WARNING: DHA (and fish oil) Impede Critical Cardiac Enzymes

For over a decade I have warned physicians and their patients about the dangers of fish oil supplementation. Independent experiments have shown that fish oil (EPA/DHA) DISPLACES the Critical Parent Omega-6 in tissue and mitochondria.

The IOWA Screening Study (2010) conclusively showed that PEOs improved arterial flexible and increased blood flow. What amazed physicians was that patients previously taking fish oil, and then converting to PEOs had an even better outcome – fish oil worsened their arterial health. I intuitively knew this because Parent omega-6 is what capillaries and intima (inner lining of the arteries) are comprised of, and its derivatives cause increased blood flow through natural vasodilation, etc.; consequently, the cardiovascular system would significantly benefit. But I wasn’t aware that Fish Oil would impede critical cardiac mitochondrial enzymes, too. Now, with a recently published paper, we can explain precisely why that pathway is impaired. Anything impairing enzymatic activity is critical; never forget that the way poisons work is by blocking respiratory enzymes, killing you. The newly published 2018 medical paper (Sullivan, E. Madison, et al., “Docosahexaenoic acid lowers cardiac mitochondrial enzyme activity by replacing linoleic acid in the phospholipidome,” Journal of Biological Chemistry, 2018, 293: 466-2018 Jan 12;293(2):466-483) is not surprising to those that follow my work.

The opening paragraph regarding diabetic patients is shocking because diabetes is the #1 epidemic in the world, with no end in sight. Diabetic patients have 2Xs-4Xs the amount of cardiovascular disease. Fish oil has previously been reported to increasing fasting blood sugar levels in diabetics. Now, we see a marker in diabetic patients of elevated DHA! While extended use of Fish Oil should not be considered, this is especially true of diabetic patients. The researchers reported:

- “Here, we first confirmed that cardiac DHA levels are elevated in diabetic humans relative to controls (by 1.7-fold).
- “Here we used dietary intervention as a tool to study remodeling of mitochondrial\ phospholipids on respiratory enzyme activity.

I had previously written about how cardiolipin (CL) in the mitochondria (cellular powerhouses) require significant amounts of fully functional Parent omega-6 (LA)—the only EFA that should be in its membrane. Diabetic patients with CVD are depleted in Parent omega-6, lowering critical cardiac enzymatic activity. The study showed:
• “[I]n diabetic cardiomyopathies, CL acyl chains undergo modifications that broadly include the **depletion of the most abundant CL species, (18:2),[Parent omega-6]….”

This paper explains that the lipid membrane is a combination of various lipids including saturated fats, cholesterol, etc. (Note: 25%-33% of tissue cell membranes are Parent omega-6 / -3) **DHA energetically impedes the required structure!**

• “Replacement of (18:2),CL [Parent omega-6] with (22:6),CL [DHA] **prevents the formation of lipid domains** by influencing the Gibbs free energy of lipid-lipid mixing.”

Thankfully, the impairment can be easily remedied.

• “(18:2),CL [Parent omega-6] rescues the major remodeling in the cardiolipin lipidome induced by long-term intake of DHA.”

• **Cardiac mitochondrial complex I, IV and V activities are rescued upon introduction of (18:2), [Parent omega-6] CL into the mitochondria of mice consuming DHA.”**

The authors stress, confirming again, that a “high-fat diet had no impact on the cardiac enzymatic activity:

• “These results were in agreement with our previous work that showed a **high fat diet had no influence on mouse cardiac enzyme activity** upon moderate remodeling of phospholipids.”

The authors warn that DHA can lower important liver enzymes, too”

• “In contrast [to the high-fat diet,], remodeling of the phospholipidome upon dietary supplementation with EPA and DHA led to diminished enzymatic activity of select complexes. These results were consistent with a study showing that **fish oil can lower liver mitochondrial complex IV and V activities.”**

Next, the study proved unequivocally that that is isn’t simply the lack of dietary intake of Parent omega-6 causing the cardiac problems, but the replacement of Parent omega-6 caused by the supra-physiologic overdose of fish oils EPA/DHA.

• “These results demonstrate mechanistically that it is **not the loss of linoleic acid alone** that drives the impairment in enzyme function since the Western diet alone did
not impair enzyme activities. **Instead, it was the replacement of linoleic acid with DHA that promoted the reduction in activities**…. Our data suggest that linoleic acid [Parent omega-6] is **key for cardiac mitochondrial enzymatic activity**….

- “The (18:2)₄ [Parent omega-6] CL rescue data advances the field by highlighting the **importance of n-6 PUFAs in mitochondrial membranes.**”

The paper clearly illustrates with detailed graphs that cardiac enzymatic impairment can reach close to 50% decrease in activity. Sadly, many noteworthy scholarly papers are lost in a sea of mediocrity and for that reason I felt compelled to share it with you. I hope you will find this paper useful in your practice.
Docosahexaenoic acid lowers cardiac mitochondrial enzyme activity 
by replacing linoleic acid in the phospholipidome

E. Madison Sullivan\textsuperscript{a,b,*}, Edward Ross Pennington\textsuperscript{a,b,c,*}, Genevieve C. Sparagna\textsuperscript{d}, Maria J. Torres \textsuperscript{b}, P. Darrell Neufer\textsuperscript{e,c}, Mitchel Harris\textsuperscript{c}, James Washington\textsuperscript{a,b}, Ethan J. Anderson\textsuperscript{1}, Tonya N. Zeczycki\textsuperscript{1b}, David A. Brown\textsuperscript{g}, and Saame Raza Shaikh\textsuperscript{a,b,c}

\textsuperscript{a}Department of Biochemistry & Molecular Biology, \textsuperscript{b}East Carolina Diabetes & Obesity Institute, Brody School of Medicine, East Carolina University, 115 Heart Drive, Greenville, NC 27834, USA
\textsuperscript{c}Department of Nutrition, Gillings School of Global Public Health and School of Medicine, The University of North Carolina at Chapel Hill
\textsuperscript{d}Department of Medicine, Division of Cardiology, University of Colorado Denver Anschutz Medical Campus, Aurora, CO 80045, USA
\textsuperscript{e}Department of Physiology, Brody School of Medicine, East Carolina University, 115 Heart Drive, Greenville, NC 27834, USA
\textsuperscript{f}Department of Pharmaceutical Sciences and Experimental Therapeutics, College of Pharmacy, Fraternal Order of Eagles Diabetes Research Center, University of Iowa, Iowa City, IA 52242, USA
\textsuperscript{g}Department of Human Nutrition, Foods, and Exercise, Virginia Tech Corporate Research Center, 1981 Kraft Drive, Blacksburg, VA 24060, USA

*Both authors contributed equally

\textbf{Running Title:} DHA remolds the cardiac lipidome to lower enzyme activity

To whom correspondence should be addressed: 
Saame Raza Shaikh, The University of North Carolina at Chapel Hill 
Telephone: 919-843-4348 
Email: shaikhsa@email.unc.edu

