HDL and Apolipoprotein A-I Protect Erythrocytes Against the Generation of Procoagulant Activity

Richard M. Epand, Alan Stafford, Bryan Leon, Philippa E. Lock, Ewan M. Tytler, Jere P. Segrest, G.M. Anantharamaiah

Abstract The appearance of anionic lipids on the extracellular surface of cells is required for the formation of the procoagulant complex that leads to the activation of prothrombin. Procoagulant activity would be expected to be inhibited by substances that stabilize the membrane structure and hence inhibit the transbilayer diffusion of phosphatidylserine from the cytoplasmic to the extracellular surface of the plasma membrane. The generation of procoagulant activity in human erythrocytes by A23187 and Ca\(^{2+}\) is inhibited by apolipoprotein A-I, its amphipathic peptide analogues, and high-density lipoprotein (HDL). These agents do not inhibit the Ca\(^{2+}\) loading of erythrocytes by A23187, nor do they inhibit the activation of prothrombin once the cells have been incubated at 37°C with A23187 and Ca\(^{2+}\). Transbilayer diffusion of fluorescently labeled phosphatidylserine is inhibited by apolipoprotein A-I. These findings indicate that class A amphipathic helices as well as lipoprotein particles and liposomes inhibit the transbilayer diffusion of phospholipids and procoagulant activity. This activity may contribute to the protective role of HDL against arteriosclerosis and thrombosis. (Arterioscler Thromb. 1994;14:1775-1783.)

Key Words • procoagulant activity • transbilayer diffusion • apolipoprotein A-I • HDL • erythrocytes

Amphipathic helices have long been known to play an important role in the interaction of peptides and proteins with membranes. Recently, these helices have been classified on the basis of the size and charge distribution of their hydrophilic domain. In general, this classification also segregates peptides and proteins according to their function. Two of these classes are the apolipoprotein helices (class A) and the lytic helical peptides (class L). We have shown that these two classes of helices have opposite effects on several membrane properties, including their effects on the bilayer-to-hexagonal phase transition temperature, leakage of liposomes, and hemolysis. We also demonstrated that class A helices can inhibit leakage from liposomes or hemolysis of human erythrocytes caused by class L helices. This suggested that proteins containing class A helices, such as the plasma apolipoprotein A-I (apoA-I), can have a protective effect against membrane damage.

One of the important functions of blood is thrombogenesis. The final step in the coagulation cascade is the conversion of prothrombin to thrombin in the presence of factor Xa, factor Va, and Ca\(^{2+}\). This process occurs on the surface of membranes containing anionic phospholipids. Such lipids are normally found on the cytoplasmic surface of the plasma membrane, to which the blood coagulation proteins normally do not have access. However, as a result of cell lysis or the transbilayer diffusion of anionic lipids, cell membranes can initiate the activation of prothrombin. This process has been termed the generation of procoagulant activity. It has been demonstrated that gramicidin promotes transbilayer diffusion of phospholipids in erythrocyte membranes only under conditions in which it would be expected to promote H\(_{36}\) phase formation. As a consequence, agents that inhibit H\(_{36}\) formation may inhibit transbilayer diffusion. This could have physiological consequences by inhibiting the transbilayer diffusion required for procoagulant activity. The level of high-density lipoprotein (HDL) is known to be inversely correlated with the incidence of arteriosclerosis. HDL has long been regarded as important for "reverse" transport of cholesterol from peripheral tissue to the liver. We suggest that this lipoprotein may also function to stabilize membranes. We further suggest that the mechanism of this stabilization may, at least in part, be through an interaction of the class A amphipathic helical segments of apoA-I with cell membranes.

If class A helices stabilize membranes, then class L helices would be expected to cause membrane defects and promote procoagulant activity. However, we observed that several agents that promote H\(_{36}\) formation, including the peptides 18L and mastoparan as well as octanol, decanol, and dodecanol, caused considerable hemolysis at concentrations required to promote procoagulant activity. Thus, it is difficult to determine the extent of transbilayer diffusion promoted by these agents in intact erythrocytes. It is known that Ca\(^{2+}\) loading of erythrocytes leads to a loss of phospholipid transbilayer asymmetry. It has recently been found that
phosphatidylinositol 4,5-bisphosphate plays an important role in this process. The loss of asymmetry is sustained because the higher intracellular levels of Ca^{2+} inhibit translocase activity. In addition, Ca^{2+} influx leads to the formation of microvesicles, but this is not a mechanism for the loss of transmembrane asymmetry. Erythrocytes attain an enhanced permeability to Ca^{2+} when subjected to physiological shear stresses. If this Ca^{2+} influx caused the transblayer diffusion of phospholipids, it could be of pathological significance because it would promote procoagulant activity leading to thrombus formation. In this article, we demonstrate that protection from this nonregulated thrombogenesis is afforded by the presence of HDL.

