F1000Reports MEDICINE

Published: 02 July 2012

Stressing the heart of the matter: re-thinking the mechanisms underlying therapeutic effects of n-3 polyunsaturated fatty acids

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F1000 Medicine Reports 2012, **4**:13 (doi:10.3410/M4-13)

Abstract

Despite their clear therapeutic effects in coronary heart disease, use of n-3 polyunsaturated fatty acids (PUFAs) to treat other types of cardiovascular disease remains controversial, and serious obstacles exist in implementing them as a reliable and consistent drug therapy. The foremost of these is that a molecular mechanism and relevant dosages have not been firmly established in other forms of cardiovascular disease. In this brief review, we highlight the current state of knowledge regarding the mechanisms behind n-3 PUFA action in the cardiovascular system. We also propose the novel hypothesis that lipid peroxidation products derived from n-3 PUFAs may be driving much of their beneficial cardiovascular effects, particularly in the myocardium. We conclude by discussing evidence to support this hypothesis, and its possible clinical ramifications.

Introduction and context

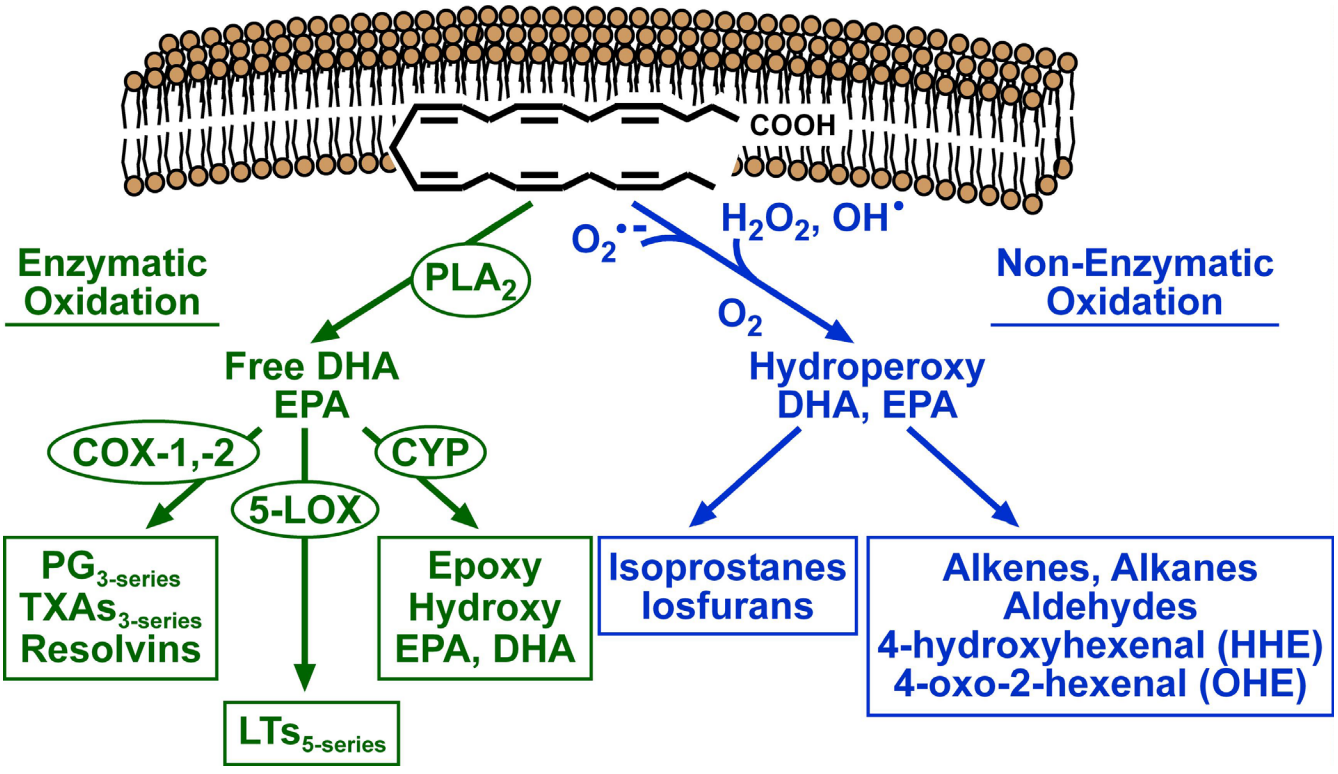
The enormous impact of cardiovascular disease on global health and economy demands that low-cost interventions, such as altered lifestyle (e.g. diet and physical activity), be rigorously implemented for prevention and treatment. Dietary intake of n-3 PUFAs, particularly fish oil, is a propitious and therapeutically achievable intervention that has been clearly shown to have a beneficial effect on the cardiovascular system. However, it is disappointing that despite significant clinical and experimental research on n-3 PUFAs and cardiovascular disease over the past 25 years, there is still no clear molecular mechanism or appropriate dosing strategy in place, making reliable and consistent therapeutic use of n-3 PUFAs extremely difficult. Thus, a coordinated effort is needed to establish a mechanism and develop proper therapeutic paradigms to make n-3 PUFAs more amenable for use in prevention and treatment of cardiovascular disease. Here, we provide a brief overview of the debate regarding the mechanism of n-3 PUFA therapy among biomedical researchers, and present a novel hypothesis that may help reconcile this controversy and unite existing, well-characterized n-3 PUFA effects with as-yet unresolved questions.

