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The regulation of blood coagulation by high-density lipoprotein particles

Cecilia Oslakovic

Doctoral Thesis



LUND UNIVERSITY

Faculty of Medicine

Academic dissertation

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Abstract		
High-density lipoprotein (HDL) has anti-atherogenic properties and the plasma level of HDL cholesterol correlates inversely with the risk of coronary artery disease. The atheroprotective functions of HDL can be explained by its function in the reverse cholesterol transport. Blood coagulation is activated in response to tissue damage and involves a series of enzymatic protein complexes that assemble on the surface of anionic phospholipids, e.g. activated platelets. Lipoproteins contain a phospholipid surface which may provide another phospholipid surface, other than platelets, that could stimulate the reactions of blood coagulation. Lipoproteins have been reported to have dual roles in the regulation of blood coagulation, therefore in this thesis the role of HDL in blood coagulation was investigated.		
HDL was studied in its ability to stimulate prothrombin activation. Anionic phospholipids lost their procoagulant function when incorporated into reconstituted HDL particles. The anionic phospholipids of these particles were unable to support binding to activated factor V (FVa). Serum was also shown to neutralize the procoagulant effect of anionic liposomes with transfer of phospholipids to both low-density lipoprotein (LDL) and HDL particles. The transfer of phospholipids was dependent on a catalytically active form of phospholipid transfer protein (PLTP). Total HDL, HDL3 and very high-density lipoprotein, all which contained endogenous PLTP, were all able to neutralize procoagulant liposomes. Addition of exogenous PLTP to either LDL or HDL2, which were both absent of endogenous PLTP, increased the neutralization of procoagulant liposomes.		
HDL has been reported to function as a cofactor to anticoagulant activated protein C (APC) in the degradation of FVa in the presence of protein S. HDL isolated by ultracentrifugation was found to stimulate the APC-mediated degradation of FVa. However, further purification of HDL by size-exclusion chromatography revealed that the stimulating activity was not a property of HDL but instead caused by contaminating anionic phospholipid membranes.		
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The regulation of blood coagulation by high-density lipoprotein particles

Cecilia Oslakovic

Doctoral Thesis



LUND UNIVERSITY Faculty of Medicine

Division of Clinical Chemistry Department of Laboratory Medicine, Malmö Faculty of Medicine Lund University 2010 The picture on the cover shows an electron microscopy picture of HDL particles made by Matthias Mörgelin.

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Till Mamma

"Research is to see what everybody else has seen, and to think what nobody else has thought" – Albert Szent-Gyorgyi

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Oslakovic C, Krisinger MJ, Andersson A, Jauhiainen M, Ehnholm C, Dahlbäck B. Anionic phospholipids lose their procoagulant properties when incorporated into high density lipoproteins. *J Biol Chem.* 2009; 284:5896-5904.

Paper II

Oslakovic C, Jauhiainen M, Ehnholm C, Dahlbäck B. The role of phospholipid transfer protein in lipoprotein-mediated neutralization of the procoagulant effect of anionic liposomes. *J Thromb Haemost*. 2010; 8: 766-72.

Paper III

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ABBREVIATIONS

ABC	ATP-binding cassette
APC	Activated protein C
apoA-I, apoB etc.	Apolipoprotein A-I, B
Arg	Arginine
ATP	Adenosine triphosphate
BPI	Bactericidal permeability increasing
	protein
CD14	Cluster of differentiation 14
CETP	Cholesteryl ester transfer protein
EGF	Epidermal growth factor
FV, FVIII etc.	Factor V, Factor VIII
FVa, FVIIIa etc.	Activated forms of FV, FVIII
Gla	γ-carboxyglutamic acid
HA-PLTP	High-activity PLTP
HDL	High-density lipoprotein
IDL	Intermediate-density lipoprotein
kDa	Kilo Dalton
kcat	Catalytic constant
K _m	Michaelis constant
LA-PLTP	Low-activity PLTP
LBP	LPS-binding protein
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LRP	LDL receptor-related protein
MSP	Membrane scaffold protein
Ox-LDL	Oxidized LDL
PC	Phosphatidylcholine

PE	Phosphatidylethanolamine	
PLTP	Phospholipid transfer protein	
PS	Phosphatidylserine	
rHDL	Reconstituted HDL	
SR-B1	Scavenger receptor B1	
TF	Tissue factor	
TFPI	Tissue factor pathway inhibitor	
ТМ	Thrombomodulin	
VHDL	Very high-density lipoprotein	
VLDL	Very low-density lipoprotein	
Vmax	Maximal rate	
VWF	Von Willebrand factor	

PREFACE

During my PhD studies I have come across both lipid metabolism and blood coagulation. This thesis will give you an introduction to both fields, with focus on one lipoprotein, HDL. In this thesis you can read how blood coagulation can be regulated by HDL particles.

I would like to take the opportunity to acknowledge those who made this thesis possible. My supervisor Björn Dahlbäck and co-supervisor Eva Norstrøm. Astra and Mike, who both helped me in different ways in my project. My collaborators in the PLTP field, Matti and Christian. My lab group, people at the Wallenberg lab and everybody I have worked with. My friends and family who are used to listen to "science talk" and always make me feel good and especially Henrik who encouraged me to do this...

BLOOD COAGULATION

Blood coagulation involves a tight cooperation between platelets and the plasma coagulation cascade. Blood coagulation is triggered in response to tissue damage and ultimately leads to the generation of thrombin and a fibrin clot^{1,2}. Platelets play an important role in blood coagulation, and are activated after vascular damage. Platelet activation involves adhesion to subendothelial structures and aggregation of platelets to form a primary hemostatic plug at the site of injury³. The coagulation cascade involves a series of reactions in which inactivated enzymes (proenzymes) become activated. The active enzymes belong to a group of proteins called serine proteases, enzymes with the amino acid serine in their active site⁴. The blood coagulation is tightly regulated by both pro- and anticoagulant mechanisms, which in normal conditions is shifted in favour of anticoagulation⁵.

Intrinsic and extrinsic pathway

Blood coagulation can be divided into two pathways, the intrinsic and extrinsic pathway, see figure 1. The intrinsic (contact) pathway is initiated by the activation of factor XII when blood is exposed to negatively charged surfaces, provided by glass (*in vitro*) or collagen (*in vivo*), in a reaction that involves prekallikrein and high molecular weight kininogen⁶. Factor XIIa also converts prekallikrein to α -kallikrein, which in turn can activate more FXII. The extrinsic (tissue factor) pathway is triggered by tissue damage and the formation of tissue factor/factor VIIa (TF/FVIIa) complex^{2,5}. The two pathways merge at the level of factor IXa (FIXa) and factor Xa (FXa), and it is the extrinsic pathway that is believed to be important *in vivo*, because FXII deficiency is not associated with bleeding^{7,8}.

Initiation, propagation, and amplification of blood coagulation

The binding of FVIIa to TF **initiates** the extrinsic pathway and the TF/FVIIa complex activates factor X and $IX^{9,10}$. Activated FIX together with its cofactor factor VIIIa constitute the tenase complex, which activates additional FXa¹¹⁻¹³. Another complex, the prothrombinase complex, activates prothrombin to thrombin and consists of FXa and its cofactor factor Va (FVa) and is discussed further below^{12,14,15}. The tenase and prothrombinase complexes constitute the **propagation** of blood coagulation with generation of thrombin. The final reaction is the generation of fibrin and a fibrin clot that will stop the bleeding. The main effector enzyme of coagulation is thrombin (factor IIa), which besides from generating a fibrin clot, also activates platelets and activates the factors V, VIII, XI, XIII and the anticoagulant protein C¹⁶. In that way, thrombin both **amplifies** and **regulates** the blood coagulation.

Regulation of the blood coagulation

Different anticoagulant pathways at different levels regulate blood coagulation. The tissue factor pathway inhibitor (TFPI) regulates the initiation phase involving TF and FVIIa and the activation of FX^{17,18}. Antithrombin is a serpin, a serine protease inhibitor, which mainly inhibits thrombin, FIXa and FXa^{19,20}. Antithrombin is a weak inhibitor by it self, but its activity is enhanced by the presence of heparin. The activated protein C system (APC) regulates the propagation phase involving FVa and FVIIIa^{5,21}. The APC system serves as one of the main anticoagulant pathway that regulates blood coagulation and is discussed further below.

Intrinsic pathway (contact activation)



Extrinsic pathway (tissue factor)

Figure 1. Schematic picture of the reactions in blood coagulation that ultimately lead to the generation of thrombin and a fibrin clot.

Platelets

Platelets are formed in the bone marrow from megakaryocytes. The production of platelets, thrombocytopoiesis, requires stimulation of various cytokines. Resting platelets have a disc-shaped structure with a diameter of about 2 μ m, and do not adhere to each other. Platelets do not contain a cell nucleus but they contain secretory granules such as dense- and α -granula and lysosomes, which are released after platelet activation³. After

stimulation with an activator, e.g. thrombin, platelets become activated and aggregate, and a primary haemostasis is formed. The released granula content consists of different signals mediators for adherence and aggregation and also different coagulation factors that trigger the coagulation cascade. The coagulation factors in the α -granules of platelets are e.g. fibrinogen, von Willebrand factor and FV^3 . The dense granules of platelets release for example polyphosphate, which has been shown to be procoagulant with activation of the contact (intrinsic) pathway of blood clotting^{22,23}. Platelets adhere both to the sub-endothelial surface and to each other (aggregation). Adherence of platelets to the sub-endothelial surface is dependent on the binding to von Willebrand factor (VWF)^{24,25}. VWF is synthesized in endothelial cells and megakaryocytes, and stored in the Weibel-Palade bodies in the endothelial cells or in the α -granules of platelets^{26,27}. VWF is released as multimers, ULVWF (Ultra Large von Willebrand Factor), and the multimers are proteolytically cleaved by the metalloprotease ADAMTS13²⁸⁻³⁰. VWF binds to the receptor glycoprotein Iba in the glycoprotein Ib-V-IX complex on platelets, and to exposed collagen^{31,32}. Thus, VWF mediates the adherence of platelets to the sub-endothelial surface. Platelet aggregation is generated by the binding of fibrinogen to the platelet integrin glycoprotein IIb/IIIa^{33,34}. Another function of platelets in blood coagulation is that they assemble the phospholipid-bound complexes, the tenase and prothrombinase complexes. The exposure of the negatively charged phospholipid, phosphatidylserine, on the plasma membrane of the platelets enables those reactions.

Phospholipids

Phospholipids are important content of the cellular plasma membrane³⁵. Phospholipids are amphipathic, thus they consist of a hydrophilic head and a hydrophobic tail. In the plasma membrane, the phospholipids form a bilayer with the hydrophobic tails towards each other and the hydrophilic head pointing either to the intracellular or extracellular interior. Most of the phospholipids in the cell membrane are constituted by glycerol-based phospholipids, glycerophospholipids, except from sphingomyelin, which is derived from sphingosine instead of glycerol³⁵. The glycerophospholipids consist of two fatty acids (tail) and one phosphate group with or without

an alcohol group (head) attached to the glycerol backbone³⁶. Three usually occurring glycerophospholipids in the plasma membrane are: phosphatidylcholine phosphatidylserine (PC), (PS) and phosphatidylethanolamine (PE), which have a choline, a serine or an ethanolamine group as their head groups, respectively³⁷. PS and PE are aminophospholipids and, it is only PS that carries a net negative charge.

Regulation of phospholipid asymmetry

In resting platelets the negatively charged phospholipids, i.e. PS, are located in the inner leaflet of the plasma membrane. Once the platelets are activated, the phospholipid asymmetry is lost, and PS is located to the outer leaflet of the membrane³⁸. This mechanism is important since the activated platelets support the binding of the prothrombinase and tenase complexes via negatively charged phospholipids, for review see reference by Zwaal et al³⁹. Thus, resting platelets are not able to support the binding of the complexes. The phospholipid asymmetry is regulated by three different activities, an aminophospholipid translocase, a floppase and a lipid scramblase⁴⁰⁻⁴³.

Aminophospholipid translocase

The adenosine triphosphate (ATP)-dependent aminophospholipid translocase transports PS and PE from the outer to inner leaflet of plasma membranes⁴⁴. The transfer is ATP-dependent, and one ATP is consumed per lipid transported^{45,46}. The activity is inhibited by Ca^{2+} , making the translocase an important regulator during activation of cells^{46,47}. The translocase prefers PS over PE and other lipids^{44,46}.

Floppase

Aminophospholipids and choline-containing phospholipids are transferred from the inner to outer leaflet by a floppase, also ATP-dependent but the half-time is about 10 times longer than that of the translocase-mediated inward movement⁴⁸. Some members of the ABC transporter superfamily have well known lipid floppase activities including ABCA1, which is important in the lipoprotein metabolism as discussed below. Not all of

ABC lipid transporters are floppases, ABCR is known to be a translocase⁴⁹.