**Methods**

**Materials**

HDL was isolated from freshly drawn human blood by density gradient centrifugation. ApoA-I was then extracted and purified by reverse-phase high-performance liquid chromatography (HPLC) as previously described. Synthetic peptides were made by solid-phase synthesis using N-ter-butoxycarbonyl chemistry. Peptides were cleaved from the resin using anhydrous HF and purified by reverse-phase HPLC. Bovine blood coagulation factor Va, factor Xa, and prothrombin were purchased from Haemalogic Technologies. N-p-Tosyl-Gly-Pro-Arg-p-nitroanilide was purchased from the Sigma Chemical Co. A23187 was from Calbiochem. Bovine serum albumin (BSA) was purchased as fatty acid-free fraction V from Boehringer Mannheim. 1-Palmitoyl-2-[[7-nitro-2,1,3-benzoxyazidol-4-yl]aminocaproyl]-sn-glycero-3-phosphoserine (NBD-PS) was purchased from Avanti Polar Lipids.

**Procoagulant Activity and Hemolysis**

Human blood was freshly drawn into tubes coated with acid-citrate-dextrose or heparin. The erythrocytes were kept at 0°C for 4°C up to the incubation step at 37°C. Blood (2 to 3 mL) was centrifuged, and the cell pellet was resuspended in 35 to 40 mL of 5 mmol/L HEPES, 150 mmol/L NaCl, 1 mmol/L MgSO_4_, 5 mmol/L KCl, pH 7.4 (HEPES buffered saline [HBS]) containing 0.1 mmol/L ZnCl_2_. The ZnCl_2_ was added to protect the erythrocytes against hemolysis. The cells were again pelleted at 4500 rpm in an SS34 rotor of a Sorvall centrifuge at 4°C. The pellet was resuspended in the HBS with Zn^{2+} and centrifuged twice more. A 125-μL aliquot of the washed, packed erythrocytes was added to 23 mL of HBS containing 0.1 mmol/L ZnCl_2_ and 1.0 mmol/L CaCl_2_. A 950-μL aliquot of this erythrocyte suspension was added to A23187 and/or various peptides, proteins, or lipoproteins in an Eppendorf centrifuge tube as indicated. The final volume was adjusted to 1.00 mL, and the solution was vortexed. The order of addition of the agents did not affect the final extent of hemolysis or the generation of procoagulant activity. The mixture with the erythrocytes was then warmed to 37°C and incubated for 20 minutes with shaking. After this period, the tube was gently vortexed, and a 20-μL aliquot of the suspension was diluted with 455 μL HBS. A 20-μL aliquot of this mixture was used to measure procoagulant activity (see below). The remaining 980 μL of erythrocytes was then spun in a microfuge for 90 seconds, and the absorbance of the supernatant at 540 nm was measured in a Perkin-Elmer spectrophotometer. The absorbance was compared with that from an equivalent-size aliquot of erythrocytes that had been sonicated to completely liberate all of the hemoglobin. The sonicated sample had an absorbance at 540 nm in the range of 1.1 to 1.3. The percentage of hemolysis was always 3±2% for the results presented in this article.

The 20-μL sample taken for the assay of procoagulant activity was diluted with 60 μL HBS. To the 80 μL of this erythrocyte suspension were added 10 μL factor Xa solution, 10 μL factor Va solution, and 10 μL CaCl_2_. The tube was then warmed to 37°C and incubated for 2 minutes at 37°C. To the 10 μL prothrombin was added to commence the reaction. The final concentration of factor Xa was 0.2 U/mL, which was stored in the freezer with 1 mg/mL BSA in 40 mmol/L HEPES, 124 mmol/L NaCl, pH 7.4, diluted 1:1 with glycerol. The final concentration of factor Va was 6 mmol/L from a stock stored as factor Xa but with the addition of 2 mmol/L CaCl_2_. The final Ca^{2+} concentration was 4 mmol/L. The prothrombin concentration was 2 μmol/L from a stock stored as above, without calcium. After addition of prothrombin, the mixture was incubated for 5 minutes at 37°C. Any thrombin generated as a consequence of the appearance of anionic lipids on the extracellular surface was then assayed spectrophotometrically using the synthetic substrate N-p-tosyl-Gly-Pro-Arg-p-nitroanilide. Two 40-μL aliquots of the incubation mixture were removed. One aliquot was added to a reference cuvette containing the phosphate buffer composed of 100 mmol/L sodium phosphate, 1 mmol/L EDTA, and 1 mg/mL polyethylene glycol 6000. The polyethylene glycol is added to avoid adsorption of thrombin to surfaces. The other 40-μL aliquot was added to a sample cuvette containing 1 mmol/L N-p-tosyl-Gly-Pro-Arg-p-nitroanilide in the same phosphate buffer at 37°C. The rate of hydrolysis of this peptide was monitored by the rate of change of absorbance at 405 nm. The rate of reaction was always compared with that given by sonicated erythrocytes that had been incubated with prothrombin and factors Va and Xa as for the test samples. The value for 100% procoagulant activity was calculated by multiplying the value given by the sonicated erythrocytes by 0.75 to correct for the larger area of the external monolayer of small vesicles. The corrected value was similar to that obtained with hypotonically lysed erythrocytes but was more reproducible. The value for 100% procoagulant activity was converted into the concentration of thrombin by use of the synthetic substrate by thrombin standards. Blanks in the absence of cells generated little procoagulant activity. The Xa for thrombin-catalyzed hydrolysis of N-p-tosyl-Gly-Pro-Arg-p-nitroanilide is about 5 μmol/L/L. Using 1 mmol/L substrate, we measured the constant rate of reaction at maximum velocity over several minutes.