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\textbf{Abstract}
Cardiac mitochondrial phospholipid acyl chains regulate respiratory enzymatic activity. In several diseases, the rodent cardiac phospholipidome is extensively rearranged; however, whether specific acyl chains impair respiratory enzyme function is unknown. One unique remodeling event in the myocardium of obese and diabetic rodents is an increase in docosahexaenoic acid (DHA) levels. Here, we first confirmed that cardiac DHA levels are elevated in diabetic humans relative to controls. We then used dietary supplementation of a Western diet with DHA as a tool to promote cardiac acyl chain remodeling and to study its influence on respiratory enzyme function. DHA extensively remodeled the acyl chains of cardiolipin (CL), mono-lyso CL, phosphatidylcholine, and phosphatidylethanolamine. Moreover, DHA lowered enzyme activities of respiratory complexes I, IV, V, and I+III. Mechanistically, the reduction in enzymatic activities were not driven by a dramatic reduction in the abundance of supercomplexes. Instead, replacement of tetralinoleoyl-CL with tetracosahexaenoyl-CL in biomimetic membranes prevented formation of phospholipid domains that regulate enzyme activity. Tetracosahexaenoyl-CL inhibited domain organization due to favorable Gibbs free energy of phospholipid mixing. Furthermore, \textit{in vitro} substitution of tetralinoleoyl-CL with tetracosahexaenoyl-CL blocked complex-IV
binding. Finally, reintroduction of linoleic acid, via fusion of phospholipid vesicles to mitochondria isolated from DHA-fed mice, rescued the major losses in the mitochondrial phospholipidome and complexes I, IV, and V activities. Altogether, our results show that replacing linoleic acid with DHA lowers select enzyme activities. For instance, CL binds oxidative phosphorylation complexes I, III, IV, V, and the mobile electron carrier cytochrome c (12-17). However, it is unknown how remodeling of CL to specific fatty acids influences enzyme function.

One unique remodeling event in the mitochondrial phospholipidome is an increase in the abundance of phospholipid species containing the long chain n-3 polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA, 22:6) (6,8,9,18). The elevation of cardiac DHA in differing cardiovascular diseases is highly paradoxical since this fatty acid is considered cardioprotective in animal models with some supporting evidence in humans (19,20). A recent systematic review on the effects of n-3 PUFA supplementation in human subjects suggested that long chain n-3 PUFAs can be used for ameliorating cardiovascular disease risk factors (21). However, not all research supports health benefits of DHA. For instance, a rodent study showed that DHA did not improve cardiomyopathy induced by a Western diet (22).

Here we used dietary intervention as a tool to study remodeling of mitochondrial phospholipids on respiratory enzyme activity. We first confirmed that cardiac DHA levels are elevated in human subjects, which was on phosphatidylcholine (PC), phosphatidylethanolamine (PE), and CL since they are the most abundant phospholipids in the mitochondria by mass at 40%, 30%, and 15-20%, respectively (23). We then determined if the experimental diets impaired mitochondrial respiratory enzyme activities since CL binds several of these enzymes (14,16,24-29). Mechanistically, we investigated if DHA lowered enzyme activities due to modifications in the formation of supercomplexes, assembly of lipid domains that regulate protein activity, and phospholipid-protein binding interactions (30-32). Finally, experiments were conducted to determine if reintroduction of linoleic acid into mitochondria

**Introduction**

Mitochondria have a central role in cardiac physiology by handling differing substrates such as pyruvate and fatty acids to produce the ATP needed to maintain homeostasis. In a range of metabolic diseases such as obesity, type 2 diabetes, heart failure, ischemia-reperfusion injury, and diabetic cardiomyopathies, cardiac mitochondria are subject to considerable dysregulation (1-5). One key impairment with cardiac mitochondria is the extensive remodeling of phospholipid acyl chains, most notably of the unique mitochondrial specific phospholipid, cardiolipin (CL) (6,7). For example, in diabetic cardiomyopathies, CL acyl chains undergo modifications that broadly include the depletion of the most abundant CL species, (18:2)\(_\alpha\), as well as an increase in longer polyunsaturated acyl chains (8,9). In fact, the remodeling of mitochondrial phospholipid acyl chains is not just limited to cardiomyopathies, but is also reported with various other conditions such as aging, obesity, and Barth Syndrome (10,11).

The mechanisms by which phospholipid acyl chain remodeling promotes mitochondrial dysfunction remain unclear. Phospholipid acyl chains, particularly linoleic acid associated with CL, bind a multitude of trans-membrane and membrane associated enzymes to regulate their activity. For instance, CL binds oxidative phosphorylation complexes I, III, IV, V, and the mobile electron carrier cytochrome c (12-17). However, it is unknown how remodeling of CL to specific fatty acids influences enzyme function.

1 **Abbreviations:** (18:2)\(_\alpha\)CL (tetralinoleoyl-cardiolipin), (22:6)\(_\alpha\)CL (tetradocosahexaenoyl-cardiolipin)
Cardiolipin (CL), Docosahexaenoic Acid (DHA), Eicosapentaenoic Acid (EPA), Monolysocardiolipin (MLCL), Phosphatidylcholine (PC), Phosphatidylethanolamine (PE), n-3 Polyunsaturated Fatty Acids (n-3 PUFAs)
isolated from mice consuming DHA rescued remodeling in the phospholipidome and thereby enzymatic function.

Results

Cardiac DHA levels are elevated in human diabetics. Data from rodent studies demonstrate that cardiac DHA levels are elevated in differing diseases (9,18); however, evidence in humans is lacking. Therefore, cardiac DHA levels from non-diabetic and diabetic subjects were first assayed. Mitochondria could not be isolated since there was extremely limited tissue samples from surgery. Furthermore, fatty acid analyses had to be conducted with total extracted fatty acids due to the small amount of tissue obtained during surgery. Analyses with GC revealed that diabetics, relative to non-diabetic controls, had no changes in the levels of saturated fatty acids (Fig. 1A). The monounsaturated fatty acid 16:1 was lowered by 2.4 fold with diabetic subjects relative to the non-diabetics (Fig. 1B). N-6 PUFA levels were not modified (Fig. 1C). Analyses of the major n-3 PUFAs showed that DHA levels were increased by 1.7 fold for the diabetic subjects compared to non-diabetic controls (Fig. 1D).

Mice consuming Western diets in the absence and presence of EPA and DHA generally have impaired glucose clearance. Subsequent studies were conducted with mice. It was first established how murine experimental diets influenced fat/lean mass, glucose clearance, and fasting insulin levels. The effects of EPA and DHA were tested at two different time points, including consumption of the fatty acids for 14 weeks or for 4 weeks (Fig. 2A). Mice fed the Western diet and the EPA or DHA enriched Western diets had increased total body weights by 1.3-1.4 fold relative to the lean control (Fig. 2B). The increase in total body weight was accounted for by an increase in fat mass with the Western and EPA or DHA enriched Western diets by 1.9-2.1 fold relative to the control (Fig. 2C). Mice fed the Western+DHA diet (14 and 4 wks) and the Western+DHA diet (4 wks) had elevated lean mass compared to lean animals (Fig. 2D). Mice fed the Western diets in the absence or presence of EPA or DHA generally had diminished glucose clearance (Fig. 2E), as quantified by the area under the curve (AUC) for glucose clearance (Fig. 2F). The only exception was the Western diet+EPA (4 wks) (Fig. 2F). Mice fed the Western diets in the absence and presence of EPA (4 wks) or DHA (14 wks and 4 wks) had increased fasting insulin levels in comparison to lean mice (Fig. 2G). The Western+DHA diet (14 and 4 wks) increased fasting glucose levels compared to the control and/or the Western diet (for 14 wks) (Fig. 2H). HOMA-IR scores were consistently increased with all of the Western diets, except the WD+EPA (4 wks), relative to lean mice (Fig. 2I). There were no statistically significant differences between EPA and DHA (14 and 4 wks) for HOMA-IR scores. Altogether, the data suggested that supplementation with EPA or DHA did not improve glucose clearance although long-term intake with EPA did not increase the HOMA-IR score relative to lean mice, consistent with previous work (33).

