OMEGA-3 FATTY ACIDS, INFLAMMATION AND ANGIOGENESIS: BASIC MECHANISMS BEHIND THE CARDIOPROTECTIVE EFFECTS OF FISH AND FISH OILS

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Abstract – Atherosclerosis is now widely accepted to be an inflammatory disease, characterized by degenerative as well as proliferative changes and extracellular accumulation of lipid and cholesterol, in which an ongoing inflammatory reaction plays an important role both in initiation and progression/destabilization, converting a chronic process into an acute disorder. Neovascularization has also been recognized as an important process for the progression/destabilization of atherosclerotic plaques. In fact, vulnerable atherosclerotic plaques prone to rupture are characterized by an enlarged necrotic core, containing an increased number of *vasa vasorum*, apoptotic macrophages, and more frequent intraplaque hemorrhage. Various functional roles have been assigned to intimal microvessels, however the relationship between the process of angiogenesis and its causal association with the progression and complications of atherosclerosis are still challenging and controversial. In the past 30 years, the dietary intake of omega-3 (n-3) polyunsaturated fatty acids - mainly derived from fish - has emerged as an important way to modify cardiovascular risk through beneficial effects on all stages of atherosclerosis, including plaque angiogenesis. This review specifically focuses on the modulating effects of n-3 fatty acids on molecular events involved in early and late atherogenesis, including effects on endothelial expression of adhesion molecules, as well as pro-inflammatory and pro-angiogenic enzymes. By accumulating in endothelial membrane phospholipids, omega-3 fatty acids have been shown to decrease the transcriptional activation of several genes through an attenuation of the nuclear factor-κB system of transcription factors. This occurs secondary to decreased generation of intracellular reactive oxygen species. This series of investigations configures a clear example of nutrigenomics - i.e., how nutrients may affect gene expression, ultimately affecting a wide spectrum of human diseases.

Key words: Omega-3 fatty acids, n-3 fatty acids, adhesion molecules, endothelium, endothelial activation, atherosclerosis, cyclooxygenase-2, plaque angiogenesis, plaque rupture.

INFLAMMATION IN ATHEROSCLEROSIS

Omega-3 fatty acids have recently emerged as an example of nutrients able to modulate the expression of genes involved in inflammation and atherosclerosis. Their ability to influence such processes is therefore a good example of nutrigenomics. This review summarizes the evidence to this regard. Since a description of the molecular events underlying endothelial changes in atherosclerosis is important for an understanding of the mode of action of these nutrients, a short review of current concepts of inflammation and atherosclerosis gives the background to place their genomic effect in context.

Atherosclerosis is currently regarded as a dynamic process arising from functional inflammatory changes in the endothelium of conduit arteries (34). Here the vascular endothelium, no longer regarded as the passive lining of blood and lymphatic vessels, is now considered a dynamic organ involved in metacrine, paracrine, and endocrine functions (40). By virtue of its strategic location between plasma and the underlying tissue and its constitutive properties, it is endowed with a large array of functions that are vital for body homeostasis. Under physiological conditions, endothelial cells monitor the transport of plasma
molecules, control bidirectional processes of receptor-mediated and receptor-independent transcytosis and endocytosis, “sense” changes in hemodynamic forces and blood-borne signals, and react by synthesizing and releasing vasoactive substances with anti-thrombotic, vasodilating and anti-atherogenic properties to maintain vascular homeostasis (40). However, in the presence of persistent damaging stimuli, such as high levels of atherogenic lipoproteins or high blood pressure, high glucose, high insulin, high homocysteine or products of cigarette smoking, this balance is disrupted, and the vasculature, through a dynamic change in its immunological phenotype, becomes susceptible to atheroma formation (132). Indeed, although the inception of atherosclerosis is strongly correlated with prolonged hyperlipidemia, it has become increasingly evident that there is an active biological component in the development and progression of the disease, both with regard to lipid accumulation and to the increase in its cellular component, characterized by the accumulation of leukocytes in the intima.

In its initial stages (Fig. 1, A), atherosclerosis is characterized by the recruitment, in the intima of conduit arteries, of selected populations of white blood cells, especially monocytes and some T-lymphocytes, and, secondarily, by the gradual accumulation of lipids and extracellular matrix (Fig. 1, B). In such new localization monocytes accumulate lipid droplets first intracellularly (foam cells) and later extracellularly, due to their apoptosis, resulting in the formation of a lipid core. The endothelium and the underlying matrix for long time during atherosclerosis progression prevent the content of the highly thrombogenic lipid core from entering in contact with circulating blood. The sudden rupture of the eventually thin collagen layer overlying the enlarged intima – the so-called fibrous cap –, from the outside or - as recently appreciated - from the inside due to the rupture of vasa vasorum penetrating the intima - or - in a minority of cases – in a condition of endothelial erosion, transforms this slow process into an acute situation, with the sudden development of thrombosis (Fig. 1, C and D) (124). When this occurs in a coronary artery, in appropriate pathophysiological conditions, the consequent myocardial ischemia configures an acute coronary syndrome, i.e., acute myocardial infarction or unstable angina.

Since monocyte recruitment into the intima of large arteries is specific for atherosclerosis as compared to other forms of inflammation, it was hypothesized that these localized monocyte-endothelium interactions reflect specific molecular changes in the adhesive properties of the endothelial surface, due to the endothelial surface expression of specific endothelium-leukocyte adhesion molecules (ELAMs) ("athero-ELAMs"). The first such protein, originally identified in the rabbit hypercholesterolemic model, is vascular cell adhesion molecule (VCAM)-1, a member of the immunoglobulin superfamily. The ligand for VCAM-1 is a heterodimeric integrin receptor, very late antigen 4 (VLA4), whose leukocyte selectivity of expression, on monocytes and lymphocytes, but not on neutrophils, can explain the selectivity of monocyte recruitment in early atherogenesis (38). 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 streaks, in a temporal pattern consistent with its pathogenetic role in lesion development. Pathophysiologically relevant stimuli for VCAM-1 expression include minimally oxidized low density lipoproteins (LDL) or beta-very low density lipoproteins (VLDL), the advanced glycation end-products (AGEs) associated with diabetes, and perhaps lipoprotein(Lp)(a), homocysteine, elevated in homocystinuria and in subtler forms of congenital or acquired enzyme defects in homocysteine biosynthetic pathways, and possibly also high glucose and high insulin, occurring in diabetes and insulin resistance, respectively (95). In addition to these humoral stimuli, the endothelial gene expression of VCAM-1 also responds to hemodynamic forces, potentially explaining the localization of atherosclerosis at particular sites of the arterial vasculature (for a general review on these issues, see (36)). A causal role of VCAM-1 in atherosclerosis is shown by the fact that the expression of a hypomorphic variant of VCAM-1, due to a partial knockout of the molecule, protects against atherosclerosis in the LDL receptor knockout mouse model (32).

Once adhered, monocytes transmigrate, through the action of chemotactic stimuli also deriving from the activated endothelium, from the blood into the subendothelial intima, the innermost layer of the arterial wall, where, upon internalization of modified lipoproteins, they become macrophages able to secrete proinflammatory cytokines. These contribute to
maintaining a state of vascular inflammation, further augmenting the expression of adhesion molecules, chemoattractants and macrophage-activating factors, thus initiating a vicious circle leading to lesion progression (Fig. 1, C). Later on, the production of matrix metalloproteinases (MMPs) by the same macrophages as well as by endothelial cells may promote the erosion of the collagen layer of the fibrous plaque and ultimately lead to plaque rupture (Fig. 1, D) (90).

The stability of the atherosclerotic plaque is indeed thought to reflect the balance and interplay of various dynamic factors, including endothelial dysfunction, the proliferation of smooth muscle cells, which are mainly responsible for the synthesis of collagen, and the degradation of collagen and other elements of the extracellular matrix by MMPs, mostly a product of activated macrophages.

