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ANIONIC PHOSPHOLIPIDS LOSE THEIR PROCOAGULANT PROPERTIES WHEN INCORPORATED INTO HIGH-DENSITY LIPOPROTEINS*

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Running title: Anticoagulant properties of apolipoprotein A-I

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Blood coagulation involves a series of enzymatic protein complexes that assemble on the surface of anionic phospholipid. To investigate whether apolipoproteins affect coagulation reactions, they were included during the preparation of anionic phospholipid vesicles using a detergent solubilization–dialysis method. Apolipoprotein components of high-density lipoproteins, especially apolipoprotein A-I, had pronounced anticoagulant effect. The anionic phospholipids lost their procoagulant effect when the vesicle preparation method was performed in the presence of apolipoprotein A-I. The anionic phospholipid–apolipoprotein A-I particles were 8–10 nm in diameter and contained around 60–80 phospholipid molecules, depending on the phospholipid composition. The phospholipids of these particles were unable to support the activation of prothrombin by factor Xa in the presence of factor Va, and unable to support binding of factor Va, while binding of prothrombin and factor Xa were efficient. Phospholipid transfer protein was shown to mediate transfer of phospholipids from liposomes to apolipoprotein A-I containing reconstituted high-density lipoprotein. In addition, serum was also shown to neutralize the procoagulant effect of anionic liposomes and to efficiently mediate transfer of phospholipids from liposomes to either apolipoprotein A-I or apolipoprotein B containing particles. In conclusion, apolipoprotein A-I was found to neutralize the procoagulant properties of anionic phospholipids by arranging the phospholipids in surface areas that are too small to accommodate the prothrombinase complex.

This anionic phospholipid scavenger function may be an important mechanism to control the exposure of such phospholipids to circulating blood and thereby prevent inappropriate stimulation of blood coagulation.

The concentration of high-density lipoprotein (HDL) in plasma inversely correlates with the incidence of ischemic heart disease as well as with other atherosclerosis-related ischemic conditions(1-3). However, the molecular mechanism by which HDL prevents ischemic diseases is not fully understood. The atheroprotective functions of HDL are thought to be related to the ability of HDL to take up cholesterol from peripheral organs and to mediate the transport of excess cholesterol to the liver for excretion(4,5). In addition, recent studies reveal that HDL has various other favorable anti-atherogenic effects(6). Apolipoprotein A-I (apoA-I) is the major protein in HDL, constituting about 70% of the protein content of HDL particles. ApoA-I is synthesized in the liver and intestine as pre-pro-apoA-I. After processing, the pre- and pro-peptides are cleaved and, apoA-I is incorporated into plasma HDL particles(7). ApoA-I can exist in three different forms in plasma, either in a lipid-free/lipid-poor form, or as a component of discoidal or spherical HDL(8). Discoidal HDL usually contains 2 or 3 molecules of apoA-I and phospholipids with or without unesterified cholesterol(4,8). Reconstituted HDL (rHDL) particles can be generated from isolated apoA-I and phospholipids and have been extensively used for *in vitro* and *in vivo* studies of discoidal HDL(9).

Rupture of an atherosclerotic plaque triggers primary haemostasis events, which involve a cascade of proteolytic reactions resulting in the formation of thrombin and subsequent fibrinogen to fibrin clot conversion. The reactions occur on membrane surfaces containing the anionic phospholipid phosphatidylserine (PS), which is exposed on the surface of activated platelets. The coagulation proteins bind to the phospholipid surface and assemble into multi-molecular enzyme complexes, e.g. the tenase and prothrombinase complexes(10,11). In the tenase complex, the enzyme factor IXa (FIXa) together with its cofactor factor VIIIa (FVIIIa) activate factor X (FX) to factor Xa (FXa)(12). The prothrombinase complex (see **Fig. 5a** in discussion for a schematic picture) consists of the enzyme FXa, which with support from its cofactor factor Va (FVa) activates prothrombin(13). The enzymes and substrates bind to PS-containing membranes via their vitamin K-dependent γ -carboxy glutamic acid (Gla) rich domains(14,15) whereas the cofactors; FVa and FVIIIa bind via their C-domains(16-19).

Plasma lipoproteins have been suggested to influence the reactions of blood coagulation. Thus, very-low-density lipoprotein (VLDL) is reported to stimulate the activations of FVII and prothrombin(20,21), whereas low-density lipoprotein (LDL) potentiated activation of FX(22). In contrast, HDL was reported to function as a cofactor for the anticoagulant protein C pathway(2). In support for an anticoagulant effect of HDL, low plasma concentration of HDL was identified as a risk factor of venous thrombosis(23).

The aim of the study was to elucidate mechanisms that regulate the reaction of blood coagulation on the phospholipid surface of lipoprotein particles from human blood and to determine whether apolipoproteins affect blood coagulation reactions. Isolated apolipoproteins were used together with anionic phospholipids to generate reconstituted lipoproteins. Here we demonstrate that apoA-I has the ability to neutralize the procoagulant properties of anionic phospholipid during the generation of rHDL. The anticoagulant properties of apoA-I may be an important component of the anti-atherogenic and antithrombotic potential of HDL.

Experimental procedures

Isolation of lipoprotein fractions. Lipidemic plasma obtained from the local blood bank was thawed overnight at 4 °C. EDTA was added to a final concentration of 0.04%. Lipoproteins were isolated from plasma by sequential flotation ultracentrifugation as previously described using a Beckman centrifuge (Optima L-70K)(24). All isolated fractions were dialyzed into HN buffer (10 mM Hepes with 150 mM NaCl, pH 7.4), and stored at -20 °C. Proteins from isolated lipoprotein fractions were extracted with, at least 20-fold excess, ether/ethanol 33/67 (v/v) at room temperature overnight with continuous stirring. Precipitated proteins were collected by centrifugation at 3000 g for 10 minutes and resuspended in 6 M guanidine-HCl to the original volume of each lipoprotein fraction.

Purification of apoA-I from HDL. Extracted proteins from the HDL fraction were separated on two serially coupled S-200 Hiprep ^{26/60} size exclusion columns (GE Healthcare) using 6 M guanidine-HCl, 50 mM Tris-HCl, pH 8. ApoA-I was further purified on a Q Sepharose Fast Flow column (GE Healthcare) equilibrated in 6 M Urea, 50 mM Tris-HCl, pH 7.5. Bound proteins were eluted by a 0-300 mM linear gradient of NaCl. Fractions containing apoA-I were pooled and stored at -20 °C. Protein concentration was determined at absorbance 280 nm with a calculated extinction coefficient of 1.155 g⁻¹Lcm⁻¹(25).

