S.I : TRANSLATIONAL ASPECTS OF LIPID BIOLOGY IN THE BRAIN

Docosahexaenoic Acid (DHA) Supplementation Alters Phospholipid Species and Lipid Peroxidation Products in Adult Mouse Brain, Heart, and Plasma

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Abstract
The abundance of docosahexaenoic acid (DHA) in phospholipids in the brain and retina has generated interest to search for its role in mediating neurological functions. Besides the source of many oxylipins with pro-resolving properties, DHA also undergoes peroxidation, producing 4-hydroxyhexenal (4-HHE), although its function remains elusive. Despite wide dietary consumption, whether supplementation of DHA may alter the peroxidation products and their relationship to phospholipid species in brain and other body organs have not been explored sufficiently. In this study, adult mice were administered a control or DHA-enriched diet for 3 weeks, and phospholipid species and peroxidation products were examined in brain, heart, and plasma. Results demonstrated that this dietary regimen increased (n-3) and decreased (n-6) species to different extent in all major phospholipid classes (PC, dPE, PE-pl, PI and PS) examined. Besides changes in phospholipid species, DHA-enriched diet also showed substantial increases in 4-HHE in brain, heart, and plasma. Among different brain regions, the hippocampus responded to the DHA-enriched diet showing significant increase in 4-HHE. Considering the pro- and anti-inflammatory pathways mediated by the (n-6) and (n-3) polyunsaturated fatty acids, unveiling the ability for DHA-enriched diet to alter phospholipid species and lipid peroxidation products in the brain and in different body organs may be an important step forward towards understanding the mechanism(s) for this (n-3) fatty acid on health and diseases.

Abbreviations

4-HHE	4-Hydroxyhexenal
4-HNE	4-Hydroxynonenal
ARA	Arachidonic acid
CHD	1,3-Cyclohexanedione
cPLA2	Cytosolic phospholipases A2
CTL	Control
DHA	Docosahexaenoic acid
HO-1	Heme oxygenase-1
HPLC	High performance liquid chromatography
iPLA2	Ca^{+2}-independent phospholipases A2
LC–MS/MS	Liquid chromatography-mass spectrometry/mass spectrometry
MS	Mass spectrometry
Nrf2	Nuclear factor erythroid 2-related factor 2
PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
dPE	Diacyl-phosphatidylethanolamine
PE-p1	Alkenylacyl-phosphatidylethanolamine or phosphatidylethanolamine plasmalogen
PI	Phosphatidylinositol
PL	Phospholipids
PLA2	Phospholipases A2
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acid
UHPLC	Ultra-high performance liquid chromatography

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Introduction

Docosahexaenoic acid (DHA, 22:6n-3) is an (n-3) polyunsaturated fatty acid (PUFA) abundant in the phospholipids in the brain and retina. DHA not only plays a role in the maintenance of cell membrane fluidity, it also is involved in the production of lipid mediators important in regulating intracellular signaling activities (Belkouch et al. 2016; Jump et al. 2012; Maulucci et al. 2016; Mita et al. 2016; Sun et al. 2018). Its pleiotropic properties has generated interest in examining its role and mechanism(s) of action in health and diseases (Avallone et al. 2019; Bazan et al. 2011; Lauritzen et al. 2016). There is suggestion that abnormalities in DHA during brain development may be an underlying factor for some psychiatric disorders including attention deficit, hyperactivity, and autism spectrum disorders (Agostoni et al. 2017; Claytonet al. 2007; Hashimoto et al. 2014; Muller et al. 2015). Furthermore, studies with animal models have demonstrated ability for (n-3) PUFAs to ameliorate age-related neurodegenerative diseases and to facilitate brain recovery after ischemic stroke and traumatic brain injuries (Bazan et al. 2011; Zhang et al. 2014; Zhu et al. 2018). Aside from its effects on the central nervous system, DHA and other (n-3) PUFAs also offer beneficial effects on other body systems, including the cardiovascular system (Duda et al. 2009; Jump et al. 2012; Sakamoto et al. 2019). Subsequently, it is not surprising that DHA is widely used as a dietary supplement and a nutraceutical across different human populations (Calzada et al. 2010).

DHA and arachidonic acid (ARA, 20:4n-6) are major PUFAs linked to the *sn*-2 position of membrane phospholipids. These PUFAs are metabolically active mediated by phospholipases A2 (PLA2) and energy-dependent fatty acid CoA ligase and acyltransferases through the deacylation-reacylation cycle, also known as the "Land's cycle" (Granger et al. 2019; Sun and MacQuarrie 1989). However, depending on the cell type and conditions, different types of PLA2 are responsible for regulating the metabolism of DHA and ARA (Sun et al. 2014,2018; Yang et al. 2019a). For example, ARA in phospholipids is released through the action of cytosolic PLA2 (cPLA2), and this pathway is known to play a role in the production of prostaglandins, thromboxanes, and leukotrienes, lipid mediators which are largely pro-inflammatory (Sun et al. 2014). In contrary, the release of DHA through the Ca^{+2}-independent PLA2 (iPLA2) is linked to production of resolvins, protectins, and marisins, which are pro-resolving mediators (Bazan 2018; Serhan et al. 2015).

Besides interaction with cyclooxygenases and lipoxygenases, PUFAs are also susceptible to enzymatic and non-enzymatic reactions with oxygen-free radicals, leading to the production of reactive aldehydes, namely, 4-hydroxyhexenal (4-HHE) from DHA and 4-hydroxynonenal (4-HNE) from ARA (Long and Picklo 2010; Yang et al. 2019a). There is evidence that these lipid peroxidation products may offer important physiological functions, due mainly to their ability to form adducts with proteins, nucleic acids, and phospholipids (Cohen et al. 2013; Long and Picklo 2010; Nakagawa et al. 2014; Riahi et al. 2010). However, in light of the "Yin-Yang" mechanism for the metabolism of DHA and ARA, there is also evidence that these peroxidation products are also regulated differently (Yang et al. 2019a). For example, our study with microglial cells demonstrated the increase in 4-HNE but not 4-HHE upon stimulation of the cPLA2/ARA pathway by lipopolysaccharides (Yang et al. 2018). Increases in 4-HNE are also observed in brain associated with neuroinflammatory diseases, including stroke and spinal cord injury (Lee et al. 2012; Ungerer et al. 2020; Zhang et al. 2014). On the other hand, increases in 4-HHE have been shown upon consumption of diets enriched in DHA or fish oil (Calzada et al. 2010; Nakagawa et al. 2014; Yang et al. 2019b). Since both peroxidation products are readily detected in tissues and body fluids, there is increasing interest to examine regulation of these products in health and diseases.

