a significant (19%, p=0.01) reduction in the combined primary endpoint, which was due to a reduction in myocardial infarction and unstable angina but not, at variance from previous studies, in sudden cardiac death. This reduction appeared to be totally independent from any change in serum cholesterol (77). These results are important indirect evidence in humans for a reduction in either the progression of atherosclerosis or its propensity to acute complications, such as plaque rupture and coronary thrombosis.

In summary, based on at least one placebo-controlled prospective study of native atherosclerosis in the coronary arteries, a placebo-controlled prospective study in coronary bypass surgery grafts, and a recent large-scale study on coronary events, human studies provide stronger evidence of an antiatherogenic effect of n-3 fatty acids than do animal studies.

Putative mechanisms by which n-3 fatty acids interfere with atherogenesis

Molecular mechanisms in atherogenesis

The initial event in atherosclerosis development is a condition of endothelial dysfunction, that precedes any morphologic evidence of endothelial damage (78). A number of different stimuli (toxins, shear stress, cigarette smoking and high cholesterol levels) can trigger

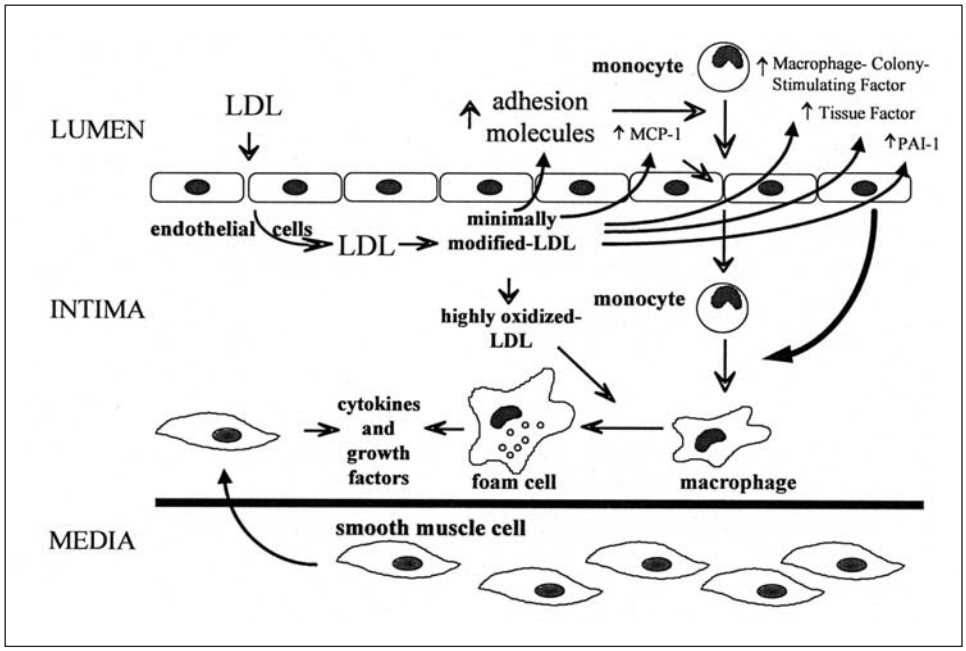

Fig. 2 - *A scheme of the modifications of the arterial intima occurring in early atherogenesis.*

endothelial dysfunction. One type of endothelial dysfunction is termed endothelial activation. In this, the endothelium modifies its phenotype in a pro-adhesive direction, triggering an increased adhesion of circulating monocytes. Their subsequent infiltration in the arterial intima is one of the first visible findings in atherosclerosis. In the intima, monocytes become activated, and begin to incorporate circulating LDL that have become oxidized through the exposure to reactive oxygen species of both endothelial and macrophagic origin; therefore, initiating the formation of the fatty streak (79) (Fig. 2).

Atherosclerosis and inflammation share similar basic mechanisms, involving the adhesion of leukocytes to the vascular endothelium in their early phases. Multiple protein families, each with a distinct function, provide "traffic signals" for leukocytes. These include:

a) the "selectin" family of adhesion molecules;
b) 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, while more recently described "chemokines", such as monocyte chemoattractant protein-1 (MCP-1) and IL-8, have selectivity for leukocyte subsets;
c) the immunoglobulin (Ig) superfamily members on the endothelium (intercellular adhesion molecule (ICAM)-1, ICAM-2, ICAM-3, and vascular cell adhesion molecule (VCAM)-1), recognizing "integrin" ligands on the leukocyte surface.

