Serum Amyloid A Protein Generates Preβ1 High-Density Lipoprotein from α-Migrating High-Density Lipoprotein[†]

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ABSTRACT: Serum amyloid A protein (SAA), an acute-phase reactant in reactive amyloidosis, has high affinity for high-density lipoprotein (HDL). When SAA is added to HDL, SAA displaces apolipoprotein A-I (apoA-I) and phospholipid from the HDL particles. These dissociated components may form preβ1-HDL because free apoA-I can associate with phospholipid to become a lipoprotein having preβ mobility. To determine whether SAA generates preβ1-HDL from α-migrating HDL, we investigated the effects of recombinant SAA on HDL subfraction concentration using nondenaturing two-dimensional gradient gel electrophoresis. When we added SAA (0.5 mg/mL) to plasma, the preβ1-HDL concentration increased by 164% (from 4.7% $\pm$ 1.3% to 12.4% $\pm$ 3.2% of apoA-I, $p < 0.005$). The increase in preβ1-HDL was proportional to the dose of SAA. When we added SAA to a column of Sepharose beads coupled to the isolated HDL (α-migrating HDL), preβ1-HDL was dissociated from the column together with the SAA-associated HDL. In summary, we demonstrate that SAA generates preβ1-HDL from α-migrating HDL. We speculate that SAA-mediated HDL remodeling may take place in inflammation.

Serum amyloid A protein (SAA)[1] is a putative precursor of amyloid A, the main component of fibrillar deposits in reactive amyloidosis (*1*). In humans, at least six isotypes of SAA have been identified (*1*−*3*). They are classified into two groups**:** the acute-phase isotypes (including SAA1 and SAA2) (*1*, *2*) and the constitutive isotype (SAA4) (*3*). Both isotypes have a high affinity for high-density lipoprotein (HDL) (*4*−*6*), but their physiological role in HDL metabolism is unknown.

When SAA is secreted into plasma, SAA displaces the apoA-I and phospholipid on HDL particles (*7*, *8*). However, the fate of dissociated components is not clearly elucidated. In vitro experiments have shown that the free apoA-I easily associates with phospholipid and becomes preβ-migrating HDL (*9*). Thus, the apoA-I and phospholipid dissociated from α-migrating HDL by SAA may form preβ1-HDL. The aim of the present study is to determine whether SAA generates preβ1-HDL from α-migrating HDL. We investigated the effect of SAA on preβ1-HDL generation in whole plasma and in isolated HDL coupled to Sepharose beads.

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[1] Abbreviations: HDL, high-density lipoprotein; SAA, serum amyloid A protein; 2D gel, two-dimensional gel; apoA-I, apolipoprotein A-I.

EXPERIMENTAL PROCEDURES

Recombinant SAA. Recombinant SAA was produced in *Escherichia coli* transfected with human SAA1α cDNA as previously described (*10*). The purified rSAA was dissolved in 0.1 M Tris (pH 7.4) and 0.05% Tween 20 (dissolving buffer). The SAA solution was stored at 4 °C until used.

Blood Samples. We used normolipidemic plasma from five healthy volunteers (three males and two females, aged 22−60 years). All subjects understood the purpose of our study and gave us their informed consent. Fasting blood was collected into precooled (ice water) glass tubes containing K_2EDTA. Plasma was obtained after centrifugation at 0 °C, 2000*g*, for 30 min and used immediately for the experiments as described below. The mean HDL-C concentration was 58 $\pm$ 15 mg/dL, and the mean apoA-I concentration was 145 $\pm$ 23 mg/dL. The experimental protocol was reviewed and approved by the Institutional Review Board at Niigata University.

Preparation of the HDL−Sepharose Column. We prepared an HDL−Sepharose column using prepacked NHS-activated Sepharose (HiTrap NHS-activated column, Amersham Pharmacia Biotech, Tokyo, Japan). HDL ($1.063 < d < 1.210$) was isolated from fresh normolipidemic plasma by sequential ultracentrifugation (*11*). The isolated HDL was extensively dialyzed against 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA containing 0.01% NaN_3 (plasma density buffer). As other investigators have reported (*12*), all of the isolated HDL ($1.063 < d < 1.210$) had the α-mobility in the agarose gel electrophoresis (data not shown). We measured protein concentration by the method of Lowry (*13*) and adjusted the final HDL concentration to 1.0 mg/mL protein

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with coupling buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3).

