Published in final edited form as:

Chem Phys Lipids. 2008 May ; 153(1): 14–23.

Mechanisms by which docosahexaenoic acid and related fatty acids reduce colon cancer risk and inflammatory disorders of the intestine

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Abstract

A growing body of epidemiological, clinical, and experimental evidence has underscored both the pharmacological potential and the nutritional value of dietary fish oil enriched in very long chain n-3 PUFAs such as docosahexaenoic acid (DHA, 22:6, n-3) and eicosapentaenoic acid (EPA, 20:5, n-3). The broad health benefits of very long chain n-3 PUFAs and the pleiotropic effects of dietary fish oil and DHA have been proposed to involve alterations in membrane structure and function, eicosanoid metabolism, gene expression and the formation of lipid peroxidation products, although a comprehensive understanding of the mechanisms of action has yet to be elucidated. In this review, we present data demonstrating that DHA selectively modulates the subcellular localization of lipidated signaling proteins depending on their transport pathway, which may be universally applied to other lipidated protein trafficking. An interesting possibility raised by the current observations is that lipidated proteins may exhibit different subcellular distribution profiles in various tissues, which contain a distinct membrane lipid composition. In addition, the current findings clearly indicate that subcellular localization of proteins with a certain trafficking pathway can be subjected to selective regulation by dietary manipulation. This form of regulated plasma membrane targeting of a select subset of upstream signaling proteins may provide cells with the flexibility to coordinate the arrangement of signaling translators on the cell surface. Ultimately, this may allow organ systems such as the colon to optimally decode, respond, and adapt to the vagaries of an ever-changing extracellular environment.

Keywords

protein targeting; trafficking; fish oil; chemoprevention; colon cancer

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Introduction

A growing body of literature supports the contention that bioactive food components containing n-3 polyunsaturated fatty acids (PUFA) are important in suppressing colon cancer. For example, there is substantial experimental, epidemiological and clinical evidence indicating that fish oil-containing diets rich in docosahexaenoic acid (DHA, 22:6 Δ 4,7,10,13,16,19) and eicosapentaenoic acid (EPA, 20:5 Δ 5,8,11,14,17) are protective against colon tumorigenesis [Anti 1992, 1994; Bartrum, 1993; Caygill, 1996; Chang, 1997, 1998; Fernandez, 1999; Rao, 2001; Cheng, 2003; Tavani, 2003; Reddy, 2005; Phillips, 2006; Courtney, 2007]. In contrast, several systematic reviews have challenged the premise that dietary fish oil reduces colon cancer risk, fueling a debate regarding the role of n-3 PUFA as chemoprotective nutrients [Lupton, 2004; MacLean, 2006]. It has been estimated that the mean dietary intake of EPA and DHA in North America is approximately 100 mg/day [Arterburn, 2006; Hibbeln, 2006]. This corresponds to a fasting blood level of ~100 μ M EPA and ~280 μ M DHA. Upon supplementation, EPA and DHA levels can exceed 700 μ M and 500 μ M, respectively [Conquer, 1998]. These changes in circulating n-3 PUFA result in the corresponding enrichment of colonocyte membrane lipids [Anti, 1992; Bartram, 1993; Hong, 2002; Chapkin, 2002].

Although the mechanism of EPA and DHA action is still not fully defined in molecular terms, it is becoming increasingly clear that these fatty acids are pleiotropic. The challenge for the future is to determine precisely how n-3 PUFA alter membrane microdomain structure and function, eicosanoid metabolism, and the formation and mobilization of reactive oxygen products and intracellular Ca^{2+} . This review will focus of recent research efforts which contribute to the development of unifying molecular mechanisms which underlie the pleiotropic protective effects of n-3 PUFA with respect to colon cancer and inflammatory diseases of the intestine.

I. Suppressive effect of n-3 PUFA on colon tumor development

The balance between proliferation and apoptosis is critical to the maintenance of steady-state number for cell populations in the colon. In general, dysregulation of this mechanism can disrupt homeostasis, resulting in clonal expansion, with the resultant over production of affected cells [Bedi, 1995; Siniscope, 1995]. The programmed induction of cell death also represents a mechanism by which colonocytes possessing DNA damage can be deleted. This “targeted” immediate apoptotic response to DNA damage is p53-dependent in contrast to “spontaneous” apoptosis which is largely a p53-independent process [Chapkin, 1999]. These protective processes can occur anywhere along the crypt axis [Bedi, 1995; Chang, 1997; Hong, 2000]. It has now been clearly established that the transformation of colonic epithelium to carcinoma is in part associated with a progressive inhibition of apoptosis [Siniscope, 1995; Bedi, 1995; Chang, 1997; Hanahan, 2000]. Importantly, we have demonstrated that measurements of apoptosis have greater prognostic value to detect dietary effects on colon tumor incidence than do measurements of cell proliferation [Chang, 1997; Hong, 2000].