For neutrophil and, probably, lymphocyte adhesion, selectins mediate initial tethering of the circulating leukocyte over the endothelium, allowing it to roll over the endothelium, considerably slowing down its speed, and allowing leukocytes to "sense" the presence of chemotactic gradients. Final firm attachment of leukocytes to the endothelium requires the interaction of integrin ligands on the leukocyte surface with Ig superfamily members, expressed on the endothelium, such as ICAM-1, ICAM-2 and VCAM-1. The multiple molecular choices available for each of these ligand-ligand interactions provide great combinatorial diversity in signals, allowing the selective responses of different leukocyte classes to inflammatory agents, the preferential recirculation patterns of lymphocyte subpopulations, or the selective binding of monocytes to the arterial endothelium during early phases of atherogenesis.

Monocyte recruitment into the intima of large arteries is specific for atherosclerosis as compared to other forms of leukocyte-endothelial interactions. Therefore, it was suggested that these localized monocyte-endothelium interactions reflect specific molecular

changes in the adhesive properties of the endothelial surface, leading to the surface expression of "atherogenic ELAMs", ie endothelium-leukocyte adhesion molecules (ELAMs) expressed in the early phases of atherosclerosis. The first such protein, originally identified in the rabbit hypercholesterolemic model, is VCAM-1, a member of the Ig superfamily, expressed on human vascular endothelium at least in two molecular forms. Both forms are able to bind a heterodimeric integrin receptor, VLA4, whose leukocyte selectivity of expression, on monocytes and lymphocytes, but not on neutrophils, can explain the selectivity of monocyte recruitment in early atherogenesis (80). Endothelial cells express VCAM-1 early during cholesterol feeding in the rabbit, before the appearance of macrophages/foam cells in the intima of developing fatty streak, in a temporal pattern consistent with its pathogenetic role in lesion development. Pathophysiologically relevant stimuli for VCAM-1 expression in atherogenesis could include minimally oxidized LDL or β-VLDL, the advanced glycosylation end-products (AGEs) associated with diabetes, lipoprotein (Lp)(a), or perhaps homocysteine, elevated in homocysteinuria and in subtler forms of congenital or acquired enzyme defects in its biosynthetic pathway. In addition to these humoral stimuli, VCAM-1 endothelial gene expression also responds to hemodynamic forces, thus potentially explaining the localization of atherosclerosis in particular points of the arterial vasculature. For a general review on these issues, see De Caterina et al (80).

The progression from fatty streak to atheroma is driven by the production of cytokines and chemo-attractants that determine the intimal accumulation of leukocytes, smooth muscle cells and fibroblasts, as well as platelet adhesion. The thin-capped, lipid-rich atheromatous plaques have a strong tendency to rupture and are at risk of complicating with thrombosis, which is usually the ultimate event leading to unstable angina and myocardial infarction (81).

Therefore, there are multiple potential points of action of n-3 fatty acids on atherogenesis. The best-characterized ones are briefly reviewed below.

Effects of n-3 fatty acids on plasma lipids

In humans, n-3 fatty acids decrease serum triglycerides, an effect that is pronounced in marked hypertriglyceridemia. VLDL-cholesterol is reduced, while LDL-cholesterol tends to be either elevated or unchanged (82, 83). In patients with mixed hyperlipidemia and in marked hypertriglyceridemia, n-3 fatty acids are a highly effective means of reducing both triglyceride and VLDL. Therefore, n-3 fatty acids appear to reduce one of the atherogenic triggers.

Effects of n-3 fatty acids on cellular responses to atherogenic triggers

Dietary intake of n-3 fatty acids, such as EPA and DHA, allows their incorporation in the phospholipids of cell membranes replacing arachidonic acid (AA). Originally, the beneficial effects of n-3 fatty acids on the cardiovascular system were attributed to their substitution of AA. Metabolites that derive from n-3 fatty acid enzymatic metabolization (through cyclooxygenase, lipoxygenase and cytochrome P-450 monoxygenase) are less pro-thrombotic and vasoconstrictive compared to the corresponding AA derivatives. Many vascular effects of n-3 fatty acids are equally shared by DHA and EPA, or possibly even more prominently shown by DHA than EPA. Since DHA, at variance from EPA, is a poor substrate for metabolization into eicosanoids, effects of n-3 fatty acids other than eicosanoid generation are likely to play a greater role in preventing atherogenesis.

