Eicosapentaenoic Acid-Enriched High-Density Lipoproteins Exhibit Anti-Atherogenic Properties

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Background: It has previously been reported that oral administration of purified eicosapentaenoic acid (EPA) generates EPA-rich high-density lipoprotein (HDL) particles with a variety of anti-inflammatory properties. In this study, the mechanism underlying the anti-atherogenic effects of EPA-rich HDL using reconstituted HDL (rHDL) was investigated.

Methods and Results: rHDL was generated by the sodium cholate dialysis method, using apolipoprotein A-1 protein, cholesterol, and various concentrations of EPA-phosphatidylcholine (PC) or egg-PC. Increased EPA-PC contents in rHDL resulted in decreased particle size. Next, the effects of rHDL containing various amounts (0–100% of total PC) of EPA-PC on vascular cell adhesion molecule-1 (VCAM-1) expression in human umbilical vein endothelial cells (HUVECs) was examined. Cytokine-stimulated VCAM-1 expression was inhibited in a dose-dependent manner based on the amount of EPA-PC in rHDL. Surprisingly, the incubation of HUVECs with EPA-rich rHDL resulted in the production of resolvin E3 (RvE3), an anti-inflammatory metabolite derived from EPA. Incubation with EPA-PC alone did not adequately induce RvE3 production, suggesting that RvE3 production requires an endothelial cell–HDL interaction. The increased anti-inflammatory effects of EPA-rich HDL may be explained by EPA itself and RvE3 production. Furthermore, the increase in EPA-PC content enhanced cholesterol efflux.

Conclusions: The EPA-enriched HDL particles exhibit cardioprotective properties via the production of anti-inflammatory lipid metabolites and the increase in cholesterol efflux.

Key Words: High-density lipoprotein (HDL); Inflammation; Lipids; Lipoproteins; Omega-3 fatty acid

Many epidemiological studies have shown an inverse association between high-density lipoprotein (HDL) cholesterol levels and coronary artery disease (CAD).^{1–3} Although HDL has multiple functions that contribute to protective effects against atherosclerosis,⁴ including anti-inflammatory, anti-oxidative, and anti-apoptotic properties, a major prospective effect of HDL is its ability to deliver cholesterol from the peripheral tissues to the liver for biliary excretion through a process known as reverse cholesterol transport. Emerging evidence has revealed a negative correlation between cholesterol efflux and the probability of CAD that is independent of the HDL cholesterol concentration.^{5,6} Thus, it is generally accepted that not only the quantity, but also the quality, of HDL are accurate predictors of CAD risk.^{7,8}

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Long-chain n-3 polyunsaturated fatty acids (PUFAs), including α -linolenic acid, eicosapentaenoic acid (EPA), and docosahexaenoic acid, possess a variety of cardioprotective properties. Supplementation with n-3 PUFA reduces triglyceride levels in both normolipidemic subjects and hypertriglyceridemic patients.⁹ In Japan, high-dose administration of EPA ethyl ester decreased the number of major coronary events in hypercholesterolemic patients (Japan EPA Lipid Intervention Study (JELIS)).^{10,11} High-dose EPA reduced the levels of triglyceride and inflammatory markers, such as lipoprotein-associated phospholipase A2 and high-sensitive reactive protein C, and improved other

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lipid parameters, including non-HDL cholesterol and ApoB in the Multicenter, Placebo-controlled, Randomized, Double-blind, 12-week Study With an Open-label Extension (MARINE) and Effect of AMR101 (Icosapent Ethyl) on Triglyceride Levels in Patients on Statins With High Triglyceride Levels (ANCHOR) studies.^{12–14} In addition, we have recently demonstrated that administration of high-dose EPA enhances the anti-inflammatory properties of HDL in Japanese patients with dyslipidemia.¹⁵ Evidence favors the possibility that EPA plays crucial roles in the prevention of cardiovascular diseases;¹⁶ however, the molecular mechanisms by which EPA exerts its anti-atherosclerotic effects remain unclear.

In this study, to determine the effects of EPA on HDL function, we generated reconstituted HDL (rHDL) particles containing various amounts of EPA and examined their effects on anti-atherosclerotic properties, such as anti-inflammatory functions and cholesterol efflux.