n-3 PUFA treatment for cardiovascular disease

Existing paradigms and controversy regarding mechanism

Compounds that exert vasodilating, anti-inflammatory, anti-thrombotic, anti-arrhythmic and heart rate-lowering effects are all effective therapies for cardiovascular disease. While n-3 PUFAs have shown promise in virtually all of these areas, results from clinical trials have been mixed, with some showing clear benefits and others showing no change compared with placebo. Excellent comprehensive reviews of these topics are found in the recent literature [1,2]. To date, the mechanisms proposed to explain these beneficial cardiovascular effects have largely focused on the ability of the two predominant n-3 PUFAs in fish oil, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), to compete with arachidonic acid in the orthodox pathways of eicosanoid synthesis (**Figure 1**). Further support for eicosanoid-mediated n-3 PUFA effects has come from the discovery of an exciting new family of EPA/DHA-derived eicosanoids by Serhan and colleagues [3].

Figure 1. Pathways of enzymatic and non-enzymatic n-3 PUFA oxidation



The pathway shown in green represents the orthodox enzymatic oxidation cascade that begins with the liberation of EPA or DHA from phospholipids in cellular membranes by phospholipase A2 (PLA2). The free fatty acid is then acted on by members of the cyclo-oxygenase (COX), 5-lipoxygenase (5-LOX) and cytochrome P450 monooxygenase (CYP) family of enzymes to form eicosanoids, all of which have various roles in vascular function and innate immunity. The pathway shown in blue depicts spontaneous oxidation of EPA or DHA by reactive oxygen species, such as superoxide ($O_2^{\bullet -}$), H_2O_2 and hydroxyl radical ($OH^{\bullet}$). Further oxidation yields lipid peroxides of EPA or DHA which, if not neutralized by endogenous antioxidant systems, undergo destabilization and fragmentation to yield reactive lipids such as isoprostanes, isofuranes, alkenes, alkanes and aldehydes. PG, prostaglandin; TXA, thromboxane; LT, leukotriene.

This family of compounds, called the specialized pro-resolving mediators, exemplified by the 'resolvin' series, were found to have potent properties that enhance the resolution phase of inflammation, in addition to other potential therapeutic effects (e.g. analgesia [4]). However, while the cardiovascular benefits of EPA/DHA-derived eicosanoids must be recognized, burgeoning experimental evidence suggests that attributing the mechanism of n-3 PUFA therapy in cardiovascular disease solely to eicosanoid-mediated effects is grossly over-simplistic.

