REVIEW

Mechanisms by Which Dietary Fatty Acids Regulate Mitochondrial Structure-Function in Health and Disease

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ABSTRACT

Mitochondria are the energy-producing organelles within a cell. Furthermore, mitochondria have a role in maintaining cellular homeostasis and proper calcium concentrations, building critical components of hormones and other signaling molecules, and controlling apoptosis. Structurally, mitochondria are unique because they have 2 membranes that allow for compartmentalization. The composition and molecular organization of these membranes are crucial to the maintenance and function of mitochondria. In this review, we first present a general overview of mitochondrial membrane biochemistry and biophysics followed by the role of different dietary saturated and unsaturated fatty acids in modulating mitochondrial membrane structure-function. We focus extensively on long-chain n–3 (ω-3) polyunsaturated fatty acids and their underlying mechanisms of action. Finally, we discuss implications of understanding molecular mechanisms by which dietary n–3 fatty acids target mitochondrial structure-function in metabolic diseases such as obesity, cardiac-ischemia reperfusion injury, obesity, type 2 diabetes, nonalcoholic fatty liver disease, and select cancers. *Adv Nutr* 2018;9:247–262.

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Mitochondrial Membranes

Mitochondria are unique structures with 2 membrane bilayers. A general overview of mitochondrial outer and inner membrane structure-function is first presented followed by a brief overview of mitochondrial biophysics. We then present a summary of dietary FAs and the underlying biochemical and biophysical mechanisms by which they influence mitochondrial function.

The outer mitochondrial membrane

The outer mitochondrial membrane (OMM) encloses the entire mitochondrion and is in contact with the cytoplasm of the cell. The OMM and inner mitochondrial membrane (IMM) create a unique environment within the mitochondria, called the intermembrane space. The OMM is exceptionally porous and contains proteins called porins (e.g., voltage-dependent anionic channels) embedded into its structure that form channels and allow molecules $\leq$5 kDa in size to diffuse freely across the membrane and into the intermembrane space (1–3). The OMM also houses proteins that translocate larger proteins by recognizing an encoded mitochondria-targeting N-terminal sequence (4–6). The OMM, similar to the plasma membrane, has high concentrations of phosphatidylcholine and phosphatidylethanolamine and a 50:50 ratio of proteins to lipids (7). Cardiolipin, one of the hallmark phospholipids of the mitochondria, is only found at ~4% of a normal functioning OMM (8–10) (**Figure 1**). However, during apoptosis, cardiolipin is released from the IMM and translocates to the OMM forming platforms for the Bax/Bid complex to bind and begin the apoptotic cascade (11–16). The OMM is associated with a multitude of functions in addition to protein transport and apoptosis, including the synthesis and elongation of FAs

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Abbreviations used: AA, arachidonic acid; ALA, α-linolenic acid; ALCAT1, acyl-CoA:lysocardiolipin acyltransferase 1; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; IR, ischemia reperfusion; LA, linoleic acid; MAM, mitochondria-associated membrane; MPTP, mitochondrial permeability transition pore; NAFLD, nonalcoholic fatty liver disease; OMM, outer mitochondrial membrane; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; $(18:2)_4$CL, tetralinoleoyl cardiolipin.

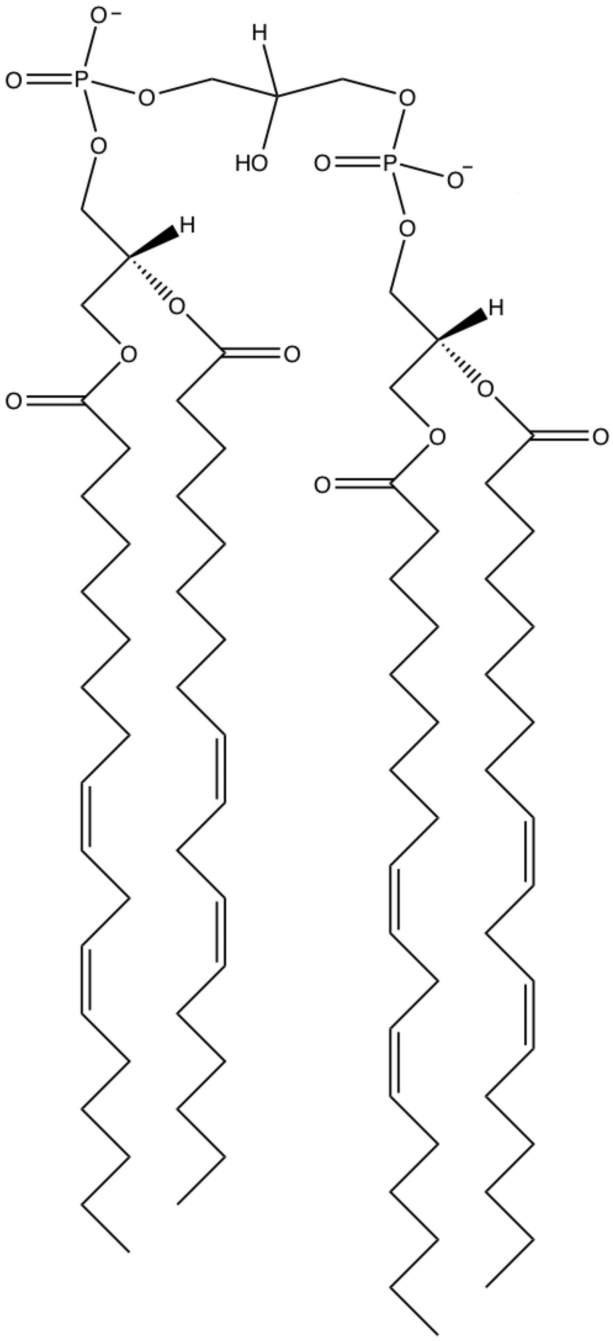

FIGURE 1 Structure of mature cardiolipin. Cardiolipin is a unique anionic phospholipid with 4 acyl chains and a small headgroup. Mature cardiac cardiolipin contains predominantly 4 linoleic acid (18:2) acyl chains under healthy conditions. Modifications to the acyl chains of cardiolipin are common in a range of diseases and can also be modified in response to dietary intake of differing FAs.

(17, 18). The OMM can also associate with the endoplasmic reticulum (ER) and create distinct mitochondria-associated membranes (MAMs) (**Figure 2**).

