8-HEPE-Concentrated Materials from Pacific Krill Improve Plasma Cholesterol Levels and Hepatic Steatosis in High Cholesterol Diet-Fed Low-Density Lipoprotein (LDL) Receptor-Deficient Mice

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Eicosapentaenoic acid (EPA), one of the N-3 polyunsaturated fatty acids (n-3 PUFAs), is a major active ingredient of fish that contributes to improve dyslipidemia. Recently, we demonstrated that 8-hydroxyeicosapentaenoic acid (8-HEPE) had a more positive effect on metabolic syndrome than EPA, and that 8-HEPE induced peroxisome proliferator-activated receptor (PPAR) activation in the liver. We investigated the effects of 8-HEPE-concentrated materials from Pacific krill on dyslipidemia and hepatic steatosis in low-density lipoprotein (LDL) receptor-deficient (LDLR-KO) mice. Eight-week-old male LDLR-KO mice were fed a Western diet (0.15% cholesterol, WD), WD supplemented with 8-HEPE-concentrated materials from Pacific krill (8-HEPE included; WD +8-HEPE), or a standard diet (SD) for eighteen weeks, respectively. Murine J774.1 macrophages were incubated in the absence or presence of 8-HEPE (50µM) or EPA (50µM). 8-HEPE-concentrated materials significantly increased the plasma high-density lipoprotein (HDL)-cholesterol level, and decreased the plasma LDL-cholesterol and hepatic triglyceride levels in WD-fed LDLR-KO mice. Moreover, the rate of Oil Red O-positive staining was higher in the liver of WD-fed LDLR-KO mice than in that of 8-HEPE + WD-fed LDLR-KO mice. 8-HEPE but not EPA significantly increased gene expression levels of ABCA1, CD36, and interleukin 6 (IL-6) in murine J774.1 macrophages compared with those in the control. These results suggest that 8-HEPE-concentrated materials improve dyslipidemia and hepatic steatosis increasing ABCA1, CD36, and IL-6 gene expressions in macrophages.

Key words 8-hydroxyeicosapentaenoic acid; dyslipidemia; N-3 polyunsaturated fatty acid

INTRODUCTION

Dyslipidemia is characterized by increased plasma triglyceride and low-density lipoprotein (LDL) cholesterol levels, and decreased high-density lipoprotein (HDL) cholesterol levels. Moreover, it increases the risk of hepatic steatosis and atherosclerosis. Omega-3 polyunsaturated fatty acids (n-3 PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are essential for human physiology, and fish and krill oils are their main dietary sources. N-3 PUFAs play a positive role in the prevention and treatment of metabolic syndrome including dyslipidemia. These beneficial effects are thought to be mainly due to the ability of these essential fatty acids to reduce plasma triglyceride levels. It was demonstrated that dried Pacific krill was a source of hydroxyeicosapentaenoic acids (HEPEs) and that 8-HEPE has a high ligand activity for peroxisome proliferator-activated receptors (PPARs). Moreover, 8-HEPE has a greater affinity for PPAR activation than EPA in vitro. We previously demonstrated that supplementation of the diet with 8-HEPE and EPA from Pacific krill had a beneficial effect on metabolic syndrome through the activation of PPARα in high-fat diet-induced obese mice. However, whether 8-HEPE-concentrated materials excluding EPA and DHA from Pacific krill improve dyslipidemia, leading to hepatic steatosis, is still unknown. We investigated this in the present study.

MATERIALS AND METHODS

Ethics This study was approved by the Animal Care Committee of Iwate Medical University. The protocol was approved by the Committee on the Ethics of Animal Experiments of Iwate Medical University (Permit Number: 26–41). All surgery was performed under sodium pentobarbital and/or isoflurane anesthesia and all efforts were made to minimize suffering.