In recent years, the direct effects on endothelial activation have been demonstrated. These include:

- the reduced production of cytokines such as IL-1 and TNF in LPS-stimulated monocytes (84);
- the reduced production of the mitogen and smooth muscle cell attractant platelet-derived growth factor (PDGF -A and -B) protein and m-RNA (85, 86);
- the reduced expression of tissue factor by monocytes (87);
- an increase of endothelial nitric oxide bioavalability (88);
- the specific downregulation of gene expression for MCP-1 (89); and
- the reduced expression of endothelial adhesion molecules, essential for monocyte adhesion to sites of inflammation and dysfunctional endothelium (90).

Research on this last aspect will be now highlighted in greater detail, since it provides a potentially comprehensive explanation of the behavior of these agents as modulators of gene expression.

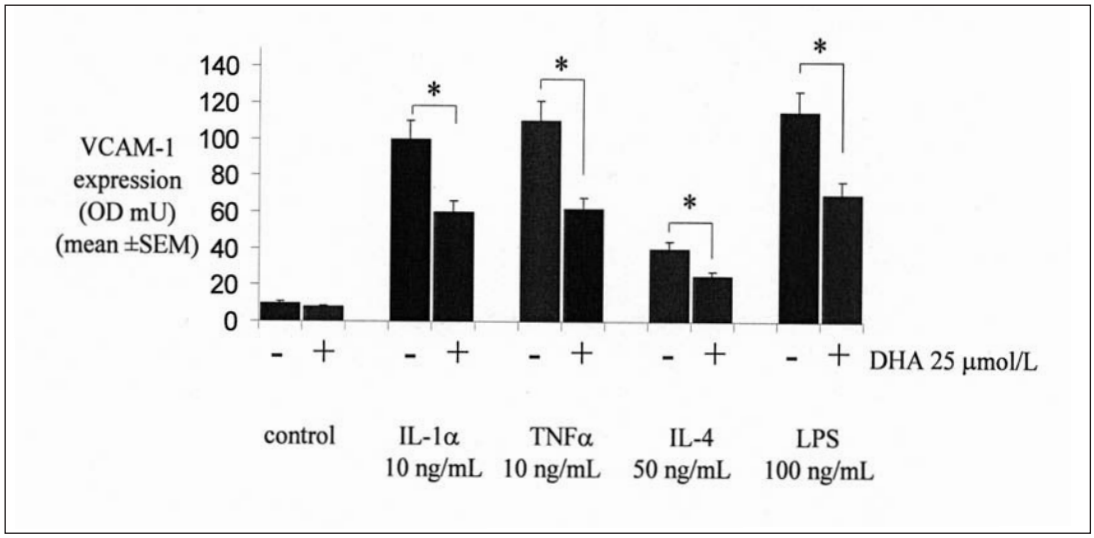

Fig. 3 - *The inhibition of adhesion molecule expression by DHA, occurring with diverse stimuli, including IL-1α and IL-1β, TNF-α, IL-4 and LPS. Asterisks denote significant differences at p<0.01. From De Caterina, et al, unpublished.*