Phospholipid vesicle preparation. Natural phospholipids (PL), phosphatidylserine (PS, brain extract), phosphatidylethanolamine (PE, egg extract), and phosphatidylcholine (PC, egg extract) were purchased from Avanti Polar Lipids (Alabaster, AL) and dissolved in 10/90 (v/v) methanol/chloroform solution. Lipids were mixed, dried under N₂-gas and resuspended in HN buffer at room temperature. A trace amount of ¹⁴C-radiolabelled PC (GE Healthcare) was added to the lipid mixture when necessary. Lipids were then solubilized by adding *n*-Octyl- β -D-glucopyranoside (Calbiochem) to a final concentration of 200 mM. Solubilized lipids and apoA-I were mixed, 50/50 (v/v), and dialyzed against at least 1000-fold excess of TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5) or HN buffer at

room temperature using 12-14 000 MW cut off membranes (Spectra/Por). During the generation of liposomes, solubilized lipids were dialyzed as mentioned above.

Characterization of rHDL with apoA-I. Generated rHDL particles, with apoA-I, were isolated on Superose 6 10/300 GL (GE Healthcare) having HN buffer as running buffer. The column was connected to an ÄKTA fast performance liquid chromatography system (Amersham Pharmacia Biotech) and calibrated according to the manufacturer's instructions using thyroglobulin, ferritin, aldolase, ovalbumin, and ribonuclease (GE Healthcare). rHDL particles were characterized for phospholipid composition by scintillation counting (Liquid scintillation counter, Wallac 1410, Pharmacia) and for protein composition by the apoA-I ELISA (see below).

ELISA Assay for apoA-I. ApoA-I was detected in rHDL particles using an ELISA method. Wells (96-well plates, MaxiSorp, Nunc) were coated with 10 µg/mL of rabbit anti-apoA-I polyclonal antibody (Dako, Denmark) overnight at 4 °C. Plates were blocked with 3% fish gelatin (Norland Products) for 2 h at room temperature. The apoA-I standard (plasma purified apoA-I dialyzed against TBS at 4 °C using 3500 MW cut off membranes (Spectra/Por)) and samples to be tested were diluted in TBS, pH 7.4, with 1% BSA (Sigma-Aldrich) and 1% Triton X-100 (Sigma-Aldrich) and placed in the plates for 2 h at room temperature. The plates were washed 3 times in TBS, pH 7.4, with 0.1% Triton X-100. Biotinylated mouse anti-apoA-I monoclonal antibody (in house made monoclonal antibody raised against apoA-I, using standard procedures(26)) was then diluted to 1 µg/mL in TBS pH 7.4, 1% BSA and 0.1% Triton X-100 and added on plates for 1 h at room temperature followed by wash. Streptavidin-avidin complex with horseradish peroxidase (Dako, Denmark) was prepared according to manufacturer's instructions and diluted in TBS, pH 7.4, with 1% BSA and 0.1% Triton X-100, and added to the plates. The plates were incubated for 30 min at room temperature and after washing developed with peroxide and o-phenylenediamine dihydrochloride (Dako, Denmark). Reaction was terminated with 1 M H₂SO₄ and absorbance at 490 nm was measured with a microplate reader (EL808; BioTek Instruments) with Deltasoft 3 software.

SDS/PAGE. Protein samples were loaded onto 15% Tricine-SDS/PAGE gels(27) under non-reducing conditions. Gels were developed using common silver staining procedure(28).

Prothrombinase assay. Phospholipid containing samples in HNBSACa (HN with 5 mg/mL BSA and 5 mM CaCl₂) were mixed with factor V (FV, purified from plasma as described(29) with minor modifications(30)) and FXa (Kordia, Leiden, Netherlands) to concentrations of 420 pM and 5 nM respectively. FV was activated by addition of thrombin (Haematologic Technologies, Inc, Essex Junction, VT) to final concentration 3 units/L for 3 minutes at 37 °C and the activation was terminated by addition of hirudin (Pentapharm, Basel, Switzerland) to final concentration 8 units/L. Samples (60 µL) were transferred to a 96-well plate (Sero-well, Sterilin) and mixed with 40 µL HNBSACa. The reaction was initiated by addition of 20 µL prothrombin (Kordia, Leiden, Netherlands) to final concentration 0.5 µM, and incubated at 37 °C for 2 minutes. The reaction was stopped by addition of 100 µL EDTA buffer (50 mM Tris-HCl, 100 mM NaCl, 100 mM EDTA, 1% PEG6000, pH 7.9). Samples were further diluted 1:75 in 100 mM EDTA buffer before detection of generated thrombin. Aliquots of 150 µL were mixed with 50 µL of a synthetic substrate, S-2238 (kindly provided by Chromogenix, Milan, Italy, final concentration 0.5 mM), and absorbance at 405 nm was measured continuously for 15 minutes with a microplate reader. Final concentrations of proteins during the activation of prothrombin were; FVa 210 pM, FXa 2.5 nM and prothrombin 0.5 µM. During the 2 min activation time, using a phospholipid concentration ≤ 5 µM, the thrombin generation was within the linear range. The amount of thrombin generated in the assay was calculated using a standard curve generated from a thrombin titration (150 µL thrombin dilution and 50 µL S-2238) with known amounts of protein.

Prothrombinase activation without FVa. Assay was done as described for prothrombinase assay, with the following changes. FXa was used at 20 times higher concentration (final concentration 50 nM) and, FV, thrombin and hirudin were replaced with HNBSACa. The reaction with prothrombin was prolonged to 5 min at 37 °C, and stopped as described. Samples were then diluted only 1:15 in

100 mM EDTA buffer before detection of generated thrombin.

Surface Plasmon Resonance Analysis. Human plasma derived prothrombin and FXa were both purchased from Haematologic Technologies, Inc, Essex Junction, VT. Recombinant annexin V was from BD Biosciences Pharmingen, San Jose, CA. Human FVa was purified from plasma as described(31). Prethrombin-1 was prepared essentially as described previously(32). Briefly, prothrombin (2.0 mg/mL) was incubated with 10 U/mL thrombin for 2 hours at 37 °C. Prethrombin-1 was isolated by chromatography on a column with DEAE-sephadex A-50 in 0.2 M Tris-HCl, pH 8.0 and eluted with a linear gradient of NaCl (0-0.3 M, 600 mL of each vessel). To check purity of the prethrombin-1, fractions were run under reducing conditions on SDS acryl amide gel and stained with silver stain. Prethrombin-1 pool activity was measured with thrombin substrate (S-2238) after activation with snake venom Echis carinatus (from Sigma-Aldrich). Prothrombin, FVa and FXa binding to isolated rHDL particles of different phospholipid composition was quantified by surface plasmon resonance using a Biacore 2000 instrument (Uppsala, Sweden) at 24 °C. Annexin V was used as an additional positive membrane binding control while prethrombin-1, a Gla-less derivative of prothrombin known not to interact with membranes was used as a negative control. LI sensor chip was washed with 40 mM octyl glucoside (1 min at 20 µl/min) immediately followed by an injection of rHDL (10-20 µM phospholipid) for 17 min at a 3 µl/min flow rate in HN running buffer. Binding responses proceeded to saturation and for typical immobilizations were between 680-1460 RU. Weakly adhering rHDL were removed with five consecutive 10 mM EDTA pH 8.0 injections (2 min at 20 µl/min). For protein binding experiments running buffer was changed to HNBSACa (HN with 10 mg/mL BSA and 5 mM CaCl₂) and flow cells were equilibrated until the baseline stabilized to less than 0.05 RU/min. Equilibrium response data was collected for each protein at several concentrations typically spanning 10-fold above and below the K_d of the interaction. A control flow cell containing rHDL with 100% PC was used to subtract response units (RU) due to refractive index of the protein solution and any instrument noise. No binding was detected in the control flow cell for any of the

