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# Newly developed reconstituted high-density lipoprotein containing sphingosine-1-phosphate induces endothelial tube formation

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**Abstract**

Reconstituted high-density lipoprotein (rHDL) has been shown to produce a rapid regression of atherosclerosis in animal models and humans. Sphingosine-1-phosphate (S1P), which is a bioactive lipid in HDL, plays a role in mitogenesis, endothelial cell motility, and cell survival, as well as organization and differentiation into a vessel. In this study, we examined the direct role of a newly developed rHDL, [POPC(1-palmitoyl-2-oleoyl phosphatidylcholine)/S1P/apolipoproteinA–I(A–I)]rHDL containing S1P in tube formation in endothelial cells (ECs) as well as cholesterol efflux in macrophage. The effect of (POPC/S1P/A–I)rHDL on cholesterol efflux in macrophage was similar to that of conventional rHDL, (POPC/A–I)rHDL. In addition, (POPC/S1P/A–I)rHDL induced EC proliferation through the activation of phospho-Akt and phospho-extracellular-signal-regulated kinases (p-ERK) 1/2 and EC tube formation, and this effect was blocked by inhibitors of Akt, ERK and endothelial nitric-oxide synthase (eNOS). In addition, (POPC/S1P/A–I)rHDL-induced p-ERK1/2 activation and EC tube formation can be mainly attributed to S1P-stimutated signaling through $S1P_2$ and $S1P_3$ as determined by an anti-sense strategy. In conclusion, (POPC/S1P/A–I)rHDL induces cholesterol efflux independently of S1P but has additional S1P-mediated effects on EC tube formation mediated by Akt/ERK/NO through $S1P_2$ and $S1P_3$. In the future, these new discs may be useful for the treatment of atherosclerotic and ischemic cardiovascular disease, such as acute coronary syndrome and atherosclerosis obliterans.


*Keywords:* Reconstituted high density lipoprotein; Sphingosine-1-phosphate; Tube formation; Extracellular-signal-regulated kinases

## 1. Introduction

High-density lipoproteins (HDL) are a heterogeneous group of small, dense lipoproteins. A low HDL level is one of the strongest predictors of coronary risk [1]. The negative correlation between coronary artery disease (CAD) and plasma HDL-cholesterol has been attributed to the ability of HDL to take up cellular cholesterol from the periphery and to mediate the transport of excess cholesterol to the liver [2].

HDL plays a crucial role in reverse-cholesterol transport [3]. A key factor in this process is the interaction between lipid depleted of apolipoproteinA–I (A–I) and the transmembrane protein ABCA1, which results in cellular cholesterol efflux. In addition, HDL has pleiotropic effects, in that it promotes anti-inflammation, including inhibition of the expression of adhesion molecules and monocyte chemotaxis, and anti-coagulation [4]. Although HDL is a target in the treatment of atherosclerotic CAD [5], there are currently only a limited number of therapeutic options to promote HDL. However, several exciting therapeutic strategies have recently been developed and are currently the focus of intense research such as the infusion of A–I [6,7] or reconstituted(r)HDL, which may act as cholesterol acceptors [5]. rHDL that mimic the structure of A–I have anti-atherogenic effects in animal models [8–10]. A–I mimetic peptides have also been shown

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to inhibit inflammation [11] and thrombus formation [12]. In addition, Nissen et al. reported that 45 patients suffering from acute coronary syndrome received weekly infusions of A–I-Milano-phospholipid complexes over a period of 5 weeks. In patients who received the infusions, there was a significant regression in plaque size compared with baseline using intracoronary vascular ultrasound. Despite the small number of patients involved, this study has focused considerable attention on A–I-infusion as a therapeutic concept [13], and more attention has been paid to rHDL for the treatment of coronary atherosclerosis.

Sphingosine-1-phosphate (S1P), which is contained in HDL, is known to exhibit a wide variety of biological activities in many mammalian and other vertebrate cell types. S1P induces cell survival signaling and is involved in migration, proliferation, angiogenesis [14] and cytoskeletal changes in endothelial cells (ECs) mediated by a specific family of G protein-coupled (GPCRs) sphingosine 1-phosphate receptors [$S1P_1$ (formerly endothelial differentiation gene-1 [Edg-1]), $S1P_2$ (Edg-5), $S1P_3$ (Edg-3), $S1P_4$ (Edg-6) and $S1P_5$ (Edg-8)] [15]. Therefore, we developed new rHDL, [(POPC(1-palmitoyl-2-oleoyl phosphatidylcholine)/S1P/A–I)]rHDL containing S1P. The new rHDL may induce cholesterol efflux and may have additional effects for cell survival, including proliferation and angiogenesis. In the future, the rHDL may be useful for the treatment of atherosclerotic cardiovascular disease, such as acute coronary syndrome and atherosclerosis obliterans.

