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REVIEWS: CURRENT TOPICS

Mechanisms underlying the cardioprotective effects of omega-3 polyunsaturated fatty acids☆

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Abstract

Typical omega 3 polyunsaturated fatty acids (n-3 PUFAs) are docosahexaenoic acid and eicosapentaenoic acid in the form of fish oils and α linolenic acid from flaxseed oil. Epidemiological studies suggested the benefits of n-3 PUFA on cardiovascular health. Intervention studies confirmed that the consumption of n-3 PUFA provided benefits for primary and secondary prevention of cardiovascular disease. Evidence from cellular and molecular research studies indicates that the cardioprotective effects of n-3 PUFA result from a synergism between multiple, intricate mechanisms that involve antiinflammation, proresolving lipid mediators, modulation of cardiac ion channels, reduction of triglycerides, influence on membrane microdomains and downstream cell signaling pathways and antithrombotic and antiarrhythmic effects. n-3 PUFAs inhibit inflammatory signaling pathways (nuclear factor-κ B activity) and down-regulate fatty acid (FA) synthesis gene expression (sterol regulatory element binding protein-1c) and up-regulate gene expression involved in FA oxidation (peroxisome proliferator-activated receptor α). This review examines the various mechanisms by which n-3 PUFA exert beneficial effects against CVD.
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Keywords: Omega 3 polyunsaturated fatty acids; Cardiovascular disease; Atherosclerosis

1. Introduction

Cardiovascular disease (CVD) in the US continues to be the leading cause of death and accounts for 36% of all deaths [1]. Furthermore, CVD constitutes the largest proportion of economic burden with an estimated impact of $475 billion in 2009. Despite the staggering statistics, CVD death rates have in fact reduced 26% from 1995 to 2005 [2]. Through lifestyle changes, education and therapeutics, the prevalence of classic CVD reversible risk factors, i.e., smoking, high blood pressure, high total cholesterol and low-density lipoprotein (LDL) cholesterol, have decreased over the last 25 years. However, other independent emerging risk factors, such as hyperglycemia [3,4], postprandial hypertriglyceridemia [5], hyperinsulinemia [6], oxidative stress [7], endothelial dysfunction [8], total and small dense LDL cholesterol [9,10], abdominal obesity [11], elevated plasma homocysteine and asymmetric dimethylarginine [12,13], low-level endotoxemia [14,15] and elevated circulating concentrations of inflammatory markers, such as C-reactive protein (CRP), interleukin-6 (IL-6), fibrinogen and serum amyloid A (SAA) [16], have surfaced. It is highly possible that CVD mortality rates in the US could rise again due to the escalating aging population coupled with the increasing incidence of obesity [17] and type 2 diabetes [18], which share many of the emerging risk factors for CVD.

An aspect of CVD research focuses on the cardioprotective effects of fish oils and of individual omega 3 polyunsaturated fatty acids (n-3 PUFA), or more specifically, eicosapentaenoic acid (EPA; 20:5 n-3), docosahexaenoic acid (DHA; 22:6 n-3) and α linolenic acid (ALA; 18:3 n-3). Many large-scale studies, including primary and secondary prevention clinical trials and metaanalysis of cohorts, have concluded that consumption of fatty fish, fish oils or individual n-3 PUFA is an effective dietary strategy to lower CVD morbidity, mortality, as well as classic and emerging risk factors listed above [19–34]. In addition, n-3 PUFA have been shown to improve a number of cardiac hemodynamic factors such as blood pressure [35,36], left ventricular diastolic filling [37], heart rate [38,39] and endothelial function [40,41]. The cardioprotective effects of n-3 PUFA also include arrhythmia prevention [42], plasma triacylglycerol reduction [43], vascular relaxation improvement [41], antiinflammatory responses [44], platelet aggregation inhibition [45], enhancement of plaque stability [46] and antiatherosclerotic effects [20]. Unlike cardiac pharmaceuticals, n-3 PUFAs have fewer side effects [47] and are generally recognized as safe (GRAS) by the US Food and Drug Administration. The American Heart Association/American College of Cardiology recommends the dietary intake of (1) 1 g of n-3 PUFA (EPA and

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DHA) per day in the form of fish or fish oils for secondary prevention for individuals with existing coronary and other vascular diseases, (2) 2 to 4 g of n-3 PUFA per day for the treatment of hypertriglyceridemia and (3) one serving of oily fish twice per week (~0.5 g of n-3 PUFA per day) for individuals without documented coronary heart disease [48]. The International Society for the Study of Fatty Acids and Lipids also recommends at least 0.5 g per day of EPA plus DHA for cardioprotective benefits in healthy adults [49]. Americans consume an average of approximately 1.6 g total n-3 PUFA per day, of which EPA and DHA accounts for only 0.1 to 0.2 g and the balance is made up of ALA (18:3 n-3) from plant sources [50]. This is clearly less than the recommended amounts.

Consumption of high amounts of saturated fatty acid (SFA), trans fatty acid (FA) and omega-6 (n-6) PUFA and low amounts of n-3 PUFA (approx n-6:n-3 PUFA ratio of 16:1) is a pattern often observed in a typical Western diet; this is very different from the pattern found in the diets of our ancestors, who presumably had a n-6:n-3 PUFA ratio of ~1 [51]. Consequently, cells must adapt to this surplus (n-6) and deficiency of (n-3) specific dietary PUFA. n-3 and n-6 PUFAs regulate a number of transcription factors and interact with nuclear receptors such as peroxisome proliferator-activated receptors (PPARs), liver X receptor (LXR), hepatocyte nuclear factor-4α, nuclear factor-κB (NF-κB) and sterol regulatory element binding protein (SREBP), all of which influence inflammatory responses and lipid metabolism. An imbalance of dietary n-6:n-3 PUFA ratio may result in altered gene regulation and expression in downstream pathways resulting in altered protein expression and activity that can negatively affect cell membrane composition and fluidity and organ function. Multiple mechanisms by which n-3 PUFA exert their cardioprotective effects have been proposed. This review will discuss the cardioprotective roles of n-3 PUFA in antiinflammatory processes, inflammation-resolving capabilities, regulation of transcription factors, acute-phase reactant (APR) suppression capacities, hypotriglyceridemic effects and influence on cell membrane properties and vascular function.