NHS-activated Sepharose was washed with ice-cold 1 mM HCl (6 column volumes) before coupling. One column volume (1 mL) of HDL solution was injected onto the column and incubated at room temperature for 30 min. The unreacted sites were blocked at room temperature with (1) six column volumes of 0.5 M ethanolamine and 0.5 M NaCl, pH 8.3 (high-pH buffer) and (2) six column volumes of 0.1 M acetate and 0.5 M NaCl, pH 4.0 (low-pH buffer). We replaced the buffer in the column with high-pH buffer and incubated it for 60 min. After the blocking procedure, unbound ligand was washed out by several cycles of low- and high-pH buffers. Finally, the column was equilibrated with phosphate-buffered saline (PBS). In some experiments, HDL2 ($1.063 < d < 1.125$) and HDL3 ($1.125 < d < 1.210$) were isolated by ultracentrifugation, and columns of HDL2−Sepharose and HDL3−Sepharose were prepared in the same way.

Experimental Procedures. We examined the effect of SAA on preβ1-HDL generation in two distinct ways. In the first series of experiments, plasma was mixed with SAA (at the concentrations indicated) in an ice−water bath. In control experiments, plasma was mixed with dissolving buffer (0.1 M Tris and 0.05% Tween 20, pH 7.4), or the same buffer containing nonimmune human IgG (Wako Pure Chemical, Osaka, Japan; final concentration 0.5 mg/mL). The mixture was tapped gently a few times and centrifuged in a refrigerated microfuge (model 1710, Kubota, Tokyo, Japan) at 0 °C, 6000*g* for 1 min. The supernatant was immediately electrophoresed on a nondenaturing two-dimensional polyacrylamide gradient gel (native 2D gel) as described below.

In the second series of experiments, SAA (0.5 mg/mL) was injected onto the HDL−Sepharose column at a flow rate of 1.25 mL/min. The eluted material was collected and analyzed by native 2D gel electrophoresis.

Native 2D Gel Electrophoresis. The distribution of apoA-I and SAA among HDL subfractions was analyzed by native 2D gel electrophoresis as described previously (*14*−*16*). Briefly, an aliquot of sample (containing 20 μL of plasma) was applied to an 0.75% agarose gel on Gelbond (FMC, Rockland, ME). Agarose gel electrophoresis was carried out in 50 mM barbital buffer (pH 8.6), at 0 °C, 200 V, until a bromophenol blue−bovine serum albumin marker had migrated 8 cm from the origin. Then, agarose gel pieces were transferred to a 2−15% polyacrylamide gradient gel. Further separation was made at 0 °C, for 2000 V·h. Separated HDL subfractions were electroblotted onto a nitrocellulose sheet (pore size 0.45 μm, Sartorius, Göttingen, Germany). The distribution of apoA-I-containing lipoproteins was detected by anti-human apoA-I goat antibodies (Daiichi Pure Chemicals, Tokyo, Japan) labeled with ^{125}I (NEN Research Products, Boston, MA). To quantitate apoA-I within individual HDL subfraction, we cut each area from the nitrocellulose sheet and determined its radioactivity by γ radiation spectrometry. The amount of apoA-I was proportional to radioactivity. Concentrations of HDL subfractions were expressed as the percentage of apoA-I to plasma apoA-I (*14*). We previously documented that the anti-apoA-I antibody reacted equivalently with each HDL subfraction (*14*−*16*). The distribution of SAA-containing lipoproteins was detected by anti-human SAA rabbit antibodies and alkaline phosphatase-labeled secondary antibodies (*6*).

Gel-Filtration Chromatography. Particle size distribution was analyzed by gel filtration chromatography on a Superose 6 column (1 × 30 cm) (Amersham Pharmacia Biotech). By use of a fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech), elution was carried out by PBS containing 0.01% EDTA (pH 7.4) and 0.1% bovine serum albumin at flow rate of 0.5 mL/min. Control plasma was diluted with running buffer and run as a reference. The collected fractions that were dissociated from the HDL−Sepharose column were applied to the FPLC system. ApoA-I and SAA concentrations in each fraction were determined by enzyme-linked immunosorbent assay. Phospholipid concentration was determined by an enzymatic method that was previously developed for phospholipid measurement in cerebrospinal fluid (*17*).