In a major step toward understanding why n-3 PUFA suppress colon cancer, we determined the effect of fish oil feeding on genetic signatures during colon cancer initiation and progression. Specifically, we contrasted the biological properties of three types of commonly consumed dietary fats, i.e., corn oil/n-6 PUFA, fish oil/n-3 PUFA and olive oil/n-9 monounsaturated fat, following carcinogen treatment at both the initiation and post-initiation stages of colon tumor development. We found that only the consumption of n-3 PUFA exerted a protective effect at the initiation (DNA adduct formation) and promotional (apoptosis and aberrant crypt foci levels) stages (Table 1) [Davidson, 2004]

A very exciting outcome of our studies was the demonstration that fish oil (containing both DHA and EPA) fed rats have higher levels of colonic apoptosis, conferring resistance to

alkylation and oxidation-induced DNA damage [Hong, 2000, 2005; Bancroft, 2003; Sanders, 2004; Leyk, 2005]. The protective effect of n-3 PUFA was exerted at both the initiation and post-initiation stages of carcinogenesis [Chang, 1997, 1998; Davidson, 2004]. This important observation identifies a clear mechanism by which dietary n-3 PUFA exert a protective modulatory effect on colonocyte deletion in the whole animal. These data are consistent with recent observations indicating that the intake of n-3 PUFA (EPA 100 mg/d and DHA 400 mg/d for 2 years) promotes apoptosis of colonic mucosa in humans [Cheng, 2003]; and supplementation with EPA (2 g/day for 3 months) significantly increased apoptosis in normal colonic mucosa in subjects with a history of colorectal adenomas [Courtney, 2007].

From a mechanistic standpoint, we hypothesized that fish oil-induced up-regulation of apoptosis would be coincident with a down-regulation of bcl-2, a well known anti-apoptotic mediator. This is based on the fact that PGE₂ is known to inhibit apoptosis in part by induction of bcl-2 [Sheng, 1998] and we have previously demonstrated that fish oil downregulates PGE₂ and COX-II in colonic mucosa [Lee, 1993; Lupton 1999]. Tissues were collected from rats consuming diets containing either corn oil or fish oil at 3–12 h after carcinogen (azoxymethane) injection. Fish oil decreased bcl-2 expression ($p < 0.05$) and increased apoptosis ($p < 0.05$) in the top third of the distal colon (Table 2).

Finally, we have demonstrated that the pleiotropic bioactive components generated by fermentable fiber (butyrate) and fish oil (DHA and perhaps EPA) work coordinately to protect against colon tumorigenesis, primarily by increasing apoptosis rather than decreasing cell proliferation *in vivo* [Chang, 1998; Davidson, 2000; Hong, 2000]. Although it has been well documented that butyrate is an inhibitor of histone deacetylases and can activate the Fas receptor mediated “extrinsic” death pathway [Smith, 1998; Fan, 1999], the role of these mechanisms in the induction of colonocyte apoptosis may be a secondary consequence of its ability to promote cellular oxidation [Archer, 1998; Mariadason, 2000]. For example, serving as the primary energy source for colonic epithelial cells, butyrate induces cellular reactive oxygen species (ROS) generation [Benard, 1997; Giardina, 1998; Smith, 1998]. This is relevant because DHA is an oxidatively susceptible lipid due to its high degree of unsaturation [Gardner, 1989; Chapkin, 2002; Hong, 2002].

II. Molecular mechanisms by which DHA modulates apoptosis

The “intrinsic” apoptotic pathway channels cell death signals through the mitochondrion which serves as a damage/oxidative stress sensor and monitor of metabolic status [Costantini, 2000; Fulda, 2001]. Consistent with previous reports linking oxidative stress and apoptosis, there is mounting evidence that *cis*-unsaturated fatty acids, particularly DHA, induce this pathway via the generation of ROS such as superoxide/hydrogen peroxide, and in particular, phospholipid hydroperoxides (PLOOH), which disrupt the mitochondrial permeability transition pore (mtPTP) and trigger the release of soluble intermembrane proteins [Nomura, 1999; Wang, 2000; Kokoszka, 2001; Koumura, 2005; Engel, 2006]. For example, we have recently demonstrated that dietary DHA is incorporated into mitochondrial membrane phospholipids, thereby enhancing oxidative stress induced by butyrate metabolism [Hong, 2002; Chapkin, 2002]. In complimentary experiments, in order to elucidate the subcellular origin of oxidation induced by DHA and butyrate, immortalized mouse colonocytes (YAMC) were treated with 0–200 μ M DHA or linoleic acid (LA, n-6 PUFA control) for 72 h with or without 5 mM butyrate for the final 24 h [Ng, 2005]. Cytosolic reactive oxygen species (ROS, measured using CMH₂-DCFDA), membrane lipid oxidation (PLOOH, measured using diphenyl-1-pyrenylphosphine, DPPP), and mitochondrial membrane potential (MP, measured using rhodamine 123), were assayed by live-cell fluorescence microscopy. After 24 h of butyrate treatment, DHA primed cells exhibited a 151% increase in lipid oxidation ($p < 0.01$), compared to no butyrate treatment, which could be blocked by a mitochondria-specific antioxidant,

