Cardiovascular Protective Effects of n-3 Polyunsaturated Fatty Acids With Special Emphasis on Docosahexaenoic Acid

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Abstract. It is widely accepted that n-3 polyunsaturated fatty acids (PUFAs) rich in fish oils protect against several types of cardiovascular diseases such as myocardial infarction, arrhythmia, atherosclerosis, or hypertension. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) may be the active biological components of these effects. Although the precise cellular and molecular mechanisms underlying the beneficial effects are still uncertain, the protective effects of n-3 PUFAs are attributable to their direct effects on vascular smooth muscle cell (VSMC) functions. These n-3 PUFAs activate \( K_{\text{ATP}} \) channels and inhibit certain types of \( \text{Ca}^{2+} \) channels, probably via at least 2 distinct mechanisms. N-3 PUFAs favorably alter the eicosanoid profile and regulate cytokine-induced expression of inducible nitric oxide synthase and cyclooxygenase-2 via mechanisms involving modulation of signaling transduction events. N-3 PUFAs also modulate VSMC proliferation, migration, and apoptosis. These recent data suggest that modulation of these VSMC functions contribute to the beneficial effects of n-3 PUFAs on various cardiovascular disorders. Furthermore, recent studies strongly suggest that DHA has more potent and beneficial effects than EPA. However, many questions about the cellular and molecular mechanisms still remain to be answered.

Keywords: docosahexaenoic acid, eicosapentaenoic acid, vascular smooth muscle cell, cardiovascular disease

1. Introduction

A large number of epidemiological studies, clinical trials, and experimental animal studies has shown that fish oils and n-3 polyunsaturated fatty acids (PUFAs) protect against several types of cardiovascular diseases such as myocardial infarction, arrhythmia, atherosclerosis, or hypertension (1 – 3). It is widely accepted that eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) are the active biological components of these effects (Fig. 1). The effects of n-3 PUFAs, particularly EPA, on functions of several types of cells such cardiac myocytes, vascular cells, platelets, leukocytes, or macrophages have been explored by a number of studies in order to understand the cellular and molecular mechanisms underlying the beneficial effects.

Although the precise mechanisms are still unclear, the protective effects of n-3 PUFAs are attributable to their direct effects on vascular endothelial and smooth muscle cell (VSMC) functions. Since endothelial cells produce and release a range of vasoactive mediators modulating vascular function and homeostasis and endothelial dysfunction is critically involved in cardiovascular disease, many studies have focused on the influences on endothelial functions (2). However, in view of the importance of VSMC functions in vascular pathophysiology, their modulation may also be critically involved in the positive effect of n-3 PUFAs as well. In deed, endothelium-independent vascular effects of DHA and EPA have been reported in animals and human studies (4, 5). Furthermore, recent results strongly suggest that DHA has more potent and beneficial effects on cardiovascular disease than EPA. DHA has been shown

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to be more effective than EPA in suppressing arrhythmia induced by ischemia in rats, inhibiting thromboxane-like vasoconstrictor responses in aorta from spontaneously hypertensive rats (SHR), and retarding hypertension development in SHR (6). DHA seems to be the principal n-3 PUFAs responsible for the hypotensive effect of fish oils, which was demonstrated by the first randomized, double-blind, placebo-controlled study in humans (7). Focusing on the more recent studies, we will here discuss that the modulation of VSMC functions is involved in the cellular mechanism for the cardiovascular protective effect of n-3 PUFAs with emphasis on the difference between EPA and DHA.