Statistical Analysis. The changes in HDL subfractions were analyzed by paired Student's *t*-test. Values are expressed as mean ± standard deviation.

RESULTS

Effect of SAA on Plasma HDL Subfractions. The exogenous SAA increased the preβ1-HDL concentration in normolipidemic plasma. First, we added SAA to plasma at a final concentration of 0.5 mg/mL. The immunoblot against apoA-I clearly showed that SAA increased preβ1-HDL (Figure 1A,B). The mean preβ1-HDL concentration increased as much as 2.6-fold over the baseline plasma level (Table 1). No other HDL subfraction concentration changed significantly. As we expected, SAA was associated with α-migrating HDL but not with preβ1-HDL (Figure 1C). SAA was also associated with LDL.

In control experiments, plasma preβ1-HDL concentration did not change from the baseline (5.0% ± 1.2% of plasma apoA-I) after the addition of dissolving buffer alone (5.1% ± 1.0% of plasma apoA-I, $n = 3$) or dissolving buffer containing nonimmune IgG (4.8% ± 1.1% of plasma apoA-I, $n = 3$) The other HDL subfractions did not change significantly, either (data not shown).

Additionally, we added SAA to plasma at three different concentrations. SAA increased the preβ1-HDL concentration in a dose-dependent manner (Figure 2).

Effect of SAA on Isolated HDL Coupled to Sepharose Beads. To confirm that SAA dissociates preβ1-HDL from α-migrating HDL, we added SAA to isolated HDL coupled to Sepharose beads. The apoA-I immunoblot showed that SAA dissociated two apoA-I-containing particles from the HDL column (Figure 3A). The smaller particle showed preβ-mobility, while the larger particle showed α-mobility. It is obvious that apoA-I was recovered primarily from preβ-migrating HDL (Figure 3A). On the other hand, SAA was recovered exclusively from α-migrating HDL (Figure 3B). We simultaneously electrophoresed the eluate and control plasma in the same native 2D gel. The preβ-migrating particles in the eluate showed the same mobility as plasma preβ1-HDL (data not shown). In control experiments, neither dissolving buffer alone nor dissolving buffer with nonimmune human IgG dissociated apoA-I-containing particles (data not shown).

SAA-induced dissociation of preβ-migrating particle was not restricted to certain HDL subclass. When we added SAA

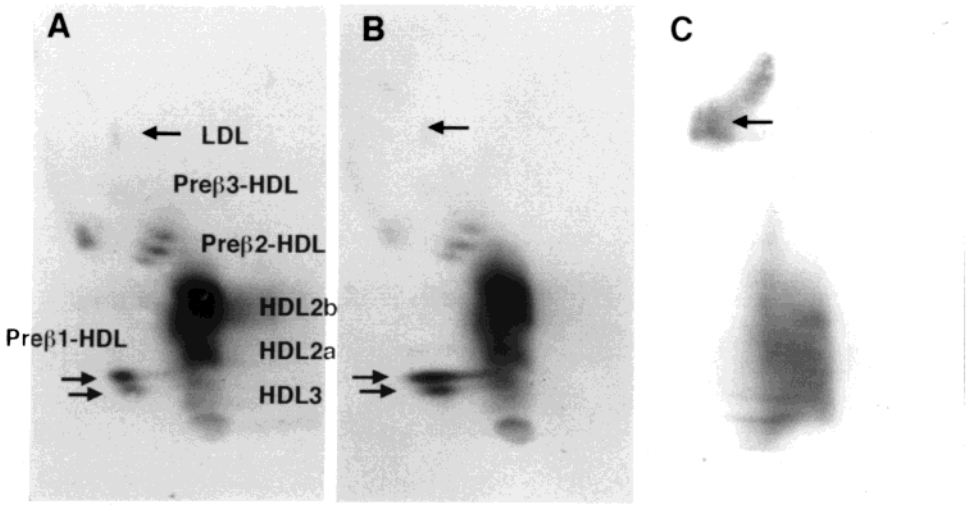

FIGURE 1: Effect of SAA on apoA-I distribution among plasma HDL subfractions. Fasting plasma from a normolipidemic volunteer was mixed with recombinant SAA at a final concentration of 0.5 mg/mL. The samples were subjected to native 2D gel electrophoresis, and the distribution of apoA-I and SAA was determined as described under Experimental Procedures. (A) Distribution of apoA-I in control plasma; (B) distribution of apoA-I after administration of SAA; (C) distribution of SAA after administration of SAA. Note that SAA increased preβ1-HDL markedly. SAA was detected in LDL and α-HDL but not in preβ-HDL.

