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REVIEWS: CURRENT TOPICS

Modulation of enzymatic activities by n-3 polyunsaturated fatty acids to support cardiovascular health

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Abstract

Epidemiological evidence from Greenland Eskimos and Japanese fishing villages suggests that eating fish oil and marine animals can prevent coronary heart disease. Dietary studies from various laboratories have similarly indicated that regular fish oil intake affects several humoral and cellular factors involved in atherogenesis and may prevent atherosclerosis, arrhythmia, thrombosis, cardiac hypertrophy and sudden cardiac death. The beneficial effects of fish oil are attributed to their n-3 polyunsaturated fatty acid (PUFA; also known as omega-3 fatty acids) content, particularly eicosapentaenoic acid (EPA; 20:5, n-3) and docosahexaenoic acid (DHA; 22:6, n-3). Dietary supplementation of DHA and EPA influences the fatty acid composition of plasma phospholipids that, in turn, may affect cardiac cell functions in vivo. Recent studies have demonstrated that long-chain omega-3 fatty acids may exert beneficial effects by affecting a wide variety of cellular signaling mechanisms. Pathways involved in calcium homeostasis in the heart may be of particular importance. L-type calcium channels, the Na^{+}–Ca^{2+} exchanger and mobilization of calcium from intracellular stores are the most obvious key signaling pathways affecting the cardiovascular system; however, recent studies now suggest that other signaling pathways involving activation of phospholipases, synthesis of eicosanoids, regulation of receptor-associated enzymes and protein kinases also play very important roles in mediating n-3 PUFA effects on cardiovascular health. This review is therefore focused on the molecular targets and signaling pathways that are regulated by n-3 PUFAs in relation to their cardioprotective effects.

Keywords: Docosahexaenoic acid; Eicosapentaenoic acid; Calcium; Phospholipases; Eicosanoids; Adrenoreceptors; Protein kinases

1. Introduction

Historically, much of the understanding of the beneficial health effects of fish oil has come from dietary studies in populations with diets rich in polyunsaturated fatty acids (PUFAs), particularly in omega-3 (n-3) PUFAs [1]. The n-3 and n-6 groups of PUFAs are different because of the presence of the first double bond in the third or sixth positions, respectively, from the methyl terminal of the aliphatic carbon chain (Fig. 1). In a typical Western diet, the ratio of n-6 to n-3 fatty acids has a range of approximately 20–30:1 instead of 1–2:1, which is believed to be present in the diets of populations consuming a diet based on fish products [2,3]. Importantly, populations consuming a Western-style n-6 PUFA diet are at a significantly greater risk of developing inflammatory diseases, including cancer and coronary heart disease (CHD), than those populations living on diets rich in fish oils containing n-3 PUFAs [4–7]. The beneficial effects of fish oils are mostly attributed to their n-3 PUFA content, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, another n-3 PUFA, α-linolenic acid (ALA), found in green leafy vegetables, flaxseed, rapeseed and walnuts, can be desaturated and elongated in the human body to EPA, docosapentaenoic acid (DPA) and then to DHA (Fig. 2). However, ALA should be used with caution as a sole source of n-3 PUFAs because recent studies using ^{13}C-labeled ALA have indicated that healthy human subjects were able to convert ALA mostly into EPA (approximately 21%) and, to a lesser

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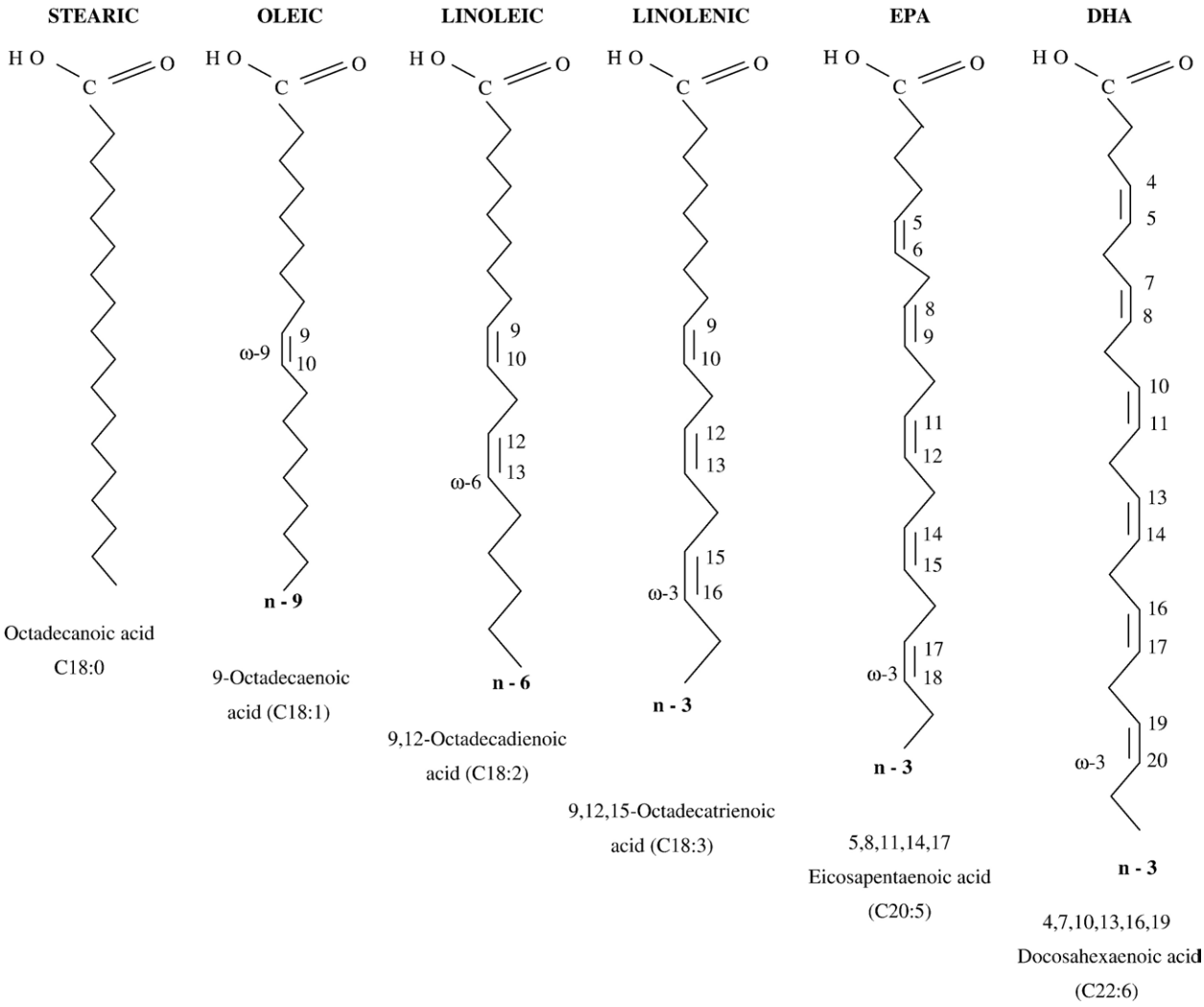

Fig. 1. Some biologically important fatty acids. Fatty acids are classified as saturated or unsaturated. Omega-3, omega-6 or omega-9 unsaturated fatty acid structures are based on the position of the last double bond at the third, sixth or ninth position from the methyl (omega) terminal of the aliphatic carbon chain.

extent, into DPA (6%), but there was very little to no enrichment in DHA [8,9]. Similarly, people with chronic illnesses were also able to convert ALA into EPA (60%) and DPA (25%) with no conversion into DHA [10]. Therefore, it is critical to consume preformed DHA in the diet to maintain an adequate membrane concentration of DHA.

The observation that Greenland Eskimos (Inuit) have a low incidence of CHD despite a high saturated fat intake has led to much scientific and public interest in the role of the various fatty acids in preventing and treating disease, particularly CHD [3]. Both epidemiological [11–13] and prospective randomized clinical trials [14–17] have reported a decrease in morbidity and mortality from heart disease in patients with diets supplemented with n-3 PUFAs. The GISSI Prevenzione Study [16] is the largest study to probe the cardiovascular benefits of n-3 PUFAs. In this study, patients, after suffering myocardial infarction, were randomized to n-3 PUFA (1 g/day), α-tocopherol (α-T; 300 mg/day), n-3 PUFAs plus α-T (1 g n-3 PUFAs+300 g/day α-T) or placebo (none) groups and treated for 3.5 years. The group receiving n-3 PUFAs alone had a significant reduction in the relative risk of death, nonfatal myocardial infarction or nonfatal stroke. Relative risk for all fatal events was 0.80. The relative risk reduction for sudden cardiac death was 45%. Vitamin E had little benefit. Another trial, the DART trial, compared three dietary interventions in 2033 post-myocardial infarction patients [17]. After 2 years of follow-up, this trial concluded that n-3 PUFA consumption significantly reduced mortality by 27%. However, it is interesting to note that most of the beneficial cardioprotective effects of eating fish or taking n-3 PUFA (EPA+DHA) supplements was seen with consuming as little as 1 g/day of n-3 PUFAs. Such a low intake of n-3 PUFAs has usually doubled the n-3 PUFA content of cell membranes, which do not further change with increased n-3 PUFA intake. These clinical trials strongly implicated n-3 PUFAs as having beneficial effects on cardiovascular health. Based on these studies, the American Heart Association (AHA) suggests