2. Essential PUFA: structure and biochemistry

Two classes of essential PUFA exist: n-3 and n-6. From the standpoint of vascular disease prevention, n-3 PUFAs are the most important and extensively studied class of essential PUFA. n-3 and n-6 PUFAs are termed "essential" FA and must be obtained from the diet because humans lack the Δ12- and Δ15-desaturases necessary to insert a double bond at the n-3 or n-6 position of an FA carbon chain. The difference between the two essential PUFA is based on the location of the first double bond of the molecule counting from the

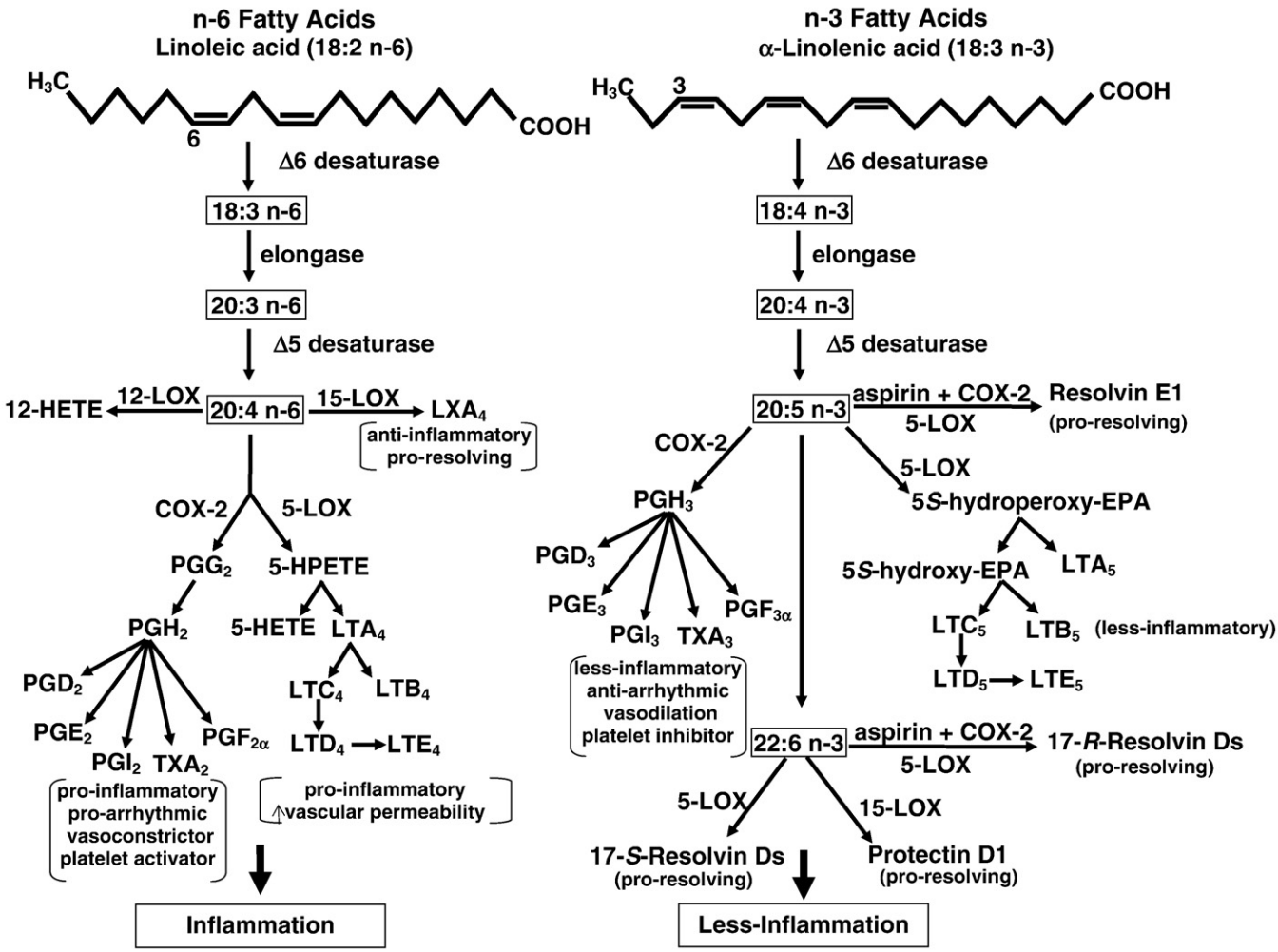

Fig. 1. The metabolism of n-3 and n-6 PUFA and the biosynthesis of their respective eicosanoid and proresolving mediators. n-3 PUFAs are generally less inflammatory than the n-6 PUFA. However, PGE_2 derived from n-6 PUFA can have an antiinflammatory effect by decreasing LTB_4 production by the inhibition of 5-LOX and increasing production of LXA_4 by stimulating 15-LOX. n-3 PUFA-derived eicosanoids have different physiological potencies than n-6 PUFA-derived eicosanoids. Abbreviations: HPETE, hydroperoxyeicosatetraenoic acid; LTA_4, leukotriene A_4; LXA_4, lipoxin A_4.

methyl end of the FA. The first double bond of the n-3 PUFA is between the third and fourth carbon atoms, while the first double bond of the n-6 PUFA is between the sixth and seventh carbon atoms. The parent FAs of the long-chain n-3 and n-6 PUFAs are ALA and linoleic acid (LA; 18:2 n-6), respectively (Fig. 1). Linoleic acid is found in the nuts, seeds and vegetable oils such as corn, sunflower, safflower, canola and soybean oil, while ALA is found in seeds of flax, rape, perilla, walnuts and chia and also in chloroplasts of leafy green vegetables. Once consumed, ALA is metabolized by Δ6 desaturation, elongation and Δ5 desaturation to yield EPA, which further undergoes elongation and Δ6 desaturation. The resulting FA is then converted to DHA via β-oxidation in the peroxisomes. Deep ocean fish are good sources of EPA, and DHA since the origin of these FAs in the aquatic ecosystem is algae [52]. Metabolism of dietary LA uses the same enzymes as in the synthesis of DHA from ALA. Linoleic acid undergoes Δ6 desaturation, elongation and Δ5 desaturation to form arachidonic acid (AA; 20:4 n-6). FAs are subsequently incorporated into triglycerides (TGs; three FAs attached to a glycerol backbone), phospholipids (PL; two FAs on a phosphatidic acid backbone) and cholesteryl esters (one FA affixed to free cholesterol). Because metabolism of LA and ALA to longer chain PUFA shares the same pathway, the two compete for the same enzymes. High intakes of LA would preferentially shift the pathway to elongation of n-6 PUFA to increase AA production and concurrently inhibit desaturation of ALA and reduce EPA and DHA formation.