The MAMs

The OMM and the ER create MAMs that are critical for cellular physiology and homeostasis (19). MAMs have been observed with electron micrographs and fluorescence microscopy. They comprise ≤20% of the OMM and are held together via tethering complexes (20–24). Subcellular MAM fractions are enriched in enzymes that are important in lipid biosynthesis, phospholipid exchange, and calcium signaling (25–30). Due to constant fission and fusion, mitochondria require a well-regulated supply of phospholipids to maintain membrane integrity. MAMs allow for phospholipid flipping between the ER and OMM, independent of ATP (31–33). There are also phospholipid-remodeling enzymes associated with the MAM, including acyl-CoA:lysocardiolipin acyltransferase 1 (ALCAT1), which remodels cardiolipin with highly oxidizable acyl chains (34, 35) (Figure 2). MAMs also appear to be intermediate destinations in pathways that lead to VLDL assembly and secretion (20, 36–38). Thus, MAMs serve as a critical metabolic and trafficking hub in lipid metabolism. Finally, MAMs are also extremely important in calcium regulation. They create calcium microdomains at contact points that facilitate the efficient uptake of calcium, which sustains mitochondria and cellular homeostasis (28). Although more research is needed on MAMs, it is clear that these structures are critical in signaling, metabolism, and organelle physiology.

The IMM

The IMM encapsulates the matrix of the mitochondria and is surrounded by the intermembrane space. There are a multitude of IMM-associated proteins, most notably those involved in oxidative phosphorylation, ATP synthesis, and protein translocation (39–41). The IMM has a unique phospholipid composition closely resembling that of a bacterial membrane with a ratio of 4:1 proteins to lipids (7). The IMM is mainly composed of phosphatidylcholine (40%), phosphatidylethanolamine (30%), and cardiolipin (15–20%). The remaining 10% of the IMM phospholipidome consists of phosphatidylinositol (5%), phosphatidylserine (3–4%), and very small quantities of other lipids including phosphatidic acid and cholesterol (42). Cardiolipin has a unique structure with 4 acyl chains that promote the formation of non-bilayer phases and high curvature that can influence cristae formation and respiratory function (43).

Since the IMM's predominant role is energy production, it is only freely permeable to small metabolites, including oxygen and water (44–46). This lack of permeability separates the matrix from the cytosolic environment of the intermembrane space. This extreme compartmentalization is necessary for energy production because ATP synthesis relies on a proton gradient created across the IMM (47, 48). In fact, ATP is produced via multiple redox reactions that occur in 5 transmembrane enzyme complexes within the IMM—complex I [NADH: ubiquinone oxidoreductase], complex II (succinate dehydrogenase), complex III (cytochrome c reductase), complex IV (cytochrome c oxidase), and complex V (ATP synthase)—and 2 mobile electron carriers (the lipid-soluble coenzyme Q and the water-soluble cytochrome c). Initially, reducing equivalents such as NADH and $FADH_2$ are oxidized by complex I and complex II, respectively. From

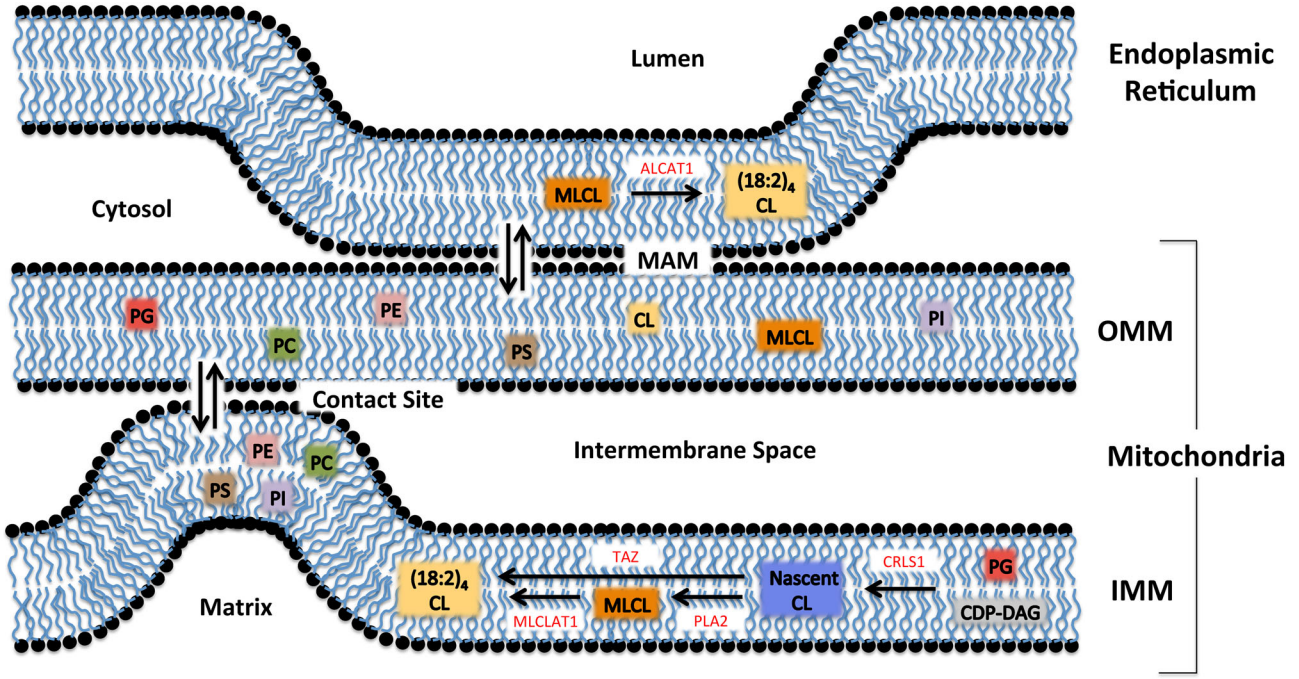

FIGURE 2 Biosynthesis of mature CL. Mature CL is largely synthesized in the IMM. Nascent CL is synthesized from PG by using CDP-DAG. The acyl chains of nascent CL are then modified to mature CL containing 4 linoleic acid acyl chains (18:2) with the enzyme TAZ. Nascent CL can also undergo acyl chain cleavage with PLA2 to produce MLCL, which can serve as a substrate for MLCLAT1 to produce mature CL. A small fraction of mature CL is produced in the endoplasmic reticulum membrane through the use of ALCAT1. For simplicity, biosynthesis of other key phospholipids is not depicted. These phospholipids (PC, PE, PS, PI, PG, and PA) are found in differing concentrations across the ER, OMM, and IMM. ALCAT1, acyl-CoA:lysocardiolipin acyltransferase 1; CDP-DAG, cytidinediphosphate-diacylglycerol; CL, cardiolipin; CRLS1, cardiolipin synthase; ER, endoplasmic reticulum; IMM, inner mitochondrial membrane; MAM, mitochondria-associated membrane; MLCL, monolyso-cardiolipin; MLCLAT1, monolyso-cardiolipin acyltransferase 1; OMM, outer mitochondrial membrane; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLA2, phospholipase A_2; PS, phosphatidylserine; TAZ, taffazin; $(18:2)_4$CL, tetralinoleoyl cardiolipin.

there, electrons are passed through coenzyme Q to complex III and then from cytochrome c to complex IV. The final electron acceptor is oxygen, located in the matrix, which is reduced to water. Electron transport through the complexes is associated with proton pumping. Complexes I, III, and IV couple electron flow and proton pumping from the matrix to the intermembrane space to create and maintain an electrochemical gradient across the IMM. Complex V uses the electrochemical gradient to produce ATP by allowing protons to re-enter the matrix and providing the work needed for phosphorylation of ADP.