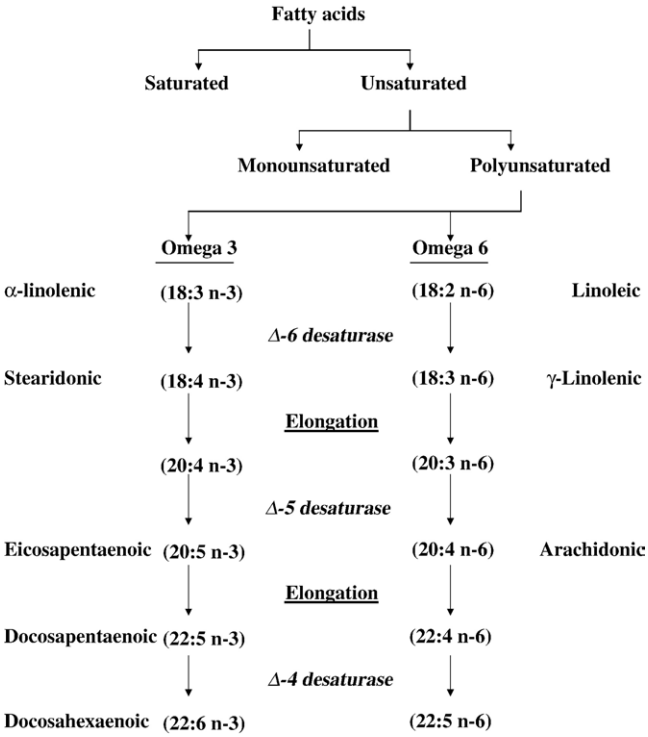

Fig. 2. Metabolic pathway of omega-6 and omega-3 fatty acid synthesis. Fatty acids are classified as saturated or unsaturated fatty acids, depending on the presence of double bonds. The unsaturated fatty acids are further divided into monounsaturated fatty acids or PUFAs. The PUFAs are either n-3 or n-6 fatty acids. ALA and linoleic acid are the precursors of n-3 and n-6 fatty acids, respectively, and are converted to different long-chain PUFAs by sequential desaturation and elongation.

that n-3 PUFAs benefit the hearts of healthy people and those at high risk of — or who have — cardiovascular disease. The AHA recommends eating a fatty fish meal at least two times a week for the general population or taking 1 g of n-3 PUFAs (EPA+DHA)/day for those with known CHD. However, the AHA has no specific recommendations for those at increased risk without known disease [18].

2. Beneficial effects of n-3 PUFAs on cardiovascular system

n-3 PUFAs exert many of their beneficial effects upon the cardiovascular system via their effects on several cellular processes. n-3 PUFAs improve the plasma lipid profile. Harris [19], in an analysis of 72 human trials, where normal subjects or hypertriglyceridemic patients were given 7 g or less of n-3 PUFAs/day for at least a 2-week period, concluded that n-3 PUFAs generally lowered triglycerides (TGs; 25–28%) in both populations. Harris [19] further noticed that n-3 PUFAs were able to lower lipoprotein cholesterol in animal studies, but there was only a minor impact on lipoprotein cholesterol levels in human studies. A recent study by Mori et al. also observed similar findings in mildly hypertriglyceridemic patients. Intake of n-3 PUFAs (4 g/day for 6 weeks) reduced TG levels by 18–20% but had a minimal impact on low-density lipoprotein cholesterol or high-density lipoprotein cholesterol (HDL-C) [20]. In contrast to these studies, long-term treatment of hypertriglyceridemic patients with n-3 PUFAs (4 g/day for 16 weeks) led to a significant reduction in TG by 47%, while TG levels rose by 16% with placebo (corn oil). This effect of n-3 PUFAs was associated with a decrease in total cholesterol: HDL ratios (20%) and a modest increase in HDL-C (13%) [21]. Similar results were also reported in another study where hypertriglyceridemic patients were treated with n-3 PUFAs (4 g/day) for 6 months [22]. It appears from different studies [23] that higher levels of n-3 PUFAs for longer duration have beneficial effects on plasma lipid profile.

n-3 PUFAs also have antiatherogenic actions. Eritsland et al. [24] reported that n-3 PUFA supplementation (4 g/day for 1 year) in post-coronary artery bypass graft patients was associated with a reduced frequency of vein graft occlusions. This n-3 PUFA effect was not linked to an influence on serum lipoproteins because serum cholesterol levels were not altered by n-3 PUFA supplementation. Furthermore, there was no association between the reduction in serum TG and vein graft occlusion. These studies, therefore, concluded that the n-3 PUFA effect on new plaque development appeared to be due to antithrombotic as well as antiatherosclerotic properties of n-3 fatty acids. Furthermore, Thies et al. [25] reported that n-3 PUFA supplementation (1–4 g/day for an average of 42 days) in heart patients prior to undergoing carotid endarterectomy resulted in a rapid incorporation of n-3 PUFAs into advanced atherosclerotic plaques, which was associated with structural changes consistent with increased plaque stability. The antiatherosclerotic effects of n-3 PUFAs appear to be mediated through their anti-inflammatory effects on platelets and endothelial cells. Platelets, through their interaction with the vascular endothelium, play a critical role in atherogenesis [26]. Mori et al. [27] observed that human consumption of n-3-PUFAs (3–4 g/day) for 3 weeks reduced platelet aggregation induced by collagen and platelet-activating factor (PAF) regardless of whether n-3 PUFAs were ingested as daily fish meals or fish oil capsules. Similarly, Agren et al. reported that consuming moderate amounts of n-3 PUFAs for 15 weeks in the form of a fish diet (0.38 g EPA+0.67 g DHA/day) or fish oil (1.33 g EPA +0.95 g DHA) also inhibited platelet aggregation but did not affect hemostatic factors. Notably, EPA-free DHA oil (1.68 g/day) was not effective in decreasing in vitro platelet aggregability [28]. The ineffectiveness of DHA implies that modulation of platelet aggregation by n-3 PUFAs may be mediated through the eicosanoid pathway (see below) rather than being a direct effect of fatty acids on platelets. Furthermore, in the patients with elevated TG levels, prolonged treatment with n-3 PUFAs (4 g/day for 7 months) was associated with reduced levels of soluble adhesion molecules (sICAM-1 and sE-selectin) [22]. Soluble adhesion molecules lack membrane-spanning and cytoplasmic

domains that are present in the membrane-bound forms, but their levels have been noted to be elevated in pathological conditions in which tissue expressions of the membrane-bound forms of adhesion molecules are known to be up-regulated [29,30]. It is difficult to measure the membrane expression of adhesion molecules in the human vasculature after n-3 PUFA supplementation. Evidence for n-3 PUFA effects on cell membrane expression of adhesion molecules is derived from in vitro experiments. DHA treatment to human adult saphenous vein endothelial cells at concentrations (10 μM) compatible with nutritional supplementation of this fatty acid to individuals consuming a normal Western diet reduced surface expression of adhesion molecules [31]. It appears from these studies that one of the beneficial effects of n-3 PUFAs on the cardiovascular system is mediated through its antiatherosclerosis properties. Furthermore, n-3 PUFAs also improve vascular functions. Treatment with EPA (1.8 g/day for 6 weeks) augments both NO-dependent and NO-independent endothelium-dependent forearm vasodilatation in patients with coronary artery disease [32]. Dietary supplementation with fish oil (5 g EPA+DHA/day for 3 weeks) significantly improved endothelium-dependent coronary vasodilation in heart transplant recipients without altering the responses to endothelium-independent vasodilation. However, these improved vascular functions play a small role in reducing hypertension. A metaregression analysis of 36 randomized trials on fish oil supplementation (mean consumption of 3.7 g/day for a median of 12 weeks) in largely overweight and hypertensive subjects showed only a small antihypertensive effect [33]. Furthermore, it is suggested that DHA is likely more favorable in lowering blood pressure and heart rate than EPA [34].

In conclusion, observational studies, human intervention trials, animal models and cell culture studies suggest that n-3 PUFAs have beneficial effects on the cardiovascular system. The molecular and cellular effects of n-3 PUFAs for mediating the beneficial cardiovascular effects are not fully known. We have attempted to review some of the key cellular and molecular pathways that are known to be modulated by n-3 PUFAs.

3. Enzymes regulating calcium ion channels

It is now well known that intracellular free calcium ions (Ca^{2+}) serve as a cofactor for several enzymatic processes required for cellular growth. Furthermore, an increase in cellular Ca^{2+} is associated with enhanced cell contraction, vasoconstriction and cell proliferation and, thus, may be involved in the development of cardiovascular diseases [35]. Among various biochemical derangements, the increase in intracellular Ca^{2+} plays a permissive role in the development of arrhythmia [36] and cardiac hypertrophy [37]. Regulation of intracellular calcium at cellular microdomain levels is described in a recent review [38] and, for simplicity, is outlined in Fig. 3. Briefly, the influx of calcium in cardiomyocytes in response to hormonal and mechanical stimulation mainly occurs through L-type Ca^{2+} channels in sarcolemmal (SL) membranes. This influx of calcium then triggers the release of Ca^{2+} from the sarcoplasmic reticulum (SR) through ryanodine-sensitive Ca^{2+} channels; this process is generally known as calcium-induced calcium release. Released Ca^{2+} is then utilized for the regulation of cellular processes, particularly its binding to troponin C, which causes conformational changes of the tropomyosin and allows the myosin head to interact with actin to generate contractile force. Ca^{2+} is then sequestered from the cytosol by the SR-Ca^{2+} ATPase pump and stored in the lumen of SR until the next event. Mitochondria are also known to store large quantities of Ca^{2+}, but their role in the contraction–relaxation cycle in the normal heart is poorly understood. In fact, these organelles are considered to serve as a Ca^{2+} sink to prevent the occurrence of intracellular Ca^{2+} overload in diseased myocardium. Therefore, under normal physiological conditions, intracellular levels of Ca^{2+} are tightly controlled by a number of key enzymes, including voltage-dependent channels, the SL-Na^{+}–Ca^{2+} exchanger, receptor-mediated calcium channels and the ryanodine receptor (RyR) and SR-Ca^{2+} ATPase pump. Any abnormalities in any of these key regulators contribute to abnormal Ca^{2+} handling and, thus, lead to cardiac dysfunction, including arrhythmia generation, hypertrophy and myocardial stunning.

