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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.

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,

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proteins. The 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 (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 complex. 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 cholesterol esters (data not shown). Taken together, these results convincingly demonstrate that the void contained anionic phospholipids but no lipoproteins.

**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 FVas, 0.5 mM APC, and 14.5 mM protein S. After 30 minutes incubation, the FVas 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 choline-phospholipids) were added to a prothrombinase assay containing 2.5 mM FXs, 210 pM FVas, 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 ± SD from repeated experiments (n = 3). Significance was determined by unpaired t test (P < 0.05). mAU05/min, milliabsorbance at 405 nm/min. PL, phospholipids.

**Figure 1A** 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 FVas, 0.5 mM APC, and 14.5 mM protein S. After 30 minutes incubation, the FVas 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 choline-phospholipids) were added to a prothrombinase assay containing 2.5 mM FXs, 210 pM FVas, 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 ± SD from repeated experiments (n = 3). Significance was determined by unpaired t test (P < 0.05). mAU05/min, milliabsorbance at 405 nm/min. PL, phospholipids.
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 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 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).

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).
FVα inactivation. FV (33.3 pM), purified from plasma (24) with minor modifications (25), was activated with 0.5 U/ml thrombin (Hematologics 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 FVα (20 pM) and either HDL (diluted 5 times during inactivation of FVα), HNBSA Ca 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 FVα). Aliquots were drawn and mixed with equal volumes of HNBSA Ca (on ice) and analyzed for FVα 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 FVα inactivation assays. The concentration of Annexin V (100 nM) was far above the reported Kᵦ (0.2 nM) for Annexin V binding to phospholipids (27). The quantitative FVα assay was unaffected by the annexin V due to the high concentration of phospholipids 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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