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Eicosapentaenoic acid administration attenuates the pro-inflammatory properties of VLDL by decreasing its susceptibility to lipoprotein lipase in macrophages

Yasutaka Jinno, Masanori Nakakuki, Hiroyuki Kawano*, Tatsuto Notsu, Kiyoshi Mizuguchi, Kazunori Imada

Development Research, Pharmaceutical Research Center, Mochida Pharmaceutical Co., Ltd., 722 Jimba-aza-Uenohara, Gotemba, Shizuoka 412-8524, Japan

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ABSTRACT

High level of plasma very low-density lipoprotein (VLDL) has been identified as a risk factor for coronary heart disease. Recent evidence suggests that excess VLDL induces inflammatory responses in macrophages and vascular endothelial cells. The Japan EPA Lipid Intervention Study (JELIS), a large scale clinical trial, demonstrated that highly purified eicosapentaenoic acid (EPA) prevented the onset of cardiovascular events in LDL-cholesterol independent fashion. In this study, we investigated the impact of EPA on pro-inflammatory properties of VLDL. Effects of VLDL prepared from mice fed 5% EPA diet for 1 week (EPA-VLDL) or mice fed normal diet (Ctrl-VLDL) on the mRNA expression of pro-inflammatory factors were examined in human THP-1 macrophages. Ctrl-VLDL increased mRNA expression of pro-inflammatory factors such as interleukin-1β and tumor necrosis factor-α in macrophages. In contrast, the increases in pro-inflammatory factors by EPA-VLDL were lower than those by Ctrl-VLDL. Moreover, EPA-VLDL-treated macrophages had less triglyceride accumulation than Ctrl-VLDL-treated macrophages. Inhibition of lipoprotein lipase (LPL) appeared to suppress inflammation and triglyceride accumulation by Ctrl-VLDL suggesting that hydrolysis of VLDL is required for the pro-inflammatory properties of VLDL. Free fatty acid release from EPA-VLDL by macrophages and purified LPL was less than that from Ctrl-VLDL. Extracellular LPL mass was decreased by EPA-VLDL. Taken together, these findings indicate that the pro-inflammatory properties of VLDL were attenuated by EPA administration via decrease in susceptibility of VLDL to LPL. It appears possible that anti-inflammatory effects of EPA on VLDL contribute to the suppression of cardiovascular risk by EPA.

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1. Introduction

Hypertriglyceridemia is an important risk factor for cardiovascular disease [1]. Very low-density lipoprotein (VLDL), a triglyceride (TG) carrier in fasting plasma, has been demonstrated to exist in human arteriosclerotic plaques [2], suggesting that VLDL is directly involved in the onset and development of arteriosclerosis. VLDL is also reported to induce foam cell formation and inflammatory cytokine production in macrophages [3–5]. Apolipoprotein CIII (apoCIII), one of the apoproteins comprising VLDL, is reported to cause inflammation through toll-like receptor (TLR) 2 [6]. The pro-inflammatory activity of VLDL is thought to be directly involved in the onset and development of arteriosclerosis, at least in past.

Although *n*-3 polyunsaturated fatty acids are known to reduce risk of cardiovascular events, eicosapentaenoic acid (EPA) is the only purified *n*-3 polyunsaturated fatty acid (*n*-3 PUFA) found to reduce the risk of cardiovascular events as a pure fatty acid. Highly purified EPA is actually being used as a drug for the treatment of hyperlipidemia and arteriosclerosis obliterans in clinics and was found to reduce the incidence of major coronary events by 19% in LDL-cholesterol-independent fashion in the Japan EPA Lipid Intervention Study (JELIS), a large scale clinical study of EPA [7]. EPA is known to have multiple anti-atherogenic effects including anti-platelet aggregation [8], anti-intimal thickening [9], and adiponectin-elevating effects [10]. In addition, EPA potently reduces the synthesis of fatty acids and triglyceride as well as the secretion of triglyceride through inhibiting the activation of SREBP-1c, a transcription factor, in the liver [11]. We have reported that administration of EPA modified the properties of VLDL including particle sizes and composition of VLDL in rats [12]. EPA is also known to have anti-inflammatory effects via eicosanoid metabolism and the NF-κB pathway [10,13]. It is important to

* Corresponding author. Tel.: +81 550 89 7881; fax: +81 550 89 8070.
E-mail address: h-kawano@mochida.co.jp (H. Kawano).

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investigate how the anti-inflammatory effects of EPA influence the pro-inflammatory properties of VLDL in attempting to clarify the mechanism of EPA-induced reduction of risk of cardiovascular events.

We report here that administration of EPA modifies the properties of VLDL and consequently attenuates its pro-inflammatory activity in THP-1 macrophages. This study is the first demonstration that a clinically used drug affects the pro-inflammatory properties of VLDL, suggesting that EPA reduces the risk of cardiovascular events in LDL-cholesterol-independent fashion as a drug with a new mechanism of action.

2. Materials and methods

2.1. Materials

Highly purified EPA ethyl ester (EPA-E) (>98%) was obtained from Nippon Suisan Kaisha, Ltd. (Tokyo). EPA-sodium salt (EPA-Na) was purchased from Nu-Chek Prep, Inc. (Elysian, MN).