One of the more intriguing and promising therapeutic potentials for n-3 PUFAs is in the treatment and prevention of heart failure [5]. Indeed, fish oil (DHA in particular) has been shown in several heart failure models to improve cardiac function and efficiency [6-9]. These findings have been supported recently by placebo-controlled clinical trials showing that daily intake of DHA/EPA for ≥ one year improved left ventricular function [10] and exercise capacity [11] in patients with established heart failure. Furthermore, clinically significant changes in left ventricular function have been reported as early as three months after initiating n-3 PUFA treatment [12]. Given that heart failure is known to result from altered cardiac energetic and structural parameters, the effects of n-3 PUFAs in enhancing these parameters has come under vigorous scrutiny by researchers.

It is clear that n-3 PUFAs are incorporated into membrane phospholipids *in vivo*. This is particularly important in highly oxidative, excitable tissues such as the heart, where phospholipid composition is critical for proper membrane structure, thereby ensuring that ion channel activity, charge separation and energy conservation are maintained. Several studies have proposed that EPA and DHA directly modify cardiomyocyte plasma membrane ion channel activity, which may partially explain their anti-arrhythmic properties [13-16]. Mitochondria are highly reliant on cardiolipin, a phospholipid unique to this organelle, to

maintain membrane electrochemical gradient and capacity for oxidative phosphorylation. Recent findings in animal models of heart failure have demonstrated that altered cardiolipin structure parallels the increase in left ventricular function seen with EPA/DHA treatment [17-19], suggesting that n-3 PUFA incorporation into cardiolipin may partially explain the improvements in cardiac function. However, from a biochemical/biophysical perspective, it is not clear how altering cardiolipin structure with EPA/DHA incorporation would enhance mitochondrial function and improve cardiac energetics. Consequently, alternative metabolic pathways and roles for n-3 PUFAs in the heart continue to be explored. In particular, there is compelling evidence that n-3 PUFAs may play a significant role in gene regulation [20-22], which may contribute to the many beneficial effects that have been observed.

Lipoxidative stress from n-3 PUFAs and adaptation in heart

Aside from the well-characterized enzymatic pathways, PUFAs are prone to oxidation from non-enzymatic (i.e. spontaneous) reactions because of their highly unsaturated structure. These reactions, initiated by reactive oxygen and nitrogen species, ultimately form lipoxidative products such as lipid peroxides, reactive aldehydes and other electrophilic lipids (**Figure 1**). Of all PUFAs, DHA is the most susceptible to lipid peroxidation due to its chemical structure [23]. To date, non-enzymatic oxidation pathways and lipoxidative products of n-3 PUFAs have been largely ignored by investigators, with a few exceptions. The reasons for this paucity of investigation are not completely clear, but could be due to either the strongly supported belief that the rate of non-enzymatic n-3 PUFA oxidation *in vivo* is negligible, or that the previously held idea that any form of lipid peroxidation is undesirable as it is unconditionally toxic. The latter idea has recently been challenged on the basis of studies described below.

In fact, evidence is accumulating that suggests that PUFA-derived 'lipoxidative stress' can be beneficial in many contexts, particularly in cells and tissues that are highly plastic and adaptable, such as the heart. Indeed, it has long been known that 4-hydroxynonenal, an aldehyde formed from n-6 PUFA oxidation, exerts bi-directional effects on the heart, characterized by a beneficial "hormetic" effect at sub-toxic concentrations (≤10μM), but toxic effects at higher concentrations [24]. The molecular mediators of these positive adaptations to "hormetic" concentrations of 4-hydroxynonenal are only beginning to be elucidated, but recent studies have pointed to the involvement of the eukaryotic translation initiating factor 2/activating transcription factor-4 (eIF2α/ATF4), NF E2-related factor-2 (Nrf2), and peroxisome proliferator-activated receptor (PPAR) family of transcription factors in up-regulating amino acid biosynthesis [25], antioxidant/anti-inflammatory genes [26,27], and mitochondrial biogenesis [28,29], respectively.