2. Intracellular calcium dynamics

As recently reviewed (1, 2), many studies in experimental animals and human subjects with hypertension have demonstrated a moderate reduction of blood pressure after supplementation of fish oils or n-3 PUFAs. Dietary administration of fish oils to genetically hypertensive rats reduces the exaggerated contractility of the vasculature in response to sympathetic nerve stimulation or vasoconstrictors. In vitro studies also have shown that both EPA and DHA have vasorelaxant effects on isolated blood vessels, indicating that the hypotensive effects are partly related to influences on vascular reactivity, possibly by both endothelium-dependent and -independent mechanisms. There are several possibilities for the endothelium-dependent mechanism, including the suppression of endothelium-derived contracting factors (EDCF) such as thromboxane A2 (TXA2) and endothelin, and enhanced release of relaxing factors such as nitric oxide (NO), prostaglandin I2 (PGI2), and hyperpolarizing factors (EDHF). The endothelium-independent mechanisms have been also suggested by several studies (1, 2). In a recent report, DHA, but not EPA, supplementation significantly enhanced dilatory responses and attenuated constrictor responses in the human forearm microcirculation, which were mediated by multiple mechanisms, predominantly endothelial-independent mechanisms (4). Similarly, EPA and DHA (1 – 100 μM) exerted the endothelium-independent vasorelaxant effects in Wistar Kyoto rats (WKY) and SHR aortae, probably through production of prostanoids that activate K+ATP channels, and through inhibition of intracellular Ca2+ release and Ca2+ channels in VSMCs (5, 8). Since intracellular Ca2+ concentration ([Ca2+]i) in VSMCs has a pivotal role as a second messenger in the mechanism of vasoconstriction, the endothelium-independent vasorelaxant effects of n-3 PUFAs can be partly attributed to the effect on intracellular Ca2+ dynamics in VSMCs.

In a recent electrophysiological study using cultured cells, n-3 PUFAs (3 – 100 μM), when applied acutely to the cells, activated a K+ current and effectively inhibited receptor-mediated non-selective cation currents, which are resistant to Ca2+ channel blockers, in rat A7r5 VSMCs stimulated with vasopressin and endothelin (9). The potency of the inhibitory effect was EPA > DHA > docosapentaenoic acid (DPA, C22:5n-3). Since n-3 PUFAs similarly inhibited the non-selective cation current induced by GTPγS, a non-hydrolyzable GTP analogue, the authors suggest that the primary site of the acute inhibitory action of n-3 PUFAs is the channel protein itself or some sites near the channels (9). These authors also reported that the long-term treatment of A7r5 VSMCs with EPA (30 μM) for 7 days suppressed the resting level and rise in [Ca2+], induced by vasoressin, endothelin, and platelet-derived growth factor (PDGF) (10). Under the current clamp condition, the resting membrane potential was significantly deeper in EPA-treated cells (–49.8 mV, mean) than in control cells (–44.6 mV, mean). In a study using VSMCs isolated from SHR, EPA (30 μM) pretreatment attenuated the angiotensin II-induced Ca2+ transient by 95% (11). We demonstrated that in VSMCs of WKY, DHA (30 μM) pretreatment for 2 days inhibited a rise in [Ca2+], induced by 5-hydroxytryptamine (5-HT), angiotensin II, and a depolarizing concentration of KCl (12). However, DHA had no effect on [Ca2+]i in VSMCs isolated from stroke-prone SHR (SHRSP), which was higher than [Ca2+]i in VSMCs of WKY. We also found that DHA
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(30 μM) pretreatment inhibited 5-HT-induced Ca^{2+} influx through both L-type and non L-type Ca^{2+} channels, while DHA had no effect on Ca^{2+} release from the internal stores in rat VSMCs (13). The mechanism of the inhibitory effect of n-3 PUFAs incorporated into the cells by adding to the culture medium may be different from that of acutely applied n-3 PUFAs (9). As illustrated in Fig. 2, these results strongly suggest that the specific inhibition by n-3 PUFAs of intracellular Ca^{2+} dynamics in VSMCs may contribute to the endothelium-independent vasorelaxant effects that had been previously observed (1, 2).