The much sought-after prevention of acute coronary events requires therefore interventions that affect such cellular and molecular mechanisms leading to both the slow formation of atherosclerotic lesions and the precipitation of plaque rupture.

INFLAMMATORY ANGIOGENESIS AS A PROMOTER OF PLAQUE GROWTH AND INSTABILITY

Recent evidence has linked “plaque angiogenesis”, the sprouting of new capillaries from pre-existing ones within the vessel wall, with progression and instability of atherosclerosis (Fig. 2) (49). Normally, the adventitial layer of human arteries contains a microvascular network, termed *vasa vasorum*, that delivers oxygen and nutrients to the outer layers of the arterial wall. In contrast to the adventitia and, occasionally, the outer media, the inner media and the intima do not contain capillaries, since the diffusion of oxygen and other nutrients, limited to 100 µm from the lumen in normal arteries, is adequate to nourish the inner media and intimal layers (84, 153). However, during the progression of atherosclerosis, largely immature neo-microvessels appear in the media and the thickened intima in more that 40% of lesions (84, 153). The possibility that such neovascularity might play a role in plaque growth and destabilization was first hypothesized by Winternitz (158) and Geringer (55) in the late 1930’s and 1940’s, respectively, and partially demonstrated by Barger in the mid 1980’s (7). Later on, several reports pointed-out that plaque neo-angiogenesis highly contributes to plaque instability, showing a positive association between the neovascularization in the intima and the media on the one hand, and symptomatic carotid occlusive disease on the other (101, 103), as well as between the density of coronary adventitial vessels reaching the intima and the extent of lumen stenosis (81). Furthermore, it has been more recently reported that the number of *vasa vasorum* is increased up to 2-fold in vulnerable plaques and up to 4-fold in ruptured plaques compared with stable plaques with severe luminal narrowing (154). The entrance of neo-vessels into the intimal space from the adventitia occurs specifically at breakpoints in the medial layer, below the sites of formation of early necrotic cores, following a typical maturation sequence: the neo-vessels first divide as they approach the core with secondary and tertiary branches, but while microvessels close to the medial wall are well formed, being accompanied by surrounding mural pericytes and smooth muscle cells, the intimal vessels near the lumen appear immature and therefore less functional, too small to conduct adequate blood flow to ischemic sites, and more prone to rupture (154). Intraplaque hemorrhage from these microvessels may contribute to plaque instability and/or expansion. A similar pattern occurs in proliferative diabetic retinopathy, where the neovessels are mechanically weak and more prone to bleed (12). Since these weak microvessels are often accompanied in their development by a recruitment of T-helper cells (119), it has been hypothesized that the T-cells release of interferon(IFN)-γ may inhibit smooth muscle cell proliferation and therefore actively contribute to the absence of mural smooth muscle cells in perforating neovessels (23, 50). Furthermore, the augmented expression of endothelial leukocyte adhesion molecules by the intimal microvascular endothelium suggests that neovessels may actively recruit inflammatory cells into the lesions (111, 112), thus allowing the establishment of a positive feedback loop whereby inflammatory cells stimulate plaque angiogenesis, and the plaque microvasculature promotes further recruitment of inflammatory cells.

The molecular mechanisms underlying plaque angiogenesis in vascular disease are now being elucidated. Hypoxia, inflammation, and mechanical forces, such as shear stress, may
Figure 1. Cellular and molecular events leading to atherosclerotic plaque formation. The illustration schematizes steps (from A to D) in the recruitment of mononuclear phagocytes into the nascent atherosclerotic plaque, and some of the functions of the endothelium and of macrophages in the mature atheroma. The steps are depicted in an approximate time sequence proceeding from left to right (for a more detailed description see related text).

Abbreviations: VCAM-1=vascular cell adhesion molecule-1; MCP-1=monocyte chemoattractant protein-1; CCR2=C-C chemokine receptor-2; ROS=reactive oxygen species; LDL=low density lipoproteins; MMP=matrix metalloproteinases.

Figure 2. Formation of neovessels. Angiogenesis occurs as an orderly series of events: 1) diseased or injured (hypoxic) tissues produce and release angiogenic growth factors (vascular endothelial growth factor (VEGF), cytokines, angiopoietin-1, etc.), that diffuse into the surrounding tissues; 2) angiogenic growth factors bind to specific receptors located on the endothelial cells. The endothelial cells then become activated, and signals are sent from the cell surface to the nucleus; 3) endothelial cells then begin to produce new molecules, including matrix metalloproteinases (MMP), which degrade the basement membrane surrounding the diseased vessels; 4) the endothelial cells begin to divide (proliferation), and then to migrate through the dissolved basement membrane towards the diseased tissue; 5) specialized molecules, such as the integrin αβ3, serve as hooks to pull the sprouting new vessel forward.

Abbreviations: Tie-1=tyrosine kinase with immunoglobulin and epidermal growth factor homology domains; Flk/KDR=fetal liver kinase-1/kinase insert domain–containing receptor; MMP=matrix metalloproteinase; PGI2=prostacyclin; TXA2=thromboxane A2.
activate endothelial cells or cause the release of growth factors or cytokines involved in the process known as abluminal sprouting – the conventional mechanism by which new vessels grow from an existing vessel (Fig. 2). Hypoxia is recognized as one of the most potent stimuli for angiogenesis, and zones of hypoxia are present within thickened atherosclerotic plaque (10). Hypoxia-induced angiogenesis is regulated by hypoxia inducible factor-1 (HIF-1), a heterodimeric transcription factor composed of an HIF-1-α and an HIF-1-β subunits. In order to respond rapidly to hypoxia, cells continuously synthesize, ubiquitinate, and degrade HIF-1-α protein under normoxic conditions. Under hypoxic conditions, such degradation is inhibited, resulting in HIF-1α accumulation, dimerization with HIF-1-β, binding to hypoxia-responsive elements within target genes, and the activation of transcription through recruitment of the coactivators p300 and CREB binding protein (CBP) (67). HIF-1 is known to activate, at the transcriptional level, the expression of more than 40 genes, including those responsible for the production of nitric oxide (nitric oxide synthase III) and vascular endothelial growth factor (VEGF) (67). The initial step in angiogenesis, in fact, involves nitric-oxide-mediated vasodilation. Vascular permeability subsequently increases in response to VEGF, allowing the extravasation of plasma proteins that lay down a provisional scaffold for migrating endothelial cells (67). Thus, it appears likely that hypoxia within the plaque core is an important trigger of proangiogenic activity in established vascular lesions that exhibit significant neointimal thickening.

On the other hand, a role for vessel wall hypoxia and plaque angiogenesis in early atherosclerotic disease has been studied, but not yet firmly established. An increased expression of proangiogenic growth factors (including VEGF) seems to precede intimal thickening in some animal models, such as the hypercholesterolemic swine, where coronary neovascularization precedes the development of endothelial dysfunction (65). Furthermore, the experimental occlusion of adventitial vasa vasorum leads to intimal lesions that resemble atherosclerosis (8). Adding to the complexity of this biology, hypoxia-independent pathways for the modulation of angiogenesis within the vessel wall have also been identified. For example, hypertension has been shown to induce vessel wall neovascularization by activation of the HIF-1 system (82), and a direct induction of VEGF expression by smooth muscle cells exposed to stretch has also been demonstrated (151). Finally, oxidative stress (79) and nicotine (30) seem to modulate growth factor gene expression in vascular cells independent of hypoxia.

That these microvessels are functionally important in atherogenesis and its complications is also illustrated by the ability of angiogenesis inhibitors, such as angiostatin and TNP-470, to reduce angiogenesis and inhibit the development of lesions in apoE knockout mice (106).