MitoQ ($p < 0.05$). LA treatment did not show any significant effect. In the absence of butyrate, DHA treatment, compared to LA, increased resting MP by 120% ($p < 0.01$). In addition, butyrate-induced MP dissipation was 21% greater in DHA primed cells as compared to LA at 6 h. This effect was reversed by preincubation with inhibitors of the mitochondrial permeability transition (MPT) pore, cyclosporin A or bongkreikic acid. The functional importance of these events is supported by the demonstration that DHA and butyrate-induced apoptosis is blocked by MitoQ. These data indicate that **DHA** and **butyrate** potentiate mitochondrial lipid oxidation and the dissipation of MP which contributes to the induction of apoptosis (Figure 1).

We have recently demonstrated a significant enrichment of EPA and DHA in mitochondrial phospholipids, e.g., cardiolipin, isolated from (i) colonic mucosa from rats fed fish oil or EPA/DHA ethyl esters, and (ii) colonocytes cultured with DHA, [Collett, 2001; Chapkin, 2002; Hong, 2002]. It is possible, therefore, that cardiolipin incorporating substantial quantities of EPA and DHA, containing 5 and 6 double bonds, respectively, would be highly susceptible to lipid peroxidation [Gardner, 1989; Chapkin, 2002]. This is noteworthy, because the accumulation of cardiolipin hydroperoxides (CL-OOH) directly triggers the release of proapoptotic factors from mitochondria [Kagan, 2005; Bayir, 2006]. Along these lines, the above mentioned observations are particularly relevant in that a mitochondria-specific antioxidant, MitoQ, blocks lipid oxidation and apoptosis induced by butyrate. Interestingly, oxidative stress regulates a broad array of signal transduction pathways that regulate mitochondrial function and apoptosis [Storz, 2005]. For example, the production of ROS/PLOOH in mitochondria is strictly regulated by L-form mitochondrial phospholipid hydroperoxide glutathione peroxidase (PHGPx), classical glutathione peroxidase (cGPx), and Mn-dependent superoxide dismutase (SOD2). Among these mitochondrial antioxidant enzymes, the L-form of PHGPx is unique because it directly reduces peroxidized phospholipids in membranes [Weitzel, 1990; Arai, 1999]. However, the precise mechanisms regulating dietary oxidative stress-induced apoptosis in the colon have not been clearly defined.

With respect to molecular triggers for apoptosis, Ca^{2+} is one of the most versatile and universal signaling mediators in cells and is required for the activation of many cellular processes. Increasing evidence indicates that alterations in the finely tuned intracellular homeostasis and compartmentalization of Ca^{2+} can lead to cell death either through apoptosis or necrosis [Berridge, 2000]. Eukaryotic cells can increase their cytosolic Ca^{2+} levels via 2 mechanisms: release of Ca^{2+} from intracellular stores or influx via plasma membrane channels. Channels located in the plasma membrane, e.g., store operated Ca^{2+} channels (SOC), receptor operated channels, and voltage operated channels, regulate the influx of Ca^{2+} into the cell. Although the importance of the endoplasmic reticulum (ER) as the major storage organelle is indisputable, growing evidence indicates that functional compartmentalization of Ca^{2+} exists within the various cellular organelles. Recent studies have identified the contributions of the mitochondria in maintaining intracellular Ca^{2+} homeostasis and cellular physiologic function [Orrenius, 2007]. In fact, it is now recognized that mitochondria play a key role in both apoptosis and necrosis by regulating energy metabolism, intracellular Ca^{2+} homeostasis, activation of caspases and the release of reactive oxygen species (ROS) [Jacobson, 2002; Fariss, 2005].

Given the central role of mitochondria in the commitment to apoptosis, we hypothesized that DHA and butyrate can interactively promote apoptosis by triggering changes in mitochondrial Ca^{2+} levels that contribute to caspase activation and colonocyte cell death. We used isogenic p53 wild type and deficient human colon tumor (HCT 116) cell lines as well as an immortalized mouse colonocyte (YAMC) cell line to determine whether or not chemoprotective nutrients modulate intracellular Ca^{2+} compartmentalization and store-operated channel (SOC) entry to induce colonocyte apoptosis [Kolar, 2007a]. The results confirm and extend our previous observations [Ng, 2005] and demonstrate that DHA and butyrate synergistically enhance both

mitochondrial Ca^{2+} accumulation and lipid peroxidation which serve as triggers for apoptosis in a p53-independent manner [Kolar, 2007b].

