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Red blood cell PUFAs reflect the phospholipid PUFA composition of major organs

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ABSTRACT

Numerous clinical trials examining the use of omega-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) on various health outcomes have been conducted, and fish oil remains one of the most widely used nutritional supplements. More recently, studies have begun to utilize the omega-3 index, defined as the sum of EPA+DHA in red blood cells (RBCs), as both a biomarker of n-3 LCPUFA exposure and a potential risk factor for coronary heart disease (CHD). Considerably less research evaluates whether RBC phospholipid fatty acids reflect the phospholipid fatty acid composition of other tissues across increasing intakes of n-3 LCPUFAs. We fed mice diets containing increasing amounts of EPA+DHA, equivalent to current recommendations by the American Heart Association on a percent of energy basis, and analyzed the phospholipid fatty acid composition of various tissues in relation to RBCs. We observed that RBCs, heart, muscle, spleen, lung, and adipose tissues all respond to dietary supplementation with EPA+DHA with increasing n-3 LCPUFA and decreasing n-6 LCPUFA levels. Furthermore, the n-3 LCPUFA profiles of all measured tissues had strong ($r > 0.7$) and significant ($p < 0.001$) correlations to RBCs. Interestingly, we also observed changes in saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) levels across various tissues in response to increased EPA+DHA intakes despite there being no change in dietary SFA and MUFA. Specifically, there were increases in RBC SFA and spleen MUFA and decreases in heart MUFA. These demonstrate that the RBC, including the omega-3 index, may serve as a marker for the relative levels of n-3 and n-6 LCPUFAs in phospholipids of certain tissues.

1. Introduction

There is increasing evidence to suggest that dietary supplementation with n-3 long chain polyunsaturated fatty acids (n-3 LCPUFAs) found in fish oil not only have cardiovascular benefit [1], but may also have potential benefit for other pathologies, including dyslipidemia [2], chronic inflammatory diseases [3], autoimmune diseases [4,5], and colon cancer [6]. Over 30 million Americans report consumption of fish oil as a dietary supplement for some purported health benefit [7]; however, FDA-approved fish oil-based pharmaceuticals, including Lovaza, Vascepa, and Epanova, are currently only indicated to lower very high serum triglyceride levels [8]. Despite widespread fish oil supplementation and extensive epidemiologic research on the health effects of n-3 LCPUFAs, results are often inconsistent for various health outcomes, such as ulcerative colitis [9,10]. One potential contributor to this problem is the lack of a standardized and universally accepted biomarker of n-3 LCPUFA exposure. Thus, many studies rely on dietary intake surveys which can be both imprecise and inaccurate.

One biomarker that has been relatively widely used is the red blood cell (RBC) content of eicosapentaenoic acid (EPA)+docosahexaenoic acid (DHA). This metric, known as the omega-3 index, has been shown to be a surrogate biomarker for cardiac tissue EPA+DHA [11,12], and further, has been proposed as a risk factor for coronary heart disease (CHD) [13]. As such, the omega-3 index has been used in several clinical trials as a biomarker for n-3 LCPUFA exposure [14–17]. The use of the omega-3 index in a recent paper that estimated worldwide omega-3 status confirms the gathering consensus on its use as a biomarker [18]. Given that many of the proposed mechanisms by which n-3 LCPUFAs exert their immunomodulatory effects at the cellular level, including

Abbreviations: %en, percent of energy; AA, arachidonic acid; CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FAME, fatty acid methyl esters; LCPUFA, long chain polyunsaturated fatty acids; PL, phospholipid; RBC, red blood cell; RT, room temperature

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gene regulation [19], the production of eicosanoids and pro-resolution molecules [3,20], and alteration of membrane organization [21,22] especially membrane composition of n-3 LCPUFAs in blood cells [23–25], it is important to identify the extent to which RBCs reflect levels of n-3 LCPUFAs in other tissues. There is limited evidence suggesting that the omega-3 index does correlate well with the EPA+DHA content of other tissues in humans [11,12,26] and mice [27].

The primary aim of this study is to identify whether RBC n-3 LCPUFAs adequately reflect the levels of n-3 LCPUFAs in various tissues of mice. Previously, we reported on the fatty acid (FA) profile of RBC phospholipids (PLs) and gastrointestinal tissue PL of mice consuming increasing intakes of EPA+DHA [27]; herein, we report on the correlations between RBC FAs and those of PLs from heart, muscle, spleen, lung, and adipose tissue. We assessed the acyl chain composition of PLs from RBCs and these tissues to determine whether RBC PL FAs (e.g. omega-3 index) are an adequate biomarker of PL FA composition of other tissues across increasing intakes of EPA+DHA.

2. Materials and methods

2.1. Experimental design

The dietary treatment and experimental design employed here has been previously described in greater detail [27]. Briefly, twenty-four SMAD3−/− mice (n=6/group) were fed *ad libitum* standard AIN-93G diet containing soybean oil (control) or one of three treatment diets containing increasing amounts of EPA+DHA. The experimental diets were calculated from the percent energy (%en) a human consuming 2000 kcal would receive from EPA+DHA following the 2002 American Heart Association recommendations [28]. The experimental diets were formulated to model a 2000 kcal human intake with either no fish or fish oil (0.0%en EPA+DHA), two servings of fish a week (0.1%en EPA+DHA), 1–2 g fish oil supplementation a day (0.675%en EPA+DHA), or 4 g prescription fish oil a day (1.8%en EPA+DHA). Mice were fed the diets for 5 wk prior to sacrifice. Tissues were collected, rinsed in cold ddH_2O to remove contaminating pools of blood, and stored in −80 °C until lipid extraction. In the current study, the fatty acid composition of PLs from heart, skeletal muscle (total skeletal muscle of the left hind limb), spleen, lung, and adipose (mesenteric adipose with removal of lymph nodes) tissues were analyzed and compared with that of RBCs previously reported [27].

