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Comparative effects of DHA and EPA on cell function

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ABSTRACT

Fish oil supplementation has been reported to be generally beneficial in autoimmune, inflammatory and cardiovascular disorders. Most researchers have attributed these beneficial effects to the high content of ω-3 fatty acids in fish oil (FO). The effects of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are not differentiated in most studies. In fact, up to 1990, purified DHA was not available for human use and there was no study regarding its effects on human immune response. In this review, the differences in the effects of these two fatty acids on cell function are discussed. Studies have shown that EPA and DHA have also different effects on leukocyte functions such as phagocytosis, chemotactic response and cytokine production. DHA and EPA modulate differently expression of genes in lymphocytes. Activation of intracellular signaling pathways involved with lymphocyte proliferation is also differently affected by these two fatty acids. In relation to insulin producing cell line RINm5F, DHA and EPA are cytotoxic at different concentrations and the proteins involved with cell death are differently modulated by these two fatty acids. Substantial improvement in the therapeutic usage of ω-3 fatty acid-rich FO will be possible with the discovery of the different mechanisms of actions of DHA and EPA.

Contents

1. Introduction

For the past 25 years our group has investigated the effects of fatty acids (FAs) on several physiological systems including the regulation of leukocyte function and insulin secretion. We have found that FAs of the same class present quite often different effects (Verlengia et al., 2004a,b). This observation includes the ω-3 polyunsaturated fatty acids (PUFA), EPA and DHA. This review summarizes the effects of EPA and DHA on several cell types such as leukocytes, endothelial cells, and pancreatic beta cells. The biophysical changes in plasma membrane induced by EPA and DHA are also presented.

Abbreviations: DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; ENaC, epithelial sodium channel; EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; FA, fatty acid; FFA, free fatty acids; FO, fish oil; GSK-3, glycogen synthase kinase; ICAM, intercellular adhesion molecule; IKK, IκB kinase; IL, interleukin; JAK, janus kinase; LDL, low density lipoprotein; MAPK, mitogen activated protein kinase; NF-κB, nuclear factor kappa B; NO, nitric oxide; PKA, protein kinase A; PKC, protein kinase C; PUFA, polyunsaturated fatty acids; STAT, signal transducer and activator of transcription); TAG, triacylglycerol; TNF, tumor necrosis factor; TLR, toll like receptor; VCAM, vascular cell adhesion molecule.

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Dietary FO has been shown to modulate a number of cell functions in animals and humans. FO supplementation presents beneficial effects on cardiovascular diseases (Calder, 2004), autoimmune and inflammatory disorders such as psoriasis (Mayser et al., 2002), rheumatoid arthritis (Goldberg & Katz, 2007; Galarraga et al., 2008) and diabetes (Woodman et al., 2003). Epidemiological studies have associated a low incidence of cancer with dietary FO (Simopoulos, 2002). These beneficial effects of FO have been attributed to their high content of ω-3 PUFA.

FOs from different sources contains variable mixtures of EPA and DHA. Most fish oils commercially available present a proportion of 2:1 for EPA and DHA. However, whether the effects of FO supplementation on immune response, for example, are due to EPA, DHA or both remain to be examined. Until 1990, purified DHA was not available and there was no study regarding its effects in humans. Most studies were performed with FOs containing a higher proportion of EPA than DHA. Despite of the high cost of DHA purification studies with DHA are required, since this FA constitutes the major ω-3 PUFA in tissues and moreover the body tends to conserve it over EPA (Kelley et al., 1998). In this review, the in vitro and in vivo effects of EPA and DHA were compared.

2. Structures, sources and intakes of docosapentaenoic acid and eicosapentaenoic acid

The spatial conformation of DHA is different from that of EPA as a result of its carbon backbone length and degree of unsaturation. EPA is a long-chain PUFA that has 20 carbon atoms and 5 double bonds (20:5). DHA has a longer chain, 22 carbon atoms and 6 double bonds (22:6).

The ω-3 PUFA are derived from α-linolenic acid (18:3) that is the precursor of EPA and DHA (Burdge & Calder, 2005). α-Linolenic acid cannot be synthesized by the human body and as it must be obtained entirely from the diet it is termed an essential FA. Increasing α-linolenic acid intake for a period of weeks to months results in an increase in the proportion of EPA in plasma lipids, erythrocytes, leukocytes, platelets and in breast milk but there is no increment of DHA (Burdge & Calder, 2005). In fact, the proportion of DHA may even decline in some pools at high α-linolenic acid intakes. Stable isotope tracer studies indicate that conversion of α-linolenic acid to EPA occurs, but is limited in male humans with further transformation to DHA being very low (Emken et al., 1994; Burdge & Wootton, 2002). The fractional conversion of α-linolenic acid to the longer chain ω-3 PUFA is greater in women possibly due to a regulatory effect of estrogen. This capacity to up-regulate α-linolenic acid conversion in women may be important for meeting the demands of the fetus and neonate for DHA.

3. Fatty acids and membrane physical properties

In cell membranes, FAs can be found either as constituent of membrane phospholipids (i.e., esterified FAs) or as free molecules (i.e., free FA — FFA). In both forms, FAs, mainly PUFA, have a remarkable contribution to the physical properties of biological membranes, including membrane organization, ion permeability, elasticity and microdomain formation. Several studies have shown the ability of ω-3 PUFA in modulating the activity of membrane-associated proteins such as ion channels and ion transporters (Bruno et al., 2007).