Lipid scramblase

The lipid scramblase activity in platelet plasma membrane is activated in response to Ca^{2+} -influx, and moves phospholipids in both directions in a Ca^{2+} -dependent manner^{50,51}. Both the translocase and the floppase works in order to maintain membrane phospholipid asymmetry, while the activation of the lipid scramblase results in its collapse. Taken together, in activated platelets the cytoplasmic Ca^{2+} level increases and leads to the activation of lipid scramblase and the inactivation of the translocase and floppase, which results in the exposure of PS on the surface of the cell.

Vitamin K-dependent proteins

The coagulation proteins FVII, FIX, FX, protein C, prothrombin and protein S belong to the vitamin K-dependent plasma proteins^{52,53}. They all have in common that they contain a N-terminal Gla domain. The Gla domain consists of about 9-12 glutamic acid residues, which are post-translationally modified into γ -carboxyglutamic acid (Gla) by vitamin K⁵⁴. The Gla-residue has a high affinity for Ca²⁺ and binding induces a conformational change that is crucial for membrane interaction⁵². Thus, the Gla-domain mediates the binding of the vitamin K-dependent proteins to the phospholipid surface of activated platelets. The Gla-domain has a high affinity to phosphatidylserine and therefore the vitamin K-dependent proteins are not able to bind to resting platelets but only to activated platelets having PS exposed on its surface.

Prothrombinase complex

The prothrombinase complex activates prothrombin, which is the final step in the formation of thrombin. The complex consists of the enzyme FXa, which together with its cofactor FVa converts prothrombin to thrombin, see figure $2^{12,14,15}$. The complex assembles on the surface of lipid membranes containing negatively charged phospholipids, e.g. activated platelets as described above. In a similar manner, the tenase complex also assembles on the surface of negatively charged phospholipids¹¹, thus both the activation of prothrombin and FX requires the presence of negatively charged phospholipids. The role of FVa as a cofactor in the prothrombinase complex can be seen when looking at the kinetic parameters, see table 1. FXa alone is a poor enzyme for the activation of prothrombin. Addition of FVa increases the Vmax of thrombin formation, and thus the efficiency (kcat/K_m) of FXa is increased. On the other hand, the presence of negatively charged phospholipid decreases the K_m for prothrombin activation. The combined addition of both phospholipids and FVa yields a highly efficient prothrombin activation complex and is necessary for a complete activation of thrombin.⁵⁵



Figure 2. Assembly of the prothrombinase complex on the surface of lipid membranes^{21,56}. Prothrombin (PT) is converted to Thrombin (T) by the enzyme FXa and its cofactor FVa.

Table 1. Kinetic constants of thrombin formation with various prothrombin-activating mixtures⁵⁵. PL = phospholipids, FIIa = thrombin. In this table, Vmax is shown as mol thrombin per second and per mol Xa, which actually is the kcat (kcat = Vmax/[Enzyme]).

Activator	K _m	Vmax	kcat/K _m
	(μM)	(mol IIa s ⁻¹ mol Xa ⁻¹)	$(M^{-1}s^{-1})$
FXa	84	0.011	131
FXa, FVa	34	6.22	1.8×10^{5}
FXa, PL	0.06	0.038	6.3×10^{5}
FXa, FVa, PL	0.21	32	$1.5 \mathrm{x10}^{8}$

Prothrombin

Prothrombin is the zymogen form of thrombin⁵⁷. Prothrombin contains different domains; a Gla-domain, two kringle domains and a serine protease domain. Activation of prothrombin is achieved by the FXa-mediated cleavage at arginine (Arg) 271 and Arg320. The resulting product, thrombin, is a 39 kDa protein and comprises two chains crosslinked by four disulphide bonds¹⁶. Prothrombin binds to phosphatidylserine-containing membranes via its Gla-domain⁵⁸.

FXa

Factor X is the zymogen form of FXa^{59,60}. Factor X is processed as a single-chain molecule, but is then converted to a light and a heavy chain by peptide cleavage. The light and heavy chains are linked together by a disulphide bond and consist of a Gla domain together with two epidermal growth factor like (EGF-like) domains and a serine protease domain respectively. Activation of FX involves the hydrolysis of a peptide bond at Arg194, at the N-terminus of the heavy chain.^{53,61} Similar to prothrombin, FXa binds to phosphatidylserine-containing membranes via its Gla-domain⁵⁸.

FVa

Factor V is a single-chain glycoprotein with a domain organization of A1-A2-B-A3-C1-C2^{62,63}. FV is activated by thrombin, with peptide cleavage at Arg709, Arg1018 and Arg1545, leading to the removal of the B domain. The fully active FVa consists of a heavy chain (A1 and A2) and a light chain (A3, C1 and C2) linked together in a calcium dependent manner, see figure 4.^{4,64,65} FVa does not contain a Gla-domain, but the protein is still capable to mediate binding to negatively charged phospholipids. The binding is mediated by the C-domains of FVa⁶⁶⁻⁶⁹.

Protein C system

The protein C anticoagulant pathway regulates the activation of prothrombin, the prothrombinase complex 5.21,56,70-72. The system consists

of activated protein C (APC) and protein S, in which protein S works as a cofactor to APC⁷³⁻⁷⁶. APC cleaves FVa at three different sites, Arg306, Arg506 and Arg679, which leads to a complete loss of procoagulant FVa activity, see figure 3 and 4^{21,77}. The cleavage at Arg506 is believed to be kinetically favoured⁷⁸. In a similar reaction, APC cleaves and inhibits FVIIIa, in which FV works as a cofactor^{21,79}. Like many of the blood coagulation proteins, APC and protein S are vitamin K-dependent proteins that bind to negatively charged phospholipids⁵⁴. This is important as the protein C system thus regulates blood coagulation directly on the phospholipid membrane surface upon which the coagulation takes place. Resistance to activated protein C is a genetic risk factor for venous thrombosis and the most common is a mutation in FV, FVR506Q (FV Leiden), in which an arginine is replaced by a glutamine^{80,81}. The mutation leads to an impaired inactivation of FVa by APC as well as impaired cofactor activity in the inactivation of FVIIIa⁸².



Figure 3. Assembly of the activated protein C system on the surface of phospholipid membranes^{21,56}. FVa (cofactor in the prothrombinase complex) is inactivated by activated protein C (APC) and protein S (PS). The inactivated form of FVa is named FVi.

Protein C

Protein C is synthesized in the liver as a single-chain molecule with the domain organisation of a Gla-domain, two EGF-like domains and a serine protease domain⁸³. In human plasma the majority of protein C consists of two polypeptide chains, the light and heavy chains, which are linked together by a disulphide bond. The Gla-domain and the EGF-like domains constitute the light chain whereas the serine protease domain can be found in the heavy chain. Protein C is activated to APC by thrombin by the thrombin-thrombomodulin complex^{70,84,85}. Thrombomodulin (TM) is a transmembrane protein present on endothelial cells and thrombin is bound to TM on intact endothelial cells. When thrombin is bound to TM, it cannot exert its procoagulant functions. The thrombin mediated activation of protein C is stimulated by an endothelial protein C receptor⁸⁶.



Figure 4. Cleavage sites in FVa during inactivation of FVa by activated protein C (APC) and Protein S (PS). APC cleaves FVa at three sites in FVa, at arginine (Arg) 306, 506 and 679.

Protein S

Human protein S is a single-chain protein with a Gla-domain, a thrombin sensitive region, four EGF-like domains and a carboxy-terminal region that is homologous to sex hormone binding globulin⁸⁷. Thus, protein S does not harbour any serine protease activity. Protein S works as a cofactor in the APC-mediated inactivation of FVa. It functions as an APC cofactor mainly for the cleavage at Arg306, while a weak cofactor activity also has been shown for the cleavage at Arg506^{88,89}. Protein S has also been shown to exert other anticoagulant properties, as it is believed to serve as a cofactor

for tissue factor pathway inhibitor in the down regulation of FX activation^{90,91}.

Phospholipid membranes

Liposomes, artificial phospholipid membranes, can be generated *in vitro* and, used in the reactions of blood coagulation. Liposomes are particles containing a lipid bilayer of phospholipids and can be generated using different methods e.g. sonication, extrusion or dialysis^{92,93}. The lipid composition can also be regulated depending on which phospholipids are used in the preparation, thus liposomes can be used as membrane surfaces in blood coagulation reactions if the negative phospholipid PS is incorporated. The dialysis method is used throughout my studies, and is based on that phospholipids are dissolved in a detergent-containing buffer. Liposomes are then formed during dialysis of the mixture, when the detergent is slowly removed.

Phospholipid membranes rich in PS can be found *in vivo* in activated platelets, as discussed above. In addition, PS is also exposed on apoptotic cells, which is important for the clearance of apoptotic cells⁹⁴⁻⁹⁷, as well as on microparticles, <1 μ m lipid particles⁹⁸⁻¹⁰⁰. In plasma, phospholipids are found in lipoprotein complexes of variable size, and it is the lipoproteins that transfer the phospholipids in plasma³⁷. The ability of lipoproteins to provide an additional surface for blood coagulation is the main subject of this thesis, thus lipoproteins will be reviewed in the next section.

LIPOPROTEINS



Figure 5. The general structure of a lipoprotein particle. Triglycerides and cholesteryl esters are located in the hydrophobic core surrounded by a hydrophilic surface of phospholipids, apolipoproteins and cholesterol. The image was originally published in Atlas of Heart Diseases: Atherosclerosis by Brewer B.H.¹⁰¹, and is reprinted here with kind permission from Springer Science and Business Media.

Lipids have many different biological functions e.g. lipids are components of cellular membranes and the main storage form of energy. In circulation, lipids are transported together with lipoproteins, due to their low solubility in water. Lipoproteins consist of a hydrophobic core with triglycerides and cholesteryl esters, surrounded by an surface monolayer of phospholipids, unesterified cholesterol and apolipoproteins, see figure 5^{102} . Apolipoproteins are the protein components of lipoproteins and functions as enzyme regulators, receptor binding partners to cell surface receptors and are important for the synthesis of lipoproteins^{102,103}. Apolipoproteins have

an amphipathic property, containing both hydrophilic and hydrophobic amphipathic α -helices^{104,105}. regions, termed In that way, the apolipoprotein both interacts with the lipids of the lipoprotein and with the aqueous environment. The first classification of lipoproteins was made by the observation that lipoproteins migrated differently in an electric field, with alpha and beta migrating mobilities^{106,107}. Later, lipoproteins were observed to have different densities during ultracentrifugation of plasma, leading to the classification into four major classes, see table 2^{108} . Lipoproteins can also be divided into either apolipoprotein B or apolipoprotein A-I containing particles, depending on their major apolipoprotein content^{109,110}.

	Density	Major	Major function	Electrophoretic
	(g/mL)	apolipoprotein		mobility
Chylomicrons	<0.94	B-48	Transport of exogenous fat	Origin
VLDL	0.94-1.006	B100	Transport of endogenous fat	Pre-β
IDL	1.006-1.019	B100	Precursor to LDL	β
LDL	1.019-1.063	B100	Transport of cholesterol	β
HDL	1.063-1.21	A-I	Reverse cholesterol transport	Pre-β, α, γ
VHDL	1.21-1.25	A-I	-	-

 Table 2. General properties of human serum lipoproteins^{102,104,111,112}

Lipoprotein metabolism

The metabolism of lipoproteins involves two metabolic pathways, the exogenous and endogenous pathways. The exogenous pathway transports dietary lipids from the intestine^{102,104}. In the intestine, the lipids are adsorbed and secreted in the blood as chylomicrons. Chylomicrons are rich in triglycerides, and contain apolipoprotein B-48 (apoB-48) as their main apolipoprotein but also apolipoprotein E (apoE). The triglyceride in the chylomicrons is catabolized by the enzyme lipoprotein lipase, to generate fatty acids that are taken up in the intestine. Surface components are transferred from chylomicrons to high-density lipoprotein (HDL), and the triglyceride-depleted chylomicrons are converted to chylomicron remnants.

The chylomicron remnant is rapidly cleared from the circulation by uptake in the liver by the LDL-receptor (LDLr) and the LDLr-related protein (LRP). The endogenous pathway transports lipids already present in the body^{102,104}, see figure 6. The liver synthesizes and secretes the triglyceriderich lipoprotein, very-low-density lipoprotein (VLDL). VLDL is also catabolized by lipoprotein lipase, and transformed to intermediate-density lipoprotein (IDL) and later also to low-density lipoprotein (LDL). During the transformation, surface components are transferred to HDL. LDL is rich in cholesterol and contains apolipoprotein B-100 (apoB-100) as its main apolipoprotein. LDL is taken up by the LDLr in the liver and peripheral tissues. HDL is the densest particle, and contains apolipoprotein A-I (apoA-I). HDL functions in the reverse cholesterol pathway, as described below.



Figure 6. Lipid metabolism and reverse cholesterol transport. Lipoproteins transport either exogenous lipids (chylomicrons) or endogenous lipids (VLDL, IDL and LDL). HDL serves as a role in the reverse cholesterol transport. The image was originally published in Atlas of Heart Diseases: Atherosclerosis by Brewer B.H.¹⁰¹, and is reprinted here with kind permission from Springer Science and Business Media.