FA concentrations of plasma, cells and tissues are responsive to n-3 PUFA consumption in a dose-dependent manner. Supplementation with dietary DHA ethyl esters and DHA triacylglycerol increased plasma and red blood cell (RBC) DHA concentrations in human adults [53–58]. Supplementation with EPA ethyl esters resulted in an increase in plasma and serum PL EPA, but DHA concentrations did not increase because of its inefficient conversion to DHA [56,59,60]. The incorporation of EPA and DHA into the PL of immune cells, that is, neutrophils, monocytes, T lymphocytes and B lymphocytes, increased as a result of fish oil consumption [61]. In the RBC, cell membrane PL became enriched with n-3 PUFA during reticulocyte maturation in the bone marrow and by direct plasma exchange via transfer of serum albumin-associated DHA and EPA containing lysophosphatidylcholine [62,63]. Docosahexaenoic acid concentration in human heart is about 10 times that of EPA (5.1% vs. 0.5%). In heart transplantation patients, supplementation with 1 g/day n-3 PUFA (20% DHA and 30% EPA) for 6 months increased EPA+DHA levels in cardiac biopsies by 110% [64]. In humans, when dietary ALA is provided in the presence of a high background of n-6 PUFA, small changes in plasma ALA concentrations, slight increases in plasma EPA and no changes in plasma DHA (due to low conversion and high oxidation rates) were observed [65,66]. Premenopausal women exhibit a better efficiency for the conversion of ALA to EPA than those found in postmenopausal women and in men. In rats, maximum incorporation of less than 1% ALA was shown to accumulate in cardiac PL within 8 weeks of a 32-week feeding study with 15.8 g of ALA/kg diet [67].

3. Mechanisms for the antiinflammatory effect of n-3 PUFA on cardiovascular health

Inflammation of the vascular wall is a key factor in the dynamic process of atherosclerosis [68]. Mediators such as oxidized LDL, lipopolysaccharide (LPS) from gram-negative bacteria, cytokines and free radical species can trigger the endothelium of the arterial wall to initiate the cascade of atherosclerosis development. The local inflammatory response by cytokine-activated endothelium results in an increased expression of leukocyte adhesion molecules, including vascular cell adhesion molecule 1 (VCAM-1), intracellular cell adhesion molecule 1 (ICAM-1) and E-selectin. Monocytes bind to the adhesion molecules on endothelial cells and subsequently transmigrate into the subendothelial space where they transform into macrophages. Macrophages are directed toward chemoattractant cytokines, such as macrophage chemoattractant protein-1 (MCP-1) secreted by the vascular wall cells in response to the oxidized LDL. These macrophages scavenge oxidized LDL, become lipid-laden and convert into foam cells. In the early stages of atherosclerosis, the accumulation of foam cells evolves into fatty streak. Lesion complications occur when smooth muscle cells in the intima divide and produce extracellular matrix molecules, such as collagen, and the smooth muscle cells in the media migrate to the intima and contribute to the formation of a fibrous cap. Thrombosis is triggered when this fibrous cap ruptures.

n-3 PUFAs have the ability to respond to inflammation in atherogenesis through direct and indirect mechanisms. A direct mechanism through which n-3 PUFA decrease inflammation includes its rapid effect on the regulation of transcription factors [69–72], and indirect modes of actions include the production of three- and five-series eicosanoids [73,74] and inflammation-resolving lipid mediators [75–82] and suppression of APRs [83–85] (Table 1).

3.1. Antithrombotic and antiinflammatory roles of n-3 PUFA

The antiinflammatory action of n-3 PUFA eicosanoids and their involvement in signaling pathways are mechanisms for their cardioprotective effects [21]. n-3 PUFA also decreased the production of several inflammatory cytokines, which will be discussed in Section

Table 1
Mechanisms involving the cardioprotective effects of n-3 PUFA

n-3 PUFA effects on CVD	Mechanisms	
Inhibition		
Inflammation	Decrease	TNF-α, IL-1β, IL-6, IL-8, CRP, SAA, PPARα, PPARγ, RXR
		Two- and four-series eicosanoids (derived from n-6 PUFA)
	Increase	Three- and five-series eicosanoids (derived from n-3 PUFA)
		Lipoxins, resolvins and protectins (derived from n-3 PUFA)
Monocyte infiltration	Decrease	MCP-1, VCAM-1, ICAM-1, E-selectin
NF-κB activation	Decrease	Degradation of I-κB via TLR4 activation
Platelet aggregation	Decrease	Two-series TX
	Increase	Three-series TX
Vasoconstriction	Decrease	Two-series TX
	Increase	Three-series TX
Arrhythmia	Decrease	Two-series PG
		Surface membrane electrical excitability
		Activity of voltage-dependent Na^+ channels
		Ca^{2+} release channels and intracellular Ca^{2+}
	Increase	Three-series PG
Stimulation		
Proresolving mediators	Increase	Lipoxins, resolvins and protectins
Stabilization of atherosclerotic plaques	Decrease	Infiltration of monocytes into the plaques
		Activity of cells, that is, macrophages within the plaques
	Increase	Incorporation of n-3 PUFA into plaques
		Production of thick fibrous cap
TG lowering	Decrease	Apo CIII
		SREBP-1c activity
		FA substrates for lipogenesis
		NEFA availability
	Increase	LPL
		FXR
		PPARα-induced oxidation
		Apo CII
		VLDL-receptor gene expression
Changes in membrane lipid composition	Decrease	Sphingomyelin content in lipid rafts
		Cholesterol and caveolin in caveolae
	Increase	Membrane fluidity