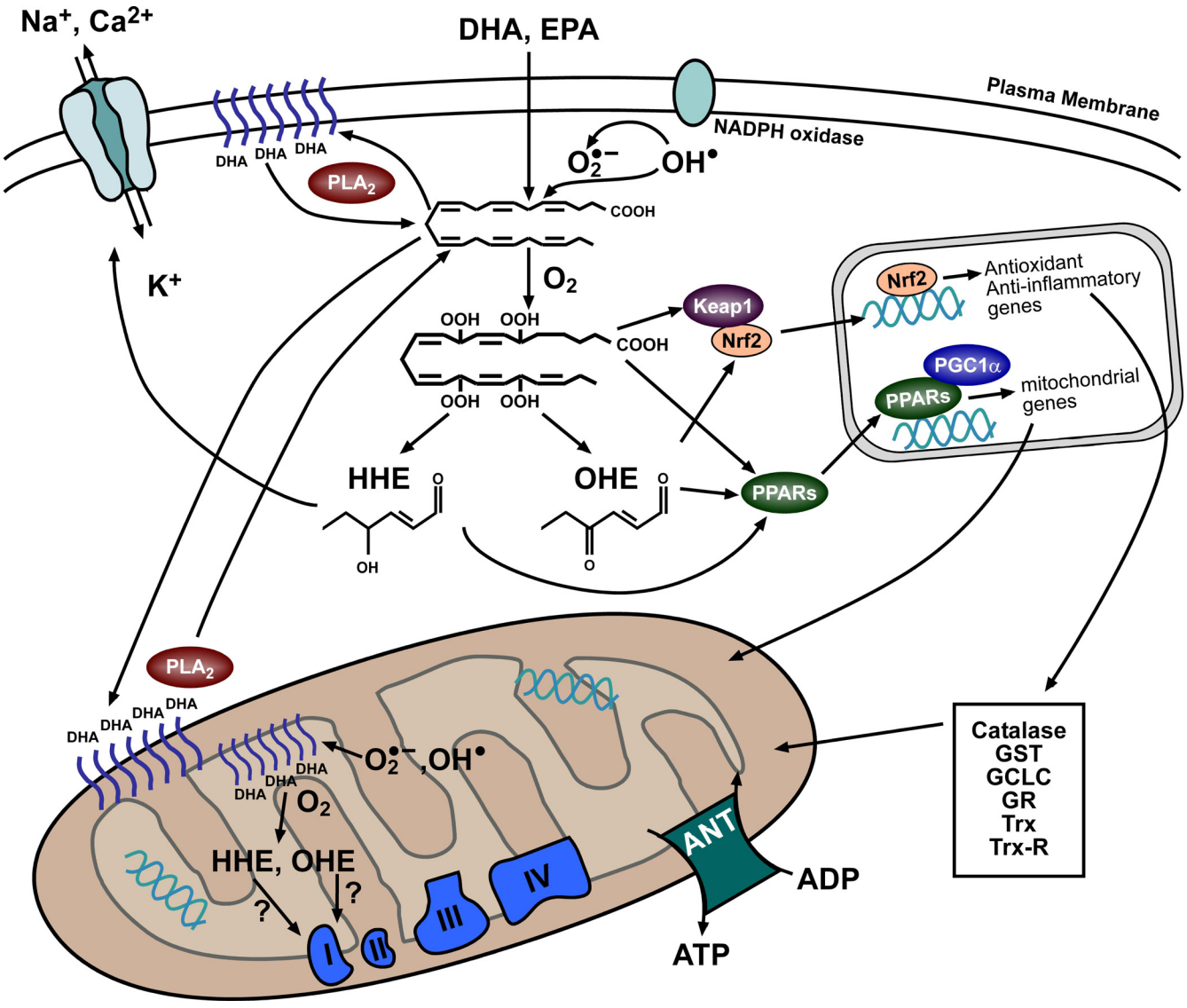
Consistent with this, Gao and co-workers demonstrated that oxidized derivatives of n-3 PUFAs up-regulate Nrf2 activity in several stable cell lines, *in vitro* [30]. Whether similar pathways of adaptation occur in heart as a result of lipoxidative products formed from n-3 PUFAs remains to be determined (**Figure 2**), but a recent study in our laboratory showed that dietary intake of n-3 PUFAs resulted in a time-dependent increase in 4-hydroxyhexenal adducts in the heart and up-regulation of Nrf2-mediated enzymes in mice that were paralleled by decreased mitochondrial reactive oxygen species production and enhanced mitochondrial tolerance to insults such as Ca^{2+} overload [31]. Several clinical studies have reported marked increases in antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase in blood of patients taking n-3 PUFAs [32-37] and in some cases, lipid peroxidation levels increased in parallel with elevation in these enzymes [38,39]. Furthermore, a number of studies in animals have reported increased expression of antioxidant/anti-inflammatory enzymes in heart following n-3 PUFA diet [40-43], and these were supported by findings from a small clinical trial showing that pre-surgical intake of fish oil suppressed NFκB activity and augmented antioxidant activity in heart tissue of patients undergoing cardiac surgery [44].