HDL and apoA-I

ApoA-I and apolipoprotein A-II (apoA-II) are the major proteins in HDL, accounting for 70% and 20% of the apolipoprotein content in HDL respectively¹¹¹. Other apolipoproteins found in HDL are apolipoprotein C-I, C-II, C-III, E and M^{111,113}. ApoA-I is one of the most abundant apolipoprotein in plasma, with a concentration of about 1-2 g/L, and functions as an activator of the enzyme lecithin-cholesterol acyltransferase (LCAT)^{114,115}. ApoA-I is synthesized in the liver and intestine as pre-pro-apoA-I, with the signal peptide as the pre-peptide¹¹⁶. After processing, the pre- and pro-peptides are cleaved and, apoA-I is secreted as a lipid-free apoA-I molecule followed by incorporation into plasma HDL particles.

Different forms of apoA-I

ApoA-I in plasma is present in three different forms, either as a lipidfree/lipid-poor apoA-I, or as a component in discoidal and spherical HDL, see figure 6¹¹⁷. Crystallization of human apoA-I revealed the structure of lipid-free apoA-I; a molecule with two antiparallel helical bundles, an N-terminal four-helix bundle and a C-terminal two-helix bundle¹¹⁸. After interactions with phospholipids, lipid-poor apoA-I is formed. Lipid-poor apoA-I takes then up more phospholipids and cholesterol and forms the discoidal HDL particle. Discoidal HDL consists of two or three apoA-I molecules and phospholipids with or without unesterified cholesterol, and has a pre- β migrating property¹¹⁹. Discoidal HDL does not contain a hydrophobic core, because of its absence of cholesteryl esters, and is shaped as a disc with a bilayer of phospholipids surrounded by apoA-I molecules. The discoidal HDL is rapidly converted to spherical HDL by LCAT, which esterifies cholesterol into cholesteryl esters^{114,115}. Spherical HDL consists of two or more apoA-I molecules together with phospholipids, unesterified cholesterol, cholesteryl esters and triglycerides. In plasma, the majority of HDL is spherical HDL, also called α -migrating HDL¹²⁰. The spherical HDL can be further divided into different HDL₂ (1.063<d<1.125 g/mL) subclasses. and HDL₃ (1.125<d<1.21 g/mL), which differ in their density¹¹⁷. There is also a subpopulation of spherical HDL called y-migrating HDL, which is HDL particles containing only apolipoprotein E (apoE)¹²¹. HDL can also be

separated up to 1.21 < d < 1.25 g/mL, forming the very-high-density lipoprotein (VHDL)¹¹¹. VHDL also contains apoA-I as its major apolipoprotein.

Reverse cholesterol transport

LDL, as mentioned above, is a cholesterol-rich lipoprotein. Besides uptake in the liver, LDL can also be taken up by scavenger-receptors present in macrophages leading to massive cholesterol deposition and cellular accumulation of cholesterol. Uptake of LDL to macrophages requires modification of LDL due to oxidation, the generation of oxidized LDL (OxLDL)¹²². When LDL is subjected to oxidative stress, the lipid molecules containing polyunsaturated fatty acids in LDL are easily oxidized and as a consequence, apoB is covalently modified by these oxidized lipids^{123,124}. Uptake of OxLDL by macrophages will lead to the formation of foam cells and the formation of an atherosclerotic lesion, an early step in the development of an atherosclerotic plaque. HDL has anti-atherogenic properties as it participates in the reverse cholesterol pathway, the removal of excess cholesterol from peripheral tissues, such as cholesterol-loaded macrophages, to the liver, see figure 6. The ATP-binding cassette transporter A1 (ABCA1) pathway mediates the uptake of cholesterol to lipid-poor apoA-I to form pre- β , discoidal HDL¹²⁵. This protein is mutated in patients with Tangier disease and, the outcome is a deficiency in HDL due to hypercatabolism of the discoidal HDL particles together with a great loss in apoA-I-mediated cellular cholesterol efflux¹²⁶⁻¹²⁸. The cholesterol in discoidal HDL is then esterified by LCAT to form mature spherical HDL^{114,115}. Spherical HDL can also mediate cholesterol efflux by the scavenger receptor B1 (SR-B1) and passive diffusion pathways¹²⁵ as well as by other ATP-binding cassette transporters¹²⁹. The cholesteryl ester in HDL is either transported directly to the liver for uptake by scavenger receptor SR-B1 or transferred to VLDL-IDL-LDL lipoproteins by cholesteryl ester transfer protein (CETP). LDL is strongly correlated with the risk of cardio vascular disease¹³⁰, and is often called the "bad cholesterol" because of its atherogenic properties described above. HDL, on the other hand, is often called the "good cholesterol", and HDL is inversely correlated with the incidence of cardio vascular disease^{131,132}. The reverse cholesterol transport plays a central role in the protection of atherosclerosis by HDL. HDL also exerts other anti-atherogenic functions, such as antioxidant action (inhibition of oxidation of LDL) and anti-inflammatory actions (inhibition of expression of endothelial cell adhesion molecules)¹³³⁻¹³⁵.

Reconstituted HDL

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 particles¹³⁶. The structure of apoA-I in discoidal HDL or rHDL has been extensively studied, for review see references by Oda, M.N and Nath, $A^{137,138}$. Because apoA-I consists of amphipathic α helices it will, together with phospholipids, adapt a lipid-bound state. Different models have shown that apoA-I can adopt different conformations in rHDL¹³⁹. The "picket fence" model features two apoA-I monomers with short antiparallell helices arranged perpendicular to the plane of the bilayer^{140,141}. The "molecular belt" model features two apoA-I monomers extended around the bilayer¹⁴²⁻¹⁴⁴. A variant of the molecular belt model is the "hairpin" model, which features the two apoA-I monomers on opposite sides of the bilayer¹⁴⁵. A crystal structure of a truncated form of apoA-I (Δ 1-43 apoA-I), in which the first 43 amino acids are deleted, suggested a lipid-bound conformation similar to the belt conformation¹⁴⁶. The belt conformation is now the most widely accepted model, but still there is no crystal structure of a complete rHDL particle. In vitro, rHDL can be generated by the dialysis method described in the phospholipid section above. Apolipoproteins (e.g. apoA-I) are then included in the detergent-dissolved phospholipid mixture, and rHDL are formed by dialysis in which the detergent is removed¹³⁶. The generated rHDL particle adapts a disc-shaped form, with the apolipoprotein surrounding the phospholipids. A schematic picture of an rHDL particle can be seen in figure 7.



Figure 7. A simplified model of rHDL generated with phospholipids and apoA- $I^{137-139}$. The disc-shaped particle consists of a lipid bilayer surrounded by apoA-I. 2 apoA-I molecules are usually incorporated per rHDL particle and, the molecular belt model is shown here.

Nanodiscs

Nanodiscs are very similar to discoidal rHDL particles and they are created in a similar manner as rHDL but instead of using apoA-1, a truncated form of apoA-I is used (Δ 1-43 apoA-I)¹⁴⁷, for review see reference by Nath, A^{138} . MSP or "membrane scaffold protein" is used as a name for $\Delta 1-43$ apoA-I, after its ability to self-assemble into discoidal nanoparticles in the presence of synthetic phospholipids. By changing the length of the amphipathic helical part in the MSP sequence, the size of nanodiscs can be controlled with average size from 9.5 to 12.8 nm¹⁴⁸. The structure of nanodiscs has been studied using molecular dynamics simulations and solidstate NMR techniques, and the analyses indicated that nanodiscs adapt the double-belt model more than the picket fence model^{149,150}. Nanodiscs have been used as model membranes and integral membrane proteins have been incorporated into nanodiscs as well¹³⁸. As model membranes, nanodiscs have been used for studying the initiating reactions of blood coagulation between tissue factor (TF) and factor VIIa (FVIIa)¹⁵¹. 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. Nanodiscs have also been studied in their ability to assemble the prothrombinase complex. Data suggest that the complex can be assembled on nanodiscs that have been engineered to encompass at least 12 nm in diameter¹⁵².

Lipid transfer proteins

In human plasma, lipids are transferred between lipoprotein particles by specific lipid transfer proteins: cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP). The main function of CETP in plasma is to transfer cholesteryl esters from HDL to VLDL and LDL, but CETP has also been shown to have phospholipid transfer activity^{153,154}. Both PLTP and CETP are able to mediate the transfer of phospholipids from phosphatidylcholine (PC)-containing HDL particles to LDL, but unlike PLTP, CETP is unable to transfer phospholipids from PC-containing liposomes to HDL¹⁵⁵. The two main functions of PLTP are phospholipid transfer activity and HDL conversion, as described in more detail below and in the review by Huuskonen et al¹⁵⁶. PLTP and CETP display sequence homology (21.7%), and are both included in the lipopolysaccharide (LPS)binding/lipid transfer protein family together with LPS-binding protein (LBP) and bactericidal permeability increasing protein (BPI)¹⁵⁷. PLTP and CETP activities are both present in many different species, but the mouse lacks CETP activity¹⁵⁸. Phospholipid transfer/exchange activities have also been demonstrated for LBP and soluble CD14¹⁵⁹.

PLTP

PLTP was originally named Lipid-Transfer Protein-II and is a 476 amino acid hydrophobic glycoprotein¹⁵³. It contains six potential N-glycosylation sites, which explains the difference between the calculated molecular weight of 54 kDa and the apparent molecular weight of 80 kDa observed by sodium dodecylsulfate-polyacrylamide gel electrophoresis under reducing conditions¹⁵⁷. PLTP in plasma is to some extent associated with HDL¹⁶⁰⁻¹⁶², and its interaction with HDL/apoA-I has been verified^{161,163,164}. PLTP binds both apoA-I and apoA-II, and the PLTP binding domain might be located in the amino terminal region of apoA-I¹⁶⁴. Molecular model of the PLTP structure, based on sequence alignments of the LPS-binding/lipid transfer protein family and the BPI crystal structure, predicts a boomerang-shaped two-domain molecule with conserved lipid-binding pockets consisting of apolar residues in both domains¹⁶³. Site-directed mutagenesis of PLTP reveals that the both lipid-binding pockets are important for the PLTP-

mediated phospholipid transfer and, that the C-terminal pocket may be involved in the association of PLTP with HDL¹⁶³.

Phospholipid transfer activity

PLTP facilitates both the transfer of phospholipids between HDL and other triglyceride-rich lipoproteins and the transfer of phospholipids between phospholipid vesicles and HDL^{160,165}. PLTP mediates transfer of phospholipids with low head specificity. group although phosphatidylethanolamine is transferred 2-3 times slower¹⁶⁶. The transfer mechanism is believed to involve a ternary complex between donor and acceptor particles¹⁶⁷. Recently though, new data support a shuttle mechanism in which PLTP physically transports lipids between lipoprotein particles without the formation of a ternary complex¹⁶⁸. Besides the phospholipid transfer activity, PLTP also has the ability to transfer diacylglycerol¹⁶⁹, free cholesterol¹⁷⁰, α -tocopherol (vitamin E)¹⁷¹, and LPS^{172,173} between lipoproteins and cells.

A deficiency of PLTP in humans has not been described yet, but PLTP knock-out mouse models have illustrated the importance of PLTP-mediated phospholipid transfer *in vivo*¹⁷⁴⁻¹⁷⁶. The levels of HDL and apoA-I in PLTP knock-out mice were reduced by 60-70% and the plasma showed total absence of ability to transfer various phospholipids species in an *in vitro* assay¹⁷⁴. The decrease in HDL and apoA-I levels could be explained by the hypercatabolism of the protein-rich HDL particles depleted in phosphatidylcholine¹⁷⁵. When the PLTP knock-out mice were fed a high fat diet, there was an accumulation of the surface components of triglyceride-rich lipoproteins, highlighting the importance of PLTP in the transfer of surface remnants from triglyceride-rich lipoproteins to nascent discoidal HDL particles¹⁷⁴. The fact that the plasma phospholipid transfer activity *in vivo* is mainly due to PLTP was shown by Kawano et al. PLTP knock-out mice were bred with human CETP transgenic mice and, the phospholipid transfer activity was similar to that of PLTP knock-out mice alone¹⁷⁶.

HDL conversion

The second function of PLTP in lipoprotein metabolism is the ability to cause HDL remodelling^{177,178}. The conversion of HDL by PLTP includes
transformation of HDL into larger and smaller particles. PLTP can act on both HDL₃ and HDL₂ particles, and is accompanied by the generation of pre- β migrating HDL particles and larger HDL particles^{177,179,180}. The mechanism of the remodelling of HDL is believed to involve particle fusion in which PLTP mediates the fusion of two HDL particles¹⁸¹⁻¹⁸³, and triglyceride-enrichment of HDL has been shown to enhance their remodelling¹⁸⁴. The generation of pre- β migrating HDL particles from HDL remodelling by PLTP plays an important role in the reverse cholesterol transport, where pre- β migrating HDL particles act as the major cholesterol acceptors. HDL can also be remodelled by other plasma factors such as LCAT, CETP, and hepatic lipase¹²⁰.