6. Eicosanoids are derived from 20-carbon PUFA, such as AA and EPA, which are physiologically active compounds that act locally as signaling molecules through G-protein-linked receptors (Fig. 1). If AA is predominantly incorporated in the cell membrane PL, then phospholipase A_2 (PLA_2) releases AA from the membrane PL in response to external stimuli, such as an injury or acute or chronic infection. Free AA serves as a substrate for the enzymes cyclooxygenases (COX) to produce two-series prostaglandins (PGE_2), prostacyclins (PGI_2) and thromboxanes (TXA_2), while 5-lipoxygenases (5-LOXs) catalyzes the oxygenation reaction of free AA to four-series leukotrienes and hydroxyl eicosatetraenoic acids (HETEs). Generally, the n-6 PUFA-derived eicosanoids are proinflammatory. Depending upon which enzyme catalyzes the oxygenation (COX or LOX), these signaling molecules elicit a wide range of responses, including vasoconstriction, vasodilation, activation of leukocytes, stimulation of platelet aggregation and generation of reactive oxygen species. 12-HETE formed from AA in the presence of 12-LOX increase inflammatory cytokine production [tumor necrosis factor α (TNF-α), IL-1 and IL-6]. In a study with healthy men, AA supplementation significantly increased PGE_2 and LTB_4 production [86]. PGE_2 at low concentration is proinflammatory by eliciting fever, pain and vasodilation and increase vascular permeability and edema [87]. But at a higher concentration, PGE_2 is antiinflammatory as it decreases LTB_4 production via inhibition of 5-LOX and stimulates lipoxin (LXA_4) synthesis through 15-LOX. PGE_2 can also stimulate COX-2 and stimulate its own production in fibroblasts and IL-6 by macrophages. Lipoxins are antiinflammatory as it can inhibit NF-κB activation, leukocyte migration, as well as decrease expression of cytokines and adhesion molecules [82].

Eicosanoids produced from DHA and EPA are generally less inflammatory than their AA-derived eicosanoid counterparts [88,89], serve as vasodilators and inhibit platelet aggregation. n-3 PUFA can reduce the production of AA-derived eicosanoids by competing with AA for incorporation into cell membrane PL, by release of free AA by PLA_2 or by inhibiting the enzymes COX-2 and 5-LOX (Fig. 1). This would shift the production of inflammatory eicosanoids derived from n-6 PUFA to n-3 PUFA. Eicosapentaenoic acid can suppress COX-2, thereby decreasing two-series PG and TX production and increasing the three-series PG, PGI and TX. Eicosapentaenoic acid can also inhibit 5-LOX, which decreases production of four-series LT but increases five-series LT. Docosahexaenoic acid on the other hand inhibited only COX-2 activity in vitro. However, supplementation of DHA to healthy men decreased production of both PGE_2, and LTB_4 [90]. Whether the decrease in LTB_4 in this study resulted from the direct inhibition of 5-LOX by DHA or it was caused by the EPA formed by retroconversion cannot be determined from the information available. Docosahexaenoic acid also decreased the ex vivo secretion of inflammatory cytokines, TNF-α and IL-1β by the peripheral blood mononuclear cell (PBMC) stimulated by LPS.

n-3 PUFA and AA compete for the same enzymes (COX-2/5-LOX) that catalyze the formation of their respective eicosanoids; therefore, high dietary intakes of n-6 PUFA would result in a dominant incorporation of AA (vs. n-3 PUFA) in cell membrane PL and preferentially convert AA to proinflammatory eicosanoids [73,74]. This would ultimately shift eicosanoid production equilibrium toward proinflammation. Although derivation of proinflammatory eicosanoids from AA is a natural response to physiological and pathological stimuli, consequences of consistent and long-term production of these eicosanoids from high n-6 PUFA dietary intakes could progress to chronic diseases such as atherosclerosis. Therefore, one possible resolution to this problem is a higher consumption of n-3 PUFA in the diet, that is, decreasing dietary n-6:n-3 ratio, which would result in a more favorable antiinflammatory state through the reduction of proinflammatory eicosanoid production capacity of monocytes, neutrophils, eosinophils, platelets and endothelial cells [44,74,91,92].

In addition to the changes in the concentrations of inflammatory eicosanoids, fish oil supplementation also decreased plasma as well the ex vivo production of a number of inflammatory cytokines including IL-1β, IL-6, IL-8 and TNF-α. These findings have recently been reviewed [93]. A number of studies have examined the effects of individual long-chain n-3 PUFA on the ex vivo production and plasma concentrations of inflammatory cytokines, and the results have been variable. Thus, we found that DHA supplementation (6.0 g/day) to healthy men decreased the ex vivo production of IL-1β and TNF-α after 90 days but not after 45 days of supplementation in healthy young men [90]. In a subsequent study with DHA (3 g/day, 90 days), we found DHA decreased the plasma concentrations of IL-6 and granulocyte macrophage colony-stimulating factor (GM-CSF) and the number of circulating granulocytes in hypertriglyceridemic men [94]. Docosahexaenoic acid supplements of 0.7 g/day for 12 weeks or of 4.7 g/day for 4 weeks in healthy subjects did not reduce the ex vivo production of TNF-α, IL-1β, IL-6 and IL-8 [95,96]. Similarly, EPA supplements of 4.7 g/day for 4 weeks or 4.05 g/day for 12 weeks to healthy men did not alter the ex vivo production of inflammatory cytokines [96]. Supplementing EPA or DHA (4.0 g/day, 6 weeks) to type 2 diabetic patients did not alter plasma concentrations of IL-6 and TNF-α [97]. Our observation regarding the decrease in the production of inflammatory cytokines is supported by a decrease in the symptoms of inflammatory diseases and the concentrations of inflammatory cytokines in a number of studies after fish oil supplementations [98]. As discussed in Section 3.3, n-3 PUFA decrease the expression of NF-κB, which regulates the expression of inflammatory cytokines. Overall, there is plenty of information indicating that n-3 PUFAs decrease the production of inflammatory cytokines. The discrepancies between the results studies discussed above may be due to differences in study protocols, diets, amounts and durations of n-3 PUFA supplement, age and health status of the subjects and the methods used. Effects of n-3 PUFA on the concentrations of APR proteins will be discussed in Section 3.4.