3.1. Orientation of fatty acid acyl chains and membrane organization

Even with the lack of direct comparison in the literature about the differences between DHA and EPA on membrane organization, it is not expected that these FAs have the same behavior or effects on membrane structure. It is well-established that the organization level of biological membranes is dependent on acyl chain length and unsaturation degree. As compared with saturated or monounsaturated FAs, PUFA are considered highly disordered (Stillwell & Wassall, 2003). Measurements with a wide variety of biophysical techniques corroborate this idea (Niebylski & Salem, 1994; Holte et al., 1995). For example, Mitchell and Litman (1998), using time-resolved fluorescence and 1,6-diphenyl-1,3,5-hexatriene fluorescence decay lifetime in large unilammelar vesicles showed a decrease in membrane order, i.e. organization, with increasing unsaturation from one to six carbon–carbon double bounds, in one or both phospholipid acyl chains.

Eldho et al. (2003), using NMR and X-ray diffraction, concluded that the differences in the physical properties of membranes due to the loss of one acyl chain double bond between DHA and docosapentaenoic acid (DPA) are mainly caused by differences in conformational freedom and rate of structural transitions. The highest membrane disordering effects observed for DHA were attributed to its higher flexibility. Despite the fact that double bonds are rigid structures, the conformational flexibility arises from lower potential energy barriers for rotation of carbon–carbon single bonds.

The incorporation of free PUFA into phospholipid membranes seems to modulate the membrane physical properties in a way that is similar to that of esterified fatty acids. Onuki et al. (2006), using fluorescence measurements in unilamellar vesicles, showed that free PUFA are able to change the packing properties of phospholipid membranes by reducing the van der Walls interactions between phospholipid acyl chains. The FA potency in causing membrane structure disorganization was: stearic acid < oleic acid < EPA ≤ DHA.

3.2. Effects of docosapentaenoic acid and eicosapentaenoic acid on membrane ion permeability

A crucial function of biological membranes and their constituents is generating and maintaining ion concentration gradients between cytoplasm and extracellular medium. Processes such as ATP production require proton diffusion and gradient generation through mitochondrial membranes. On the other hand, in some metabolic circumstances, the organism needs to increase heat production, requiring proton gradient dissipation through mitochondrial membranes. Changes on ion permeability seem to be directly dependent upon the unsaturation degree of FAs. Ehringer et al. (1990) showed that DHA has a more pronounced effect on membrane ion permeability than linolenic acid, despite similar effects of both FAs on membrane fluidity.

Substantially different effects of EPA and DHA on proton conductance and permeability measurements were observed in planar lipid bilayer preparations (Table 1); for detailed method description, please see Brunaldi et al. (2005). DHA, for example, rendered the lipid bilayers completely (100%) selective to protons, whereas EPA increased proton selectivity to about 60%. The "flip-flop model" proposed by Kamp and Hamilton (1992) for the protein-independent diffusion of FFA across phospholipid membranes may be responsible for coupling the translocation of both H^+ and FFA; however, structural differences between EPA and DHA molecules may be responsible for the difference observed in the effects of these FA on proton selectivity. The higher degree of unsaturation of DHA molecule could result in a more important effect on membrane disorganization, increasing formation of aqueous defects and, as a consequence, increasing proton permeability through proton passive pathways, such as water wires and/or water cluster-contact mechanism (Haines, 2001; Decoursey, 2003). The existence of more double bonds in DHA than EPA could create higher number of bends in the acyl chain and conformations, with consequent shortening the DHA molecule. Depending on the phospholipid acyl chain and the FA, this shortening of effect is known to create an optimal association between FA length and membrane leaflet thickness, enhancing FA flip-flop rate (Wojtczak & Wieckowski, 1999).

3.3. Fatty acids and membrane microdomain formation

A new conceptual framework on the basic structure of biological membranes has emerged in the last decade or so. Instead of a more or less homogeneous mixture of lipids and proteins as proposed in the fluid mosaic model (Singer, 2004), the membrane can be best seen as a mosaic

Table 1
Effect of EPA and DHA on proton selectivity, conductance (G_H^+) and permeability (P_H^+) of diphytanoylphosphatidylcholine black lipid membranes.

Free fatty acids	Selectivity	G_H^+ (nS cm^{-2})	P_H^+ (cm s^{-1})
None (control)	0.18 ± 0.03	1.90 ± 0.30	$4.20\times10^{-6}\pm9.17\times10^{-7}$
EPA (50 μM)	0.58 ± 0.10	33.20 ± 6.90	$7.28\times10^{-5}\pm2.00\times10^{-5}$
DHA (50 μM)	1.00 ± 0.04	480.00 ± 130.30	$1.05\times10^{-3}\pm3.12\times10^{-4}$

All values are shown as average ± standard error of mean of at least 4 experiments.

of heterogeneous domains composed of different structures and physicochemical properties. These domains are in the range from microscopic (nm) to macroscopic (μm) size (Edidin, 2001, 2003). This heterogeneous feature of membranes is the basis of the "raft lipid hypothesis".