High activity (HA) and low activity (LA) PLTP

Two forms of PLTP have been characterized in human plasma, one being catalytically active (HA-PLTP), whereas the other one has low activity (LA-PLTP)^{162,185,186}. The fact that PLTP exists in two forms might explain the lack of association between PLTP mass and transfer activity^{187,188}. The different forms of PLTP are associated with macromolecular complexes of different size; HA-PLTP has an average molecular mass of 160 kDa and copurifies with apoE, while LA-PLTP is 520 kDa in size and in complex with apoA-I¹⁸⁵.

PRESENT INVESTIGATION

Lipoproteins have been reported to regulate blood coagulation in different ways¹⁸⁹⁻¹⁹⁴. The presence of a phospholipid monolayer in lipoproteins may provide another phospholipid surface, other than platelets, that could stimulate the reactions of blood coagulation. However, the main part of the phospholipid content in lipoproteins is constituted by the neutral phospholipid, phosphatidylcholine (around 70 % depending on lipoprotein class)¹⁹⁵. In addition, HDL is reported to only contain about 1 % of the negatively charged phospholipid, phosphatidylserine¹⁹⁵. VLDL has been reported to support FXa-, and FXa/FVa-mediated FVII activation¹⁸⁹ as well as activation of prothrombin by the prothrombinase complex¹⁹⁰, whereas LDL has been shown to support the activation of FX by the tenase complex¹⁹¹. On the other hand, HDL has been reported to exert anticoagulant functions. HDL has been reported to enhance the anticoagulant activities of plasma protein S and activated protein C^{192} . In addition, HDL deficiency has been reported to be associated with venous thrombosis in men^{196,197}. Taken together, dual roles of lipoproteins in blood coagulation have been reported, and the current investigation strives to elucidate the role of HDL in blood coagulation. Papers I and II show how the prothrombinase complex can be regulated by HDL particles, while paper III reevaluates the role of HDL in the APC system.

Paper I

Lipoproteins can be isolated from human plasma by sequential ultracentrifugation, a common method for lipoprotein isolation¹⁹⁸. The method takes advantages of the fact that lipoproteins can be separated regarding to their density. Further purification and validation of the

lipoproteins, especially LDL and HDL, can be achieved by gel filtration – separation by size. Intact lipoproteins isolated by ultracentrifugation were studied in their ability to support prothrombin activation. None of the intact lipoproteins were able to stimulate prothrombin activation. A small stimulatory activity was present in the isolated HDL preparation but was found not to be associated with HDL after further purification by gel filtration. Extracted apolipoproteins, from the isolated lipoproteins, were then used together with anionic phospholipids to create reconstituted lipoproteins and, tested in their ability to stimulate prothrombin activation. Reconstituted lipoproteins with apolipoproteins from high-density lipoprotein had a pronounced anticoagulant effect, with a decrease in thrombin formation. More precisely, apoA-I was shown to cause a decrease in thrombin formation when used in reconstituted lipoprotein particles. In order to study these particles, reconstituted HDL (rHDL) particles were generated consisting of only apoA-I and natural phospholipids. Reconstituted HDL particles, containing different amounts of anionic phospholipids, were unable to support activation of prothrombin and, unable to mediate binding of the cofactor FVa. HDL was also investigated for its ability to take up anionic phospholipids. Serum was able to transfer phospholipids from anionic liposomes to both apoA-I- and apoBcontaining particles, thereby causing a strong attenuation of the procoagulant effect of anionic phospholipids. A model of the neutralization of procoagulant phospholipids by HDL was proposed and could be explained by the fact that the rHDL particle is too small (about 8 nm) to accommodate a complete prothrombinase complex, mainly because of deficient binding of FVa.

Paper II

This paper is based on the findings in paper I, where serum was shown to cause neutralization of procoagulant liposomes. The lipid transfer protein, PLTP, was studied in its role in the neutralization of procoagulant liposomes. The neutralization was dependent on lipoprotein-enriched serum and more precisely on PLTP containing lipoprotein fractions. PLTP protein was absent in LDL while present in apoA-I containing particles

HDL and VHDL, as judged by immunoblotting. Even though phospholipids were transferred to both LDL and HDL particles in serum¹⁹⁹, the addition of LDL to procoagulant liposomes alone was not sufficient to cause a neutralization. On the other hand, addition of exogenous PLTP to the LDL-liposome mixture caused an increase in the transfer of phospholipids as well as a more pronounced neutralization of the procoagulant phospholipids. The neutralizing activity in serum was dependent on PLTP, as judged by immunoprecipitation of PLTP from VHDL. The neutralizing activity could be regained by the addition of a catalytically active form of PLTP, HA-PLTP, and a low activity form of PLTP had no effect.

Paper III

This study is based on the reported function of HDL to enhance the activities of APC and protein S in the degradation of FVa¹⁹². The fact that HDL was shown to be unable to accommodate a complete prothrombinase complex¹⁹⁹, the ability to accommodate an even bigger complex like the APC complex was investigated. Thus, the role of HDL in the APC system was reevaluated. HDL, isolated by ultracentrifugation, was able to stimulate inactivation of FVa in the presence of APC and protein S as previously reported¹⁹². However, further purification of HDL by gel filtration revealed that the stimulating activity was not a property of HDL but instead caused by the presence of contaminating anionic phospholipids eluting in the void volume of the separation. This effect was first observed in paper I where HDL, isolated from ultracentrifugation, was found to stimulate prothrombin activation. After further separation of HDL by gel filtration, the activity was shown not to be associated with HDL. Both the anti- and procoagulant activities in the ultracentrifuged HDL fraction were now further characterized. Both activities were blocked by the addition of annexin V and in addition the procoagulant activity was also blocked by the presence of phospholipase A_2 . The void fractions were absent in both apoB content and cholesteryl esters. Taken together, these results demonstrated that HDL is not able to stimulate neither inactivation of FVa by APC complex nor the activation of prothrombin by the prothrombinase complex. The

previously reported anticoagulant activity of HDL¹⁹² is instead caused by contaminating anionic phospholipid membranes.

Future Perspective

We now report that HDL is not able to activate prothrombin to thrombin as well as not able to inactivate FVa by the presence of APC and protein S. However, HDL can participate in the regulation of blood coagulation by neutralizing procoagulant liposomes, with uptake of anionic phospholipids to HDL where it cannot stimulate the activation of prothrombin due to the small surface area. The future perspective might be to investigate the role of HDL in a more biological setting. The anionic phospholipid scavenger function of HDL might be an important mechanism to control the exposure of other anionic phospholipid membranes like circulating microparticles or apoptotic cells. Microparticles are released into circulation upon activation of various cells^{98,200,201}. For instance, activation of platelets can cause the generation of platelet-derived microparticles²⁰². Common features of microparticles are that they are less than 1 µm in size and highly procoagulant due to the presence of anionic phospholipids in their outer membrane^{98,203}. Microparticles are reported to be elevated in various conditions with vascular dysfunction and inflammation^{98,204}. Microparticles do contain a phospholipid surface similar to liposomes, which have been used in our studies so far. The difference between microparticles and liposomes is that microparticles also contain proteins from which they originate. Thus, platelet-derived microparticles contain proteins from platelets. In conclusion, a microparticle might be a more complex phospholipid-containing particle and different from a liposome. The apoA-I- and PLTP-mediated neutralization of anionic phospholipids may be one of the mechanisms to control the exposure of this type of phospholipids to circulating blood, and needs further investigation.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Blodet i kroppen har många uppgifter, så som att transportera syre till alla organ i kroppen. I normala förhållanden cirkulerar blodet flytande, men vid en skada måste blödningen förhindras och blodet levrar sig. Levringen av blodet (blodkoagulering) innefattar en rad olika reaktioner som slutligen leder till genereringen av proteinet trombin och ett nätverk av fibrin. I fibrinnätverket fastnar blodplättarna vilket medför att en så kallad plugg bildas och hindrar blödningen. Blodkoaguleringen sker på blodplättarnas yta. Reaktionerna som slutligen leder till genereringen av trombin involverar en rad olika proteiner som aktiveras. Trombin är den aktiva formen av protrombin. Enzymet faktor Xa och dess kofaktor faktor Va (FVa) vilka så aktiverar protrombin, tillsammans utgör det kallade protrombinaskomplexet. Protrombinaskomplexet regleras och inhiberas utav ett annat komplex, aktiverat protein C-komplex (APC-komplex). APCkomplexet inhiberar kofaktorn FVa, vilket gör att FVa inte längre fungerar och protrombinaskomplexet blir ofullständigt. Både protrombinas- och APC-komplexen samlas på blodplättarnas yta. Blodplättarnas yta består av en särskild komponent som är avgörande för komplexens aktivitet, fosfolipiden fosfatidylserin. Fosfolipider finns i alla cellers membran, dock är det främst i blodplättarna som fosfatidylserin finns tillgänglig på ytan. Fosfatidylserin är en negativt laddad fosfolipid som i normala fall inte finns på ytan av celler. När blodkoaguleringen aktiveras, exponeras dock fosfatidylserin på blodplättarnas yta och möjliggör att de olika proteinerna i protrombinaskomplexet kan binda till blodplättarna.

Fosfolipider finns även på ytan av lipoproteiner, partiklar i vår kropp som transporterar fett. Fetter är svårlösliga i vatten och i vår kropp

transporteras fetter i särskilda partiklar, lipoproteiner, som har förmågan att binda fetter i dess inre kärna samtidigt som de har en vattenlöslig yta som exponeras mot blodet. Lipoproteiner kan delas upp i olika grupper beroende på dess densitet. LDL (low-density lipoprotein) och HDL (high-density lipoprotein) är de två vanligaste partiklarna och brukar kallas för det onda respektive det goda kolesterolet. Detta p.g.a. att LDL ger upphov till åderförkalkning i blodkärlen, medan HDL istället motverkar detta. Lipoproteinernas yta består av olika fosfolipider därutav fosfatidylserin, som skulle kunna göra det möjligt att binda protrombinas- och APCkomplexen. Lipoproteiner har visats sig kunna ha olika delaktighet i regleringen av blodkoagulering. I den här avhandlingen fokuseras det på HDL, och hur blodkoaguleringen kan regleras av HDL. Det är viktigt att veta hur blodkoaguleringen regleras då det alltid ska finnas en balans mellan anti- och prokoagulanta reaktioner.

HDL kan isoleras från blodet men kan även göras i laboratoriet. Att göra HDL i laboratoriet kallas för att rekonstruera HDL (rHDL) och metoden har egenskapen att valfria fosfolipider kan inkorporeras, dvs. i vårt fall användes ett högt antal fosfatidylserin för att studera blodkoaguleringen. De negativt laddade fosfolipiderna i rHDL förlorade sin egenskap att stimulera aktiveringen av protrombin när de inkorporerades i rHDL partiklar (Artikel I). Dessa negativt laddade fosfolipider, fosfatidylserin, kunde inte binda kofaktorn FVa och därmed var inte protrombinaskomplexet fullständigt och aktiveringen av protrombin nedreglerades. Ytan av HDL är för liten för att ett helt protrombinaskomplex ska samlas. HDL, isolerat från blodet, kunde inte heller aktivera protrombin. HDL visade sig även kunna ta upp fosfatidylserin och på så sätt hindra aktiveringen av protrombin (Artikel **II**). Denna upptagelse av fosfolipider var beroende av ett protein, PLTP (phospholipid transfer protein) (Artikel II). HDLs förmåga att ta upp fosfatidylserin kan vara ett sätt att reglera blodkoaguleringen. I blodet kan fria fosfolipider med fosfatidylserin, mikropartiklar, frisättas och utgöra en ökad risk för levring av blodet. HDL kan därmed reglera blodkoaguleringen genom att ta upp fosfatidylserin och på så sätt hindra oönskad koagulering.

I den sista studien (**Artikel III**) undersöktes om HDL kunde utgöra en fosfolipidyta för APC-komplexet, dvs. om inaktiveringen av FVa kan ske

på ytan av HDL, vilket tidigare har visats. Utifrån resultaten från artikel I och II kan inte aktiveringen av protrombin ske på ytan av HDL. Det verkar därför ologiskt att ett större komplex som APC-komplexet skulle kunna samlas på ytan av HDL. HDL, isolerat från blodet, hade faktiskt förmågan att inaktivera FVa m.h.a. APC-komplexet. Dock, genom vidare rening av HDL visade det sig att denna aktivitet inte var associerad med HDL. HDL-preparationen var nämligen oren och förorenad med fria negativa fosfolipider. Detta framhäver betydelsen att alltid använda ett rent prov av HDL.

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PAPER I

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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 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.