3.2. Inflammation-resolving effects of n-3 PUFA

Impairment in the resolution of vascular inflammation can promote atherosclerosis development [99]. Resolution of inflammation is a programmed normal response that enables the body to control inflammation and minimize tissue damage by limiting neutrophil and eosinophil infiltration and nonphlogistic phagocytic removal of apoptotic cells [78]. Most macrophages exit injured/infected sites via lymphatics and the inflammation subsides; however, under certain pathological conditions, inflammatory responses do not subside and lead to tissue injury. Using lipidomics and informatics with liquid chromatography–UV–tandem mass spectrometry-based analysis, inflammation-resolving mediators LXA_4 derived from AA and resolvins and protectins derived from EPA and DHA were identified and characterized [75–77,79–81]. The biological activities of these mediators are thought to be another antiinflammatory mechanism by which n-3 as well as n-6 PUFA, to some extent, exerts their cardioprotective effects.

The proresolving oxygenated metabolite is derived from AA catalyzed by 15-LOX and aspirin-acetylated COX-2. They have been shown to be expressed during the resolution phase of inflammation by inhibiting the expression of chemokines, cytokines and adhesion molecules, NF-κB activation and neutrophil migration [82]. In a study in transgenic rabbits, overexpression of 15-LOX increased the levels of LXA_4 and reduced atherosclerosis through protection of lipid deposition in the vessel wall [100]. In another study, biosynthesis of LXA_4, through the overexpression of 15-LOX in mice, significantly lowered macrophage-produced cytokines including IL-1α, IL-1β, TNF-α, interferon-gamma (IFN-γ) and MCP-1, thus, controlling local inflammation and the development/progression of atherosclerosis [99].

Other potent oxygenated metabolites found during the resolution phase of inflammation are (1) resolvins E and D series that are formed from EPA and DHA, respectively, by aspirin-acetylated COX-2 in vascular endothelial cells and 5-LOX in leukocytes and (2) protectins formed from DHA by leukocytes and other cell types by 15-LOX [76,78,79,81,101]. Resolvins are antiinflammatory through the inhibition of neutrophil transmigration and infiltration by initiating apoptosis and proinflammatory mediator synthesis [81]. Protectins are another potent antiinflammatory bioactive compound with the capacity to block neutrophil recruitment and activation, inhibit COX-2 expression and inhibit TNF-α secretion in an ischemic stroke animal model and cultured neuronal cells [101]. Protectins, along with resolvins and lipoxins, can reduce neutrophil recruitment during the resolution phase of inflammation by increasing the expression of chemokine receptor 5 (CCR5) on apoptotic neutrophils, thus, facilitating binding of CCR5 ligands, that is, CCL3 (macrophage inflammatory protein-1α) and CCL5 (RANTES). Engulfment of these chemoattracting agents is then removed by macrophages and results in chemokine clearance to limit further neutrophil infiltration (regulated by lipoxin, resolvin and protectin) to the inflamed site. In addition, both resolvins and protectins have the ability to decrease the production of proinflammatory markers involved in atherosclerosis. When human aortic endothelial cells were incubated with resolvin D1 or protectin D1, MCP-1 and IL-8 were down-regulated by both metabolites, but only protectin D1 decreased expression of VCAM-1 [99]. A lack in the biosynthesis of resolvins and protectins from n-3 PUFA will prolong local proinflammation and fuel atherosclerosis progression. Therefore, attenuation of atherosclerotic progression can be achieved through a synergistic modulation of AA-, EPA- and DHA-derived mediators (lipoxins, resolvins and protectins), which can facilitate restoration of inflamed tissues back to homeostasis.

3.3. Regulation of transcription factors by n-3 PUFA

PUFA can affect gene expression by modulating gene transcription, mRNA processing and decay and stimulating posttranslational protein modifications [70–72]. When nonesterified FAs (NEFAs) enter the cell, they are immediately converted by acyl-CoA synthetases to fatty acyl CoA thioesters (FA-CoAs). The FA-CoAs can then be esterified to TG, PL and cholesterol esters or used to synthesize secondary signaling molecules (prostanoids and leukotrienes). PUFA in the cell can bind to nuclear receptors or transcription factors involved in lipid metabolism. PUFA also have the ability to regulate the expression of genes involved in inflammation.

3.3.1. Nuclear factor-κB

Activation of NF-κB transcription factor plays a key role in the regulation of the expression of genes involved in inflammatory responses and has been implicated in a number of cardiac-related disease states [102,103]. Regulation of target genes starts in the cytoplasm where NF-κB activity is sequestered by its association with the protein inhibitor-κB (I-κB). Once I-κB is phosphorylated in response to an inflammatory stimulus (cytokines, viruses, LPS), I-κB is released, thereby releasing NF-κB and allowing its translocation into the nucleus to modulate genes involved in inflammatory signaling pathways. Nuclear factor-κB increases expression of cytokines (IL-1β, IL-2, IL-6, IL-12, TNF-α, GM-CSF), chemokines (MCP-1, MIP-1α) and inducible effector enzymes [inducible nitric oxide synthase (iNOS), COX-2, PLA_2]. Activated NF-κB has been detected in fibrotic-thickened intima in the atherosclerotic vessel wall leading to the progression of atherosclerotic lesions [104]. Furthermore, in endothelial cell cultures, NF-κB has been shown to activate the expression of ICAM-1 and VCAM-1 and to modulate endothelial cell MCP-1 that signals leukocytes to atherosclerotic lesions [103]. A reversal of such inflammation-related gene expression has been demonstrated by inhibiting the NF-κB signal transduction pathway in a gene knockdown mice model via direct gene delivery of short hairpin RNA against NF-κB p65 [105]. The silencing of NF-κB resulted in a decrease in cardiac mass and improved cardiac function.

Similarly, n-3 PUFA can decrease the expression of target genes involved in inflammation through NF-κB. Zhao et al. [106] found that EPA decreased TNF-α expression through the prevention of NF-κB activation by impeding I-κB phosphorylation and therefore preventing NF-κB translocation into the nucleus. This supports the study by Novak et al. [107], which showed n-3 PUFA inhibited murine macrophage TNF-α production following LPS stimulation via inactivation of NF-κB secondary to inhibition of I-κB phosphorylation. Furthermore, in an ischemic brain injury mice model, DHA inhibited ischemia–reperfusion-induced NF-κB-DNA binding activity and decreased COX-2 expression and therefore prostanoid synthesis [101]. Proresolving oxygenated product of EPA, resolvin E1, also has the ability to terminate NF-κB activation and cytokine production by binding to the G-protein-coupled receptor chemokine-like receptor 1 (Chem R23) in dendritic cells. This suggests that this ligand-receptor binding is a counterregulatory response and may be another antiinflammatory mechanism of n-3 PUFA [75]. Taken together, it appears that one of the multiple cardioprotective mechanisms of n-3 PUFA is through a decrease in transcription of inflammatory cytokines, adhesion molecules and COX-2 genes through the inactivation of NF-κB signal transduction pathway.