3. NO Production

Besides the vasodilating effect as endothelium-derived relaxing factor (EDRF), NO has inhibitory effects on platelet aggregation and adhesion, leukocyte adhesion, and VSMC proliferation/migration and is involved in the pathophysiology of cardiovascular disorders. NO is produced from the metabolism of L-arginine by a family of enzymes, NO synthase (NOS). There are three isoforms of NOS, neuronal NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). eNOS is restricted to the endothelium, while iNOS is expressed in several types of cells including VSMCs following stimulation with cytokines such as interleukin-1β (IL-1β) (14).

Studies in vitro have suggested that endothelium-dependent relaxation of vessel preparation by fish oils or n-3 PUFAs is due to the enhancement of NO release (1, 2). This is supported by the fact that EPA (>30 μM) stimulates NO production by endothelial cells in situ and induces endothelium-dependent relaxation of bovine coronary arteries (15). However, DHA and DPA (60 μM) had no effect. The immunostaining analysis of eNOS using cultured bovine endothelial cells revealed eNOS to be co-localized with caveolin-1 in the cell membrane at a resting state, while EPA induced Ca^{2+}-independent activation and translocation of eNOS to the cytosol and its dissociation from caveolin. This effect was comparable to that of the eNOS translocation induced by a [Ca^{2+}]-elevating agonist, bradykinin (10 μM) (15).

An early study using cultured cells has shown that EPA also potentiates the release of NO from IL-1β-stimulated human and rat VSMCs in a concentration-dependent manner with a significant effect at concentrations higher than 3 μM and 30 μM, respectively (16). In agreement with this study, we have recently reported that DHA (>10 μM), when added to the incubation medium, increases IL-1β-induced NO production by rat VSMCs (17). Although less potent than DHA, EPA, but not arachidonic acid, also showed the stimulatory effect, suggesting that this effect is common to n-3 PUFAs. The effect of DHA was not much remarkable, but substantial especially when IL-1β concentration was low. The effect of DHA was accompanied by potentiation of IL-1β-induced iNOS protein (Fig. 3) and mRNA expressions through a mechanism involving activation of the p44/42 mitogen-activated protein (MAP) kinase signaling cascade (Fig. 4). These results clearly indicate that DHA enhanced the upstream signaling pathways leading to iNOS translation in IL-1β-stimulated VSMCs.

NO production and iNOS expression in VSMCs are implicated in atherosclerotic vascular disease or hypertension (14). At the site of local vascular lesions such as atherosclerosis, IL-1β, and tumor necrosis factor-α are actively produced and released from activated macrophages, which may induce iNOS expression in VSMCs. Up-regulation of iNOS in VSMCs, therefore, may primarily function as a defensive and compensatory mechanism for endothelial dysfunction, by preventing the further development of pathological conditions, although excess amounts of NO radicals may cause cytotoxicity or apoptosis. It should be emphasized that recent experimental gene therapies by transferring an NOS gene including the iNOS gene to cardiovascular beds of various animal models of cardiovascular diseases show encouraging results and provide a novel and promising...
therapeutic strategy for treating cardiovascular diseases (18). Therefore, n-3 PUFAs may poteniate the primary function of iNOS-derived NO by the mild and persistent enhancing effect on iNOS induction in VSMCs.

4. Eicosanoid production

The beneficial effects of fish oils or n-3 PUFAs have been discussed largely with regard to arachidonic acid metabolism in the vessel wall as well as platelets (1, 2). EPA is a substrate of cyclooxygenase (COX) in the arachidonate cascade, resulting in the conversion to biological active PGI₂, with equipotent activities to PGI₁, and inactive TXA₁ (Fig. 5). DHA is not a direct substrate, rather an inhibitor in vitro, of COX, although DHA and EPA can be interconverted to each other. Administration of fish oils containing EPA and DHA increases the n-3/n-6 PUFA ratio in the membrane phospholipids, the sum of PGI₁ and PGI₂, and effectively reduces TXA₂ production in human studies and animal experiments in vivo (1, 2). The origin of PGI₂/PGI₁ and TXA₂ is considered predominantly to be endothelial cells and platelets, respectively. The in vivo increase in PGI₂/PGI₁ after n-3 PUFA supplementation may be explained, at least in part, by the retroconversion of DHA to EPA. DHA has been also found to antagonize prostanoid TP receptors in platelets and aorta (2).