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The concentration of high density lipoprotein (HDL)² 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 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 (FVIIIa) activate factor X (FX) to factor Xa (FXa) (12). The prothrombinase complex (see Fig. 5*A* in discussion for a schematic picture) consists of the enzyme FXa, which

² The abbreviations used are: HDL, high density lipoprotein; apoA-I, apolipoprotein A-I; rHDL, reconstituted high density lipoprotein; PS, phosphati-dylserine; FIXa, activated factor IX; FVIIa, activated factor VII; FX, factor X; FXa, activated factor X; FVa, activated factor V; FVIIa, activated factor VIII; FX, factor V; GIa, -, carboxy glutamic acid; VLDL, very low density lipoprotein; LDL, low density lipoprotein; PS, phosphatidylcholine; BSA, bovine serum albumin; PLTP, phospholipid transfer protein; VHDL, very high density lipoprotein; TF, tissue factor; AGCA1, ATP-binding cassette transporter; TBS, Tris-buffered saline; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; RU, response unit.



The on-line version of this article (available at http://www.jbc.org) contains a supplemental figure.

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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) 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—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 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 \times g for 10 min 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 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

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¹⁴C-radiolabeled PC (GE Healthcare) was added to the lipid mixture when necessary. The lipids were then solubilized by adding *n*-octyl-β-*p*-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 ÄKTA 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 $1 \mu g/ml$ 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). The reaction was terminated with $1 \text{ M} \text{H}_2\text{SO}_4$, 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 μ l) were transferred to a 96-well plate (Sero-well, Sterilin) and mixed with 40 μ l of HNBSACa. The reaction was initiated by the addition of 20 µl prothrombin (Kordia, Leiden, The Netherlands) to final concentration 0.5 μ M and incubated at 37 °C for 2 min. The reaction was stopped by the addition of 100 μ l 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 μ l were mixed with 50 μ l 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 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 μ M. During the 2-min activation time, using a phospholipid concentration of $\leq 5 \ \mu$ M, 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 μ l 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 Hematologic 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 м, 600 ml of each vessel). To check the purity of the prethrombin-1, the 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, whereas 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 μ M phospholipid) for 17 min at a 3 μ l/min flow rate in HN running buffer. Binding responses proceeded to sat-

uration 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 μ l/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 base line stabilized to less than 0.05 RU/min. Equilibrium response data were 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 RUs because of the 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 50 mM NaOH pH 11.5 (for FVa), which returned the base line to the value prior to introducing protein. Equilibrium data $(R_{\rm eq})$ 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 nonspecific protein-lipid and protein-protein interactions (34). The 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 μ M PL) and 100 μ M 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 μ M 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 μ M 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 μ M 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



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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), and then the detergent was removed by dialysis. The lipid-apolipoprotein comthen the deletion was remove by using a more than a product the places were then used as phospholipid source in a prothrombinase reaction (final phospholipid concentration, $5 \mu \omega$) 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 (\blacksquare), LDL (▲), HDL (\square), VHDL (△), and lipoprotein-free plasma (●). *B*, extracted proteins from HDL were separated on S200 Hiprep chromatography in 6 M guanidine HCI. Upper panel, fractions were mixed with detergent-solubilized PL (10:40:50 PS/PE/PC) and dialyzed and analyzed by nonreduced 15% Tricine-SDS/PAGE (silver-stained). Lower panel, dialyzed samples were used as phospholipid source in a pro-thrombinase reaction (right y axis, \bullet). The thrombin generation was normalized to a control reaction with liposomes (5 μ M) formed in the absence of apolipoproteins. Protein elution profile (left y axis,) was measured at absorbance 280 nm. Final concentrations of proteins during the 2 min of activation of prothrombin were; FVa, 210 pm; FXa, 2.5 nm; and prothrombin, 0.5 μM

Elution volume (mL)

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

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nrombin

(%)

of control

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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						
ароА-I (µм) ^a	0.4 ± 0.1	0.5 ± 0.07	0.6 ± 0.06	0.3 ± 0.07						
Phospholipid (µм) ^b	14.5 ± 2.2	19.7 ± 3.3	20.0 ± 2.0	18.2 ± 1.7						
PL/apoA-Î	38.1 ± 12.7	39.2 ± 8.8	31.6 ± 3.7	63.0 ± 8.3						
Stokes diameter (nm) ^c	8.2 ± 0.4	9.5 ± 0.0	10.1 ± 0.5	7.9 ± 0.0						

^a By apoA-I enzyme-linked immunosorbent assay.
^b By scintillation counting of [¹⁴C]PC.

By size exclusion chromatography.

PTase +FVa Thrombin (nM/min) 60 50 40 30 20 n. 0.0 0.2 0.6 0.8 1.0 0.4 PL (μM) В PTase -FVa Thrombin (nM/min) 01 0.2 0.4 0.0 0.6 0.8 1.0 1.2 PL (μΜ)

FIGURE 2. Anionic phospholipids when incorporated in rHDL have reduced procoagulant activity. Isolated rHDL (~40:1 PL/apoA-I molar ratio) or control liposomes (III) 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. The values are expressed as the means \pm S.E. from three independent rHDL preparations. Final concentrations of proteins during activation of prothrombin were: 210 pM FVa, 2.5 nM FXa, 0.5 μM prothrombin using a 2-min activation time (A) and 50 nm FXa, 0.5 μm prothrombin, using a 5-min activation time (B). Please note the different scales of the x axes (thrombin generation) in A and B.

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



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600 700

8

80

1.5

10

100

2.0

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 ~25kDa 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).

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

1.0

Factor Xa (µM)

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0.5

0.0

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efficiencies. The SPR response curves for 10:40:50 rHDL are shown after backaround 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 μ M). All of the proteins were injected in duplicate. Note: RU (y axis) is proportional to mass (1 RU = 1pg/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 (~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. The responses obtained at equi librium 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 $B_{\rm max}$ 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 the y axis scale differences in B, C, and D. See "Experimental Procedures" for more details.

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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

The K_d 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 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, 90 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 a naverage phospholipit molecular mass of 0.77 kDa and assuming 1 RU of protein = 0.22 pg/mm².

		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
	μм	fmol	mol protein/mol rHDL	μм	fmol	mol protein/mol rHDL	μм	fmol	mol protein/mol rHDL
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

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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 $B_{\rm max}$ 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 because a Glaless 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. 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 ($B_{\rm max}$), 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 with FXa bound with slightly lower affinity and $B_{\rm max}$. 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 (data not shown), demonstrating that the neutralization process was time-dependent. Next, the preincubated 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, 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

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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 (**A**) or absence (**④**) of PLTP (final PLTP activity 1000 nmol/ml/h). As a control, labeled liposomes were incubated with PLTP but without rHDL (**⑤**). The samples were separated on Superose 6 10/300 GL, and eluted fractions were analyzed by scintillation counting. *B*, human serum (**□**) or BSA (**A**) 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. The values are expressed as the means $\pm 5.D$. from duplicates and are representative from repeated experiments. *C*, the samples in *B* were separated on Superose 6 10/300 GL, and eluted fractions were analyzed by scintillation counting.

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 investi-

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gated. 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-1, a truncated form of apoA-1 (Δ I-43 apoA-1) 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.

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 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 \sim 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 ${\sim}2$ 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, \sim 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).

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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 prothrombinnase 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 because of deficient binding of FVa.

The combined assembly of FXa, FVa, and prothrombin on a rHDL particle thus seems unfeasible (Fig. 5*B*).

The rHDL used here were made with natural phospholipids and showed a ~2-fold lower total phospholipid/rHDL ratio (80 phospholipid/rHDL) compared with 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 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 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 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-

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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 wildtype 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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SUPPLEMENTARY

Figure legend

<u>Figure 1</u>. *rHDL particles consist of 2 apoA-1 molecules per particle*. Chemical cross-linking of apoA-1, 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 μ g) is shown in lane 1. Cross-linked rHDL particles with 10:40:50 (0.6 μ g apoA-I), 50:0:50 (0.8 μ g apoA-I) and 75:0:25 (1 μ g 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



PAPER II

Oslakovic C, Jauhiainen M, Ehnholm C, Dahlbäck B. The role of phospholipid transfer protein in lipoprotein-mediated neutralization of the procoagulant effect of anionic liposomes. *J Thromb Haemost.* 2010; 8: 766-72.

ORIGINAL ARTICLE

The role of phospholipid transfer protein in lipoproteinmediated neutralization of the procoagulant effect of anionic liposomes

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Summary. Background: Serum has the ability to neutralize the procoagulant properties of anionic liposomes, with transfer of phospholipids (PLs) to both high-density lipoprotein (HDL) and low-density lipoprotein (LDL) particles. Phospholipid transfer protein (PLTP) mediates transfer of PLs between HDL and other lipoproteins and conversion of HDL into larger and smaller particles. Objectives: To examine the role of PLTP in the neutralization of procoagulant liposomes. Methods: Procoagulant liposomes were incubated with different lipoproteins in the presence or absence of PLTP, and then tested for their ability to stimulate thrombin formation. Results and Conclusions: In the absence of added PLTP, the lipoprotein-enriched fraction, total HDL, HDL3 and very high-density lipoprotein (VHDL) were all able to neutralize the procoagulant properties of the liposomes. In these samples, endogenous PLTP was present, as judged by Western blotting. In contrast, no PLTP was present in LDL, HDL2 and lipoprotein-deficient serum, all of which displayed no ability to neutralize the procoagulant liposomes. The phospholipid (PL) transfer activity was dependent on both enzyme (PLTP) and PL acceptor (lipoproteins). After treatment of the VHDL fraction with antiserum against PLTP, the neutralization of procoagulant activity was reduced, but could be regained by the addition of active PLTP. The neutralizing activity was dependent on a catalytically active form of PLTP, and addition of a low activity form of PLTP had no effect. In conclusion, PLTP was found to mediate transfer of anionic PLs to HDL and LDL, thereby neutralizing the effect of

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procoagulant liposomes, resulting in a reduction of procoagulant activity.

Keywords: anionic phospholipid, apoA-I, coagulation, HDL, phosphatidylserine, phospholipid transfer, PLTP, procoagulant, prothrombinase.

Introduction

Blood coagulation is activated in response to tissue damage (i.e. rupture of an atherosclerotic plaque) and ultimately leads to the generation of thrombin and a fibrin clot[1,2]. Many of the reactions (e.g. the prothrombin activation) occur on activated platelets having the anionic phospholipid (PL), phosphatidylserine (PS), exposed on their surface. The activation of prothrombin to thrombin is mediated by the enzyme factor Xa (FXa) and its cofactor FVa, which together with the anionic PL comprise the prothrombinase complex [3]. Several different anticoagulant pathways, for example the protein C pathway, antithrombin, and TFPI (tissue factor pathway inhibitor), regulate blood coagulation. The regulation of blood coagulation by lipoproteins, including the effect of lipids and the different lipoprotein fractions, has been studied [4-9]. High-density lipoprotein (HDL) has several atheroprotective properties; it mediates reverse cholesterol transport and has antioxidant, antiinflammatory and antithrombotic functions [10,11]. We have recently shown that human serum has the ability to neutralize procoagulant liposomes by transferring anionic PLs to both HDL and low-density lipoprotein (LDL) [12]. Moreover, reconstituted HDL (rHDL) using apolipoprotein A-I (apoA-I) and anionic PL was unable to accommodate the prothrombinase complex because in the rHDL, the anionic PLs were arranged in surface areas that were too small to bind FVa. These results suggest that HDL and apoA-I have an anticoagulant effect by functioning as a scavenger for anionic PL [12].

Several proteins are known to participate in the transfer of PLs. For example, the plasma protein phospholipid transfer

protein (PLTP) mediates transfer of PLs between different lipoproteins in plasma, whereas transfer of PLs from cells to HDL is mediated by ABCA1 (ATP-binding cassette transporter 1) [10]. PLTP is a 476 amino acid hydrophobic glycoprotein. It contains six potential N-glycosylation sites, which explains the difference between the calculated molecular weight of 54 kDa and the apparent molecular weight of 80 kDa in SDS-PAGE [13]. PLTP displays sequence homology to cholesteryl ester transfer protein (CETP), lipopolysaccharide (LPS) binding protein (LBP) and bactericidal permeability increasing protein (BPI) (20-26%), which are included in the LPS-binding/lipid transfer protein family [13]. The main function of CETP in plasma is to transfer cholesteryl esters from HDL to VLDL and LDL but CETP has also been shown to have PL transfer activity [14,15]. PLTP in plasma is to some extent associated with HDL, and its interaction with HDL/ apoA-I has been verified [16-18]. The molecular model of the PLTP structure predicts a boomerang-shaped two-domain molecule with conserved lipid-binding pockets consisting of apolar residues in both domains [16].

The two main functions of PLTP are phospholipid transfer activity and HDL conversion [19]. PLTP facilitates the transfer of PLs between HDL and other lipoproteins [20] as well as between PL vesicles and HDL [21]. PLTP mediates transfer of PLs with low head group specificity [22]. The conversion of HDL by PLTP includes transformation of HDL into larger and smaller particles [23,24]. During the conversion process, lipid-poor apoA-I is released and proteolytically cleaved by PLTP [25]. Other plasma factors, besides PLTP, that are known to remodel HDL include LCAT (lecithin:cholesterol acyltransferase), CETP and hepatic lipase[26]. Two forms of PLTP have been characterized, one being catalytically active (HA-PLTP) whereas the other one has low activity (LA-PLTP) [27,28]. The physiological role of LA-PLTP is unresolved.