proteins tested. The immobilized rHDL surface could be regenerated by removing membrane-bound protein with an injection of 10 mM EDTA pH 8.0 (for prothrombin, FXa and annexin V) or 50mM NaOH pH 11.5 (for FVa), which returned the baseline to the value prior to introducing protein. Equilibrium data (R_{eq}) was fitted to a one-site binding hyperbola according to the relationship $R_{eq} = B_{max} \cdot C / (K_d + C)$, where B_{max} is the binding at saturation, C corresponds to the injected analyte concentration and K_d is the equilibrium dissociation constant. An excess concentration of Ca²⁺ (5 mM) was included to avoid limiting membrane affinity(33) and BSA (0.1%) was included to block any non-specific protein-lipid and protein-protein interactions(34). Experiments were carried out with replicate analyte concentrations.

Liposome uptake to rHDL. Equal volumes of apoA-I containing rHDL particles with 30:1 PL/apoA-I molar ratio (10:40:50 PS/PE/PC, 800 µM PL) and 100 µM ¹⁴C-PC labeled liposomes (10:40:50 PS/PE/PC) were mixed. Samples were then incubated in the presence or absence of purified human phospholipid transfer protein (PLTP)(35) (final PL-transfer activity 1000 nmol/mL/h) at 37 °C for 24 h. As a control, 50 µM ¹⁴C-PC labeled liposomes (10:40:50 PS/PE/PC) were incubated with PLTP as above. Samples were then separated on Superose 6 10/300 GL using TBS as running buffer. Eluted fractions were analyzed for radioactivity by scintillation counting.

Liposome phospholipid uptake by lipoproteins in human serum. Equal volumes of human serum (from healthy volunteer) and 50 µM ¹⁴C-PC labeled liposomes (10:40:50 PS/PE/PC) were incubated at 37 °C for 24 h. Samples were then separated on Superose 6 10/300 GL using TBS as running buffer. Eluted fractions were analyzed for radioactivity by scintillation counting. As a control, equal volumes of 40 mg/mL fatty acid-free BSA (Sigma-Aldrich) and 50 µM ¹⁴C-PC labeled liposomes (10:40:50 PS/PE/PC) were incubated at 37 °C for 24 h. Before addition to the gel filtration column, samples were also used as source of phospholipid in a prothrombinase assay to test for procoagulant phospholipid activity (as described above).

RESULTS

Lipoproteins are unable to support prothrombinase reaction. Isolated chylomicrons/VLDL, LDL, HDL, and VHDL (very-high-density lipoprotein) were tested for their ability to support prothrombinase activity. The enzyme FXa and its cofactor FVa were incubated with intact lipoprotein particles or with liposomes generated from anionic phospholipids. Prothrombin was added and the generation of thrombin determined. None of the intact lipoproteins were able to stimulate prothrombin activation (data not shown). A small stimulatory activity was observed in the isolated HDL preparation, but was found not to be associated with the HDL particle when the HDL was further purified by gel filtration chromatography and fractions tested in the prothrombinase assay. The stimulatory activity eluted in the void of the column and not in fractions containing the HDL particles. Thus, it was concluded that none of the isolated lipoproteins were able to support the activation of prothrombin by the FXa-FVa complex.

Anionic phospholipids lose procoagulant properties when incorporated into reconstituted HDL. The extracted apolipoproteins were then used together with anionic phospholipids to generate reconstituted lipoproteins. These lipoproteins were tested in the prothrombinase reaction to elucidate whether the anionic phospholipids retained their ability to support the prothrombin activation when incorporated into lipoproteins. The extracted protein components from HDL and VHDL completely inhibited the ability of the anionic phospholipids to support prothrombin activation, whereas protein extracts from VLDL and LDL had no such effect (**Fig. 1a**). To identify which protein was responsible for the inhibiting effect, the extracted HDL proteins were fractionated on gel filtration chromatography in the presence of 6 M guanidine-HCl. The proteins were then used together with anionic phospholipids to reconstitute lipoproteins that were tested in the prothrombinase reaction. The inhibitory activity was found to be associated with an approximately 25-kDa protein (**Fig. 1b**), which after further purification on Q Sepharose was identified as apoA-I.

To further investigate the anticoagulant effects of apoA-I, rHDL was generated from purified apoA-I and natural phospholipids (10:40:50 PS/PE/PC), using a molar PL/apoA-I ratio of 260:1 and the rHDL was isolated on a Superose 6 column. The isolated rHDL particles had a Stokes diameter of 8 nm and the molar PL/apoA-I ratio was determined to be around 38:1 (**Table 1**). The isolated rHDL are discoidal and cross-linking experiments suggested that each disc contained two apoA-I molecules (see supplement) and thus, 38 phospholipids were contained per leaflet of the membrane bilayer. The isolated rHDL did not stimulate prothrombin activation to the same extent as control liposomes (10% PS), which were highly efficient in supporting prothrombin activation (**Fig. 2a**). When FXa was used without its cofactor FVa, the rHDL did support activation of prothrombin, similar to control liposomes (**Fig. 2b**). The isolated rHDL preparations were also tested in a tenase reaction with FIXa and FVIIIa but also in this case, the rHDL did not stimulate the reaction, in contrast to control liposomes that were highly efficient (data not shown).

rHDL particles with higher PS content (75:0:25 and 50:0:50 PS/PE/PC) were also tested. The isolated rHDL particles had the same PL/apoA-I molar ratios and slightly larger Stokes diameter than the rHDL particles with 10% PS (**Table 1**). However, despite their higher PS content, the rHDL particles did not support prothrombin activation in the presence of FVa to the same extent as control liposomes (10:40:50 PS/PE/PC) (**Fig. 2a**). In the absence of FVa, the rHDL particles were capable of prothrombin activation, to a similar extent as control liposomes (**Fig. 2b**). Liposomes with high PS content are known to aggregate, fuse and collapse in the presence of calcium(36). For that reason the control liposomes used in prothrombinase assay were those containing 10% PS. In contrast, rHDL are stable in the presence of calcium as judged by size exclusion chromatography on a Superose 6 column (data not shown).