Our recent study demonstrated the effects of maternal DHA-enriched diet to alleviate stress-induced behavioral deficits in offspring mice (Matsui et al. 2018). Subsequently, a follow-up study using the same dietary regimen showed that administration of the DHA-enriched diet to pregnant mothers resulted in altered fatty acid composition in all brain regions, but increase in 4-HHE levels mainly in the cerebral cortex and hippocampus in the offspring pups (Yang,et al. 2019b). In addition to the brain tissue, this study also demonstrated substantial changes in fatty acid composition and levels of lipid peroxidation products in the heart and plasma (Yang et al. 2019b). Considering that (n-3) and (n-6) PUFAs are linked to different classes of membrane phospholipids, an important goal in this study is to determine whether the DHA-supplemented diet may alter lipid peroxidation products and molecular species in different phospholipids in adult mice administered with a control or DHA-enriched diet. In addition to examining the changes in the brain, this study also included determination of lipid peroxidation products and phospholipids species in the heart and plasma.

Materials and Methods

Chemicals and Reagents

4-Hydroxyhexenal (4-HHE, 1 mg in 100 µL of ethanol), 4-hydroxynonenal (4-HNE, 1 mg in 100 µL of ethanol),

and 4-hydroxy hexenal-d$_3$ (4-HHE-d$_3$, 100 µg in 100 µL of methyl acetate) were purchased from Cayman Chemical Co. (Ann Arbor, MI). 1,3-Cyclohexanedione (CHD, 97%), ammonium acetate (HPLC grade), acetic acid (ACS grade), and formic acid (mass spectrometry grade) were purchased from Sigma-Aldrich (St. Louis, MO). C18 Sep-Pak cartridges (1 mL, 100 mg) were obtained from Waters Corporation (Milford, MA). Phospholipid removal cartridges (Phree™, 1 mL) were purchased from Phenomenex Inc. (Torrance, CA). All solvents (HPLC grade) used for sample preparation, UHPLC, and MS analysis were obtained from Thermo Fisher Scientific Inc. (Fair Lawn, NJ).

Animals and Diets

Three-month-old male mice (C57Bl/6J) were purchased from Jackson Laboratories (Bar Harbor, ME) and were given a nutritionally complete, control (CTL) diet (modified AIN-93G #103619) from Dyets Inc. (Bethleham, PA). The CTL diet contained no preformed DHA (Supplemental Table S1), but did contain sufficient amounts of alpha-linolenic acid (ALA,18:3n-3) to meet normal brain DHA requirements (Domenichiello et al. 2015). At the beginning of the study, animals ($n = 12$) were randomly divided into two groups. The control group ($n = 5$ mice) remained on the CTL diet, whereas the experimental group ($n = 7$ mice) was fed a diet containing 1%, by weight, DHA (#103598) for 3 weeks. The reason for the 3-week diet is because this period of time was used in a study in which adult mice fed a fish oil diet with a similar amount of DHA resulted in substantial changes in fatty acids and lipid peroxidation products in different body organs (Nakagawa et al. 2014). The composition of the control and DHA diets have been described previously (Matsui et al. 2018; Yang et al. 2019b) as well as the final fatty acid profiles (Supplemental Tables S1 and S2). The diets were stabilized against auto-oxidation with 0.02 g tertiary-butylhydroquinone/100 g fat. All experiments were approved by the University of Missouri Animal Care and Use Committee (#8945) and were performed in compliance with the National Institutes of Health (NIH) guidelines for the Care and Use of Laboratory Animals.

Sample Preparation

After the feeding regimen, animals were anesthetized with isoflurane and blood was obtained by heart puncture using heparin as an anticoagulant, followed by perfusion with physiological saline. The brain was dissected to obtain left and right cerebral cortices, striatum, hippocampus, and cerebellum. In addition, the perfused heart was also obtained. The blood was centrifuged at 16,100×g for 5–8 min to obtain plasma and was transferred to Eppendorf tubes and frozen at − 80 °C until use. Samples were placed in sterilized aluminum foil, immediately flash frozen in liquid nitrogen, and stored at − 80 °C until use.

For the experiments using LC–MS/MS to determine 4-HHE and 4-HNE, the left cerebral cortex, hippocampus, striatum, and cerebellum, along with the heart were weighed and homogenized for in eightfold volumes of HPLC water using a bullet blender (Next Advance, Inc., Averill Park, NY) as described previously (Yang et al. 2019b). The heart tissue was first pulverized in liquid nitrogen prior to homogenization, and the homogenates were centrifuged at 16,100×g for 20 min at 4 °C. The supernatant was collected and stored at − 80 °C prior to use. Total protein concentration in the supernatant was determined using the Bicinchoninic Acid Assay (Sigma-Fisher, St. Louis, MO).