Methods

Materials

Human apolipoprotein A-1 (apoA-1), 1,2-Dieicosapentaenoyl-sn-glycero-3-phosphocholine, and 1,2-Diacyl-sn-glycero-3-phosphocholine from egg yolk were purchased from Sigma-Aldrich (St. Louis, MO, USA). Tumor necrosis factor- α (TNF- α) was purchased from WAKO (Osaka, Japan). Antibodies against 5-lipoxygenase, 12-lipoxygenase, and 15-lipoxygenase were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Preparation of rHDL

The rHDL particles were prepared using a previously described sodium cholate dialysis method.¹⁷ Briefly, the required amounts of phosphatidylcholine (PC) and cholesterol were mixed and dried under a N₂ gas stream. The dried mixture was solved in tris(hydroxymethyl)amino-methane (Tris)-buffered saline (TBS; 8.2 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 8.0) and supplemented with 19 mmol/L sodium deoxycholate until the solution was clear. ApoA-1 was added to the solution to make a final PC:cholesterol:apoA-1 molar ratio of 160:8:1. The mixture was incubated at 37°C for 1 h and dialyzed against TBS for 3 days to remove sodium deoxycholate. Protein concentration was measured by using the Bradford protein assay.

The samples were subjected to non-denaturing 4–20% gradient polyacrylamide gel (Bio-Rad, Hercules, CA, USA) electrophoresis and stained with Coomassie Brilliant Blue to visualize the rHDL particles. Particle size was assigned by a comparison with protein standards using a High

Figure 1. Native polyacrylamide gel electrophoresis (PAGE) analysis of reconstituted high-density lipoprotein (rHDL) prepared at different eicosapentaenoic acid-phosphatidylcholine (EPA-PC) molar ratios in a 4–20% polyacrylamide gel. Standard proteins of known hydrodynamic diameters were used for this analysis. Samples (1.0 μ g proteins) were separated by non-denaturing gel electrophoresis and stained with Coomassie Brilliant Blue.

Molecular Weight Calibration Kit (GE Healthcare, Buckinghamshire, England).

Vascular Cell Adhesion Molecule-1 Expression

Vascular adhesion molecule-1 (VCAM-1) expression was examined by using a previously described method.¹⁵ Briefly, human umbilical vein endothelial cells (HUVECs) were plated in serum-deprived RPMI1640 medium supplemented with 1% fetal bovine serum (FBS). HUVECs were incubated with RPMI1640 supplemented with 1% FBS, 100 μ g/mL rHDL and 10 ng/mL TNF- α for 4 h. VCAM-1 expression was measured by using real-time polymerase chain reaction (PCR). All data were normalized to β -actin expression.

Cholesterol Efflux Assay

The cholesterol efflux was determined by using a commercially available assay kit (Abcam, Cambridge, England) according to the manufacturer's instructions. Briefly, J774.1 cells were pre-incubated for 16 h with the labeling reagent

Table. Fatty Acid Compositions of the rHDL Used in This Study, as Examined Using GC-MS

Fatty acid	EPA-HDL (100)	EPA-HDL (80)	EPA-HDL (20)	Egg HDL
Palmitate (μ mol/L)	25.5	373.0	1,192.7	1,602.3
Stearate (μ mol/L)	13.6	129.9	400.5	507.9
Oleate (μ mol/L)	20.1	355.1	1,228.9	1,529.3
Linoleate (μ mol/L)	5.9	169.1	581.2	715.0
Arachidonate (μ mol/L)	11.7	48.0	125.2	138.9
Eicosapentaenoate (μ mol/L)	3,007.2	3,502.8	611.1	6.2

The rHDL preparation contained EPA-PC:egg-PC ratios of 100:0 (EPA-HDL (100)) to 0:100 (egg HDL). EPA, eicosapentaenoic acid; GC-MS, gas chromatography-mass spectrometry; rHDL, reconstituted high-density lipoprotein.