Anionic phospholipids in reconstituted HDL are unable to bind FVa. To more precisely understand an underlying mechanism why the rHDL particles could not efficiently support prothrombinase activity, the rHDL binding abilities of prothrombin, FXa and FVa were

evaluated individually using a surface plasmon resonance approach. Isolated rHDL particles were immobilized on a biosensor surface. As anticipated, membrane binding was reversible, and for the Gla-containing proteins also Ca^{2+} dependent, as any remaining protein was completely removed from the rHDL particle surface with EDTA (data not shown). Prothrombin and FXa bound rHDL particles, whereas FVa showed relatively insignificant binding to the phospholipid containing particles when analyzed at a protein concentration approaching their respective B_{max} concentrations (**Fig. 3a**). Furthermore, annexin V, which binds negatively charged phospholipids in the absence of a Gla-domain(37), was used as an additional rHDL membrane binding control and bound rHDL efficiently (**Fig. 3a**). The observed binding of proteins to the rHDL was also membrane specific as a Gla-less derivative of prothrombin, prethrombin-1, was unable to bind (**Fig. 3a**). Binding affinity and binding saturation determinations for the proteins for the three rHDL preparations were strikingly different between the cofactor and the two vitamin K dependent proteins. (**Fig. 3b-d, Table 2**). Binding affinity for prothrombin and FXa were within the affinity range previously reported for Gla proteins using liposomes of similar phospholipid composition(38). On the contrary, FVa clearly showed a weaker affinity to all rHDL tested compared to liposomes of similar composition(39). The FVa preparation used was considered valid as judged from binding experiments using immobilized liposome (10:40:50, PS/PE/PC) that bound FVa efficiently (data not shown). From the amount of protein bound to rHDL at saturation (B_{max}), a stoichiometry was calculated in terms of bound molecules of clotting protein per molecule rHDL (**Table 2**). Approximately 1.4-4.7 molecules of FXa bound per rHDL particle, whereas FVa bound only 0.009-0.11 per rHDL particle (equivalent to 1 FVa per 9-110 rHDL particles). This difference was most pronounced for the 10:40:50 rHDL particles, where the cofactor had a 155-fold lower binding occupancy relative to the enzyme at saturation. Prothrombin as compared to FXa bound with slightly lower affinity and B_{max} . These qualitative parameters clearly reveal that FVa, due to its poor interaction with rHDL, is the major

factor responsible for the poor prothrombinase activity when rHDL is used as a membrane surface.

Transfer of anionic phospholipid from liposomes to reconstituted HDL stimulated by PLTP. ApoA-I was then studied in its ability to act as a scavenger for phospholipids in the presence of PLTP. rHDL was generated from purified apoA-I and natural phospholipids (10:40:50 PS/PE/PC), using a molar PL/apoA-I ratio of 30:1. In the presence of PLTP, phospholipids were transferred from liposomes to apoA-I containing particles (**Fig. 4a**). Spontaneous transfer of phospholipids was also seen in the absence of PLTP, but to a non-significant extent.

Transfer of anionic phospholipid from liposomes to lipoproteins in serum associated with loss of procoagulant properties. The phospholipid scavenger function of apoA-I and other lipoproteins was then tested in human serum, to which liposomes were added. After incubation of the liposomes (10:40:50, PS/PE/PC) with serum at 37 °C for 24 h, the ability of the liposomes to stimulate thrombin formation was lost. In contrast, control liposomes and BSA incubated with liposomes containing the same concentration of phospholipids were highly efficient in supporting prothrombin activation (**Fig. 4b**). When the serum-liposome mixture was tested in the prothrombinase assay immediately after mixing, the liposomes were as active in the prothrombinase assay as control liposomes (data not shown), demonstrating that the neutralization process was time dependent. Next, the pre-incubated liposomes mixtures were subjected to size exclusion chromatography to monitor the transfer of phospholipids (**Fig. 4c**). We consistently found low recovery of liposomes after the size exclusion chromatography, suggesting that the liposomes due to their large size (dialysis method yields large liposomes) adhered to the matrix. However, this was not a problem after transfer of the ^{14}C -PC to the different lipoproteins in serum. In the incubated serum sample, phospholipids were transferred to 20 nm (12.7 mL elution volume) and 8 nm (16.2 mL elution volume) particles, corresponding to apolipoprotein B (apoB) and apoA-I containing particles. In the liposome mixture containing BSA a small amount of labeled PC was recovered in the albumin peak, which eluted much later (17.6 mL elution volume) than the apoB and apoA-I

containing peaks, suggesting that albumin is not the preferred acceptor in serum for phospholipids.

DISCUSSION

PS is an important anionic phospholipid in the reactions of blood coagulation and inappropriate exposure of PS to circulating blood may result in a hypercoagulable state. We now demonstrate that PS loses its procoagulant properties when incorporated into rHDL and propose that this may be an important scavenger mechanism mediated by apoA-I. The use of rHDL to study the functional properties of discoidal HDL is well established, and involves the incorporation of phosphatidylcholine with apoA-I(9). To our knowledge, anionic phospholipids have not previously been used to prepare rHDL and their effects on the prothrombinase reaction investigated. However, the initiating reactions of blood coagulation between tissue factor (TF) and factor VIIa (FVIIa) have been studied using nanodisc technology (40). Nanodiscs are created in a similar manner as rHDL but instead of using apoA-I, a truncated form of apoA-I ($\Delta 1-43$ apoA-I) is used. In the TF-FVIIa study, the membrane protein TF was incorporated into the nanodiscs and the FVIIa-mediated FX activation studied. It was found that the nanodiscs could fully support the TF-FVIIa enzyme complex. This study is different from our study as the TF, in contrast to FVa, is a transmembrane protein and incorporated into the phospholipid layer of the nanodiscs.

A lot of research has focused on the anti-atherogenic and anti-inflammatory properties of apoA-I and HDL and their roles in reverse cholesterol transport and prevention of atherosclerosis(6). The study by Deguchi *et al*, which demonstrated that venous thrombosis patients have significantly lower levels of HDL and apoA-I, suggests that apoA-I may also protect against venous thrombosis(23). The now described anticoagulant properties of apoA-I may be an important mechanism by which apoA-I protects against both venous and arterial thrombosis.

Membrane localization and ensuing function of the vitamin K dependent proteins, as well as the cofactors (FVa and FVIIIa), is primarily dependent on the availability of PS and to a lesser extent PE. rHDL particles used in this study were made using a phospholipid mixture

containing either 10, 50 or 75% PS. Assuming an equal phospholipid distribution during reconstitution, these rHDL particles correspondingly have ~4, 20 and 24 PS molecules per apoA-I (or per leaflet of the membrane bilayer). It has been estimated previously, in experiments using a soluble form of PS or liposomes, that approximately 2 and 5 PS molecules are required to bind FVa(19) and the Gla-domain(40,41) to a membrane surface, respectively. Purely based on availability of PS, the 10% PS containing rHDL particles surely seem inadequate to allow the formation of two protein binding sites, let alone 3 (e.g. for enzyme, cofactor and substrate) required for a prothrombinase or tenase reaction. The higher PS containing rHDL particles do allow multiple proteins to bind as was shown with FXa (4.7 molecules/rHDL or $4.7/2 = 2.4$ molecules/leaflet of the rHDL), indicating that the rHDL leaflet surface area provided, ~50 nm²(42), is sufficiently large to accommodate 2-3 FXa molecules. Our prothrombin activation experiments using only FXa (in the absence of FVa) are in line with the SPR data, in that 2 or more Gla proteins can bind rHDL. FVa binding to rHDL would not seem to be limited by the availability of PS, however a bilayer area limitation may pose a problem and impede binding. A recent crystal structure of APC inactivated FVa shows that the membrane contact regions of the C1 and C2 domains require a combined width of 5.7 nm(43), which approached the rHDL bilayer diameter of 8 nm. If this x-ray derived model has relevance to membrane bound FVa, it is conceivable that rHDL will impose a size restriction for FVa binding. In addition, a recent docking model of FXa-FVa showed that the Gla-EGF1 membrane contact area is spatially separated from the C1-C2 area indicating that a significant greater area, likely more than provided by the rHDL, is required to allow FXa-FVa membrane binding(44). The combined assembly of FXa, FVa and prothrombin on a rHDL particle thus seems unfeasible (**Fig. 5b**).