An important site for the modulating effects of FAs in biological membranes arises from their ability to remodel specialized membrane microdomains such as lipid rafts and caveolae (Ma et al., 2004). Lipid rafts are liquid-ordered membrane phase structures, 10–200 nm in size, that are characterized by a tight packing of cholesterol and sphingolipids, whereas caveolae constitutes an specialized microdomain enriched with caveolin-1 protein. Both structures are directly influenced by the cholesterol concentration into the membranes. Incorporation of PUFAs into these microdomains decreases the microdomain cholesterol content and the corresponding liquid-ordered membrane phase structures. There is an incompatibility between the highly flexible structure of PUFA and the rigid moiety of cholesterol as has been demonstrated by biophysical studies using membrane models (Pitman et al., 2004; Harroun et al., 2006; Shaikh et al., 2006; Marrink et al., 2008). These findings are in agreement with in vivo studies. For example, Ma et al. (2004) and Fan et al. (2003) showed that dietary EPA and DHA are able to remodel the content of colonic caveolae and mouse T-cell lipid rafts by decreasing microdomain-cholesterol amount.

In opposition to highly ordered lipid raft microdomains, Niu and Litman (2002) showed that the cholesterol partition coefficient measured in unilamellar vesicles is lower when the membrane is composed by phospholipids with DHA-esterified as compared to other phospholipids. This idea is corroborated by X-ray diffraction, NMR (Brzustowicz et al., 2002) and molecular dynamics simulations that showed a reduced solubility of cholesterol in DHA-containing membranes. When DHA-containing phospholipid is incorporated into membranes that contain sphingolipids and cholesterol as lipid raft components, they enhance cholesterol segregation into sphingolipid/cholesterol/phospholipid rafts and move away DHA-rich microdomains that exclude the sterol (Wassall & Stillwell, 2008). Thus, the main feature of their model is the coexistence of highly ordered lipid domains and highly disordered DHA-rich domains. These authors also hypothesized that some of the health benefits associated with DHA and other PUFA could be directly associated to the coexistence of these membrane domains, acting on the movement of signaling proteins in and/or out of rafts due to changes in membrane organization after DHA incorporation that comes from the diet.

3.4. Modulation of ion channels by eicosapentaenoic acid and docosapentaenoic acid

The effects of ω-3 PUFA on ion channel function have been increasingly investigated and recognized. Nonetheless, these studies usually do not attempt at comparing the effects of different ω-3 PUFAs, but rather to choose either EPA or DHA as the prototypical ω-3 PUFA to be compared with other FA classes. Most studies have been performed with a mixture of these two FA, assuming that both have similar effects. However, differences in potency and even in actions of DHA and EPA have been reported.

In CA1 neurons isolated from the rat hippocampus, both EPA and DHA had an inhibitory effect on sodium and calcium currents by inducing a shift in the inactivation response observed in more negative potential membranes (Vreugdenhil et al., 1996). However, DHA was more potent than EPA to induce this effect (2.1 and 4 μM, respectively). EPA and DHA similarly stimulated desensitization of P2X inward currents in rat nodose ganglion neurons (Eto et al., 2006). DHA blocked neuronal voltage-gated potassium channels transfected into fibroblasts (Poling & LeSage, 1995) and voltage-gated potassium currents in rat olfactory receptor neurons (Seebungkert & Lynch, 2002), while it had a facilitatory effect on NMDA-glutamate receptors in rat pyramidal neurons (Nishikawa et al., 1994), suggesting that DHA may also have stimulatory actions in neurons. If this trend would also be observed with EPA treatment remains to be further investigated.

ω-3 PUFA intake has been associated with protective effect against fatal cardiac arrhythmias (Leaf et al., 1996). Xiao et al. investigated the molecular basis of this action and have identified several sarcolemmal ion channels as targets of ω-3 PUFA in rat ventricular myocytes. Although their studies have mainly focused on EPA, they have shown that ω-3 PUFA in physiological concentrations (<10 μM) inhibit voltage-dependent sodium channels (Xiao et al., 1995) and L-type calcium channels (Hallaq et al., 1992; Xiao et al., 1997). Also, ω-3 PUFA shift the inactivation curve of cardiac sodium channels towards more negative membrane potentials (Xiao et al., 1995). This effect seems to involve a direct interaction of the FAs with the channel as both EPA and DHA dislodge, from the ventricular myocyte membrane, a radioactive derivative of batrachotoxin — that binds sodium channels — with the same apparent affinity (Kang & Leaf, 1996). EPA binds to the cardiac sodium channel in its inactive state, prolonging its recovery to the closed excitable state (Xiao et al., 1998). This is probably one important mechanism underlying the negative chronotropic effect of ω-3 PUFA and thus of their protective action against arrhythmias. In this sense, ω-3 PUFA antiarrhythmic action seems to be restricted to contractile fibres, as DHA has only a very subtle effect on HCN4 pacemaker currents (Xiao et al., 2005), thus preventing the occurrence of re-entrant arrhythmias.

Studies with mutants for the α-subunit of the channel have shown that the Asn406 residue is required for the inhibitory effect of EPA (Xiao et al., 2001). When only the α-subunit is expressed in HEK293t cells, EPA has a much more prominent inhibitory effect than DHA at 5 μM concentration (82 vs. 52%, respectively) (Xiao et al., 1998). However, when the β-subunit is co-expressed, this difference in inhibitory potency is greatly reduced (58% for EPA and 53% for DHA) and the residual inhibitory effect of saturated and monounsaturated FAs is abolished (Xiao et al., 2000). Interestingly, in primary neonatal rat ventricular myocytes, EPA and DHA (5 μM) have similar inhibitory effects (by 51%) (Xiao et al., 1995).