3.3.2. Peroxisome proliferator-activated receptors

Peroxisome proliferator-activated receptors, which include the isoforms PPARα, PPARγ and PPARδ, are a group of nuclear receptors encoded by different genes. The PPAR isoforms are ligand-regulated nuclear transcription factors that form heterodimers with retinoid X receptor (RXR) and bind to peroxisome proliferator response elements in the promoter region of target genes involved in lipid metabolism and inflammation and subsequently modulate their expression. Peroxisome proliferator-activated receptor α and γ activation has the ability to inhibit expression of proinflammatory genes by inhibiting NF-κB activation [108–111]. Peroxisome proliferator-activated receptor α activators can improve cardiovascular risk factors and are antiatherosclerotic through antiinflammatory effects in vascular smooth muscle cells (VSMCs) by inhibiting cytokine-induced VCAM-1 expression [112], and PPARγ has antiatherogenic and antiinflammatory properties in monocyte/macrophages, endothelial cells, adipocytes and VSMCs through its ability to decrease IL-1β, IL-6 and TNF-α release into circulation when activated [113].

Eicosapentaenoic acid and DHA have been implicated as PPARα/γ agonists and inhibit NF-κB binding activity. Recent results from computational methods, that is, molecular dynamics simulation, confirm very high affinity binding of DHA to PPARs and RXR [114]. This suggests a direct mechanism of n-3 PUFA in regulating target genes and antiinflammatory effects. Also, n-3 PUFA can activate PPARα, thereby increasing expression of FA oxidation genes and resulting in a decrease in hepatic and plasma TG, which would have an overall beneficial cardioprotective effect for hypertriglyceridemic patients.

3.3.3. Toll-like receptor 4

Inflammatory responses as a result of chronic and unresolved infection lead to epithelial barrier dysfunction. This results in low-level endotoxemia that can contribute to the progression of atherosclerosis [14,15,115]. Chronic inflammation is also a contributor for atherosclerotic plaque rupture, which is the leading cause of fatal coronary thrombi [116–118]. Circulating endotoxins, such as LPS from gram-negative bacteria, bind and activate toll-like receptor 4 (TLR4) on immune cells, including macrophages infiltrating atherosclerotic lesions [119], VSMCs [120,121], adipose tissue [122] and

coronary artery endothelial cells [123]. Furthermore, it has been proposed to be a key receptor in the development of atherosclerosis [124]. Toll-like receptor 4 generates downstream signaling cascades that lead to NF-κB activation and expression of COX-2, inflammatory cytokines and adhesion molecules. Low-level circulating endotoxin has been shown to result in a three- to four-fold increase in proinflammatory cytokine IL-8 and chemokine MCP-1 production in human saphenous veins [115]. In human vascular aortic smooth muscle cells, LPS has been shown to induce TLR4 expression, increase expression of iNOS, thereby increasing NO production, induce vascular endothelial growth factor and enhance ICAM-1 and VCAM-1 expression [125]. However, DHA and EPA can interfere with TLR4 activation by LPS or free SFA [126]. In this murine monocyte/macrophage cell culture model, free lauric acid activated TLR4 and induced NF-κB activation and expression of COX-2, iNOS and IL-1α. However, PUFA inhibited COX-2 expression induced by LPS, SFA or constitutively active TLR4. This observation that n-3 PUFA failed to inhibit COX-2 expression induced by activation of signaling components downstream from TLR4 suggests that the n-3 PUFA directly acts on TLR4.

3.4. Effect of n-3 PUFA on APRs

Acute-phase reactants are proteins whose concentrations increase or decrease by 25% during injury or inflammatory states. Chronic activation of APR can have adverse consequences on health. APR include ceruloplasmin, C3 component of complements, haptoglobin, ferritin, α-1 antitrypsin, albumin, transferrin, apolipoprotein CIII (Apo CIII), CRP, fibrinogen and SAA. Among these APR, elevated levels of fibrinogen, Apo CIII, CRP and SAA are considered predictors of CVD risk [16,127–130]. n-3 PUFA supplementation has been shown to have no or modest effects on fibrinogen levels in humans [131–134]. The Apo CIII-lowering effects of n-3 PUFA has been shown in hypertriglyceridemic men [94], but not in normolipidemic adult subjects [135]. Furthermore, some human studies have observed a decrease in circulating CRP and SAA concentrations with n-3 PUFA consumption [136–151], while others have reported no change [152–159]. The variance between studies may be attributed to a shorter duration of n-3 PUFA supplementation, low n-3 PUFA intakes or subjects with low baseline CRP concentrations. The difference may also be due to a polymorphic variant on the *APOC3* gene promoter [160].

The mode of action by which n-3 PUFA decreases APR concentration has mostly focused on its effect on CRP. C-reactive protein is a stronger predictor of cardiovascular events than LDL cholesterol [161,162]. The major source of CRP is the hepatocytes, and its synthesis is regulated by IL-6 and IL-1 [163]. Increased circulating concentrations indicate pathogenesis of atherosclerosis and inflammation. The mechanisms by which n-3 PUFA decreases CRP may be through the inactivation of TLR4 and NF-κB and IL-6/IL-1 expression as discussed above. Another possibility is through the farnesoid X receptor (FXR), the nuclear receptor for bile acids. Farnesoid X receptor is a member of the nuclear hormone receptor superfamily that functions as a ligand-activated transcription factor. When activated, FXR can inhibit VSMC inflammation and migration through down-regulation of IL-1-induced iNOS and COX-2 expression [164]. Docosahexaenoic acid, as well as AA and LA, are ligands for FXR [165]. Recently, Zhang et al. [83] demonstrated that an FXR ligand, GW4064, suppressed IL-6-induced CRP expression in human Hep3B cells. This was confirmed by using FXR short interfering RNA (siRNA), which abolished this inhibitory effect and therefore enhanced IL-6 to induce CRP expression. They also observed that in livers of mice, FXR ligand WAY-362450 decreased LPS-induced serum amyloid A3 (SAA3) and serum amyloid P (SAP) mRNA levels — SAA and SAP are the major acute-phase proteins in mice. This effect was confirmed in FXR knockout mice, where SAA mRNA levels remained constant.