There is direct evidence that suggests n-3 PUFAs are very potent agents in regulating intracellular calcium levels. Research in various laboratories has demonstrated that calcium transport in isolated cardiac myocytes from fish-oil-fed rats and mice was altered [39,40]. Earlier studies have shown that EPA and DHA (5 μM) can prevent arrhythmias, fibrillation and contracture in isolated rat cardiac myocytes induced by toxic concentrations of ouabain [41,42], a cardiac glycoside that binds to the α-subunit of membrane-bound Na, K-ATPase [43]. The primary action of cardiac glycosides is to inhibit this enzyme, which is also known as the sodium pump. With inhibition of this enzyme, sodium ions accumulate in the cell and intracellular concentrations of potassium decrease. Increased intracellular sodium activity favors the accumulation of calcium ions in the cell via the Na^{+}–Ca^{2+} antiport system. Addition of either oxygenase inhibitors or antioxidants did not alter the effects of n-3 PUFAs on ouabain-induced cardiac arrhythmia [41]. This observation suggests that n-3 PUFA incorporation into the phospholipids of cell membranes may have prevented the toxicity caused by ouabain, and its presence was associated with fewer rises in cytosolic free calcium [41]. Furthermore, EPA (2–10 μM) exhibited a similar protective effect against ouabain toxicity when cardiomyocytes were incubated for 3–5 days in the presence of these n-3 PUFAs [44]. Two other PUFAs, linoleic acid (18:2, n-6) and linolenic acid (18:3, n-3), also exhibited similar but less potent effects compared with EPA. In contrast, neither oleic acid (18:1, n-9) nor saturated fatty acids (18;0, 14:0, 12:0) affected contraction rate [44]. These studies have shown that the beneficial effects

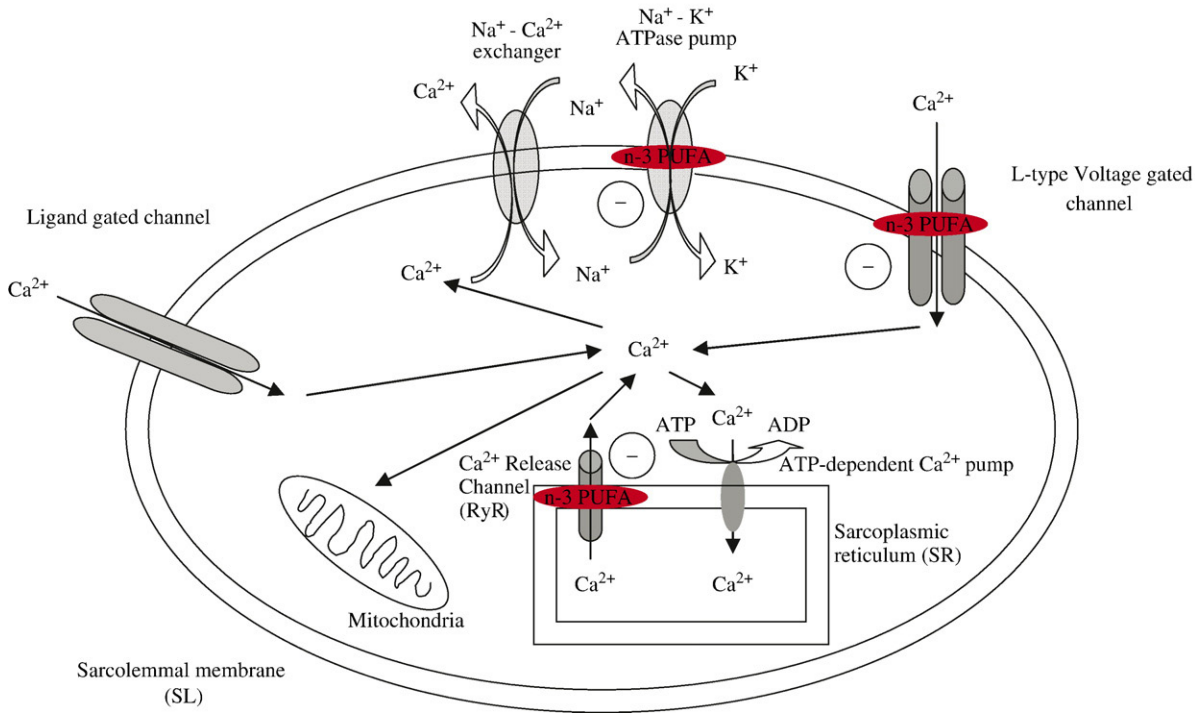

Fig. 3. Effect of n-3 PUFAs on intracellular calcium regulation. The influx of calcium in cardiomyocytes in response to hormonal and mechanical stimulation mainly occurs through L-type Ca^{2+} channels and ligand-gated channels in SL membranes. This influx of calcium then triggers the release of Ca^{2+} from the SR through ryanodine-sensitive Ca^{2+} channels (RyR). Released Ca^{2+} is then utilized for regulation of cellular processes. The excess Ca^{2+} is then either sequestered from the cytosol by the SR-Ca^{2+} ATPase pump and stored in the lumen of SR until the next event or pumped out through the Na^{+}–Ca^{2+} exchanger. The concentration of Na^{+} is then balanced by the Na^{+}–K^{+} ATPase pump. Mitochondria also store large quantities of Ca^{2+}. n-3 PUFAs inhibit (shown as negative marks in circle) rises in intracellular calcium by acting on multiple sites as shown in red (see text for details).

of fish oil in preventing fatal arrhythmias in myocardial ischemia are at least in part mediated by modulating the dihydropyridine-sensitive L-type calcium current [44]. Consistent with this observation, treatment of cardiomyocytes with DHA or EPA prevented the increase in calcium influx by Bay K8644, an established agonist for L-type calcium channels [44], strongly suggesting that L-type calcium channels are the target site for these fatty acids. Similarly, it has been demonstrated that n-3 PUFAs modulate calcium current through the L-type calcium channels, and these effects occur within minutes of adding EPA or DHA to the perfusing medium of the cultured cardiac myocytes [45]. These results were further supported by studies where DHA (10 μM) pretreatment of rat cardiomyocytes acutely (20 min) or chronically (72 h) prevented intracellular rise in Ca^{2+} only when stimulated with endothelin-1 or KCl or exposed to anoxic conditions, suggesting that DHA, as a free fatty acid, binds to dihydropyridine sites only when the channel is in an active state [46]. Furthermore, Hirafuji et al. [47] demonstrated that DHA can also suppress Ca^{2+} influx through the L-type voltage-dependent channels in vascular smooth muscle cells (VSMCs). Since intracellular Ca^{2+} plays an important role in regulating vascular tone, it suggests that the suppressive effect of DHA on Ca^{2+} influx in VSMCs may contribute to the beneficial properties of DHA in cardiovascular disorders [48]. Xiao et al. [49] also confirmed reductions of voltage-gated L-type Ca^{2+} currents by n-3 PUFAs in adult and neonatal rat ventricular myocytes. Moreover, it has also been shown that the delayed rectifier K^{+} channel is inhibited by n-3 PUFAs [50]. The combined effect of this is suggested to reduce electrical excitability, making arrhythmias less likely [51]. Both EPA and DHA are known to be antiarrhythmic. They depress surface membrane electrical excitability [51,52] and inhibit spontaneous release of Ca^{2+} from overloaded cardiac SR [53]. The effect of n-3 PUFAs on the L-type Ca^{2+} channel appears to be due to their direct binding to the channel proteins. This fact is supported by a recent study investigating the link between n-3 PUFA content of the plasma membrane and ion channel activity [54], which suggested that n-3 PUFA concentrations required for antiarrhythmic action were too low to produce a significant change in the overall arrangement of the phospholipids within cardiac membranes. Similarly, the effect is quickly reversed when free PUFAs are extracted from the cells by adding delipidated BSA to the bathing medium [55]. These observations imply that n-3 PUFAs are neither fully incorporated into membrane phospholipids nor covalently bound to any constituents of the myocyte to produce the antiarrhythmic effect [55]. These studies, therefore, suggest that n-3 PUFAs exert antiarrhythmic effects by direct interactions with SL ion channels rather than indirectly by perturbing membrane phospholipid packing.

Furthermore, Xiao et al. [56] demonstrated that n-3 PUFAs lose their blocking effects on the human myocardial Na^+ channel α-subunit when it is transiently expressed in HEK293 cells, where the asparagine in the 406 position in D1,S6 was substituted by a lysine. These investigators further demonstrated that cells transfected with a mutant Na^+ channel α-subunit, which is persistently active, were potently inhibited by n-3 PUFA treatment and that the activation was restored after an n-3 PUFA washout [57]. However, in the studies performed by Xiao et al. [56,57], n-6 PUFAs [linoleic acid, linolenic acid and arachidonic acid (AA)], but not oleic acid (n-9) or saturated fatty acids, were also able to inhibit the Na^+ channel. These observations provide evidence that a specific binding site for the both the n-6 and n-3 PUFAs exists on the Na^+ channel and that these fatty acid bindings result in the modulatory effects on the ion channel currents. As n-3 PUFAs have other specific effects on cardiac-related enzymatic activities, modulation of the Na^+ current can be an important effect by n-3 PUFAs together with other potential cardiac benefits.

In addition to the effects on L-type Ca^{2+} channels, there is strong evidence that part of the antiarrhythmic action of PUFAs is mediated through inhibition of the Ca^{2+}-release mechanism of the SR. Consistent with this suggestion, it has been shown that EPA (10 μM) exerted part of its antiarrhythmic action by directly interacting with the RyR in rat cardiomyocytes [58]. Similarly, another recent study demonstrated that the anticancer drug doxorubicin binds to the RyR in rat cardiomyocytes and induces a rise in intracellular Ca^{2+}. The doxorubicin effect was blocked by DHA (10 μM) treatment, further suggesting that n-3 PUFAs interact directly with RyR and prevent arrhythmia [59]. It is apparent from these few studies that both EPA and DHA [58–60] have regulatory effects on RyR-mediated Ca^{2+} release. However, these effects also appeared to be nonspecific, as other fatty acids, including oleic acid [60] and AA [61], are also capable of binding to RyR and inhibiting Ca^{2+} release.