**The role of COX-2 as potential regulator of inflammatory angiogenesis and plaque instability**

Due to the ability of prostanoids to contribute to the complex process of neovascularization (54), the proinflammatory enzyme cyclooxygenase(COX)-2 is now regarded as a critical inducer of angiogenesis (85). Prostanoids include prostaglandin(PG)D2, PGF2α, prostacyclin (PGI2), and thromboxane(TX) A2, and their biosynthesis involves 3 sequential enzyme-catalyzed steps (Fig. 3): (1) agonist-induced phospholipase activation, leading to the release of arachidonic acid (AA) from membrane phospholipid pools; (2) cyclooxygenase(COX)-catalyzed oxygenation of the free fatty acid to generate the cyclic endoperoxide PGH2; and (3) enzymatic rearrangement of the PGH2 structure to yield one of several bioactive derivatives. While the first 2 steps are shared by virtually all human cell types, the expression of downstream prostanoid synthases displays considerable cell type specificity (137).

Prostanoids mediate angiogenesis through multiple mechanisms, including: (1) the induction of VEGF production (93); (2) the stimulation of endothelial cell sprouting, migration and tube formation (58, 83, 107); (3) the enhancement of endothelial cell survival, through the upregulation of the anti-apoptotic protein B-cell lymphoma(Bcl)-2 (92) and the activation of phosphatidylinositol(PI3)-kinase(K)-Akt pathway (4, 44); (4) the up-regulation of MMP-2 (21, 24) and MMP-9 (116), which are required for vascular invasion; (5) the promotion of angiogenic functions of the αβ3 integrin (46, 47); (6) the activation of the epidermal growth factor receptor (EGFR), causing downstream angiogenic events (115); and (7) the decreased production of the endogenous angiogenesis inhibitor interleukin(IL)-12 (128).
Several reports have demonstrated the presence of both mRNA and protein for COX-2 in macrophages and smooth muscle cells of atherosclerotic plaques, as well as of COX-1 and COX-2 in the endothelium of both atherosclerotic and healthy vessels (127, 141), thus suggesting a potential pathogenetic role for COX-2 activity. Furthermore, the finding of a co-localization of COX-2, MMP-9 and membrane type(MT)-1 MMP in the endothelial lining of vasa vasorum in human atherosclerotic aortas suggests a COX-2 mediated promotion of plaque angiogenesis (69).

Besides contributing to plaque neovascularization (11), several other mechanisms have been hypothesized by which COX-2 inhibition may prevent atherothrombosis. COX-2 directly contributes to plaque instability, favoring MMP release in macrophages, as observed in vitro (31) and in vivo in atherosclerotic lesions obtained from patients with symptomatic carotid artery disease, where the simultaneous overexpression of functionally coupled COX-2, PG synthase and MMP has been reported (26). Secondly, PGs exert potent actions on vascular smooth muscle cells, regulating contractility (48, 113), cholesterol metabolism (159), and proliferation (160). The increased expression of COX-2 may thus contribute to the accumulation of lipids in lesional smooth muscle cells, besides macrophages, favoring the formation of smooth muscle cell- and macrophage-derived foam cells within the atheroma. Conversely, anti-proliferative and anti-migratory actions of COX-2 products on smooth muscle cells (160) contribute to the evolution of a lesion toward a smooth muscle cell-depleted and macrophage-enriched plaque phenotype, and thus a more vulnerable plaque (71).

In spite of these well-documented proatherogenic functions (25), the usefulness of inhibiting COX-2 activity is still controversial (140). In murine models of atherosclerosis, treatment with selective COX-2 inhibitors has been reported to decrease, increase or have no impact on atherosclerosis (9, 17, 18, 117, 125). These studies suggest that the role of COX-2 in atherosclerosis is complex and may vary with lesion stage or the animal model. A critical review of the experimental design of the studies so far reported reveals several plausible explanations for the variability of the results. Studies reporting a beneficial effect of selective COX-2 inhibition on atherosclerosis mostly looked at the impact of treatment on early atherosclerotic lesion formation, whereas studies showing no effect evaluated the impact on advanced lesions. Thus, one can hypothesize that COX-2 contribution in atherogenesis varies temporally according to the stage of atherosclerotic lesion formation. In addition, clinical consequences of COX-2 inhibition strongly depends on the inhibition of production of anti-thrombotic COX-2-dependent mediators, such as prostacyclin, explaining the overall adverse vascular effects of selective COX-2 inhibitors (coxibs).

**CARDIOVASCULAR PROTECTION BY OMEGA-3 FATTY ACIDS**

Polyunsaturated fatty acids (PUFA): structure, source and metabolism

Polyunsaturated fatty acids (PUFA) are organic acids naturally containing more than one double bonds in their aliphatic chain. It is the number and the position of double bonds within the hydrocarbon chain that give PUFA their name and their physical and physiological properties. Biologically relevant families of PUFA are the omega-3 and the omega-6 fatty acids. In the omega-3 fatty acids the terminal double bond is on the third carbon (from the methyl end of the hydrocarbon chain, Fig. 4). In this respect, they are structurally distinct from the more commonly encountered omega-6 fatty acids in which the terminal double bond is on the sixth carbon atom.

The simplest members of omega-6 and omega-3 families are linoleic (18:2n-6, LA) and α-linolenic (18:3n-3, ALA) acids respectively (Fig. 4). Since the double bond in the n-3 or n-6 position cannot be inserted into fatty acids by animal enzymes (but only by vegetarianΔ12 - and Δ15-desaturase), LA and ALA acids represent “essential nutrients” for mammals, as was shown in the late 1920’s when omega-3 and omega-6 deprivation experiments performed in rats identified the “essential fatty acid deficiency” syndrome as similar to other “essential nutrient deficiencies” syndromes (19, 20). It is estimated that minimum human requirements for these fatty acids are 1 and 0.2% of daily energy intake for omega-6 and omega-3 fatty acids, respectively (42). Because they are synthesized by plants, plant tissues and oils are good sources of LA and ALA. Green plant tissues are especially rich in ALA, which typically comprises 55% of the fatty acids present in green vegetables. However such tissues are not rich in fat, and therefore this
Figure 3. Metabolism of omega-3 fatty acids versus omega-6 fatty acids by cyclooxygenases and lipoxygenases. FA=fatty acid(s); PGH$_2$=prostaglandin H$_2$; PGI$_2$=prostacyclin; TXA$_2$=thromboxane A$_2$; PGD$_2$=prostaglandin D$_2$; PGE$_2$=prostaglandin E$_2$; PGF$_{2\alpha}$=prostaglandin F$_{2\alpha}$; HPETE=hydroperoxy eicosatetraenoic acids; EET=epoxyeicosatrienoic acids; LT=leukotrienes; PGI$_3$=prostaglandin I$_3$; PGA$_3$=prostaglandin A$_3$; TXA$_3$=thromboxane A$_3$; PGF$_{3\alpha}$=prostaglandin F$_{3\alpha}$; GSH=glutathione; PLA$_2$= phospholipases A$_2$; AA=arachidonic acid; EPA=eicosapentaenoic acid; DHA=docosahexaenoic acid.

Figure 4. Chemical structure and pathway of conversion of linoleic and $\alpha$-linolenic acid into longer derivative.
source does not make a sufficient contribution to the minimum intake of this fatty acid in humans. In contrast, some plant oils, such as corn, sunflower and rapeseed oil, can make a significant contribution to the intakes of these fatty acids and of their longer derivatives. In fact, although mammalian cells cannot synthesize LA and ALA, they can metabolize them by further desaturation and elongation (Fig. 4). Linoleic acid can be converted into γ-linolenic acid (18:3n-6) by Δ6-desaturase, and then γ-linolenic acid can be elongated (by elongase) to dihomo-γ-linolenic acid (20:3n-6). Dihomo-γ-linolenic acid can be desaturated further by Δ5-desaturase to finally yield arachidonic acid (20:4n-6, AA). Using the same series of enzymes, ALA is converted into eicosapentaenoic acid (20:5n-3, EPA) (Fig 4). The further conversion of EPA into docosahexaenoic acid (22:6n-3, DHA) involves a first addition of two carbon atoms to form docosapentaenoic acid (22:5n-3, DPA), the addition of two further carbon atoms to produce 24:5n-3, and a desaturation to form 24:6n-3. The removal of two carbon atoms from 24:6n-3, by limited β-oxidation, yields DHA (139).