The aim of the present study was to elucidate the role of PLTP in lipoprotein-mediated neutralization of procoagulant liposomes. Here we demonstrate that HDL-associated PLTP has the ability to neutralize the procoagulant properties of anionic liposomes by transferring the PLs to the lipoprotein particles. This transfer may have a biologically important role for the transfer of circulating anionic PLs from microparticles and apoptotic cells to the lipoproteins, thereby neutralizing their procoagulant properties.

Materials and methods

Isolation of lipoprotein fractions

Human citrate plasma was obtained from the local blood bank. Lipoproteins were isolated by sequential flotation ultracentrifugation, thus generating LDL ($1.0068 < d < 1.068 \text{ g mL}^{-1}$), total HDL ($1.068 < d < 1.21 \text{ g mL}^{-1}$), HDL₂ ($1.063 < d < 1.125 \text{ g mL}^{-1}$), HDL₃ ($1.125 < d < 1.21 \text{ g mL}^{-1}$) and very high-density lipoprotein (VHDL) ($1.21 < d < 1.25 \text{ g mL}^{-1}$), as described earlier [12]. From 250 mL of plasma, around 10 mL of each lipoprotein fraction were isolated, having

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absorbances at 280 nm of \approx 7 (LDL), \approx 9 (total HDL) and \approx 4 (VHDL). Serum was generated from citrated plasma by addition of 20 mM CaCl₂ followed by incubation at 37 °C for 1 h and overnight at 4 °C. The generated serum was separated from the clot by centrifugation at 5000 ×g for 10 min. Lipoprotein-deficient serum (LPDS, d > 1.25 g mL⁻¹) and lipoprotein-enriched fraction (LPEF, d < 1.25 g mL⁻¹) were obtained by ultracentrifugation using a density of 1.25 g mL⁻¹.

Purification of plasma PLTP, plasma LA- and HA-PLTP

Purification of total plasma PLTP as well as the high activity and low activity forms of PLTP from human plasma was performed as described [23,29]. The final PLTP activity used in the experiments was 1000 nmol mL⁻¹ h⁻¹. Similar amounts of LA-and HA-PLTP protein were used in the assays.

Preparation of liposomes

Natural PL, phosphatidylserine (PS, brain extract), phosphatidylethanolamine (PE, egg extract) and phosphatidylcholine (PC, egg extract) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Liposomes, 10/40/50 PS:PE:PC, were prepared as previously described[12]. Briefly, lipids were mixed, dried under N₂ gas, and resuspended in HBS buffer (10 mM Hepes, 150 mM NaCl, pH 7.4) with *n*-octyl- β -D-glucopyranoside, at room temperature. Solubilized lipids were dialyzed against HBS buffer at room temperature to generate liposomes. A trace amount of ¹⁴C-radiolabelled PC (GE Healthcare, Chalfont St Giles, UK) was added to the lipid mixture when necessary.

Separation of lipoproteins by size exclusion chromatography

Lipoprotein-containing samples, with labelled liposomes, were separated on Superose 6 10/300 GL (GE Healthcare) using HBS buffer with 0.1 mg mL⁻¹ BSA (Sigma-Aldrich, St. Louis, MO, USA) as running buffer. The eluted fractions were analyzed for radioactivity by scintillation counting (liquid scintillation counter; Wallac 1410, Perkin Elmer, Waltham, MA, USA).

Immunoblotting analysis of PLTP

PLTP-containing samples were separated on 8% SDS/PAGE under reducing (10 mM dithiothreitol) conditions followed by transfer to Biotrace PVDF membrane (Pall Corporation, Port Washington, NY, USA). PLTP protein (≈80 kDa) was visualized using PLTP polyclonal rabbit antibody (R180, IgG fraction of an in-house polyclonal antibody raised against PLTP using standard procedures) followed by HRP-conjugated goat antirabbit polyclonal antibody (Dako, Glostrup, Denmark). Membranes were developed by enhanced chemiluminescence using Immobilon Western chemiluminescent HRP substrate (Millipore Corporation, Billerica, MA, USA) and a CCD camera (LAS-3000; Fujifilm, Tokyo, Japan). Intensities of bands were quantified using ImageGauge 4.1 (Fujifilm).

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Measurement of procoagulant PL neutralizing activity with prothrombinase assay

Isolated lipoproteins (see figure legends for final concentrations) or HBS buffer with fatty acid-free BSA (3.75 mg mL⁻¹ final concentration) as control, were incubated with procoagulant liposomes (10:40:50 PS/PE/PC, 56 µM) in the presence or absence of PLTP (final PLTP activity, 1000 nmol mL⁻¹ h⁻¹) at 37 °C for 24 h. Samples were then analyzed for their ability to stimulate thrombin formation, as previously described, and thereby diluted 10 times during prothrombin activation[12]. Briefly, in the presence of the liposome-lipoprotein mixtures (as defined in each experiment), the activation of prothrombin by FXa and its cofactor FVa during 2 min incubation at 37 °C was determined. After activation of prothrombin, samples were diluted 183 times and the amount of thrombin formed was measured using a synthetic substrate, S-2238. The absorbance at 405 nm was followed continuously for 15 min using a microplate reader (Tecan Infinite F200; Männedorf, Switzerland) with Magellan6 software. The final concentrations of protein components during activation of prothrombin were: FVa 210 pM, FXa 2.5 nM, and prothrombin 0.5 µM.

Immunoprecipitation of PLTP with anti-PLTP antibodies

Isolated VHDL (500 μ L, 4 mg mL⁻¹) was precleared using 20 μ L protein A-Sepharose 4B conjugated beads (Invitrogen; Paisley, UK) at 4 °C for 1 h. The beads were pelleted by centrifugation, and the supernatant (90 μ L) was incubated with 10 μ L of PLTP polyclonal rabbit antibody (R180 IgG fraction, 100 μ g) or as a control phosphate-buffered saline (PBS, HyClone, Thermo Scientific; Waltham, MA, USA) at 4 °C for 1 h. Protein A-Sepharose beads were added (20 μ L) to each immunoprecipitation (IP) sample and further incubated at 4 °C for 1 h. The beads were pelleted by centrifugation, and the PLTP-depleted supernatant was analyzed for PLTP (SDS/PAGE immunoblotting), and for its ability to neutralize procoagulant PL.

Statistics

All statistical analyses were performed with GRAPHPAD PRISM 4.0 (GraphPad Software, San Diego, CA, USA) by using an unpaired *t*-test. Results are expressed as means, $n \ge 3$. *P*-values (two-tailed) below 0.05 are considered statistically significant; ***P < 0.001, **P < 0.01, *P < 0.05.

Results

Neutralization of procoagulant activity of liposomes by lipoproteins

We have recently shown that serum can neutralize the procoagulant effect of anionic liposomes by mediating transfer of PLs from liposomes to either apo A-I- or apolipoprotein B-containing particles [12]. To elucidate the underlying mech-



Fig. 1. Procoagulant PL neutralizing effect of isolated lipoproteins. Ultracentrifuged fractions from either serum (A) including LPDS (5.6 mg mL⁻¹) and LPEF (3.7 mg mL⁻¹) or plasma (B) including LDL (2.6 mg mL⁻¹), HDL (3.4 mg mL⁻¹) and VHDL (1.5 mg mL⁻¹), were incubated with liposomes (10/40/50 PS:PE:PC, 56 µM) at 37 °C for 24 h. As control, fatty acid-free BSA (3.75 mg mL⁻¹) was incubated with liposomes. Final concentrations are given in brackets. Samples were then tested for their ability to stimulate thrombin formation, and thereby diluted 10 times during prothrombin activation. Final concentrations during activation of prothrombin were: 210 pM FVa, 2.5 nM FXa, 0.5 µM prothrombin using a 2 min activation time. Values are expressed as means, n = 4. Significance was determined by unpaired *t*-test (***P < 0.001, **P < 0.01, *P < 0.05.

anism, serum was ultracentrifuged in order to separate LPDS $(d > 1.25 \text{ g mL}^{-1})$ from LPEF $(d < 1.25 \text{ g mL}^{-1})$. These two fractions were analyzed for their ability to neutralize the procoagulant activity of anionic liposomes (10/40/50, PS:PE:PC). Thus, the fractions were mixed with the procoagulant liposomes, incubated at 37 °C for 24 h, and then tested in the prothrombinase reaction to elucidate the remaining procoagulant activity. LPDS did not affect the procoagulant activity of the liposomes, while LPEF demonstrated a strong neutralizing effect, as demonstrated by a low rate of thrombin generation (Fig. 1A). The amount of thrombin formed after 2 min incubation of the prothrombinase reaction is shown, but the rate of prothrombin activation was equally low during at least 15 min incubation (not shown). This demonstrated that the lipoproteins had stably neutralized the procoagulant properties of the liposomes and that the low rate of prothrombin activation was not due to temporary inhibition.



Fig. 2. Western blot analysis of PLTP content in ultracentrifuged fractions. Isolated lipoprotein fractions (1 µg) were subjected to 8% SDS/ PAGE (reduced), followed by transfer to PVDF membrane. PLTP protein (≈80 kDa) was visualized using anti-PLTP rabbit polyclonal antibody.

To further define the neutralizing activity, the individual lipoproteins LDL, HDL and VHDL were analyzed for neutralizing activity. HDL and VHDL both demonstrated strong neutralizing effect, whereas neither LDL nor the BSA-buffer control affected the procoagulant properties of the anionic liposomes (Fig. 1B). These results indicate that the procoagulant neutralization activity is associated with the lipoproteins, and more precisely located in apoA-I-containing lipoprotein particles.

Immunoblotting of PLTP in lipoprotein fractions

To investigate the importance of PLTP for the procoagulant neutralizing activity, the PLTP content in the isolated lipoproteins was analyzed by Western blotting. PLTP was detected in LPEF, HDL and VHDL but not in LDL and in LPDS (Fig. 2A). The PLTP content parallels the neutralizing activity of these fractions (Fig. 1). Total HDL can be divided into two major subpopulations, HDL₂ (1.063 < d < 1.125 g mL⁻¹) and HDL₃ (1.125 < d < 1.21 g mL⁻¹), which were found to display different PLTP content. HDL₃, which is the denser form of HDL, contained PLTP, while HDL₂ did not (Fig. 2B).

Importance of PLTP for neutralization of procoagulant activity of anionic liposomes by lipoprotein fractions

Because neither PLTP protein nor procoagulant neutralizing activity was present in LDL and LPDS, the effect of added exogenous PLTP was tested. The lipoprotein fractions were incubated with radiolabelled anionic liposomes (56 μ M) at 37 °C for 24 h in the presence or absence of purified plasma PLTP (1000 nmol mL⁻¹ h⁻¹). Addition of PLTP to LDL increased the transfer of labelled PLs from the liposomes to the LDL particles, and in parallel the neutralizing activity increased (Fig. 3). In contrast, addition of PLTP to LPDS or BSA had no effect on procoagulant liposomes (Fig. 3A). Moreover, incubation of control liposomes with BSA did neither affect their procoagulant activity nor their elution profile on the gel filtration (Fig. 3). These results indicate that both PLTP and a PL-acceptor (lipoproteins) have to be present for adequate transfer of PLs and neutralizing activity.



Fig. 3. Effects of added PLTP on the ability of isolated lipoproteins to neutralize procoagulant liposomes. (A) Isolated lipoprotein fractions (same final concentrations as in Fig. 1) or fatty acid-free BSA (3.75 mg mL⁻¹) as control were incubated with liposomes (10/40/50 PS:PE:PC, 56 µM) at 37 °C for 24 h in the presence (○) or absence (●) of PLTP (1000 nmol mL⁻¹ h⁻¹). Final concentrations are given in brackets. Samples were then tested for their ability to stimulate thrombin formation, and thereby diluted 10 times during prothrombin activation. Final concentrations during activation of prothrombin were: 210 pM FVa, 2.5 nM FXa, 0.5 μ M prothrombin using a 2 min activation time. Values are expressed as means, n = 4. Significance was determined by unpaired *t*-test (***P < 0.001, **P < 0.01, *P < 0.05). (B) LDL was incubated with labelled liposomes as in (A), in the presence (
) or absence (
) of PLTP (1000 nmol mL⁻¹ h⁻¹). Samples were then separated on superose 6 10/300 GL, and eluted fractions were analyzed by scintillation counting. Values are expressed as means, n = 3. Liposomes (10/40/50 PS:PE:PC, 56 μ M) were incubated with fatty acid-free BSA (3.75 mg mL⁻¹) for 24 h as control (V) and analyzed by gelfiltration chromatography.