## 2. Material and methods

### 2.1. Materials

The following antibodies and reagents were purchased: A–I (Calbiochem); PD98059, 2-(2′-amino-3′-methoxyphenyl) oxanaphthalen-4-one, a specific inhibitor of extracellular-signal-regulated kinases (ERK) (PD98059) (New England BioLabs); S1P and L-NAME (*N*ω-Nitro-L-arginine methyl ester hydrochloride) (Sigma); Akt inhibitor (1L-6-Hydroxymethyl-chio-inositol-2-(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate)(Merck); antibodies for Akt, phospho (p)-Akt, ERK1/2 and p-ERK1/2 ($Thr^{202}/Tyr^{204}$) (Cell Signaling Technology) and for $S1P_2$ and $S1P_3$ (Santa Cruz); cDNAs of S1P receptors, $S1P_1$, $S1P_2$ and $S1P_3$ (UMR cDNA Resource Center, Rolla, MO); and pCMV-RasN17 (dominant-negative type) (Clontech, Palo Alto, CA).

### 2.2. Cell culture and transfection

Human coronary artery ECs (HCECs) were purchased from Clonetics. ECs were cultured in media supplemented with 5% fetal bovine serum (FBS), penicillin/streptomycin, endothelial cell growth supplement (Takara Co., Osaka, Japan) at 37 °C under 5% $CO_2$. Chinese hamster ovary (CHO) cells were grown in Dulbecco's modified Eagle's essential medium (DMEM)(Gibco-BRL) with 10% FBS and penicillin/streptomycin at 37 °C under 5% $CO_2$. In the experiments, cells supplemented without cell growth supplement were used. CHO cells and HCECs were transfected using the Lipofectamin 2000 liposomal reagent according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). The efficiency of transfection in each experiment was estimated by comparison to transfection with pEGFP(enhanced green fluorescence protein).

### 2.3. Oligodeoxynucleotides

Expression of $S1P_2$ and $S1P_3$ was down-regulated by transfection with 18-mer phosphothioate oligonucleotides as described previously [16]. Antisense $S1P_2$, 5′-CGAGTACAAGCTGCCCAT-3′; sense $S1P_2$, 5′-ATGGGCAGCTTGTACTCG-3′; scrambled $S1P_2$, 5′-ACGTAGGGCTTGCCATTG-3′; antisense $S1P_3$; 5′-CGGGAG GGCAGTTGCCAT-3′; sense $S1P_3$, 5′-ATGGCAACTGCCCTCCCG-3′; scrambled $S1P_3$, 5′-ATCCGTCAAGCGGGGGTG-3′ were synthesized by Sigma–Aldrich Japan K.K. Genosys Division. $1 \times 10^6$ HCECs were transfected with oligos at a final concentration of 1 μM using Oligofectamine (Invitrogen Corp., Carlsbad, CA).

### 2.4. Preparation of (POPC/A–I)rHDL, (POPC/S1P/A–I)rHDL and HDL

A–I was purified from ultracentrifugally isolated HDL as described elsewhere [17–19]. Discoidal rHDL containing POPC (Avanti Polar Lipids, Alabaster, AL) and A–I, (POPC/A–I)rHDL, (initial POPC/A–I molar ratio 100/1) or POPC, S1P (Cayman Chemical Company, Ann Arbor, MI) and A–I, (POPC/S1P/A–I)rHDL, (initial POPC/S1P/A–I molar ratio 89/11/1) were prepared by the cholate dialysis method [20]. Briefly, to prepare discoidal rHDL containing POPC, S-1-P and apoA–I, POPC (100 mg/ml) and S-1-P (10 mg/ml) were dissolved in chloroform/methanol (2/1, v/v). Aliquots of POPC and S1P (containing 4.8 and 0.3 mg, respectively) were added to clean dry test tubes, and then dried under nitrogen to obtain a thin film on the tube wall. Sodium cholate (102 μl of a 30 mg/ml solution) was then added to each tube with vortexing. The tubes were placed on ice and vortexed for approximately 20 s at 10 min intervals until they were optically clear (approximately 1 h). At this time 2 mg of purified apoA–I in Tris-buffered saline (10 mM Tris–HCl, 150 mM NaCl, pH 7.4) containing 1 mM EDTA-$Na_2$ and 0.01% (w/v) $NaN_3$ (TBS) was added to each tube. The rHDL was incubated at 4 °C overnight, and then pooled and dialysed against 5 × 1l TBS over 5 days. Prior to use, rHDL was dialysed against 3 × 1l endotoxin-free phosphate-buffered saline (PBS) [21]. The final molar ratios of the discoidal (POPC/A–I)rHDL and discoidal (POPC/S1P/A–I)rHDL were 92.5/1.0 and 89/11/1, respectively. Particle diameters were determined by electrophoresis on 3–40% non-denaturing gradient gels. For the