In mammals, these pathways of desaturation and elongation occur mainly in the liver, where Δ6-desaturase constitutes the rate-limiting enzyme (161). The preferred substrate for Δ6-desaturase is ALA (161); however, being LA much more abundant than ALA in most human diets, metabolism of n-6 fatty acids is quantitatively more important. Furthermore, recent studies have revealed that the conversion of ALA into its longer-chain derivatives is, altogether, scarcely efficient in adult humans, especially in males (14, 16). This means that the direct intake of the long-chain derivatives EPA and DHA is by far the easiest way to increase the concentration of such fatty acids in human tissues.

A good dietary source of long-chain n-3 fatty acids is fish. Fish can be classified into “lean” fish, that store fat as triacylglycerols in the liver (for example cod), or “fatty” fish that store fat as triacylglycerols in the flesh (e.g., mackerel, herring, salmon and tuna). The oil obtained from the fatty fish flesh or from the liver of lean fish is termed ‘fish oil’, and has the distinctive characteristic of being rich in long-chain omega-3 fatty acids. Different oily fish (and therefore different fish oils) contain different amounts of EPA and DHA (Table 1) and this seems to be related to the fish dietary habits and metabolic characteristics (64, 91), as well as to season, water temperature and phase in the breeding cycle (147).

In general, the non-esterified fatty acids (NEFA) present in the diet are rapidly and efficiently absorbed entering cells via FA transporters, where are rapidly converted to FA acyl-CoA thioesters before undergoing three main metabolic fates. First, they contribute to ATP production by the classical β-oxidation pathway. In men, following the ingestion of [13C]-labelled PUFA of different length and degree of unsaturation, the recovery of 13CO2 in breath ranges between 20–30% of the proportion ingested, in the following order: about 20% for n-9, 25% for n-6 and 30% for n-3 PUFAs (15). Secondly, FA acyl-CoA is also a substrate for the synthesis of neutral (triglycerides, cholesterol esters) and polar (phospholipids, sphingolipids, etc) lipids. In human adipose tissues, it has been calculated that the percent content of LA is about 11.0% versus 0.7% of ALA (15). Thirdly, as above described, FA acyl-CoA may be subjected to a complex series of elongation/desaturation reactions to generate long chain PUFA. Since the biosynthesis of FA and phospholipids occurs in the endoplasmic reticulum, the intermediates of PUFA metabolism can either be incorporated into phospholipids or become the substrate for a further elongation/desaturation reaction. When in the membranes, PUFA contribute to membrane fluidity, which is an important determinant of the correct hormone-receptor binding. A good example for this is represented by insulin receptors. Insulin resistance is associated with plasma membrane stiffening, a condition which should limit the number of insulin receptors. On the opposite, increased cell membrane fluidity by increasing PUFA concentration may result in an enhanced number of insulin receptors and in an increased affinity of insulin to its receptors, and hence in reduced insulin resistance (33). PUFA esterified in phospholipids also however exert important signaling activities. When esterified in the sn-2 position of phospholipids, the n-6 FA AA as well as the n-3 FA EPA and DHA can be released through the action phospholipase A2 and metabolized through “orthodox” and “unorthodox” pathways (Fig. 3). The “orthodox pathway” involves reactions catalyzed by COX and lipoxygenases (LO) to eicosanoids, including PGs, TXs, and leukotrienes (LT), which are mediators of a vast number of biologic effects (see below). AA is the precursor of the prostanoids of the 2-series (including PGI2 and...
activities (129) (Fig. 3), whereas EPA is the precursor of prostanoids of the 3-series (PGI3 and TXA3). Increasing the content of n-3 FA in the diet causes a partial substitution of the FA of the n-6 series, especially decreasing the relative proportions of AA in cell membrane phospholipids. This causes a net decrease in the production of prostanoids (because n-3 FA are worse substrates for the metabolizing enzymes) and favors the synthesis of generally less biologically active prostanoids, especially TXA3, which, contrary to AA-derived TXA2, has weak platelet-aggregating and vasoconstricting activity. The results of these changes in eicosanoid production are vasodilatation and inhibition of platelet aggregation. In leukocytes and monocytes, AA and EPA are substrates of 5-LO for the synthesis of LT. LTB4, derived from AA, has potent chemotactic and leukocyte-activating properties, whereas sulfido-peptide LT (LTC4, LTD4, LTE4) have vasoconstrictive effects and can increase vascular permeability. Through 5-LO, EPA gives rise to LT of the 5-series, namely LTB5, LTC5, LTD5, and LTE5, which have weaker proinflammatory and vasoconstrictive activities than those of the 4-series. On the contrary, in endothelial cells, AA and EPA are precursors of the almost equipotent PGI2 and PGI3, respectively, both endowed with anti-aggregating and vasodilating properties.

In addition to these metabolic pathways, the existence and the beneficial contribution of an “unorthodox” generation of alternative eicosanoid derivatives of n-3 FA has been recently appreciated. Such alternative compounds are typically produced during the resolution of self-limited inflammation. These compounds were first identified by Serhan et al. with the trivial name of resolvins (resolution phase interaction products), to emphasize their original isolation and production during the resolution phase of acute inflammation and to signify the frequent contribution of the transcellular biosynthesis of these new mediators. The production of resolvins is mediated by the serial combined activities of acetylated COX-2 (or cytochrome P-450 monoxygenase) and 5-LO on EPA, to produce the E-series resolvins (RV) E1 and E2, and on DHA, to produce the 17R D-series resolvins (RvD1 through RvD4). Upon tissue specialization, DHA can be also metabolized by 15-LO to produce the 17S D-series resolvins, with potent anti-inflammatory activities (129) (Fig. 3).

In addition, the formation of another set of compounds, formed by the nitration of unsaturated fatty acids, called nitrolipids, has been shown to occur in vivo and to have potent biological actions. Such derivatives are known as nitro-fatty acids (NO2-FA) and display both proinflammatory and anti-inflammatory activities (53). They are biologically active prostanoids, especially TXA3, which, contrary to AA-derived TXA2, has weak platelet-aggregating and vasoconstricting activity. The results of these changes in eicosanoid production are vasodilatation and inhibition of platelet aggregation. In leukocytes and monocytes, AA and EPA are substrates of 5-LO for the synthesis of LT. LTB4, derived from AA, has potent chemotactic and leukocyte-activating properties, whereas sulfido-peptide LT (LTC4, LTD4, LTE4) have vasoconstrictive effects and can increase vascular permeability. Through 5-LO, EPA gives rise to LT of the 5-series, namely LTB5, LTC5, LTD5, and LTE5, which have weaker proinflammatory and vasoconstrictive activities than those of the 4-series. On the contrary, in endothelial cells, AA and EPA are precursors of the almost equipotent PGI2 and PGI3, respectively, both endowed with anti-aggregating and vasodilating properties.

