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ScienceDirect
Journal of Nutritional Biochemistry 54 (2018) 57–65
Journal of Nutritional Biochemistry

Selective enrichment of n-3 fatty acids in human plasma lipid motifs following intake of marine fish☆

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Received 22 August 2017; received in revised form 6 November 2017; accepted 7 November 2017

Abstract

Plasma levels of n-3 long chain polyunsaturated fatty acids (LCPUFA) are associated with a reduction in risk of cardiovascular disease and other chronic, age-related diseases like Alzheimer's disease. In this work, we tested the hypothesis that n-3 LCPUFA fatty acids in human plasma are incorporated into selective lipid species following intake of n-3 LCPUFA rich marine fish. To test this hypothesis, we performed lipidomic analysis on plasma samples from a clinical trial in which participants consumed increasing amounts of farmed Atlantic salmon (*Salmo salar*). Under basal conditions, n-3 and n-6 LCPUFA were selectively incorporated into plasma phosphatidylcholine (PC) species containing saturated fatty acids (SFA) versus unsaturated fatty acids as the complementary fatty acids. LCPUFA were incorporated into selective triacylglycerol (TAG) species with complementary diacylglyceryl environments of 34:1 or 34:2 (for 20:5 and 22:5) and 36:2>36:3>36:4 and 36:1 (for 20:4 and 22:6). High n-3 LCPUFA marine fish intake resulted in selective increases of PC SFA_n-3 LCPUFA species and LCPUFA-containing TAG species. Changes in cholesteryl esters and phosphatidylethanolamines also occurred following fish intake. Our results highlight the importance of discriminating phospholipid and TAG species and dietary background when evaluating lipidomic outcomes and disease associations.
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Keywords: Polyunsaturated fatty acids; Lipidomics; Nutrition; Cholesterol; Dietary lipids

1. Introduction

N-3 polyunsaturated fatty acids (PUFA) are associated with a number of health benefits including reduction in risk of cardiovascular disease (CVD) and other chronic, age-related diseases like Alzheimer's disease [1–4]. For CVD risk reduction, the 2015 Dietary Guidelines for Americans recommend intake of fish enriched in n-3 long chain PUFA (LCPUFA) like docosahexaenoic acid (DHA; 22:6n-3) and eicosapentaenoic acid (EPA; 20:5n-3) [5]. Mechanistic studies indicate that n-3 LCPUFA supplementation has pleiotropic effects including lowering serum triacylglcyerol (TAG) levels, modifying lipoprotein type and content, competitively blocking the formation of prothrombogenic and proinflammatory arachidonic acid (ARA; 20:4n-6)-derived eicosanoids, acting as substrates for anti-inflammatory and anti-thrombogenic docosanoids and resolvins, and modifying ion channel or cellular receptor function [6].

N-3 LCPUFA levels in plasma phospholipids (PLs) are used as potential biomarkers of disease [3,7,8]. Recent data demonstrate that the multiple pools within the blood including plasma phospholipid fatty acids (PLFA), red blood cell, platelets, and white blood cells demonstrate selective dose-dependent, time-dependent and n-3 LCPUFA dependent responses to fish oil supplementation [7]. Previous studies from our research team indicate that the PLFA content of DHA and EPA exhibits different saturation capacity following dose-dependent intake of farmed Atlantic salmon, a high n-3 LCPUFA containing fish [9].

Advances in lipidomic methodologies allow for a greater understanding of the role of lipid species in health and disease and the development of more predictive biomarkers of disease. Current studies have identified elevations in PUFA containing PC lipids, but there are relatively few data at present that define the extent to which elevated n-3 fish intake modifies the plasma lipidome or that identify the separate, molecular lipid species into which n-3 PUFA are incorporated, *e.g.* PC 18:0_22:6 *vs* PC 18:1_22:6 [10–12]. A greater

Abbreviations: ARA, arachidonic acid; BHT, butylated hydroxytoluene; CE, cholesteryl esters; CVD, cardiovascular disease; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; IS, internal standard; LCPUFA, long chain PUFA; LPC, *lyso* PC; MS, mass spectrometry; NLS, neutral loss scan; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIS, Precursor Ion Scan; PL, phospholipid; PUFA, polyunsaturated fatty acid; SPE, solid phase extraction; SM, sphingomyelin; TAG, triacylglycerol; VLDL, very low density lipoprotein.

☆ Funding: This work was supported by National Institute of Food and Agriculture, U.S. Department of Agriculture, under award number 2014-67017-21758 and through USDA-ARS project 5450-51000-048-00D.

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https://doi.org/10.1016/j.jnutbio.2017.11.002
0955-2863/Published by Elsevier Inc.

understanding of how these lipidomic endpoints are altered with n-3 intake along with more detailed biomarker identification analyses will allow development of appropriate nutritional therapies for disease prevention and treatment.