Treating the heart with stress: are lipoxidative products of n-3 PUFAs mediators of their therapeutic effect?

The context and background provided by the findings outlined above allude to a provocative question: is it possible that, to some (or even a large) extent, the cardiovascular benefits derived from n-3 PUFAs result from the 'lipoxidative stress' that they cause? If so, it would follow that any physiological state resulting in sustained increases in cellular and tissue reactive oxygen species (e.g. cardiovascular and metabolic diseases) would drive increased lipoxidative product formation when presented with EPA and DHA. This is undoubtedly a controversial hypothesis because it contradicts existing paradigms regarding reactive oxygen species and disease, but we offer the following evidence in support of this.

First, recent reports demonstrated that oxidized DHA has the highest affinity and PPAR-activating effect of any PPAR ligand tested, including all members of the fibrate and glitazone drug classes [45,46]. This finding could have broad clinical implications because it indicates that DHA peroxidation *in vivo* would greatly enhance its potency as a PPAR activator. Secondly, an

Figure 2. Proposed intracellular effects of n-3 PUFA-derived peroxides and reactive aldehydes



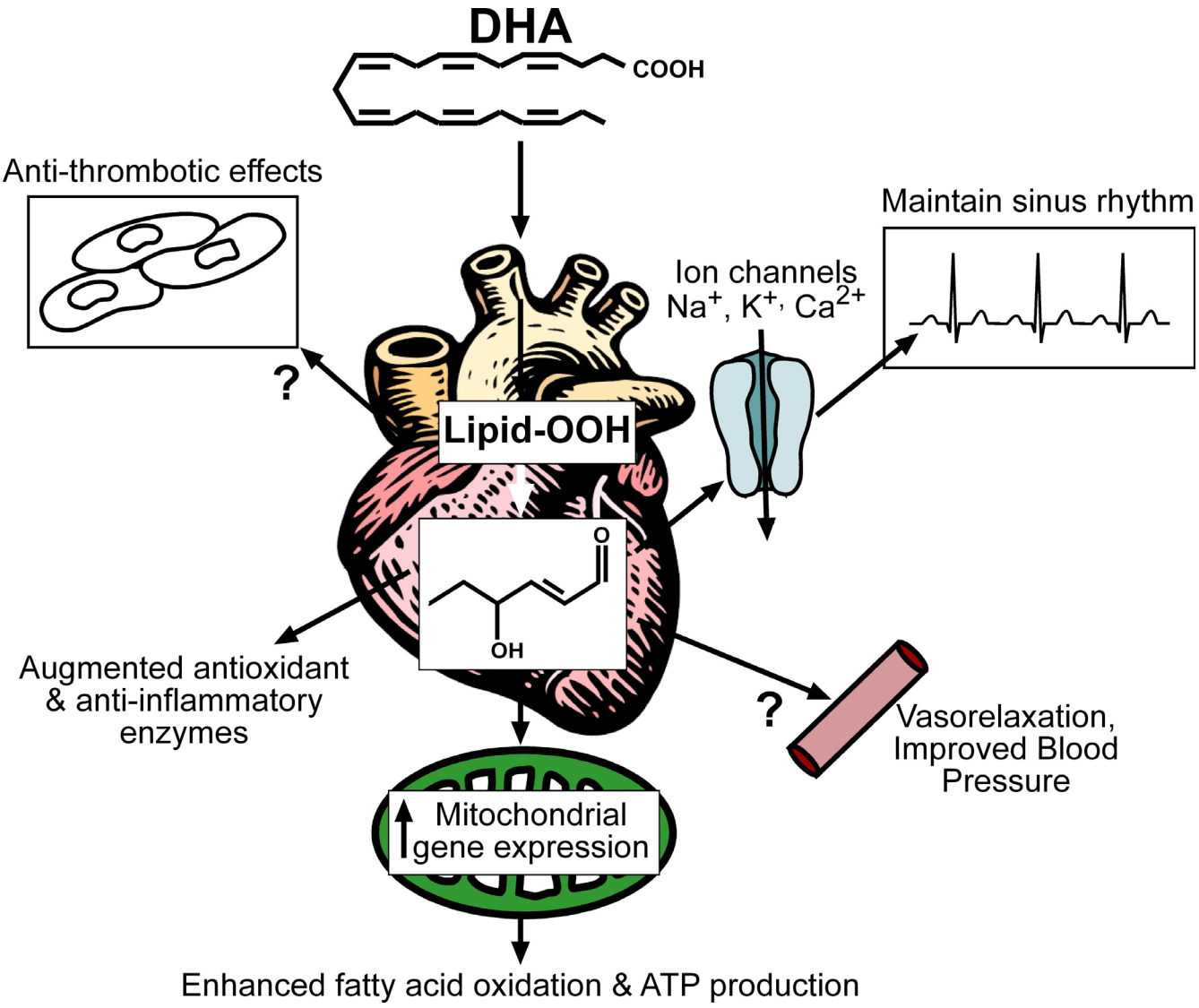
Upon entering cells, DHA and EPA are either used for oxidative metabolism (not shown) or esterified and used for phospholipids within membranes (shown here in both plasma and mitochondrial membranes). Liberation of DHA and EPA from the phospholipids is catalyzed by Phospholipase A2 (PLA2). In their free fatty acid form, the n-3 PUFAs are oxidized directly by reactive oxygen species coming from