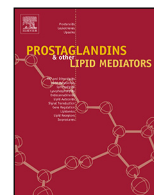
Prostaglandins & other Lipid Mediators 113–115 (2014) 21–29

Contents lists available at ScienceDirect

Prostaglandins and Other Lipid Mediators

journal homepage: www.elsevier.com/locate/prostaglandins



Comparison of the effects of long-chain omega-3 fatty acid supplementation on plasma levels of free and esterified oxylipins

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ARTICLE INFO

Article history:
Available online 29 May 2014

Keywords:
Eicosanoids
Epoxides
Diols
EPA
DHA
PUFA
Arachidonic acid
Omega-3 fatty acids

ABSTRACT

Introduction: It is believed that many of the beneficial effects of long-chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) are mediated by their oxidized metabolites, the oxylipins. The formation and biological role of many cytochrome P450 and lipoxygenase derived hydroxy, epoxy and dihydroxy FA, particularly of oxylipins esterified in polar lipids and triglycerides remain unclear. In this study, we compared the impact of twelve weeks of LC n-3 PUFA supplementation on the patterns of free and total (sum of esterified and free) hydroxy, epoxy and dihydroxy FAs.

Subjects and methods: Subjects (5 male; 5 female) between 46 and 70 years were supplemented with 1.1 g/d of eicosapentaenoic acid (EPA) and 0.74 g/d docosahexaenoic acid (DHA) as ethyl esters. Blood samples were drawn before and after twelve weeks of treatment. Oxylipins in plasma were analyzed by LC–MS directly for free oxylipins and after saponification. Relative FA composition in erythrocyte membranes was analyzed by GC.

Results: LC n-3 PUFA treatment led to a significant increase in EPA (200%) and DHA (23%) in erythrocyte membranes. Of the oxylipins measured in plasma, total and free EPA-derived metabolites were highly increased (70–150%), while total AA-derived metabolites were decreased on average by 30%. There was no effect on DHA-metabolites. Concentrations of total hydroxy and epoxy FAs in plasma were considerably higher compared to free hydroxy and epoxy FAs (up to 350 times), while levels of most free dihydroxy FAs were in a similar range to total dihydroxy FAs. However, the individual ratios between total and free plasma oxylipins remained unchanged after LC n-3 PUFA treatment.

Discussion and conclusions: LC n-3 PUFA supplementation causes a shift in the levels of circulating oxylipins, having the strongest impact on EPA-derived epoxy, dihydroxy and hydroxy FA. The unchanged ratio of free and esterified oxylipins in plasma indicates that both concentrations are valuable biomarkers for assessing the individual status of these lipid mediators.

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1. Introduction

An increased intake of the long-chain omega-3 fatty acids (LC n-3 PUFAs) eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) has been associated with a reduced risk for several chronic inflammatory diseases such as rheumatoid arthritis [15], neuropsychiatric diseases [25,13], atherosclerosis and cardiovascular diseases [7,17]. However, the underlying molecular mechanisms by which LC n-3 PUFA exert their effects are versatile and far away from being understood. It is believed that many actions of LC n-3 PUFAs are mediated by their bioactive lipid metabolites [21,30,16], the oxylipins. Both, LC n-6 and n-3 PUFAs undergo oxygenation in the mammalian body giving rise to a large number of active lipid mediators [38,27]. These oxylipins can be formed enzymatically by cyclooxygenases [24], lipoxygenases (5-LOX, 12-LOX and 15-LOX) [26,22], and cytochrome P450 enzymes (CYPs, e.g. CYP4, CYP2C and CYP2J2) [23,5] or are formed during FA autoxidation [39,35,19].

Oxylipins are involved in the regulation of various biological processes, for example inflammation, pain, cell proliferation, apoptosis, angiogenesis, blood coagulation and blood vessel permeability [2,4]. Compared to oxylipins derived from arachidonic

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http://dx.doi.org/10.1016/j.prostaglandins.2014.05.002
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acid (AA, C20:4 n-6) by cyclooxygenase and 5-lipoxygenase action (i.e. prostaglandins and leukotrienes), only limited information is available about the formation and biological role of hydroxy, epoxy and dihydroxy FA derived from LC n-3 PUFAs. Recent studies have shown that particularly epoxy FA from LC n-3 PUFAs possess highly potent anti-arrhythmic [36], vasodilatory [1] and anti-thrombotic effects [11]. Hydroxylated FAs serve as precursors for pro-resolving signaling molecules, i.e. resolvins and (neuro)protectins which actively mediate the resolution of inflammation [38,31,30].