Other nuclear receptors to which EPA and DHA are ligands for are the LXRα and LXRβ [166–168]. Liver X receptor ligands have been shown to delay atherosclerotic development in mouse models and inhibit atherosclerotic lesion progression [169,170]. Liver X receptors are involved in cholesterol and FA metabolism and have been proposed as a target for therapeutic intervention for CVD. Activated LXRs have the potential to decrease atherosclerotic risk because they can inhibit intestinal cholesterol absorption, promote bile acid synthesis in the liver and stimulate cholesterol efflux in macrophages [171–174]. More recently, synthetic LXR ligands have been demonstrated to inhibit IL-6-induced CRP expression in human hepatocytes [84,85].

4. Effect of n-3 PUFA on cell membrane properties

A physicochemical mechanism by which n-3 PUFAs prevent CVD may start with the changes in the properties of the cell membrane as a result of n-3 PUFA incorporation. The type and amount of dietary FAs can alter the content of the membrane PL FA [175,176] and directly affect cell membrane properties, such as fluidity [177]. This can lead to modifications in the way transmembrane proteins, such as receptors, interact with their ligands [178]. The number and location of double bonds and the length of the acyl chains will also affect properties that may influence FA preference of enzymes and membrane proteins that modulate intracellular signaling pathways and other physiological functions significantly. For example, 18:0 in both the sn-1 and sn-2 positions (18:0–18:0) of a phosphatidylcholine (PC) molecule has a melting point of 55°C and is, therefore, solid at physiological temperatures. This is too rigid for physiological functions; however, the temperature at which the molecule transitions from the solid-to-liquid crystalline phase for 18:0 to 18:2 PC is −16°C [179]. Recently, determination of the energetics of rotation about the carbon–carbon bonds of DHA through molecular modeling of membrane PL bilayers revealed that the DHA acyl chain is highly flexible and dynamic [180]. This computational experimentation infers that DHA can closely intercalate between the grooves of cell membrane bound proteins and may provide a molecular explanation as to why low concentrations of DHA containing lipids could induce such large effects on membrane-associated protein systems. Furthermore, Ma et al. [181] found that n-3 PUFA can alter plasma membrane microdomains called lipid rafts and caveolae that function as signaling platforms that regulate cholesterol transport, signal transduction and endocytosis. When n-3 PUFA is introduced, the microdomain lipid composition is altered: the sphingomyelin content in lipid rafts and the cholesterol and caveolin in caveolae are reduced. The effect of n-3 PUFA-induced cholesterol reduction is a result of poor incorporation of cholesterol into long-chain n-3 PUFA containing PL bilayer [182]. Ultimately, the incorporation of n-3 PUFA can lead to changes in membrane properties, protein functionality and microdomain localization of signaling proteins, thus, resulting in the modulation of downstream cellular signaling pathways [178].

Membrane effects on ion channel conductance by n-3 PUFA have been studied. Intravenous and dietary administration of n-3 PUFA have demonstrated antiarrhythmic effects in animals [183–186], cells [187–190] and humans [31,191–196]. n-3 PUFAs have been shown to prevent arrhythmias through multiple mechanisms. One direct mechanism is that n-3 PUFA reduced membrane electrical excitability and activity of voltage-dependent Na^+ channels in cardiomyocytes. This is mediated through an increase in the threshold of depolarizing current required to initiate an action potential and by prolonging the refractory period following an action potential [42,190]. Fatal arrhythmias are caused by not only dysfunctional Na^+ channels but also by cytosolic free Ca^{2+} variability [176]. Hallaq et al. [197] found that in isolated neonatal rat cardiomyocytes subjected to arrhythmogenic stress caused by glycoside ouabain toxicity, EPA and DHA

exhibited a modulatory action on L-type Ca^{2+} channels, which resulted in lowered cytosolic free Ca^{2+} and Ca^{2+} influx rate; however, AA led to Ca^{2+} overload during a period of ischemic stress. It is possible that n-3 PUFAs directly bind to the ion channel proteins and therefore modulate ion channel activity. A single-point mutation at the 406 of D1-S6 region of the α-subunit Na^{+} channel decreased sensitivity to n-3 PUFA, indicating a binding to a specific location on the Na^{+} channel protein [198]. Alpha linolenic acid has also been implicated to have antiarrhythmic effects similarly to those of EPA and DHA by increasing the threshold for arrhythmia in cardiomyocytes [199]. Ander et al. [200] demonstrated that ALA in a flaxseed-rich diet is antiarrhythmic in hypercholesterolemic rabbits possibly through shortening of the action potential. This study also delineated that this effect was mediated specifically through ALA and not due to the in vivo conversion of ALA to EPA and DHA.

5. Effect of n-3 PUFA on vascular endothelial and smooth muscle cells

n-3 PUFAs have beneficial effects on vascular endothelial function by decreasing endothelial activation. Endothelial cells express ICAM-1, VCAM-1, E-selectin and P-selectin that are involved in leukocyte recruitment and platelet adhesion during thrombosis and inflammation and also contribute to early phases of atherogenesis. Cytokine-induced endothelial activation has been shown to increase the expression of genes for ICAM-1, VCAM-1 and E-selectin, and n-3 PUFA have been shown to inhibit the production of inflammatory cytokines that activate the endothelium. De Caterina et al. [201] found that culturing endothelial cells with DHA following challenges with IL-1, IL-4, tumor necrosis factor (TNF-α) or LPS decreased expression of VCAM-1, ICAM-1 and E-selectin and secretion of IL-6, and IL-8. In addition, ICAM-1, VCAM-1 and E-selectin mRNA levels were decreased in human umbilical vein endothelial cells stimulated with IL-1β by DHA and EPA [202]. Treatment with n-3 PUFA also decreased the expression of adhesion molecule in human monocytes [203] and murine macrophages [204]. A decrease in expression of adhesion molecules by n-3 PUFA would decrease adhesion and migration of monocytes to the endothelium thereby mitigating atherosclerosis development and inflammation.