Overall, the aforementioned mitochondrial membranes play an important role in the organelle's functions and in maintaining cellular homeostasis. The composition of these membranes is very dynamic, especially the specialized IMM, and is tightly regulated but can be altered through the intake of dietary FAs. Alterations in the composition of membrane phospholipids will influence the biophysical properties of the membrane and thereby protein function (49).

Mitochondrial Membrane Biophysics

Mitochondria are cellular organelles containing 2 structurally and functionally distinct membranes (50–52). The biophysical organization of the outer and inner membranes of the mitochondria is of great importance because both membranes regulate the trafficking of ions, metabolites, and many small molecules between the cellular cytosol and mitochondrial matrix. Any impairment in the biophysical organization of these membranes could severely affect mitochondrial bioenergetics and cellular homeostasis. Given that the IMM is the major site of oxidative phosphorylation and ATP synthesis, we focus on the biophysical organization of the IMM.

Biophysical organization of the IMM

The structure of the IMM is extensively folded and compartmentalized by invaginations of the inner membrane termed "cristae," which provide a large amount of surface for biochemical reactions such as cellular respiration and ATP generation (53–55). Within the IMM, 2 distinct regions have been observed: the inner boundary membrane and the cristae membrane. The inner boundary membrane is adjacent to the OMM, whereas the cristae membrane represents invaginations that protrude into the mitochondrial matrix (54). These convoluted structures of the IMM were established by pioneering studies on mitochondrial ultrastructure. These studies led to the proposal of several hypothetical models describing the biophysical organization of the IMM. To date,

3 models of the IMM have been proposed, including the "baffle model," the "septa model," and the "crista junction" model (54). The baffle model was initially proposed in the early 1950s by Palade (56), who confirmed the existence of the outer and inner membranes of the mitochondria and originally described cristae as being invaginations with rather broad openings (50). An alternate interpretation of the IMM structure was proposed around the same time by Sjöstrand (51, 57), who termed the "septa model." Sjöstrand suggested that sheets of the inner membrane are divided by septa, which separate the matrix into several distinct compartments.

More recent EM tomography studies have shown that cristae are attached to the inner boundary membrane by narrow tubular openings, which have been termed "crista junctions" (58–62). It is thought that crista junctions play a critical role in compartmentalizing the IMM, because they may limit the diffusion of metabolites, such as protons and ADP, between the intermembrane space and the intracristal space (59). Indeed, crista junctions could contribute to the regulation of oxidative phosphorylation (OXPHOS), and ultimately ATP production, because 1 of the key limiting metabolites is ADP. Although more structural details with regard to crista junctions have become available, the functional significance of these structures remains unknown. Therefore, investigating the mechanistic and functional relation between these various IMM structures and dietary fatty acids is critical for better understanding mitochondrial function in health and disease.

Cardiolipin structure

The biophysical organization of the IMM is highly regulated by cardiolipin, a unique anionic, polyunsaturated phospholipid consisting of 2 phosphatidic acid moieties linked by a central glycerol backbone (Figure 1). Under normal physiologic conditions, cardiolipin may only carry 1 negative charge at a time because the phosphates of cardiolipin are diastereotopically inequivalent, and thus ionize at 2 different pH levels ($pK_1 = \sim 2.8$, $pK_2 = \sim 7.5–9.5$) (63–66). This allows cardiolipin to trap protons within its headgroup and thereby localize the proton pool near the surface of the IMM. The bioenergetic importance of cardiolipin to function as a proton trap may be to supply a greater buffering capacity at the membrane-water interface (66). In theory, cardiolipin could bridge the gap between the proton donor and the proton acceptor, because highly mobile cardiolipin would laterally shuttle protons from OXPHOS protein complexes to the ATP synthase machinery (66).

Cardiolipin is predominantly found in membranes capable of generating an electrical potential, such as bacterial and mitochondrial membranes (67). In mitochondria, cardiolipin is almost exclusively localized to the inner membrane where it is synthesized and required for membrane structure, membrane fusion and fission, protein function, and apoptosis (43, 68–72). The functional importance of cardiolipin within the IMM arises from its ability to directly influence lipid molecular organization and thereby cluster proteins that require specific lipid microenvironments, including key enzymes involved in OXPHOS (43, 70, 71, 73–75). Previous studies have clearly shown the importance of cardiolipin within the IMM by showing that cardiolipin clusters respiratory enzymes in order to augment efficient electron channeling for optimal OXPHOS activity (66, 70, 71, 73, 75–77). However, due to cardiolipin's polyunsaturated nature, and its close association with OXPHOS, it is extremely susceptible to oxidative damage often arising from reactive oxygen species (ROS).

Many studies have suggested cardiolipin's acyl chain specificity to be critical for optimal mitochondrial function. In the mammalian heart, linoleic acid (LA; 18:2) constitutes 80–90% of total cardiolipin acyl chains, where tetralinoleoyl cardiolipin [$(18:2)_4$CL] is the most abundant species (Figure 1) (78). The abundance of $(18:2)_4$CL within the IMM suggests an important role in structure-function. Indeed, cardiolipin enriched in tetralinoleic acid $(18:2)_4$ binds with high affinity to a multitude of proteins and enzymes within the IMM, including key respiratory chain enzymes (79–86).

The $(18:2)_4$-rich composition of cardiolipin is achieved by an acyl chain remodeling process that occurs within the IMM. Details of the synthesis of mature cardiolipin are presented in Figure 2. Briefly, de novo cardiolipin biosynthesis occurs in a series of steps on the inner face of the inner membrane from phosphatidylglycerol (PG) and cytidinediphosphate-diacylglycerol (CDP-DAG) via cardiolipin synthase (87). Nascent cardiolipin must be remodeled into a composition enriched in LA by enzyme-dependent remodeling processes within the IMM. A small fraction of mature cardiolipin can also be synthesized in the ER membrane (Figure 2). Environmental and genetic factors, such as diet and mutations in essential remodeling enzymes, influence the bioavailability and abundance of $(18:2)_4$CL within the IMM (78, 88, 89).