Table 1: Effect of Exogenous SAA on HDL Subfraction Concentrations in Plasma[a]

HDL subfraction	% of plasma apoA-I	
	baseline	+SAA (0.5 mg/mL)
preβ3-HDL	0.8 ± 0.2	0.8 ± 0.3
preβ2-HDL	3.6 ± 0.5	4.1 ± 1.7
preβ1-HDL	4.7 ± 1.3	12.4 ± 3.2[b]
HDL2b	28.0 ± 10.9	24.1 ± 6.9
HDL2a	40.1 ± 5.3	35.8 ± 6.4
HDL3	21.3 ± 6.6	21.5 ± 5.7

[a] SAA (in 0.1 M Tris-HCl, pH 7.4, and 0.05% Tween 20) was added to plasma obtained from normolipidemic subjects at a final concentration of 0.5 mg/mL. HDL subfraction concentrations were quantitated by use of a native 2D gel as described under Experimental Procedures. Values are mean ± SD for five healthy volunteers. [b] $p < 0.005$ vs baseline.

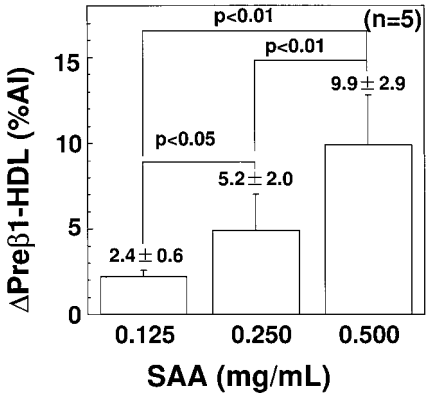

FIGURE 2: Dose-dependent generation of preβ1-HDL by SAA. Fasting plasma from a normolipidemic volunteer was mixed with recombinant SAA at the concentrations indicated. The samples were subjected to native 2D gel electrophoresis, and preβ1-HDL concentration was determined as described under Experimental Procedures.

solution to an HDL2−Sepharose column, small preβ-migrating particle was detected in the eluate (Figure 4A). When we added SAA solution to an HDL3−Sepharose column, both preβ1-HDL and small preβ-migrating particle were detected in the eluate (Figure 4B). In both columns, α-migrating particle was dissociated.

Gel filtration chromatography also showed that the larger and smaller apoA-I-containing particles were dissociated from the HDL-column by SAA. In the eluate, the larger apoA-I-containing particle was detected at the same position of plasma HDL (Figure 5). SAA and phospholipid were also detected in this particle. However, phospholipid content of the larger particle was much less than that of plasma HDL. On the other hand, the smaller apoA-I-containing particle was detected in the eluate but not in plasma. This second apoA-I peak did not exactly correspond to the greatest peak of phospholipid (Figure 5).

DISCUSSION

This study indicates that SAA generates preβ1-HDL from α-migrating HDL. We found that exogenous SAA increased preβ1-HDL concentration in plasma in a dose-dependent manner (Figure 2). Moreover, SAA immediately dissociated preβ1-HDL from ultracentrifugally isolated HDL coupled to Sepharose beads (Figure 3).

The dissociation of apoA-I from α-migrating HDL is a common phenomenon in apolipoprotein-mediated HDL remodeling. Labeur et al. (*18*) reported that exogenous apoA-II, or its carboxyl-terminal (C-terminal) helical peptide, dissociated apoA-I from α-migrating HDL. In their experiment, apoA-II or its C-terminal peptide was able to replace up to 40% of apoA-I on α-migrating HDL. As shown in the present study, SAA (one of the apolipoproteins) also dissociated apoA-I from α-migrating HDL (Figures 1−3). The earlier study showed that SAA can replace up to 80% of the apoA-I on HDL (*7*). In non-apolipoprotein-mediated HDL remodeling, as well as in the apolipoprotein-mediated HDL remodeling, several factors including hepatic lipase (*19*), cholesteryl ester transfer protein (*20*), and phospholipid transfer protein (*21*) can also dissociate apoA-I from α-migrating HDL.