As judged by Western blot analysis, HDL_2 did not contain detectable endogenous PLTP, as compared with HDL_3 (Fig. 2B). Total HDL, HDL_2 and HDL_3 were compared using the same protein concentration (1.7 mg mL⁻¹) for their ability to neutralize procoagulant liposomes, and as expected, HDL_2 (having no or low endogenous PLTP) was not able to neutralize procoagulant PLs (Fig. 4A). In contrast, HDL_3 and total HDL demonstrated strong neutralizing activity (Fig. 4A). Addition of exogenous PLTP to HDL₂ increased the transfer of PLs from the liposomes to the HDL₂ particles and the neutralization of procoagulant PLs (Fig. 4). These results are in line with the results obtained with LDL and



Fig. 4. Effects of added PLTP on the ability of isolated HDL fractions to neutralize procoagulant liposomes. (A) Lipoprotein fractions (1.7 mg mL⁻¹) or fatty acid-free BSA (3.75 mg mL⁻¹) as control were incubated with liposomes (10/40/50 PS:PE:PC, 56 μ M) at 37 °C for 24 h in the presence (\bigcirc) or absence (\bigcirc) of PLTP (1000 nmol mL⁻¹ h⁻¹). Final concentrations are given in brackets. Samples were then tested for their ability to stimulate thrombin formation, and thereby diluted 10 times during prothrombin activation. Final concentrations during activation of prothrombin were: 210 pM FVa, 2.5 nM FXa, 0.5 µM prothrombin using a 2 min activation time. Values are expressed as means, n = 4. Significance was determined by unpaired *t*-test (***P < 0.001, **P < 0.01, *P < 0.05). (B) HDL₂ was incubated with labelled liposomes as in (A), in the presence (\Box) or absence (\blacksquare) of PLTP (1000 nmol mL⁻¹ h⁻¹). Samples were then separated on superose 6 10/300 GL, and eluted fractions were analyzed by scintillation counting. Values are expressed as means, n = 3. Liposomes (10/40/50 PS:PE:PC, 56 µM) were incubated with fatty acidfree BSA (3.75 mg mL⁻¹) for 24 h as control (♥) and analyzed by the gelfiltration chromatography.

indicate that no neutralization of procoagulant PLs is achieved when no PLTP is present even if a PL-acceptor is present.

Immunoprecipitation of PLTP from VHDL decreases procoagulant-neutralizing activity

Immunoprecipitation of PLTP from VHDL with specific polyclonal anti-PLTP antibody decreased the PLTP content by approximately 70% (range 55–75%, n = 3) (Fig. 5A). It was noteworthy that precipitation of PLTP did not result in any co-immunoprecipitation of apoA-I (data not shown). After precipitation, the VHDL-containing supernatant was analyzed for remaining procoagulant-liposome neutralizing activity. The



Fig. 5. Immunoprecipitation of PLTP from VHDL. PLTP from VHDL (90 μ L, 4 mg mL⁻¹) was immunoprecipitated with either specific anti-PLTP antibody (10 μ L, 100 μ g) or PBS as control (10 μ L). Supernatant (20 μ L) was analyzed for PLTP protein on Western blot (A). Supernatant (final dilution of 2.7 times) was also incubated with liposomes (10/40/50 PS:PE:PC, 56 μ M) in the absence or presence of PLTP (including HAand LA-PLTP) at 37 °C for 24 h. Samples were then tested for their ability to stimulate thrombin formation (B and C), and thereby diluted 10 times during prothrombin activation. Values are expressed as relative thrombin formation compared with thrombin formed by control liposomes. Final concentrations during activation of prothrombin were: 210 pM FVa, 2.5 nM FXa, 0.5 μ M prothrombin using a 2 min activation time. Values are expressed as means, n = 5 in (B) and n = 3 in (C) with a representative Western blot, n = 3 in (A). Significance was determined by unpaired *t*-test (***P < 0.001, **P < 0.01, *P < 0.05).

neutralizing activity was decreased in samples treated with PLTP antibody compared with samples treated with PBS, as judged by the increase in thrombin formation for samples treated with PLTP antibody (Fig. 5B). When PLTP was added to the VHDL supernatant after the IP, the neutralizing activity was regained (Fig. 5C). The two different activity forms of PLTP, HA-PLTP and LA-PLTP, were added to the VHDL supernatant after the IP and the procoagulant liposome-neutralizing activity tested. HA-PLTP had about the same neutralizing activity as the purified plasma PLTP, while LA-PLTP had no neutralizing activity (Fig. 5C). These data demonstrate that the high activity form of PLTP is needed for the neutralizing effect.

Discussion

We recently reported that serum neutralizes the procoagulant activity of anionic liposomes and efficiently mediates transfer of PLs from liposomes to apoA-I- and apoB-containing particles [12]. The role of PLTP in HDL metabolism has been extensively studied, and functions such as PL-transfer activity and HDL conversion are carefully characterized [19]. This prompted us to investigate in more detail the importance of PLTP for the neutralization of anionic liposomes. We now demonstrate that PLTP has the ability to transfer anionic PLs to HDL and LDL particles, and thereby to neutralize the procoagulant properties of these PLs.

The neutralizing activity of procoagulant liposomes in serum/plasma was found in the lipoprotein-enriched fraction and more precisely in total HDL, HDL₃ and VHDL from plasma. VHDL, which is the densest form of HDL, also contains apolipoprotein A-I as its major apolipoprotein. PLTP protein was found predominately in total HDL, HDL₃ and VHDL, whereas the PLTP content of HDL₂ was low or absent. This indicates that both the neutralizing activity and the PLTP protein are found in the denser forms of HDL and not in the mature form of HDL, HDL₂, suggesting that PLTP is important for the neutralizing activity. The neutralization of the procoagulant liposomes was a time-dependent process and required incubation at 37 °C for at least 20 h to reach completion, which is consistent with a PLTP-mediated enzymatic reaction. Addition of PLTP to LDL and HDL₂ increased the transfer of PLs from the liposomes to the lipoprotein particles. The small amount of transfer of PLs that was observed in the absence of added PLTP, as judged by size exclusion chromatography (Fig. 3B and 4B), indicated that either a spontaneous transfer of PLs occurred or that LDL and HDL₂ contained a small amount of active PLTP, which, however, was not detected by Western blot analysis.

We previously showed that the mechanism by which the uptake of the anionic PLs by HDL results in the neutralization of its procoagulant properties is that the anionic PL is arranged in a surface area, which is too small to accommodate a fully active prothrombinase complex [12]. We now observed that incorporation of the anionic PL into LDL also results in the neutralization of the procoagulant properties of the phospholipid. LDL is somewhat larger than HDL (20 nm vs. 8-10 nm) [30], and the mechanism by which incorporation of the anionic PLs into LDL results in neutralization of the procoagulant properties of the phospholipid is not clarified. Although the surface area of LDL is larger than that of HDL, it is likely that the prothrombinase complex cannot assemble on the surface of the LDL particles, although direct binding studies have not been performed. The anticoagulant protein TFPI (tissue factor pathway inhibitor) is known to be associated with LDL [31] and could hypothetically inhibit the binding of the prothrombinase complex to the LDL particle. However, we found this not to be the case because addition of polyclonal antibodies against TFPI did neither increase the prothrombin activation in

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the presence of LDL nor affect the ability of LDL to neutralize the procoagulant liposomes (data not shown).

Two forms of PLTP have recently been characterized, HAand LA-PLTP, with different phospholipid transfer activities, the underlying molecular difference between HA- and LA-PLTP being unknown [27,28]. The procoagulant neutralizing activity of the lipoproteins was fully dependent on the phospholipid transfer ability and accordingly, LA-PLTP had no neutralizing activity. The fact that PLTP is associated with HDL, and possibly with apoA-I/apoE [17,32], may explain why the IP of PLTP from VHDL was not completely efficient. The PLTP still present after the IP possibly is associated with lipoproteins in such a way that the epitopes for the antibodies are hidden.

Possibly, there may be additional proteins, other than PLTP, involved in mediating the transfer of procoagulant PLs to lipoproteins. Phospholipid transfer/exchange activities have been demonstrated for LBP, soluble CD14 [33], and CETP [34]. Both PLTP and CETP are able to mediate the transfer of PLs from PC-containing HDL particles to LDL, but unlike PLTP, CETP is unable to transfer PLs from PC-containing liposomes to HDL [34]. Whether these proteins have any impact in our system remains to be elucidated. Furthermore, there are also intracellular phospholipid transfer proteins present in mammals, such as phosphatidylcholine transfer protein, phosphatidylinositol transfer protein and the non-specific lipid transfer protein, but they were not investigated by the present experimental setup [35].

The levels of HDL cholesterol and apoA-I in patients with venous thromboembolic disease (VTE) or recurrent VTE have been studied [36,37]. In both studies, high levels of apoA-I and HDL cholesterol were found to be associated with decreased risk of VTE. Deguchi *et al.* also addressed the genetic influence contributing to the observed dyslipoproteinemia in VTE patients. A CETP allele (CETP *Taq*I B), which conveys elevated CETP activity, was found to be less common in VTE patients than controls and associated with VTE [36]. It would be highly interesting to study the importance of plasma phospholipid transfer proteins, like PLTP, in VTE and other thrombotic events.

In conclusion, we have shown that procoagulant anionic liposomes, when incubated with human serum, are transferred to HDL and LDL particles and as a result their procoagulant activity is neutralized [12]. The present study demonstrates that the anionic PL-transfer and procoagulant neutralizing activity are mainly mediated by PLTP. We also show that HA-PLTP, the high activity form of PLTP, but not the low activity form LA-PLTP, is responsible for this neutralizing activity. This novel function of PLTP may be an important mechanism to control the exposure of anionic PLs to circulating blood, thereby preventing inappropriate stimulation of coagulation.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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PAPER III

Oslakovic C, Norstrøm E, Dahlbäck B. Re-evaluation of the role of HDL in the anticoagulant activated protein C system. *J Clin Invest*. 2010; 120(5): 1396-1399.



Reevaluation of the role of HDL in the anticoagulant activated protein C system in humans

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HDL has anti-atherogenic properties, and plasma levels of HDL cholesterol correlate inversely with risk of coronary artery disease. HDL reportedly functions as a cofactor to the anticoagulant activated protein C (APC) in the degradation of factor Va (FVa). The aim of the present study was to elucidate the mechanism by which HDL functions as cofactor to APC. Consistent with a previous report, HDL isolated from human plasma by ultracentrifugation was found to stimulate APC-mediated degradation of FVa. However, further purification of HDL by gel filtration revealed that the stimulating activity was not a property of HDL. Instead, the stimulating activity eluted completely separately from HDL in the high-molecular-weight void volume fractions. The active portion of these fractions stimulated FVa degradation by APC and supported the assembly of factor Xa and FVa into a functional prothrombinase complex. Both the procoagulant and anticoagulant activities were blocked by addition of annexin V, suggesting that the active portion was negatively charged phospholipid membranes. These results demonstrate that HDL does not stimulate the APC/protein S effect and that the activity previously reported to be a property of HDL is instead caused by contaminating negatively charged phospholipid membranes.

Introduction

HDL is an apolipoprotein A-I–containing lipoprotein with atheroprotective functions, and the levels of HDL cholesterol inversely correlate with the risk of coronary artery disease (1, 2). HDL is the key component in reverse cholesterol transport, which removes excess cholesterol from peripheral tissues for secretion through the liver (3, 4). HDL has also been reported to have antioxidant, antiinflammatory, anticoagulant, and antithrombotic properties (5).

Blood coagulation involves a series of enzymatic activations, which are tightly regulated by both procoagulant and anticoagulant mechanisms (6, 7). The activation of prothrombin, which is the final reaction of the coagulation cascade, takes place on the surface of negatively charged phospholipid membranes, where the enzyme factor Xa (FXa) and its cofactor factor Va (FVa) assemble to form the prothrombinase complex (8). The protein C anticoagulant pathway regulates this complex. Thus, activated protein C (APC) cleaves and inhibits FVa (7, 9, 10). In a similar reaction, APC inhibits factor VIIIa (FVIIIa), which is a cofactor to factor IXa (FIXa) in the activation of factor X (FX) (10). The anticoagulant activity of APC is stimulated by its cofactor protein S (11). APC and protein S are vitamin K-dependent proteins and bind to negatively charged phospholipids (12). This is important, as the protein C system regulates coagulation directly on the phospholipid surface upon which coagulation takes place.

Plasma lipoproteins have been reported to stimulate the reactions of coagulation (13–19). Moyer et al. suggested that lipoproteins support prothrombinase and other procoagulant reactions (17). However, we recently demonstrated that the supporting activity present in lipoprotein preparations is not a property of the lipoproteins, but rather is caused by a high-molecular-weight contaminant, presumably negatively charged

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In 1999, Griffin et al. reported that HDL enhances the activities of protein S and APC (14). Specifically, HDL was shown to enhance the inactivation of FVa by APC and protein S in an experimental setup that did not include addition of negatively charged phospholipids. As HDL is unable to support the binding of FVa to its surface, we were interested in elucidating the possible mechanism by which HDL could support the inactivation of FVa by APC.