Cardiolipin microdomains

Membrane microdomains often form as a function of favorable physiochemical properties of lipids (90–94). This phenomenon is observed in the plasma membrane of cells where lipid rafts, which are enriched in cholesterol and sphingolipids, exist as highly ordered regions that serve to compartmentalize cellular processes (92, 94). However, the idea of "raft-like" mitochondrial microdomains remains debated because the concentration of lipids required for lipid rafts (i.e., cholesterol and sphingolipids) is very low (95). This does not rule out the notion that specific lipids (i.e., cardiolipin) may localize to concentrate proteins into confined signaling centers.

Due to cardiolipin's unique physicochemical properties, it can induce negative membrane curvature and form nonbilayer phases, such as the inverted-hexagonal phase (96–99). The ability of cardiolipin to organize into the inverted-hexagonal phase suggests that cardiolipin may promote specific localized structures within the IMM and implicate its potential role in the formation of mitochondrial microdomains. Indeed, some laboratories have suggested that cardiolipin-enriched microdomains exist within

mitochondria as essential activating platforms for mitochondrial fusion and fission and apoptosis (15, 97, 100). Experiments that used lipid monolayers and bilayers have shown that specific mitochondrial proteins, such as mitochondrial creatine kinase and cytochrome c, tightly interact with cardiolipin and induce segregation into microdomains (101–104). These proteins are found at mitochondrial contact sites as well as the surface of the IMM, where the assembly of enriched domains of cardiolipin plays an important role in mitochondria-mediated apoptosis and OXPHOS. However, the notion that mitochondria contain localized subdomains enriched in cardiolipin is still in its infancy. Future studies need to address how these microdomains may cluster proteins, such as respiratory protein complexes, for optimal ATP production and enhanced mitochondrial signaling (70, 71, 73, 74, 105, 106).

Dietary FAs

SFAs

Dietary FAs will influence the composition of mitochondrial membranes and thereby function. The 2 most abundant SFA acyl chains within cellular membranes are palmitic (16:0) and stearic (18:0) acids (107, 108). Although the body can synthesize SFAs, they are consumed from a variety of substances, including butter, milk, palm oil, coconut oil, and ground beef or beef tallow. These foods also contain shorter SFAs, including lauric acid (12:0) acid and myristic acid (14:0).

SFAs play important roles in the membranes of a multitude of organelles. Due to their structure, SFAs can pack tightly together and increase the viscosity of membranes. They can also interact with cholesterol and create lipid raft domains within the plasma membrane, which are important in cellular signaling (109–112). In mitochondrial membranes, the abundance of SFAs is significantly lower than the amount of unsaturated FAs. However, an increase in dietary intake of SFAs can increase the amount of FAs such as palmitic and stearic acids within the mitochondrial membranes, leading to potential impairments (113). In one study, SFAs were increased in rat liver mitochondria, particularly in cardiolipin, which led to the impaired release of cytochrome c (114). Although significantly less studied than unsaturated FAs in relation to mitochondria, SFAs likely play an important role in regulating mitochondrial membrane structure and function. Elucidating how SFAs influence mitochondrial biophysical organization is an area for future investigation. One possibility is that SFAs may counter-regulate the effects of unsaturated FAs by perturbing the diffusion of protein complexes and thereby their ability to execute optimal respiratory function (**Figure 3**). This would be driven by the ability of SFAs to increase membrane microviscosity and potentially increase the thickness of the membrane bilayer.

n–6 PUFAs

n–6 PUFAs play an essential role in membrane structure and function. Generally, due to their multiple double bonds, n–6 PUFAs decrease the viscosity of the membrane (115). They do not pack as tightly as SFAs and will not form liquid ordered microdomains (116). LA (18:2) is the predominant n–6 PUFA in the Western diet. LA is obtained through the diet from vegetable oils, such as sunflower, soybean, corn, and canola oils, as well as walnuts and seeds. Dietary n–6 PUFAs will incorporate into mitochondrial phospholipids. Without LA incorporation into cardiolipin's acyl chains, the function of respiratory and other membrane-associated proteins may be diminished (117–119). In many diseases, including cardiovascular diseases, diabetes, and Barth syndrome, the loss of LA in cardiolipin is detrimental to the overall function of the mitochondria (120, 121). However, one research group, by using a yeast model concluded that unremodeled cardiolipin (i.e., containing a significant amount of saturated acyl chains) is functionally equivalent to fully remodeled cardiolipin (containing predominately PUFA acyl chains that are 18:2) (122). In addition, another study that used a yeast model determined that a decrease in the cardiolipin-to–monolyso-cardiolipin ratio caused deficiencies in respiration (123). Although innumerable studies have been conducted on the role of cardiolipin remodeling, its role in mitochondrial bioenergetics still requires further investigation.

Dietary arachidonic acid (AA; 20:4) is mainly obtained through the consumption of chicken, eggs, and beef. AA will also incorporate into mitochondrial membranes, although it is controversial if it is beneficial to the function of the mitochondria (124, 125). For example, rats were supplemented with an AA-enriched diet for 10 wk, which led to the incorporation of AA at the expense of LA acyl chains, particularly in cardiolipin. This replacement delayed the opening of the mitochondrial permeability transition pore (MPTP) and reduced the risk of apoptosis but did not alter mitochondrial respiration (125). Conversely, a study showed that AA induced the opening of the MPTP but did not cause depolarization or respiratory inhibition. When AA was added to MH1C1 rat hepatoma cells, it rapidly opened the MPTP and induced cytochrome c release and apoptosis (126). In addition, in studies in which the FFA form of AA is incorporated into the mitochondria, there are uncoupling effects during state 4 respiration and an overall inhibition in uncoupled respiration. There is also a decrease in complex I and complex III activity as well as an increase in ROS production and membrane permeability (124). Overall, n–6 PUFAs have differing effects on mitochondrial membranes. LA is the most abundant acyl chain in mitochondrial membranes and is required for optimal function and AA is also found in mitochondrial membrane phospholipids but to a lesser extent. The role of AA in mitochondrial membranes is still unclear and requires further scientific exploration, particularly in the context of n–3 PUFAs, which lower n–6 PUFAs (127, 128).

n–3 PUFAs

The most notable n–3 PUFAs are α-linolenic acid (ALA; 18:3), EPA (20:5), and DHA (22:6). ALA is a short-chain n–3 PUFA and an essential FA that is obtained through the consumption of flaxseed, canola, and walnut oils. ALA can undergo elongation and desaturation to produce long-chain