Based on the findings described above, we propose a pathway for the induction of apoptosis in colonic epithelium that involves the synergistic action of two bioactive molecules, DHA and butyrate, on enhanced mitochondrial ROS/PLOOH and Ca^{2+} accumulation (Figure 2). Our results indicate that the effects of individual chemoprotective nutrients (DHA and butyrate) may not be as important as the nutritional combination. We propose that the failure to address the interaction between fat and fiber may explain why the chemoprotective effects of n-3 PUFA and butyrate may be partly obscured in human studies [Alberts, 2000; Peters, 2003; Park, 2005; MacLean, 2006].

III. Effects of DHA and EPA on membrane structure and function, and membrane-associated proteins

EPA and DHA affect diverse physiological processes including cell membrane structure/function and eicosanoid-dependent signaling [Smith, 2005; Chapkin, 2007a,b], thereby providing significant protection against a variety of apparently unrelated human diseases [Stulnig, 2003; Lupton, 2004; Chapkin, 2007a]. n-3 PUFA are rapidly incorporated into cells, primarily into membrane phospholipids at the *sn*-2 position [Chapkin, 1991; Stillwell, 2003], and in general, the cellular level is readily influenced by diet [Katan, 1997]. Increasing evidence suggests that DHA is a unique fatty acid because it significantly alters basic properties of cell membranes, including acyl chain order and fluidity, phase behavior, elastic compressibility, ion permeability, fusion, rapid flip-flop and resident protein function [Stillwell, 2003; Shaikh, 2003]. The presence of long chain n-3 PUFA in membrane phospholipids imparts unique physicochemical properties to cellular membranes and DHA-induced alterations in membrane structure and function have been proposed to underlie its pleiotropic salutary effects [Ehringer, 1990; Hashimoto, 2001, 2006; Ma, 2004ab; Seo, 2006; Stillwell, 2006; Chapkin, 2007ab].

A. Effects on membrane microdomains and protein compartmentalization

The plasma membranes of all eukaryotic cells contain specific detergent-resistant domains in which key signal transduction proteins are localized. These regions are classified as “lipid rafts”, which are composed mostly of cholesterol and sphingolipids and therefore do not integrate well into the fluid phospholipid bilayers causing them to form microdomains [Hancock, 2006]. In addition, caveolae represent a subtype of lipid raft that form flask-shaped membrane invaginations containing the structural protein caveolin-1 [Anderson, 1998]. Because of its polyunsaturation, DHA and possibly EPA are sterically incompatible with sphingolipid and cholesterol and, therefore, appear to alter lipid raft behavior and protein function [Stulnig, 2003; Shaikh, 2004; Chen, 2007]. Many cytosolic proteins covalently modified with saturated palmitate (16:0) preferentially partition into liquid-ordered microdomains such as lipid rafts and caveolae, which is critical for their function (Figure 3). PUFA have been shown to selectively displace acylated signaling proteins from lipid rafts and caveolae (liquid ordered phases in the plasma membrane), influencing downstream signaling events [Stulnig, 1998, 2001; Zeyda, 2002, 2005; Webb, 2000; Liang, 2001]. Since dietary n-3 PUFA are incorporated into colonocytes [Hong, 2002], we have demonstrated that PUFA classes (n-6 vs n-3) differentially modulate colonocyte membrane microdomains [Ma, 2004ab; Chapkin, 2005]. Specifically, dietary n-3 PUFA-induced perturbations in colonocyte membrane microdomain (caveolae) lipid composition can alter signaling protein microdomain localization and function in the colon [Ma, 2004ab; Chapkin, 2005; Stillwell, 2006]. Of relevance to colonic apoptosis and tumor development, we have recently demonstrated that DHA suppresses the activation of Ras, a 21 kDa guanine nucleotide binding protein, by limiting its microlocalization to the lipid raft/caveolae [Ma, 2004ab]. This is significant because the

high frequency of Ras mutations/overexpression in human and animal model adenomas and adenocarcinomas directly drives colonic tumor development [Hoshino, 1999; Sebolt-Leopold, 1999]. Furthermore, since oncogenic Ras generates a powerful anti-apoptotic signal in the colon [Bissonnette, 2000; Sears, 2002; Liu, 2005, 2006], capable of blocking *extrinsic* death (Fas) receptor mediated signaling [Peli, 1999], its antagonism will promote apoptosis and have therapeutic value. Our findings highlight a novel modality by which n-3 PUFA influence membrane micro-organization and biochemical makeup, thereby modulating biological responses.

