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Combination of n-3 polyunsaturated fatty acids reduces atherogenesis in apolipoprotein E-deficient mice by inhibiting macrophage activation

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ABSTRACT

Background and aims: Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are major components of n-3 polyunsaturated fatty acids (n-3 PUFAs) which inhibit atherogenesis, although few studies have examined the effects of the combination of EPA and DHA on atherogenesis. The aim of this study was to investigate whether DHA has additional anti-atherosclerotic effects when combined with EPA.
Methods: Male 8-week-old apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice were fed a western-type diet supplemented with different amounts of EPA and DHA; EPA (2.5%, w/w), low-dose EPA + DHA (2.5%, w/w), or high-dose EPA + DHA (5%, w/w) for 20 weeks. The control group was fed a western-type diet containing no n-3 PUFA. Histological and gene expression analysis were performed in atherosclerotic lesions in the aorta. To address the mechanisms, RAW264.7 cells were used.
Results: All n-3 PUFA treatments significantly attenuated the development and destabilization of atherosclerotic plaques compared with the control. The anti-atherosclerotic effects were enhanced in the high-dose EPA + DHA group ($p < 0.001$), whereas the pure EPA group and low-dose EPA + DHA group showed similar results. EPA and DHA additively attenuated the expression of inflammatory molecules in RAW264.7 cells stimulated with LPS. DHA or EPA + DHA suppressed LPS-induced toll-like receptor 4 (TLR4) expression in lipid rafts on RAW264.7 cells ($p < 0.05$). Lipid raft disruption by methyl-β-cyclodextrin suppressed mRNA expression of inflammatory molecules in LPS-stimulated macrophages.
Conclusion: n-3 PUFAs suppressed atherogenesis. DHA combined with EPA had additional anti-inflammatory effects and inhibited atherogenesis in *Apoe*$^{-/-}$ mice. The reduction of TLR4 expression in lipid rafts in macrophages by DHA might be involved in this mechanism, at least partially.

Abbreviations: Apoe$^{-/-}$, apolipoprotein E-deficient; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LPS, lipopolysaccharide; Mac3, macrophage antigen 3; MβCD, methyl-beta-cyclodextrin; MCP1, monocyte chemoattractant protein 1; MMP9, matrix metalloproteinase 9; n-3 PUFA, n-3 polyunsaturated fatty acid; Tfr, transferrin receptor; TLR4, toll-like receptor 4; TNFα, tumor necrosis factor α; VCAM1, vascular cell adhesion molecule 1.

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1. Introduction

Accumulating evidence has demonstrated that higher consumption of fish oil reduces the risk of cardiovascular disease [1–3]. n-3 polyunsaturated fatty acids (n-3 PUFAs) are major components of fish oil and contribute to the prevention of cardiovascular events [4]. Previous studies including our own have revealed anti-atherosclerotic effects of n-3 PUFAs [5–8]. We reported that highly purified eicosapentaenoic acid (EPA) reduces and stabilizes atherosclerotic lesions in apolipoprotein E-deficient (*Apoe*$^{-/-}$) mice by inhibiting activation of macrophages [8]. Also, several studies reported that low levels of docosahexaenoic acid (DHA), one of the major n-3 PUFAs, are related to cardiovascular disease [9–11]. These studies suggested that both EPA and DHA have anti-atherosclerotic properties. On the other hand, there are some studies that indicate differential effects of EPA and DHA [12,13]. Few studies have investigated the effects of the combination of EPA and DHA on atherogenesis.

Atherosclerosis is a chronic inflammatory disease in which macrophages play an important role [14,15]. Previous studies have demonstrated that n-3 PUFAs inhibit the accumulation and activation of macrophages [8,16]. Many cellular and molecular mechanisms are involved in n-3 PUFA-associated anti-atherosclerotic effects [2,17–19]. Recently, lipid rafts, which are subdomains of the plasma membrane that contain high concentrations of cholesterol and glycosphingolipids, have attracted much attention as a signaling platform. Lipid rafts on the cell surface take part in signal transduction initiated by complex protein-protein interactions [20]. However, the role of n-3 PUFAs in macrophage activation through lipid rafts remains unclear.

In this study, we fed a western type diet supplemented with different combinations and amounts of n-3 PUFAs to *Apoe*$^{-/-}$ mice and examined the anti-atherosclerotic effects of n-3 PUFAs. We also examined the hypothesis that n-3 PUFAs inhibit inflammatory activation of macrophages by reducing toll-like receptor 4 (TLR4) expression in lipid rafts as an underlying mechanism by which n-3 PUFAs reduce atherogenesis. The results of our study suggested that n-3 PUFAs inhibited atherogenesis and that DHA showed additional anti-atherosclerotic effects when combined with EPA in *Apoe*$^{-/-}$ mice. The reduction of macrophage activation through the inhibition of TLR4 localization in lipid rafts by DHA partially contributed to this result.