Modulation of endothelial-leukocyte interactions by n-3 fatty acids

We used human adult saphenous vein endothelial cells activated by cytokines, in an *in vitro* model of these early steps in atherogenesis, first, assessing the effects of various fatty acids on the surface expression of endothelial leukocyte adhesion molecules, and, subsequently, characterizing mechanisms and functional relevance of these effects. One n-3 fatty acid, DHA, when added to cultured endothelial cells, hours to days before the stimulation with cytokines, early enough to allow a significant incorporation of this fatty acid in cell membrane phospholipids, significantly inhibited events connected with endothelial activation. These included 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 (90, 91). Therefore, this inhibition could be demonstrated with IL-1α and IL-1β, TNF-α, IL-4 and LPS (Fig. 3). Inhibition of adhesion molecule expression occurred in a range of DHA concentrations compatible with nutritional supplementation of this fatty acid to normal Western diets. In addition, this inhibition occurred at any time point after the appearance of the cytokine effect, modifying the specific kinetics of the surface expression of adhesion molecules, and was strictly related in its magnitude to the extent of incorporation into total cell lipids. The extent of VCAM-1 inhibitory effect paralleled the incorporation of DHA and the overall increase in incorporation of n-3 fatty acids and was inversely related to the content of n-6 fatty acids. Following the fate of 14C-labelled DHA into cell phospholipids, we could show a significant incorporation of DHA into the phosphatidyl ethanolamine pool. This is a specific and not the most abundant phospholipid pool, likely in the inner plasma membrane; and therefore, possibly in a strategic position to alter intracellular signal transduction pathways. This effect was not limited to the expression of transmembrane molecules involved in leukocyte recruitment. It also appeared to occur for other cytokine-activated products, such as the soluble proteins IL-6 and IL-8, involved in either the amplification of the inflammatory response (IL-6), or in the specific chemoattraction for granulocytes (IL-8). The effect was also accompanied by a functional counterpart, i.e. a reduced monocyte or monocytoid cell adhesion to cytokine activated endothelium. Compared to DHA, EPA was a weaker inhibitor of the expression of these molecules and of monocyte adhesion, although still more potent than other fatty acids. We also showed that DHA effects on VCAM-1 expression are accompanied by parallel reductions in VCAM-1 mRNA steady state levels, as assessed by Northern blot analysis (90, 91). Similar results, in experiments with remarkably similar design, were later reported by Weber et al (92). The authors also carried these investigations one step further, demonstrating an inhibition by DHA of the activation of the NF-κB system of transcription factors (92), which controls the coordi-

nated expression of adhesion molecules and of leukocyte-specific chemoattractants upon cytokine stimulation (93, 94).

We further analyzed endothelial effects of various fatty acids differing in chain length, number, position (n-3 vs. n-6 vs. n-9) and *cis/trans* configuration of the double bonds. Using VCAM-1 surface expression as a readout, we concluded that:

a) saturated fatty acids are inactive;
b) potency of polyunsaturated fatty acids increases with the number of unsaturations;
c) potency does not depend on chain length;
d) the single double bond present in the monounsaturated fatty acid oleic acid is indeed sufficient to produce all the effects obtainable with higher unsaturated fatty acids, albeit at higher concentrations;
e) for such an effect to occur, even the configuration (*cis* vs. *trans*) of the double bond does not really matter, since oleic acid (19:1 n-9 *cis*) and its *trans* stereoisomer elaidic acid are of equal potency (95). In addition, inhibition of NF-κB activation could be reproduced upon incubation of endothelial cells with oleic acid (96).

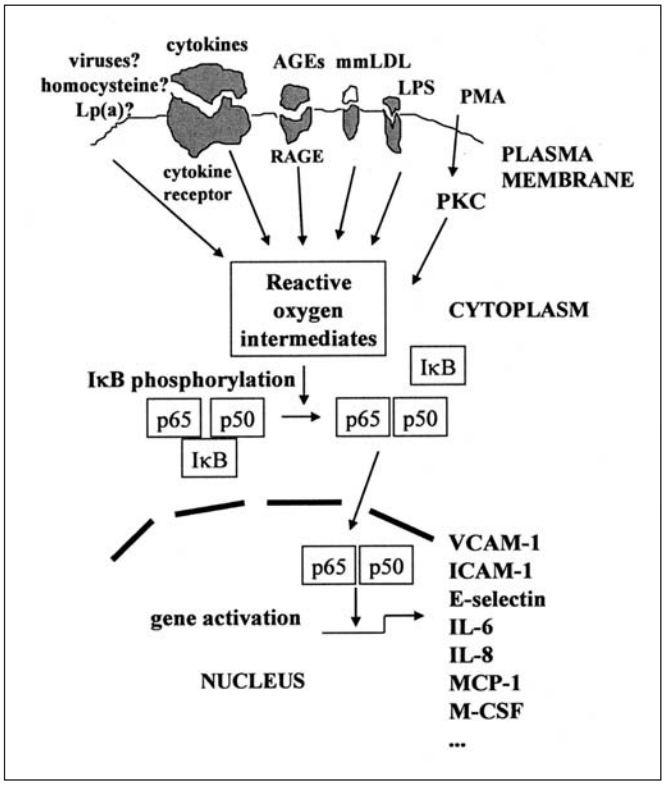

Fig. 4 - *A scheme of the intracellular signal transduction pathways leading to increased gene expression of target genes upon endothelial cell exposure to atherogenic triggers. Redrawn, modified, from (93).*

Possible molecular mechanisms by which unsaturated fatty acids inhibit endothelial activation

