Anionic Phospholipids Lose Their Procoagulant Properties When Incorporated into High Density Lipoproteins*

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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 a 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, whereas 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 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 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 antiatherogenic 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 two or three 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 hemostasis 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 (FVIIa) 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

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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; FVIIa, activated factor VIII; FX, factor X; FXa, activated factor X; FVa, activated factor V; FVIIIa, activated factor VIIIb; FV, factor V; Glu, γ-carboxy glutamic acid; VLDL, very low density lipoprotein; LDL, low density lipoprotein; PL, phospholipids; PE, phosphatidylethanolamine; PC, phosphatidylcholine; BSA, bovine serum albumin; PLTP, phospholipid transfer protein; VLDL, very high density lipoprotein; TF, tissue factor; ABCA1, ATP-binding cassette transporter; TBS, Tris-buffered saline; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; RU, response unit.
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-carboxyl 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) potentiates 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 anti-thrombotic potential of HDL.

**EXPERIMENTAL PROCEDURES**

**Isolation of Lipoprotein Fractions**—Lipidmic 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 of the isolated fractions were dialyzed into HN buffer (10 mM Heps 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 min and resuspended in 6 ml 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 mM 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 mM 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−1 liter·cm−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. The lipids were mixed, dried under N2 gas, and resuspended in HN buffer at room temperature. A trace amount of 14C-radiolabeled PC (GE Healthcare) was added to the lipid mixture when necessary. The 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 molecular weight cut-off membranes (Spectra/Por). During the generation of liposomes, the 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 AKTA fast performance liquid chromatography system (Amersham Biosciences) 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 enzyme-linked immunosorbent assay (see below).

**Enzyme-linked Immunosorbent Assay for ApoA-I**—ApoA-I was detected in rHDL particles using an enzyme-linked immunosorbent assay 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. The 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 molecular weight 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 three 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 on plates for 1 h at room temperature. The 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**—The protein samples were loaded onto 15% Tricine-SDS/PAGE gels (27) under nonreducing conditions. The gels were developed using a 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, The Netherlands) to concentrations of 420 pm and 5 nm, respectively. FV was activated by the addition of thrombin (Hemato-
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logic Technologies, Inc (Essex Junction, VT) to final concentration 3 units/liter for 3 min at 37 °C, and the activation was terminated by addition of hirudin (Pentapharm, Basel, Switzerland) to final concentration 8 units/liter. The samples (60 μl) were transferred to a 96-well plate (Sero-well, Sterilin) and mixed with 40 μl of HNBSACa. The reaction was initiated by the addition of 20 μg prothrombin (Kordia, Leiden, The Netherlands) to final concentration 0.5 μM and incubated at 37 °C for 2 min. The reaction was stopped by the addition of 100 μl of EDTA buffer (50 mM Tris-HCl, 100 mM NaCl, 100 mM EDTA, 1% polyethylene glycol 6000, pH 7.9). The 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 μM), and absorbance at 405 nm was measured continuously for 15 min with a microplate reader. The 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 of ≤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 of thrombin dilution and 50 μl of S-2238) with known amounts of protein.

Prothrombinase Activation without FVa—The 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. The 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 Hemalogen 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 units/ml thrombin for 2 h 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 the purity of the prethrombin-1, the fractions were run under reducing conditions on SDS acrylamide 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). Prethrombin, 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, whereas prethrombin-1, a Glα-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 and 1460 RU. Weakly adhering rHDL were removed with five consecutive 10 mM EDTA pH 8.0 injections (2 min at 20 μl/min).

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. The 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. The samples were then separated on Superose 6 10/300 GL using TBS as running buffer. The 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. The 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 the addition to the gel filtration column, the 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 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 was 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 were 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.

**Anticoagulant Properties of Apolipoprotein A-I**

**TABLE 1**

Properties and composition of rHDL of various PL composition (PS/PE/PC)
The values are the means ± S.D. from three independent rHDL preparations.

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

<sup>a</sup> By apoA-I enzyme-linked immunosorbent assay.
<sup>b</sup> By scintillation counting of [14C]PC.
<sup>c</sup> By size exclusion chromatography.
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 ~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 supplemental Fig. 1), 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).