8-HEPE Purification from Pacific Krill Details of the purification of 8-HEPE were reported previously. Briefly, homogenized Euphausia (E.) pacifica was incubated with twice the volume of water at 20°C for 20h with 160-rpm shaking. The clarified E. pacifica solution was applied to SP700 packed into a Sepacore glass column at a flow rate of 10mL/min, which was then washed with water:ethanol (60:40) solution. 8-HEPE was eluted from SP700 in ethanol. Then, 8-HEPE and fatty acids in the eluted fraction were separated by liquid–liquid separation using water, ethanol, and hexane. Finally, 8-HEPE was collected by preparative HPLC using an InertSustain ODS-3 column (20-mm dia. × 250mm, GL Science Inc., Tokyo, Japan). The purified 8-HEPE was analyzed by LC/hybrid quadrupole time-of-flight mass spectrometry (LC/QTOfMS: Triple TOF 5600, SCIEX) using SCIEX OS Software. We used the multiple reaction monitoring (MRM) method to quantify 8-HEPE. The m/z of precursor ions and product ions of 8-HEPE was 317.2 and 155.0, respectively. We quantified the amount of 8-HEPE from a calibration curve generated using 8-HEPE purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.).

Experimental Animals LDL cholesterol receptor knock-out (LDLR-KO) mice were kindly provided by Dr. S. Ishibashi. Eight-week-old male LDLR-KO mice were fed a Western diet (0.15% cholesterol, F2WTD, Oriental Yeast Co., Ltd., Tokyo, Japan, WD), WD supplemented with 8-HEPE concentrated materials from Pacific krill (8-HEPE (100mg/kg) but not EPA or DHA; WD+8-HEPE), or a standard diet (CE-2, Nihon Clea Co., Ltd., Tokyo, Japan) for eighteen weeks, respectively. All mice were allowed to access water ad libitum and housed under a 12-h light/dark cycle. Feed was replaced weekly.
every three days. Body weight was measured before and 18 weeks after the three different types of diet had started.

**Plasma Lipid Analysis** Mice were fasted overnight (12h) with free access to water. Blood was collected by cardiac puncture under isoflurane anesthesia (2–4%). The plasma was separated by centrifugation at 1900 × g and 4°C for 10 min and stored at −80°C until required. Triglyceride, total cholesterol, LDL-cholesterol, HDL-cholesterol, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels in blood plasma were measured using DRI-CHEM slide kits (DRI-CHEM NX 500 FUJIFILM Co., Ltd., Tokyo, Japan) respectively. All kits were used according to the manufacturers’ protocols.

**Quantification of Triacylglycerol in Mouse Liver** Lipids were extracted according to the Bligh–Dyer method (BLIGH EG, DYER WJ), a rapid method of total lipid extraction and purification. The lipid extract was resuspended at 10 mg/mL in chloroform. A 0.1-µg aliquot of the resuspended extract was applied to a Chromarod (LSI Medience Corp., Tokyo, Japan). A chloroform/methanol/water (42:24:2.5) mix was drawn 5 cm up the Chromarod. After drying, hexane/diethyl ether (50:30) was drawn 10 cm up the Chromarod and lipids were detected and measured by TLC-FID (LSI Medience Corp., Tokyo, Japan). Dipalmitoyl phosphatidylcholine, 1,2-dipalmitoyl-sn-glycerol-3-phosphoethanolamine, glyceryl tripalmitate, DL-alpha-palmitin, and 1-palmitoyl-sn-glycerol-3-phosphocholine were used as lipid standards to identify the lipid class from the Rf value. We quantified the amount of triacylglycerol from a calibration curve generated using glyceryl tripalmitate.