Profiling of oxylipin patterns in response to LC n-3 PUFA supplementation in humans may assist to deduce their mechanisms of action. Moreover, it may provide novel biomarkers facilitating the investigation of the effects of LC n-3 PUFAs. Several human studies documented changes of hydroxy, epoxy and dihydroxy FA levels in serum and plasma following treatment with LC n-3 PUFA regarding free oxylipins [14,28] or total oxylipins (sum of both free and esterified) after conjugate cleavage by saponification [34,12,29,8]. Applied LC n-3 PUFA doses and treatment duration varied considerably among the long term studies: 11.0 ± 2.0 mg/kg/d (∼0.8 g/d) as ethyl esters for 4 weeks [12]; 0.84–1.74 g/d as ethyl esters for 4–8 weeks [8]; 3.0 g/d as re-esterified triglycerides for twelve weeks [28]; 4.0 g/d LC n-3 PUFAs as ethyl esters for 4 weeks [34] and 6.0 g/d EPA/DHA as ethyl esters for 3 weeks [14]. In addition to the varying study designs, the strong inter-individual differences in the blood oxylipin levels, as discussed in [27], make it complicated to compare different studies. When comparing the reported baseline levels of free serum and plasma levels against total plasma oxylipins, it becomes apparent that the major portion of hydroxy, epoxy and dihydroxy FA are found mainly esterified in the plasma [27]. Interestingly, with and without saponification most dihydroxy FA were detected in the same range indicating that no or a very low portion of these lipid mediators in plasma is found in lipids or triglycerides. LC n-3 PUFA intake causes both a change in the pattern of total oxylipins [34,12] as well as in the levels of free oxylipins [14,28,29]. However, there is no information on how the changes in esterified and free oxylipins are correlated. Therefore, the aim of this study was to determine the impact of a supplementation of LC n-3 PUFAs on the patterns of hydroxy, epoxy and dihydroxy FA species and their ratio between free and total levels in plasma. Using a targeted metabolomics approach, we simultaneously quantified 50 oxylipins in human plasma before and after twelve weeks of supplementation with EPA and DHA ethyl esters.

2. Materials and methods

This investigator initiated study was designed and conducted according to the principles of the Good Clinical Practice Guidelines laid down in the Declaration of Helsinki and was approved by an independent Research Ethics Board (Freiburg Ethics Commission International).

2.1. Subjects

Ten subjects were recruited via newspaper advertisements and screened in the Institute of Food Science and Human Nutrition at the Leibniz University Hannover, Germany. The exclusion criteria were age 30 and >75 years, body mass index >35 kg/m^2, type 1 and 2 diabetes, cancer, coronary heart disease, renal failure, liver disease, bleeding disorders, gastrointestinal disorders (e.g. Colitis ulcerosa, Crohn's disease, chronic pancreatitis, pancreatic insufficiency, coeliac disease, enterocolitis, cholestasis, short bowel syndrome), surgical operation in the gastrointestinal system (e.g. gastrectomy, stomach reduction, stomach tape or balloon), hormonal disorders (e.g. hyperthyroidism and Cushing's disease), high intake of oily fish (>2 times per week), intake of lipid-lowering drugs (statins, fibrates, ezetimibe, and niacin) or nutritional supplements (including LC n-3 PUFAs, phytosterols and polyglucosamin), intake of anticoagulant drugs. Only participants that fulfilled the criteria were included in the study population. All subjects gave their written informed consent to take part in the study.

2.2. Study design

Subjects were supplemented with LC n-3 PUFA capsules for a period of twelve weeks. Soft gelatin capsules contained re-esterified fish oil (840 mg/capsule, Dr. Loges GmbH, Winsen, Germany). The oil contained 330 mg/g EPA and 220 mg/g DHA ethyl ester. The total amount of LC n-3 PUFA ethyl esters (C20:5, C22:6, C18:3, C18:4, C20:4, C21:5, C22:5) was 640 mg/g Moreover the oil contained 2.8 mg/g tocopheroles as antioxidant. Subjects were instructed to ingest four capsules per day (two in the morning and two in the evening together with food and a glass of water). The daily intake was 1.1 g EPA ethyl ester and 0.74 g DHA ethyl ester. Subjects were requested to abstain from eating fatty fish during the intervention period, while other exercise and dietary habits should be maintained. During the two visits, baseline (t_0) and after twelve weeks of intervention (t_{12}) fasting blood was collected. Additionally, at t_0 height and weight of the subjects were measured. The subjects' compliance was assessed by a questionnaire, capsule-intake diaries and a count of left-over capsules at the end of the intervention period.

2.3. Sample collection and analysis

Blood samples were collected by venipuncture of an arm vein into EDTA-monovettes (Sarstedt, Germany) in the morning between 6:00 and 9:30 a.m. after overnight fasting. Plasma was prepared directly by centrifugation (2000 × *g*, 10 min, 10 °C), transferred into 15 mL falcon tubes (Becton Dickinson) and immediately frozen and stored at −80 °C awaiting LC–MS analysis. Other sets of EDTA-whole blood samples collected simultaneously were sent to an external laboratory (Omegametrix, Martinsried, Germany), where the FA composition in erythrocyte membranes was determined as previously described [10,20]. Results are presented as a percentage of the total identified FAs.

Oxylipins were analyzed in plasma samples as free oxylipins and after saponification to determine the sum of both free and esterified oxylipins as previously described [3]. In brief, 250 μL of plasma were mixed with an equal volume of methanol. After addition of 300 μL aqueous sodium hydroxide (10 M) the samples were incubated at 60 °C for 30 min. Following neutralization with 50% acetic acid the oxylipins were extracted by solid phase extraction and quantified by liquid chromatography-electrospray ionization tandem mass spectrometry (LC–ESI–MS) as previously described [27]. Only oxylipins which exceeded the limit of quantification in ≥95% of each sample set were used for data analysis.

2.4. Data analysis and statistics

PUFA composition in erythrocyte membranes (Fig. 1) as well as concentrations of free and total oxylipins in plasma (Table 1) is presented as mean ± standard error (SE). The sample sets were analyzed for their distribution by the Kolmogorov–Smirnov test. Differences between time points within groups were analyzed by *t*-test for paired samples. Changes in the concentration (c) were calculated individually for each subject at each time point (0 and 12) as Δ%, calculated by: $\Delta\% = 100 \times (c_{t12} - c_{t0})/c_{t0}$. Results are shown as mean change ± SE (Table 1 and Fig. S1). To compare their relative distribution, the oxylipins were grouped by substrate (Fig. S2). For the calculation, the sum of the median values of the analyzed

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