In apolipoprotein-mediated HDL remodeling, the dissociated apoA-I is detected as a lipoprotein, that is, preβ1-HDL.

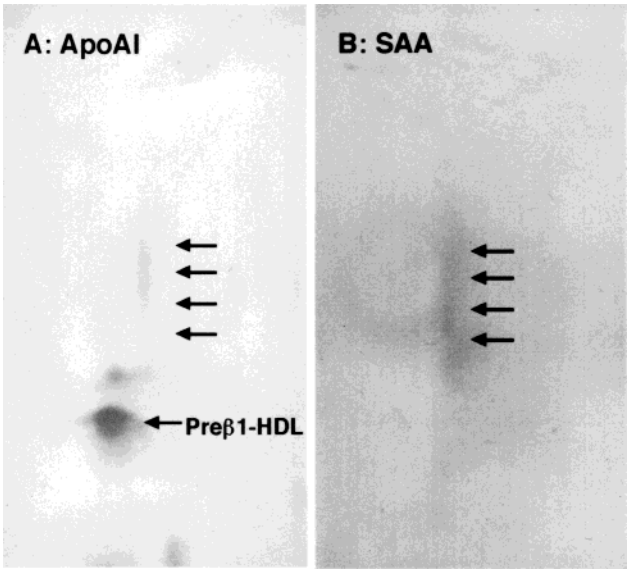

FIGURE 3: Lipoproteins dissociated from a HDL-Sepharose column by SAA. The ultracentrifugally isolated HDL was coupled to Sepharose beads (HDL column) as described under Experimental Procedures. A solution of SAA was added to the HDL column. The dissociated lipoproteins were analyzed by native 2D gel electrophoresis followed by immunoblotting. (A) ApoA-I-containing lipoproteins dissociated from the HDL column; (B) SAA-associated lipoprotein dissociated from the HDL column.

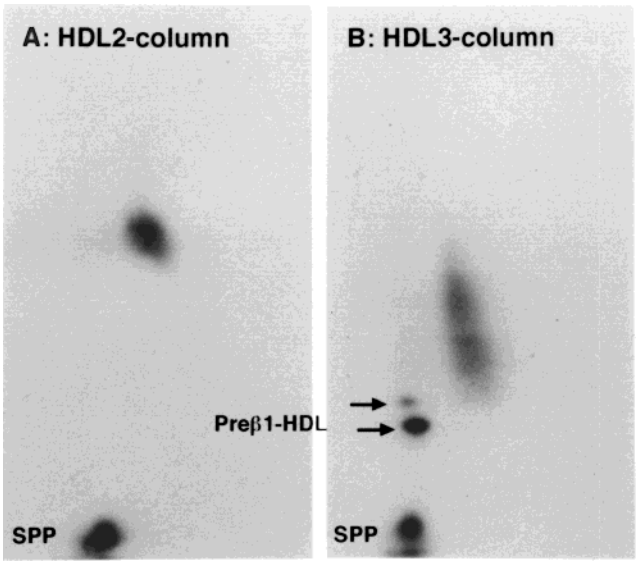

FIGURE 4: ApoA-I-containing lipoproteins dissociated from HDL2-Sepharose column and HDL3−Sepharose columns by SAA. The ultracentrifugally isolated HDL2 and HDL3 were coupled to Sepharose beads (HDL column) as described under Experimental Procedures. A solution of SAA was added to the HDL column. The dissociated lipoproteins were analyzed by native 2D gel electrophoresis followed by immunoblotting against apoA-I. (A) ApoA-I-containing lipoproteins dissociated from the HDL2 column; (B) ApoA-I-containing lipoproteins dissociated from the HDL3 column. SPP, small preβ-migrating particle.