LC–MS/MS Analysis of 4-HHE and 4-HNE

The sample preparation stage involved three main steps: phospholipids removal, derivatization, and desalting as previously described (Sun et al. 2019; Yang et al. 2019b). Briefly, phospholipids were removed via solid phase extraction using a 1 mL Phree™ cartridge. This step removes phospholipids and helps eliminate matrix effects to allow for lower limits of detection of the peroxidation products. The bed was loaded with 30 µL of homogenized sample and an equal volume of internal standard (4-HHE-d$_3$, 1000 ng/mL). Acetonitrile (500 µL) was spiked with 1% formic acid and was used to elute the analyte of interest. The clear elute was dried under a stream of nitrogen and used for derivatization with a freshly prepared acidified CHD reagent. The mixture was incubated at 60 °C for 1 h and then cooled on ice prior to desalting using a C18 SPE cartridge. The C18 cartridge was pre-conditioned with methanol (0.7 mL) and equilibrated with water (0.7 mL). The cooled derivatized mixture was loaded onto the cartridge, washed twice with water (0.7 mL) and followed by 5% acetonitrile in water (0.7 mL). The derivatized analytes of interest (4-HHE, 4-HNE, and 4-HHE-d3 derivatives) were eluted with 100% acetonitrile (0.7 mL) and dried with a steady stream of nitrogen gas. The samples were reconstituted in 40% methanol in water with 0.1% formic acid (300 µL) and were ready for LC–MS/MS analysis. A Waters Acquity UHPLC system equipped with a quaternary solvent manager was used in conjunction with a C18 reverse phase column (Luna Omega, Phenomenex). A previously developed and validated UHPLC-MS/MS method with a 7 min LC gradient including equilibration was used for the separation and detection of the 4-HHE and 4-HNE derivatives (Yang et al. 2019b). A Waters Xevo, TQ-S triple-quadrupole mass spectrometer with an electrospray ionization source operated in the positive-ion mode was used. Multiple reaction monitoring transition m/z 326.3 ≥ 216.1 Da, m/z 284.2→216.1 Da and 287.2 → 216.1 Da were chosen for

simultaneous monitoring of 4-HNE, 4-HHE , and 4-HHE-d$_3$ (internal standard) derivatives, respectively.

Analysis of Phospholipid Species

Sample Preparation

Four randomly selected frozen samples (half of the left cerebral cortex, half of the heart tissue, and plasma) from the control and DHA groups were sent to Han's laboratory (University of Texas Health Science Center at San Antonio, TX) for analysis of phospholipid species. The reason for selecting only cerebral cortex was because a substantial amount of brain tissue is required for this type of lipid analysis, and in our previous study, similar changes in fatty acid composition (i.e., decrease in ARA and increase in DHA) were observed in all brain regions (Yang et al. 2019b). Tissue samples (~ 10 mg) were pulverize and homogenized in 0.5 mL of ice-cold diluted PBS (0.1x) with a Potter–Elvehjem tissue grinder. Protein assay on individual homogenates was conducted. An aliquot of homogenate was transferred to a disposable glass test tube. A mixture of lipid internal standards for quantification of all reported lipid classes was added to the tube based on the tissue protein content (Wang et al. 2017). Lipid extraction was performed by a modified Bligh and Dyer method as previously described (Wang and Han 2014). All of the lipid extracts were flushed with nitrogen, capped, and stored at − 20 °C.

Mass Spectrometric Analysis of Lipids

Mass spectrometric analysis of lipids was performed with an Altis triple-quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) or a Q-Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an automated nanospray device (Triversa Nanomate, Advion Biosciences, Ithaca, NY) and operated with the Xcalibur software as previously described (Han et al. 2008; Yang et al. 2009). Identification and quantification of all reported lipid molecular species were performed using an in-house automated software program (Wang et al. 2017; Yang et al. 2009). All lipid levels were normalized to sample protein content.

Statistical Analysis

For the determination of 4-HHE and 4-HNE levels via LC–MS/MS, three biological replicates of each tissue sample, and three analytical replicates of each biological replicate were completed for each sample. Statistical analyses were performed with GraphPad Prism (version 8.3; GraphPad Prism Software Inc., San Diego, CA). Results are expressed as the mean ± standard error of mean (SEM).

Depending on the experiments, samples were analyzed by non-parametric t-tests or one-way ANOVA to compare among control and DHA diet groups. Differences were considered significant at $p < 0.05$ for all analyses.

Results

Feeding mice with Control and DHA-Enriched Diets

In this study, adult mice were given control and 1% DHA-enriched diets for 3 weeks. Fresh diets were provided every other day, and body weights of each mouse was obtained every 4 days. As shown in Fig. 1, there was no significant change in body weights between the two groups during the 3-week period with the control and DHA-enriched diets.

Determination of 4-HHE and 4-HNE

Levels of 4-HHE and 4-HNE in cerebral cortex, hippocampus, striatum, and cerebellum in control and DHA supplement groups were determined. Among the different brain regions, only hippocampus showed a small but significant ($p < 0.01$) increase in 4-HHE (Fig. 2). Although there was an apparent increase in 4-HHE in the cerebral cortex, this change was not significant (p = 0.12) due to the large variances. Except for the striatum which showed a small but significant ($p < 0.05$) increase in 4-HNE levels in the DHA-enriched diet group, no apparent changes in 4-HNE levels were found in other brain regions (Fig. 2).

In the heart tissue, levels of 4-HNE were higher than levels of 4-HHE in the control group (Fig. 3a). Nevertheless, supplement with the DHA-enriched diet resulted in an
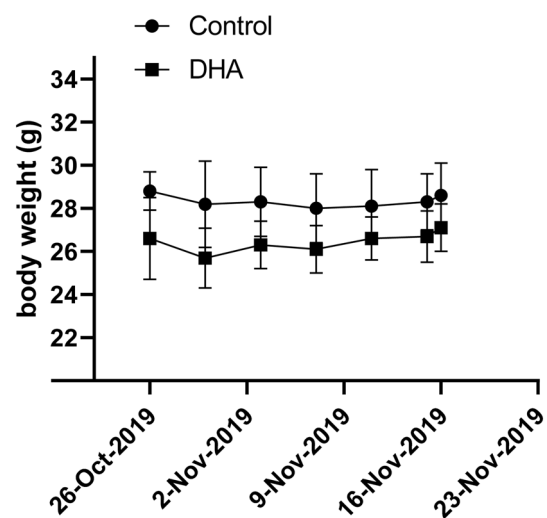

Fig. 1 Body weights of mice in the control and DHA group over the time of the study