B. Effects on protein cytosol-to-membrane translocation

In some cases, cytosolic signaling proteins are transiently recruited to and released from the cytoplasmic surface of cellular membranes. Regulation of dynamic membrane translocation of these proteins is critical for their function, and has been shown to be affected by n-3 PUFA. For example in Con A-stimulated human peripheral blood mononuclear cells, DHA dose-dependently promoted membrane translocation of ADP-ribosylation factor (ARF), but only to the detergent-soluble non-raft membranes. This was associated with the dose-dependent activation of phospholipase D (PLD) by DHA [Diaz, 2002]. In addition, DHA increased membrane translocation of protein kinase C (PKC) α , β , and ξ in unstimulated cells, while dose-dependently suppressing membrane translocation in stimulated cells.

We examined the separate effects of dietary corn oil (devoid on n-3 PUFA), fish oil, containing EPA and DHA, and highly purified DHA ethyl ester on the membrane subdomain distribution of critical signal transducing molecules (PKC θ , LAT, and Fas/CD95) in mouse splenic T lymphocytes [Fan, 2004]. Fish oil and DHA ethyl ester feeding increased phospholipid n-3 fatty acyl content while reducing sphingomyelin content in T cell lipid rafts. In addition, both DHA-containing diets inhibited raft recruitment and downstream signaling of PKC θ in stimulated T cells, decreasing lymphoproliferation. In complimentary studies, the effects of EPA and DHA (vs untreated control) on membrane translocation and downstream signaling of PKC isoforms in Jurkat T cells were examined [Denys, 2005]. EPA and DHA inhibited phorbol ester-induced membrane translocation of PKC α and PKC ϵ , but not PKC δ , which was associated with the inhibition of downstream signaling. This may in part be attributed to the ability of certain long chain PUFA to alter ER-Golgi protein trafficking [Shaikh, 2007]. Collectively, these data support the hypothesis that PUFA classes (n-6 vs n-3) differentially modulate the dynamic translocation of signaling proteins. The mechanisms by which n-3 fatty acids alter plasma membrane translocation remain to be elucidated.

C. Effects on lipidated protein targeting via vesicular transport

Many signaling proteins must associate with membranes to function properly. For this purpose, cytosolic proteins that are initially synthesized as soluble proteins are co- or post-translationally modified with specific lipid anchors or contain special domains with high affinity for membranes (Figure 3). Since membrane localization of these otherwise soluble proteins is mediated by interactions between lipid anchors of proteins and cell membranes, it is conceivable that their membrane localization and hence function are sensitive to n-3 PUFA-induced changes in the cellular lipid environment. However, while the importance of post-translational protein lipidation to provide membrane anchors and targeting signals is well established and our understanding of protein transport mechanisms has substantially progressed, the potential regulatory role of the cellular lipid environment in the subcellular targeting of lipidated proteins has not been well appreciated.

Recently, our laboratory has demonstrated that DHA significantly inhibited plasma membrane targeting of Ras isoforms and Src-related tyrosine kinases using green fluorescent protein (GFP) fusion chimeras and quantitative fluorescence imaging of living colonic epithelial cells

[Seo, 2006]. DHA selectively decreased the plasma membrane targeting of cytoplasmic lipidated protein cargo of the exocytic pathway, which involves constant budding and fusion of transport vesicles. However, the vesicular transport-independent trafficking of lipidated proteins as well as the general secretory vesicular traffic in colonic epithelial cells was unaffected DHA. Interestingly, this DHA effect appears to be only dependent on the protein trafficking route, irrespective of the types of membrane anchors as well as the functional status of lipidated proteins. It is noteworthy that DHA enrichment in cellular membranes was essential to elicit this inhibitory effects. These data are significant because overactivation of Ras proteins either by mutation, protooncogene overexpression, or chronic up-regulation of their upstream receptors contributes to deleterious cellular malfunction including oncogenesis and inflammatory diseases of the colon [Bos, 1989; Egan, 1989; Manges, 1992; Kim, 1997]. For example, Ras effectors induce cyclin D1 and COX-2 resulting in hyperproliferation and apoptotic resistance. In addition, Src-related nonreceptor tyrosine kinases such as Lck play a key role in T cell-mediated immune responses and inflammatory diseases [Lowell, 2004]. Therefore, the selective suppression of plasma membrane targeting of lipidated signaling proteins by n-3 PUFA may constitute a novel modality of the mechanisms responsible for the long appreciated chemoprotective and immunomodulatory effects of dietary fish oil and DHA.

