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Journal of Nutritional Biochemistry 35 (2016) 58–65

Journal of Nutritional Biochemistry

Addition of aspirin to a fish oil-rich diet decreases inflammation and atherosclerosis in ApoE-null mice☆,☆☆

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Received 21 December 2015; received in revised form 25 April 2016; accepted 25 May 2016

Abstract

Aspirin (ASA) is known to alter the production of potent inflammatory lipid mediators, but whether it interacts with omega-3 fatty acids (FAs) from fish oil to affect atherosclerosis has not been determined. The goal was to investigate the impact of a fish oil-enriched diet alone and in combination with ASA on the production of lipid mediators and atherosclerosis. ApoE$^{-/-}$ female mice were fed for 13 weeks one of the four following diets: omega-3 FA deficient (OD), omega-3 FA rich (OR) (1.8 g omega-3 FAs/kg·diet per day), omega-3 FA rich plus ASA (ORA) (0.1 g ASA/kg·diet per day) or an omega-3 FA deficient plus ASA (ODA) with supplement levels equivalent to human doses. Plasma lipids, atherosclerosis, markers of inflammation, hepatic gene expression and aortic lipid mediators were determined. Hepatic omega-3 FAs were markedly higher in OR (9.9-fold) and ORA (7-fold) groups. Mice in both OR and ORA groups had 40% less plasma cholesterol in very low-density lipoprotein-cholesterol and low-density lipoprotein fractions, but aortic plaque area formation was only significantly lower in the ORA group (5.5%) compared to the OD group (2.5%). Plasma PCSK9 protein levels were approximately 70% lower in the OR and ORA groups. Proinflammatory aortic lipid mediators were 50%–70% lower in the ODA group than in the OD group and more than 50% lower in the ORA group. In summary, less aortic plaque lesions and aortic proinflammatory lipid mediators were observed in mice on the fish oil diet plus ASA vs. just the fish oil diet.
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Keywords: Atherosclerosis; Inflammation; Fish oil; Omega-3 fatty acids; Aspirin

1. Introduction

It is well known that elevated plasma levels of lipids and proinflammatory cytokines are strongly associated with cardiovascular disease (CVD) [1–3]. Based on numerous animal and human studies, omega-3 fatty acids (FAs), which are enriched in fish oils, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are considered to be atheroprotective, because of their potent lipid-lowering and anti-inflammatory effects [4,5]. Acetylsalicylic acid (ASA), also commonly known as aspirin, is a cyclooxygenase 1, 2 (COX-1, 2) inhibitor and has also been shown to be useful in the prevention of CVD. This has largely been attributed to its antiplatelet effect [6], but possibly also because of its general anti-inflammatory and pro-resolving properties from the biosynthesis of aspirin-triggered lipid mediators (LMs) derived from essential FAs [7,8]. Recent clinical trials have found that treatment with fish oils in combination with ASA led to a significant reduction in inflammatory cytokines and that these two agents can work synergistically in lowering CVD risk [9,10].

Although it is generally agreed that treatment with fish oils plus ASA may result in beneficial lipid and anti-inflammatory changes, the

Abbreviations: AA, arachidonic acid; Apo, apolipoprotein; ASA, acetylsalicylic acid/aspirin; COX, cyclooxygenase; CVD, cardiovascular diseases; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAs, fatty acids; HDHA, hydroxydocosahexaenoic acid; HDL-C, high-density lipoprotein cholesterol; HEPE, hydroxyeicosapentaenoic acid; HETE, hydroxyeicosatetraenoic acid; LDL-C, low-density lipoprotein cholesterol; LM, lipid mediator; LT, leukotriene; LX, lipoxin; MaR, maresin; PCSK9, proprotein convertase subtilisin/kexin type 9; PD, protectin; PG, prostaglandin; Rv, resolving; SPMs, specialized pro-resolution mediators; Tx, thromboxane; VLDL-C, very low-density lipoprotein cholesterol.

☆ Funding sources: This study is supported by intramural research funding of the National Heart, Lung, and Blood Institute. LC-MS-MS profiling was supported by the National Institutes of Health (R01GM038765 and P01GM095467) to Charles N. Serhan.

☆☆ Author disclosures: A.V. Sorokin, Z.-H. Yang, B.L. Vaisman, S. Thacker, Z.-X. Yu, M. Sampson, C.N. Serhan, and A.T. Remaley have no conflicts of interest to disclose.