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Epidemiologic and experimental evidence for atheroprotective effects of omega-3 FA

The cardioprotective effects of omega-3 FA have long been recognized. The original observation is dated almost 50 years ago, when Hugh Sinclair published his seminal observations on the negative effects of some essential fatty acids deficiency on the development of cardiovascular disease (136). He strengthened his hypothesis noting the low incidence of mortality from coronary heart disease in Greenland Eskimos, a population consuming a high fat diet, but rich in omega-3 FA (136). Late in the seventies, Sinclair’s group, as well as Dyerberg and others confirmed the positive association between the high dietary intake of EPA and DHA of Greenland Inuits and the lower rate of death from acute myocardium infarction compared with a Danish population, although these two groups consumed similar amount of total fat (about 42% of total calories) and showed comparable levels of blood cholesterol (51). Afterwards, similar observation were also done for Eastern populations such as the Japanese (162) and the Chinese (163), consuming traditional diets rich in oily fish. In Western populations with a generally low intake of omega-3 FA, both protective effects (2, 45, 80, 109, 110) and no effects (5, 70, 104, 131) of omega-3 FA on cardiovascular disease have been reported. This can be explained by the difficulty in maintaining constant feeding habits in a population during long observational studies and by the competing influence of other dietary principles, including saturated or other unsaturated fatty acids.
The epidemiological association between dietary omega-3 FA and protection from cardiovascular disease can be attributed, at least in part, to a decreased extent of atherosclerosis. Many experimental studies have clearly demonstrated that omega-3 fatty acids favorably affect many of the factors involved in the development of atherosclerosis (Table 2). Numerous animal studies have shown decreased atherosclerosis in animals treated with omega-3 fatty acids (reviewed in (39, 41, 138)). Furthermore, evidence has been obtained about such effects in humans, through autopsy studies in Alaskan natives (consuming high amounts of fish-derived products) and non-natives, mostly consuming Western-type diets. In the study by Newman and coworkers, the magnitude of difference in fatty streak development appears larger in younger age groups (108), suggesting an effect of diet mainly in the early events leading to fully developed atherosclerotic lesions. Furthermore, a study of omega-3 fatty acids supplementation after coronary bypass surgery indicated that such treatment significantly reduces vein graft stenosis (52), a process which may be regarded as an accelerated form of atherosclerosis.

Table 1. EPA and DHA contents of various seafoods and other foods ac

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<th>Food</th>
<th>Omega-6 LA (mg/100g)</th>
<th>Omega-6 AA (mg/100g)</th>
<th>Omega-3 ALA (mg/100g)</th>
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*: data reported as mg/100 g  
*: traces or nothing  
c*: content of n-6, n-3 FA may slightly vary according to species, sources and analytical methods.

Table 2. Effects of omega-3 polyunsaturated (PUFA) fatty acids on factors involved in the development of atherosclerosis

<table>
<thead>
<tr>
<th>Proatherosclerotic factor</th>
<th>Effect of omega-3 PUFA</th>
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<tr>
<td>Plasma triacylglycerol concentration</td>
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<td>Production of growth factors</td>
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<td>Cell surface expression of adhesion molecules</td>
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<td>Cardiac arrhythmias</td>
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<td>Atherosclerotic plaque stability</td>
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Modulation of endothelial-leukocyte interactions and endothelial activation by omega-3 FA

Our own research has disclosed potential mechanistic explanations for the preventive or therapeutic use of omega-3 FA. We hypothesized that omega-3 FA modulate atherogenesis by affecting processes of early atherosclerotic development leading to the formation of fatty streaks. Such processes are mostly comprised - as illustrated above - under the name of “endothelial activation”. We used human adult saphenous vein endothelial cells or human umbilical vein endothelial cells activated by cytokines, as 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 such effects. In particular, we observed that DHA, when added to cultured endothelial cells hours to days before the stimulation with cytokines, early enough to allow a significant incorporation in cell membrane phospholipids, significantly inhibited events connected with endothelial activation, 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 (36, 37). The inhibition of adhesion molecule expression occurred in a range of DHA concentrations compatible with nutritional supplementation of this FA to a normal Western diet, occurred at any time point after the appearance of the cytokine effect, modifying the kinetics of surface expression of adhesion molecules, and was strictly related in magnitude to the extent of incorporation into total cell lipids. Indeed, the extent of VCAM-1 inhibitory effect paralleled the incorporation of DHA in cellular phospholipids, and was inversely related to the content of omega-6 FA (36, 37). This effect was not limited to the expression of transmembrane molecules involved in leukocyte recruitment, but appeared to occur also 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 chemotraction for granulocytes (IL-8), and was accompanied by a functional counterpart, i.e. a reduced monocyte or monocytoid cell adhesion to cytokine-activated endothelium (36, 37). Experiments following the fate of $^{14}C$-labelled DHA into cell phospholipids showed a significant incorporation of DHA into the phosphatidyl ethanolamine pool, i.e. in a specific and not particularly abundant phospholipid pool, likely in the inner plasma membrane, and therefore in a possibly strategic position to alter intracellular signal transduction pathways. To investigate the mechanism of DHA action on endothelial activation, we analyzed the effects of various FA (differing in chain length, number, position - omega-3 vs omega-6 vs omega-9 - and cis/trans configuration of the double bond) on the stimulated expression of VCAM-1. We observed the lack of any effect by saturated FA, and an increase of potency of PUFA in parallel with the number of unsaturations, independently of the chain length and configuration (cis vs trans) of the double bond (35). In the same experimental conditions we also measured the intracellular cytokine-induced production of reactive oxygen species (ROS) believed to act as critical second messengers in the signaling pathway leading to the activation of adhesion molecule expression and hence to endothelial activation. In parallel with the modulation of VCAM-1 expression, a reduction of intracellular ROS release was also evident, proportional to the number of double bonds in the fatty acid chain (98). This suggested that a property related to fatty acid peroxidability (the presence of multiple double bonds), usually regarded as a detrimental consequence of PUFA enrichment of cell membranes, is probably also directly related to the inhibition of ROS release/production, an event crucial for cell responsiveness to cytokines. Whether however PUFA protective effects occur through a direct quenching of free ROS on the double bonds or indirectly, through the production of metabolically active oxidized products, is still a matter of debate.

These data are in not in contrast with the possibility that a product of omega-3 FA oxidation inhibits cytokine-induced VCAM-1 expression through a peroxisome proliferators activated receptor (PPAR)$\alpha$-dependent mechanism, as observed by Sethi and cowoker (130). PPARs are ligand-activated transcription factors belonging to the nuclear receptor superfamily, which also includes the steroid and thyroid hormone receptors (96). Originally cloned in an attempt to identify the molecular mediators of peroxisome proliferation in the liver of rodents (73), after their identification also in endothelial cells (72) many experimental data have accumulated regarding their function as regulators of lipid and glucose metabolism and of inflammatory gene expression in vascular cells (96, 164). The PPAR family is comprised of three members, $\alpha$, $\gamma$ and $\beta/\delta$. PPAR$\alpha$ is found in tissues where fatty acid catabolism is important (the liver, the kidney, the heart, and the muscle), and regulates genes that are involved in lipid and lipoprotein metabolism (155). PPAR$\alpha$ natural ligands include polyunsaturated fatty acids (DHA and EPA), oxidized phospholipids, lipoprotein lipolytic products and synthetic ligands such as fenofibrate and gemfibrozil, clinically used to treat patients with elevated serum triglycerides (43). PPAR$\gamma$, conversely, controls adipocyte differentiation and lipid storage (148). PPAR$\gamma$
natural ligands are the PGD2 derivative 15-deoxy-
Δ12,14-prostaglandin J2 and forms of oxidized linoleic acid, 9- and 13(S)-hydroxyoctadecadienoic acid (HODE). PPARβ/δ is expressed in many tissues and, like PPARα, plays a role in lipid metabolism by stimulating fatty acid oxidation in the heart and the skeletal muscle (13). It is known that synthetic PPARα agonists decrease the transcription of VCAM-1 and the adhesion of monocytes to cytokine-activated endothelial cells (97). Sethy and coworkers observed that oxidized EPA, activating PPARα, reduces cytokine- and LPS-induced NF-κB activation (102), VCAM-1 expression and the adhesion of monocytes in vivo. This effect was seen to occur in a PPARα-dependent fashion because oxidized EPA did not show any effect in LPS-treated PPARα-deficient mice (130).