Figure 2. EPA-rich rHDL inhibited cytokine-stimulated vascular adhesion molecule-1 (VCAM-1) expression. **(A)** Concentration-dependent rHDL-induced suppression of tumor necrosis factor (TNF)- α -induced VCAM-1 expression. Human umbilical vein endothelial cells (HUVECs) were pre-incubated with the indicated concentrations of rHDL containing EPA-PC:egg-PC ratios of 100:0 overnight and stimulated with TNF- α for 4 h. The relative VCAM-1 expression levels were analyzed by using real-time polymerase chain reaction. * P <0.05, ** P <0.01. P -values were determined by analysis of variance (ANOVA) with Tukey's multiple comparisons post-hoc test. **(B)** Inhibitory effects of EPA-PC concentration in rHDL on HUVECs. Increase in EPA-PC contents in rHDL enhanced the suppression of VCAM-1 expression. rHDL was supplemented at 10 μg protein/mL. * P <0.05, **** P <0.0001. P -values were determined by using an ANOVA with Tukey's multiple comparisons post-hoc test. Abbreviations as in Figure 1.

Figure 3. Effects of EPA-PC contents in rHDL on resolvin E3 production. **(A–C)** HUVECs were pre-treated with rHDL containing different EPA-PC molar ratios and stimulated with TNF- α for 4 h. The samples were analyzed with LC-MS/MS to measure the resolvin E3 **(A)**, 18-hydroxyeicosapentaenoic acid (18-HEPE) **(B)**, and free-EPA levels **(C)** in HUVECs. All data represent mean $\pm$ SD. **(D)** HUVECs were stimulated with TNF- α for 4 h, and subjected to Western blots for the analysis of 5-lipoxygenase, 12-lipoxygenase, and 15-lipoxygenases. β -actin was used as the loading control. Abbreviations as in Figures 1,2.

and equilibration buffer (provided in the kit). The cells were washed with the medium and incubated with rHDL for 4 h. Media were removed and the cells were lysed in cell lysis buffer (provided in the kit). Cholesterol efflux into the medium was expressed as a percentage using the following formula: fluorescence intensity (FI) in the medium/(FI in the medium+FI in the cells)×100.

Fatty Acid Profiling

Fatty acids were purified from HDL and methylated by using a commercially available kit (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol. The concentrations of methylated fatty acids were measured using gas chromatography-mass spectrometry (GC-MS). The GC-MS conditions used for the measurements in this study are described in a previous study.¹⁸

Measurement of Lipid Mediators

Incubations were terminated by adding a 2× volume of ice-cold methanol. Then, the deuterated internal standards, leukotriene B₄-d₄ (d₄-LTB₄), d₈-5-hydroxyicosatetraenoic acid (d₈-5-HETE), d₄-prostaglandin E₂ (d₄-PGE₂), and d₅-resolvin D₂ (d₅-RvD₂), which represented each chromatographic region of the identified LMs, were added to the samples (500 pg each) to facilitate quantification. Samples underwent solid phase extraction (SPE) on C18 columns and were subjected to liquid chromatography-mass spectrometry (LC-MS/MS). The system consisted of a Qtrap 6500 (Sciex, Framingham, MA, USA), equipped with a Shimadzu LC-30AD HPLC system. A ZORBAX Eclipse Plus C18 column (100 mm×4.6 mm, 3.5 μm; Agilent Technologies, Santa Clara, CA, USA) was used with a gradient of methanol/water/acetic acid from 55:45:0.01 (v/v/v) to 98:2:0.01 at a flow rate of 0.4 mL/min. A multiple reaction monitoring (MRM) method was developed for the signature ion pairs Q1 (parent ion)/Q3 (characteristic fragment ion) of each molecule to monitor and quantify the levels of targeted LMs. The LMs were identified using published criteria, such as LC retention time, specific fragmentation patterns, and diagnostic fragmentation ions. The samples were quantified based on the peak area of the MRM chromatograph, and linear calibration curves were obtained with authentic standards for each compound.¹⁹

Statistical Analysis

A one-way analysis of variance (ANOVA) with Tukey's post-hoc test was used to analyze the comparisons as indicated. All statistical analyses were performed using GraphPad Prism software.

Results

Preparation and Characterization of rHDL

Discoidal rHDL was prepared by mixing apoA-1 and free cholesterol to create different amounts of EPA-PC. Egg-PC, free cholesterol, and apoA-1 produced rHDL particles with diameters of ~10 nm (Figure 1). In contrast, the increasing amounts of EPA-PC in rHDL gradually reduced the rHDL particle size, suggesting that EPA-PC is responsible for the HDL particle size.