The rHDL used here were made with natural phospholipids and showed a ~2-fold lower total phospholipid/rHDL ratio (80 phospholipid/rHDL) compared to rHDL reconstituted with synthetic phospholipid (130-160 phospholipid/rHDL, data not shown). However, the use of natural phospholipids does not fully explain the

low ratio as there was a recent report on rHDL particles of synthetic phospholipids with 35:1 molar ratio (PL/apoA-I), which also adopts a diameter around 8 nm(45). We have also observed that rHDL particles made with only phosphatidylcholine have higher number of phospholipid molecules per HDL particle than the combinations of phosphatidylcholine and phosphatidylserine (**Table 1**). Thus, the number of phospholipid molecules per particle seems to depend on the type of phospholipid that is used, i.e. if the rHDL contains only phosphatidylcholine or if phosphatidylserine is included, if the phospholipid is natural or synthetic, and presumably also on the method to prepare the rHDL particles.

Anionic phospholipids are exposed to circulation during activation of various cells, e.g. platelets, and during apoptosis. Microparticles are also rich in anionic phospholipids and capable of supporting coagulation(46). The apoA-I mediated binding of anionic phospholipids may be one of the mechanisms to control the exposure of this type of phospholipid to circulating blood. Several enzymes are known to participate in the transfer of phospholipids between different compartments. For example, PLTP mediates transfer of phospholipids between different lipoproteins in plasma, whereas transfer of phospholipids from cells to HDL is mediated by ATP-binding cassette transporter 1 (ABCA1). PLTP is involved in the remodeling of HDL and is responsible for the majority of phospholipid-transfer activity in plasma. PLTP acts on apoA-I- as well as apoE-containing particles and is secreted by macrophages, where it is highly expressed(4,47). ABCA1 plays an important role in HDL metabolism where it transports free cholesterol and phospholipids from macrophages to lipid-poor apoA-I, thus generating discoidal pre- β HDL(4). ABCA1 also functions in early steps of HDL biogenesis in the liver and intestine, and targeted ABCA1 deficiency in these tissues leads to severe hypo α -lipoproteinemia(48). A recently proposed mechanism of the ABCA1-mediated efflux of

cellular lipids to apoA-I involves membrane bending and blebbing off induced by ABCA1 lipid translocase activity(5). PS has been suggested to be a preferred substrate for translocation, and PS has been shown to redistribute from the cytoplasmic side to the exoplasmic plasma membrane leaflet in ABCA1 expressing cells(49). Recently, the role of PLTP in the transport of vitamin E from lipoproteins to erythrocytes was studied in a mouse model(50). It was shown that vitamin E accumulated in circulating erythrocytes from PLTP-deficient mice and that these erythrocytes displayed fewer externalized PS molecules and decreased procoagulant activity than wild-type controls. Our experimental setting is quite different since we look at the transport of PS molecules already exposed at the surface of liposomes to HDL particles, and not the transfer of PS between the inner- and outer leaflet of the membrane bilayer. Whether there is an impact of vitamin E in our system remains to be elucidated.

Here we show that PLTP can mediate transfer of phospholipids from liposomes to apoA-I containing rHDL. Furthermore, we also show that serum has the potential to transfer phospholipids from liposomes to either apoA-I or apoB containing particles, and thereby causing strong attenuation of the procoagulant effect of anionic phospholipids. Our demonstration that the procoagulant properties of the anionic phospholipids are lost when incorporated into apoA-I containing HDL particles show that apoA-I can function as a scavenger for anionic phospholipids possibly mediated by PLTP, which is a previously unrecognized anticoagulant property of this apolipoprotein. The uptake of anionic phospholipids by apoA-I may involve the phospholipid transporters PLTP and ABCA1, and other mechanisms yet to be defined. Our findings here are physiologically relevant and suggest HDL to be an important therapeutic target to be considered in the context of coagulation process.

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FOOTNOTES

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The abbreviations used are: HDL, high-density lipoprotein; apoA-I, apolipoprotein A-I; rHDL, reconstituted high-density lipoprotein; PS, phosphatidylserine; FIXa, activated factor IX; FVIIIa, activated factor VIII; FX, factor X; FXa, activated factor X; FVa, activated factor V; Gla, γ -carboxy glutamic acid; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; EDTA, ethylenediaminetetraacetic acid; PL, phospholipids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; FV, factor V; BSA, bovine serum albumin; PLTP, phospholipid transfer protein; VHDL, very-high-density lipoprotein; apoB, apolipoprotein B; TF, tissue factor; FVIIa, activated factor VII; ABCA1, ATP-binding cassette transporter.

FIGURE LEGENDS

Figure 1. *Effect of apoA-I on anionic phospholipid neutralizing activity.* A) The apolipoprotein extracts from the isolated lipoproteins were diluted as indicated and mixed with detergent-solubilized PL (10:40:50 PS/PE/PC) where after the detergent was removed by dialysis. The lipid-apolipoprotein complexes were then used as phospholipid source in a prothrombinase reaction (final phospholipid concentration was 5 μ M) in which FXa with support from its cofactor FVa activates prothrombin to thrombin. Liposomes formed in the absence of apolipoprotein extracts were used as a control. The protein extracts were from a chylomicrons-VLDL mixture (■), LDL (▲), HDL (□), VHDL (△), and lipoprotein-free plasma (●). B) Extracted proteins from HDL were separated on S200 Hiprep chromatography in 6 M guanidine-HCl. Upper) Fractions were mixed with detergent-solubilized PL (10:40:50 PS/PE/PC) and dialyzed and analyzed by non-reduced 15% Tricine-SDS/PAGE (silver-stained). Lower) Dialyzed samples were used as phospholipid source in a prothrombinase reaction (right y-axis, ●). The thrombin generation was normalized to a control reaction with liposomes (5 μ M) formed in absence of apolipoproteins. Protein elution profile (left y-axis, □) was measured at absorbance 280 nm. Final concentrations of proteins during the 2 min activation of prothrombin were; FVa 210 pM, FXa 2.5 nM, prothrombin 0.5 μ M.