Murine cardiac mitochondrial CL and monolysosyl-CL acyl chains are dramatically remodeled in response to Western diets in the absence and presence of EPA and DHA. We next established how the Western diets, particularly in the presence of EPA and DHA, targeted CL and monolysosyl-CL (MLCL) acyl chains. An example of the raw LC/MS data are presented in Fig. S1A-D. LC/MS analyses are presented as heat maps in Figure 3. CL species were classified as either major (Fig. 3A), intermediate (Fig. 3B), or minor (Fig. 3C) in terms of abundance. MLCL levels are shown in Fig. 3D.

The most abundant CL species, (18:2)\textsubscript{n}, was robustly reduced with all of the Western diets by 4.3-20.0 fold (Fig. 3A, Fig. S2A-F). The Western diet, in the absence of EPA (4 and 14 wks) or DHA (4 and 14 wks), also increased a range of other major CL species relative to the control and the Western diet (Fig. 3A). CL species containing 22:6 were elevated in response to EPA intervention and robustly with DHA intervention (4 and 14 wks). The increase in differing CL species came at the expense of lowering a range of CL species, notably containing 18:2 and 20:4 acyl chains (Fig. 3A). Several intermediate and minor CL species were also increased or decreased with the Western diet, in the absence or presence of EPA or DHA, when compared to the control by 2.0-14.0 fold (Fig. 3B, 3C). There were also some differences between EPA and DHA (Fig. S2A-F).
DHA remodels the cardiac lipidome to lower enzyme activity

Murine cardiac mitochondrial PC and PE acyl chains are remodeled in response to a Western diet in the absence and presence of EPA and DHA. LC/MS analyses were also conducted with PC and PE phospholipids. Generally, the Western diet in the absence and presence of EPA (14 wks and 4 wks) and DHA (14 and 4 wks) lowered the abundance of PCs containing 16:0, 18:1, 18:2, and 20:3 acyl chains by 1-2-fold compared to the lean controls (Fig. 4A). For some species such as (18:2)_{PC}, the effects were more robust with DHA (14 and 4 wks) compared to EPA (14 and 4 wks) (Fig 4A). DHA also notably increased the levels of (18:2)(20:4)PC compared to control, Western diet, and Western diet+EPA fed mice. Similar trends were observed with PEs, where the Western diet in the absence or presence of EPA (14 and 4 wks) and DHA (14 and 4 wks) lowered species containing 16:0, 18:1, and 18:2 acyl chains (Fig. 4B). DHA had stronger effects than EPA on some PEs (Fig. 4B).

Cardiac mitochondrial respiratory enzyme activities are decreased with the EPA and DHA enriched Western diets. Subsequent experiments addressed if the aforementioned changes in the mitochondrial phospholipidome were associated with impaired respiratory enzyme activities. We focused on long-term of intake of EPA and DHA (14 wks). Strikingly, mice that consumed the Western diet did not have a reduction in enzyme activities and combined enzyme activities when compared to the lean control (Fig. 5A-G). The Western+EPA and Western+DHA diets (14 wks) respectively reduced complex I activity by 1.8 and 1.9 fold relative to the control diet (Fig. 5A). The EPA and DHA containing diets had no effect on complex II (Fig. 5B) and complex III (Fig. 5C) activities. Complex IV activity was lowered with the EPA or DHA enriched Western diets relative to the control and/or Western diet by 1.8-2.7 fold (Fig. 5D). The Western+DHA diet (14 wks) also modestly reduced complex V activity by 0.6 fold relative to the control (Fig. 5E). The Western+DHA diet decreased the combined activities of complex I+III by 2.0 fold compared to the control (Fig. 5F). There was no effect of EPA and DHA enriched Western diets on complex II+III activities (Fig. 5G).

EPA and DHA do not robustly suppress formation of the major supercomplexes. The next study addressed if decreased enzyme specific activities with EPA or DHA (14 wks) were mechanistically driven by a reduction in mitochondrial supercomplex formation or in the expression levels of the complexes (34). Long-term intake of EPA or DHA had no major effect on the majority of the supercomplexes, which are depicted as supercomplexes 1-5 (Fig. S3A-F) (35). The notable exception was that mice consuming the Western+DHA diet had a reduction in the amount of supercomplex 4 when compared to the control and Western diets by 2.5 and 2.7 fold, respectively (Fig. S3E). Blue native (BN)-PAGE analysis also revealed that there were no changes in complexes I, II, III, IV, and V expression in the Western and EPA or DHA enriched Western diets compared to the lean control (data not shown).

Replacement of (18:2)_{CL} with (22:6)_{CL} prevents the formation of lipid domains by influencing the Gibbs free energy of lipid-lipid mixing. Given that DHA did not robustly impair the abundance of the majority of supercomplexes, we investigated another potential mechanism by which respiratory enzyme activity could be lowered with DHA. We used a biophysical approach to determine if DHA could target the formation of CL domains, which control phospholipid-protein binding that is critical for optimal enzyme function (36). Biomimetic mitochondrial membranes modeling the composition of the cardiac inner mitochondrial membrane were used since they allow for a controlled model system. (22:6)_{CL} was specifically investigated given that it is a symmetric phospholipid and was elevated in response to DHA in the diet (Fig. 3A, Fig. S2F). Cytochrome c was added to the biomimetic membranes since it known to induce domain formation in giant unilamellar vesicles (37).

Imaging revealed that (18:2)_{CL} promoted the formation of phase separated domains (as visualized by NAO), which were diminished upon replacement of (18:2)_{CL} with (22:6)_{CL} (Fig. 6A). A range of phase separated domain areas were measured with the NAO fluorescent probe and are presented as a frequency distribution. A Gaussian fit was applied to the frequency distributions with larger areas reflecting greater coverage of the NAO fluorophore on the perimeter.
of the giant unilamellar vesicles (Fig. 6B). Visual inspection of the frequency distributions of the domains were clearly different between (18:2)₄CL and (22:6)₄CL (Fig. 6B). Quantification of the average area occupied by the NAO probe showed that DHA prevented formation of phase separated domains (Fig. 6C).

To explain how (22:6)₄CL prevented domain formation, monolayers of the same lipid mixtures modeling the inner mitochondrial membrane were constructed containing either (18:2)₄CL (Fig. 6D) or (22:6)₄CL (Fig. 6E) in the absence or presence of cytochrome c. This model system allowed us to investigate phospholipid mixing properties that are central for the formation of phase separated lipid domains (38). The monolayer pressure area isotherms (Fig. 6D, 6E) were then used to calculate the change in excess area per molecule in response to cytochrome c. The excess area per molecule values provide a quantitative change at the angstrom level on how phospholipids are mixing or de-mixing (i.e. lipid-lipid miscibility) (39).