We measured the fatty acid compositions of PC in rHDL using GC-MS analysis. The amount of EPA was increased in proportion to the EPA-PC contents in rHDL, while the amounts of other fatty acids, including palmitate, stearate, oleate, linoleate, and arachidonate, were reduced

Figure 4. Effects of EPA-PC contents in rHDL on cholesterol efflux. **(A)** Dose-dependent effects of rHDL on cholesterol efflux. Cholesterol efflux from macrophages was measured over 4 h following stimulation with the rHDL particles containing EPA-PC:egg-PC ratios of 100:0. **P*<0.05, ***P*<0.01, *****P*<0.0001. *P*-values were determined by ANOVA with Tukey's multiple comparisons post-hoc test. **(B)** Cholesterol efflux from macrophages in response to rHDL particles. Increase in EPA-PC contents in rHDL enhanced the cholesterol efflux capacity of rHDL. rHDL was supplemented at 100 μg protein/mL. **P*<0.05. *P*-values were determined by ANOVA with Tukey's multiple comparisons post-hoc test. Abbreviations as in Figures 1,2.

(Table).

Anti-Inflammatory Properties of rHDL Containing EPA

We next examined the effects of EPA on the anti-inflammatory properties of rHDL. First, HUVECs were pre-incubated with various concentrations of rHDL containing EPA-PC:egg-PC ratios of 100:0 and stimulated with TNF-α for 4 h. VCAM-1 expression was dose-dependently inhibited by rHDL (Figure 2A). Next, we used rHDL particles with various EPA-PC contents to examine whether rHDL enriched in EPA-PC exhibited increased anti-inflammatory activity. As shown in Figure 2B, rHDL highly enriched with EPA efficiently blocked TNF-α-stimulated VCAM-1 expression, whereas rHDL with a low amount of EPA-PC was less effective.

Role of Resolvin E3 in the Anti-Inflammatory Effects

Based on growing evidence, specialized pro-resolving mediators (SPMs), particularly the resolvin family derived from long-chain n-3 fatty acids, such as EPA and DHA,²⁰⁻²²

actively stimulate the resolution of inflammation. Among the resolvins, E-series resolvins are known as EPA metabolites. We hypothesized that the SPMs were involved in the anti-inflammatory properties of EPA-rich rHDL. Treatment with EPA-rich rHDL dramatically increased the amount of resolvin E3 (RvE3) in HUVECs, depending on the EPA content in rHDL (**Figure 3A**, columns 4–6), while TNF- α stimulation had no effect on RvE3 production (**Figure 3A**, columns 7–9). Egg HDL supplementation did not increase RvE3 production because of the low amount of EPA in egg HDL (**Table**). Surprisingly, the addition of liposomes containing EPA-PC failed to effectively generate RvE3 compared to EPA-rHDL (**Figure 3A**, column 3), suggesting that HDL particles are required for efficient RvE3 production from endothelial cells, and that liposomes are not sufficient to support resolvin production.

The intermediate metabolite, 18-hydroxyeicosapentaenoic acid (18-HEPE), is derived from EPA and further converted to RvE3 by 15-lipoxygenase. Consistent with the RvE3 levels, the levels of 18-HEPE and free EPA also increased in response to EPA-rHDL supplementation (**Figure 3B,C**). Finally, we determined whether HUVECs possessed the lipoxygenases required for RvE3 synthesis by using Western blotting. All lipoxygenases were expressed in HUVECs, and there was no difference between TNF- α -stimulated and unstimulated cells (**Figure 3D**).

Effects of the Phospholipid Composition of rHDL on Cholesterol Efflux

Based on the evidence presented above, we concluded that EPA-enriched rHDL has anti-inflammatory properties. A major function of HDL is to protect against atherosclerosis via cholesterol efflux.⁵ Thus, we explored the roles of EPA-PC in rHDL in cholesterol efflux. First, we confirmed that cholesterol efflux from macrophages was dependent on the rHDL dose (**Figure 4A**). Furthermore, increasing EPA-PC concentrations enhanced cholesterol efflux ability (**Figure 4B**), suggesting a crucial role for EPA-PC in rHDL in cholesterol efflux activity.