Preβ1-HDL, putative discoid-shaped HDL, is the initial acceptor of cellular cholesterol (*22*). Preβ1-HDL is rich in apoA-I and phospholipid but poor in core lipid (cholesteryl ester and triglyceride) (*22*). When apoA-II or its C-terminal peptide was added to isolated HDL, preβ1-HDL appeared in the native 2D gel (*18*). In their study, phospholipid was detected at the preβ1-HDL position by one-dimensional

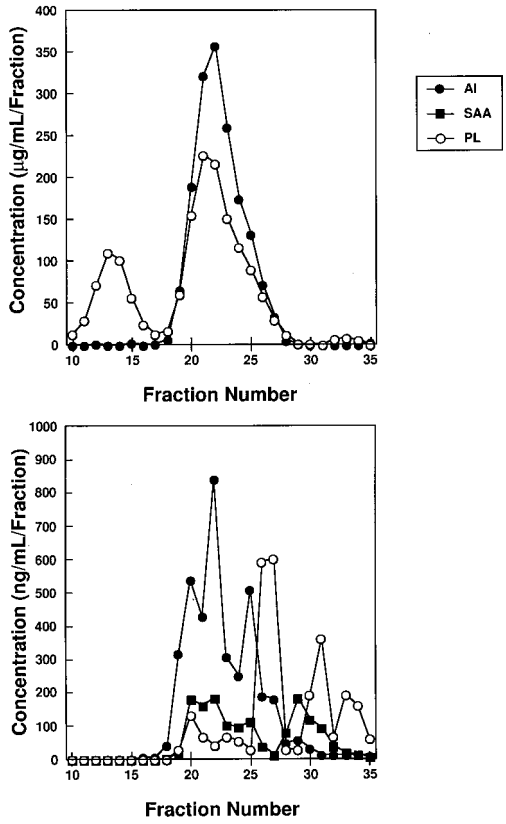

FIGURE 5: Gel filtration of dissociated lipoproteins from HDL−Sepharose column by SAA. The eluate from an HDL−Sepharose column was fractionated by gel filtration. ApoA-I, SAA, and phospholipid concentrations were determined as desribed under Experimental Procedures. Upper panel, plasma; lower panel, dissociated lipoproteins by SAA.

agarose gel electrophoresis (*18*). In our experiments with SAA and isolated HDL, α-migrating HDL provided the only source for the lipid components of preβ1-HDL. Therefore, both apoA-I and the phospholipid of preβ1-HDL are considered to have come from α-migrating HDL. In the non-apolipoprotein-mediated HDL remodeling, preβ1-HDL is also generated in vitro (*19*) and in vivo (*16, 23, 24*).

Why did SAA dissociate not only preβ1-HDL but also SAA-associated HDL from the HDL column? The α-migrating HDL is considered to have more than two apoA-I molecules per particle (*25*). Therefore, some apoA-I may bind to the Sepharose beads (the fixed apoA-I), while some apoA-I on the same HDL particle may not (the free apoA-I). If SAA displaces the free apoA-I, preβ1-HDL is likely to be dissociated from the column. In this case, the SAA-associated HDL could stay on the column by means of the fixed apoA-I. On the other hand, if SAA displaces the fixed apoA-I, the SAA-associated HDL cannot stay on the column. In this case, the SAA-associated HDL loses the fixed apoA-I. In the immunoblot against SAA, SAA was present only in the α-migrating HDL (Figure 3). In addition, gel-filtration chromatography revealed that the larger apoA-I-containing particle had less phospholipid than plasma HDL and eluted with SAA (Figure 5). These findings support our hypothesis.

We failed to purify preβ1-HDL by gel-filtration chromatography because preβ1-HDL is unstable for dilution. Asztalos et al. (26) diluted plasma 0, 4, and 8 times with Tris−tricine buffer and examined HDL subfraction changes using native 2D gel electrophoresis (*26*). As plasma was diluted,

preβ1-HDL decreased and small preβ-migrating particle increased (*26*). In the 8 times diluted plasma, all the preβ-migrating HDL were the smaller particles. In the present study, small preβ-migrating particle was detected in the experiments with HDL2− and HDL3−Sepharose columns (Figure 4). In addition, the peak of the smaller apoA-I-containing particle, which was separated by gel filtration, did not exactly correspond to the greatest phospholipid peak (Figure 5). Probably, apoA-I and phospholipid of preβ1-HDL were dissociated again due to sample dilution. We could not label preβ1-HDL to investigate its metabolism. More study is needed to elucidate the physiological significance of preβ1-HDL generation by SAA.

In summary, this study indicates that SAA generates preβ1-HDL from α-migrating HDL. We speculate that the displacement of apoA-I from HDL by apolipoproteins might be an important source for plasma preβ1-HDL in circulation.

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