Modulation of inflammatory angiogenesis and plaque stability by omega-3 FA

A recent study has disclosed a further potential role for omega-3 FA in promoting a decrease in the risk of atherosclerotic plaques rupture in patients awaiting carotid endarterectomy (146). In this study, plaques from patients taking fish oil featured a clear incorporation of omega-3 FA into plaque lipids, and this correlated with a reduced macrophage infiltration and thicker fibrous caps compared with plaques from patients assuming sunflower oil-enriched control capsules (146). In the attempt to find out the mechanistic basis of such plaque-stabilizing effect, an anti-angiogenic activity as well as a reduction of MMP release by omega-3 FA might well be invoked. We specifically looked at the effects of omega-3 FA on angiogenesis and MMP expression in atherosclerosis. Studies of this kind are present in the literature in the area of cancer and tumor angiogenesis, from which a number of paradigms are more and more nowadays transferred to cardiovascular medicine (105). In the early 1990’s, Rose and Connolly found that dietary omega-3 FA inhibited the growth and development of metastases from transplanted MDA-MB-435 human breast cancer cells in the athymic nude mice (28). The invasive potential of these cells, when evaluated in vitro, also appeared to be reduced when the cells were incubated with EPA and DHA (123). The same authors subsequently expanded these results showing that the effect of DHA on tumor growth could be explained by the reduction of tumor angiogenesis (microvessel count) and the production of PGE2, known to affect the release of MMP (122). In this study, the release of MMP was not assessed, but using similar cancer cell transplantation in the nude mice model, Suzuki and coworkers showed that the inhibition of lung metastases of a colon cancer cell line by EPA and DHA was associated with a reduced activity of MMP-9 (143). Such a reduction in MMP-9 activity was also found by Harris and coworkers in the uterus, the placenta and the liver of rats fed diets enriched with DHA (59). These authors explained their findings according to the known competition hypothesis of omega-3 FA with omega-6 FA (mostly AA) for being substrates for COX. This would have consequently changed the production of PGE2, thus in turn affecting MMP activity. Such in vivo anti-angiogenic activities of omega-3 FA have been confirmed in in vitro models of angiogenesis. It has been recently reported that omega-3 FA reduced the induction of endothelial angiogenic markers such as the expression of angiopeptin-2 and related receptor Tie-2, as well as the expression of VEGF receptor 1 and 2 as induced by VEGF and basic fibroblast growth factor (bFGF) (144). Accordingly, Kanayas et al. (76), Tsuji et al. (150), Suzuki et al. (152) and Szymczak et al. (144), who independently tested bovine and human endothelial cells treated with omega-3 FA in different angiogenesis models, found an inhibition of tube formation and maturation. Similar results were confirmed in our laboratory using human umbilical vein endothelial cells (HUVEC). By performing Matrigel assays, which allow to detect the formation of capillaries in a tri-dimensional collagen matrix, we observed that the exposure of HUVEC to DHA reduced the vascular endothelial tube-like formation induced by VEGF and phorbol-myristate acetate (PMA), as determined by the reduced number of branching points formed by DHA-treated HUVEC on the matrix surface (100). Such anti-angiogenic effects have been even more elegantly confirmed in a recent work by Connor et al. showing that, with the increase of the omega-3 FA tissue levels as obtained in mice by dietary or genetic means, a decrease in the avascular area of hypoxic retinas was observed as the result of both reduced vaso-oblitration and retinal neovascularization (29).

In agreement with what observed by Jones and coworkers (74), we also found an anti-angiogenic effect after cell treatment with NS-398, an inhibitor of COX-2 activity, thus further
supporting the hypothesis of a pro-angiogenic role of COX-2 metabolites. This made the hypothesis plausible that DHA might exert an anti-angiogenic effect also reducing COX-2 expression.

**COX-2 gene expression: a pivotal role for NF-κB**

There are two known isoforms of COX, COX-1 and -2. The gene for COX-1, located on human chromosome 9, is approximately 22 kb in length with 11 exons, and is transcribed as a 2.8 kb mRNA. COX-1 is a glycoprotein of 576 amino acids with an apparent molecular mass of 69 kDa. There are several putative transcriptional regulatory elements in the promoter region, including two Sp1 motifs, two activator protein (AP)-2 sites, a nuclear factor for interleukin-6 (NF-IL-6) motif, and a GATA site, but the lack of a TATA box, linked to the high frequency of GC repeats, makes COX-1 a typical housekeeping gene (156). It is indeed constitutively expressed in many tissues including the vascular endothelium, monocytes, platelets, renal collecting tubules and seminal vesicles (145). The gene for COX-2, located on human chromosome 1, is approximately 8.3 kb long with 10 exons, and is transcribed as 4.6, 4.0, and 2.8 kb mRNAs variants (68). The gene structures of COX-1 and COX-2 demonstrate remarkable conservation of exon–intron junctions (145). The differences between COX-1 and COX-2 genes consist on the following: (a) the first intron in COX-1 is lost in COX-2 and (b) the introns in the COX-2 gene are shorter than those in the COX-1 gene. The largest exon in the COX-2 gene encodes the entire 3’-untranslated region (3’-UTR), containing 23 copies of the ‘ATTTA’ RNA instability element (118). Sequence analysis of the 5’-flanking region has shown several potential transcription regulatory elements, including a TATA box, a nuclear factor (NF)-IL6 motif, two AP-2 sites, three Sp1 sites, two NF-κB sites and a cyclic adenosine mono-phosphate response element (CRE) (Fig. 5). However only a limited number of elements, namely CRE, NF-IL6 and NF-κB, are known to be critically involved in the regulation of the endothelial COX-2 gene expression (145).

NF-κB comprises a family of redox-sensitive transcription factors recognized as critical regulators of the expression of many pro-inflammatory genes, such as those encoding for adhesion molecules (VCAM-1, ICAM-1 and E-selectin), chemokines, cytokines, and enzymes producing inflammatory mediators, such as COX-2 (114). Sequencing of the endothelial COX-2 promoter region has revealed the presence of two NF-κB consensus sites (145), and their importance in COX-2 induction has been clearly shown by studies using deleted and site-mutated promoter constructs (145). Active NF-κB complexes are dimers of various combinations of the Rel family polypeptides, consisting of p50 (NF-κB1), p52 (NF-κB2), c-Rel, v-Rel, Rel A (p65), and Rel B (63). In resting cells, NF-κB is retained in the cytoplasm by binding to one of the inhibitory IκB proteins (IκBα, IκBβ, IκBe, p105, and p100), which blocks the nuclear translocation of NF-κB (63). NF-κB is activated in response to a wide variety of pro-inflammatory and pro-angiogenic stimuli, each promoting the dissociation of IκB through its phosphorylation, followed by ubiquitination and degradation (Fig. 5). Thus, the unmasking of the nuclear localization sequence of NF-κB allows NF-κB to enter the nucleus and bind to κB-regulatory elements (114). The phosphorylation of IκB is catalyzed by an IκB kinase(IKK) complex (63). The core of the IKK complex consists of a heterodimer of IκKα and IKKβ, and two IKKγ subunits. IκKα and IKKβ mediate the phosphorylation of IκB, whereas IKKγ links the core to the upstream signaling molecules (Fig. 5) (63). Also the activation of the IKK complex is dependent on phosphorylation, and multiple upstream kinases, some of which are redox-sensitive, have been suggested to act as IKK kinases (63).