Figure 2. *Anionic phospholipids when incorporated in rHDL have reduced procoagulant activity.* Isolated rHDL (\approx 40:1 PL/apoA-I molar ratio) or control liposomes (■) were added at increasing phospholipid concentrations to a prothrombinase reaction with the presence (A) or absence of FVa (B). Different compositions of phospholipids (10:40:50 (▲), 50:0:50 (▼) or 75:0:25 (●) PS/PE/PC) were used to prepare the rHDL. Control liposomes consisted of 10:40:50 PS/PE/PC. Values are expressed as means \pm SEM from three independent rHDL preparations. Final concentrations of proteins during activation of prothrombin were; A) FVa 210 pM, FXa 2.5 nM, prothrombin 0.5 μ M, using a 2 min activation time and B) FXa 50 nM, prothrombin 0.5 μ M, using a 5 min activation time. Please note the different scales of the X-axes (thrombin generation) in A and B.

Figure 3. *Binding of prothrombin, FXa, FVa and annexin V to isolated rHDL of varying phospholipid composition.* A) Proteins at concentrations approaching their respective B_{\max} of the 10:40:50 PS/PE/PC rHDL interaction (10 μ M Prothrombin, 2 μ M FXa, 100 nM FVa and 100 nM annexin V) were injected over a 10:40:50 rHDL surface to gauge their relative binding efficiencies. The SPR response curves for 10:40:50 rHDL are shown after background correction to the control coated with 0:0:100 rHDL. Binding to the control surface was not apparent and no evidence of non-specific binding was evident from an injection of Gla-less, prothrombin-1 (10 μ M). All proteins were injected in duplicate. Note: response unit

(RU, y-axis) is proportional to mass ($1 \text{ RU} = 1 \text{ pg/mm}^2$) and thus binding responses do not take into account the large mass differences between proteins analyzed. MW (KDa) of FVa; 168, prothrombin; 72, prothrombin-1; 50, FXa; 46 and annexin V; 36. Steady state binding of either B) prothrombin, C) FVa or D) FXa to isolated rHDL (~30:1 PL/apoA-I molar ratio) composed of either 10:40:50 (■), 50:0:50 (▲) or 75:0:25 (▼) PS/PE/PC was measured as in A) using the indicated protein concentrations shown. Responses obtained at equilibrium were used to generate a binding isotherm fitted to a one-site binding hyperbola using nonlinear least squares analysis. Binding isotherms were used to determine K_d and B_{max} reported in Table 2. Responses (RU) were converted to fmol to allow a comparison of molar binding to be made easily between the proteins. Note y-axis scale differences in B), C) and D). See Material and Methods for more details.

Figure 4. *ApoA-I can act as scavenger for phospholipids.* A) rHDL (30:1 PL/apoA-I molar ratio, 10:40:50 PS/PE/PC) was mixed with labeled liposomes (10:40:50 PS/PE/PC) and incubated at 37 °C for 24 h in the presence (▲) or absence (●) of PLTP (final PLTP activity 1000 nmol/mL/h). As a control, labeled liposomes were incubated with PLTP but without rHDL (■). Samples were separated on Superose 6 10/300 GL and eluted fractions were analyzed by scintillation counting. B) Human serum (□) or BSA (▲) were incubated with labeled liposomes (10:40:50 PS/PE/PC) at 37 °C for 24 h and studied in their ability to stimulate thrombin formation at different phospholipid concentrations (calculated from the radioactivity). As a control, labeled liposomes (10:40:50 PS/PE/PC, ■) were used at different phospholipid concentrations. Values are expressed as means \pm SD from duplicates, and representative from repeated experiments. C) Samples in B were separated on Superose 6 10/300 GL and eluted fractions were analyzed by scintillation counting.

Figure 5. *Schematic model of phospholipid-bound prothrombinase complex.* A) On the surface of activated platelets or model liposome membranes, the enzyme FXa together with its cofactor FVa form the prothrombinase complex that activates the substrate prothrombin. B) Proposed model of an rHDL particle that although it contains anionic phospholipids is unable to assemble a prothrombinase complex mainly due to deficient binding of FVa.

Table 1. Properties and composition of rHDL of various PL composition (PS/PE/PC).

PS/PE/PC	10/40/50	50/0/50	75/0/25	0/0/100
ApoA-I (μM) ^a	0.4 \pm 0.1	0.5 \pm 0.07	0.6 \pm 0.06	0.3 \pm 0.07
Phospholipid (μM) ^b	14.5 \pm 2.2	19.7 \pm 3.3	20.0 \pm 2.0	18.2 \pm 1.7
PL/apoA-I	38.1 \pm 12.7	39.2 \pm 8.8	31.6 \pm 3.7	63.0 \pm 8.3
Stokes diameter (nm) ^c	8.2 \pm 0.4	9.5 \pm 0.0	10.1 \pm 0.5	7.9 \pm 0.0

Mean \pm SD from 3 independent rHDL preparations.

^a By apoA-I ELISA. ^b By scintillation counting of ¹⁴C-PC. ^c By size exclusion chromatography.

Table 2. Affinities, saturation (B_{max}) and stoichiometries (n/n) of the binding of prothrombin, FXa and FVa to rHDL of various PL composition (PS/PE/PC), as analyzed by surface plasmon resonance.

K_d (μM) ^a , B_{max} (fmol) ^{a,b} , n/n (mol protein/mol rHDL) ^{b,c}									
	10/40/50			50/0/50			75/0/25		
	K_d	B_{max}	n/n	K_d	B_{max}	n/n	K_d	B_{max}	n/n
Prothrombin	3.5	4.2	0.3	2.4	9.7	0.9	1.8	13.9	1.2
FXa	1.3	21.3	1.4	0.70	42.8	4.0	0.45	54.1	4.7
FVa	0.075	0.15	0.009	0.050	0.64	0.06	0.045	1.2	0.11

^a SD is less than 10% of all reported values. Except for K_d (FVa;10/40/50): 0.075 \pm 0.031 μM SD was larger as binding site occupancy was very low.

^b based on MW (KDa) of prothrombin; 72, FXa; 46, FVa; 168 and assuming 1 RU protein = 1 pg/mm².

^c based on MW (KDa) of rHDL 10/40/50; 89, 50/0/50; 103 and 75/0/25; 96 as calculated from a representative preparation as stated in Table 1 and assuming an average phospholipid MW of 0.77 KDa and assuming 1RU phospholipid = 0.92 pg/mm².