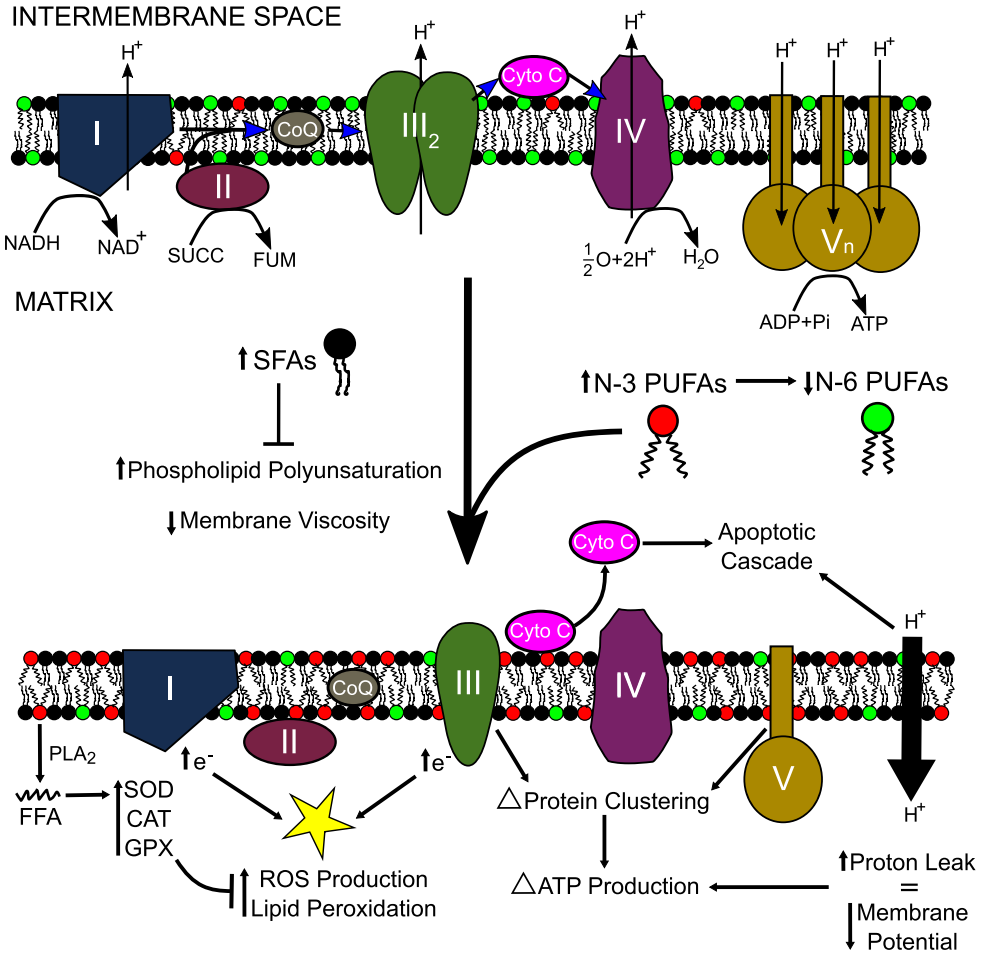

FIGURE 3 Model depicting how dietary SFA, n–6 PUFA, and n–3 PUFA acyl chains target IMM structure-function. An increase in n–3 PUFA acyl chains within the IMM will replace the n–6 PUFA acyl chains and thereby influence microviscosity. This may cause the membrane to become "leaky" and allow for more proton leak back into the matrix. In addition, an increase in SFAs will decrease polyunsaturation and increase viscosity. n–3 PUFA incorporation into the membrane may also alter protein clustering and enzyme activity, which may alter the amount of ATP produced. Respiratory enzymes are influenced by the increase in n–3 PUFAs and may allow more electrons to escape during oxidative phosphorylation, which will lead to an increase in ROS production and peroxidation. However, n–3 PUFAs can also be cleaved from the membrane via PLA_2 and increase the antioxidant capacity of the mitochondria. In addition, an increase in n–3 PUFAs may release cytochrome c, starting the apoptotic cascade as seen in some cancer models. Overall, FAs through the diet likely have a wide range of different roles within the IMM that require further investigation in both healthy and diseased states. CAT, catalase; CoQ, coenzyme Q; Cyto C, cytochrome c; FUM, fumarate; GPX, glutathione peroxidase; IMM, inner mitochondrial membrane; Pi, inorganic phosphate; PLA_2, phospholipase A_2; SOD, superoxide dismutase; SUCC, succinate.

n–3 PUFAs such as EPA and DHA (129). Elongation and desaturation from ALA are highly inefficient, so most long-chain n–3 PUFAs come from dietary sources such as marine oils or as dietary supplements.

Long-chain n–3 PUFAs play an important role in the structure and function of cellular membranes (130). Due to their extreme chain length and multiple double bonds, they dramatically decrease membrane viscosity (130). EPA and DHA can incorporate into the plasma membrane of a multitude of cell types, particularly immune cells, and disrupt plasma membrane raft domains and cellular signaling (131–136). Presumably, similar mechanisms exist by which EPA and DHA target the organization of mitochondrial membranes. Indeed, there is evidence that n–3 PUFAs incorporate into the cardiac mitochondrial phospholipidome (137).

Effects of Dietary FAs on Mitochondrial Function Independent of Changes in Mitochondrial Membrane Organization

This review focused on how dietary FAs regulate mitochondrial membrane structure-function. However, it is essential to recognize that dietary FAs can influence mitochondrial function independent of changes in the composition of the OMM and IMM. Here we briefly describe how dietary FAs directly influence mitochondrial function through metabolic gene regulation. Long-chain SFAs and unsaturated FAs act as ligands for PPARs, a superfamily of nuclear transcription factors responsible for upregulating genes that influence mitochondria and peroxisome function. These nuclear transcription factors (PPARα, PPARβ/δ, and PPARγ) heterodimerize with retinoid X-receptor (RXR), promoting

target gene transcription through DNA binding of the peroxisome proliferator response element (PPRE) in the nucleus (138, 139).

Endogenous ligands for PPARs, such as PUFAs, eicosanoids, and other lipid metabolites, can also activate nuclear transcription factors. Furthermore, PPAR-induced gene transcription differs by subtype and tissue distribution, with PPARα and PPARβ/δ primarily responsible for regulating β-oxidation genes in hepatocytes, cardiomyocytes, myocytes, and enterocytes (140, 141), and PPARγ is responsible for adipocyte differentiation and immune cell metabolism, differentiation, and function, respectively (142, 143). Within the mitochondria, activation of PPARs by long-chain FAs induces gene expression of several key mitochondrial enzymes, including carnitine palmitoyltransferase (CPT) enzyme I and II, located on the OMM and IMM, respectively; acetyl-CoA synthetase (ACS); and numerous other β-oxidation enzymes (144). Thus, long-chain FA activation of PPARs induces their own metabolism through this transcriptionally regulated pathway, directly influencing mitochondrial function by increasing FA oxidation.