Fig. 2 Levels of 4-HHE and 4-HNE in mouse brain regions after feeding with a control or DHA-enriched (1%) diet for 3 weeks. Brain tissues including cerebral cortex, hippocampus, striatum, and cerebellum were dissected and homogenized as described in text. Levels of 4-HHE and 4-HNE were determined by LC–MS/MS protocol as described in text. Data are normalized to tissue weight. Results represent the mean ± SEM of control ($n=5$) and DHA ($n=7$) samples. Analysis using a two-tail unpaired t-test indicated significance between DHA group and controls. $*p < 0.05$; $**p < 0.01$
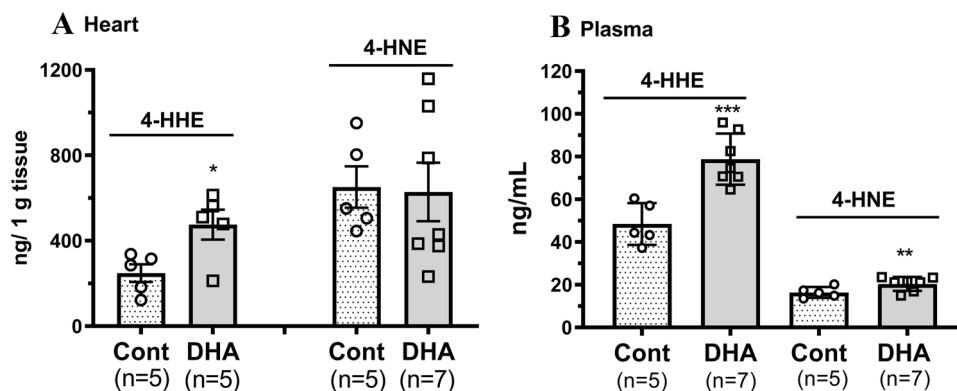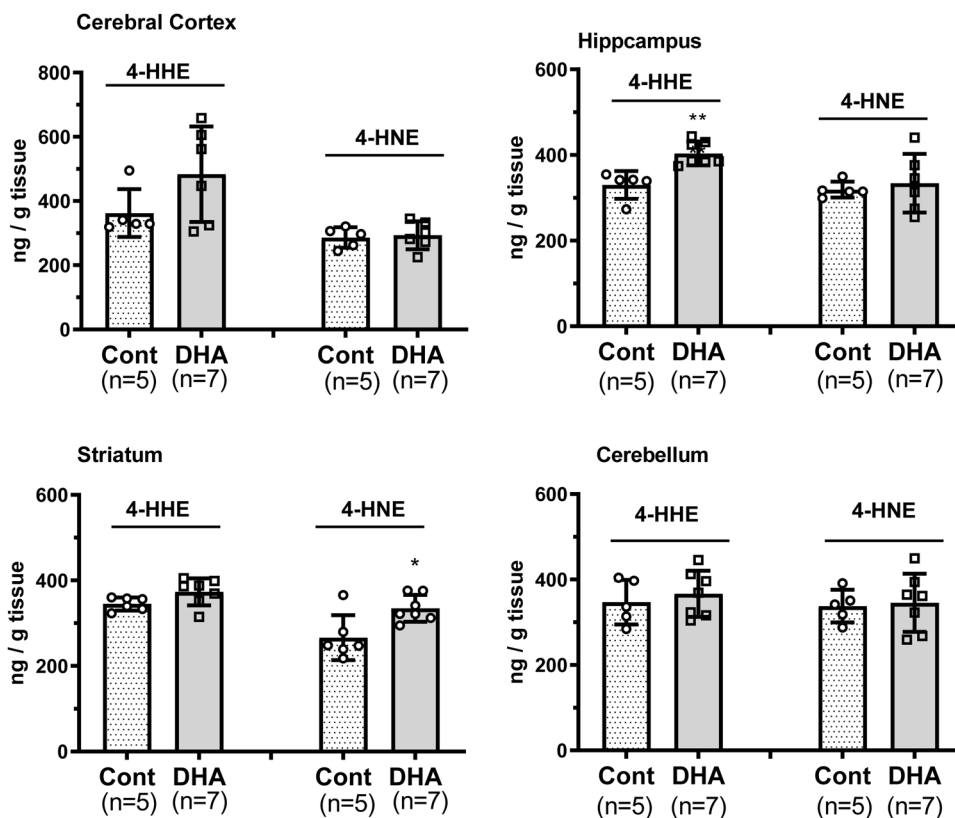

Fig. 3 Levels of 4-HHE and 4-HNE in mouse (**a**) heart and (**b**) plasma after feeding with a control or DHA-enriched diet for 3 weeks. Procedures for processing the heart and plasma and protocol for LC–MS/MS determination of 4-HHE and 4-HNE are described in text. Results represent the mean ± SEM of control ($n=5$) and DHA ($n=7$) samples. Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$

almost twofold increase in 4-HHE ($p < 0.05$) as compared to the control group (Fig. 3a). Again, DHA-enriched diet did not alter 4-HNE levels in the heart tissue (Fig. 3a).

In the plasma samples, there were higher levels of 4-HHE as compared to 4-HNE in the control group (Fig. 3b). Similarly, levels of 4-HHE in the DHA-enriched group were increased significantly ($p < 0.001$) as compared to the controls. In the plasma, the level of 4-HNE in the DHA-enriched group showed a small but significant increase ($p < 0.01$) (Fig. 3b).