ω-3 PUFAs can affect intracellular calcium homeostasis in cardiac myocytes by exerting effects on both extracellular calcium influx and on calcium-induced calcium release from the sarcoplasmic reticulum. EPA and DHA inhibit extracellular calcium influx through L-type calcium channels (Xiao et al., 1997). Initially it was thought that this effect was exclusively due to direct binding of the ω-3 PUFA to the channel, as both EPA and DHA noncompetitively inhibited the specific binding of [H^3]-nitrendipine to L-type channels (Hallaq et al., 1992), but recent findings have shown that PKA activation by EPA contribute to its inhibitory effect on L-type calcium currents (Szentandrassy et al., 2007). Along this line, it is interesting to note that EPA stimulates epithelial sodium channels (ENaC) in a cAMP-dependent fashion, to which effect the interaction of ENaC with a membrane-bound PKA binding protein is required (Mies et al., 2007).

EPA and DHA seem to also affect sarcoplasmic reticulum function in ventricular myocytes. Both EPA and DHA decrease calcium release from intracellular calcium stores, reducing the amplitude and frequency of spontaneous calcium sparks and of contractile events, and in contrast increase the amount of calcium stored in the sarcoplasmic reticulum (Negretti et al., 2000). This effect is due to direct inhibition of ryanodine receptors, which are the calcium-induced calcium release channels found in the sarcoplasmic reticulum membrane of cardiac myocytes (Honen et al., 2003) Contrary to what is observed with the sarcolemmal L-type

calcium channels, ryanodine receptors inhibition does not seem to involve PKA activation (Swan et al., 2003). EPA shortens the duration of spontaneous calcium sparks, an effect that is not observed with DHA (Honen et al., 2003). In addition, EPA increases the speed of relaxation of ventricular myocytes (Szentandrassy et al., 2007). This is linked to an effect of ω-3 PUFAs on voltage-dependent potassium currents that are responsible for the repolarization of the cardiac action potential. In fact, many types of potassium current are involved in the repolarization of the cardiac myocyte, several of which are modulated by ω-3 PUFAs. DHA inhibits transient outward (I_{to}) potassium currents but stimulates the steady-state outward current, both effects being enhanced by DHA peroxidation (Jude et al., 2003). DHA has been shown to increase the slowly activating delayed rectifier potassium current (I_{Ks}), upon which EPA had no inhibitory effect, although it decreased the velocity of activation (Doolan et al., 2002). In addition, DHA and arachidonic acid inhibit human *ether-a-go-go*-related gene (HERG) channels (Guizy et al., 2005).

These findings, taken together, point to an effect of ω-3 PUFAs to both shorten cardiac action potential duration — through inhibition of sodium and calcium channels and stimulation of I_{Ks} currents — and to lengthen action potentials — via inhibition of I_{to} and HERG channels. Actually, DHA slightly prolongs the cardiac action potential (Macleod et al., 1998). In any case, the inhibition by ω-3 PUFAs of several conductance that participate in the depolarizing and repolarizing steps of the action potential implies an increase in the refractory period of cardiac cells, which would obviously preclude the appearance of spontaneous asynchronous electrical activities in the cardiac muscle.

4. Endothelial cell function

The dysfunction of the vascular endothelium triggers the initial development of atherogenesis. This "activation" changes the hemodynamic equilibrium and causes oxidative and inflammatory reactions, which result in leukocyte adhesion to vascular endothelium (Ross, 1993). This is the first stage in the atherosclerotic process, being followed by migration of mononuclear cells into the subendothelial space and the transformation of macrophages into foam cells through the uptake of oxidized low-density lipoproteins (LDL-ox) and, thereafter, through cholesterol enrichment. Leukocyte adhesion to the endothelium is rendered possible by the appearance of adhesion molecules at the surface of neutrophils, monocytes, and endothelial cells (Homem de Bittencourt & Curi, 2001). The phenomenon is reversible at this stage, as the inhibition of adhesion molecule expression prevents in endothelial dysfunction and halts the development of atherosclerotic lesions.

In cultured human endothelial cells activated by pro-inflammatory cytokines, De Caterina et al. (1994, 2000) showed that preincubation for 24 h with DHA (10 μmol/L) caused a reduction in expression of vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), and E-selectin. According to these authors, DHA interference probably occurred at pretranslational level. This FA probably inhibited NF-κB activation and hence expression of adhesion molecules and monocyte adhesion to the vessel wall. In cultures of human endothelial cells preincubated for 24 h with the FAs (Weber et al., 1995), DHA (20 μmol/L) but not EPA inhibited VCAM-1 expression through a reduction in the mRNA levels of this adhesion molecule.