There are two isoforms of COX, that is, COX-1 and COX-2. COX-1 is constitutively expressed in most cells, whereas COX-2 is induced by stimulation with various growth factors and cytokines such as IL-1β. IL-1β also induces COX-2 in VSMCs, resulting in the increase in the major arachidonate metabolite, PGI₁ (19). Although the arachidonate metabolite PGE₂ is normally considered to be a pro-inflammatory mediator, PGI₂/PGI₁ regulates vasoconstriction, platelet aggregation/adhesion, leukocyte adhesion, and VSMC proliferation/migration, sharing these cardioprotective effects with NO. In certain pathophysiological situations, therefore, COX-2 and iNOS co-expressed in VSMCs may function in concert to potentiate the defensive and compensatory mechanism at the site of vascular injury, particularly in atherosclerosis or thrombosis.

Supporting the increased PGI₂/PGI₁ production in
vivo, it is reported that PGI₂ production is enhanced in rat VSMCs cultured with a triglycerol (TG) emulsified form of EPA (EPA-TG, >80 μM) to simulate them under the in vivo condition, whereas DHA-TG has no effect (20). Since EPA-TG did not alter COX-1 and 10% fetal calf serum-induced COX-2 protein expressions, they concluded that low levels of lipid peroxides activate COX activities. A more recent study demonstrated that IL-1β-induced PGE₂ production and expressions of the type IIA secreted phospholipase A₂ and COX-2 genes in rat VSMCs were inhibited by preloading cells with EPA and DHA (50 μM) for 24 h, while these were enhanced with arachidonic acid (21). COX-2 protein expression was not evaluated in this study. Interestingly, the authors also showed that IL-1β-induced p44/42 MAP kinase phosphorylation is enhanced by arachidonic acid and slightly enhanced by DHA, but not by EPA. However, in contrast to their study, we have recently observed that when simultaneously added to the culture medium, DHA (30 μM) and, to a lesser extent, EPA-TG inhibited VSMC proliferation through modulating various steps of the signal transduction pathway by PDGF (24). These included the inhibitions of PDGF binding on its receptors and activation of protein kinase C. EPA-TG also suppressed c-fos mRNA expression, one of the immediate early genes, through partly inhibiting c-fos transcription. EPA (20 μM), but not DHA, also significantly inhibited basal and angiotensin

5. Proliferation, migration, and apoptosis

Human and experimental animal studies have shown that supplementation of n-3 PUFAs or fish oils significantly inhibit the development of atherosclerotic lesions, via mechanisms independent of their lipid lowering effect (23). Since VSMC proliferation and migration play an important role in the pathogenesis of atherosclerosis, or postangioplasty restenosis as well as hypertension, these mechanisms can be proposed to include the direct effect on VSMC proliferation and migration. In fact, EPA-TG (>40 μM) and, to a lesser degree, DHA-TG inhibited VSMC proliferation through modulating various steps of the signal transduction pathway by PDGF (24). These included the inhibitions of PDGF binding on its receptors and activation of protein kinase C. EPA-TG also suppressed c-fos mRNA expression, one of the immediate early genes, through partly inhibiting c-fos transcription. EPA (20 μM), but not DHA, also significantly inhibited basal and angiotensin