IV. Mechanisms by which DHA affects lipidated protein targeting

The exact mechanism(s) whereby DHA suppresses the plasma membrane targeting of lipidated protein via the exocytic pathway remains elusive. Although this DHA effect seems universal to lipidated proteins traveling through the secretory pathway, DHA does not appear to alter the bulk flow of secretory vesicular traffic since the plasma membrane delivery of the conventional transmembrane protein cargo remained unaffected [Seo, 2006] (Figure 4). Furthermore, it is unlikely that DHA induces recycling from the cell surface via endocytosis since the selectivity of DHA effects cannot be accounted for in this manner. However, potential explanations can be proposed based on previous findings from our laboratory and other groups as discussed below.

A. Alterations in protein palmitoylation

The role of palmitate (16:0) in protein plasma membrane targeting seems to be more than just increasing membrane binding affinity but rather operating as an actual targeting signal directing proteins to the plasma membrane [Greaves, 2007]. Recent work revealed that a dynamic de/repalmitoylation cycle is essential for maintaining the plasma membrane localization of Ras [Rocks, 2005, 2006]. We have demonstrated that Ras palmitoylation is unaffected by DHA in colonocytes [Collett, 2001]. Given that palmitoylation is the last step of Ras posttranslational modification, requiring the prior processing of the CAAX motif including farnesylation, this further indicates that DHA does not compromise the overall posttranslational processing of Ras proteins. This is supported by the lack of a DHA effect on farnesyltransferase activity, HMG-CoA reductase expression and Ras farnesylation state [Collett, 2001; Davidson, 1999]. However, DHA-induced changes in de/repalmitoylation kinetics may have not been detected by measurements of steady-state palmitoylation status in previous studies [Resh, 2006a,b,c]. Clearly, further progress in the identification and characterization of protein palmitoyltransferases and acyl protein thioesterases is required to clarify whether DHA influences the dynamics of de/repalmitoylation [Fernandez-Hernando, 2006; Mitchell, 2006; Linder, 2007].

An alternative possibility concerning protein lipidation is that DHA may be directly acylated to proteins in place of palmitate, thereby altering the membrane binding properties and subcellular localization of normally palmitoylated proteins. Although palmitate is the predominant fatty acid linked to cysteine residues of proteins, it appears that some proteins can be S-acylated with other acyl chains with different chain lengths and a varying degree of

unsaturation [Bizzozero, 1986; Casey, 1994; Fujimoto, 1993; Hallak, 1994; Muszbek, 1993, 1999; O'Brien, 1987]. However, the efficiency of protein acylation with PUFAs appears extremely low relative to palmitate [DeMar, 1997; O'Brien, 1987; Stone, 1979], even in the PUFA-enriched environment such as cells incubated with PUFAs [Liang, 2001] and the biological significance and *in vivo* consequences of heterogeneous S-acylation of proteins remain under debate [Stulnig, 2001]. Our findings that the palmitoylation state of Ras is unaffected by DHA in colonocytes indicates that proteins modified with DHA, if any, must constitute only a minor fraction [Collett, 2001; Davidson, 1999], arguing that neither dramatic alterations in protein localization profile nor the differential effects on palmitoylated proteins observed can be accounted for by S-acylation of proteins with DHA [Seo, 2006]. More detailed exploration of the potential alterations in protein palmitoylation by DHA, either inhibition or substitution with DHA, will require further advances in the characterization of protein palmitoyl transferases.

B. Alteration of lipid-lipid and lipid-protein interactions

Given that alterations in protein modification are unlikely to provide sufficient explanations for the selectivity and magnitude of DHA effects, it seems reasonable to conclude that the unique properties of the DHA-enriched cellular lipid environment, i.e., lipid-lipid and lipid-protein interactions, are primarily responsible for the effects of DHA on lipidated protein localization.

We propose that DHA-enriched membranes do not provide an environment conducive to forward transport of newly synthesized lipidated proteins. Since the vesicular transport-independent targeting of lipidated proteins and the bulk flow of secretory membrane traffic are unaffected by DHA, it is most likely that lipidated cytoplasmic cargos are discriminated against in DHA-enriched secretory apparatus during sorting into transport vesicles (Figure 4). The unique membrane properties of DHA may involve subtle changes in the profile of heterogeneous transport vesicle/carrier populations in terms of lipid composition, size, curvature, and budding efficiency. Reduced plasma membrane delivery of lipidated proteins but not transmembrane proteins may result from the facilitated formation of certain types of transport vesicles in which packaging of membrane-embedded cargo is unaffected but lipidated peripheral proteins are not efficiently anchored on the cytoplasmic surface. Our finding that Ras plasma membrane targeting was inhibited only when DHA was sufficiently enriched in membranes to substantially increase membrane unsaturation [Seo, 2006] supports our hypothesis that alterations in membrane physical properties are involved. However, further studies are required to establish 1) differential membrane binding affinities of lipid-anchored proteins as a function of membrane fatty acyl composition, 2) heterogeneous lateral lipid-protein organization in DHA-enriched ER and Golgi/trans Golgi network (TGN) membranes allowing for the formation of discrete microdomains, and 3) DHA-induced alterations in the transport vesicle/carrier population in relation to lipid composition and lipidated protein partitioning.