Nitric oxide (NO) plays a pivotal role in the pathophysiology of atherosclerosis and a reduction in NO production may represent a very early step in atherogenesis (Roch-Arveiller & Couderc, 2000). Omura et al. (2001) demonstrated that EPA (30 and 60 μM) increased NO production by triggering eNOS activation through Ca^2/calmodulin system in bovine endothelial cells in situ. EPA stimulated the translocation of eNOS to cytosol and its dissociation from caveolin, preventing the enzyme activity inhibition. Caveolin blocks eNOS activity by its association with the enzyme in caveolae (Michel et al., 1997). Caveolin is also a FA-binding protein and has an important role in FA transport between membrane compartments (Okamoto et al., 1998). EPA can bind to caveolin, which may in turn assist caveolin to associate with eNOS, and thereby activate eNOS. However, it is still unclear why EPA may activate eNOS while its closely-related FAs (DPA and DHA) do not. The incorporation of EPA and/or DHA in membrane phospholipids causes an increase in membrane fluidity that raises NO synthesis and secretion. Hashimoto et al. (1999) observed that in rat endothelial cells cultured for 72 h with EPA and DHA (both at 5 mM) there was a significant reduction in membrane cholesterol levels, and that the effect of DHA was more pronounced. This may be due to differences in the conformational structure of these fatty acids as described before.

Vascular smooth muscle cells (VSMC) have the potential to proliferate and to accumulate lipids. Cyclins and cyclin-dependent kinases control eukaryotic cell cycle progression and thus the proliferation of these cells. VSMC cultured with EPA-triacylglycerol and DHA-triacylglycerol for 13 h showed a reduction in DNA synthesis through inhibition of G1 cyclins and cyclin-dependent kinases and stopped the progression from G1 to S phases (Terano et al., 1997). This explains part of the anti-atherosclerotic effect of FOs.

4.1. Endothelium mitogen activated protein kinase (MAPK) pathway

MAPKs play an important role in TNF-α induced pro-inflammatory effects. Xue et al. (2006) investigated the effects of EPA and DHA on MAPKs in the endothelium. They evaluated the intracellular signaling mechanisms in the inhibition of endothelial activation by ω-3 PUFA. Kinase activities were measured in TNF-α-activated endothelial cells from human umbilical vein (HUVEC). EPA or DHA alone significantly reduced the TNF-α-induced activation of p38 and JNK kinases at 20 μM concentration, but EPA was a more potent inhibitor than DHA. In contrast, both EPA and DHA significantly counteracted the TNF-α-mediated deactivation of extracellular signal-regulated kinase (ERK) 1/2. Both EPA and DHA significantly attenuated the TNF-α-induced expression of p38 and ERK1/2 mRNA. DHA, but not EPA, also reduced the TNF-α-induced JNK mRNA expression.

5. Comparative effects of eicosapentaenoic acid and docosapentaenoic acid on leukocyte function

5.1. In vivo studies

Some studies have shown that dietary FO does compromise host resistance to infections by certain pathogens such as influenza virus (Byleveld et al., 1999), *Listeria monocytogenes* (Fritsche et al., 1997) and *Salmonela typhimurium* (Chang et al., 1992). On the other hand, Anderson and Fritsche (2002) concluded that there are few human clinical trials involving ω-3 FAs and human infectious disease.

There are only few studies that investigated whether EPA or DHA or both are responsible for the reduction of host resistance to infections. A study of Oarada et al. (2003) compared the effect of DHA and EPA-rich FOs on host resistance to *Paracoccidioides brasiliensis* infection. In this study, mice fed with palm oil supplemented with DHA showed reduced antifungal activity in the spleen and liver, as compared with mice fed with palm oil or soybean oil without supplementation with DHA. Mice fed with DHA-supplemented soybean oil also showed reduced antifungal activity in the liver, but the intensity of reduction was less pronounced. This reduction in antifungal activity was not observed with EPA-supplemented palm or EPA-supplemented soybean oil. These results are indicative that oral intakes of large amounts of DHA in combination with saturated or monounsaturated fatty acids reduced host resistance to this fungus. On the other hand, EPA did not cause significant effect in the combinations with other FAs mentioned above. The authors concluded that excessive dietary DHA supplementation has potentially adverse effects on humans with a traditionally high consumption of saturated and monounsaturated fatty acid-rich dietary oils.

Kew et al. (2004) reported the effects of supplementation with EPA-rich and DHA-rich oils on several functions of neutrophils, monocytes, and lymphocytes in healthy humans. The FA composition of plasma and neutrophil phospholipids was dramatically altered by supplementation

with EPA (4.7 g per day) or DHA (4.9 g per day) for 4 weeks. The DHA-rich oil contained some docosapentaenoic acid (DPA) but the plasma phospholipids and neutrophil lipids in the DHA group were not enriched in DPA. The authors suggest that DPA was retroconverted to EPA because there was an increase in EPA contents in the group supplemented with DHA-rich oil. However the dominant influence on fatty acid composition in this study was that of the major fatty acid in the supplement.

Kew et al. (2004) observed that the effects of EPA on leukocyte function differed notably from those of DHA. DHA supplementation (4 g per day) decreased T lymphocyte activation, as assessed by expression of CD69, whereas EPA supplementation had no significant effect. Neither the EPA-rich oil nor the DHA-rich oil had any significant effect on monocyte or neutrophil phagocytosis, cytokine production, and adhesion molecule expression by peripheral blood mononuclear cells. Therefore, the authors concluded that supplementation with DHA, but not with EPA, suppressed T lymphocyte activation. In a study from our group (Gorjao et al., 2006), ten healthy male volunteers were given 3 g per day of a DHA-rich fish oil (1.62 g DHA per day) for 8 weeks. FO supplementation altered the FA composition of lymphocytes, resulting in an increase of the ω-3/ω-6 ratio from 0.18 to 0.62. Supplementation with DHA-rich FO promoted an increase of 40% in ConA-dependent lymphocyte proliferative capacity determined by [2-^{14}C] thymidine incorporation. This enhanced response was abolished after a 2-month wash-out period. These conflicting observations may be associated to the higher dose of DHA used in the study by Kew et al. (4.91 g per day) as compared with our study(1.62 g per day) and the age of the volunteers: 25–45 years (our study) and 23–65 (study by Kew). Other explanation to this discrepancy is that Kew et al. evaluated the expression of CD69 (a cell surface marker, which expression is rapidly upregulated in response to stimulation) being associated with lymphocyte activation. On the other hand, we assessed cell division that is not necessarily correlated with CD69 expression.