In biomimetic mitochondrial monolayers containing (18:2)₄CL, the change in excess area per molecule was positive, indicating unfavorable lipid mixing (i.e. formation of phase separated domains) (Fig. 6F). Upon replacement of (18:2)₄CL with (22:6)₄CL, this value became negative, indicating favorable mixing between phospholipids (i.e. no formation of phase separated domains). We further calculated the change in Gibbs free energy of mixing upon the addition of cytochrome c. (18:2)₄CL had a positive change in Gibbs free energy of mixing, indicating unfavorable mixing of phospholipids (Fig. 6G). Replacement of (18:2)₄CL with (22:6)₄CL shifted the Gibbs free energy of mixing to a favorable negative value. Thus, these results established that (18:2)₄CL promoted phase separated domains due to unfavorable lipid mixing. In contrast, the lack of domains in the presence of (22:6)₄CL was driven by favorable Gibbs free energy of mixing between phospholipids.

Replacement of (18:2)₄CL with (22:6)₄CL decreases the major remodeling in the cardiolipin lipidome induced by long-term intake of DHA. The last set of experiments tested the possibility that the replacement of the predominant CL fatty acid, linoleic acid (18:2) with DHA (22:6), was driving the reduction in enzyme activity. Therefore, we fused cardiac mitochondria isolated from mice consuming the Western+DHA (14 wks) diet with small unilamellar vesicles comprised of (18:2)₄CL. We ensured that (18:2)₄CL fusion did not influence the fatty acids associated with PC or PE. LC/MS heat maps show no alterations in any of the acyl chains associated with PC (Fig S4A) and PE (Fig. S4B) upon fusion with (18:2)₄CL.

CL and MLCL were analyzed in response to the fusion. LC/MS analyses are presented as heat maps in Figure 8 for CL species that were classified as either major (Fig. 8A), intermediate (Fig. 8B), or minor (Fig. 8C) in terms of abundance. MLCL was also analyzed (Fig. 8D). Several intermediate and minor CL species were altered in the (18:2)₄CL mitochondrial fusion when compared to control and Western+DHA diets by 1.7-10.0 fold (Fig. 8B, 8C). Notably, the most abundant CL species, (18:2)₄CL, was increased back to control levels with the fusion of (18:2)₄CL
DHA remodels the cardiac lipidome to lower enzyme activity by 12.6 fold relative to the Western+DHA (14 wks) diet (Fig. 8A). In addition, fusion with (18:2)\textsubscript{CL} increased (18:1)(18:2)\textsubscript{CL} by 4.3 fold relative to the Western+DHA (14 wks) diet (Fig. 8A). The fusion with (18:2)\textsubscript{CL} also decreased several other CL species by 3.1-4.7 fold relative to the Western+DHA (14 wks) diet (Fig. 8A). Relative to the control, fusion with (18:2)\textsubscript{CL} still displayed decreased levels of some CL species such as (18:1)(18:2)\textsubscript{CL} (Fig. 8A).

**Cardiac mitochondrial complex I, IV and V activities are rescued upon introduction of (18:2)\textsubscript{CL} into the mitochondria of mice consuming DHA.** We determined if improvement in the CL lipidome of DHA-fed mice upon fusion of (18:2)\textsubscript{CL} rescued the impaired oxidative phosphorylation enzyme activities. The fusion of (18:2)\textsubscript{CL} to control samples had no effect on enzyme activity (data not shown). The fusion of (18:2)\textsubscript{CL} to mitochondria isolated from Western+DHA (14 wks) rescued complex I activity (Fig. 9A). The fusion of (18:2)\textsubscript{CL} with the Western+DHA (14 wks) diet did not influence complex II activity (Fig. 9B) and complex III activity was reduced relative to the control diet by 2.5 fold (Fig. 9C). (18:2)\textsubscript{CL} fusion rescued complex IV and complex V activities by 2.4 and 2.5 fold relative to the Western+DHA diet (Fig. 9D-E). Complex II and II+III activities were not influenced by the introduction of (18:2)\textsubscript{CL} (Fig. 9F, G).

**Discussion**

The rationale for the study was based on previous data showing rearrangement of cardiac mitochondrial phospholipid acyl chains, particularly CL, in several diseases like cardiomyopathies and diabetes, as well as aging (8-11,18). In such scenarios, decreased mitochondrial supercomplex formation, decreased mitochondrial respiratory function, increased oxidative stress and elevated cardiac DHA acyl chains levels are generally observed (18,45-54).

Herein, we first confirmed mouse studies by demonstrating that DHA levels were elevated in human type II diabetics. A limitation of this study was that we were not able to assay if DHA was specifically associated with the polar lipid pool, particularly CL. This was due to a very limited tissue sample size obtained during surgery.

Given that the concentration of free fatty acids is significantly low in the heart (55), it is likely that DHA levels represent polar and neutral lipids. We also acknowledge that the size of the samples, which were very difficult to obtain, was small. Thus, future studies will need to further confirm if DHA levels, particularly in CL, are elevated in human subjects while controlling for various confounding variables.

Following the human study, we performed experiments in a murine model to determine the potential underlying mechanisms by which DHA acyl chains influence mitochondrial enzymatic activity. A striking finding from the mouse study was that the Western diet in the absence of EPA or DHA dramatically remodeled the lipidome of the three major phospholipids of the mitochondria, but had no effect on enzyme activity, even upon a strong reduction in (18:2)\textsubscript{CL} levels. These results were in agreement with our previous work that showed a high fat diet had no influence on mouse cardiac enzyme activity upon moderate remodeling of phospholipids (56). In contrast, remodeling of the phospholipidome upon dietary supplementation with EPA and DHA led to diminished enzymatic activity of select complexes. These results were consistent with a study showing that fish oil can lower liver mitochondrial complex IV and V activities (57).

DHA potentially lowers cardiac mitochondrial enzyme activities by influencing domain organization and protein-lipid binding. The data suggest two potential mechanisms, which are not mutually exclusive, by which DHA lowered respiratory enzyme function. One possibility was that DHA prevented CL domain formation. Studies with biomimetic membranes specifically showed that (22:6)\textsubscript{CL} had a stronger effect than (18:2)\textsubscript{CL} on domain formation, which was driven by the ability of DHA acyl chains to promote phospholipid mixing. The other possibility was that DHA prevented binding of linoleic acyl chains to specific complexes.

There are several binding sites within complex I and IV that could be influenced by DHA. There are 9 binding sites for CL in bovine complex I and depletion of CL from complex I renders the enzyme inactive (24,25). Similarly, CL is required for complex IV activity with 4 known CL binding sites of which two are considered high
affinity. The high affinity CL binding sites are associated with the regulation of electron transport and the loss of CL at these sites lowers enzymatic activity (26,27). The two low affinity sites are also important in the structural integrity of the complex in its dimer form (28,58). Thus, DHA acyl chains may lower enzyme function due to diminished binding of linoleic acid to either of the two complexes. Indeed, studies to determine the strength of the phospholipid-protein interactions with complex IV revealed that biomimetic membranes containing (22:6)ω3CL did not bind to complex IV while those containing (18:2)ω6CL exhibited significant interactions with complex IV (presumably both the low and high affinity CL-binding sites). Disruption of (22:6)ω3CL binding to complex IV could result in reduced conformational flexibility or electron transport, effectively reducing enzyme activity. Subsequent studies will need to test binding of other DHA-containing CL species with complex IV and other complexes. It was beyond the scope of this study to test CL binding kinetics with all of the complexes.