Many agents that activate mitogen activated protein kinases (MAPK) also induce an overproduction of ROS besides activating NF-κB, suggesting a cross-talk between these pathways (Fig. 5). For example, IL-1 has been shown to induce COX-2 through a mechanism mediated by the activation of the extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 MAPK (57). Lysophosphatidylcholine, a component of oxidized LDL, activates COX-2 expression by p38 MAP kinase activation, the proangiogenic transcription factors cAMP-responsive element binding protein (CREB) and activating transcription factor (ATF)-1 (121). Finally, phorbol myristate acetate (PMA), an activator of protein kinase C (PKC), induces COX-2 primarily through the activation of extracellular regulated kinase (ERK)1/2 (66). However, the exact molecular site of action of these MAPK
Figure 5. A simplified scheme of signaling pathways leading to COX-2 gene induction by NF-κB activation. The binding of growth factors, IL-1 or LPS to their respective receptors induces the activation of specific signaling pathways - including the production of reactive oxygen species (ROS) (including H₂O₂) through the activation of NAD(P)H oxidase, which leads to NF-κB-inducing kinase (NIK) activation, IκB kinase (IκB) degradation, and the nuclear translocation of p65-p50. In the nucleus, NF-κB, upon binding to the respective consensus sequences, critically contributes to COX-2 and to the induction of other proinflammatory genes, such as VCAM-1, ICAM-1, MCP-1, etc.

Abbreviations: TLR4=toll-like receptor-4; IL-1 R=IL-1 receptor; PKC=protein kinase C; MyD88=myeloid differentiation factor; IRAK=IL-1 receptor-associated kinase; TRAF=TNF receptor-associated factor; AP-2=activator protein-2; NF-IL-6=nuclear factor for interleukin-6; CRE=cyclic adenosine mono-phosphate response element.

activities along the pathway leading to NF-κB activation, as well as the site of interference with ROS production, likely triggered by the activation of nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase (Fig. 5), are still elusive.

Effects of omega-3 fatty acids on endothelial COX-2 expression

The effect of omega-3 FAs on COX-2 expression and activity in the vascular tissue has been extensively studied, but the findings are not always in agreement. In the murine monocytic cell line RAW264.7, DHA (86, 87), but not EPA (6), reduced the stimulated COX-2 expression. Similarly, results obtained using cultured endothelial cells have been variable. Gilbert et al. showed that DHA treatment of bovine aortic endothelial cells potentiated COX-2 expression induced by PMA (56). Conversely EPA, but not DHA, greatly decreased IL-1β-induced COX-2 expression in human microvascular endothelial cells (1). Using both human saphenous and umbilical vein endothelial cells (HSVEC and HUVEC, respectively), we have observed that DHA treatment before IL-1 or PMA stimulation reduces COX-2 expression (Fig 6, A) and activity (99). The regulation of COX-2 expression involves both transcriptional and post-transcriptional events. Post-transcriptional regulation of COX-2 is dependent, in part, on “UAAAU” instability sequences contained within the 3'-untranslated region (UTR) of the coding mRNA (27). Having observed that DHA reduced COX-2 steady-state mRNA levels at Northern analysis, in the attempt to understand the site of DHA interference we first explored the possibility that DHA might increase COX-2 mRNA stability, setting up experiments to measure the messenger half-life in the presence of actinomycin-D as a transcription blocker. We observed no changes in COX-2 mRNA half-life when the endothelium was exposed to DHA, thus inferring a transcriptional effect for DHA.
Accordingly, transient transfection experiments using full-length human COX-2 promoter constructs showed that DHA inhibited total promoter activity independent of the pro-inflammatory stimuli used. Furthermore, by the use of COX-2 promoter constructs either deleted or site-mutated at specific transcriptionally active sites, we observed that DHA inhibitory effect was abrogated only when promoter sequences were lacking, by deletion or site-mutation, functional NF-κB sites. This, together with the observed reduced activation of NF-κB (as assessed by electrophoretic mobility shift assay (EMSA)) and the reduced nuclear translocation of p65, suggested an interference by DHA with the activated cytoplasmic signaling pathway leading to NF-κB activation.

It is well known that omega-3 FAs, modifying lipid composition, alter membrane lipid microdomains, such as lipid rafts and caveolae, involved in the compartmentalization, modulation, and integration of cell signaling components (33, 94, 142). In our experimental conditions, DHA accumulates into membrane phospholipids. Therefore, in the attempt to explore which molecular target(s) upstream of NF-κB was (were) affected by DHA, we first focused our attention on the NADP(H) oxidase, an enzyme system producing ROS involved in the activation of NF-κB by IL-1 (88), the assembly and activation of which is potentially sensitive to membrane phospholipid composition (120). In resting conditions, NADPH oxidase complex consists of several subunits: two of them, p22phox (where phox stands for phagocytic oxidase) and glycoprotein (gp)91phox (NOX2 – NOX stands for NADPH oxidase – in the endothelium) are integral membrane proteins, while the p47phox and p67phox subunits are located in the cytosol. Upon cell stimulation, p47phox is phosphorylated and, in complex with p67phox and the small GTP-binding protein Rac, moves to the plasma membrane to form the active enzyme complex (126). We demonstrated that DHA leads to decreased p47phox membrane translocation, together with diminished NAD(P)H oxidase activity and intracellular ROS production (Fig. 6, B and C). Since PKCs, besides being implicated in NADPH oxidase activation, also mediate COX-2 expression (22), and since PKCe activity is specifically involved in the activation of NF-κB by ERK1/2 induction (89), we next explored the possibility that PKCs could be another molecular switch affected by DHA. We monitored the membrane translocation of the main PKC isoforms in endothelial cells stimulated by PMA and IL-1 in the presence of DHA. All such isoforms were activated by PMA, as demonstrated by their translocation to plasma membrane, but only the translocation of PKCe was reduced by DHA treatment (Fig. 6D). We therefore concluded that DHA, possibly modifying the plasma membrane lipid composition and hence membrane microdomain organization, inhibits at least two molecular switches both involved in COX-2 expression, the activation of NAD(P)H oxidase and the activation of PKCe. An integrative molecular model of dietary omega-3 FA interference with IL-1 signaling pathway leading to adhesion molecules and COX-2 induction in endothelial cells is proposed in Fig. 7.

The omega-6/omega-3 FA ratio as possible explanation for the health effects of fish and fish oil

Several authors tend to explain the health effect of omega-3 FA in terms of a balance between total omega-6 and omega-3 FA, rather than in term of absolute amount of each single molecule. In the most simplistic interpretation, a very high omega-6/omega-3 ratio is considered detrimental for human health, while a value as much as possibly close to 1 is considered protective (61). Historically, Simopoulos, in a pivotal study dated 1991, was the first to define the importance of the omega-6/omega-3 FA ratio (133). Her study is particularly interesting since it includes the anthropological data according to which, in the Palaeolithic period (about 40,000 years ago), the human diet was much lower in saturated FA and contained small but roughly equal amounts of omega-6 and omega-3 FA. Unfortunately, no data are available on the health status of our ancient progenitors. However it is undeniable that a dramatic change in human diet and lifestyle occurred over the past 10,000 years, first with the agricultural revolution, which introduced refined cereals and grains rich in omega-6 FA in the diet, and then (in the last 150 years) with the large introduction of vegetable seed oil consumption, similarly rich in omega-6 FA. The result is the current omega-6/omega-3 FA ratio, which in some Western diet ranges between 15:1 and 20:1 (134, 135). Several clinical intervention studies support the view that decreasing the omega-6/omega-3 FA ratio results in an increased protection against degenerative diseases (reviewed in (134, 135)).
Figure 6. DHA reduces COX-2 expression by inhibiting NADPH oxidase and PKCε activation. Panels A show the immunocytochemical analysis of the effect of docosahexaenoic acid (DHA) on the stimulated expression of cyclooxygenase(COX)-2 in endothelial cells. Human saphenous vein endothelial cell (HSVEC) were grown in 24-well plates for 20 h, and treated with 25 µmol/L DHA for 48 h before stimulation with interleukin(IL)-1α 10 ng/mL for 16 h. After incubations, monolayers were fixed and immunostained with an anti-COX-2 monoclonal antibody. Immunocytochemistry with a non-immune IgG, as a control, revealed no staining (not shown). A reduction of COX-2 expression occurs in cells pre-exposed to DHA (right column).