2. Materials and methods

2.1. Animals

Eight-week-old male *Apoe*$^{-/-}$ mice (C57BL/6 background) were divided into four groups and fed a western-type diet supplemented with 2.5% (w/w) eicosapentaenoic acid ethyl ester (EPA group, 4 g/kg/day), 2.5% (w/w) omega-3-acid ethyl ester (low-dose EPA + DHA group, EPA 1.9 g/kg/day and DHA 1.5 g/kg/day), 5% (w/w) omega-3-acid ethyl ester (high-dose EPA + DHA group, EPA 3.7 g/kg/day and DHA 3 g/kg/day), or no n-3 PUFA (control group) for 20 weeks. Eicosapentaenoic acid ethyl ester was purchased from Yoshindo Inc., and omega-3-acid ethyl ester was provided by Takeda Pharmaceutical Company, Ltd. All mice were kept in microisolator cages under a 12-h light/dark cycle. All experimental procedures and protocols were approved by the Animal Care and Use Committee of Tokushima University and complied with the "Guide for the Care and Use of Laboratory Animals" [21].

2.2. Analysis of blood pressure and serum lipid levels

Blood pressure was measured using a tail-cuff system (BP-98A, Softron). We measured blood pressure in conscious mice for three times and used the averaged value for comparison. At the time of sacrifice, blood was collected, and serum was stored at −80 °C until required. Commercially available kits were used for the measurement of total cholesterol, free fatty acids and triglyceride levels (Wako Diagnostics).

2.3. Analyses of atherosclerotic lesions

The severity of atherosclerotic lesions in the aorta and characterization of atherosclerotic plaques were examined as we reported previously [22]. In brief, atherosclerotic lesions in the aortic arch was determined by en face Sudan IV staining and characterization of plaques were performed by frozen section histological analyses in the aortic root. Lipid deposition in atherosclerotic plaques was determined by oil red O staining. Accumulation of macrophages and the expression of matrix metalloproteinase 9 (MMP9), vascular cell adhesion molecule 1 (VCAM1), and monocyte chemoattractant protein 1 (MCP1) in plaques were analyzed by immunohistochemical staining. Detailed methods for immunostaining were described in Supplementary Methods. The ratio of positive area to plaque area was calculated in three valve lesions in the aortic root by using Photoshop CS3 (Adobe Systems) and used for comparison.

2.4. Measurement of concentration of n-3 PUFAs in abdominal aorta and cell

The concentrations of EPA and DHA in the abdominal aorta and cells were measured by modified liquid chromatography-tandem mass spectrometry (LC/MS/MS) at CMIC Pharma Science Co., Ltd. (Osaka, Japan) [23,24]. Abdominal aortas and cell pellets were accurately weighted and fractured in liquid nitrogen using a medical scissors. For lipid extraction, samples were solubilized in 490 μL chloroform/methanol (1:1, v/v) and 10 μL BHT solution (10 mg/mL in ethanol). Extracted solutions were used for measurement of concentration of fatty acids. Standard solutions of EPA and DHA (Cayman Chemical Co.) were used for calibration curves. The following solutions were transferred to glass tubes: 20 μL of supernatant of homogenized samples or phosphate buffered saline (used for calibration curves), internal standard solution (10 μg/mL; [2H5]-EPA and [2H5]-DHA), and 200 μL of acetonitrile/6N HCl (90/10, v/v). The tubes were capped, incubated at 100 °C for 45 min, and cooled to room temperature. Then, 200 μL of methanol/10N NaOH (90/10, v/v) was added; and the tubes were capped, incubated at 100 °C for 45 min, and cooled to room temperature. Liquid/liquid extraction was performed using hexane. The upper layer was evaporated under a stream of nitrogen gas at 40 °C, reconstituted with 35 μL acetonitrile, and injected into an optimized LC/MS/MS system. LC was performed using an ACQUITY UPLC (Waters) and an API4000 triple quadrupole tandem mass spectrometer (AB Sciex). An ACQUITY UPLC HSS T3 analytical column (2.1 × 150 mm, particle size 1.8 μm; Waters) was used to separate the fatty acids. An atmospheric pressure chemical ionization source was operated in negative ionization and multiple reaction monitoring (MRM) mode. The MRM transitions for precursor to product ion were as follows: arachidonic acid, m/z 303-259; EPA, m/z 301-257 and DHA, m/z 327-283. Other parameters were adjusted to optimum values.

2.5. Cell cultures

RAW264.7 cells, originally purchased from the American Type Culture Collection, were cultured in DMEM (containing 4 g/L glucose) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin in CO2 incubator (5% CO_2 at 37 °C). Cells were pretreated with 3 μM EPA, 3 μM DHA, or EPA + DHA (3 μM each) for