In conclusion, these studies suggest that n-3 PUFAs directly interact with calcium regulatory enzymes in cardiomyoctes and inhibit a rise in intracellular Ca^{2+}, which prevents arrhythmia generation and development of pathological hypertrophy in cardiac cells. In addition to ion channels, there are other cellular processes, including various active phospholipases, receptor-bound enzymes and protein kinases, that also play an important role in regulating intracellular calcium and other cellular processes (described below). The activities of these enzyme systems are also modulated by n-3 PUFAs to beneficially affect the cardiovascular system.

4. Enzymes regulating phospholipid degradation

n-3 PUFAs have been shown to influence all major classes of phospholipases, including phospholipase C (PLC), phospholipase D (PLD) and phospholipase A_2 (PLA_2). Activation or inhibition of these phospholipases by n-3 PUFAs affects calcium mobilization from intracellular stores, mainly by generating second messengers from phospholipid degradation (Fig. 4). For example, activation of phosphatidylinositol-4,5-bisphosphate (PIP_2)-specific PLC-β through receptor (or nonreceptor)-mediated activation of a GTP-binding protein (G-protein) pathway or PLC-γ through receptor-mediated activation of a tyrosine kinase pathway causes phosphatidylinositol (PI) degradation [62]. Activation of PLC-β or PLC-γ results in the formation of the putative Ca^{2+}-releasing compound inositol 1,4,5-trisphosphate (IP_3) and the activator of the protein kinase C (PKC) isoenzymes, 1,2-diacylglycerol (DAG). IP_3 binds to the RyR to induce a rise in intracellular Ca^{2+} (see Fig. 4). To date, 11 mammalian PLC isozymes have been identified, all of which are single polypeptide chains and can be divided into four types: PLC-β, PLC-γ, PLC-δ and PLC-ε. PLC-β, PLC-δ and PLC-ε are activated by G-protein-coupled receptors, receptor tyrosine kinase and the ras pathway, respectively [63]. These PLC isozymes display differences in structure and activation [63,64], and it is possible that the specific cardiac effects may depend on the type, quantity and activity of the PLC isozyme present at the SL membrane. For example, PLC-δ_1 is the most abundant isozyme in normal rat heart tissue compared to PLC-β or PLC-γ [65]. PLC-ε, which is primarily expressed in lungs and hearts, is regulated by a number of regulators, including the Ras family of GTPases, Rho A, Gα and Gβγ [66]. Activation of PLC contributes to many processes believed to be involved in arrhythmia, hypertrophy and atherogenesis. Expression of leukocyte adhesion molecules and infiltration of blood cells to the vasculature, platelet aggregation [67], mechanical stress effects on the endothelium [68], secretion of endothelium-derived factors [69], mitogenic responses of VSMCs [70], smooth muscle contraction [71,72] and formation of relaxing and contracting factors by endothelial cells [73] are partially mediated by activation of the PI cycle. Inhibition of PLC activities using pharmacological inhibitors has been shown to improve myocardial recovery after ischemia–reperfusion [74] and to attenuate arrhythmia induced by receptor activation [75] in isolated rat hearts.

Studies from various laboratories demonstrated that n-3 PUFAs provide beneficial effects on the cardiovascular system through their effects on PLC activities. Woodcock et al. [76] showed that feeding rats for 8 weeks with n-3 and n-6 PUFAs caused a depression of total release of inositol phosphates in left atrial tissue in the presence or absence of norepinephrine; however, the effect of n-3 PUFAs on decreasing the release of inositol phosphates was significantly greater than that of n-6 PUFA-fed rats. Similarly, IP_3 release in cardiac myocytes from fish-oil-fed pigs (50 g/kg diet for 6 weeks) after stimulation with adrenergic agonists was significantly reduced compared to myocytes isolated from pigs fed a similar amount of beef tallow [77]. In other studies, EPA-treated cells (214 μM for 4–5 days) stimulated

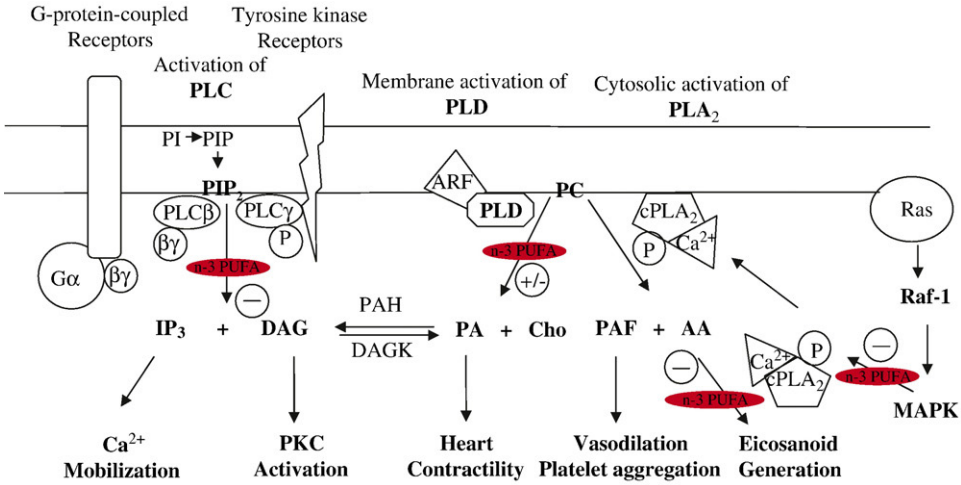

Fig. 4. Regulation of phospholipase activities by n-3 PUFAs in cardiovascular functions. The major classes of phospholipases, including PLC, PLD and PLA_2, generate mediators for regulating cardiovascular functions. Activation of PLC-β through receptor-mediated activation of G-protein or by PLC-γ through receptor-mediated activation of tyrosine kinases causes PIP_2 degradation. This results in the formation of the putative Ca^{2+}-releasing compound inositol IP_3 and the activator of the PKC isoenzymes, 1,2-DAG. Activation of PLD through ARF causes degradation of PC into PA and free choline (Cho). PA and DAG can be interconverted by PA hydrolase (PAH) or DAG kinase (DAGK), respectively. Activation of PLA_2 through calcium (Ca^{2+})- and Ras–Raf-1–MAP kinase-mediated phosphorylation results in degradation of PC and release of AA and lysophosphatidylcholine, which is converted into PAF. AA is then utilized for eicosanoid synthesis through COX, LOX and cytochrome P450 pathways. n-3 PUFAs regulate the generation of phospholipid-derived signaling molecules via their effects on phospholipase activities (shown in red), and thus, signaling pathways involved in the cardiovascular functions (see text for details) can be modulated by n-3 PUFAs.

with phenylephrine or treated with GTPγ after permeabilizing exhibited reduced IP_3 production [78]. Similar effects were not found when the cells were treated with equivalent concentrations of either saturated or n-6 PUFAs. Furthermore, DHA but not AA or EPA also selectively suppressed activation of PLC by TNFα [79]. One possible mechanism by which n-3 PUFAs exert their effects on PLC activation is that incorporation of n-3 PUFAs results in modification of the fatty acyl composition of the membrane phospholipids. The studies by Salem et al. [80] indicated that EPA-containing phospholipids are concentrated in the microenvironment of membrane-bound proteins. These studies suggest that n-3 PUFAs could profoundly influence cellular signalling pathways and possibly affect receptor-mediated PLC at the level of the agonist–receptor, receptor–G-protein coupling or G-protein–PLC-β interaction [80].

Another class of phospholipases that could have an effect on cellular calcium is PLD. PLD is also regarded as one of the key enzymes to influence cardiac function in normal hearts [81]. It has two isozymes, and both PLD_1 and PLD_2 are present in cardiomyocytes. PLD_1 is localized in the Golgi apparatus and nuclei [82], whereas PLD_2 is mostly present in the SL membranes [83]. PLD hydrolyzes phosphatidylcholine (PC) and produces phosphatidic acid (PA) and phosphocholine. PA is important in heart function as it has been shown to stimulate the SL and SR Ca^{2+} transport system to increase intracellular Ca^{2+} in adult cardiomyocytes and augment contractile activity in the normal heart [84,85]. It also acts as a mediator in cardiac hypertrophy [86]. PA was also shown to activate PLC-ε and generate IP_3 formation [87]. In addition to this, PA also influences other cardiac signaling pathways after its hydrolysis by PA phosphohydrolase to DAG, a potent activator of PKC [88]. Results from Dhalla's laboratory conclude that PLD activities are differently altered in congestive heart failure; PLD_1 activities are decreased whereas PLD_2 activities are increased in viable left ventricle tissues [89]. The significance of these changes in the failing heart is currently not known [81]. Several investigators have demonstrated that PLD activity can be modulated by fatty acids. *cis*-Unsaturated fatty acids, including oleic acid, linoleic acid, linolenic acid and AA, have been shown to stimulate PLD activity in SL membranes, whereas saturated and *trans*-isomers of fatty acids have very few or no effects [90]. At present, there is not much known about the effect of n-3 PUFAs on PLD activities in heart tissue. Diaz et al. [91] have demonstrated that DHA stimulates PLD_1 activity in human peripheral blood monocytes. Dai et al. [90] suggested that activation of PLD in heart SL membranes by unsaturated fatty acids is related to their preferentially partitioning into fluid domains, which causes disordering in the membranes, while saturated or *trans*-unsaturated fatty acids partition into gel domain without causing disarray. Consistent with this, we have demonstrated that both oleic acid and DHA partitioned into detergent-soluble membrane domains in rat cardiomyocytes [92]. Diaz et al. [91] further demonstrated that DHA treatment of monocytes disorganizes their membranes and strongly displaces PLD_1 from detergent-insoluble membrane domains to detergent-soluble membrane domains, where PLD_1 binded to its cofactor [adenine ribosylating factor (ARF)] and became activated. It is not known if a similar mechanism of action for n-3 PUFA activation of PLD exists

in cardiomyocytes. However, based on DHA distribution in lipid domains, it is expected that n-3 PUFAs may have a stimulatory effect on PLD activation in the heart and may play a physiological role in the regulation of DAG production by activating PLD and PA phosphohydrolase activity, as previously suggested [93].