2.2. Animals and VLDL preparation

Male C57BL/6J mice (CLEA Japan, Inc. Tokyo) at 11–18 weeks of age were given free access to water and one of the following two diets. The control group was fed control diet (fish meal–free F1, Funabashi Farm, Chiba). The EPA group was fed F1 supplemented with 5% (w/w) EPA-E. After 1 week administration, mice had blood withdrawn under anesthesia. EDTA was used as anticoagulant. The $d < 1.006$ g/mL fraction of lipoprotein (VLDL) was prepared from pooled plasma by density gradient ultracentrifugation as previously described [12]. VLDL was dialyzed and concentrated using a centrifugal filter (Millipore, Billerica, MA). The contents of protein, triglyceride (TG), total cholesterol (TC), and phospholipid (PL) were determined with commercially available kits. Fatty acid composition of VLDL was analyzed by gas chromatography. All animal experiments were carried out in accordance with the guidelines for the use and care of laboratory animals of Mochida Pharmaceutical.

2.3. Cell culture

Human THP-1 monocytes obtained from American Type Culture Collection (ATCC, Manssas, VA) were maintained in RPMI1640 medium (Sigma, St Louis, MO) containing antibiotics and inactivated 10% (v/v) fetal bovine serum (Nichirei, Tokyo). Monocytes were seeded at 1.0×10^5 cells/well in 96-well plates and triggered to differentiate into macrophages by the addition of 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma) for 4 days, followed by overnight incubation with VLDL at 100 μg protein/mL or 300 μg TG/mL. As a vehicle group, the same volumes of phosphate-buffered saline (PBS, Sigma) were used. EPA-Na was dissolved in ethanol and added to medium containing 0.1% (w/v) bovine serum albumin (Sigma) at 10–50 μM. Orlistat (Sigma) was dissolved in dimethyl sulfoxide (DMSO) and added to medium at 2 μM.

2.4. Gene expression analysis

Total RNA was extracted using TRIzol® regent (Invitrogen, Carsbad, CA) and purified with the Purelink Micro-to-Midi total RNA purification system (Invitrogen). cDNA was generated using a SuperScript™ III first-strand synthesis system (Invitrogen). Quantitative real-time polymerase chain reaction was performed on duplicate samples using the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) with TaqMan® gene expression assay kits (interleukin-1β (IL-1β) Hs00174097_m1, tumor necrosis factor-α (TNF-α) Hs00174128_m1, monocyte chemotactic protein-1 (MCP-1) Hs00234140_m1 and as an internal control; peptidylprolyl isomerase B (Hs00168719_m1)).

2.5. Cellular lipid analysis

Cellular lipid was extracted as described in Ref. [14] and the cellular contents of TG and TC were determined with commercially available kits. Residual cells were dissolved in 0.5 M NaOH and cellular protein contents were determined. Cellular lipid contents were normalized by cellular total protein content.

2.6. Assay of susceptibility of VLDL to lipoprotein lipase

To determine the susceptibility of VLDL to lipoprotein lipase (LPL), VLDL specimens containing 300 μg TG/mL were incubated for the indicated times in THP-1 macrophage-plated cultures or with purified bovine LPL (Sigma) bound to heparan sulfate proteoglycans (HSPG, Sigma). HSPG-bound LPL was prepared according to Ref. [15]. After incubation, the reaction solutions were mixed with 1% Triton X-100 (Sigma) and cellular protein contents were determined by the above-described method. Free fatty acid released from VLDL was determined with a commercially available kit. Background values were assessed in the absence of macrophages or purified LPL, respectively.

For determination of extracellular LPL mass, 10 U/mL heparin (Mochida Pharmaceutical Co., Tokyo) was added to the medium at 30 minutes before the end of the incubation period. The amount of human LPL-immunoreactive mass was determined in the medium by enzyme-linked immunosorbent assay using the Markit-M LPL kit (DS Pharma, Osaka). LPL mass was normalized to total level of cell protein.

2.7. Analysis of lipid contents, particle size, and apolipoprotein composition in VLDL

TG, TC, and PL contents of VLDL were determined and normalized to protein content. VLDL particle size was analyzed by high performance liquid chromatography (HPLC) at Skylight Biotech (Tokyo). For analysis of apolipoprotein composition of VLDL, 40–50 μg VLDL protein was loaded onto IPG strip gels (pH 3–5.6, GE Healthcare, Buckinghamshire, UK) in an isoelectric focusing system. Isoelectric focusing gels were fixed and stained with Coomassie brilliant blue, and band intensities were quantified using ImageQuant TL software (version 7.0, GE Healthcare).

2.8. Statistical analysis

Results are presented as the means ± S.E.M. Data were assessed using the *t*-test for two-group comparison. Findings of $p < 0.05$ were considered significant ($^*p < 0.05$; $^{**}p < 0.01$).

3. Results

3.1. Effects of EPA administration on plasma lipids and fatty acid composition of VLDL in mice

Plasma levels of TG, TC, and PL significantly decreased in mice fed 5% (w/w) EPA-E diet compared with mice fed normal diet ($p < 0.01$) (Fig. 1A). No difference was noted in body weight between these two groups (data not shown). The content of EPA (C20:5 *n*3) markedly increased in VLDL (EPA-VLDL) prepared from mice fed EPA-E diet compared with that (Ctrl-VLDL) prepared from mice fed normal diet (Fig. 1B).

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