Complex V activity was also lowered with DHA, which could be due to a specific disruption in CL domain formation. Complex V forms dimers and CL is specifically needed to assemble the enzymes into larger oligomeric structures, which effects energy efficiency (16,29,59). It is possible that DHA, due to unique conformational flexibility, imparted disorder on the bilayer that lead to an impairment in the formation of higher order complex V oligomers that rely on the microdomain environment.

The effects of DHA on domain organization could also explain the reduction in complex I+III activity. Perhaps DHA lowered the ability of coenzyme q, the mobile electron carrier from complex I to complex III, to diffuse appropriately as the domains were disrupted. This would then explain why modifications to complex I and III activity alone did not recapitulate the results with complex I to III electron transfer. It is also conceivable that DHA impaired other aspects of membrane biophysical organization such as bending rigidity that could impede electron flow (60).

There are likely additional mechanisms by which DHA could lower respiratory enzyme function. These would include the possibility that an increase in acyl chain unsaturation with dietary DHA could manipulate CL turnover and thereby protein activity (61). In addition, DHA may have indirect effects on enzymatic responses by influencing the expression and activity of phospholipase A2γ, which cleaves select fatty acids from CL to regulate mitochondrial bioenergetics (62).

Despite the clear impact that mitochondrial membrane phospholipid remodeling was having on respiratory complex activity, it is noteworthy that coupled ADP-stimulated respiration was not altered with EPA and DHA (data not shown). This effect mirrored our previous study where EPA/DHA supplementation in high fat-fed mice did not significantly alter maximal respiration in the heart (63). This fact illustrates the complexity of mitochondrial bioenergetics and suggests that while discrete respiratory complexes may be altered individually, or even in aggregate (i.e. supercomplexes), that there are additional factors that impinge on respiratory function as a whole when mitochondria are intact and fully coupled. Altogether, the data provide a roadmap for future studies that will require purification of differing respiratory enzymes to study how DHA acyl chains disrupt enzyme activity in the context of CL-protein binding and formation of CL domains.

**The paradox on DHA.** In conditions such as obesity and type 2 diabetes, that are linked to cardiovascular diseases, pathological remodeling of CL with EPA and/or DHA increases oxidative damage that contributes to mitochondrial dysfunction (64). For instance, a study showed that DHA levels in C2C12 cells were elevated upon upregulation of ALCAT1 (a lyso-CL acyltransferase), which remodels CL to species containing highly oxidizable acyl chains and increased oxidative stress (64). A deficiency in ALCAT1 in a murine model protected against diet induced obesity and insulin resistance (64). Shotgun lipidomic studies by Han et. al. also showed a significant decrease in CL abundance and a profound remodeling of the remaining CL species to include DHA acyl chains in rat diabetic myocardium (9,18).

In contrast, many studies demonstrate that consumption of EPA/DHA can have beneficial effects in obesity and type 2 diabetes through
pleiotropic mechanisms. The mechanisms for improvements with EPA and DHA consist of enhancing insulin signaling, maintaining glucose metabolism, reversing dyslipidemia, and resolving inflammatory signals via targeting of transcription factors and gene expression (65-68). For instance, in a similar study by our group, supplementation of EPA and DHA in high fat diet led to increased levels of 4-hydroxyhexenal (HHE) adducts in the myocardium, products of EPA/DHA specific lipid peroxidation (63). In parallel with the increase in HHE-adducts, antioxidant gene expression increased while mitochondrial ROS production decreased in the heart, suggesting that high fat diet supplementation with EPA/DHA caused a beneficial ‘hormetic’ response in the heart. Similar effects were also seen to some extent in a recent small clinical trial of fish oil in patients prior to cardiac surgery. Anderson et al, showed that patients consuming 4 g/day of EPA/DHA ethyl esters (Lovaza™) had increased myocardial PPARγ activation, upregulated fatty acid metabolic gene expression, greater expression/activity of antioxidant as well as anti-inflammatory enzymes, and increased palmitoylcarnitine supported respiration (69).

The role of linoleic acid in the myocardium.
Introduction of linoleic acid in CL vesicles rescued the decrement in the phospholipidomic profile and enzyme activities of complexes I, IV, and V in response to DHA. These results demonstrate mechanistically that it is not the loss of linoleic acid alone that drives the impairment in enzyme function since the Western diet alone did not impair enzyme activities. Instead, it was the replacement of linoleic acid with DHA that promoted the reduction in activities. The notable exception was complex III activity, which was not restored upon introduction of linoleic acid. Perhaps DHA was bound to complex III in a manner that precluded displacement by linoleic acid associated with CL. Several of the known complex III CL-specific binding sites are buried deep within protein cavities, presumably making displacement of the DHA-containing CL difficult due to structural constraints (15).

The (18:2)ω6 CL rescue data advances the field by highlighting the importance of n-6 PUFAs in mitochondrial membranes. There is considerable debate about the effects of n-6 PUFAs, notably linoleic acid, on cardiovascular health. Initially, linoleic acid was hypothesized to have benefits for cardiovascular outcomes since it lowers serum cholesterol (70,71). However, recent studies challenge this notion about increasing the intake of linoleic acid at the expense of saturated fatty acids (72). For instance, several meta-analyses show that increasing the dietary consumption of n-6 PUFAs, at the expense of saturated fatty acids, does not lower the risk of death from cardiovascular diseases (73-75). In addition to n-6 PUFAs, the role of n-3 PUFAs in cardiovascular diseases is still controversial. Meta-analyses on the consumption of EPA and DHA and coronary heart disease indicate that n-3 PUFAs may be associated with lowered cardiovascular risk (76,77). On the other hand, a recent clinical trial showed that daily treatment with n-3 PUFAs did not decrease the prevalence of cardiovascular mortality or morbidity in patients with multiple cardiovascular risk factors (78). Our data suggest that linoleic acid is key for cardiac mitochondrial enzymatic activity and its replacement, at least with select doses of DHA, could promote impairments.

It is conceivable that EPA and/or DHA could improve cardiac enzyme activities in other model systems. In this model, we did not find that DHA was improving fasting insulin or glucose clearance in contrast to other studies (33,79). Thus, perhaps DHA could improve enzyme activities under conditions in which whole body metabolism is improved. Furthermore, DHA in the triglyceride or free fatty acid form may have different effects on the mitochondrial phospholipidome and enzymatic activity, which was beyond the scope of this study.

Conclusions. The data demonstrate that remodeling of the murine cardiac mitochondrial phospholipidome in the absence of DHA had no influence on mitochondrial enzyme activities. In contrast, remodeling of the phospholipidome with DHA, in particular, lead to a reduction in complex I, IV, V, and I+III activities, potentially through mechanisms involving the formation of lipid domains and phospholipid-protein binding. These results suggest that increased levels of DHA in the myocardium in differing diseases may be targeting enzymatic activity. Furthermore, the impairments with DHA in the lipidome were generally rescued.
with the introduction of linoleic acid accompanied by an improvement in the activities of select complexes. This has implications for future studies on the balance between linoleic acid and n-3 PUFAs in the heart.

**Experimental Procedures**

**Human subjects.** The Institutional Review Board of East Carolina University approved all aspects of human tissue and data collection for this study. The cohorts evaluated were type 2 diabetic and non-diabetic patients at Vidant Hospital undergoing elective coronary artery bypass graft surgery. The demographic and clinical data pertaining to the patients are shown in Table S1. The subjects were grouped either as non-diabetic or diabetic according to two major variables: 1) clinical diagnosis of diabetes; and 2) glycated hemoglobin (HbA1c) values of ≥ 6.1 extending up to approximately 1 year prior to surgery. It is standard of care at Vidant Hospital to give all diabetic patients intra-venous insulin when admitted for surgery and all other pre-operative diabetic medications are noted in Table S1. Patients with enlarged atria, history of arrhythmia, or left ventricular ejection fractions < 30% were excluded from this study.