PLA_2 is another physiologically important enzyme whose activity is also modulated by n-3 PUFAs. PLA_2 catalyzes the hydrolysis of fatty acids from the sn-2 position of membrane phospholipids, resulting in the production of proinflammatory AA-derived eicosanoids and PAF. A recent study suggests that PLA_2 activity is involved in the activation of calcium channels via generation of lysophospholipids [94]; however, most of the effects of PLA_2 believed to be involved in inflammation [95], atherogenesis [96] and cardiac functions [97] are mediated through eicosanoid generation [98]. PLA_2 has several isozymes, which are broadly classified according to their sequence homology: activation by calcium and cellular localization as secreted ($sPLA_2$), cytosolic ($cPLA_2$) and calcium independent ($iPLA_2$) [99,100]. $sPLA_2$ is highly inducible in response to cytokines [101–106], has no strict fatty acid selectivity [107] and is involved in cardiac inflammation, ischemia and atherosclerosis [108,109]. $cPLA_2$ is activated by submicromolar concentrations of Ca^{2+} and phosphorylation by MAP kinase [110,111]. It is widely distributed in most tissues and displays selectivity for arachidonyl in the sn-2 position of phospholipids [100,112–114]. $iPLA_2$, which also has no selectivity for phospholipid–fatty acid substrate [115], represents 80% of the total PLA_2 activity in normal myocardium, and its activity is increased during ischemia–reperfusion [116]. In addition to these PLA_2s, two other minor classes of PLA_2 include PAF acetylhydrolase and lysozomal PLA_2 [100]. Most of n-3 PUFAs' effects on PLA_2-mediated signaling events are related to the fatty acyl composition of phospholipids, the PLA_2 substrate [117]. There is not much known about a direct regulatory effect of n-3 PUFAs on PLA_2 activity. n-3 PUFAs easily incorporated into membrane phospholipids on the sn-2 position, where AA is usually present. Hydrolysis of n-3 PUFAs containing phospholipids by PLA_2 then generates free DHA or EPA. n-3 PUFAs block PLA_2 effects by inhibiting AA release, therefore generating fewer inflammatory eicosanoids. On the other hand, EPA released from PLA_2 activity generates anti-inflammatory eicosanoids through the cyclooxygenase (COX) and lipoxygenase (LOX) pathways (see below).

5. Enzymes regulating eicosanoid generation

Fatty acids released from the sn-2 position of the membrane phospholipids by the action of PLA_2 (described above) are substrates for COXs, LOXs or cytochrome P450 monooxygenases (CYPs) and generate enzymatically oxidized, biologically active fatty acid products (Fig. 5) that are collectively called eicosanoids [118,119]. COX catalyzes AA oxygenation to prostanoids and thromboxanes (TXAs) [120]. The LOX pathway catalyzes the AA conversion to leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs) [121,122], and CYP-mediated oxidation of AA yields a variety of eicosanoids, including epoxides, midchain hydroxy fatty acids, ω-hydroxy fatty acids and dihydroxy fatty acids [123,124]. These eicosanoids regulate cardiovascular functions through multiple mechanisms. For example, the COX products are modulators of thromboregulatory, inflammatory and chemotaxic responses [118]; the LOX products are involved in vascular permeability, vasoconstriction and bronchoconstriction [125]; and epoxy derivatives of AA are reported to modulate calcium signaling, channel activity, transporter function and mitosis and to impact hypertension [124].

COX is the rate-limiting enzyme for the conversion of AA to various prostaglandins (PGs) and TXAs. There are two major isoforms of COX: COX-1 is constitutively expressed in most tissues, whereas COX-2 is undetectable in many tissues under basal conditions, but its expression can be induced rapidly in response to inflammatory and mitogenic stimuli [118]. Recently, an alternative splice variant of COX-1, which is selectively inhibited by acetaminophen, has been identified and termed COX-3 [126]; however, its expression in cardiac tissues is not known. COX-1 and COX-2 expression in VSMCs, vascular endothelial cells and inflammatory cells, including monocytes and macrophages, causes synthesis and release of PG and TXA. PGs and TXAs are modulators of vascular tone and hemostasis under normal physiological conditions; however, under pathological conditions, increased production from expression/activation of COX-2 of these eicosanoids mediates proatherogenic, proinflammatory effects and causes impairments in the cardiovascular system [127–130]. The production of these eicosanoids is regulated by the availability of AA. n-3 PUFAs compete with AA for COX enzymes, and both EPA and DHA are poor substrates compared to AA for the COX and LOX enzymes [131–134]. Corey et al. [135] suggested that DHA is resistant to COX-1 enzymatic oxidation and, therefore, functions as an inhibitor and not as a substrate. Smith [136] described that purified COX-1 and COX-2 catalyze AA with comparable efficiencies, whereas EPA is a very poor substrate for purified COX-1, and cells expressing only COX-1 form little or no oxygenated product from EPA, whereas COX-2 oxygenates EPA with only 30% efficiency of that of AA. Based on several studies, both DHA and EPA are generally regarded as inhibitors for enzymatic activity of both COX isoforms [137,138]. Furthermore, n-3 PUFAs also directly reduce IL-1-induced expression of COX-2 in endothelial cells [139] and in macrophages [140]. However, DHA was found to enhance COX-2 expression in VSMCs [141], and Gilbert et al. [142] showed that DHA treatment of bovine aortic endothelial cells potentiates COX-2 expression induced by phorbol myristate acetate (PMA). This enhancement of COX-2 expression by DHA is suggested to contribute to the cardioprotective effects, probably by

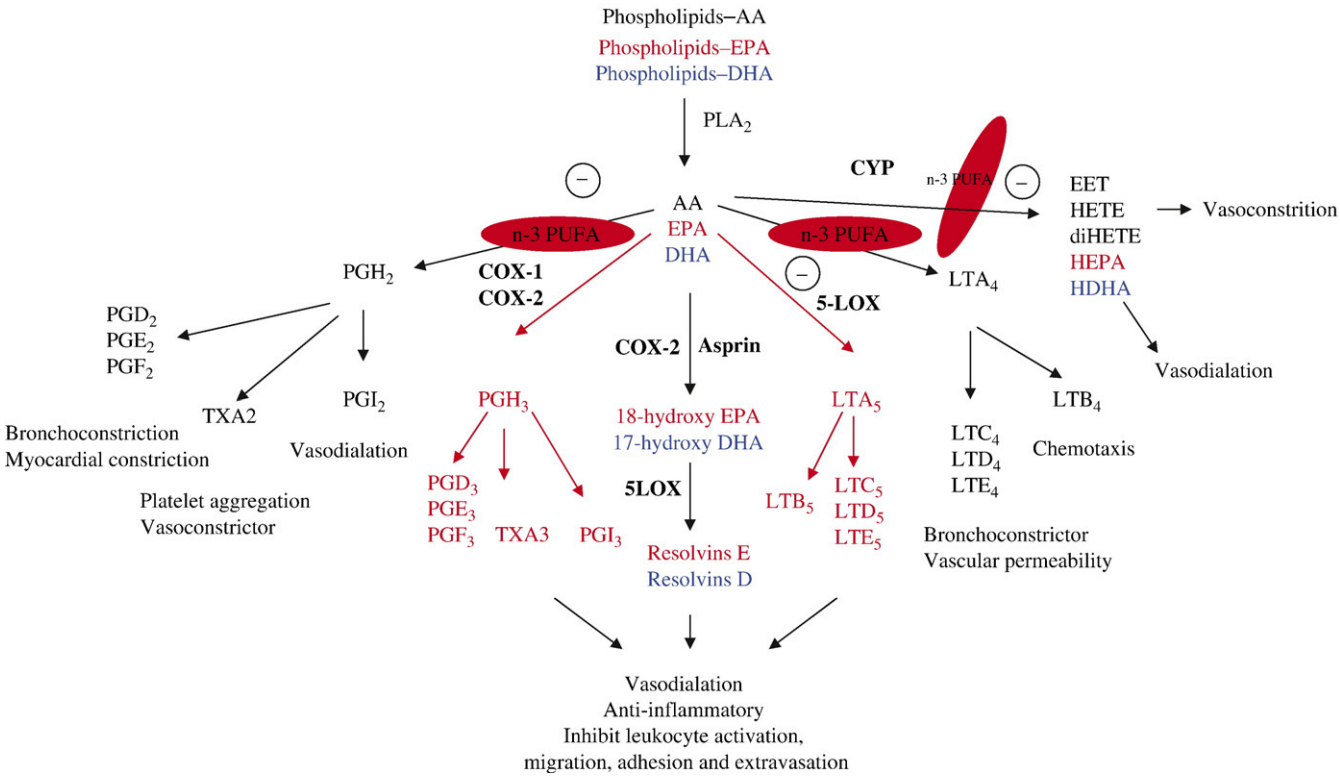

Fig. 5. Generation of n-3 and n-6 PUFA-derived eicosanoids and their effects in cardiovascular functions. Phospholipids containing AA, EPA or DHA are degraded into free fatty acids by the action of PLA_2. AA, EPA and DHA are substrates for COX, LOX or CYP. COX-1 and COX-2 catalyze oxygenation of AA into 2-series PGs and TXAs and oxygenation of EPA into 3-series PGs and TXAs. The LOX pathway catalyzes conversion of AA to 4-series LTs and conversion of EPA into 5-series LTs. CYP mediates oxidation of AA, EPA or DHA into their corresponding epoxy fatty acids and hydroxy fatty acids. Eicosanoids derived from n-6 fatty acids are inflammatory and induce vasoconstriction. n-3 PUFAs not only inhibit COX, LOX and CYP activities but also generate eicosanoids with anti-inflammatory and vasodilator functions (see text for details). In endothelial cells, COX-2 enzymes convert fatty acids into hydroxy fatty acids in the presence of aspirin. These are released from the endothelium and are rapidly converted by 5-LOX in adherent leukocytes into bioactive compounds called resolvins (resolution phase interaction product). Resolvins derived from EPA are designated as E series (RvE), and those derived from DHA are called D series.

releasing PGI_2/PGI_3 as a compensatory mechanism for the endothelial dysfunction [141]. Furthermore, n-3 PUFAs also exert some inhibition of platelet TXA production. EPA is a worse substrate for platelet COX than AA [143], and DHA can competitively inhibit eicosanoid biosynthesis from AA [144].