Discussion

According to the study results, the phospholipid composition of rHDL altered the cholesterol efflux and anti-inflammatory effects of HDL. For the first time, the E-series resolvins were shown to be involved in the anti-inflammatory effects of HDL, and the production of the E-series resolvins depended on the interaction between HDL and endothelial cells. Moreover, the increasing EPA-PC contents in rHDL decreased the particle size, suggesting that EPA-rich rHDL forms saddle-shaped, minimal surface particles in high-fluidity membranes, which is consistent with the findings from a previous study.²³

There are possible mechanisms by which EPA-PC decreases rHDL particle size. Because EPA-PC increases membrane fluidity, the lipid bilayer in EPA-rich rHDL forms a saddle surface, which results in smaller rHDL particles. It is also possible that conformational changes in ApoA-1 or the number of ApoA-1 molecules, depending on EPA-PC amounts, may influence HDL particle size. It will be important to further investigate the role of EPA-PC contents on HDL particle size.

The unsaturated fatty acid composition of the phospholipids in HDL was shown to be associated with a significant suppression of VCAM-1 expression in several studies.^{15,24}

In the present study, RvE3, a potent specialized pro-resolving mediator,²² was produced after the interaction between EPA-rich rHDL and endothelial cells. RvE3 was biosynthesized from EPA by P450 or aspirin-acetylated cyclooxygenase-2, followed by 15-lipoxygenase activity.²⁵ TNF- α stimulation had no effects on lipoxygenase expression, and EPA-rich rHDL induced RvE3 production, suggesting that RvE3 production is directly reflected by the amount of EPA.

Surprisingly, the HDL structure appeared to be necessary for the efficient conversion of EPA-PC to 18-HEPE and RvE3 by endothelial cells. Addition of EPA-PC liposomes slightly increases the intracellular amounts of EPA, 18-HEPE, and RvE3 in cells, probably through passive fusion of the plasma membrane and liposomes.²⁶ Notably, the addition of EPA-HDL remarkably increases EPA, 18-HEPE, and RvE3 levels. Moreover, because the production of EPA, 18-HEPE, and RvE3 is proportional to the EPA content in HDL, they are generated from PC in rHDL but not in cells. Therefore, we speculate that HDL is incorporated into cells using active transport machinery. It will be worthwhile to investigate in future studies whether HDL is actively incorporated into the cells.

We previously reported that orally administrated EPA was efficiently incorporated into the HDL particles to generate EPA-rich HDL.¹⁵ In addition, it is widely recognized that anti-inflammatory effects of E-series resolvins are nearly 1,000-fold greater than those of EPA.²² Taken together, dietary EPA consumption that improves cardiovascular function may be associated with RvE3 production from the EPA-incorporated HDL.

As shown in recent studies, smaller HDL particles are highly efficient in ATP-binding cassette transporter 1-(ABCA1) dependent cholesterol efflux,^{27,28} providing strong evidence for the enhanced cholesterol efflux ability of EPA-rich HDL reported here. Du et al suggested that the capacity of small HDL species for ABCA1-dependent cell surface binding may be related to their ability to promote cholesterol efflux.²⁸ Conversely, in the study by Marmillot et al, the cholesterol efflux capacity of saturated lipid-based rHDL was higher than that of rHDL incorporating unsaturated lipids.²⁹ However, results from all studies are not easily comparable because the experimental conditions, such as cell type, rHDL concentration, and duration of incubation with rHDL and cells, varied. Notably, the phospholipid composition in rHDL affects its cholesterol efflux capacity. We cannot exclude the possibility that apoA-1 was released from rHDL and was converted to lipid-poor or lipid-free apoA-1. Further studies are required to investigate the roles of the EPA in HDL in cholesterol efflux.

Study Limitation

The oral EPA therapy in daily practices is unable to achieve the equivalent concentrations of EPA, which was observed in rHDL used in this study. However, our findings may indicate that the functions of HDL are potentially affected by its fatty acid composition in phospholipids. Further studies are required to confirm the present study findings in clinical samples.

Conclusions

The EPA content in HDL influences the size of HDL particles, cholesterol efflux, and cytokine-induced VCAM-1

expression by producing E-series resolvins. Our findings provide novel evidence that allows us to better understand the mechanism by which dietary EPA consumption improves HDL function and reduces cardiovascular risks.

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Disclosure

The authors declare no conflicts of interest.

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