**Cell Treatment and RT-PCR** Murine J774.1 macrophages (JCRB0018, Japanese Cancer Research Bank, National Institutes of Biomedical Innovation, Health and Nutrition.) were seeded on 6-well plates (1 × 10⁶ cells/well) and maintained in RPMI1640 medium supplemented with 10% fetal bovine serum (FBS) in a humidified incubator at 37°C and 5% CO₂ for 24 h. Then, the cells were incubated in RPMI1640 without FBS at 37°C and 5% CO₂ for 24 h for cell cycle synchronization. Thereafter, the cells were incubated in 10% FBS/RPMI1640 with 8-HEPE (50 µM), EPA (50 µM), or a volume of ethanol equal to that of the vehicle, as reported by Yamada et al. with minor modification. After 24 h, the cells were stimulated with 50 µg/mL of human oxidized low-density lipoprotein (Ox-LDL, AlfaAesar, Lancashire, U.K.) for 18 h. The cells were harvested and homogenized in Sepasol-RNA I Super G (Nacalai, Kyoto, Japan), and the total RNAs were extracted according to the manufacturer’s instructions. After treatment with deoxyribonuclease (DNase) (Nippon Gene, Tokyo, Japan), cDNAs were synthesized with Moloney Murine Leukemia Virus reverse transcriptase (Nippon Gene) and used for real-time PCR with GeneAce SYBR qPCR Mix a Low ROX (Nippon Gene). Real-time PCR was performed on ABI Prism7500 (Applied Biosystems, Foster City, CA, U.S.A.). The sequences of oligonucleotide primers used are presented in the supplementary materials. The Ct values of the target genes were normalized with the corresponding Ct value of 18S ribosomal RNA (rRNA), and the relative expression level was estimated by 2^−ΔΔCt methods.

**Histology** The livers perfused with saline (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) were fixed with 3.7–4.3% (w/w) formalin (Mildform® 10N, Wako Pure Chemical Corporation, Osaka, Japan) and embedded in O.C.T. compound (Sakura Finetechanical Co., Ltd., Tokyo, Japan) after immersion in 20% sucrose in phosphate buffered saline (PBS). Then, 6-µm-thick sections were cut using a cryostat (Leica CM3050 S; Leica Microsystems, Germany) and stained with Oil Red O (Sigma, St. Louis, MO, U.S.A.) and hematoxylin. The preparations were photographed using a Keyence microscope BZ-X710 (Keyence, Osaka, Japan).

**Data Analysis** All data are shown as the mean ± standard error (S.E.). An ANOVA with Bonferroni’s test was used for the statistical analysis of multiple comparisons of data. p < 0.05 was considered significant.

**RESULTS**

**Effect of 8-HEPE-Concentrated Materials on Body Weight and Lipid Levels** Body weight significantly increased in LDLR-KO mice fed standard diet (SD) and WD + 8-HEPE for 18 weeks (Fig. 1A). In contrast, LDLR-KO mice fed WD for 18 weeks did not show any change in body weight. LDL-cholesterol, total cholesterol, and triglyceride levels in blood plasma were increased in WD-fed compared with SD-fed LDLR-KO mice (Figs. 1B–E). 8-HEPE concentrated materials significantly increased HDL-cholesterol levels but decreased LDL-cholesterol levels in blood plasma of WD-fed LDLR-KO mice (Figs. 1B, C). In contrast, total cholesterol
and triglyceride levels in blood plasma did not differ between LDLR-KO mice fed with WD and WD + 8-HEPE (Figs. 1D, E).

Effect of 8-HEPE-Concentrated Materials on Plasma AST and ALT Levels, and Hepatic Triglyceride Levels

Plasma AST and ALT levels tended to increase in WD-fed compared with SD-fed LDLR-KO mice (Figs. 2A, B). 8-HEPE-concentrated materials did not affect the plasma AST and ALT levels in WD-fed LDLR-KO mice. Interestingly, 8-HEPE-concentrated materials significantly decreased hepatic triglyceride levels in WD-fed LDLR-KO mice (Fig. 2C). Moreover, Oil Red O histological staining as a marker of fat accumulation in the liver demonstrated that rates of Oil Red O-positive staining were higher in the liver of WD-fed LDLR-KO mice than in that of 8-HEPE + WD-fed LDLR-KO mice (Fig. 2D).

Effects of 8-HEPE on Gene Expressions (ABCA1, CD36, and Interleukin 6 (IL-6)) of Murine J774.1 Macrophages

8-HEPE but not EPA significantly increased gene expression levels of ABCA1, CD36, and IL-6 in murine J774.1 macrophages compared with those in the control (Figs. 3A–C). Moreover, compared with 8-HEPE, EPA significantly decreased expression levels of all genes tested in OxLDL and treated murine J774.1 macrophages (Figs. 3A–C).