sources such as nicotinamide adenine dinucleotide phosphate (NADPH) oxidase or mitochondrial electron transport system. The lipid peroxides formed in this manner are then more reactive and their potency as transcriptional activators is increased, as depicted by the hydroperoxy-DHA reacting with PPARs and Nrf2. Sustained increases of these lipid peroxides can lead to a build-up of their reactive aldehyde derivatives such as 4-hydroxyhexenal and 4-oxo-2-hexenal (OHE), and these can also cause transcriptional activation or react with functional proteins directly. Activation of PPARs would be expected to cause increased mitochondrial gene expression and lipid oxidation capacity, particularly in the heart. Nrf2 activation would lead to increased expression of antioxidant/anti-inflammatory genes. Possible outcomes of the direct protein modifications include changes in ion channel conductance (shown in plasma membrane above) or in altered activity of mitochondrial respiratory enzymes. Keap1, Kelch-like ECH-associated protein 1; PGC1α, PPARγ coactivator-1α; ANT, adenine nucleotide translocase; GST, glutathione S-transferase; GCLC, γ-glutamylcystein ligase catalytic subunit; GR, glutathione reductase; Trx, thioredoxin; Trx-R; thioredoxin reductase.

interesting study by Judé and colleagues showed that the electrophysiological effects of DHA on the transient outward current in cardiomyocytes were only present when the DHA was oxidized [47]. This finding led the authors to speculate that perhaps much of the electrophysiological effects that investigators have attributed to DHA were actually coming from lipoxidative products derived from it, since a large amount

of DHA oxidation occurs spontaneously upon exposure to air (~30%).