Given that there are dose-dependent and fatty acid-dependent changes in the PLFA pool of n-3 LCPUFA following n-3 supplementation, we tested the hypothesis that n-3 LCPUFA are incorporated into lipid species with selective complementary fatty acids in human plasma. To test this hypothesis we analyzed archival plasma samples derived from a randomized, cross-over designed clinical trial in which participants were fed increasing levels of n-3 LCPUFA rich farmed Atlantic salmon (*Salmo salar*) [8]. Our data demonstrate that n-3 PUFA are incorporated into selective PC and TAG structural motifs in human plasma and that these PC and TAG species are elevated in response to elevated n-3 intake.

2. Materials and methods

2.1. Study design and participants

The current work is an ancillary assessment of a study which evaluated the dose–response effects of the consumption of farmed Atlantic salmon. Complete details of the initial trial are provided elsewhere [8]. Briefly, the study was a cross-over designed feeding trial in which participants were randomly assigned to receive three doses of farmed Atlantic salmon twice weekly over 4-week treatment periods with a 4–8 week washout between treatments. The dietary treatments consisted of farmed Atlantic salmon supplied by Cooke Aquaculture (Blacks Harbor, New Brunswick, Canada). In this secondary outcomes analysis we assessed the plasma lipidomic responses to fish consumption at two doses (90 g and180 g). Consumption of these levels of salmon led to a daily intake of ARA of 79 mg and 158 mg, EPA of 158 mg and 316 mg, docosapentaenoic acid (DPA; 22:5n-3) of 73 mg and 146 mg, and DHA of 149 and 299 mg, for the 90 g and180 g portions, respectively [8].

Eligible participants were recruited from the Greater Grand Forks Area, Grand Forks, ND. Sixty-one volunteers were screened for study participation. Twenty-two volunteers eligible for participation were initially randomized to treatment, although 3 withdrew prior to study initiation. Of the 19 participants completing the trial, samples from 9 participants are included in this study. The archival samples from these participants represent those from which baseline and post treatment samples for participants completing both the 90 g and 180 g treatments. The current samples are derived from men (n=5) and women (n=4) with an average age of 50.1±2.4 y (mean ± SE) and an average body mass index of 29.2±1.0 kg/m^2. The trial was performed at the USDA, ARS, Grand Forks Human Nutrition Research Center (GFHNRC), Grand Forks, ND. Approval for the trial was obtained from the Institutional Review Board at the University of North Dakota. All study participants provided informed consent prior to initiation of the study. The trial was registered at www.clinicaltrials.gov as NCT01183520.

Blood samples were obtained on days 0 and 29 of each treatment period after an overnight fast. Blood samples were centrifuged to obtain plasma. Plasma samples were aliquoted and stored at −80 °C until analysis.

2.2. Lipidomic analyses

2.2.1. Nomenclature

Lipid nomenclature according to Liebisch et al. was used [13].

2.2.2. Chemicals

The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA): chloroform, potassium chloride, butylated hydroxytoluene (BHT), ammonium chloride, ammonium acetate. Methanol was purchased from (Avantor Performance Materials, Inc., Center Valley, PA, USA or EMD Chemicals, Billerica, MA, USA) and used without further purification; 18 MΩ water was used. TAG and cholesterol ester (CE) species were ordered from Nu-Chek (Elysian, MN, USA) and stored until use following the manufacturer's guidelines. HPLC-MS grade methanol was ordered from). HPLC-MS grade chloroform, BHT, hexane, and HPLC-MS grade ammonium acetate were ordered from Sigma Aldrich (St. Louis, MO, USA) and used without further purification. Activated silicic acid (Clarkson Chromatography Products, South Williamsport, PA, USA) was preconditioned overnight in chloroform.

2.2.3. Plasma sample preparation

For PL analysis, an organic extract of the human plasma was prepared based upon the procedure of Folch et al. [14] as was used in our previous work [15]. The resulting organic extract was reconstituted in 1 mL of chloroform: methanol (1:1) to yield the crude extract. For the purpose of mass spectrometric analysis the crude extract was further diluted in a mobile phase (MP) (chloroform: methanol 1:1, 10 mM ammonium acetate, 50 μM BHT) (see below). The crude extracts were stored at−27 °C prior further dilution.

Since quantitation of TAG species was performed using an MS^1 infusion technique (see below), SPE was necessary to remove any phospholipid interferences, thus TAGs and CEs were purified by solid phase extractions over silicic acid as detailed in the supporting information [16]. Plasma samples were thawed on water ice for 2 h and vortexed to ensure homogeneity before Folch extraction [14]. TAGs and CEs were analyzed after a 200:1 dilution, relative to the initial plasma volume. A second solution diluted to 4000:1 was analyzed to check for ion suppression of more concentrated analytes like cholesteryl linoleate.

2.2.4. PL analysis

MS analysis of PLs were performed on an AB Sciex 5500 QTRAP hybrid quadrupole ion-trap mass spectrometer with a Turbo Spray source (AB Sciex, Framingham, MA, USA) operating at unit mass resolution. PL standards used in this study, including LIPID MAPS MS standards – Core H, were purchased from Avanti Polar Lipids Co. (Alabaster, AL, USA). The following compounds of certified concentration were used as the internal standards (IS) for quantitation of PCs, *lyso* PCs (LPCs), sphingomyelins (SMs), phosphatidylethanolamines (PEs) and LPEs: PC 17:0/14:1, PC 17:0/20:4, PC 21:0/22:6, LPC 17:1, PE 17:0/14:1, PE 21:0/22:6.