DHA-rich FO also caused an increase in neutrophil and monocyte phagocytosis (Gorjao et al., 2006), whereas others demonstrated that EPA-rich fish oil did not alter this cell function (Miles et al., 2004; Kew et al., 2004). The different composition of fish oils used in these studies and the doses supplemented may explain these observations.

5.2. In vitro studies

5.2.1. Cytokine production

Modulation of leukocyte cytokine production by FAs is associated with alterations of several functions of these cells such as lymphocyte proliferation, neutrophil chemotaxis and macrophage recruitment.ω-3 PUFA are well-recognized to decrease the production of pro-inflammatory cytokines being modulated by EPA and DHA at different intensities. Weldon et al. (2007) investigated the differential effects of pure EPA and DHA on cytokine expression and nuclear factor kappa B (NF-κB) activation in human THP-1 monocyte-derived macrophages. NF-κB plays a key role in regulating cytokine gene transcription. This transcription factor is located in the cytoplasm being associated with the inhibitory protein IκB. Extracellular signals can activate IκB kinase (IKK). This enzyme phosphorylates the IκBα protein, which results in dissociation of IκBα from NF-κB, and eventual degradation of IκBα by the proteosome. The most abundant form of NF-κB is the p65/p50 heterodimer, in which the p65 subunit contains the transcriptional activation domain. In resting cells, NF-κB is sequestered in the cytoplasm in an inactive state bound to IκB (Ghosh et al., 1998). Activation by LPS requires sequential phosphorylation of IκB, ubiquitination and degradation by proteosome, followed by translocation of NF-κB to nucleus. The activated NF-κB is then translocated to the nucleus where it binds to specific sequences of DNA called NF-κB response elements activating cytokine transcription. Pre-treatment with 100 μM EPA and DHA decreased LPS-stimulated THP-1 macrophage production of TNF-α, IL-1β and IL-6, compared to control cells. Both FAs also reduced TNF-α, IL-1β and IL-6 mRNA expression. However, in all cases, the effect of DHA was significantly more potent than that of EPA. Furthermore, a low dose (25 μM) of DHA had a greater inhibitory effect than that of EPA on IL-1β and IL-6 production by macrophages following LPS stimulation. LPS-induced NF-κB/DNA binding was down-regulated by EPA and DHA in THP-1 macrophages. On the other hand, only DHA significantly decreased macrophage nuclear p65 expression and increased cytoplasmic IκB expression. Therefore, the findings reported above suggest that DHA may be more effective than EPA in alleviating LPS-induced pro-inflammatory cytokine production by macrophages, an effect possibly mediated by NF-κB.

EPA competes with arachidonic acid and acts as a substrate for cyclooxigenase and lipoxygenase enzymes being converted to eicosanoids, while DHA does not. Lokesh et al. (1988) reported that DHA-enriched murine peritoneal macrophages produced less pro-inflammatory leukotriene B_4 than EPA-enriched cells, but cytokine production was not measured. This study suggests that the effects of DHA on pro-inflammatory cytokine production might occur downstream of altered eicosanoid synthesis.

In contrast with the results obtained in macrophages, Khalfoun et al. (1997) reported a more potent reduction in IL-6 production by lymphocytes treated with EPA when compared with DHA. We reported that EPA and DHA promote an inhibitory effect on IL-2, IL-10 and IFN-γ production by Jurkat cells, a leukemic lineage of human T lymphocyte (Verlengia et al., 2004b). In a lineage of human B lymphocytes (Raji cells), EPA and DHA decreased IL-10, TNF-α and IFN-γ production (Verlengia et al., 2004a). In these studies, the suppressive effect of EPA on cytokine production was always more pronounced as compared to DHA.

5.2.2. Intracellular signaling

As described above, DHA has a more potent effect on NF-κB activation in THP-1 cells as compared to EPA. Komatsu et al. (2003) observed that DHA was a more potent inhibitor of NF-κB-mediated nitric oxide production in RAW264 macrophages, compared to EPA, and this effect was associated with reduction of oxidative stress and increased levels of the antioxidant glutathione (GSH).

IL-2 plays an essential role in lymphocyte proliferation (Wang et al., 2004). We have recently shown that DHA and EPA decrease the stimulatory effect of IL-2 on human lymphocyte proliferation, increasing the percentage of cells in G1 phase and decreasing the proportion of cells in S and G2/M phases (Gorjao et al., 2007). This effect is associated to inhibition of phosphorylation of some proteins activated by this cytokine after a short period of treatment (1 h). The IL-2-induced heterodimerization of β and γ chains of the IL-2 receptor results in intermolecular transphosphorylation of their corresponding receptor associated Janus kinase 1 (JAK1) and JAK3 (Russell et al., 1994; Kirken et al., 1995). Transcription factor members of the STAT (signal transducer and activator of transcription) family are activated by JAK. Tyrosine phosphorylation of STAT5 allows src homology 2 domain-mediated homodimerization or heterodimerization, with a resultant induction of nuclear migration and sequence-specific DNA binding by the STATs (Darnell et al., 1994). Both DHA and EPA decreased JAK1, JAK3, STAT5, ERK1/2 and Akt phosphorylation induced by IL-2, but the effects of DHA were more pronounced.