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http://dx.doi.org/10.1016/j.jnutbio.2016.05.012
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mechanisms for this synergy are not fully understood. Some studies have suggested that the fish oil treatment effect on CVD is distinct from its effect on lowering plasma triglycerides and cholesterol and instead may be related to its ability to decrease the inflammatory response [11]. One possible interaction between ASA and fish oil is the ability of ASA to alter the production of specialized pro-resolution mediators (SPMs), such as resolvins, protectins and their aspirin-triggered forms [12], which are all derived from EPA and DHA. SPMs are key molecules involved in the resolution of the inflammatory process and may also have antiatherosclerotic properties [13]. The acetylation of COX-2 by ASA inhibits prostaglandin formation but still allows for the production of 15*R*-hydroxyeicosatetraenoic acid (HETE) from arachidonic acid (AA) and 17*R*-hydroxydocosahexaenoic acid (HDHA) from DHA [7,14]. Further conversion of these by leukocytes, for example, can lead to the production of aspirin-triggered lipoxins, which have potent anti-inflammatory actions [15] or from 17*R*-HDHA in the case of the D-series resolvins.

Thus, the endogenous biosynthesis and metabolism of prostaglandins and SPMs from EPA and DHA may differ in the presence and absence of ASA, and therefore, ASA could significantly alter the anti-atherogenic effect of fish oil supplementation.

2. Materials and methods

2.1. Animals and diets

ApoE$^{-/-}$ mice on C57BL/6 background were obtained from Jackson Laboratory (strain 002052) at 12 weeks of age. The diets were obtained from the Teklad, Harlan Laboratories Inc., and the composition is shown in Supplemental Table 1. The mass ratio of EPA/DHA of the omega-3 FAs used in this study was approximately 1.5. After 2 weeks on an omega-3 FA Deficient (OD) diet, mice were randomly assigned to one of three experimental diets ($n=10$): (1) OD diet, (2) omega-3 FA-rich (OR) diet (1.8 g omega-3 FAs/kg·diet per day), or (3) omega-3 FA-rich + ASA (ORA) diet (0.1 g ASA/kg·diet per day) for another 13-week feeding period. Additional diet, (4) omega-3 FA deficient + ASA (ODA) ($n=4$) was included later under the same experiment conditions. Supplementation with EPA + DHA and ASA was calculated according to a metabolic body weight formula [16] to produce a human (70 kg) equivalent dose of 4 g/day of EPA + DHA and 100 mg/day of ASA. All feeds were stored in vacuum at 4 °C and were changed twice a week. All procedures were approved by the Animal Care and Use Committee of the NHLBI (#H-0050R2). At the end of the 13-week feeding period, blood and organ samples were collected at sacrifice without fasting.

2.2. Analyses of the hepatic FA composition

Liver lipids were extracted by the Folch method [17]. In brief, a portion of liver sample in each mouse was homogenized with chloroform/methanol (2:1; vol/vol), followed by washing with 0.9% NaCl solution after recovered the liquid phase by centrifuge. Hepatic lipid aliquots were heated at 100 °C for 1 h with methanol containing 14% BF_3 to generate FA methyl esters (FAMEs). An aliquot of FAME from each sample was injected onto a DB-FFAP fused silica capillary column (30 m×0.25 mm i.d.×0.25 μm; J&W Scientific, Folsom, CA, USA) on HP-5890 (series II) gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA) coupled with a flame ionization detector. Injector and detector temperatures were set to 250 °C, and the oven temperature program was as follows: 130 to 175 °C at 4 °C/min, 175 to 210 °C at 1 °C/min, and then to 245 °C at 30 °C/min, with a final hold for 15 min. The FAME of each FA was identified by comparison with the retention times of a standard mixture containing 28 FAME (462; Nu-Chek-Prep, Elysian, MN, USA). The concentrations were calculated by comparing the integrated areas of each FA peak in the gas chromatograms with that of the known amount of internal standard (C22:3n−3 free FA) added in the sample prior to total lipid extraction. The data were expressed as the proportion of each FA in the weight of the total identified FAs in each sample (wt%).

2.3. Analyses of the plasma lipids

Plasma collected in heparinized capillary tubes from the retro-orbital sinus was analyzed for total cholesterol (TC), triglycerides (TGs), phospholipids (PL), free cholesterol (FC) and cholesteryl esters (CEs) using enzymatic methods [18]. Plasma lipoprotein profile from pooled plasma ($n=10$) was analyzed after separation by fast protein liquid chromatography (FPLC) chromatography [18].

2.4. Assessment of atherosclerotic lesions

After 13 weeks on the diets, the extent of atherosclerosis as the percentage of the aortic surface covered by lesions was assessed using an *en face* analysis [19]. A second assessment of atherosclerosis in the aortic root and ascending aorta was histologically performed [20], using 5 slides/10 sections per animal ($n=7$). Slides were stained with hematoxylin and Movat, and immunofluorescent staining was performed on frozen sections of the aortic sinus after 15 min of fixation in 4% paraformaldehyde. Nonspecific binding was blocked with 10% goat serum in phosphate-buffered saline, and sections were incubated with 1:50 rat anti-mouse monocyte + macrophage (MOMA-2) or 1:500 rat anti-mouse vascular cell adhesion molecule (VCAM-1) primary antibodies (Abcam Inc., USA) overnight at 4 °C. Slides were then incubated with a FITC-conjugated goat anti-rat secondary antibody (1:200 IgG; Jackson Immuno-Research Lab, USA) for 1 h at room temperature. Histopathological categorization of lesion severity by developmental stage was done in a blind manner by two investigators according to the Reddick classification [21,22].