Panels B: HSVEC were pretreated with 25 µmol/L DHA for 48 h and then stimulated with 10 ng/mL IL-1α for 1 h. Monolayers were then washed and loaded for 30 min with reduced 2',7'-dichlorodihydrofluorescein diacetate (DCF). This is a non-polar compound that readily diffuses into the cells, where it is hydrolyzed by intracellular esterases to the non-fluorescent derivative, which - being polar - is trapped within the cells. In the presence of intracellular hydrogen peroxide (H₂O₂) or some of its downstream products, this compound is oxidized to the highly fluorescent compound 2',7'-dichlorofluorescein. The green fluorescence measured is therefore proportional to the H₂O₂ produced. DHA treatment reduces the fluorescence intensity and therefore, by inference, the production of reactive oxygen species (panel in the right column).

Panel C: HSVEC were pretreated with 25 µmol/L DHA for 48 h and then stimulated with 10 ng/mL IL-1α for 20 min. Subcellular soluble and particulate (membrane) fractions were isolated, and Western blots were performed with an antibody specific for p47phox. Values are in units of optical density (OD). The blot depicted is representative of 2 similar ones.

Panel D: HSVEC were pretreated with 25 µmol/L DHA for 48 h and then stimulated with 10 nmol/L PMA for 20 min. Subcellular soluble and particulate (membrane) fractions were isolated, and Western blots were performed using an anti-PKCε antibody. Values of PKC translocations are reported as units of OD at densitometric analysis. The blot is representative of 2 similar ones. Modified from ref. (99).
Figure 7. Proposed integrative molecular model of dietary omega-3 fatty acid interference with IL-1 signaling pathways, leading to adhesion molecules and COX-2 induction in endothelial cells. IL-1 binds to the IL-1 receptor type I (IL-1RI), which heterodimerizes with the IL-1 receptor accessory protein (IL-1RacP). The IL-1R-associated kinases (IRAK) are then recruited and associated by the adapter proteins myeloid differentiation factor(MyD)88 and Toll-interacting protein (Tollip). The signaling pathway also includes the production of reactive oxygen species (ROS, namely H$_2$O$_2$) through the activation of NAD(P)H oxidase by IRAK activation, as well as the activation of PKC, both contributing to nuclear factor(NF)-κB activation. DHA, by interfering with the production of ROS (through the inhibition of p47phox translocation and/or the scavenging of ROS by its multiple double bonds, prevents the formation of H$_2$O$_2$, thus limiting all the downstream cascade leading to COX-2 gene expression. Furthermore, DHA reduces PKCε activation, thus inhibiting ERK1/2 activation, also leading to NF-κB activation and COX-2 expression. Finally, cytokine-induced ROS can attack DHA and transform it into an oxidized derivative able activate PPARα and thereby further inhibit NF-κB through a mechanism of trans-repression (149).

Abbreviations: TRAF=TNF receptor-associated factor; TAK-1=TGFβ-activated kinase 1; TAB-2=TAK1-binding protein 2; NIK=NF-κB-inducing kinase; IKK=IkB kinase; PPARα=peroxisome-proliferators activated receptor. Modified from (99).

et al., aimed at evaluating the protective effect of Mediterranean dietary pattern in the secondary prevention of coronary heart disease, the replacement of corn oil (high in LA) with olive and canola oil (low in LA) to reach a 4:1 ratio of LA/ALA led to a 70% decrease in total mortality. Similarly, in the study by Ambring et al, when healthy subjects were fed with a typical Swedish diet (serum omega-6/omega-3 FA ratio of 4.7:1), they showed increased numbers of circulating leukocytes and platelets as well as VEGF levels compared with individuals who ate a Mediterranean-style diet with a measured serum omega-6/omega-3 FA ratio of 2.6:1 (3). Several cellular and animal models also support the negative effects of a high omega-6/omega-3 FA ratio in the diet. For example, the adenovirus-mediated introduction of the Caenorhabditis elegans fat-1 gene encoding an omega-3 FA desaturase into mammalian cells has been shown
to quickly and effectively elevate the cellular omega-3 PUFA contents and dramatically balance the ratio of omega-6/omega-3 PUFA. In particular, the heterologous expression of the fat-1 gene in rat cardiac myocytes rendered the cells capable of converting various omega-6 PUFA into the corresponding omega-3 PUFA, and changed the omega-6/omega-3 FA ratio from about 15:1 to 1:1 (77). The same group also showed that transgenic mice expressing the C. elegans fat-1 gene showed an increase in omega-3 FA and a reduction in omega-6 FA in all organs and tissues, and appeared to be normal and healthy for four generations (78), as well as less predisposed to degenerative and angiogenic-related disease (29).

An opposing view considers that the omega-6/omega-3 FA ratio is of little value from a theoretical and experimental point of view, creating confusion in the field and diluting the main interest represented by increasing the dietary intake of omega-3 FAs (60, 62, 157). According to these authors, “without knowledge of the absolute value of the numerator and the denominator, the meaning of a given ratio, whether as a biomarker or dietary target, will be impossible to discern” (60). This conclusion is the result of several arguments, such as: (i) there are several independent and nutritionally unrelated strategies to decrease the omega-6/omega-3 FA ratio; (ii) apparently, the omega-6/omega-3 FA ratio does not distinguish among the different classes of PUFA, putting on the same level the LA:ALA versus the AA:EPA/DHA ratio, which is questionable from a functional and biochemical point of view; (iii) evidence suggesting that an increased intake of omega-3 FA reduces the risk for CVD is strong, while it is still a matter of opinion that the same effect can be obtained by decreasing the levels of omega-6 FA. As a good example, it is worthwhile to note that the percentage of all deaths from cardiovascular disease in Europe and United States versus Japan is 45% and 12%, respectively, corresponding to a significant difference in the concentration of EPA in thrombocyte phospholipids (0.5% versus 1.6%), while the percentage of AA is similar in the two groups (26% versus 21%) (134). In other words, the difference in EPA concentration gives the same indication as the omega-6/omega-3 FA ratio; therefore, there is no apparent need to look at this ratio, but simply to the omega-3 FA concentration.

CONCLUSIONS AND PERSPECTIVES

The vascular endothelium plays a key role in the progression of cardiovascular disease, as well as in the maintenance of solid cancer cell cords, orchestrating the neovascularization of atherosclerotic plaque and cancer tissue growth. On the other hand, omega-3 FAs have emerged as an effective tool in the primary and secondary prevention of cardiovascular disease and some forms of cancer. Although they exert multiple actions (75), the transcriptional control of several endothelial pro-inflammatory genes, including those encoding for adhesion molecules, chemokines and other soluble cytokines in the endothelium, likely plays an important role. The recent findings showing inhibition of COX-2 expression and activity by omega-3 FA allows a deeper understanding of the therapeutic potential of these nutrients, being COX-2 overexpression pathogenetically involved in different inflammatory/angiogenic diseases besides atherosclerosis (from rheumatoid arthritis to inflammatory bowel disease and - possibly - Alzheimer's disease). Far from being only a source of energy and building blocks of our body tissues, omega-3 FA appear therefore to finely tune the response of our genes to dangerous environmental challenges, by curbing physiological responses without abrogating them totally. By decreasing the endothelial responsiveness to pro-inflammatory, pro-atherogenic and pro-angiogenic stimuli, omega-3 FA appear to impact molecular events not targeted by any other drugs or interventions, thus allowing us to propose their therapeutic role complementary to those of already implemented pharmacologic treatments in inflammatory diseases, including atherosclerosis. Many effects of omega-3 FA are clear examples of nutrigenomics, i.e. how selected nutrients affect the expression of our genes, thus modulating the risk of human diseases.

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Other articles in this theme issue include references (165-176).

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