In addition to COX enzymes, n-3 PUFAs are unique in interfering with the production and/or activity of LTs [145], which have recently emerged as potentially important pathogenetic factors in atherosclerosis, most likely through the chemoattractive properties of LTB_4 [146]. The enzyme 5-LOX induces the production of LTs. 5-LOX with 5-LOX-activating protein catalyzes oxidation of AA into LTA_4, which, upon hydrolyzation, is converted into LTB_4 or conjugated with glutathione to form cysteinyl LTs [122,147]. The vascular effects of the AA-derived 4-series cysteinyl LTs ($CysLTC_4$, $CysLTD_4$ and $CysLTE_4$) include vasoconstriction, endothelium constriction and extravasation of plasma, whereas AA-derived LTB_4 actively recruits leukocytes and induces degranulation and lysozomal enzyme release by neutrophils [148–152]. In contrast to AA, 5-LOX activity induces the 5-series cysteinyl LTs ($CysLTC_5$, $CysLTD_5$ and $CysLTE_5$) from EPA [153–156]. 5-Series LTs possess reduced proinflammatory and vasoactive potencies [156]. LOX is abundantly expressed in atherosclerotic lesions, and macrophages represent one of the major sources of 5-LOX [157,158]. It is therefore expected that EPA treatment may result in reduced inflammation at atherosclerotic sites because of a decrease in 4-series LTs. The LTs exert their actions via four subclasses of 7-transmembrane G-protein-coupled cell surface receptors: BLT_1 and BLT_2, which represent receptors for LTB_4, whereas $CysLT_1$ and $CysLT_2$ receptors are activated by the cysteinyl LTs [153,159]. BLT_1 receptors are not present in healthy arteries, but their expression is induced in atherosclerotic arteries [160]. BLT_1 receptors are also expressed in human VSMCs [160], which are involved in cell migration and proliferation in response to LTB_4 [161]. Similarly, it has been demonstrated that cysLT binding sites are increased in atherosclerotic vessels [162] and $ApoE^{-/-}$ mice, a widely used model for studying atherosclerosis, which display an increased cysLT receptor expression in the aorta compared with nonatherosclerotic mice [163]. Stimulation of human endothelial cells with AA-derived cysLTs leads to an increase in intracellular calcium [161], the release of vasoactive factors [164–166] and induction of gene

expression [167]. In cultured human umbilical vein endothelial cells and coronary artery smooth muscle cells (SMCs), the predominantly expressed cysLT receptor is of the $CysLT_2$ subtype [161,168]. It is important to note that EPA is a preferred substrate for 5-LOX, resulting in the release of EPA-derived 5-series cysLTs at the expense of AA-derived 4-series cysLTs [169]. 5-Series cysLTs are also known to be less potent vasoconstrictors and also antagonize 4-series cysLT function [170]. Furthermore, EPA-derived LTB_5 have approximately 20- to 50-fold less binding activity to LTB_4 receptors on human leukocyte membranes [171]. In rabbits, EPA suppressed an *E. coli* hemolysin-induced increase in vascular leakage in a dose-dependent (50–200 nmol/L) manner [170]. This effect was accompanied by a decrease in 4-series LT generation and a dose-dependent appearance of 5-series LTs. Furthermore, EPA also fully antagonized AA-induced amplification of hemolysin-induced vascular leakage. In another study, posttransplant intravenous alimentation of fish oil (9 g/kg body weight/day) prolonged survival in rats following allograft heart transplantation when compared to similar levels of soybean infusion [172]. This effect was also accompanied with generation of 5-series LTs. Based on few observations demonstrating inhibitory cellular effects of EPA-derived cysLTs compared to those of AA-derived cysLTs, it can be speculated that EPA-derived cysLTs may be poor agonists to cysLT receptors. Overall, it can be concluded from the above observations that n-3 PUFAs have inhibitory effects on the AA-derived 5-LOX pathway, which lead to a reduction in inflammatory conditions and inhibition of the development of atherosclerosis.

In addition to COXs and 5-LOXs, another class of n-3 PUFA-derived oxidized products, resolvins, have recently been characterized for their cardiobeneficial properties [173]. The resolvins are produced from transcellular reactions where aspirin triggers conversion of EPA or DHA by COX-2 enzyme in vascular endothelial cells into 18-hydroxyeicosapentaenoic acid (18-HEPA) or 17-hydroxydocosahexaenoic acid (17-HDHA), respectively, which are released from endothelium and rapidly converted by 5-LOX in adherent leukocytes into resolvins (resolution phase interaction products) [173,174]. Resolvins derived from EPA are designated as E series (RvE), and those derived from DHA are termed as D series (RvD, also known as neuroprotectin for anti-inflammatory effects in the brain). Beneficial effects of resolvins are demonstrated in the classical GISSI study showing improvements in cardiovascular patients receiving 1 g n-3 PUFA/day [16]. These patients also took low doses of aspirin, whose effects were not accounted for in the published analysis. Apparently, acylation of COX-2 by aspirin inhibits synthesis of PG from AA, but this enzyme remains active for hydroxylation of EPA and DHA [173,174]. Resolvins derived from EPA and DHA possess very potent anti-inflammatory activity by affecting activation, migration, adhesion and transendothelial migration of leukocytes [173,175]. Serhan et al. suggest that endogenous generation of resolvins provides a mechanism for the beneficial effects of n-3 PUFAs whereby endothelial cells that cover an enormous surface area of the vasculature can substantially contribute to reducing inflammation and provide cardiac health benefits [173].

As described above, fatty acids are also converted to both epoxy and hydroxy fatty acids by cytochrome P450-linked monooxygenases (CYPs). The research on the role of CYP on cardiovascular health has recently increased [124]. AA is metabolized by CYP epoxygenase to epoxyeicosatrienoic acid (EET) and by CYP hydroxylase to 20-HETE [176,177]. The vascular endothelium further metabolizes EET by epoxide hydrolase to the respective regioisomers of diHETEs [178]. The roles of these metabolites in maintaining cardiac, renal and pulmonary homeostasis are numerous and complex [124]. EETs increase intracellular calcium concentrations by causing the opening of calcium-activated potassium channels, thus increasing calcium entry via voltage-sensitive channels [179]. 20-HETE, on the other hand, is a potent vasoconstrictor of small arteries in canine and porcine microvessels but has little effect on large arteries with increases in intracellular calcium concentration [178]. In contrast, epoxydocosapentaenoic acid, a product of DHA from CYP epoxygenase activity, potently dilates coronary microvessels. This is suggested to be the most potent fatty epoxide known to activate calcium-activated potassium channels in coronary SMCs [124]. EPA and DHA are also oxidized by CYP hydrolase to corresponding 20-HEPA and 22-HDHA, respectively [180]. These oxidized products from n-3 PUFAs directly inhibit 20-HETE production from AA [181]. Based on these observations, it can be speculated that oxidized products of n-3 PUFAs from CYP activity may contribute to some of the hypotensive effects of dietary fish oils.

6. Enzymes/proteins involved in the adrenergic system

Acylation of n-3 PUFAs into the plasma membrane phospholipids may influence signal transduction pathways by affecting membrane proteins, including affinities and densities of receptors and guanine nucleotide (GTP) binding proteins and activities of adenylate cyclase (AC), guanylate cyclase and cyclic nucleotide phosphodiesterase (PDE) enzymes [182–186]. There is considerable evidence that changes in the fatty acid composition of the phospholipids may alter the agonist–receptor binding characteristics, thus influencing the receptor-mediated signaling pathway [185]. Adrenergic receptors play an important role in regulating contractility and/or heart rates (for review, see Ref. [187]). β-adrenergic receptors are coupled to G_s-protein–adenyl cyclase pathways, whereas α1-adrenergic receptors are coupled to the $G_{q/11}$-protein–PLC pathway. There are reports that small amounts of α2-adrenergic receptors are also present in human hearts, but the signaling pathway coupled to this receptor has not been well characterized [187]. Incorporation of n-3 PUFAs into cell membranes affects

both β- and α-adrenergic systems. A few studies have demonstrated that increasing DHA content in the phospholipids of isolated rat cardiomyocytes resulted in a significantly higher positive chronotropic effect on stimulation of the β-adrenergic receptors with isoproterenol. This effect of DHA appeared to be due to a decreased affinity of the β-receptors for the ligand without alteration of the number of β-receptor binding sites, which also caused a significant decrease in cyclic AMP (cAMP) production [188,189]. Furthermore, it has also been shown that incorporation of n-3 PUFAs into membrane phospholipids was associated with a decreased affinity of the α1-adrenoceptors for their antagonist ligand [^{3}H]prazosin in heart muscle [185]. Kang and Leaf [190] have shown that free unsaturated fatty acids virtually inhibit the binding of [^{3}H]benzoyl-2,5-[3*H*]batrachotoxinin (BTXB) to its receptor on the Na^+ channel protein of neonatal rat cardiac myocytes. The inhibition by fatty acids of [^{3}H] BTXB binding was dose dependent, saturable, reversible and allosteric; this inhibition occurred at pharmacologically relevant concentrations [190]. These findings suggest that unsaturated fatty acids bind to a specific receptor site through interaction of both the unsaturated hydrocarbon chain and the carboxyl group with appropriate domains of the cardiac Na^+ channel protein [190].