The following parameters were the same for all applied mass spectrometric experiments for PLs: ion source temperature 200 °C, curtain gas 20 L/min, ion source gas 15 L/min, declustering potential 80 V and entrance potential 10 V. Parameters specific for each experiment are listed below. PCs were analyzed using a combination of methods precursor ion scan (PIS) of mass m/z 184 in positive mode (PIS m/z+184) and MS^3 fragmentation performed in negative mode as it was described by Zacek et al. [15]. Combination of both methods enabled identification and quantitation of isobaric PCs including the positional isomers [15]. For the PIS m/z+184 experiment, the collision energy was 35 V, ion spray voltage 5200 V and scanning rate 1000 Da/s. Sample infusion rate was 7 μL/min. The quantitative ratio of isobaric species and positional isomers were determined by MS^3 scan in negative mode using following parameters: ion spray voltage−4500 V, declustering potential−100 V, entrance potential−10 V, collision energy−32 V, excitation amplitude 0.180 V, excitation time 25 ms and scanning rate 10,000 Da/s. Dynamic filling time of the linear trap was used. Sample infusion rate was 10 μL/min. The first precursor corresponded to mass of [PC + acetate]$^-$ and the second precursor corresponded to [PC-15]$^-$. The MS^3 targets were selected for PCs with an abundance greater than 0.5% of the base peak height in PIS of m/z+184.

PEs were analyzed using a neutral loss scan (NLS) for losses of 141 units in positive mode (NLS 141). This scan allowed the determination of PEs as [M + H]$^+$ ions on a level of a lipid species including a sum of carbons and double bonds of the attached pair of the fatty acids (*e.g.* PE 34:2) [13]. LPEs were quantified only relatively with respect to the PE 12:0/13:0 due to the lack of commercially available LPE IS. Collision energy for the NLS 141 experiment was 27 V, ion spray voltage 5200 V and scanning rate 1000 Da/s. Sample infusion rate was 10 μL/min. Our methodology was not able to distinguish PE containing an ether linkage *versus* a vinyl ether linkage.

Data were collected using Analyst® ver. 1.6 software (AB Sciex, Toronto, Ontario) and processed in PeakView® ver. 2.0 (AB Sciex, Toronto, Ontario) and self-programmed Excel macros (Microsoft Office 2010, Microsoft). Processing of the PIS m/z+184 and NLS 141 was carried out by LipidView™ software. For analysis, a 20 μL portion of the reconstituted crude extract (see above) was diluted in 970 μL of MP along with 10 μL of a mixture of PE ISs (Solution 1). This solution was used for quantitation of PEs and LPEs using NLS 141 and for determination of isobaric and isomeric PCs signal distribution determination using MS^3 fragmentation. Subsequently a 25 μL portion of this solution was diluted in 500 μL of MP with 5 μL of a mixture of PC and LPC standards. This more dilute solution was used for quantitation of PCs, LPCs, and SMs using PIS m/z+184. The methods are not capable of determining of the position of double bonds in the fatty acids. To discriminate n-3 and n-6 fatty acids we used results from our previous study where the position was determined using fatty acid methyl ester analysis by gas chromatography [8].

For PCs and PEs, ISs were employed for the quantitation of lipid species within a given mass range. SMs were measured using the PC IS and corrected for the difference in molar response between PC and SM using a correction factor determined with the commercially available SM d18:1/16:0 and PC 12:0/13:0 species. Final concentrations were also corrected for the ionization efficiency change with molecular mass. For the purpose of this correction other compounds besides those mentioned above were used LPC 13:0, PE 12:0/13:0 and PE 17:0/20:4. Results obtained by MS^3 fragmentation experiments were corrected for the contribution of a lipid species with different isotopic composition (mostly containing two 13C) using method described by Zacek et al. [15].

Components of the plasma sample overlapping with IS were subtracted. Correction for the suppression of ionization efficiency, caused by the increasing number of carbons in the PC molecule, was applied within the mass range. The equation correcting the ionization efficiency was determined for PCs, LPCs and PEs by measuring equimolar mixture of ISs of each lipid species.

2.2.5. TAG and CE analyses

For analysis of TAGs and CEs, infusion electrospray mass spectrometry was performed on an AB Sciex 5500 QTRAP (AB Sciex, Framingham, MA, USA) operating in two different scan modes. The electrospray source was operated in positive mode under the following source parameters: Spray Voltage (IS) = 5500 V; Curtain gas=20 V; Gas 1 (GS1) = 20; Gas 2 (GS2) = 15, Source Temperature (TEM) = 200 °C; Collison Gas = High. For brutto structure determination and quantitation, the enhanced mass

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