Our group also studied the effects of DHA and EPA on lipid raft distribution in human lymphocytes after 1 h treatment. Lipid rafts in plasma membrane were labeled by using Vybrant Lipid Raft Labeling Kit (Molecular Probes, Eugene, OR) containing red-fluorescent Alexa fluor 594 conjugate of cholera toxin subunit B (CT-B). This CT-B conjugate binds to the pentasaccharide chain of plasma membrane ganglioside G_{M1}, which selectively partitions into lipid rafts (Janes et al., 1999). Cells were visualized by fluorescence on a Nikon E1000 microscope (Nikon Inc., Melville, NY, USA). An alteration of lipid raft content was induced by DHA, but it did not occur in lymphocytes treated with EPA. Lipid rafts play an important role in a variety of

cellular processes including the compartmentalization of cell-signaling events (Brown & London, 1998; Jury & Kabouridis, 2004). Li et al. (2005) showed that IL-2 receptor is located in lipid rafts and that any alteration in these microdomains may alter intracellular pathways activated by this receptor. Therefore, the effect of DHA on IL-2 activated signaling pathways (Gorjao et al., 2007) is associated to a decrease in membrane lipid raft content (data not shown). The mechanism involved in the effect induced by EPA in these signaling pathways is still unclear.

5.2.3. Gene expression

The modulating effects of DHA and EPA on immune function also occur by changes in gene expression. Verlengia et al. (2004b) have shown, using a macroarray technique (in which 83 genes were evaluated using membranes from Clontech Laboratories), that the expression of genes related to signal transduction, cell survival, apoptosis and cytokine production was altered by the treatment of cultured Jurkat cells with 12.5 μmol/L EPA or DHA for 24 h. Of the genes which expression was changed by the FAs, DHA raised the expression of 62%, whereas EPA up-regulated 33% of them. Only 6% of the genes investigated were similarly regulated by both FAs. DHA had a stimulatory effect on expression of genes related to defense and repair. In contrast, EPA augmented the expression of other genes, such as the proto-oncogenes myc, c-*jun*, and p53-associated gene. These results indicate that the molecular mechanisms underlying the modulatory effect of these two ω-3 PUFA on T-lymphocytes are different.

In Raji cells (a human B-lymphocyte cell line) treated as described above, our group showed that the effect of EPA on gene expression was more pronounced (25.9% of genes investigated were altered) when compared with DHA (8.4% of genes investigated were changed). Only 3% of the genes investigated were similarly up-regulated by both FAs. The up-regulation of the cell-cycle genes by EPA may be associated to the pronounced increase in cell proliferation induced by this FA when compared with DHA (Verlengia et al., 2004a).

Rahman et al. (2008) compared the effects of highly purified EPA or DHA on receptor activator of NF-kB (RANKL) in osteoclastogenesis using RAW 264.7 murine macrophage. DHA was found to inhibit osteoclast differentiation, activation and function more potently than EPA at 50 μM. This finding was associated with stronger inhibition of expression, by DHA as compared to EPA, of osteoclast-specific genes such as tartrate resistant acid phosphatase, cathepsin K, calcitonin receptor, matrix metalloproteinase-9, c-Fos and TNF-α. Pre-treatment of RAW264.7 cells with DHA caused a more pronounced decrease in NF-kB and p38 MAPK activation than EPA. These findings suggest that DHA may be much more effective than EPA in alleviating RANKL induced pro-inflammatory cytokine production and intracellular signaling activation, thereby decreasing osteoclast activation and bone resorption.

6. Effects of omega-3 polyunsaturated fatty acids in cell dysfunction induced by diabetes

Most studies have tested the influence of ω-3 FA supplementation on different parameters of the diabetic condition such as endothelial dysfunction (Das, 2000) and insulin resistance (Ramel et al., 2008) and sensitivity (Andersen et al., 2008). On the other hand, there is strong epidemiological evidence for a protective effect of long-chain ω-3 PUFA on type 2 diabetes risk. The prevalence of type 2 diabetes is low in populations such as in Greenland (Martinsen et al., 2006) and Alaskan Eskimos (Schraer et al., 1988). These populations are known to have a very high intake of ω-3 FAs in the diet (De Caterina et al., 2007). However, it is difficult to compare the consumption of isolated EPA or DHA in the habitual diet because both fatty acids are provided by the same alimentary sources.

The first published human study examining the contribution of ω-3 FA intake on type 1 diabetes was a case-control study from Norway. This study showed that children with diabetes were less likely to have been given cod liver oil during infancy than children without diabetes (Stene & Joner, 2003). This was a large case-control study conducted after the conclusion of the same group that vitamin D or ω-3 PUFAs (EPA and DHA) or both in the cod liver oil had a protective effect against Type I diabetes (Stene et al., 2000).