The consumption of high-fat diets and resultant elevated endogenous FFAs have previously been shown to increase mitochondrial biogenesis through a PPARδ-mediated mechanism involving increased PPARγ co-activator 1 (PGC-1) in rat muscle. However, the increase in mitochondrial biogenesis did not result in improved insulin resistance caused by consuming a high-fat diet (145). Compared with low-fat diets, the prolonged consumption of a high-fat diet in Wistar rats resulted in obesity, dyslipidemia, insulin resistance, and skeletal muscle mitochondrial dysfunction, with notable significant differences in phosphorylation efficiency and mitochondrial respiration (146). These findings support numerous investigations in rodent models that show that high-fat diet consumption results in greater β-oxidation, likely mediated by PPAR activation, concomitant with greater free radical and oxidant production (147). These direct mitochondrial effects of high-fat diets likely contribute to mitochondrial, cellular, and systemic metabolic dysfunction (147). Taken together, it is essential to recognize that dietary FAs can target mitochondrial function independent of changes in structural membrane organization.

Effects of Long-Chain n–3 PUFAs on Mitochondrial Membrane Structure-Function

The mitochondrial phospholipidome is remodeled with the consumption of n–3 PUFAs

As mentioned previously, the composition of the IMM is highly regulated due to its unique structure and function in maintaining the electrochemical gradient required for OXPHOS. However, the composition of the IMM is subject to change in response to dietary FA consumption (88). Many studies have addressed how n–3 PUFAs target mitochondrial membrane properties in health and disease (125, 137, 148–156). In **Table 1**, we present an overview of key studies showing the effects of n–3 PUFAs on mitochondrial membrane organization and function (i.e., membrane potential, respiration, individual complex activities, and ROS production). Although most of the studies differ in methodology, the results on membrane structure are similar. An increase in n–3 PUFA concentrations leads to an increase in EPA and/or DHA incorporation into mitochondrial membranes at the expense of n–6 PUFAs (Figure 3). Incorporation of n–3 PUFAs into mitochondrial membranes also alters the biophysical organization by decreasing membrane viscosity and potentially changing lipid-lipid and lipid-protein interactions (Figure 3).

Although some studies show that n–3 PUFAs influence membrane viscosity, the field has not moved beyond to establish how EPA and DHA acyl chains are targeting the formation of mitochondrial microdomains, protein-phospholipid binding, and protein-protein clustering. Our laboratory has very recently shown that DHA, upon incorporation into cardiolipin, can prevent formation of mitochondrial microdomains and binding of cardiolipin to complex IV (150). However, more work is needed in this area.

ROS production and antioxidant capacity are mechanistic targets of n–3 PUFAs

The mitochondria are an important source of ROS within the cell. ROS occur when electrons "escape" or leak from the enzyme complexes associated with OXPHOS. The main contributors to electron leakage are complexes I and III (157). Once these electrons escape, they interact with oxygen to produce a superoxide. In normal-functioning mitochondria, these superoxides interact with redox enzymes within the matrix of the mitochondria, reducing the superoxide to water. However, superoxides can still damage the mitochondria, by peroxidizing phospholipid acyl chains, mitochondrial DNA, and proteins (158). The peroxidation of phospholipids within the IMM can lead to a disorganization of the membrane, an accumulation of toxic products, and increased levels of free radicals, enhancing mitochondrial damage.

Acyl chains such as EPA and DHA are highly susceptible to peroxidation via ROS due to their tremendous level of unsaturation (Figure 3). Dietary supplementation of EPA or DHA increases the levels of n–3 PUFA acyl chains within mitochondrial membranes, which leads to membrane disorganization and potentially increased electron leakage. Several studies show that an increase in the polyunsaturation of phospholipids, particularly cardiolipin, increases the production of ROS (153, 159). When ROS production overwhelms the antioxidant capacity of the mitochondria, phospholipase A_2 is activated and enhances the release of peroxidized fatty acyl chains from membrane phospholipids, resulting in membrane disorganization and mitochondrial damage (160). This mechanism of increased oxidative stress, resulting in mitochondrial dysfunction and thereby apoptosis, is implicated in the anticancer properties of n–3 PUFAs, as discussed below.

Although, the presence of n–3 PUFA acyl chains within the IMM can increase ROS production, this does not always translate to increased mitochondrial dysfunction. Figure 3 shows how n–3 PUFAs mechanistically can increase

TABLE 1 Summary of key studies on n–3 PUFAs and mitochondrial membrane structure-function[1]

Supplementation	Organism/tissue	Membrane alterations	Functional endpoints	Reference
Diet containing DHA at 2.5% total caloric intake for 10 wk	Male Wistar rats/cardiac mitochondria	↑ Amount of DHA and displaced AA and LA	↓ In $(18:2)_4$CL, no effect on respiration	(125)
100 μM n–3 PUFA treatment for 72 h	H9c2 cardiac myoblasts	↑ Highly unsaturated CL containing n–3 PUFAs	↑ High-molecular-weight CL and ↓ low-molecular-weight CL	(137)
Fusion of mitochondria with 18:0/22:6 PC vesicles	4-wk-old male mice/liver mitochondria	↓ In DHA and viscosity	↓ RCI and membrane potential and ↑ proton movement	(148)
10% Menhaden oil for 3 wk	2-mo or 21-mo CBA/ca female mice/liver mitochondria	↑ In EPA and DHA and ↓ in AA; DHA was primarily associated with PE and PC	↑ Age-related mitochondrial dysfunction	(148)
DHA ethyl esters (2% of energy)	C57BL/6 obese mice	↓ Formation of membrane domains	↓ In enzyme activity	(150)
n–3 Complete: 2 g EPA and 1 g DHA/d for 12 wk	Recreationally active men aged 22.7 ± 0.8 y/subsarcolemmal and intermyofibrillar mitochondria	↑ In EPA/DHA in PC and PE but not in CL	↑ Sensitivity to ADP and ROS but not in oxidant products	(151)
Diet containing DHA or EPA at 2.5% total caloric intake for 8 wk	Male Wistar rats/cardiac mitochondria	↑ EPA/DHA and ↓ AA	↓ Ca^{2+}-induced opening of the MPTP and swelling	(152)
11.5 g Menhaden fish oil/100 g for 2 wk	Male Sprague-Dawley rats/colonic crypt mitochondria	↑ In unsaturation index of CL, PE, and PC	↑ ROS- initiated apoptotic cascade	(153)
20% wt:vol Menhaden fish oil for 4 wk	Male Sprague-Dawley rats/renal cortical mitochondria	↓ In OA, LA, and AA and ↑ in EPA and DHA	↑ In PLA_2 activity and mitochondrial damage via ROS; ↓ in state 3 respiration and complex I	(154)
15% (wt:wt) fish-oil or EPA and DHA pure ethyl ester diet for 2 wk	Male Sprague-Dawley rats/colonic crypt mitochondria	↑ In EPA and DHA at the expense of AA and LA	↓ In membrane potential and ↑ in caspase 3 activity	(155)
20% Fish-oil diet–12% tuna oil and 8% sardine oil for 12 wk	Wistar male rats/liver mitochondria	↓ In viscosity	↑ Membrane potential, respiration, and complex V activity; ↓ complex III and IV activity	(156)

