SUPPLEMENTAL MATERIAL

Materials

Purified factors Va, Xa, and prothrombin were obtained from Haematologic Technologies Inc., Essex Junction, VT, Chromogenic substrate PefaChrome TH from Pentapharm Ltd., Basel, Switzerland, S2765 from DiaPharma, West Chester, OH, L-α-Phosphatidylcholine (PC) (chicken egg) and L-α-phosphatidylserine (PS) (Porcine brain) from Avanti Polar Lipids, Alabaster AL. L-3-PC-1,2-di [1-^{14}C]oleoyl from Amersham Biosciences. For some studies, L-α-PS (bovine brain) and L-α-PC (bovine brain)from Sigma-Aldrich Co. Phospholipid vesicles containing PC and PS in the molar ratio 9:1 for prothrombinase assays were prepared by sonication and centrifugation as described [1], normal human plasma for clotting assays was purchased from George King Biomedical Inc., Overland Park, KA. HDL was either isolated in the Analytical Laboratory of Dr. Joseph L. Witztum at the University of California, San Diego from fresh fasting human plasma using classical sequential flotation techniques, at density values of 1.063 to 1.21 g/mL, as described [2,3] or purchased from Intracel Resources, Frederick, MD. Anti-ApoA-I antibody came from Meridian Life Science Inc, Saco, ME and was covalently linked to Sepharose BrCN beads (GE Healthcare, Parsippany, NJ). HDL was subfractionated using density gradient centrifugation as described [2] with the following modifications. 40 aliquots were collected from the centrifuged tube containing the density gradient, and the density of each aliquot was measured by density meter (Mettler-Toledo, Inc., Hightstown, NJ) at room temperature, with variations from 1.091 to 1.296 g/mL. Fractions with the density between 1.091 and 1.125 g/mL were designated as HDL2 and fractions with the density between 1.125 and 1.21 g/mL were HDL3.

Superose 6 Chromatography and Determination of the Concentration of ApoA-I and Lipids in Superose 6 HDL fractions

Purified HDL was subjected to chromatography using a Superose 6 HR column (0.5 x 28 cm) (GE Healthcare, Parsippany, NJ) at a flow rate of 0.5 ml/min at room temperature.
Five hundred µl fractions were collected and used for protein, apoA-I antigen, and lipid assays and for clotting assays. Assay reagents were sourced as follows: Cholesterol (Wako Chemicals, Richmond, VA), choline phospholipid (Wako Chemicals), and apoA-I (DiaSorine, Stillwater, MN). Total protein was determined with the BCA kit from Pierce, Rockford, IL.

Assay for ability of HDL to enhance anticoagulant activity of APC:Protein S

To determine APC:Protein S enhancing anticoagulant activity of HDL, prothrombin time clotting assays were performed using an Amelung KC 4A micro apparatus (Sigma Diagnostics, St. Louis, MO). 7.5 µL normal plasma was mixed with 5 µL fibrinogen (267 nM), 25 µL APC (22 nM) or buffer (Tris buffered saline (TBS) containing 2% BSA), 25 µl of protein S (88 nM) or buffer, and 25 µl of Superose 6 column fractions or buffer, and this mixture was incubated 3 min at 37°C. Clotting times were then measured after addition of 25 µl containing 1:40 dilution of recombinant human tissue factor (Innovin; Baxter-Dade, Dade, FL), 30 mM $CaCl_2$, and TBS containing 2% BSA. Enhancement of APC:Protein S anticoagulant activity, defined as a "Normalized Ratio", was calculated as the ratio of the prothrombin times plus and minus APC:Protein S, normalized to 1.0 for control values measured in the absence of Superose 6 column fractions.

Prothrombinase activity assays

Prothrombin activation in the presence of Superose 6 column fractions was determined using purified factor Xa (0.7 nM final) plus factor Va (15.5 nM final). Factor Xa and factor Va, were mixed with Superose 6 subfractions at room temperature for 5 min before addition of prothrombin (0.76 µM final). Then the rate of thrombin (IIa) formation was quantified by

measuring thrombin based on the rate of substrate (PefaChrome-TH, 0.4 mM final) hydrolysis monitored as Absorbance change at 405 nm.