In order to ascertain mechanisms for these effects we demonstrated the inhibition of NF-κB activation by DHA (the most potent fatty acid inhibitor of endothelial activation) in parallel with measurements of hydrogen peroxide production by cultured endothelial cells. This reactive oxygen species (or one of more of its downstream unstable products) appears to be a critical mediator of NF-κB activation (Fig. 4). We had previously shown that treatment of endothelial cells with polyethylene glycol (PEG)-complexed superoxide dismutase (a cell membrane-permeable form of this enzyme, which catalyzes the conversion of superoxide anion to hydrogen peroxide) does not much affect VCAM-1 m-RNA production. On the contrary, a treatment with PEG-catalase, which acts by accelerating the degradation of hydrogen peroxide, quenches endothelial activation (80). This suggested that hydrogen peroxide (or some of its downstream products) is more relevant than upstream products (i.e. superoxide anion) in the activation of NF-κB. We also assessed the production of intracellular hydrogen peroxide (and/or its downstream products) by dichloro-fluoresceine before or after stimulation with IL-1 or TNF. In both these experimental systems, we could document a reduction in baseline production of reactive oxygen species after cell membrane enrichment with DHA, and an even more pronounced dampening of the increase produced by stimulation with cytokines. Saturated fatty acids served as a negative control in these experiments. Therefore, our current understanding of these phenomena is that a property related to fatty acid peroxidability (the presence of multiple double bonds), usually regarded as a detrimental consequence of polyunsaturated fatty acid enrichment of cell membranes, is directly related to this putatively favorable outcome (Fig. 5).

These results have spurred the reappraisal of how the action of fatty acids on endothelial cells could modulate, not only general phenomena such as atherogene-

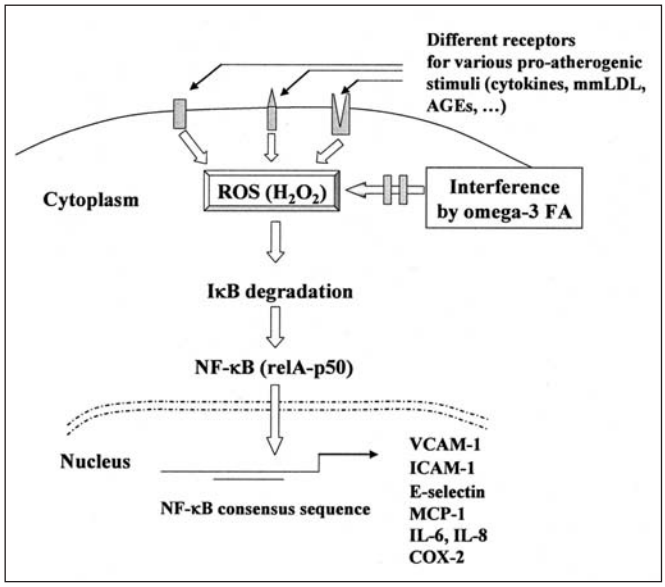

Fig. 5 - *A scheme of the intracellular signal transduction pathways leading to increased gene expression of target genes upon endothelial cell exposure to atherogenic triggers. Redrawn, modified, from (93).*

sis but also, potentially, inflammation or some immune responses. Since all these effects occurred even in the presence of inhibitors of metabolic conversion of fatty acids to eicosanoids, they provide a novel explanation for the modulating effect of n-3 fatty acids in atherogenesis, distinct from the now outdated hypothesis of substrate substitution (97). The results with oleic acid also might partially explain the beneficial effects of olive-oil rich ("Mediterranean") diets on atherogenesis. Notably, incorporation of oleic acid likely occurred at the expense of saturated fatty acids; therefore, disclosing the potentially additive effects of n-3 fatty acids, which mostly substitute less unsaturated fatty acids in the membrane phospholipid pools. If extended to cell types different from endothelial cells such as the monocyte-macrophage that also undergo the "activation" phenomena when stimulated by cytokines or LPS, such effects could provide a coherent explanation for several previous observations such as the inhibition of cytokine formation by LPS-activated macrophages (84). These effects might be associated closely with the peroxidability of polyunsaturated fatty acid.

Future research will further elucidate molecular aspects of these phenomena (90) and expand the greater scope of this research line to explain the many biological effects of unsaturated fatty acids as modulators of biological responses to cytokines.

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