[1] AA, arachidonic acid (20:4); CL, cardiolipin; LA, linoleic acid (18:2); MPTP, mitochondrial permeability transition pore; OA, oleic acid (18:1); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PLA_2, phospholipase A_2; RCI, Respiratory Control Index; ROS, reactive oxygen species; $(18:2)_4$CL, tetralinoleoyl cardiolipin; ↑, increase/increased; ↓, decrease/decreased.

antioxidant potential and increase ROS production within the mitochondrial matrix. Several literature reports show that supplementation with n–3 PUFAs increases the antioxidant capacity of the cell through a variety of mechanisms (161–165). For instance, n–3 PUFA supplementation increased the concentrations of oxidative stress products, including lipid hydroperoxides and malondialdehyde. However, superoxide dismutase activity was upregulated, which increased mitochondrial antioxidant capacity and thereby maintaining protein function (161). In addition, in HepG2 cells supplemented with DHA, there was no increase in lipid peroxidation but an increase in glutathione-related antioxidant enzyme activities and superoxide dismutase activity (162). DHA has also been implicated in several studies for inhibiting free radical generators, including NADPH oxidases (165). The role of n–3 PUFAs and their antioxidant capacity is relevant in a multitude of disease states and warrants more extensive research (164).

Consumption of n–3 PUFAs influences mitochondrial protein activity

The IMM houses a multitude of proteins that perform a variety of functions, most notably of those involved in OXPHOS. The IMM is a sea of proteins with imbedded phospholipids with a protein to phospholipid ratio of 4:1 (7). Despite a high concentration of proteins within the IMM, phospholipids play a crucial role in maintaining protein organization and function.

Cardiolipin binds to a multitude of IMM proteins and aides in their biophysical organization and function. This includes binding to individual electron transport chain (ETC) enzymes as well as promoting the formation of supercomplexes. For example, there are 9 binding sites for cardiolipin in bovine complex I (166). Complex III has 6 cardiolipin binding sites that have functional and structural importance. Structural studies suggest that cardiolipin binds to sites on complex III and aids in proton uptake (81, 167). Cardiolipin is also functionally required for complex IV activity. There are 4 cardiolipin binding sites in complex IV: 2 are "high affinity" and 2 are "low affinity." The high-affinity cardiolipin binding sites are associated with the regulation of electron activity and the low affinity sites are important in structural integrity of the complex in its dimer form (80, 168–171). In addition, complex V dimers require cardiolipin for assembly into larger oligomeric structures (79, 172, 173). As well as binding to individual complexes, cardiolipin is also considered to function as a "glue" by holding respiratory supercomplexes together (174).

Cardiolipin undergoes remodeling in the mitochondria from its nascent form to a highly regulated species that is in the cardiac tissue ~90% tetra-linoleic or $(18:2)_4$ (Figure 2). It is well documented that reductions in the concentration of

$(18:2)_4$CL in the IMM will lead to supercomplex dissociation, loss of ATP production, and structural abnormalities, most notably seen in Barth syndrome (120, 121, 175). Along with a loss of content, alterations in acyl chain species of cardiolipin may also result in a disorganization of the membrane and thereby decreased respiratory enzyme activity and supercomplex formation. For example, in Barth syndrome, respiratory supercomplexes are destabilized due to cardiolipin acyl chain remodeling and decreased cardiolipin concentrations (176). However, the importance of cardiolipin concentration compared with cardiolipin acyl chain remodeling in supercomplex destabilization has yet to be definitively determined.

The consumption of n–3 PUFAs also targets enzymatic activity, which could be through a direct effect on respiratory enzymes, formation of supercomplexes, or both. In 1 study, rats were fed a 30% fish-oil diet for 12 wk, which resulted in a decrease in complex II+III and complex IV activities (156). Several other studies showed that an increase in n–3 PUFA consumption reduced ATP production and various states of respiration (148, 152). Other laboratories reported no loss of respiratory function with n–3 PUFA supplementation (125, 151). A recent study from our group showed that DHA ethyl esters lowered respiratory enzyme activities of complexes I, IV, V, and I+III. The underlying mechanism was driven by replacement of LA by DHA in the mitochondrial phospholipidome (150). Although an increase in n–3 PUFAs disorganizes the mitochondrial membrane and can potentially alter protein function, studies are overall inconsistent. More standardized experiments need to be conducted on the role of n–3 PUFA acyl chains and their effects on mitochondrial enzyme functions.

Effects of Long-Chain n–3 PUFAs on Diseased Mitochondrial Membranes and Function

Ischemia reperfusion injury

Cardiac ischemia reperfusion (IR) injury is characterized by an acute loss of blood flow followed by the reintroduction of blood and oxygen. The pathogenesis of IR injury is complex and involves multiple pathways, including ROS and inflammation (177, 178). In the mitochondria, IR is characterized by a distinct loss in cardiolipin, energy dysregulation, and overabundance of ROS. Several studies suggest that treatment with n–3 PUFAs improves myocardial resistance to IR injury and reduces infarct size, although the mechanisms remain elusive (179–182).

n–3 PUFAs could improve IR injury through differing mechanisms, either before ischemia or during the reperfusion phase. Several laboratories report that chronic n–3 PUFA supplementation reduces the severity of IR injury and decreases myocardial infarct size. These studies also showed that n–3 PUFA treatment upregulates mitochondrial antioxidant activity, including superoxide dismutase, catalase, and glutathione peroxidase, which combat lipid peroxidation during IR injury (161, 180, 183, 184). Chronic n–3 PUFA treatment may be associated with a reinforced antioxidant defense system that protects the mitochondria from ROS produced during the reperfusion phase of IR injury. Acute treatment with n–3 PUFAs during the reperfusion phase of IR injury has also been explored. One study gave a bolus of n–3 PUFA TGs to rats after ischemia but before reperfusion. The results indicated that acute treatment with an EPA-to-DHA ratio of 6:1 improved the outcome of IR injury by reducing vascular inflammation and oxidative stress (185). Another laboratory found that infusion with DHA diminished cardiac damage and increased antioxidant protection (186).