DISCUSSION

We demonstrated that 8-HEPE-concentrated materials significantly increased the plasma HDL-cholesterol level, and decreased the plasma LDL-cholesterol and hepatic triglyceride levels in WD-fed LDLR-KO mice. Moreover, rates of Oil Red O-positive staining were higher in the liver of WD-fed LDLR-KO mice than in that of 8-HEPE + WD-fed LDLR-KO mice. These results suggest that 8-HEPE-concentrated materials improve dyslipidemia and hepatic steatosis in WD-fed LDLR-KO mice. It is known that dyslipidemia increases the risk of hepatic steatosis and atherosclerosis. Several studies have suggested that n-3 PUFAs, especially EPA and DHA, play a positive role in the prevention and treatment of the pathologies associated with metabolic syndrome including dyslipidemia. These beneficial effects are thought to be mainly due to the ability of n-3 PUFAs to reduce plasma triglyceride levels. We demonstrated that 8-HEPE-concentrated materials significantly increased the plasma HDL-cholesterol level but did not reduce the plasma triglyceride level. Several factors are associated with increases in HDL cholesterol. One of them is ABCA1, which transports phosphorylids and free cholesterol from macrophages to lipid-free apoA-I, leading to the generation of HDL particles. N-3 PUFAs participate in macrophage-specific cholesterol efflux in mice and humans. Interestingly, EPA impairs ABCA1-mediated cholesterol efflux in both human and murine macrophages. In fact, EPA did not stimulate HDL-mediated cholesterol efflux from macrophages, suggesting that it does not promote ABCA1/apoA-I mediated cholesterol efflux from macrophages. 8-HEPE but not EPA significantly increased gene expression of ABCA1 in murine J774.1 macrophages (Fig. 3A). Moreover, compared with 8-HEPE, EPA did not increase but decreased ABCA1 gene expressions in OxLDL-treated murine J774.1 macrophages. Rayner et al. showed that antagonism of microRNA-33 increases ABCA1 expression levels, leading to increases in circulating HDL cholesterol in LDLR-KO mice, suggesting that 8-HEPE increases the circulating HDL cholesterol through increased ABCA1 gene expression in macrophages. EPA did not increase gene expression of ABCA1 in murine J774.1 macrophages, whereas it improved dyslipidemia in WD-fed LDLR-KO mice. Several studies demonstrated that EPA decreased plasma triglycerides through decreases in expressions of cholesterol- and triglyceride-synthesizing enzymes. In addition, it increased β-oxidation of fatty acids, and inhibited phosphatidic acid phosphatase and diacylglycerol acyltransferase, leading to the reduction of triglyceride and/or very low-density lipoprotein (VLDL) synthesis. Moreover, EPA increased the expression of LPL, leading to increased triglyceride removal from circulating VLDL. Therefore, EPA might improve dyslipidemia in WD-fed LDLR-KO mice via a different mechanism from 8-HEPE. 8-HEPE decreased plasma triglycerides in high-fat diet-fed mice, whereas it failed to decrease them in WD-fed LDLR-KO mice in this study. In contrast, EPA failed to decrease plasma triglycerides in high-fat diet-fed mice, whereas it decreased them in WD-fed LDLR-KO mice. Moreover, plasma triglyceride levels were approximately three times greater in WD-fed LDLR-KO compared with high-fat diet-fed mice in this study. These results suggest that 8-HEPE-concentrated materials have beneficial effects on plasma lipid levels through increased ABCA1 gene expression and decreased triglyceride levels in WD-fed LDLR-KO mice.
results suggest that model-dependent differences in the effect of 8-HEPE and EPA on plasma triglycerides may exist. 8-HEPE-concentrated materials significantly decreased plasma LDL-cholesterol in WD-fed LDLR-KO mice. It is known that n-3 PUFAs such as EPA inhibits cholesteryl ester transfer protein (CETP) expression, leading to increased HDL- and decreased LDL-cholesterol in humans. However, CETP expression is low in mice, suggesting that CETP hardly participated in the regulation of the plasma LDL-cholesterol level in this study. Previous studies suggested that ABCA1-mediated cholesterol efflux from peripheral cells plays a critical role in HDL formation and the reverse cholesterol transport (RCT) pathway, leading to the improvement of plasma lipid profiles. Ren et al. suggested that augmentation of ABCA1 expression in macrophages enhanced RCT, leading to increased HDL and decreased LDL-cholesterol levels. 8-HEPE-concentrated materials increased plasma HDL-cholesterol levels. Moreover, 8-HEPE significantly increased gene expression levels of ABCA1 in murine J774.1 macrophages compared with those in the control, suggesting that 8-HEPE-concentrated materials induced enhancement of RCT via increased plasma HDL cholesterol contributes to plasma LDL cholesterol lowering.