**Human atrial appendage biopsy, tissue processing, and fatty acid analyses.** After median sternotomy, and prior to institution of cardiopulmonary bypass, a purse-string suture was placed in the right atrial appendage to allow for placement of the venous cannula. A sample of the appendage directly superior to the purse-string was dissected and immediately rinsed in ice-cold saline to remove excess blood, trimmed of the epicardial layer and pericardial fat, and frozen in liquid N2. Total fatty acids from the atrial appendage were extracted with organic solvents (HPLC grade, Sigma) and analyzed by gas chromatography (GC) as previously described (80). Stringent precautions were taken to prevent oxidation (80).

**Animals and diets.** All experiments were conducted in accordance with guidelines established by the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996) and with prior approval by the Animal Care and Use Committee at East Carolina University and at The University of North Carolina at Chapel Hill. Male C57BL/6 mice (Charles River, Wilmington, MA), 5 weeks old, were fed a low fat control diet, a Western diet (42% of kcal from milkfat), or a Western diet with 2% kcal from either EPA or DHA ethyl esters for 14 weeks (Envigo Inc. Indianapolis, IN). Short-term intervention with EPA and DHA was also tested, which entailed feeding mice the Western diet for 10 weeks followed by 4 weeks of the Western diet with either EPA or DHA. EPA and DHA ethyl esters (Cayman Chemicals, Ann Arbor, MI) were greater than 93% purity and were routinely tested for oxidation prior to and during the course of the study. The composition of the experimental diets is presented in Table S2. Mice were housed on a 12:12 h light-dark cycle with free access to water. Mice were sacrificed via isoflurane inhalation followed by cervical dislocation.

**Metabolic profiling.** Mice were fasted for 5 hrs prior to the administration of an intraperitoneal glucose injection (2.5 g/kg fat-free mass) of a 50% dextrose saline solution (Hospira Inc, Lake Forest, IL) (81). Blood glucose was monitored from the tail vein using an AlphaTrak 2 animal glucometer (Abbott Laboratories, Chicago, IL) at 0, 15, 30, 60, and 90 mins post-injection (82). Values were normalized to fasting blood glucose levels and glucose tolerance was assessed by calculating the corresponding area under the curve (AUC). Blood samples, collected after the 5 hr fast using microcapillary tubes, were used to quantify insulin levels with an Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL). Fat and lean masses were determined with whole-body EchoMRI (Active Field Resources, LLC, Houston, TX).

**Isolation of mitochondria.** Mitochondrial isolations were performed on ice and all instruments and buffers were chilled to 4 °C before isolation using our established protocols (56). Cardiac tissue was removed and rinsed in mitochondrial isolation medium (MIM) containing 300 mM sucrose, 10 mM Na-HEPES (pH=7.2) and 1 mM EGTA (Sigma-Aldrich, St. Louis, MO). The tissue was minced for 5 mins and diluted in MIM+BSA (1mg/ml BSA) (pH=7.4). Tissue was then subjected to homogenization with a Teflon Potter homogenizer. The homogenate was
centrifuged at 800 x g for 10 mins and the supernatant was centrifuged at 12,000 x g for 15 mins. The mitochondrial pellet was resuspended in MIM and stored at -80 °C. Protein content was determined using a BCA protein quantification assay (Thermo Fisher Scientific, Waltham, MA).

Electrospray ionization mass spectrometry. Phospholipid molecular species were determined in lipid extracts (0.2 mg) of mitochondrial protein by liquid chromatography with electrospray ionization mass spectrometry (LC/MS) and analyzed as previously described (83).

Small unilamellar vesicle formation and fusion with mitochondria. Small unilamellar vesicles (SUVs) were generated using 0.025 mg of (18:2)2CL (Avanti Polar Lipids, Abalaster, AL) (84). Multilamellar vesicles (MLVs) were first constructed as previously shown (85). SUVs were then formed by the sonication of the MLVs using a Branson Digital Sonifier. SUVs were added to 1 mg of total mitochondrial protein and allowed to gently shake for an hour at 4°C. Excess SUVs were then removed from the fused mitochondria and the mitochondrial pellet was resuspended and stored at -80°C.

Blue-native PAGE for quantifying supercomplex formation. BN-PAGE was performed as previously described (56). Briefly, pelletted mitochondria were resuspended in Native PAGE Sample Buffer (Life Technologies, Carlsbad, CA) and solubilized using an 8:1 digitonin (Sigma-Aldrich, St. Louis, MO) to protein ratio. After solubilization and centrifugation, the supernatants were collected, and protein content was determined via a BCA protein quantification assay (Thermo Fisher Scientific, Waltham, MA). Samples were combined with 5% G-250 sample additive (Life Technologies, Carlsbad, CA) and loaded onto the 3-12% Bis-Tris gel (Life Technologies, Carlsbad, CA). The gel was run on ice at 150 V for 3 hours. Gels were fixed using 40% methanol and 10% acetic acid (Sigma-Aldrich, St. Louis, MO) and destained in 8% acetic acid. Gels were imaged and quantified using the ChemiDoc Imaging System (Bio-Rad, Hercules, CA). Data were normalized to the total amount of protein in each sample.

Construction of biomimetic mitochondrial giant unilamellar vesicles (GUVs) to quantify domain organization. Biomimetic mitochondrial GUVs were constructed by co-dissolving lipids - 39.9 mol% 18:0-22:6 PC, 30.0 mol% 16:0-20:4 PE, 20 mol% (18:2)2CL or (22:6)2CL, 5 mol% 18:1-18:1 phoshatidylinositol (PI), 3 mol% 18:1-18:1 phosphatidylerine (PS), and 2 mol% cholesterol (Chol), with the CL-specific fluorescent probe nonyl acridine orange (NAO) (0.1 mol%), in chloroform (0.5 mg/mL). (22:6)2CL was a custom synthesis from Avanti Polar Lipids. The levels of PC, PE, CL, PI, PS, and Chol approximated ratios found in the inner mitochondrial membrane. 5.0 mg of total lipid was spread onto the conductive side of an indium tin oxide coated glass slide. The lipid-coated slide was subjected to dark vacuum overnight to remove excess solvent. Once the lipid film was dried, a GUV electroformation chamber was assembled as described (86).

GUVs were constructed by electroformation at room temperature as previously described (39). To promote microdomain formation, cytochrome c was added at a 29:1 lipid to protein ratio. Following sample preparation, vesicles were drawn into a rectangular micro-capillary tube, mounted onto a microscope slide, and imaged at 23 °C.

Confocal microscopy and image analysis. Imaging was conducted with an Olympus FV1000 Confocal Microscope using a 60X 1.35NA oil immersion objective (Olympus, Waltham, MA). The NAO probe was excited with an Argon laser at 488 nm. All acquired images were of GUV equatorial cross-sections. Analysis of lipid domains was conducted with NIH ImageJ software as shown previously (39).