The membrane phospholipid fatty acid composition can also influence the anchoring and/or mobility of the different G-protein subunits: the α-monomer and the βγ-heterodimer. However, at present, there are only a few myocardial studies available dealing with the effects of dietary PUFAs on G-protein function. It is shown in adipocytes that the G-protein, which couples inhibitory receptors to AC, was affected specifically by free EPA, resulting in the inhibition of AC activity [191]. Nevertheless, these studies imply that n-3 PUFAs, after incorporation in phospholipids of cardiac tissues, could potentially alter signaling through a G-protein-dependent mechanism. Consistent with studies by Price and Tisdale [191], Courtois et al. [192] have shown previously that enrichment of rat cardiomyocytes with EPA or DHA, which results in alteration of the phospholipid fatty acid composition, was able to affect the efficiency of the β-adrenoceptor–adenylyl cyclase pathway.

The n-3 PUFAs also modify the activities of other membrane proteins, particularly adenylyl and guanylyl cyclases, which control the cyclic nucleotide intracellular levels. Cyclic nucleotide PDEs play an important role in intracellular signal transduction and provide the major means through which intracellular cyclic GMP (cGMP) and cAMP signals are diminished by degradation. PDEs are subdivided into 11 broad families (PDE1 to PDE11) based on their tissue distribution, biochemical properties and sensitivity to chemical inhibitors. In cardiac myocytes, multiple PDE isozymes from at least five different families (PDE1, PDE2, PDE3, PDE4 and PDE5) have been described [193]. Dubois et al. [182] have shown that dietary manipulations can affect the heart cyclic nucleotide PDE activity. In particular, n-6 and n-3 PUFA-enriched diets have been shown to decrease the activities of cAMP-PDE more potently than saturated fatty acids in both particulate and soluble fractions of rat heart, whereas cGMP hydrolysis remained unaffected by various diets [182]. These investigators have further shown that the enrichment of cardiomyocytes by EPA or DHA (100 μM) increases the intracellular concentration of cGMP and cAMP. The cGMP level is more drastically increased by fatty acids than the cAMP level, particularly by EPA [194]. Another important factor in these studies was that growing cardiomyocytes in a DHA- or EPA-supplemented medium decreased their cGMP-PDE specific activity as compared to nontreated cells. A possible mechanism involved in the lowering of cGMP-PDE activity of cardiomyocytes by EPA or DHA enrichment might be a direct interaction between nonesterified fatty acids and the PDE enzyme. In an in vitro system, DHA was shown to inhibit the cytosolic PDE activity of an adult rat heart with IC_{50} values of 115 and 58 μM for cAMP and cGMP-PDE, respectively [194]. PDE2 isoenzyme activity measured in EPA- and DHA-enriched cells exhibited different sensitivity to cGMP compared to control cells [195]. It appears that the enrichment of cells with EPA or DHA modifies the degree to which PDE2 isozyme activity is subject to regulatory control by cGMP. The opposing effects of cGMP and cAMP on the inotropic response in the heart are believed to converge at the level of the L-type Ca^{2+} current, which plays a crucial role in regulating cardiac functions [196,197]. It is clear from these studies that n-3 PUFAs can affect cellular levels of cAMP and cGMP by affecting both cyclase and PDE activities, which may indirectly affect calcium mobilization from L-type calcium channels and, thus, play a role in preventing abnormal cardiac contractility and arrhythmia generation. Effects of other PUFAs were not addressed in these studies [194,195]; it is therefore not clear if regulation of PDEs specifically modulated by n-3 PUFAs or n-6 PUFAs also has similar effects.

7. Enzymes with protein kinase activities

In addition to their effects on adrenergic receptors and intracellular calcium, n-3 PUFAs also affect kinase-mediated serine/threonine and tyrosine phosphorylation of cellular proteins (Fig. 6). Protein phosphorylation by protein kinases plays an essential role in signal transduction between the plasma membrane and nucleus. Furthermore, protein phosphorylation also plays a key role in regulating Ca^{2+} influx in cardiomyocytes [198]. Modulation of Ca^{2+} channel activity by serine/threonine phosphorylation by cAMP-dependent protein kinase and PKC has been well established [197,199]. Recent studies have also shown that tyrosine kinase inhibitors inhibited the basal calcium currents in cardiomyocytes, suggesting that constitutively activated tyrosine kinases also up-regulate Ca^{2+} channel activity [200,201]. Consistent with this study, c-src, a nonreceptor tyrosine kinase, has been found to be directly associated with

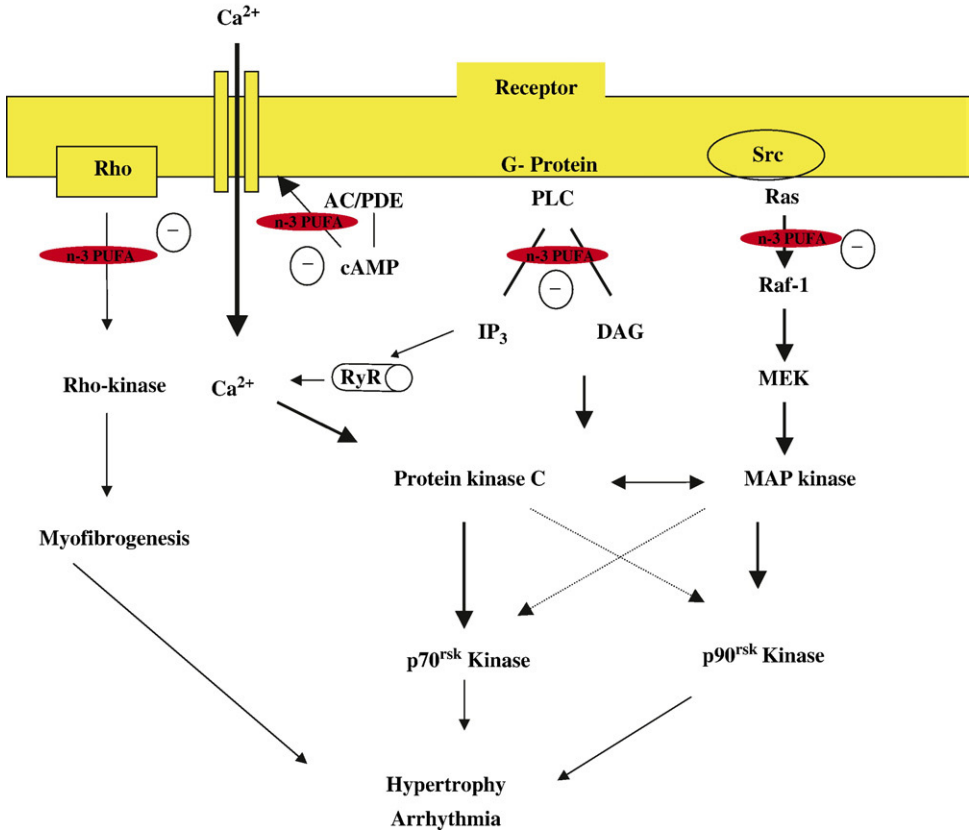

Fig. 6. Protein kinase targets of n-3 PUFAs for regulating cardiovascular functions. n-3 PUFAs have multiple potential sites for modulating signal transduction pathways to alter cardiac functions. n-3 PUFAs can prevent a rise in receptor-induced cytosolic calcium by directly affecting G-protein-mediated activation of PLC and generation of second messengers: inositol IP_3 and DAG. Intracellular Ca^{2+} levels can also be regulated by n-3 PUFAs by modulating AC and PDE activities, which control cellular cAMP concentrations. Ca^{2+} and DAG are potent activators of PKC; therefore, n-3 PUFAs can prevent activation of PKC, which consequently affects downstream pathways for protein synthesis involving activation of p^{70rsk} kinase and phosphorylation of S6 ribosomal proteins. Another potential target of n-3 PUFAs includes decreased acylation and translocation of src (tyrosine kinase) and ras (small G-protein), which then potentially affect activation of Raf–mitogen-activated kinase (MAP) pathways. As a consequence of these effects, protein synthesis through activation of p^{90rsk} kinase would be affected. Other important sites for n-3 PUFAs involve prevention of myofibrillogenesis by affecting small G-protein (Rho) and its downstream signaling through Rho kinase and MLC phosphorylation. n-3 PUFAs could affect these pathways in parallel, or one pathway may be more affected than the others, depending on their incorporation into plasma membrane phospholipids. A regulatory effect of n-3 PUFAs on these kinases can be beneficial to the cardiovascular system, especially in preventing arrhythmia and cardiac hypertrophy.

the α-subunit of Ca^{2+} channels in SMCs [202]. n-3 PUFAs potentially inhibit src-mediated signaling by displacing the protein from lipid rafts. In human retina endothelial cells, incorporation of DHA (50–100 μM) into fatty acyl chains of phospholipids in caveolae/lipid rafts resulted in displacement of src [203]. Both G-protein-coupled receptors and tyrosine kinases are involved in the activation of c-src [204,205]. Downstream signaling of c-src includes formation of complexes with Shc, Grb2 and Sos and activation of ras, raf-1 and the MAP kinase cascade [206]. Studies have shown that microinjection of H-ras into cardiac myocytes elicits a strong growth response and hypertrophic gene expression [207], whereas the targeted expression of H-ras in transgenic mice increases heart size concomitantly with an increase in myofibrillar organization [208,209]. n-3 PUFAs can also affect ras-mediated signaling, as we have recently demonstrated in a study showing that DHA treatment of cardiomyocytes results in displacement of ras from lipid rafts [210]. Downstream ras activation in cardiomyocytes leads to activation of MAP kinase [211]. A coordinated activation of the ras–MAP kinase pathway is involved in the expression of a number of genes that encode atrial natriuretic peptide (ANP), α and β myosin heavy chain and SERCA2, followed by increased protein synthesis without DNA synthesis [212,213]. Activation of MAP kinase has been reported to be necessary for phenylephrine-induced transactivation of c-fos and fetal-type genes such as ANP [214]. MAP kinase is known to participate in the development of cardiac hypertrophy via phosphorylating p^{90rsk} kinase in cardiac myocytes [215–217]. p^{90rsk}, which catalyzes the phosphorylation of ribosomal subunit protein S6. MAP kinase also participates in the transcriptional regulation of c-fos by phosphorylation of serum response factors [216]. In a recent study, we have demonstrated that DHA (5 μM for 24 h) treatment of rat cardiomyocytes inhibited phenylephrine-induced cardiac hypertrophy by inhibiting the Ras-

mediated raf-1–MAP kinase–p^{90rsk} activation pathway [210]. Inhibition of phenylephrine-induced cardiac hypertrophy was not seen with similar doses of other fatty acids (EPA, oleic acid, linolenic acid and AA).