Hepatic steatosis is characterized by the accumulation of triglyceride lipid droplets in the hepatocyte cytoplasm. 8-HEPE-concentrated materials decreased Oil Red O-positive staining along with decreased hepatic triglyceride levels in LDLR-KO mice fed WD (Fig. 2), suggesting that they improve hepatic steatosis in LDLR-KO mice fed WD. The accumulation of hepatic triglycerides depends on the non-esterified fatty acids derived from the plasma or newly synthesized from glucose in the liver. The rate of fatty acid uptake from plasma into cells depends on the fatty acid concentration in plasma and hepatocellular capacity for fatty acid uptake. 8-HEPE-concentrated materials activate hepatic PPARα, leading to the improvement of hepatic steatosis. Fatty acids synthesized from glucose in the liver also play a role in the development of hepatic steatosis. Muscle and liver insulin resistance promotes the accumulation of specific lipid metabolites. In insulin resistance, it continues to promote lipogenesis within the liver, leading to the development of hepatic steatosis. 8-HEPE increased IL-6 gene expression in macrophages (Fig. 3B). It is known that IL-6 signaling leads to a signal transducer and activator of...
transcription 3 (STAT3)-dependent upregulation of SOCS3 that, in turn, induces insulin resistance in the liver. In contrast, Cai et al. demonstrated that pu-erh tea extract ameliorated insulin resistance and hepatic steatosis in high-fat diet-fed mice through enhancement of the IL-6-STAT3 signaling pathway. Awazawa et al. also reported that IL-6 derived from macrophages contributed to the enhancement of hepatic insulin sensitivity through adiponectin. Moreover, 8-HEPE made adipocytes smaller in high-fat diet-induced obese mice, suggesting increased adiponectin. Therefore, 8-HEPE may contribute to improve hepatic steatosis through increased free fatty acid accumulation in macrophages, increased fatty acid oxidation, and the improvement of insulin sensitivity in the liver by increasing CD36 gene expression in macrophages, hepatic PPARα activation, and increased IL-6 gene expressions in macrophages and the inhibition of adipocyte hypertrophy, respectively.

8-HEPE-concentrated materials slightly increased plasma ALT and AST levels in WD-fed LDLR-KO mice. In this study, 8-HEPE at doses of approximately 10 mg/kg/d was administered orally to WD-fed LDLR-KO mice because EPA at doses of approximately 15–30 mg/kg/d is administered orally to patients with hyperlipidemia in clinical settings. We examined the cytotoxic effects of 8-HEPE and EPA on NIH/3T3 cells using the tetrazolium (MTT) assay. Cytotoxicity of 8-HEPE was similar to that of EPA in NIH/3T3 cells. (see Supplementary materials). Therefore, 8-HEPE might have a minimal cytotoxic effect on the mouse liver.

In conclusion, we first demonstrated that 8-HEPE-concentrated materials significantly increased the plasma HDL-cholesterol level and decreased the plasma LDL-cholesterol and hepatic triglyceride levels in WD-fed LDLR-KO mice. Moreover, 8-HEPE but not EPA significantly increased gene expression levels of ABCA1, CD36, and IL-6 in murine J774.1 macrophages compared with those in the control. These results suggest that 8-HEPE-concentrated materials improve dyslipidemia and hepatic steatosis, in part, by increasing ABCA1, CD36, and IL-6 gene expressions in macrophages.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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