![Diagram of eicosanoid profile in VSMCs as a possible mechanism for their cardiovascular protective effects. Supplementation of fish oils containing n-3 PUFAs may increase the n-3/n-6 PUFA ratio in the membrane phospholipids, the sum of PGI₂ and PGI₃, and effectively reduces TXA₂ production in vivo. Furthermore, DHA may antagonize prostanoid TP receptors in platelets and blood vessels. PGI synthase, prostaglandin I synthase; TX synthase, thromboxane synthase; LPO, lipid peroxides.](image)
II- and phorbol ester-induced DNA synthesis in rat VSMCs, but did not affect PDGF-stimulated DNA synthesis (25). The authors also showed that EPA exerted specific inhibition of exaggerated growth of VSMCs of SHR through the suppression of transforming growth factor-β mRNA expression and cyclin-dependent kinase 2 activity. Thus, EPA seems to have more potent anti-proliferative effect than DHA. However, it has been reported that a low concentration (0.33 – 3.3 μM) of EPA and DHA equipotently block the proliferation induced by TXA₂, 5-HT, and PDGF (26, 27) in canine VSMCs. When EPA and DHA were combined in the ratio they were present in fish oils, there was a synergistic interaction in the inhibitory effect. Furthermore, these n-3 PUFAs suppressed 5-HT-induced upregulation of 5-HT₂ receptor mRNA expression (27). N-3 PUFA pretreatment also inhibited PDGF-induced migration of human (28) and rat A7r5 (10) VSMCs. Thus, n-3 PUFAs have been reported to regulate VSMC proliferation/migration induced by several mitogens via multiple mechanisms, as summarized in Fig. 6.

VSMC apoptosis plays an important role in cardiovascular diseases by influencing vascular remodeling, restenosis or plaque rupture, although the precise role still remains unclear (29). DHA (40 μM) has been demonstrated to induce apoptosis through translocation of plasma membrane phosphatidylserine and disruption of mitochondrial transmembrane potential, followed by increased bax expression and caspase 3 activation in rat VSMCs (30). DHA also induced apoptosis through more than 2 distinct mechanisms: p38 MAP kinase-dependent pathways that regulate peroxisome proliferator-activated receptor-α and p38 MAP kinase-independent pathways via dissipation of mitochondrial transmembrane potential and cytochrome c release (31). These studies suggest that by triggering VSMC apoptosis, DHA may play a pathophysiological role in cardiovascular diseases. N-3 PUFAs may also modulate VSMC proliferation, migration, or apoptosis indirectly by modulating eicosanoid and NO production at the site of vascular injury (Fig. 6).

6. Mechanisms of cellular actions

PUFAs are major components of membrane phospholipids and play a key role in membrane functions. When added to the culture medium, n-3 PUFAs can be easily incorporated into the membrane phospholipids or triglycerides and increase membrane cholesterol efflux in VSMCs (32). It is reported that DHA has a greater effect than EPA in increasing membrane fluidity of endothelial cells cultured from rat thoracic aorta (33). The greater effect of DHA was considered to be due to its greater ability to decrease membrane cholesterol content and/or the cholesterol/phospholipid molar ratio and also to its greater ability to elevate the unsaturated index in the plasma membrane. These physicochemical alterations in the membrane properties may directly or...
indirectly influence functions of membrane-bound proteins such as receptors, GTP binding proteins, ion channels, and various enzymes. Since signaling events such as the MAP kinase cascade is initiated also at the plasma membrane and modified by the membrane lipid microenvironment, the alteration in the membrane properties may affect downstream signaling pathways after receptor stimulation or expression of ion channel proteins.

In addition, ion channels seem to be modulated by n-3 PUFAs by another mechanism that does not require their incorporation into the membrane lipid pools. The inhibitory effects of acutely applied n-3 PUFAs on receptor-mediated non-selective cation current in agonist-stimulated rat A7r5 VSMC are acutely irreversible after washing out by a buffer containing albumin, suggesting that incorporation of n-3 PUFA is not required for the ion channel inhibition (9). The authors suggested that the primary site of the acute inhibitory action of n-3 PUFAs is the channel protein itself or some site near the channels. Interestingly, a single point mutation in the α subunit of human myocardial Na+ channel transiently expressed in human embryonic kidney 293 cells significantly decreased the inhibitory effect of n-3 PUFAs on the voltage-gated Na’ currents, which may play a significant role in the antiarrhythmic actions of n-3 PUFAs (34). Although this interactive or binding site is sensitive not only to n-3 PUFAs, but also to monounsaturated and saturated fatty acids, such an approach may provide a new insight into the mechanism through which n-3 PUFAs modulate ion channel activity.