Analysis of Phospholipid Species

The major phospholipid classes in the cerebral cortex are diacyl-PE, alkenylacyl-PE (PE-pl, also PE plamalogen), PC, PS, and PI (Fig. 4a). Analysis also detected small amounts of
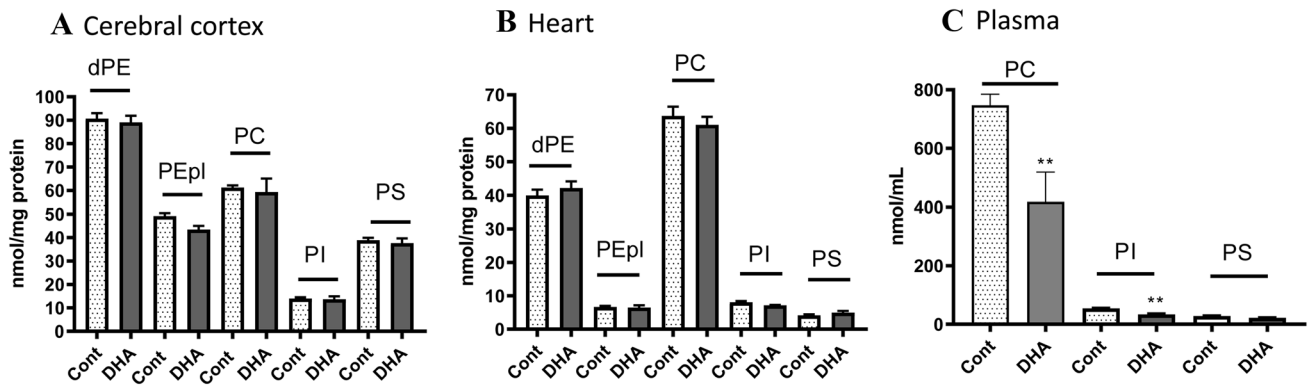
Fig. 4 Levels of phospholipids (diacyl-PE, PE-pl, PC, PI, and PS) in mouse **a** cerebral cortex, **b** heart, and **c** plasma after feeding a control and DHA-enriched diet for 3 weeks. Lipids were extracted and analyzed by the shotgun lipidomics platform as described in text. Data are expressed as nmol/mg protein and are mean ± SEM of control ($n = 4$) and DHA ($n = 4$) samples. Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. Abbreviations: *dPE* diacyl-phosphatidylethanolamine, *PE-pl* alkenylacyl-phosphatidylethanolamine or ethanolamine plasmalogen, *PC* phosphatidylcholine, *PI* phosphatidylinositol, *PS* phosphatidylserine

alkylacyl-PE and alkenylacyl-PC species (data not shown). The major phospholipids in the heart tissue are PC and diacyl-PE, and unlike those in the cerebral cortex, only low levels of PE-pl, PI, and PS were detected (Fig. 4b). In the plasma, although PC was the major phospholipid, low levels of PI and PS were detected (Fig. 4c). However, the levels of diacyl-PE in plasma were too low to allow proper analysis of its molecular species. Levels of individual phospholipid classes in the cerebral cortex and heart were not different when comparing the DHA supplement group with the control group (Fig. 4). Nevertheless, levels of PC and PI in plasma were significantly lower ($p < 0.01$) in the DHA supplement group as compared with the control group (Fig. 4c).

In the cerebral cortex, analysis of the diacyl-PE species containing (n-6) PUFAs showed primarily the 18:0/20:4 species, and those with (n-3) were primarily 18:0/22:6 (Table S3 in the supplemental material). For PE-pl, where linkage in the *sn*-1 position is in alkenyl (ake) form, the species with (n-6) were comprised mainly of 18:0 ake/20:4 and 18:0 ake/22:4, and the (n-3) species were mainly 16:0 ake/22:6. For PC, the (n-6) species were primarily 16:0/20:4 and 18:0/20:4, and the (n-3) species were mainly 16:0/22:6 and 18:0/22:6. For PI, there were high levels of (n-6) species, mainly 18:0/20:4, and low levels of the (n-3) species, mainly 16:0/22:6. For PS, there were low levels of (n-6) species, mainly 18:0/20:4, and high levels of (n-3) species, mainly 18:0/22:6.

Among the diacyl-PE species in the cerebral cortex, there were more (n-3) species than (n-6) species, whereas in PE-pl, the (n-6) species were higher than (n-3) species (Fig. 5a). PC had similar levels of (n-6) and (n-3) species, whereas PI contained high (n-6) species and PS contained high (n-3) species (Fig. 5a). In all phospholipid species analyzed in the cerebral cortex, the DHA-enriched group showed a trend for increase in (n-3) species and a decrease in (n-6) species. In fact, significant ($p < 0.05$) decreases in the (n-6) species were observed in dPE, PE-pl, and PS. When the ratios for (n-6)/(n-3) species in the control and DHA-enriched group were determined, all three major phospholipid classes (diacyl-PE, PE-pl, and PC) showed significant decreases ($p < 0.05$, $p < 0.01$) in (n-6) to (n-3) ratios in the DHA-enriched group as compared with the control group (Fig. 5b).

Analysis of phospholipid species in the heart tissue showed significant larger effects of DHA supplementation to all phospholipid classes. Among the phospholipid classes analyzed (diacyl-PE, PC, PI and PS), the DHA-enriched group showed significant decreases in (n-6) species ($p < 0.001$), and significant increases in the (n-3) species ($p < 0.05$) in all classes examined (Fig. 6). These results clearly indicate that a 3-week DHA-enriched diet is sufficient to cause alterations of membrane phospholipids in the heart tissue.

In the plasma, PC with (n-6) PUFA is the predominant phospholipid species, and DHA-enriched diet resulted in a large and significant decrease ($p < 0.001$) in this phospholipid species (Fig. 7). For PI in plasma, DHA-enriched diet resulted a large decrease in (n-6) and a small increase in (n-3) species ($p < 0.001$, $p < 0.01$, respectively) (Fig. 7). Despite low levels, PS in plasma also showed a decrease in (n-6) and only a small increase in (n-3) species in this phospholipid ($p < 0.01$, $p < 0.05$, respectively) (Fig. 7).