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 nonspecific binding was evident from an injection of Gla-less, prethrombin-1 (10 μM). All of the proteins were injected in duplicate. Note: RU (y axis) is proportional to mass (1 RU = 1 pg/mm²), and thus binding responses do not take into account the large mass differences between proteins analyzed. Molecular masses of FVa, 168 kDa; prothrombin, 72 kDa; prethrombin-1, 50 kDa; FXa, 46 kDa; and annexin V, 36 kDa. Steady state binding of either prothrombin (B), FVa (C), or FXa (D) to isolated rHDL (75:0:25 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. The 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 Kd and Bmax 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 the y axis scale differences in B, C, and D. See “Experimental Procedures” for more details.
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 Ca²⁺-dependent, because 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 Bₘₐₓ 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 because a Gla-less derivative of prothrombin, prothrombin-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. 3, B–D, and 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 with 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 (Bₘₐₓ), 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 with FXa bound with slightly lower affinity and Bₘₐₓ. These qualitative parameters clearly reveal that FVa, because of 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 nonsignificant 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 or 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 preincubated liposomes 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, because of their large size (dialysis method yields large liposomes), adhered to the matrix. However, this was not a problem after transfer of the ¹⁴C-PC to the different lipoproteins in serum. In the incubated serum sample, phospholipids were transferred to 20 nm (12.7 ml of elution volume) and 8 nm (16.2 ml of elution volume) particles, corresponding to apolipoprotein B- 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 of elution volume) than the apolipoprotein B- 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

## TABLE 2

| Protein     | 10:40:50 | 50:0:50 | 75:0:25 |
|-------------|----------|---------|---------|
| Kᵣ (µm)    | Bₘₐₓ (fmol mol protein/mol rHDL) | n/n | Kᵣ (µm) | Bₘₐₓ (fmol mol protein/mol rHDL) | n/n | Kᵣ (µm) | Bₘₐₓ (fmol mol protein/mol rHDL) | n/n |
| Prothrombin | 3.5      | 213.3   | 0.3     | 2.4     | 97.9    | 0.9     | 1.8     | 13.9    | 1.2    |
| FXa         | 1.3      | 213.3   | 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   |

Affinities, saturation (Bₘₐₓ) 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

The Kᵣ value (S.D.) is less than 10% of all reported values. Except for Kᵣ (FVa;10/40/50), 0.075 ± 0.031 µm S.D. was larger because the binding site occupancy was very low. The Bₘₐₓ values are based on molecular masses of prothrombin, 72 kDa; FXa, 46 kDa; and FVa, 168 kDa and assuming 1 RU of protein = 1 pg/mm². The n/n values are based on molecular mass of rHDL 10/40/50, 89 kDa; 50/0/50, 103 kDa; and 75/0/25, 96 kDa as calculated from a representative preparation as stated in Table I and assuming an average phospholipid molecular mass of 0.77 kDa and assuming 1 RU phospholipid = 0.92 pg/mm².
values are expressed as the means ± S.D. from duplicates and are representative from repeated experiments.

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. (23), 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. 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 FVIIa), 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 ~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 three (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 two or three FXa molecules. Our prothrombin activation experiments using only FXa (in the absence of FVa) are in line with the SPR data, in that two 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 activated protein C-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).

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 have been 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 (Δ1–43 apoA-I) is used. In the TF-FVIIa study, the membrane protein TF was incorporated into the nanodiscs, and the FVIIa-mediated FX activation was studied. It was found that the nanodiscs could fully support the TF-FVIIa enzyme complex. This study is different from our study because the TF, in contrast to FVa, is a transmembrane protein and is incorporated into the phospholipid layer of the nanodiscs.
activation of various cells, phospholipid is natural or synthetic, and presumably also on phosphatidylcholine or if phosphatidylserine is included, if the of phospholipid molecules per particle seems to depend on the ecules per HDL particle than the combinations of phosphati-
phosphatidylcholine have higher number of phospholipid mol-
We have also observed that rHDL particles made with only synthetic phospholipid (130–160 phospholipid/rHDL; data not shown). However, the use of natural phospholipids does not fully explain the low ratio because there was a recent report on rHDL particles of synthetic phospholipids with a 35:1 molar ratio (PL/apoA-I) that also adopt a diameter around 8 nm (45).

We have also observed that rHDL particles made with only phosphatidycholine have higher number of phospholipid molecules per HDL particle than the combinations of phosphati-
dycholine 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 HDL contains only phosphatidycholine or if phosphatidylserine is included, if the phospholipid is natural or synthetic, and presumably also on the method used 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 phospho-
lipids 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 because 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 apolipoprotein B-containing particles, 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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