Furthermore, in VSMCs, platelet-derived growth factor (PDGF) binding resulted in activation of the MAP kinase pathway, leading to enhanced expression of c-fos [218]. PDGF is considered to play a critical role in the development of atherosclerotic lesions by stimulating migration and proliferation of VSMCs [219]. Physiologically relevant concentrations (30–300 μM) of EPA as well as other n-3 PUFAs (DHA, DPA) inhibited migration and proliferation of SMCs [218,220]. This effect occurs via EPA-mediated suppression of binding of PDGF to SMCs and also by suppressing expression of c-fos mRNA by PDGF or PMA in a dose-dependent manner [218,220]. This suggests that EPA may contribute to prevention of atherosclerosis by inhibiting PDGF-mediated MAP kinase activation. This indirect evidence suggests that activation of MAP kinase could be seriously altered by n-3 PUFAs by modulating upstream activation events. MAP kinase is directly regulated by raf-1 kinase, also known as MAP kinase kinase [221]. Raf-1 can be activated by cellular phospholipids [222]. It is therefore possible that phospholipids containing EPA or DHA could alter the activation of MAP kinase. This effect could have a direct impact on the activation of p^{90rsk} and, hence, protein synthesis in cardiac tissue, leading to prevention of cardiac hypertrophy. Therefore, it appears that a blockade of the ras–MAP pathway by n-3 PUFAs could be an effective therapeutic strategy in treating the contractile defects associated with cardiac hypertrophy and failure.

Another pathway that is particularly involved in increased expression of cellular proteins during cardiac hypertrophy is dependent on the activation of Rho kinase. Studies have demonstrated that increased myosin light chain (MLC) and atrial natriuretic factor gene expression, which may be induced by Gq-coupled α1-adrenoceptors, is mediated by activation of Rho, a small G-protein [223]. Rho-GTP plays a crucial role in cytoskeletal regulation, mediating cellular events such as cell morphology, cell motility and cytokinesis [223,224]. Rho is required for actin stress fiber and focal adhesion complex formation [225]. Various putative downstream effectors of Rho have been identified, including Rho kinase [225,226]. Known substrates of Rho kinase are MLC and the myosin binding subunit of MLC phosphatase [227,228]. Phosphorylation of these proteins by Rho kinase alters the sensitivity of smooth muscle myosin to Ca^{2+} [229,230]. It therefore appears that Rho and Rho kinase might play roles in the organization of actin–myosin (myofibrillogenesis) in cardiomyocytes during hypertrophy. Although there is no direct evidence that n-3 PUFAs are involved in the regulation of Rho-kinase activation, this Rho-kinase-dependent pathway may be a potential site for n-3 PUFA regulation. It is possible that n-3 PUFAs may affect fatty acylation of Rho, thereby altering its translocation to plasma membrane and its interaction with Rho kinase [231]. This could lead to the prevention of myofibrillogenesis in cardiomyocytes during hypertrophy.

In addition to the protein kinases described above, the role of PKC is also crucial in normal cardiac physiology and in various disease states [232,233]. Activation of PKC is known to affect multiple cardiovascular functions, including vascular permeability, cell migration and growth [234,235]; extracellular matrix production [236–238] and expression of various cytokines [239,240]; ion conductance and transport activity [241]; intracellular calcium homeostasis and properties of contractile proteins [242]; ischemic preconditioning of the heart [243]; genesis of arrhythmias [244]; and induction of cardiac hypertrophy [245,246]. The three groups of the PKC family of kinases comprise approximately 13 different isozymes [conventional (PKCα, PKCβ I, PKCβ II, PKCγ), novel (PKCδ, PKCε, PKCθ, PKCη, PKCμ) and atypical (PKCζ, PKCι, PKCν, PKCλ)]. It is suggested that the individual isoforms of PKC become active in a distinct manner from transducer signals on the cell surface to the responsive elements of the nuclear DNA [247]. Once activated, PKC isozymes translocate from the cytoplasm to discrete subcellular membrane sites [248]. Many observations suggest that different isoforms of PKC are recruited to the membranes by different stimuli, phosphorylate different sets of cellular substrates and may regulate different cellular functions. As described above, elevated intracellular Ca^{2+} and generation of DAG as a result of PLC activity have profound effects on PKC activation [249]. PKC activation then leads to activation of specific pathways. For example, PKC-dependent pathways are responsible for load-induced p^{70rsk} kinase activation and induce hypertrophy [250]. There is strong evidence that n-3 PUFAs modulate the translocation and activation of PKC in cardiac tissues. For example, acute incorporation of EPA (10–25 μM for 20 min) into VSMC phospholipids inhibits intracellular calcium mobilization and PKC activation [251]. DHA (5 μM for 24 h) has also been shown to reduce activation of membrane-bound PKC in isolated cardiomyocytes [252]. The effect of n-3 PUFAs on PKC occurs through multiple mechanisms. It is suggested that the function of PKC isozymes may be affected by alteration of the molecular species of DAG due to changed fatty acid composition of the phospholipid source or directly by free fatty acids [253,254]. Consistent with this, dietary fish oil significantly alters the fatty acid composition of myocardial DAG and results in inhibition of PKCα, PKCβ and PKCε translocation in mice [255]. This observation suggests that dietary fish oil may attenuate cardiac hypertrophy with improvements in cardiac function and survival in mice via modification of the molecular species composition of myocardial DAG and the consequent inhibition of PKC redistribution [255]. Similarly, incorporation of EPA into phospholipids of VSMCs significantly inhibited PKC activity [221,254,256]. On the other hand, free fatty acids have variable potencies for PKC activation [257–259]. Indirect evidence for the modulation of PKC activity by nonesterified PUFAs was found in cardiac

myocytes by studying a purinergically induced Ca^{2+} response [260]. It has also been shown that EPA directly suppresses the activation and/or translocation of PKC in VSMCs [221,256]. Furthermore, studies in a cellular system demonstrated that DHA differs from all other n-3 or n-6 PUFAs and is a highly potent inhibitor of phosphatidylserine- and diolein-stimulated PKC in rat colon cells [261]. In conclusion, the evidence overall suggests that n-3 PUFAs affect translocation and activation of PKC. This effect of n-3 PUFAs on PKC may result in the alteration of PKC-mediated signaling to inhibit various cardiac diseases. The regulation of PKC activities is therefore clearly a potential target for n-3 PUFAs to prevent cardiovascular diseases.

8. Conclusion

It can be concluded from the research presented in this review that n-3 PUFAs possess potent beneficial effects on the cardiovascular system. It appears that n-3 PUFAs modulate enzymatic activities through several mechanisms. n-3 PUFAs directly interact with calcium channels, enzymes of eicosanoid pathways and protein kinases to provide protective effects. n-3 PUFAs can also be incorporated into phospholipids and, therefore, change affinities of receptors to their ligands, as well as their interaction with downstream signaling proteins. n-3 PUFA-containing phospholipids alter phospholipase activities and also generate PL-derived signaling molecules with altered activities. n-3 PUFAs also regulate COX, LOX and CYP enzyme activities and generate cardioprotective eicosanoids.

The studies for elucidating the mechanisms by which n-3 PUFAs induce their effects are largely performed in cell culture and animal models with variable doses of n-3 PUFAs. The concentration of n-3 PUFAs in plasma of humans consuming a regular Western diet ranges from 8 to 12 μM [31]; however, consuming moderate to high fish intake for several months can raise plasma n-3 PUFAs levels to 200–400 μM [262,263]. Similarly, the plasma concentration of DHA can reach up to 30 μM in rats consuming a diet containing 5% DHA for 14 weeks [264]. It therefore appears that, in many cellular studies, the concentration of n-3 PUFAs appears to be within achievable physiological ranges; however, the cellular system lacks the complexities of the biological system. In addition, cellular and animal systems have often shown excessive levels (up to 10-fold) of enrichment of fatty acids, whereas in most human studies, the levels of n-3 PUFAs can only be increased by approximately 2-fold. Furthermore, studies performed in animal models may not be directly applicable to human situations because disease pathology in animal models often progresses differently than in the human system. Therefore, caution should be taken in extrapolating on these pathways to explain the effects of n-3 PUFAs in humans. It is also important to note that some of the n-3 PUFA effects can also be induced by n-6 PUFAs. However, n-6 PUFA metabolism generates inflammatory mediators (eicosanoids) that may have potentially harmful effects on cardiac patients. In contrast, n-3 PUFAs inhibit the generation of inflammatory mediators, and therefore, the use of n-3 PUFAs will be more beneficial overall than the use of n-6 PUFAs. It should also be realized that n-3 PUFAs may not be able to affect all these cellular pathways simultaneously. It is also possible that there may be individual variation, based on health status, and more than one pathway may be operating at a given time. Similarly, there may also be differences in how n-3 PUFAs (DHA vs. EPA) affect cellular pathways under different health situations. In conclusion, it is evident from the studies presented in this review that fish oil n-3 PUFAs may be a dietary agent that modifies the development of cardiac functions; therefore, they are an attractive preventive agent for cardiovascular problems in normal healthy humans. n-3 PUFAs can also be an attractive therapeutic strategy for treating cardiovascular abnormalities in some heart patients.

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