The unique properties of DHA-enriched membranes have been well studied using both model and biological membranes in view of the broad health benefits of dietary fish oil and DHA [Stillwell, 2000, 2003]. The presence of six *cis*-double bonds confers DHA exceptional conformational flexibility of the acyl chain to bend, tilt, and back-fold within membrane bilayers [Feller, 2002; Gawrisch, 2003]. Consequently, membranes containing bulky, disordered DHA have looser lipid packing relative to saturated membranes [Eldho, 2003; Holte, 1995; Huster, 1997, 1998; Mitchell, 1998a,b]. The less cohesive acyl chain packing in DHA-enriched membranes will likely weaken van der Waals chain-chain interactions between lipid anchors of proteins and membrane phospholipids, thereby lowering binding affinity of lipidated proteins relative to the more tightly packed saturated membranes. In addition, the

presence of DHA renders membranes easily deformable elastically [Koenig, 1997; Smaby, 1997], prompting speculation that the local concentration of DHA may influence vesicle budding and fusion efficiency. In fact, the fusogenic effect of DHA has been documented [Ehringer, 1990]. Collectively, the formation of DHA-enriched transport vesicles that are relatively depleted in lipidated peripheral proteins but bud and fuse more efficiently than saturated vesicles may underlie the reduced plasma membrane delivery of lipidated proteins.

C. Examples of DHA-enriched membranes and altered protein trafficking

As discussed below, several observations support the primary hypothesis that a DHA-enriched cellular lipid environment attenuates anterograde (forward) transport of lipidated proteins.

Polarized DHA distribution in cell membranes has been well documented in certain cell types naturally enriched in DHA, such as retinal rod photoreceptor cells, brain nerve cells, and sperm [Breckenridge, 1973; Connor, 1998; Cotman, 1969; Fleischer, 1965; Poulos, 1973; Stone, 1979]. DHA is particularly concentrated (up to 60 mol%) in the specialized and functionally most active regions such as rod outer segments and synaptic vesicles in the brain, and sperm tails. Studies of photoreceptor membrane biogenesis provide evidence suggesting that there exist different local concentrations of DHA in TGN membranes as well as distinct protein partitioning therein, allowing for active sorting into discrete post-Golgi vesicles *en route* to specific destinations. Rodriguez de Turco and co-workers demonstrated that newly synthesized docosahexaenoyl phospholipids are sequestered and co-transported by rhodopsin-bearing post-Golgi vesicles upon transport from the ER of inner segments, where their biosynthesis takes place, to rod outer segments [Rodriguez de Turco, 1997]. Most notably, the segregation and association of newly synthesized docosahexaenoyl phospholipids and rhodopsin appeared to occur on the TGN membranes prior to their exit and subsequent vectorial co-transport on post-Golgi vesicles to rod outer segments.

In adrenocortical cells, in which docosapentaenoic acid (22:5, n-3) and DHA only account for 6.9 and 0.1 mol% of total cellular lipids, respectively, distinct types of coated vesicles, presumably originating from the Golgi, have been isolated. These coated vesicles differed in their size, density, and notably 22:5 and DHA profile, indicating the presence of heterogeneous intracellular vesicles with different DHA distribution [de Paillerets, 1987].

Analysis of lipid composition and membrane molecular order of isolated small exfoliated vesicles (50–200 nm in diameter) from T27A leukemia cells revealed that DHA enrichment significantly mitigated or reversed the characteristic differences between the parent plasma membrane and shed microdomain vesicles [Williams, 1998, 1999]. Given that exfoliated vesicles normally arise from non-random regions of the cell surface [Armstrong, 1988; Beaudoin, 1991; Black, 1980; Taylor, 1988; van Blitterswijk, 1979], these data clearly indicate that cellular lipid environment can influence the lipid composition and structure of vesicles formed therein.

Interesting effects of DHA have been observed on the localization and activation of regulators of vesicular traffic, ARF and PLD [Diaz, 2002]. ARF, a Ras-related small GTPase, regulates coated vesicle biogenesis by controlling assembly of coat proteins and by activating its effector, PLD, which also participates in regulation of membrane traffic and actin remodeling via phosphatidic acid and indirectly via phosphatidylinositol 4,5-bisphosphate [Brown, 1993; Cockcroft, 2001; Roth, 1999]. In human peripheral blood mononuclear cells, DHA enrichment of cell membranes dose-dependently increased ARF membrane translocation and PLD activation only in the disordered nonraft membrane fractions. In view of the role of ARF and PLD in vesicle formation, these findings of increased ARF translocation and PLD activation only in specific regions of DHA-enriched membranes indicate that vesicle budding efficiency depends on the local lipid composition in relation to DHA content.