Discussion

In many studies, including our own, there is evidence for the ability for DHA-enriched or diet with (n-3) fatty acids to alter fatty acid composition in brain and other body organs
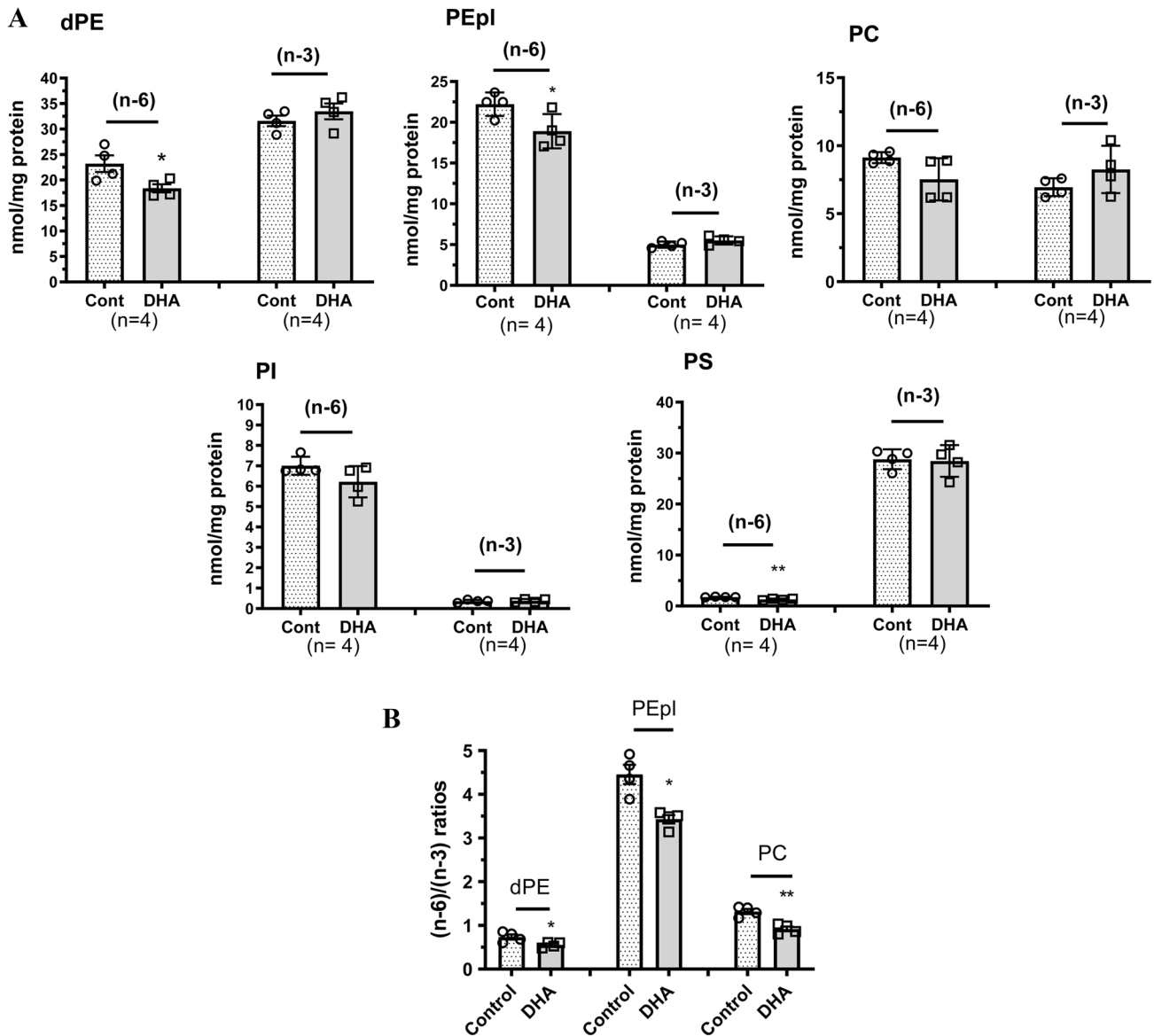
Fig. 5 **a** Levels of (n-3) and (n-6) phospholipid species (dPE, PE-pl, PC, PI and PS) in mouse cerebral cortex after feeding a control and DHA-enriched diet for 3 weeks. Lipids were extracted and analyzed by the shotgun lipidomics platform as described in text. In each phospholipid class, species with fatty acids containing (n-3) or (n-6) were grouped and expressed as nmol/mg protein. **b** Ratios of (n-6)/(n-3) phospholipid species of dPE, PE-pl, and PC from control and DHA group in the cortex using the data from (**a**). Data are mean $\pm$ SEM of control ($n=4$) and DHA ($n=4$) samples. Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. $*p < 0.05$; $**p < 0.01$

(Barceló-Coblijn et al. 2005; Nakagawa et al. 2014; Yang et al. 2019a). In a study by Murphy's group, they further demonstrated increases in (n-3) and decreases in (n-6) fatty acids in different phospholipid classes in brain, heart and liver upon feeding rats with fish oil diet for 8 weeks (Barceló-Coblijn et al. 2005). Unlike the peripheral organs, linoleic acid (18:2n-6) is low in the brain tissue, and thus ARA and DHA contributed to the major (n-6) and (n-3) PUFAs linked to the *sn*-2 position of phospholipids. Despite that both PUFAs are abundant in the brain tissue,