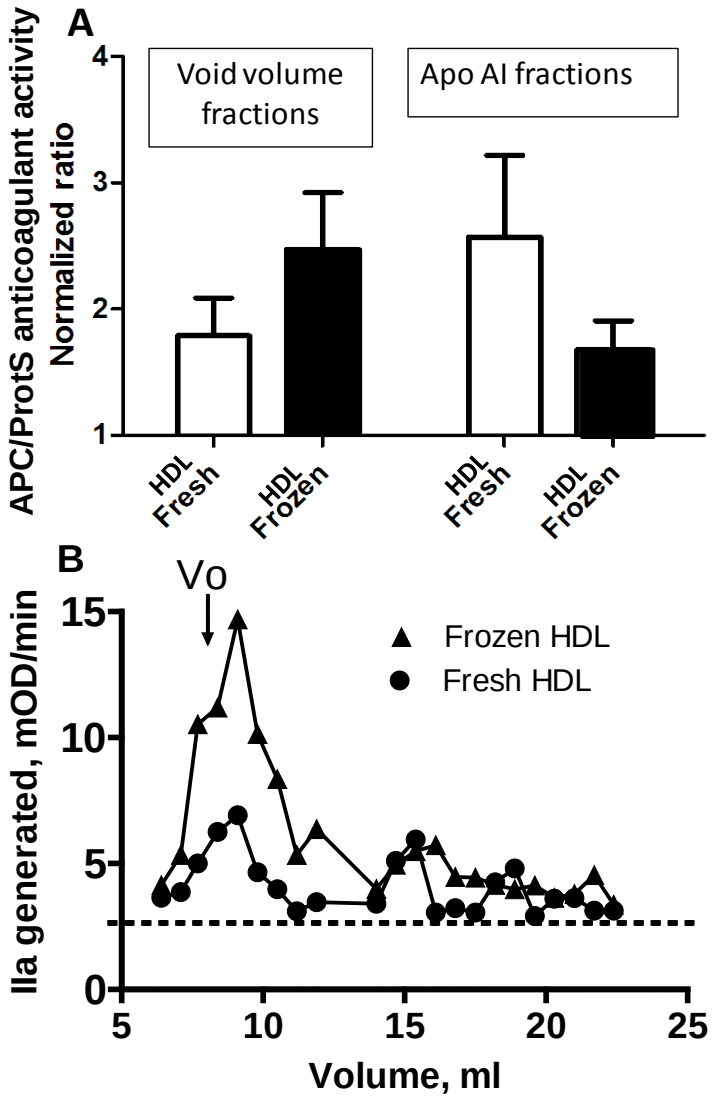
References for Materials

(1) Yegneswaran S, Nguyen PM, Gale AJ, Griffin JH. Prothrombin amino terminal region helps protect coagulation factor Va from proteolytic inactivation by activated protein C. *Thromb Haemost.* 2009;101:55-61.

(2) Deguchi H, Fernandez JA, Hackeng TM, Banka CL, Griffin JH. Cardiolipin is a normal component of human plasma lipoproteins. *Proc Natl Acad Sci U S A.* 2000;97:1743-1748.

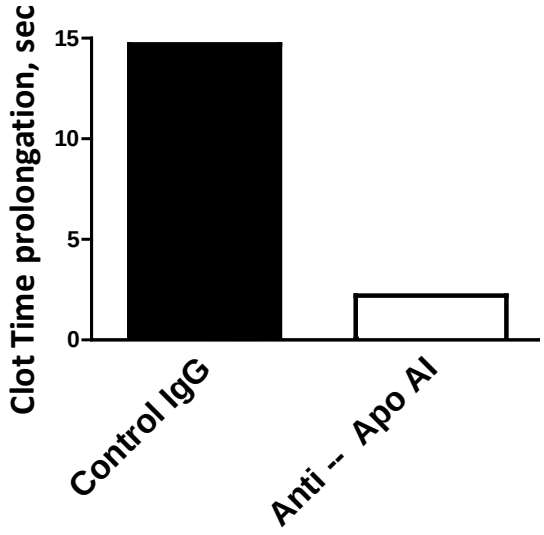
(3) HAVEL RJ, EDER HA, BRAGDON JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest.* 1955;34:1345-1353.

Supplemental Figures



Suppl. Figure I. Effects of freezing and thawing on the anticoagulant activity and procoagulant prothrombinase activity of HDL. **A.** Fresh and frozen-thawed HDL (-20º C) were chromatographed onto a Superose 6 column. Pooled fractions from the column void volume and from the fractions containing Apo AI from fresh and frozen-thawed HDL preparations were tested for APC/Protein S cofactor activity using clotting assays. The bars are the mean values and SD of three separated chromatography runs. **B.** Fractions from the Superpose 6 column

(from fresh and frozen-thawed HDL) were tested for procoagulant activity using a prothrombinase assay.



Suppl. Figure II. Effect of immunoadsorption of Apo AI on the anticoagulant activity of Superpose 6 fractions. Pools of apo AI-containing fractions from Superose 6 chromatography were immunoadsorbed with immobilized anti-ApoAI antibody or control IgG. Then, the ability of the immunoabsorbed fractions to prolong clot times in the presence of APC/Protein S was determined.