2.2. Phospholipid isolation and analysis of fatty acid methyl esters (FAMEs)

An expanded methods section for the PL extraction and analysis was previously described [27]. Briefly, total lipid was extracted using a modified Rose and Oklander extraction [29], PLs were isolated using Isolute-XL® SPE aminopropyl columns (500 mg; Biotage, Charlotte, NC) from a procedure modified by Agren et al. [30], and FAMEs generated using acidified methanol described by Burdge et al. [31]. Gas chromatography of phospholipid FAMEs were analyzed at OmegaQuant Analytics, LLC (Sioux Falls, SD). Gas chromatography was performed using a GC2010 Gas Chromatograph (Shimadzu, Columbia, MD) equipped with a SP2560, 100-m column (Supelco, Bellefonte, PA) using hydrogen as carrier gas. Referenced against a standard of fatty acids characteristic of erythrocytes, phospholipid fatty acids were identified and calculated as a percentage of total identified fatty acids after response factor correction. Reproducibility was previously reported in Pickens et al. [32].

2.3. RNA extraction for real-time qRT-PCR

RNA was extracted from tissues using Trizol reagent (Invitrogen Life Technologies Corp., Carlsbad, CA, 15596-018, lot 1217839). Heart, skeletal muscle, spleen, and adipose tissues were removed from storage at −80 °C, weighed, and crushed with 500 µl of Trizol on dry ice using a sterile mortar and pestle. Crushed tissue was transferred into a 14 mL polypropylene tube with 500 µl of Trizol. In the tube, 1 mL of Trizol per 100 mg of tissue was added and the mixture was homogenized in short bursts. Homogenized tissue samples were incubated for 5 min at room temperature (RT), transferred to a clean 1.5 mL Eppendorf tube, and centrifuged at 12,000 × *g* for 10 min at 4 °C. The resulting supernatant was transferred to a clean Eppendorf tube and 200 µl of chloroform was added, shaken vigorously for 15 sec., and incubated for 2–3 min at RT. After incubation, samples were again centrifuged at 12,000 × *g* for 15 min at 4 °C. The upper aqueous phase was added to new 1.5 mL Eppendorf tubes containing 0.5 mL of isopropanol, shaken, and incubated at RT for 10 min. Samples were again centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernant was removed, the resulting RNA pellet washed with 1 mL of 75% ethanol, and vortexed until dissolved. The samples were then centrifuged at 7,500 × *g* for 5 min at 4 °C. Ethanol was removed and tube left open for 5–10 min allowing for any remaining ethanol to evaporate. The RNA pellet was re-suspended in DEPC H_2O (10–12 µl depending on pellet size). Estimation of RNA quality and quantity was determined with an Aligent 2100 Bioanalyzer (Aligent Technologies, Carlsbad, CA). Extracted RNA was then stored at −80 °C until analysis.

2.4. Real-time qRT-PCR and analysis of relative cytokine gene expression

According to manufacturer instructions, total RNA extracted from tissues was converted into cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA). Reverse transcriptase was performed on a Mastercycler Pro (Eppendorf, Hauppauge, NY). The quantity of cDNA was determined using an Aligent 2100 Bioanalyzer (Ailgent Technologies, Carlsbad, CA) and adjusted with RNase-free ddH_2O to a final working concentration of 10 ng/µl. All cDNA dilutions were stored at −20 °C until use in real-time qRT-PCR reactions. Relative fold changes in genetic expression of were quantitated in tissues from EPA+DHA supplemented and control mice by using real-time qRT-PCR. To detect relative gene expression, IL-1β (mm00434228_m1), IFN-γ (mm00801778_m1), IL-6 (mm00446190_m1), and TNF-α (mm00443258_m1) Taqman assays were used (Applied Biosystems, Waltham, MA). Results were normalized using the GAPDH (p/n: 4352339E-0506006) housekeeping gene. Real-time qRT-PCR was performed with an ABI 7900HT Sequence Detection System (Thermo-Fisher Scientific, Waltham, MA) by using an 2× Taqman Master Mix (Applied Biosystems) at the MSU Genomics Core facility. All assays were performed in triplicate, and the relative expression for each gene was calculated by using the comparative cycle threshold (ΔΔCT) method (Applied Biosystems, Waltham, MA). A non-parametric *t*-test was employed for fold change analysis and conducted using Prism (GraphPad Prism, La Jolla, CA).

2.5. Statistical analysis

To evaluate whether RBC n-3 and n-6 fatty acids correlate with those of other tissues, Spearman correlation coefficients were calculated for EPA+DHA (the omega-3 index), EPA alone, DHA alone, docosapentaenoic acid (n-3) (DPA n-3) alone, and arachidonic acid (AA) alone using data for all dietary treatments. The data (mean ± SD) for the PL FA composition for all tissues and