it is well recognized that they undergo different metabolic pathways and participate in different reactions: ARA is the precursor for multiple reactions leading to lipid mediators that are involved in pro-inflammatory functions, whereas DHA are known for production of oxylipins that are pro-resolving (Yang et al. 2019a). Recent studies also demonstrated involvement of both PUFAs in lipid peroxidation and production of 4-HNE from ARA and 4-HHE from DHA. Although the physiological role of these reactive aldehydes has not been clearly understood, there is increasing evidence
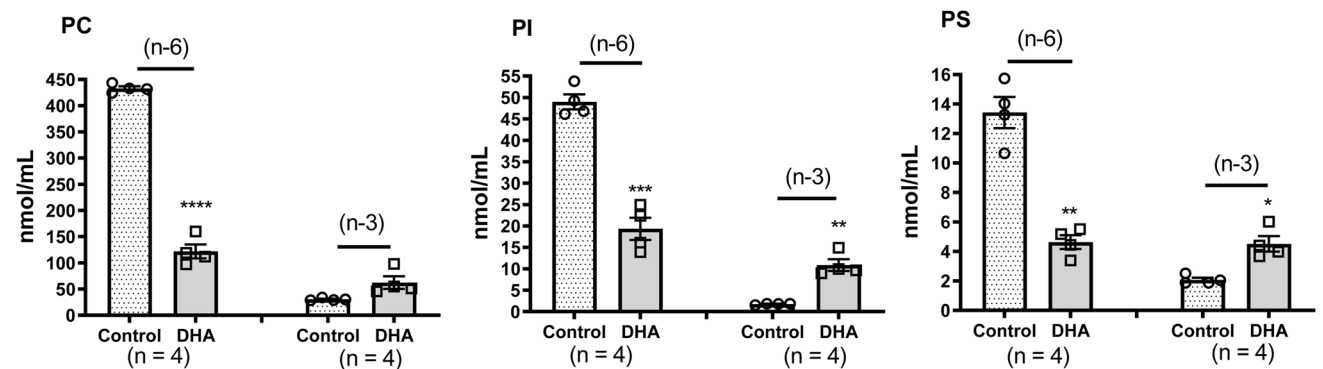
Fig. 6 Levels of (n-3) and (n-6) phospholipid species (dPE, PC, PI and PS) in mouse heart after feeding a control and DHA-enriched diet for 3 weeks. In each phospholipid class, species with fatty acids containing (n-3) or (n-6) were grouped and expressed as nmol/mg protein. Data are mean ± SEM of control ($n = 4$) and DHA ($n = 4$) samples. Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$
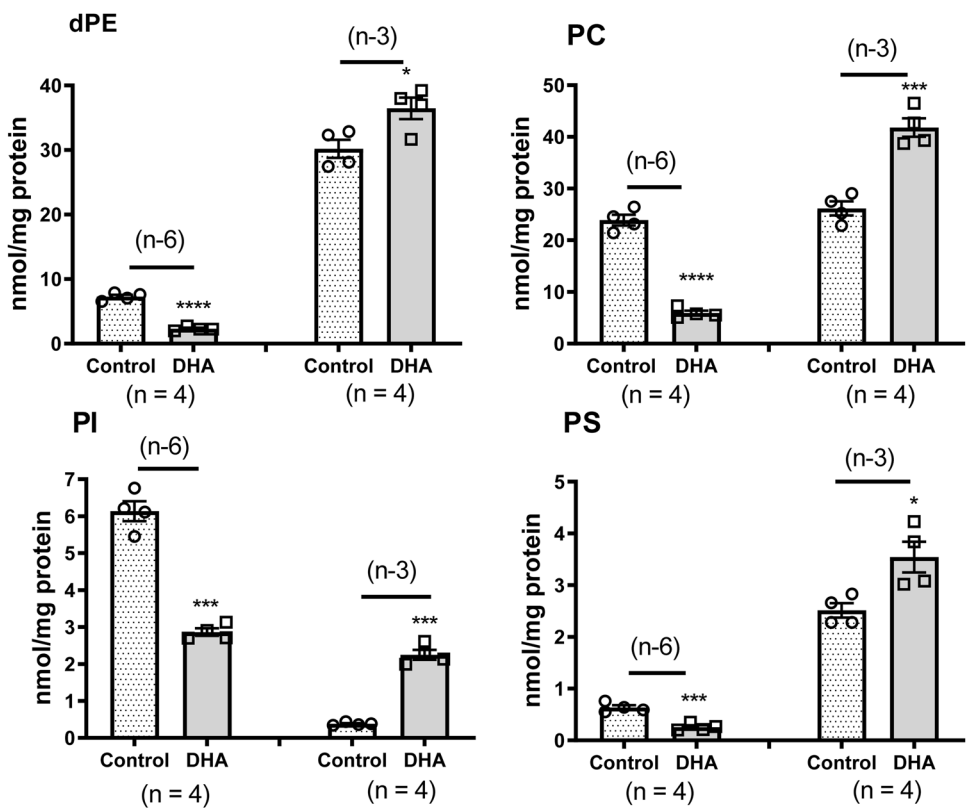

Fig. 7 Levels of (n-3) and (n-6) phospholipid species (PC, PI, and PS) in mouse plasma after feeding a control and DHA-enriched diet for 3 weeks. In each phospholipid class, species with fatty acids containing (n-3) or (n-6) were grouped and expressed as nmol/mg protein. Data are mean ± SEM of control ($n = 4$) and DHA ($n = 4$) samples. Analysis using two-tail unpaired t-test indicated significance between DHA group and controls. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

for their engagement in specific cellular metabolism (Long and Picklo 2010). In our previous study with the maternal mouse model, weaning pups nursed by mothers with the DHA-enriched diet showed increase in 4-HHE mainly in the cerebral cortex and hippocampus, despite that changes in fatty acid composition were observed in all brain regions, including cerebellum (Yang et al. 2019b). In this study with adult mice, a 3-week supplement with DHA-enriched diet again showed increase in 4-HHE in the hippocampus. These results are in agreement with the contention that the

hippocampus is responsive to the DHA-enriched diet in producing more 4-HHE. Interestingly, despite that supplement of DHA-enriched or fish oil diet resulted in a decrease in ARA in all brain regions, there was no obvious decrease in 4-HNE in these brain regions. The study by Nakagawa's group reported changes in fatty acids and peroxidation products (4-HHE and 4-HNE) in plasma and in multiple organs after feeding adult mice with fish oil for 3 weeks (Nakagawa et al. 2014). In their study, besides increase in 4-HHE, a significant decrease in 4-HNE was observed in the plasma. The

discrepancy between our results and those by Nakagawa's group is not known. In a study by Calzada et al. healthy human subjects given DHA supplement ranging from 200 to 1600 mg/day for 2 weeks also showed a dose-dependent increase in 4-HHE (but not 4-HNE) in plasma (Calzada et al. 2010). Therefore, these studies with animal models and humans provided consensus that DHA-enriched and fish oil diets are associated with increase in 4-HHE. Taken together, these results also show that determination of 4-HHE levels in plasma can be a useful biomarker for assessing DHA consumption. Since there is a concern that the increase in 4-HHE due to DHA-enriched or fish oil diets may be due to contamination of this peroxidation product in the diet, an attempt was made to determine the 4-HHE and 4-HNE content in the control and DHA-enriched diets used in our experiments. Our results indicated no detectable 4-HHE in the diets although trace amounts of 4-HNE (control 7.62 ± 0.21 versus DHA 5.73 ± 0.15 ng/g) were detected in both control and DHA-enriched diet. The rationale for detecting 4-HNE in both control and DHA-enriched diets is not clear but since we could not detect 4-HHE, these results clearly show that the increase in 4-HHE levels in brain and other organs upon feeding the DHA-enriched diet is a physiological phenomenon. Several studies with cell and animal models have demonstrated the hormetic effects of 4-HHE through upregulation of the Nrf2 pathway and production of heme oxygenase-1, a potent antioxidant enzyme (Geng et al. 2020; Ishikado et al. 2010; Nakagawa et al. 2014; Yang et al. 2018). However, more studies are needed to examine possible physiological roles of these lipid peroxidation products in the heart and other body systems (Long and Picklo 2010).