Although a multitude of studies have measured the effects of n–3 PUFAs before and during IR treatment, little is known about their mechanisms of action in relation to the IMM. Several reports suggest that n–3 PUFAs, particularly DHA, incorporate into the membrane and regulate Ca^{2+} channels and delay the Ca^{2+}-dependent MPTP opening. The MPTP opening is associated with cardiovascular diseases, including IR injury, and interventions to target its opening are of significant clinical relevance (152, 187). However, some laboratories suggest that supplementation with EPA or DHA has no effect on MPTP opening in diseased hearts, only in healthy heart tissue (188). Likewise, as mentioned above, supplementation with EPA or DHA can potentially alter enzymatic activities, which could increase the adverse effects of IR injury. More research is needed on n–3 PUFAs and IR injury in addition to other cardiovascular diseases, such as heart failure (78). This is highly relevant given the debate about n–3 PUFA efficacy for cardiovascular diseases (78, 189).

Obesity and type 2 diabetes

Several literature reports suggest that the consumption of n–3 PUFAs can have beneficial effects for obesity and type 2 diabetes (190). The mechanisms for improvements consist of improving insulin signaling, preventing alterations in glucose metabolism, and reversing dyslipidemia via targeting of transcription factors and gene expression (191, 192). n–3 PUFAs also modulate inflammation by decreasing the n–6-to-n–3 PUFA ratio, which reduces the burden of these diseases through n–6-derived lipid mediators (127, 128). Although much is known about the role of n–3 PUFAs in regulating gene expression in these diseases, less is known about how n–3 PUFAs modulate mitochondrial membranes during obesity and type 2 diabetes. One set of experiments showed that an increase in DHA intake partially prevented mitochondrial dysfunction induced by insulin resistance in cardiac mitochondria (193).

Some studies suggest that an increase in n–3 PUFAs within mitochondrial membranes will modulate proton conductance and enhance proton uncoupling, leading to limited weight gain or increased weight loss (194, 195). However, evidence that n–3 PUFAs affect weight loss is lacking and at times contradictory (196, 197). Paradoxically, some studies showed that pathological remodeling of cardiolipin will increase oxidative damage that contributes to mitochondrial dysfunction in obesity and type 2 diabetes. For instance, Li et al. (35) showed that DHA concentrations in C2C12 cells

were elevated upon upregulation of ALCAT1 and increased oxidative stress. ALCAT1 is relevant because a murine ALCAT1 knockout model was shown to protect against diet-induced obesity and insulin resistance (35). In addition, shotgun lipidomic studies showed a significant decrease in cardiolipin abundance and a profound remodeling of the remaining cardiolipin species to include DHA acyl chains in rat diabetic myocardium (198, 199). These studies suggest that an increase in n–3 PUFA acyl chains within mitochondrial membranes could be detrimental for mitochondrial function.

The mechanisms by which n–3 PUFAs regulate mitochondrial membranes and protein function are still vastly unknown in obesity and type 2 diabetes. However, an increase in acyl chain unsaturation may result in membrane disorganization, which would lead to altered lipid-lipid interactions and protein-lipid interactions and potentially mitochondrial dysfunction (Figure 3). It is likely that there is a fine balance between detrimental and beneficial effects of n–3 PUFAs, which will require extensive experimentation as a function of dose of EPA and DHA.

Nonalcoholic fatty liver disease

Nonalcoholic fatty liver disease (NAFLD) is characterized by an increase in the accumulation of lipids in the liver and can lead to fibrosis and cirrhosis. NAFLD is also associated with insulin resistance, oxidative stress, inflammation, and altered lipid metabolism (200). NAFLD impairs mitochondrial dysfunction, which can occur in a number of ways, including lipid and mitochondrial DNA peroxidation, ultrastructure abnormalities, reduced ATP stores, and alterations in respiratory chain enzyme activities (201–207). Several studies and meta-analyses suggest that an increase in n–3 PUFA intake can reduce the effects of NAFLD by regulating gene transcription, reducing inflammation markers, and increasing β-oxidation (208–217). In fact, recent clinical data suggest that supplementation with n–3 PUFAs positively affects plasma lipid profiles, improves liver histology, and decreases inflammatory markers in patients with NAFLD (218, 219).

Although much is known on the role of n–3 PUFAs in abating the effects of NAFLD, little is known on their role in mitochondrial membranes during the development of this disease. For example, incubating HepG2 cells (a steatotic hepatocyte model) with 50 μmol EPA or DHA/L rescued the reduction in mitofusin 2, a multifunctional protein that participates in proliferation and metabolism, which caused an increase in the length of mitochondrial tubules (220). This study also showed that n–3 PUFAs increased ATP production and decreased ROS production (220). The role of n–3 PUFA incorporation into mitochondrial membranes during NAFLD warrants more scientific exploration.

Cancers

There are data to suggest that the consumption of n–3 PUFAs decreases the risk of various cancers, including breast cancer, colon cancer, prostate cancer, and leukemias (221–224). The mechanisms by which n–3 PUFAs destroy cancer cells are multifaceted. These mechanisms include suppression of AA-derived eicosanoids by decreasing the n–6-to-n–3 PUFA ratio, influencing transcription factors and gene expression, increasing oxidative stress, and altering cellular membrane organization (132, 225, 226).

Supplementation with n–3 PUFAs increases the polyunsaturation index and decreases the membrane potential of the IMM, thereby priming the cell to undergo apoptosis (153, 227–229). In HL-60 (promyelocytic leukemia) cells, supplementation with n–3 PUFAs led to induction of the apoptosis cascade by activating various caspase enzymes and releasing cytochrome c from the IMM (223). In addition, n–3 PUFA supplementation also decreased the membrane potential effectively depolarizing the mitochondria (223). As another example, DHA, with the addition of butyrate, induced apoptosis in colonocytes, which protected against tumorigenesis (221). Apoptosis is mediated through Ca^{2+}-mediated pathways, enhancing phospholipid oxidation, and increasing proton leak across the IMM (221, 222). n–3 PUFAs may also have a role in improving outcomes in cancer linked to obesity through anti-inflammatory and pro-resolving mechanisms (230, 231).

Conversely, it has been suggested that n–3 PUFAs increase the antioxidant capacity of the cell by upregulating antioxidant enzyme production, thereby suppressing inflammation, ROS production, and carcinogenesis (224). These pathways are potentially contradictory and further research needs to be done to determine the primary role of n–3 PUFAs in cancer suppression. One likely mechanism is DHA influencing mitochondrial membrane dynamics and thereby interactions with cytochrome c.

Conclusions and Future Directions

Mitochondria have a central role in cellular function across a range of tissues in health and disease. The underlying mechanisms by which SFAs and unsaturated FAs influence mitochondrial membrane structure are in their infancy. However, there are clear indications that dietary FAs remodel the mitochondrial phospholipidome, particularly in dysfunctional mitochondria, which influences a range of mitochondrial responses. More research is needed in FA structure and membrane biophysics to elucidate how FAs go beyond disrupting membrane microviscosity to regulate a range of mechanisms, including MAM formation, microdomain distribution, phospholipid-protein binding, protein activity, antioxidant capacity, apoptosis, and respiration.

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