Quantification of the Gibbs free energy of lipid mixing. To quantify the Gibbs free energy of lipid-lipid mixing, biomimetic mitochondrial monolayers were generated by co-dissolving lipids (40 mol% 18:0-22:6 PC, 30.0 mol% 16:0-20:4 PE, 20 mol% (18:2)2CL or (22:6)2CL, 5 mol% 18:1-18:1PI, 3 mol% 18:1-18:1PS, and 2 mol% Chol) in chloroform (2.5 mg/mL). Lipid monolayers were constructed by spotting approximately 9.0 nmol on a subphase of 10 mM sodium phosphate buffer (pH 7.4). Biomimetic mitochondrial monolayers were analyzed in the absence and presence of
cytochrome c. Excess chloroform was allowed to evaporate for 10 min prior to cytochrome addition and monolayer compression. Pressure-area isotherms were generated and analyzed as previously described (39). The excess area per molecule and Gibbs free energy were calculated to quantify lipid-lipid mixing, and are presented as the change upon the addition of cytochrome c (39). All lipid mixtures were acquired multiple times to ensure reproducibility.

**Synthesis of large unilamellar vesicles (LUVs).** LUVs were constructed at room temperature, as previously described (39), using the same lipid mixture described above for GUV and monolayer studies containing either (18:2)₄CL or (22:6)₄CL.

**Surface plasmon resonance (SPR).** SPR data were recorded on an OpenSPR system (Nicoya Lifesciences). For all experiments, PBS (10 mM Na₂HPO₄, 2.7 mM KCl, and 137 mM NaCl, pH 7.4) was used as the running buffer. Solubilized complex IV in 25 mM Tris-HCl buffer (pH 7.8), 5 mM EDTA, and 39 mM n-dodecyl β-D-maltoside (Sigma-Aldrich) was diluted to 100 µg/mL in activation buffer and immobilized on a gold-plated carboxyl-functionalized biosensor chip according to manufacturer instructions using carbodiimide crosslink chemistry (Nicoya Lifesciences). After immobilization and blocking (10 min), LUVs in differing concentrations were injected (200 µL) and allowed to interact with the sensor for 10 min at a pump speed of 20 µL/min. Complete dissociation of LUV’s from the immobilized complex IV was observed after 15 min. Interactions between complex IV and the LUVs were determined in triplicate using three different chips to eliminate the possibility of chip-to-chip variability. Sensorgrams obtained for the (18:2)₄CL were globally fit to a one-site binding model using TraceDrawer (Nicoya Lifesciences) to determine the association constant (kₐss, M⁻¹ sec⁻¹), dissociation constant (kₐff, sec⁻¹), and the equilibrium binding constant (Kₐ, µM). Rate constants were obtained from global fits using 4-5 concentrations of LUVs and are presented as average ± S.D. from three separate experiments.

**Mitochondrial kinetic assays.** Kinetic assays were conducted using a UV-Vis 1800 Spectrophotometer at 37°C as previously demonstrated (56). Complex I activity was measured by monitoring the oxidation of 0.8 mM NADH (Sigma-Aldrich, St. Louis, MO) at 340 nm. Complex II activity was measured by the reduction of 80 µM dichlorophenolindophenol (Sigma-Aldrich, St. Louis, MO) at 600 nm. Complex III activity was assayed by monitoring the reduction of 40 µM cytochrome c (Sigma-Aldrich, St. Louis, MO) at 550 nm. Complex IV activity was measured by monitoring the oxidation of 10 µM reduced cytochrome c (Sigma-Aldrich, St. Louis, MO) at 550 nm. Complex V activity was measured by the oxidation of 1 mM NADH (Sigma-Aldrich, St. Louis, MO) at 340 nm. The activity of complexes I+III and II+III were measured by monitoring the reduction of 40 µM cytochrome c at 550 nm. Citrate Synthase activity was assayed using 0.1 mM 5,5-dithio-bis-(2-nitrobenzoic acid) (Sigma-Aldrich, St. Louis, MO). Each reaction was performed in duplicate and all activities were normalized to citrate synthase activity.

**Statistical analyses.** Data are presented as average ± S.D. All data are from multiple independent experiments, as indicated in the figure legends. Each independent experiment consisted of 1 mouse per diet group. The human data were analyzed with an unpaired one-tail t-test since previous studies have established an increase in DHA levels in the myocardium (18); therefore, the null hypothesis for this study was to test if diabetics would increase DHA levels. The mouse data were normally distributed based on a Kolmogorov-Smirnov test. Thus, these results were analyzed with parametric statistics using GraphPad Prism 7 software. Statistical significance was established using a one-way ANOVA followed by a post-hoc Bonferroni test. P < 0.05 was considered significant.
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Conflicts of Interest: The authors declare no conflicts of interest with the contents of this article.

Author Contributions: E.M.S and E.R.P. contributed equally by designing experiments, conducting studies, analyzing data, and writing the manuscript; G.S. conducted MS studies; M.J.T. conducted insulin/glucose testing and Echo MRI, wrote parts of the manuscript; E.J.A. provided isolated cardiac tissue and wrote parts of the manuscript; M.H. conducted fatty acid analyses; P.D.N. designed experiments, wrote parts of the manuscript, and provided intellectual expertise; J.W. conducted surface plasmon resonance studies; T.N.Z. designed experiments, wrote parts of the manuscript, and provided intellectual expertise; D.A.B. designed experiments, and provided intellectual expertise; S.R.S. designed experiments, analyzed data, wrote parts of the manuscript, and assumes responsibility for the entire project.

2 The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.
DHA remodels the cardiac lipidome to lower enzyme activity

References


Table 1. Binding constants for biomimetic mitochondrial membranes containing (18:2)₄CL with complex IV. Large unilamellar vesicles were constructed containing either (18:2)₄CL or (22:6)₄CL. Vesicles were then used for binding studies with immobilized complex IV using surface plasmon resonance. Data are average ± S.D. from 3 independent experiments, n.d. indicates not detectable.

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<th>Constant</th>
<th>(18:2)₄CL</th>
<th>(22:6)₄CL</th>
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<td>kₚₚ</td>
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<td>kᵪᵡ</td>
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<td>Kᵤ</td>
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Figure 1. Type 2 diabetic subjects have elevated cardiac DHA (22:6) levels relative to controls. Relative percentage of (A) saturated fatty acids (SFAs), (B) monounsaturated fatty acids (MUFAs), (C) n-6 polyunsaturated fatty acids (PUFAs) and (D) n-3 PUFAs for control (CON) and type 2 diabetics (T2D). Values represent total fatty acids that were extracted from human tissue. Data are the average ± S.D. from 7-8 subjects per group. Asterisks indicate significance from control (CON) (*p<0.05).

Figure 2. Metabolic profile of mice consuming a Western diet in the absence or presence of EPA and DHA for differing time points. (A) Schematic of the diet feeding schedule for mice consuming the control (CON), Western diet (WD), and WD containing EPA or DHA ethyl esters. (B) Body weights at the end of the 14 week feeding period. (C) Fat and (D) lean mass determined by Echo-MRI. (E) Whole-body glucose tolerance test, performed by intraperitoneal injection of glucose after a 5 hr fast. (F) Area under the curve (AUC), calculated by integration of the raw GTT curves shown in E. (G) Fasting insulin and (H) fasting glucose levels. (I) HOMA-IR scores. Data are the average ± S.D. from 8-20 independent experiments. Asterisks indicate significance from control (CON) (*p<0.05; **p<0.01; ***p<0.001, ****p<0.0001). Horizontal bars with asterisks indicate significance between treatments. For simplicity, statistical significance is not indicated between time points (14 and 4 wks).