In this study, we examine changes in phospholipid species in the brain, heart and plasma after feeding with DHA-enriched diet for 3 weeks. In agreement with the presence of the blood brain barrier, uptake of fatty acids to the brain and their turnover are slower as compared to peripheral tissues such as heart (Patrick et al. 2005). Phospholipid classes in the cerebral cortex are comprised primarily of dPE, PE-pl, PC, PI and PS (Fig. 4). Analysis of the molecular species of dPE, PE-pl, and PC in this brain region indicate small increase in (n-3) and decrease in (n-6) species for these phospholipids (Fig. 5a). Although determination of (n-6)/(n-3) ratios showed significant decrease for dPE, PE-pl and PC, it is obvious that a longer feeding time is required to obtain significant outcome (Bascoul-Colombo et al. 2016; Lamaziere et al. 2011). In this study, it is of interest that PE-pl showed a high level of (n-6) species, and the decrease in (n-3)/(n-6) ratios in PE-pl was greater for diacyl-PE and PC. In a study by Rosenberger et al. a 2-h i.v. injection of labeled hexadecanol (precursor for the alkyl group) showed a distribution of 97% in the gray matter and only 3% in myelin (Rosenberger et al. 2002). This study demonstrated at least two pools of ether phospholipids, a static pool in the myelin and a dynamic pool in the synaptic area. Their findings together with the present results highlight the active metabolic function of plasmalogens in brain as well as their ability to participate in membrane signaling activity (Su et al. 2019). Future investigation should examine whether dietary DHA may alter molecular species of this PE-pl in different brain regions and subcellular fractions.

Upon examining the molecular species of dPE and PC in heart, results showed significant decreases ($p < 0.001$) in (n-6) and concomitant increases ($p < 0.05$, 0.001, respectively) in (n-3) species in both phospholipids comparing the DHA-enriched diet group with controls (Fig. 6). In a previous study by Murphy's group, analysis of fatty acids in phospholipid classes (PC, PE, PI and PS) in rat heart after an 8-week feeding with fish oil also indicated increase in (n-3) and decrease in (n-6) fatty acids in all phospholipid classes (Barceló-Coblijn et al. 2005). In another study, feeding rats with different levels of fish oil diet also showed a time- and dose-dependent decrease in (n-6) and increase in (n-3) phospholipids in the heart tissue (Owen et al. 2004). As indicated in the review by Oppedisano et al. (n-3) fatty acids could offer vaso- and cardio-protective effects through modulation of membrane phospholipids and improve cardiac mitochondrial functions (Oppedisano et al. 2020). In our study, it seems that a 3-week DHA-enriched diet is sufficient to change phospholipid species and peroxidation products in the heart tissue.

PC is the major phospholipid in plasma, and in the control group, plasma PC are comprised with high levels of (n-6) species and only low levels of (n-3) species (Fig. 7). In this study, plasma obtained from DHA-enriched diet showed more than 70% decrease in the PC (n-6) species but only a small increase in the (n-3) species. As a result, the total level of plasma PC in the plasma from the DHA-enriched diet group was declined by almost 50% as compared with the control group (Fig. 4c).

In agreement with earlier studies (Bascoul-Colombo et al. 2016; Sun et al. 1988), PI in brain showed high proportions of 20:4n-6, and PS with high proportions of 22:6 n-3 (Fig. 5a). It is also recognized that PI and PS have distinctive functions, i.e., PI is involved in inositol metabolism associated with second messengers and intracellular calcium mobilization (Lin et al. 1993), whereas PS plays a role in membrane trafficking and endocytosis (Varga et al. 2020). In the study here, the 3-week DHA-enriched diet did not greatly alter the (n-6) and (n-3) species in PI and PS in the cerebral cortex (Fig. 5a), but this dietary regimen resulted in more than 50% decrease in (n-6) species and a concomitant increase in (n-3) species in both PI and PS in the heart (Fig. 6). Considering the diverse functions of these two phospholipids, it is possible that a change in their fatty acid composition may link to its interaction to different enzymes

and thus resulting in altered ability to regulate myocardial functions.

Our results indicate that DHA-enriched diet not only decrease phospholipid species containing 20:4(n-6), but also other (n-6) fatty acids, such as 20:4 (n-6) and 22:4 (n-6). These results suggest that changes in the (n-6) fatty acid pool are likely to occur at the initiation point of fatty acid elongation and desaturation. This is in agreement with the study showing that DHA could suppress metabolism of linoleic acid (18:2 n-6) and its ability to form elongation products, including 20:4(n-6) and 22:4(n-6) (Sullivan et al. 2018). Nevertheless, considering that phospholipid species with 20:4 (n-6) undergo active metabolism through the diacylation-reacylation cycle, and its release by cytosolic phospholipase A2 is linked to inflammatory pathways, the ability for DHA-enriched diet to decrease phospholipids with (n-6) species may be advantageous in suppressing cell membranes against inflammatory responses.

Conclusions

Albeit to different extents, feeding adult mice a DHA-enriched diet for 3 weeks resulted in changes in (n-6) and (n-3) phospholipid species and alteration of lipid peroxidation products in the brain, heart and plasma. The increase in (n-3) phospholipids due to DHA-enriched diet is associated with the increase in 4-HHE but not 4-HNE. In brain, the increase in 4-HHE appears to be mainly observed in the hippocampus, suggesting higher lipid peroxidation activity in this brain region. The decrease in (n-6) species in the heart tissue suggests the possibility that the DHA dietary regimen can mitigate ARA metabolism associated with inflammatory and oxidative activity.

Compliance with Ethical Standards

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