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Oslakovic, Cecilia; Norstrøm, Eva; Dahlbäck, Björn

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LUND UNIVERSITY

PO Box 117
221 00 Lund
+46 46-222 00 00



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

Cecilia Oslakovic, Eva Norström, and Björn Dahlbäck

Department of Laboratory Medicine, Division of Clinical Chemistry, Lund University, University Hospital, Malmö, Sweden.

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 athero-protective 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, anti-inflammatory, 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

phospholipid membrane fragments, which can be separated from the lipoproteins by gel filtration chromatography (13). There are at least two reasons why plasma HDL cannot support the prothrombinase reaction, one being that the content of phosphatidylserine in the circulating HDL is very low (20), the other being that the surface area is too small to bind FVa and accommodate the prothrombinase complex (13).

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.

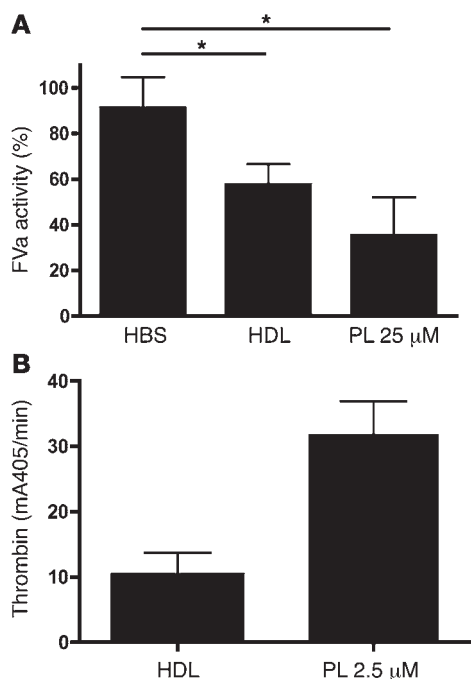
We now demonstrate that the enhancement of anticoagulant activities of APC and protein S observed associated with HDL is not a property of HDL, but rather is caused by contaminating negatively charged phospholipid membranes present in HDL prepared by ultracentrifugation. Thus, HDL enhances neither the 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 decreased to 35% in the positive control containing APC,

Conflict of interest: The authors have declared that no conflict of interest exists.

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**Figure 1**

Anti- and procoagulant activities of HDL prepared by ultracentrifugation. (A) HDL (final concentrations of 2 mg/ml and 680 μ M 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 μ M 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 μ M choline-phospholipids) were added to a prothrombinase assay containing 2.5 nM FXa, 210 pM FVa, and 0.5 μ M 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 μ M 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.

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 μ 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-

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, 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

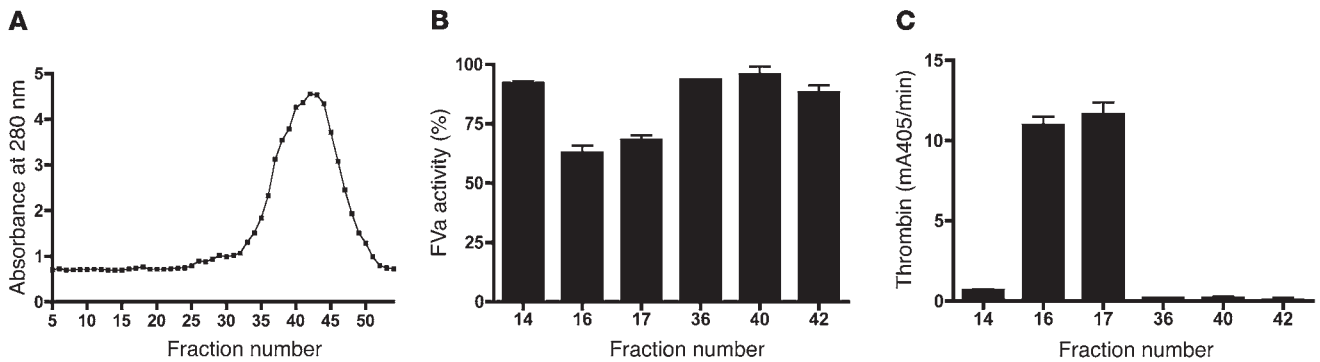


Figure 2

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 μM 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 ± 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), phosphatidyletha-

nolamine (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 μM 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 μM prothrombin.

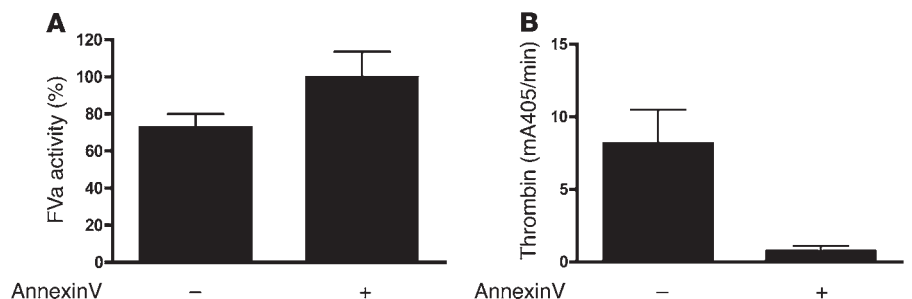


Figure 3

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, final concentrations were 210 pM FVa, 2.5 nM FXa, 0.5 μM 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 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 A₂ 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-

ids used (50 μM) (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 ± SD when possible. *P* values (2-tailed) less than 0.05 were considered statistically significant.

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Address correspondence to: Björn Dahlbäck, Wallenberg Laboratory, Entrance 46, Floor 6, University Hospital, S-20502 Malmö, Sweden. Phone: 46.40.331501; Fax: 46.40.337044; E-mail: bjorn.dahlback@med.lu.se.

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