It is worth noting that an effective concentration (30 μM) of DHA or EPA in VSMCs isolated from WKY had no significant effects on Ca2+ influx and iNOS/COX-2 expression in VSMCs isolated from SHRSP (Ref. 12 and M. Hirafuji et al., unpublished data). The reason why VSMCs of SHRSP are resistant to these n-3 PUFAs is unclear. It may be related to the alterations in uptake, intracellular transport, metabolism, or composition of PUFA, or in function of target molecules in the plasma membrane of VSMCs derived from SHRSP. Regardless of the mechanisms responsible for the cellular effects of n-3 PUFAs seen in VSMCs of normotensive WKY, it remains to be clarified why VSMCs of SHRSP are refractory to n-3 PUFAs.

There are some differences in the cellular effects of EPA and DHA in VSMCs, partly due to the differences in chain length and number of double bonds. Differential effects of EPA and DHA have been also shown not only in blood pressure and vascular reactivity, but also in heart rate and lipid/lipoprotein metabolism in humans (35). However, it is unclear whether these differences have relevance to the different potency between DHA and EPA in modulating VSMC functions. Conflicting results concerning the potency of EPA and DHA may be partly due to the difference in the form applied to the cells, that is, free acid form, esterified form, albumin-bound form, or TG-emulsified form.

7. Conclusion

There are accumulating evidences that support the modulation of VSMC functions as one of the possible mechanisms underlying the cardiovascular protective effects of DHA and EPA. These n-3 PUFAs activate K’_{ATP} channels and inhibit certain types of Ca2+ channels, probably via at least 2 distinct mechanisms. The suppression of VSMC excitability may partly explain the reduction of vascular response and endothelium-independent vasorelaxant effects of n-3 PUFAs. The effects on ion channel activities by n-3 PUFAs may further affect the subsequent signaling cascade, resulting in the modulation of other cellular functions such as proliferation or migration. N-3 PUFAs alter the
eicosanoid profile by decreasing TXA₂ but also by enhancing PGI₂/PGI₁ production. N-3 PUFA s also modulate IL-1β-induced expression of iNOS and COX-2 via a mechanism involving the p44/42 MAP kinase signaling cascade. Since these enzymes synthesize important mediators, NO and eicosanoids, respectively, these effects may have particular relevance to the protective effect against local vascular injury such as atherosclerosis or thrombosis. N-3 PUFA s also modulate VSMC proliferation/migration or apoptosis directly or indirectly through the autocrine effect of these mediators at the site of vascular injury. As illustrated in Fig. 7, all these cellular effects seem to be favorable to the beneficial effects of n-3 PUFA s on cardiovascular diseases. Nevertheless, many questions still remain to be answered as to whether the effects may be exerted merely through the physicochemical changes in the membrane properties, through interacting with specific target proteins in the plasma membrane, or through interacting with other functional cytoplasmic or nuclear molecules.

Recent results strongly suggest that DHA has more potent and beneficial effect on cardiovascular diseases than EPA. DHA has been also reported to prevent and be effective for treating senile dementia, depression, certain visual dysfunction, arthritis, diabetes mellitus, and some cancers, some of which EPA is not effective for. It should be noted that DHA, a natural occurring fatty acid, has so many diverse beneficial effects without recommended dose. The progress in studies for the potent and beneficial effect on cardiovascular diseases. Nevertheless, many questions still remain to be answered as to whether the effects may be exerted merely through the physicochemical changes in the membrane properties, through interacting with specific target proteins in the plasma membrane, or through interacting with other functional cytoplasmic or nuclear molecules.

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