We now demonstrate that the enhancement of anticoagulant activities of APC and protein S observed associated with HDL is not a property of HDL, but rather is caused by contaminating negatively charged phospholipid membranes present in HDL prepared by ultracentrifugation. Thus, HDL enhances neither the prothrombinase reaction nor the APC-mediated degradation of FVa.

Results and Discussion

Anticoagulant activity of HDL prepared by ultracentrifugation. HDL isolated by ultracentrifugation was tested for its ability to enhance the inactivation of FVa by APC and protein S, following the protocol of Griffin et al. (14). After 30 minutes incubation of the HDL with FVa, APC, and protein S (no extra negatively charged phospholipid liposomes added), the remaining FVa activity was quantified by a prothrombinase assay. HDL was found to enhance the APC-mediated inactivation of FVa, the remaining FVa activity being 58% of the control without APC (Figure 1A). In the absence of added HDL, but with APC and protein S, the FVa activity decreased to 92%, while the FVa activity ity decreased to 35% in the positive control containing APC,

brief report



protein S, and negatively charged liposomes (25 μ M, 10:20:70 PS/PE/PC). We obtained similar results with 3 other independent HDL preparations (data not shown). These results are in agreement with those reported by Griffin et al. (14).

To analyze whether the HDL preparation could be used as potential source of procoagulant phospholipids, we analyzed its ability to stimulate thrombin formation in a prothrombinase assay containing no other liposomes. HDL was found to stimulate the formation of thrombin, the amount of thrombin formed being one-third of that generated in the control with $2.5 \,\mu$ M liposomes (Figure 1B). Similar results were obtained with 3 other HDL preparations (data not shown).

Size exclusion chromatography separates anticoagulant activity from HDL. To elucidate whether the anti- and procoagulant effects were associated with HDL, HDL was further purified on a Superose 6 column. The fractions were tested for their ability to enhance FVa inactivation and prothrombin activation. The activity that enhanced FVa inactivation eluted at fractions 16-17, corresponding to the void volume of the chromatography, whereas HDL eluted later, peaking at fraction 42 (Figure 2). A trace amount of apolipoprotein B-containing lipoproteins that were present in the HDL preparation eluted clearly separated from the void, peaking at fraction 29, as judged by Western blotting against apolipoprotein B (data not shown). Fractions 16-17 were also found to stimulate prothrombin activation, whereas the HDL-containing fractions stimulated neither prothrombin activation nor FVa inactivation (Figure 2). Thus, the stimulatory effects that were observed in HDL preparations isolated by ultracentrifugation were not associated with HDL itself, but rather with a high-molecular-weight component eluting in the void of the column. This suggests that the observed procoagulant/anticoagulant activities in HDL samples from ultracentrifuged plasma were not due to HDL, but rather caused by a contaminant that had the characteristics of anionic phospholipids (see below). Re-chromatography of the HDL peak did not generate new activ-

Figure 1

Anti- and procoagulant activities of HDL prepared by ultracentrifugation. (A) HDL (final concentrations of 2 mg/ml and 680 µM choline-phospholipids) was tested in a FVa inactivation assay including 20 pM FVa, 0.5 nM APC, and 14.5 nM protein S. After 30 minutes incubation, the FVa activity was measured in a prothrombinase assay. Liposomes (10:20:70 PS/PE/PC) at 25 μM and HBS buffer were used as positive and negative controls, respectively. Values are expressed as percent of controls without APC. (B) HDL (final concentrations of 1 mg/ml and 340 µM cholinephospholipids) were added to a prothrombinase assay containing 2.5 nM FXa, 210 pM FVa, and 0.5 µM prothrombin. After 2 minutes incubation at 37°C, the reaction was stopped, and the amount of thrombin formed was determined as described in Methods. Liposomes (10:40:50 PS/PE/PC) at a concentration of 2.5 µM were used as positive control. Values are expressed as mean \pm SD from repeated experiments (n = 3). Significance was determined by unpaired t test (*P < 0.05). mA405/min, milliabsorbance at 405 nm/min. PL, phospholipids.

ity in the leading fractions. Moreover, mixing of the void and HDL fractions yielded activity (data not shown). These results indicate that there was no transfer of phospholipids in and out from the HDL particles (data not shown).

The procoagulant/anticoagulant activities of HDL batches caused by contaminating anionic phospholipid membranes. To clarify whether the procoagulant/anticoagulant activities that eluted in the void volume of the column were caused by anionic phospholipids, 100 nM annexin V was added to the samples before testing. After incubation of fractions 16-17 with annexin V (100 nM), we determined the remaining pro- and anticoagulant activities (Figure 3). The presence of annexin V completely blocked both activities, indicating that the stimulation was mediated by the presence of anionic phospholipids. Similar results were obtained when the ultracentrifuged HDL (prior to size exclusion chromatography) was incubated with annexin V, indicating that anionic phospholipids contaminated the HDL batches (data not shown). To further demonstrate that anionic phospholipids constituted the active principle, the void was incubated with 20 nM phospholipase A2 for 15 minutes at 37°C and retested in the prothrombinase assay. The activity was completely blocked by the phospholipase A2, treatment (data not shown). The void fractions contained cholesterol but no cholesteryl esters (data not shown). Taken together, these results convincingly demonstrate that the void contained anionic phospholipids but no lipoproteins.

HDL from human plasma has been shown to contain about 1% of the anionic phospholipid phosphatidylserine (20). Even if reconstituted HDL (rHDL) is made to contain anionic phospholipids, the surface area of HDL is too small for binding of proteins involved in prothrombinase complex and FVa inactivation (13). Similar conclusions have been derived from experiments using a specialized form of rHDL particles, the nanodiscs. Nanodiscs, which in many respects are similar to rHDL, are created using a truncated form of apoA-I (Δ 1-43 apoA-I) called membrane scaffold protein (MSP) (21). The diameter of these nanodiscs is similar to that of discoidal HDL (about 8 nm), and they are unable to assemble a prothrombinase complex. However, by using a larger MSP, nanodiscs with diameter up to 12 nm can be generated (21, 22). At this size, the nanodiscs are shown to assemble a fully active prothrombinase complex, demonstrating that the surface area of the membrane is crucially important for the assembly of the prothrombinase

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Figure 2

Size exclusion chromatography of HDL. HDL was separated on Superose 6 10/300 GL. After loading of the 1.5-ml sample, fractions of 0.35 ml were collected, analyzed for protein (**A**), and tested for their ability to stimulate inactivation of FVa in the presence of APC and protein S (**B**) or prothrombin activation (**C**). Final concentrations during inactivation of FVa were 20 pM FVa, 0.5 nM APC, 14.5 nM protein S, using a 30-minute inactivation time. Fractions were diluted 5-fold during inactivation of FVa and values are expressed as percent of controls without APC. In the prothrombin activation, final concentrations were 210 pM FVa, 2.5 nM FXa, 0.5 μ M prothrombin, using a 2-minute activation time. The samples were then diluted and the generated thrombin tested as described in Methods. The tested fractions were diluted 10-fold during activation of prothrombin. Values in **B** and **C** are expressed as mean \pm SD from repeated experiments (*n* = 2).

complex (23). These results further strengthen our hypothesis that circulating HDL cannot support the assembly of either a prothrombinase complex or the FVa inactivation complex.

Even though we now show that HDL does not function as a cofactor to APC, HDL can participate in the regulation of procoagulant reactions. We recently demonstrated that circulating HDL has the capacity to neutralize procoagulant liposomes (13). The mechanism is that the anionic phospholipids are transferred from the liposomes into HDL, where it cannot stimulate the reactions of coagulation due to the small surface area.

In conclusion, we now report that HDL, contrary to what has been reported, does not function as a cofactor to APC and protein S in the inactivation of FVa. The previously identified stimulating activity of isolated HDL was not an intrinsic property of HDL, but rather was caused by contaminating anionic

phospholipid membranes, possibly microparticles or membrane fragments of disrupted cells. This highlights the importance of using a highly purified HDL preparation for characterization of its regulation of blood coagulation rather than HDL prepared by ultracentrifugation only.

Methods

Isolation of HDL HDL (1.068 < density < 1.21 g/ml) was isolated from human plasma, obtained from the local blood bank, using sequential flotation ultracentrifugation (13), dialyzed against HBS (10 mM HEPES, 150 mM NaCl, pH 7.4), and stored at -20°C. Phospholipids were quantified using Phospholipids B kit (Wako Chemicals). The protein concentration (absorbance at 280 nm) of the HDL batch used for the presented experiment was 10 mg/ml, and phospholipid content was 3,400 µM.

Liposomes. Natural phospholipids, phosphatidylserine (PS; brain extract), phosphatidylethanolamine (PE; egg extract), and phosphatidylcholine (PC; egg extract) were from Avanti Polar Lipids. Liposomes were prepared as previously described (13).

Separation of HDL by size exclusion chromatography. HDL was concentrated about 10 times using 3000 MWCO Amicon Ultra (Millipore) and gel-filtered on Superose 6 10/300 GL (GE Healthcare), and HBS with 0.1 mg/ml BSA (Sigma-Aldrich) was used as running buffer.

Prothrombinase assay. HDL (diluted 10 times during prothrombin activation) or liposomes 10:40:50 PS/PE/PC (2.5 μ M during prothrombin activation) were analyzed for their ability to stimulate thrombin formation (13). Briefly, prothrombin was activated by FXa and its cofactor FVa for 2 minutes at 37°C in the presence of liposomes or HDL. Aliquots were diluted 183 times in EDTA buffer (50 mM Tris-HCl, 100 mM NaCl, 100 mM EDTA, 1% polyethylene glycol 6000 [PEG6000], pH 7.9) and thrombin measured with S-2238. Final concentrations during activation of prothrombin were 210 pM FVa, 2.5 nM FXa, and 0.5 μ M prothrombin.



Figure 3

Annexin V inhibits both anti- and procoagulant activities. Fractions 16–17 from the void of the size exclusion chromatography of HDL were incubated in the absence or presence of 100 nM annexin V with 2.5 mM CaCl₂ at 25°C for 15 minutes. Remaining anti- and procoagulant activities were tested using a FVa inactivation assay (**A**) or prothrombinase assay (**B**). (**A**) Final concentrations during inactivation of FVa were 20 pM FVa, 0.5 nM APC, 14.5 nM protein S, using a 30-minute inactivation time. Fractions were diluted 5-fold during inactivation of FVa, and values are expressed as percent of controls without APC. (**B**) In the prothrombin activation time. The samples were then diluted and the generated thrombin tested as described in Methods. The tested fractions were diluted 10-fold during the activation of prothrombin. Values are expressed as mean ± SD from repeated experiments (*n* = 2).





FVa inactivation. FV (33.3 pM), purified from plasma (24) with minor modifications (25), was activated with 0.5 U/ml thrombin (Hematologic Technologies Inc.) for 5 minutes at 37°C, and the activation was terminated by addition of 1.5 U/ml hirudin (Pentapharm). According to the protocol described by Griffin et al. (14), APC (0.5 nM, prepared as described previously; ref. 26) and protein S (14.5 nM; Kordia) were incubated for 30 minutes at 37°C with FVa (20 pM) and either HDL (diluted 5 times during inactivation of FVa), HNBSACa buffer (25 mM HEPES, 150 mM NaCl, 5 mg/ml BSA, 5 mM CaCl₂, pH 7.5), or control liposomes (10:20:70 PS/PE/PC) (25 μM during inactivation of FVa). Aliquots were drawn and mixed with equal volumes of HNBSACa (on ice) and analyzed for FVa activity using the prothrombinase assay, while diluted 1.7-fold (13), to which FXa (5 nM), liposomes (10:0:90 PS/PE/PC) (50 μM), and prothrombin (0.5 μM) were added. After incubation at 37°C for 2 minutes, aliquots were drawn, diluted 100 times, and tested for thrombin with S-2238.

Annexin V and phospholipase A_2 inhibition experiments. Phospholipid-containing samples were incubated with 100 nM Annexin V (BD Biosciences – Pharmingen) in the presence of 2.5 mM CaCl₂ at 25°C for 15 minutes and tested in prothrombinase and FVa inactivation assays. The concentration of annexin V (100 nM) was far above the reported K_d (<0.2 nM) for annexin V binding to phospholipids (27). The quantitative FVa assay was unaffected by the annexin V due to the high concentration of phospholip-

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ids used (50 μ M) (data not shown). The void (fraction 18) was incubated in the presence and absence of 20 nM phospholipase A₂ (from bee venom; Sigma-Aldrich) at 37°C for 15 minutes in the presence of 2.5 mM CaCl₂ and the procoagulant activity tested by the prothrombinase assay.

Statistics. Statistical analysis (unpaired t test) was performed using GraphPad Prism 4.0 (GraphPad Software). Results are expressed as mean \pm SD when possible. *P* values (2-tailed) less than 0.05 were considered statistically significant.

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