A balance between the concentrations of vasoconstrictors (TXA_2, PGH_2, endothelin-1) and vasodilators (NO, endothelium-derived hyperpolarizing factor, PGI) that are produced by the endothelium determines the vascular tone. The vasorelaxant effect of DHA has been attributed to the decreases in Ca^{2+} influx in VSMCs [205]. As discussed above, n-3 PUFA can modify eicosanoid production to favor vasodilation and antithrombotic actions. It has also been suggested that n-3 PUFAs increase endothelium-dependent relaxation through an enhancement of NO release [206]. NO inhibits platelet aggregation and adhesion, leukocyte adhesion and smooth muscle cell proliferation. Hirafuji et al. [207] showed that DHA, and to a lesser degree, EPA and not AA, enhanced IL-1β-induced NO production and increased iNOS mRNA and protein expression through a mechanism involving p44/42 mitogen-activated protein kinase signaling pathway in rat VSMCs. Furthermore, DHA-suppressed ischemia induced arrhythmia in hypertensive rats through the inhibition of TX-like vasoconstrictor responses in the aorta [208].

Another n-3 PUFA antiatherosclerotic mechanism is its effect on VSMC. Exaggerated VSMC growth results in arterial damage and is an important component in the pathogenesis of atherosclerosis [209,210]. Eicosapentaenoic acid, and DHA to a lesser extent, can affect vascular function through the inhibition in VSMC growth and proliferation at various steps of the signal transduction pathway of growth factors [209–211]. Terano et al. [212] found that EPA prevented binding of platelet-derived growth factor to its receptor and activation of protein kinase C and suppressed c-fos mRNA expression, an early growth gene, via inhibition of c-fos transcription. Furthermore, EPA and DHA demonstrated an inhibition of DNA synthesis through cyclins and cyclin-dependent kinases, which control eukaryotic cell cycle progression [210]. Eicosapentaenoic acid also has been shown to have a suppressing effect on transforming growth factor-β and VSMC growth in spontaneously hypertensive rats [209].

6. Effects of n-3 PUFA on blood TGs

Elevated fasting and postprandial plasma TG levels increase inflammation and are independent risk factors for CVD. n-3 PUFA supplementation decreased concentrations TGs and of inflammatory markers. Thus, DHA supplementation reduced both the fasting and postprandial TGs by more than 25% in hypertriglyceridemic men [94]. Furthermore, DHA also decreased the concentrations of atherogenic small dense LDL particles, total LDL particles and the remnant chylomicron particles [58,94]. As discussed earlier, DHA supplementation decreased the circulating concentrations of Apo CIII, which inhibits the activity of lipoprotein lipase (LPL) that controls TG clearance from blood. Thus, a reduction in the concentration of Apo CIII means increased activity of LPL and hence increased clearance of plasma TG. Apo CIII-rich lipoproteins also enhance monocyte adhesion to vascular endothelial cells [130]. Plasma concentration of Apo CIII is therefore considered an other emerging lipoprotein-associated marker for CVD risk [160]. n-3 PUFA also regulate Apo CIII through their effects on PPARα, which down-regulates Apo CIII expression [213], and NF-κb, which up-regulates Apo CIII expression [214].

n-3 PUFA can also decrease TG concentration through the inhibition of hepatic very low-density lipoprotein (VLDL)-TG synthesis and secretion that is secondary to a decrease in TG synthesis. This decrease in VLDL-TG secretion may be due to the decrease in the expression of hepatic gene transcription factor, SREBP-1c, which is the key switch in controlling lipogenesis. The LXRα/RXRα heterodimer regulates the expression of SREBP-1c by 2 LXR response elements (LXREs) in the SREBP-1c promoter. n-3 PUFA-mediated suppression of SREBP-1c promoter activity is possibly due to the prevention of the binding of the LXR/RXR heterodimer to the LXREs in the SREBP-1c promoter region so as to decrease SREBP-1c expression [215]. This in turn would diminish the synthesis of acetyl-CoA carboxylase and FA synthase. The net effect is a decrease in FA synthesis. The TG lowering effect may also be due to the simultaneous increase in mitochondrial and/or peroxisomal β-oxidation, which may be a direct result of increased PPARα-induced increase in acyl-CoA oxidase gene expression and therefore lead to reduced FA substrate for TG synthesis [45]. Another nuclear receptor with TG lowering potential is FXR. Docosahexaenoic acid is an FXR ligand and has been shown to suppress the expression of hepatic lipase and Apo CIII and increase Apo CII and VLDL-receptor gene expression in HepG2 cells [216–219]. Another TG lowering mechanism by n-3 PUFA include the decreased activity of key enzymes in TG biosynthesis, such as phosphatidic acid phosphohydrolase or diacylglycerol (DG) acyltransferase that catalyzes phosphatidate to DG and DG to TG, respectively. An overall decrease in TG production by n-3 PUFA in adipose tissue would ultimately lead to decreased serum NEFA transport.

7. Concluding remarks

Based on the results from cellular and molecular studies, the cardioprotective effects of n-3 PUFA appear to be due not through a single mode of action but to a synergism between multiple, intricate mechanisms that involve TG lowering, antiinflammatory, inflammation-resolving, regulation of transcription factors and gene expression, membrane fluidity and antiarrhythmic and antithrombotic effects. Eicosapentaenoic acid and DHA have similar yet very

distinctive cardioprotective properties. Only DHA seems to decrease blood pressure, heart rate and the number of total and small dense LDL particles. Docosahexaenoic acid also has higher potency to regulate the activity of several transcription factors than EPA. Scientific knowledge regarding the cardioprotective benefits of n-3 PUFA has been translated into nutritional guidelines for improving cardiovascular health, and it has the potential to be used for the improvement or resolution of other inflammatory diseases.

More than ever, n-3 PUFA availability has increased not only from marine vertebrate origin, but also from native and transgenic algae and plants. Cardiovascular disease in the US continues to be the leading cause of death and may rise in years to come due to the escalating aging population and increasing incidence of obesity and type 2 diabetes. Further research on the overlapping and independent mechanisms by which EPA and DHA prevent and reverse CVD is needed.

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