Figure 1

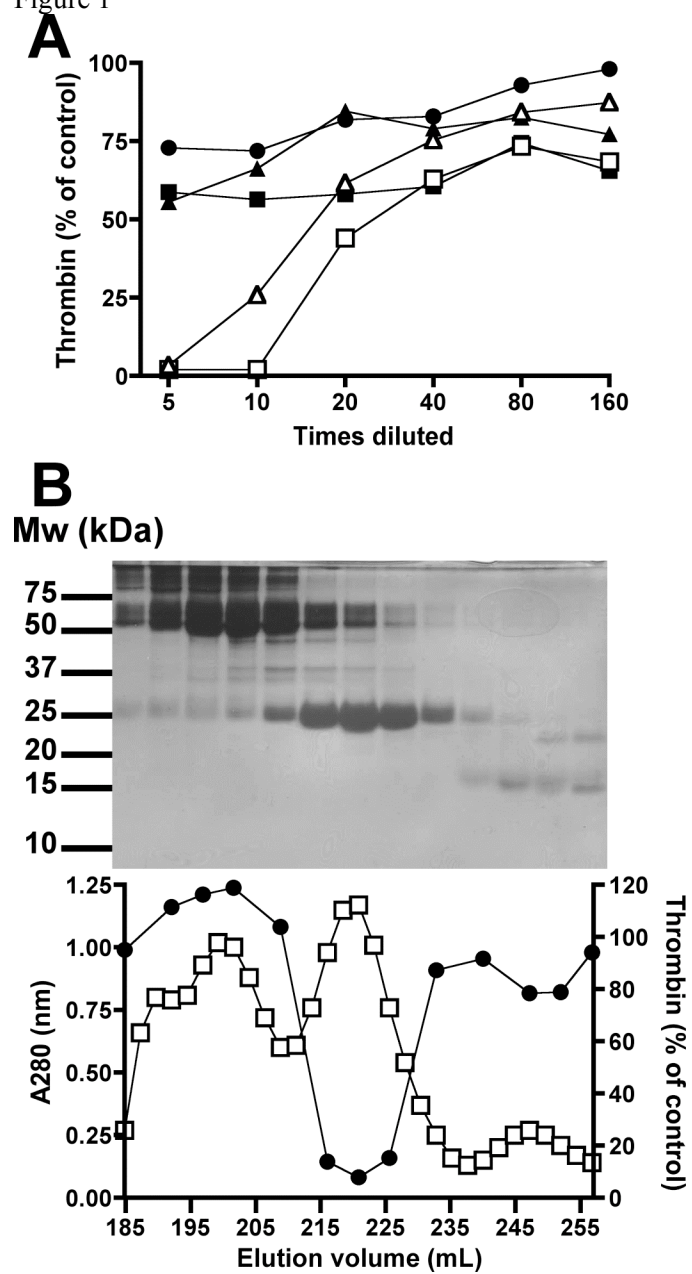


Figure 2

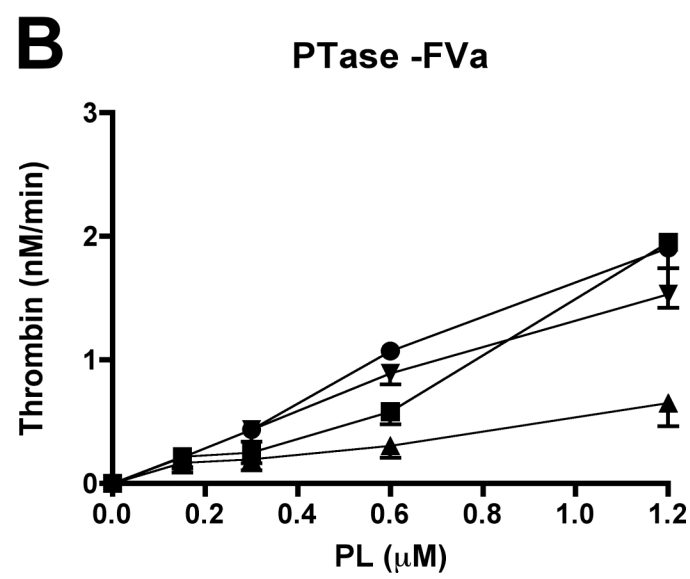
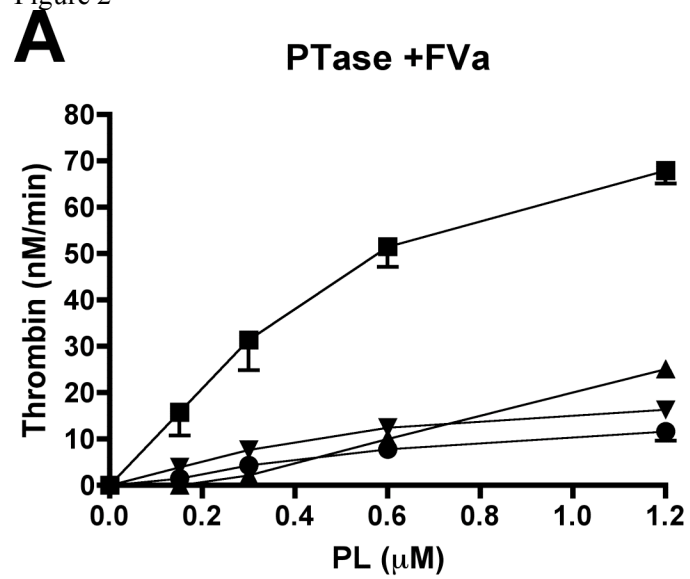