2.5. Quantification of endothelial progenitor cells by flow cytometry

Bone marrow cells were harvested by flushing femurs and tibias harvested from mice. Red blood cells were lysed and four-color flow cytometry was performed for the markers CD45 (30-F11), CD34 (MEC14.7), CD117 (2B8), CD309 (Avas12) and Linage cocktail [23] with antibodies from BioLegend (USA), using a BD LSR II flow cytometer (BD Biosciences, USA).

2.6. Measurement of cytokines, PCSK9 and COX activity

Plasma cytokines (IL-1β, IL-12p70, IFN-γ, IL-6, mKC, IL-10 and TNF-α) were measured with a Multi-Spot 96-Well-7 Spot ELISA (Meso Scale Discovery, USA) [24]. Sandwich-type ELISA from eBioscience (USA) was used to measure TGF-β and MCP-1. Plasma PCSK9 was assessed with a Mouse Proprotein Convertase 9/PCSK9 Quantikine ELISA kit (R&D Systems, Inc., USA). COX activity in tissue extracts was measured with COX Fluorescent Activity Assay Kit (Cayman Chemicals, USA).

2.7. Gene expression analysis

RNA analysis was done as previously described [25]. RNA had an A260:A230 ratio greater than 1.7, an A260:A280 ratio of approximately 2.1 ± 0.1 and a RIN number of 8.2 ± 0.2. Gene expression in liver of the mice was analyzed with Mouse Lipoprotein Signaling and Cholesterol Metabolism RT^2 Profiler PCR Array (Qiagen, catalog no. PAMM-080E) for RT-PCR with an ABI 7900HT Real-Time PCR System (Life Technologies).

Expression of *Ccl2* and *Hmgcr* was measured individually by RT-PCR with TaqMan assays, Mm00441242_m1 and Mm01282499_m1, respectively (Supplemental Table 2) for OD, OR, ORA groups. Relative expression of the genes was calculated by the comparative CT (ΔΔCT) method [26], using software provided by the manufacturer of the Array. Expression of several genes, as indicated in the text, was measured by RT-PCR with mouse β-actin and 28S rRNA genes used for normalization. Standard error of the mean was calculated with the REST 2009 software from Qiagen.

2.8. LC/MS/MS analysis of lipids

Identifications of SPMs by LC/MS/MS were done, using at least six diagnostic ions [27]. Spleen and lung tissue were removed from nonfasting mice within 5 min of sacrifice, whereas aortic tissue was isolated after approximately 10 min. Collected tissue was snapped frozen and stored at −80 °C before analysis. The LC/MS/MS system, QTrap 5500 (AB SCIEX, USA), used for the analysis was equipped with an Agilent HP1100 binary pump and diode-array detector. An Agilent Eclipse Plus C18 column was used with a gradient of methanol/water/acetic acid of 60:40:0.01 (vol/vol/vol) to 100:0:0.01 at 0.5 mL/min flow rate. To monitor and quantify LM levels, a multiple reaction monitoring method was developed with signature ion fragments for each molecule. Identification was conducted using published criteria [27] that included retention time and at least six diagnostic ions. Calibration curves were obtained using synthetic and authentic LM mixtures [d_8-5*S*-HETE, d_4-LTB_4, d_4-PGE_2, resolvin (Rv) D1, RvD2, RvD5, maresin (MaR) 1, protectin D1 (PD1), as well as their synthetic aspirin triggered epimers (AT-RvD1, AT-LXA_4) 4-HDHA, 7-HDHA, 14-HDHA, 17-HDHA, lipoxin (LX) A_4, LXB_4, LTB_4, PGD_2, PGE_2, $PGF_{2\alpha}$, thromboxane (Tx) B_2, 5-HETE, 12-HETE, 15-HETE, RvE1, RvE2, 5-hydroxyeicosapentaenoic acid (HEPE), 12-HEPE, 15-HEPE, 18-HEPE] at 2, 10, 40 and 200 pg. Quantification was carried out based on area beneath the peak of the multiple reaction monitoring transition and the linear calibration curve for each compound.

2.9. Statistical analysis

Unless otherwise indicated, all data are expressed as the means ± S.E.M.s. Overall results were first analyzed by one-way analysis of variation (ANOVA) or, where indicated, by two-factor ANOVA, using a fixed-effects model. Differences between diet groups were assessed by paired two-tailed *t* test for single comparisons, and *P* values were adjusted for when there were unequal variances. Bonferroni's post hoc test correction was done for multiple comparisons. Data were considered statistically significant when $P<.05$.

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