Norris and colleagues suggested that high consumption of total ω-3 FA is associated with low islet autoimmunity in children at increased genetic risk for type 1 diabetes (Norris et al., 2007). Recently, a TrialNet-based clinical trial, called "The Nutritional Intervention for the Prevention of Type 1 Diabetes", has been established to address the hypothesis that dietary supplementation with anti-inflammatory doses of DHA in uterus and in infancy may block early islet inflammatory events involved in the pathogenesis of type 1 diabetes. If this hypothesis is confirmed, dietary supplementation with ω-3 FAs could be recommended to prevent the development of type 1 diabetes. In spite of the importance of this observation, the effects of EPA and DHA for the development of type I diabetes were not compared yet and the results until now described still preset controversies.

6.1. Molecular aspects of fatty acids and diabetes

Cellular and molecular mechanisms that explain the fatty acid effects and the incidence of diabetes are still unclear. FAs act on both beta cell metabolism and function and on insulin-sensitive tissues such as adipose tissue and muscle (Riserus, 2008). PUFAs (e.g., linoleic acid and ω-3 FAs) suppressed lipogenic gene expression and enhanced oxidative metabolism in the liver. Conversely, saturated FAs (e.g. palmitic) presented an opposite effect (Clarke, 2004). Recently, free FAs have been identified as ligands for orphan G-protein coupled receptors (GPCRs) that mediate some effects of dietary FAs on insulin action in periphery tissues and insulin secretion by pancreatic islets. Winzell & Ahrén (2007) have suggested that free FAs play an important role in glucose homeostasis. GPR40 and GPR120 are activated by medium and long-chain FAs, whereas GPR41 and GPR43 are activated by short-chain FAs (Covington et al., 2006). Both groups of FAs are abundant in dairy fat. GPCRs are, therefore, novel targets for the studies on diabetes prevention and/or treatment. Another receptor family that binds to FA is the Toll-Like Receptors (TLR). Kim et al. (2007) suggested that TLRs mediate the association of insulin resistance to FAs and inflammation. Saturated lauric acid (12:0) initiates TLR4 signaling in macrophages. Other saturated FA (14:0, 16:0 and 18:0) also activate TLR4 that in turn triggers inflammatory responses by activating IKK/NF-κB pathway and stimulates macrophage production of cytokines (Lee et al., 2001; Lee et al., 2003; Lee et al., 2004; Shi et al., 2006). The TLR4 signaling pathway has been recognized as a key mediator of the effects induced by palmitic acid including inflammation and impaired endothelial NO signaling and insulin signal transduction (Kim et al., 2007).

6.2. Comparative effects of eicosapentaenoic acid and docosapentaenoic acid on insulin producing beta cell lines — in vitro studies

Aarnes et al. (2002) investigated the changes in viability of insulin-producing INS-1E cells incubated for 6 days with EPA and DHA at 0.07 mM concentration. There was negative effect of DHA whereas EPA did not cause significant alteration.

We have found that EPA presents low toxicity, as compared with other fatty acids even at high concentrations (0.3 and 0.4 mM), to RIN-m5F insulin secreting cells (Azevedo-Martins et al., 2006). EPA and DHA did not cause marked effect on viability of these cells (data not published). However, DHA presented very high toxicity by inducing DNA fragmentation in 35% of the cells already at 0.1 mM reaching around 100% at 0.2 mM (data not shown). EPA presented a much lower cytotoxicity at the same concentrations and time of incubation

(Azevedo-Martins et al., 2006; Simon et al., 2008). Suresh & Das found a protective effect of various FA, including EPA and DHA, on RIN cell viability. Preincubation with EPA or DHA (15 μg/mL) recovered cell viability back to around 95% and 85%, respectively (Suresh & Das, 2001).

The toxicity of FAs has been attributed to the inability of the cells to incorporate them into neutral lipid droplets (Cnop et al., 2001). ω-3 FAs are readily incorporated in to neutral lipids, when compared with oleic acid in pancreatic islet cells. DHA is more accumulated than EPA after 24 h treatment with 0.1 mM. However, the inverse relationship between cytotoxicity and the intracellular lipid accumulation was not observed for both EPA (Azevedo-Martins et al., 2006), and DHA (data not shown), as proposed for oleic and palmitic acids (Cnop et al., 2001).

Our group has investigated whether the toxic effect of the ω-3 FAs in RINm5F cell is associated with the phosphorylation state of Akt, ERK and PKC-δ (Simon et al., 2008). The regulation of these kinases was compared in three experimental protocols: (a) 4 h-exposure, (b) 4 h-exposure and a subsequent withdrawn of the FAs for a 20 h period and (c) 24 h-exposure. Although DHA activated Akt in the short-period treatment, this effect did not persist. In fact, DHA inhibited Akt phosphorylation in 24 h experiment. This finding led us to postulate that DHA cytotoxicity is probably associated to inhibition of Akt and ERK phosphorylation in the 24 h-exposure experiment. EPA promoted a late increase of Akt phosphorylation as seen in the 4/24 h experiment (b). The activation of Akt might induce anti-apoptotic effects by phosphorylation of GSK-3 (glycogen synthase kinase-3), a Bcl-2 family member involved in cell-survival (Simon et al., 2008).

7. Conclusions

DHA and EPA present different effects on several functions of leukocytes, insulin secreting cells and endothelial cells. These differences are associated with their effects on membrane physico-chemical properties, intracellular signaling pathways and gene expression. The marked differences between the effects of EPA and DHA indicate that it is an over-simplification to generalize the effects of ω-3 PUFA on cell function.

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