As mentioned above, it is now clear that 4-hydroxynonenal can exert a beneficial effect on the heart. Zhang *et al.* [26] recently showed that treating cardiomyocytes with small, sub-toxic doses (5μM) of 4-hydroxynonenal elicits protection from subsequent exposure to toxic doses (≥20 μM). This group further showed the physiological relevance of this effect by pre-treating mice with 4-hydroxynonenal prior to ischemia/reperfusion, and showed that 4-hydroxynonenal-treated mice exhibited reduced infarct size. In addition, this 4-hydroxynonenal-induced cardioprotection was lost in Nrf2 -/- mice, suggesting a fundamental requirement for Nrf2 in the 4-hydroxynonenal effect [26]. The role of Nrf2 in cardioprotection is only beginning to be understood, and this was underscored by a recent study where overexpression of Nrf2 protected against

Figure 3. Diagram of proposed clinical effects of n-3 PUFA-derived peroxides and reactive aldehydes in heart



Many of the cardiovascular effects of n-3 PUFAs have been linked to their anti-thrombotic, antioxidant/anti-inflammatory, anti-arrhythmic, and vasorelaxing activities. We propose that many of these effects are mediated by n-3 PUFA-derived peroxides and reactive aldehydes. Moreover, to this list of well-known clinical effects of n-3 PUFAs we add enhanced mitochondrial biogenesis (i.e. gene expression) and antioxidant capacity in the myocardium, which would be expected to augment fatty acid oxidation and protect mitochondria against insults such as Ca^{2+} overload. The latter effects would be expected to have particular importance in clinical scenarios such as pathological cardiac hypertrophy and heart failure, conditions known to result in, or be a result of, mitochondrial dysfunction.

pressure overload-induced cardiac hypertrophy [48]. 4-hydroxyhexenal is the n-3 PUFA alkenal equivalent to 4-hydroxynonenal, which is similar in structure but displays different reactivity. As an electrophile, it is equipotent in activating Nrf2 [27], but studies on heart mitochondria in our laboratory have shown that at physiological concentrations, 4-hydroxynonenal inhibits oxidative phosphorylation and also causes increased susceptibility to Ca^{2+} overload, whereas 4-hydroxyhexenal does not (unpublished data). From a translational perspective, this observation is important because it implies that n-3 PUFA-derived lipoxidative products may cause similar adaptations to those of n-6 PUFA-derived lipoxidative products, without as much of the corresponding cellular and mitochondrial toxicity.

Further direction

It must be emphasized that if indeed n-3 PUFA-derived lipoxidative products are partially responsible for the therapeutic effects observed, this would be manifested to the greatest extent in highly plastic and adaptable organs (i.e. organs with large antioxidant capacities, like the heart). The reason for this is because 4-hydroxyhexenal, and indeed all n-3 PUFA-derived lipoxidative products, will affect tissues and organ systems differently depending on their ability to positively adapt to mild 'lipoxidative stress.' Moreover, it is expected that this adaptation would require several days or weeks to become optimal. Despite these considerations, our contention is that when taken together, the experimental evidence outlined here and elsewhere [49,50] supports the notion that many of the broad effects of n-3 PUFAs in cardiovascular disease (**Figure 3**) can be explained by lipoxidative products derived from them, particularly reactive aldehydes such as 4-hydroxyhexenal. If further investigation confirms this observation, it is deserving of rigorous evaluation as it may allow for future development of novel n-3 PUFA therapies in cardiovascular disease and other diseases, and assist in developing relevant and proper dosing strategies for n-3 PUFA use in clinics.

Abbreviations

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PPAR, proliferator-activated receptor; PUFA, polyunsaturated fatty acid.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

Supported in part by National Institutes of Health grant HL098780 to E.J.A. The authors would like to thank Kathleen Thayne for her kind assistance with preparation of Figures.

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