Unsaturated fatty acids are known to promote fusion of natural membranes as well as liposomes [Ahkong, 1973; Lavoie, 1991; Meers, 1988] and this has been related to the curvature stress provided to membranes by inverted phase-forming lipids [Ellens, 1989]. In particular, this fusogenic effect was clearly correlated with the number of double bonds in acyl chains and was much more pronounced with DHA than α -linolenic acid (18:3, n-3) [Ehringer, 1990; Talbot, 1997]. These findings imply that local DHA concentrations in the donor and/or acceptor membranes may affect fusion rates of transport vesicles.

In conclusion, the health benefits of DHA are diverse and a plethora of nutritional studies continue to demonstrate important benefits from the consumption of omega-3 enriched oils. Recently, the U.S. Food and Drug Administration (FDA) has approved the use of a health claim on labels for foods containing DHA. As part of an ongoing commitment to provide consumers with innovative-healthy products, food companies are now scrambling to incorporate omega-3 fatty acids into a range of novel commercial foods in order to provide for the wider public consumption of DHA. It is both appropriate and timely, therefore, to precisely determine how DHA modulates cell signaling networks and reduces the risk of developing colon cancer and intestinal inflammatory disorders.

Acknowledgments

Supported in part by NIH grants CA59034, CA129444, DK71707, and P30ES09106.

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Figure 1. Induction of apoptosis in fatty acid and butyrate-treated colonocytes

Cultures contained 5 mM butyrate and 50 μ M DHA, LA or no fatty acid treatment. Apoptosis was measured by DNA fragmentation ELISA. Data represent mean absorbance at 405 nm $\pm$ SE divided by the total number of adherent cells per dish, $n=4-6$ separate wells from 1 of 4 representative experiments. Values not sharing the same letters are significantly different ($P < 0.05$). Adapted from Ng *et al* (2005).

Figure 2. Proposed molecular model of DHA and butyrate-induced apoptosis

We have previously demonstrated that butyrate induces colonocyte apoptosis via a non-mitochondrial, Fas-mediated, *extrinsic* pathway which is antagonized by activated Ras [Fan, 1999]. DHA and butyrate work coordinately in the colon to initiate a distinct *intrinsic proapoptotic cycle* involving the activation of store-operated channels (SOC), leading to rapid entry of Ca^{2+} through the plasma membrane and mitochondrial Ca^{2+} loading. This directly or indirectly increases mitochondrial phospholipid hydroperoxides (PLOOH) and triggers the opening of the permeability transition pore (PTP) and release of pro-apoptotic molecules like cytochrome C. These effects culminate in the induction of apoptosis.

Regulatory role of membrane lipid composition?

Figure 3. Modulation of protein-membrane localization by dietary DHA

Potential effects of dietary PUFA on post translational lipidation (fatty acylation and/or prenylation) and membrane composition. These effects may culminate in a shift in the localization of intracellular proteins.

Figure 4.
DHA-induced modulation of vesicular protein transport

Table 1**Effect of dietary lipid source on apoptotic index in the top third (upper tertile) of the colonic crypt**

Rats fed diets containing either n-3, n-6 or n-9 fatty acid enriched oils and injected with a colon specific carcinogen, azoxymethane or saline were terminated 10 wk later. Apoptosis was assessed by TUNEL assay on 10 rats per group. Aberrant crypt foci (ACF) were scored following methylene blue staining and high multiplicity ACF (>3 aberrant crypts per foci) were scored. n=5 rats per diet group with 20 crypts per animal scored. Means with different letters are significantly different ($p<0.05$). *Adapted from Davidson et al (2004).*

	Dietary Lipid Source		
	n-3	n-6	n-9
Apoptotic Index (%)	3.08 ± 0.52^b	1.28 ± 0.40^a	1.51 ± 0.31^a
# of High Multiplicity ACF	45.42 ± 5.9^a	65.69 ± 7.48^b	67.32 ± 7.13^b

Table 2

Fish oil doubles the apoptotic index (percentage of cells undergoing apoptosis) compared to corn oil in the top 1/3 of the colonic crypt following carcinogen exposure

The induction of apoptosis is associated with a decrease in bcl-2 in the top 1/3 of the crypt. Mean $\pm$ SE, n=15 rats/diet.

Adapted from Hong et al (2003).

Dietary Lipid Source		
	n-3	n-6
Apoptotic Index (%)	1.22 $\pm$ 0.44 ^b	0.61 $\pm$ 0.23 ^a
Bcl-2 (staining intensity)	26.01 $\pm$ 1.62 ^a	30.34 $\pm$ 1.53 ^b