Figure 3. Cardiac mitochondrial cardiolipin acyl chains are remodeled in response to a Western diet in the absence and presence of EPA and DHA. Heat maps of (A) major, (B) intermediate and (C) minor CL acyl chains for mice consuming a control (CON), Western diet (WD), WD+EPA, and WD+DHA. (D) Monolys-CL species in cardiac mitochondria are also shown. Data are the average from 5 independent experiments. Asterisks indicate significance from CON (*p<0.05). Crosses indicate significance relative to the WD (‘p<0.05) and hash tags indicate significance between EPA and DHA at 14 or 4 weeks (‘p<0.05). For simplicity, statistical significance is not indicated between time points (14 and 4 wks).

Figure 4. Cardiac mitochondrial PC and PE acyl chains are remodeled in response to a Western diet in the absence and presence of EPA and DHA. Heat maps of (A) PC and (B) PE acyl chains in cardiac mitochondria for mice consuming a control (CON), Western diet (WD), WD+EPA, and WD+DHA. Data are the average from 5 independent experiments. Asterisks indicate significance from
DHA remodels the cardiac lipidome to lower enzyme activity

CON (*p<0.05). Crosses indicate significance from WD (†p<0.05), and hash tags indicate significance between EPA and DHA at 14 or 4 weeks (‡p<0.05). For simplicity, statistical significance is not indicated between time points (14 and 4 wks).

Figure 5. EPA and DHA administration to a Western diet lowers select murine cardiac mitochondrial enzyme activities. (A) Complex I activity, measured by NADH oxidation for mice consuming a control (CON), Western diet (WD), WD+EPA, and WD+DHA. (B) Complex II activity, assessed by DCU reduction. (C) Complex III activity, assayed by cytochrome c reduction. (D) Complex IV activity, measured by cytochrome c oxidation. (E) Complex V activity, measured by the oxidation of NADH. (F) Complex I+III activity measured by NADH oxidation coupled to cytochrome c reduction. (G) Complex II+III activity assayed by succinate oxidation coupled to cytochrome c reduction. Activities were determined relative to total protein content and then normalized to citrate synthase (CS) activity. Data are the average ± S.D. from 5-6 independent experiments. Asterisks indicate significance from CON (*p<0.05, **p<0.01). Horizontal bars with asterisks indicate significance between treatments.

Figure 6. Replacement of (18:2)4CL with (22:6)4CL prevents formation of lipid domains due to a favorable Gibbs free energy of mixing. (A) Sample confocal images of biomimetic giant unilamellar vesicles containing either (18:2)4CL or (22:6)4CL. Cytochrome c (Cyt C) was added to promote phase separation and images were visualized with the CL-specific probe NAO. Biomimetic membranes modeled the composition of the inner mitochondrial membrane. (B) Frequency distribution of the differing areas occupied by the NAO probe on the perimeter of the vesicles. A Gaussian fit was applied to the data. (C) Area average occupied by the NAO fluorophore measured with NIH ImageJ software. Each dot represents a single GUV. Monolayers were also constructed to assay lipid-lipid miscibility. Sample pressure-area isotherms of biomimetic monolayers are presented containing either (D) (18:2)4CL or (E) (22:6)4CL. Lipid-lipid miscibility was quantified at a physiologically relevant surface pressure of 30 mN/m in terms of the change in (F) excess area per molecule and (G) Gibbs free energy of mixing upon cytochrome c addition. Negative values for excess area per molecule and Gibbs free energy indicate favorable lipid-lipid mixing. Positive values indicate unfavorable mixing. Data are average ± SD from 3-6 independent experiments. Asterisks indicate statistical significance relative to (18:2)4CL: *p<0.05, **p<0.01.

Figure 7: Replacement of (18:2)4CL with (22:6)4CL in biomimetic mitochondrial vesicles prevents binding to complex IV. Representative SPR sensorgrams of biomimetic LUVs containing (A) (18:2)4CL or (B) (22:6)4CL binding to complex IV. LUVs modeled the composition of the inner mitochondrial membrane. Solubilized complex IV was immobilized to gold-plated carboxyl sensor chips using EDC/NHS chemistry (Nicoya Lifesciences). Binding of varying concentrations of LUVs were measured as a function of time. Small increases in the signals of (22:6)4CL are attributed to non-specific binding interactions between the LUVs and sensor chip at high concentrations. Data in (A) were globally fit to a one-site binding model (solid black line, TraceDrawer) to determine k_on, k_off, and equilibrium dissociation constants. Binding constants (Table 1) were determined in three separate experiments using three different chips to account for chip-to-chip variability.

Figure 8. Incorporation of (18:2)4CL improves the cardiolipin lipidome of mitochondria isolated from DHA fed mice. Heat maps of (A) major, (B) intermediate and (C) minor CL acyl chains for mice consuming a control (CON), Western diet (WD), and WD+DHA. (D) Monolysoc-CL species in cardiac mitochondria are also shown. Data are the average ± S.E.M. from 3 independent experiments. Asterisks indicate significance from CON (*p<0.05). Crosses indicate significance relative to WD+DHA+(18:2)4CL (†p<0.05).
Figure 9. (18:2)αCL rescues the DHA-induced loss of complex I, IV, and V activities. (A) Complex I activity, measured by NADH oxidation for mice consuming a control (CON), Western diet (WD) and WD+DHA. (B) Complex II activity, measured by DCU reduction. (C) Complex III activity, assayed by cytochrome c reduction. (D) Complex IV activity, measured by cytochrome c oxidation. (E) Complex V activity, measured by the oxidation of NADH. (F) Complex I+III activity assayed by NADH oxidation coupled to cytochrome c reduction. (G) Complex II+III activity, assessed by succinate oxidation coupled to cytochrome c reduction. Activities were determined relative to total protein content and then normalized to citrate synthase (CS) activity. Data are the average ± S.E.M. from 4 independent experiments. Asterisks indicate significance from CON (*p<0.05, **p<0.01). Horizontal bars with asterisks indicate significance between treatments.
DHA remolds the cardiac lipidome to lower enzyme activity

Figure 1
Figure 2
DHA remodels the cardiac lipidome to lower enzyme activity

**Figure 3**

- **A.** Major CL
- **B.** Intermediate CL
- **C.** Minor CL
- **D.** MLCL
**DHA remolds the cardiac lipidome to lower enzyme activity**

**Figure 4**

A. **PC**

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nmol/mg: 40, 60

B. **PE**

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nmol/mg: 50, 100, 150
DHA remolds the cardiac lipidome to lower enzyme activity

Figure 5
DHA remodels the cardiac lipidome to lower enzyme activity

Figure 6
DHA remodels the cardiac lipidome to lower enzyme activity

**Figure 7**

A.  

B.  

![Graph A](image1.png)

![Graph B](image2.png)
DHA remodels the cardiac lipidome to lower enzyme activity

Figure 8
Figure 9
Docosahexaenoic acid lowers cardiac mitochondrial enzyme activity by replacing linoleic acid in the phospholipidome
E Madison Sullivan, Edward Ross Pennington, Genevieve C Sparagna, Maria J Torres, P Darrell Neufer, Mitchel Harris, James Washington, Ethan J Anderson, Tonya N Zeczycki, David A Brown and Saame Raza Shaikh

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