Figure 3

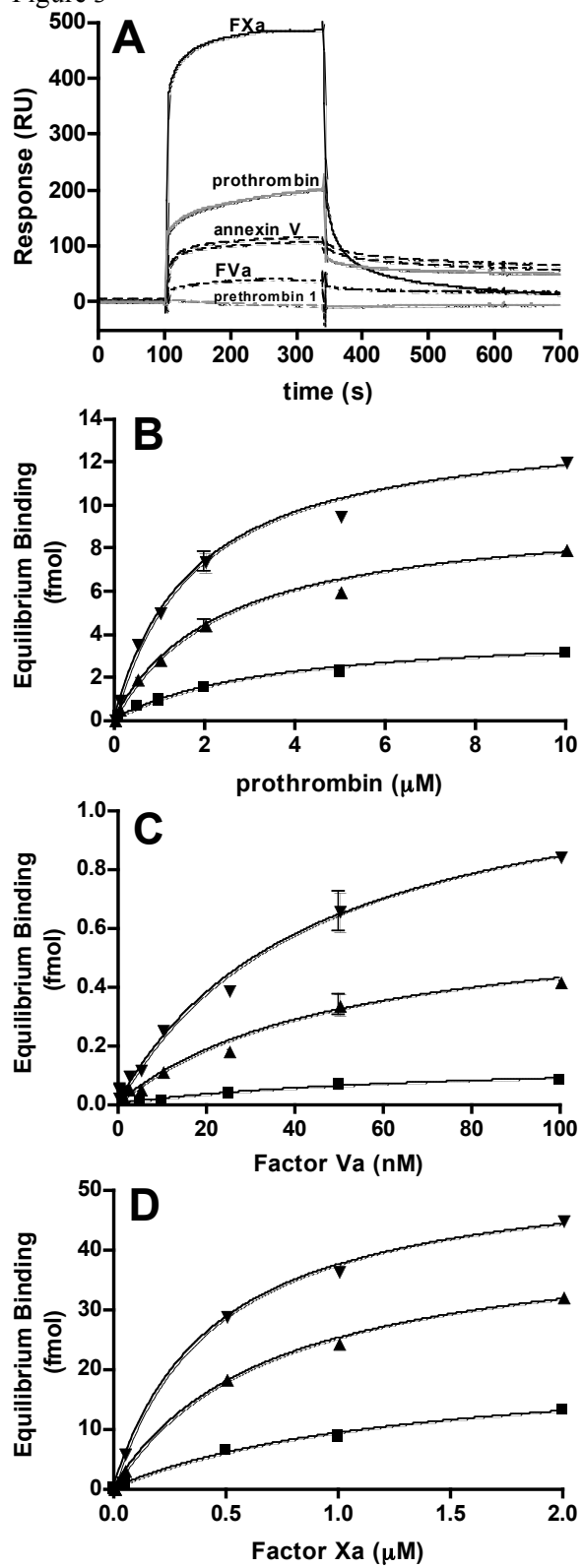


Figure 4

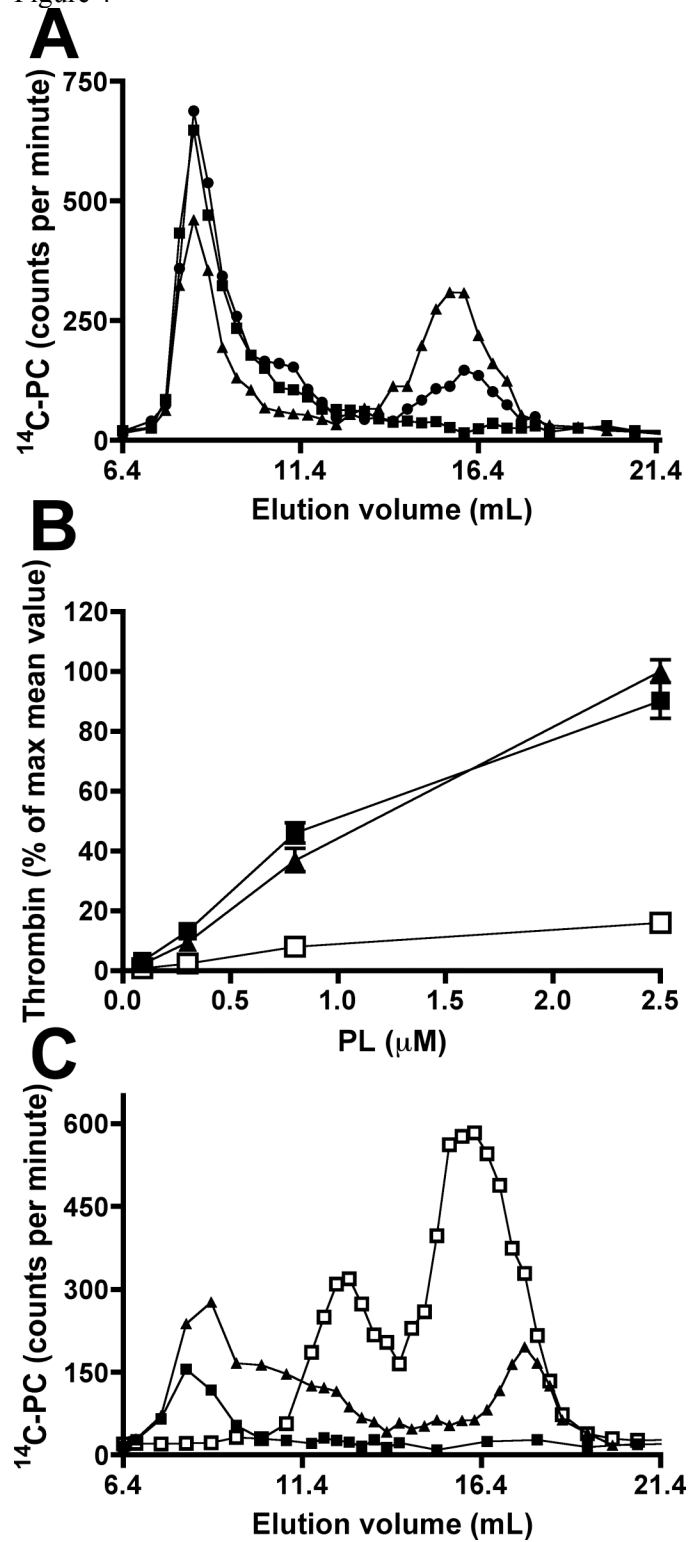
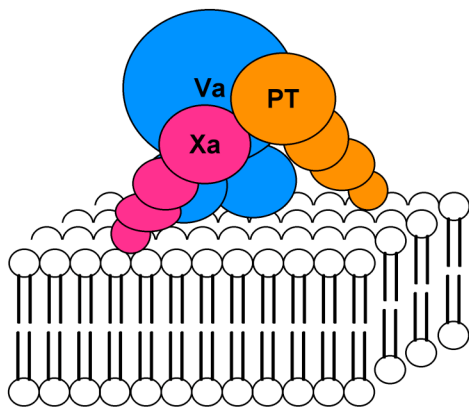
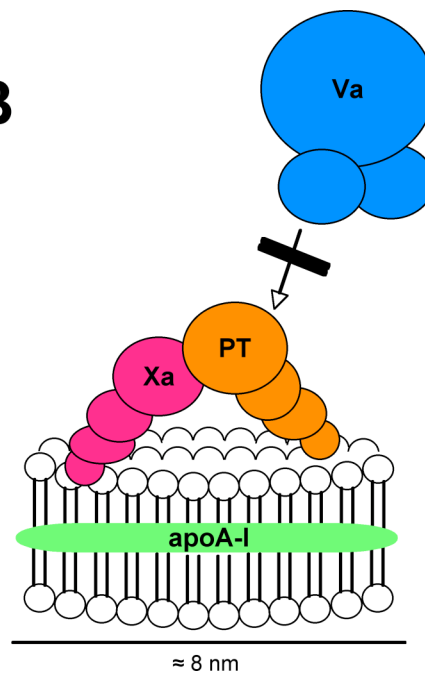


Figure 5

A



B



SUPPLEMENTARY

Figure legend

Figure 1. *rHDL particles consist of 2 apoA-I molecules per particle.* Chemical cross-linking of apoA-I, in rHDL particles, using cross-linking agent EDC (1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride, Pierce, Rockford, IL). Isolated rHDL particles were dialyzed against HN buffer (10 mM Hepes, 150 mM NaCl, pH 5) using 14 000 MW cut off membranes (Spectra/Por). Particles were cross-linked at a 1000x molar excess of EDC relative to apoA-I, and incubated at 4 °C for 24 h. Samples were analyzed by non-reduced 10% SDS/PAGE silver-stained. Lipid-free plasma apoA-I (1 µg) is shown in lane 1. Cross-linked rHDL particles with 10:40:50 (0.6 µg apoA-I), 50:0:50 (0.8 µg apoA-I) and 75:0:25 (1 µg apoA-I) PS/PE/PC (lane 2, 3 and 4 respectively). rHDL particles were prepared using 260:1 PL/apoA-I molar ratio. After cross-linking a band of about 60 kDa (2x28 kDa) appeared, indicating that rHDL particles consist of around 2 apoA-I molecules per particle.

Figure 1