**A23187 Promoted Calcium Uptake by Human Erythrocytes**

An experiment was performed to test if apoA-I affected the extent of Ca^{2+} accumulation in erythrocytes. This was done by incubating human erythrocytes in the presence of A23187 and "Ca^{2+}-. Cells were separated from the extracellular environment by centrifugation through oil. ApoA-I, which could hypothetically extract A23187 from the membrane, was also added to test its effect on Ca^{2+} accumulation in erythrocytes. For these experiments, 1 mL of blood, drawn from a healthy donor, was collected in an EDTA-treated tube. The whole blood was mixed with 10 mL HBS and centrifuged at 3000 rpm for 10 minutes at 4°C in a Sorvall HG-4L rotor. The serum and buffy coat were discarded, and the erythrocytes were washed twice more by resuspension in 10 mL HBS and recentrifugation. The washed erythrocytes were resuspended in 4 mL HBS to give a hematocrit level of 10%. Calcium accumulation was measured at 2% hematocrit in the presence of 100 nCi/mL of "CaCl_2_ (New England Nuclear) and 1 mmol/L cold CaCl_2_ in a total volume of 3.5 mL HBS, in the presence or absence of 10 μmol/L A23187, and in the presence or absence of human apoA-I or HDL. The higher concentration of A23187 was used because the concentration of erythrocytes was higher than that in the experiments above, and procoagulant activity was less but still resulted in the concentration of A23187 in the erythrocyte membrane being similar for the two experiments. Use of 1 μmol/L A23187 gave much lower uptake of Ca^{2+}.
shaking. The reaction was terminated by centrifugation of triplicate 1-mL samples through 100-μL aliquots of oil (dibutyl phthalate:dinonyl phthalate 5:1) at 16 000g for 3 minutes at room temperature.17 Aliquots (500 μL) of each supernatant were taken for scintillation counting. The remaining supernatant and the oil were removed and discarded. 4Ca incorporated into each erythrocyte pellet was extracted with 600 μL of 5% trichloroacetic acid (TCA).18 The pellets were vortexed vigorously, and TCA extraction was carried out overnight at 4°C. TCA-insoluble material was removed by centrifugation at 16 000g for 3 minutes. Aliquots (500 μL) of the TCA supernatants were taken for scintillation counting. Scintillation fluid (5 mL) was added to each vial, and 4Ca levels were determined with a Beckman LS5000TD scintillation counter. A buffer blank and triplicate standards, containing 100 nCi of 4Ca, were included to allow Ca2+ accumulation to be calculated in disintegrations per minute from the raw data on counts per minute.

Microvesiculation

Although microvesiculation of erythrocytes is not thought to contribute to phospholipid reorientation induced by Ca2+,8 we nevertheless measured the effect of apoA-I on this process. Erythrocytes treated with Ca2+ and A23187 shed microvesicles, which remain in the supernatant after centrifugation of the erythrocytes.19 Erythrocytes were washed, processed, and incubated for 20 minutes at 37°C as described in "Procoagulant Activity and Hemolysis," after which time the cells were centrifuged for 5 minutes at 500g. After centrifugation, 600 μL of the supernatant was diluted to 1.2 mL of pH 8.0 phosphate buffer, and the solution was assayed for acetylcholinesterase activity20 as a measure of microvesicle formation.21 Replacement of NaCl with KCl in the buffer suppresses microvesiculation.19 This was confirmed in the present study.

Transbilayer Diffusion of Phospholipids

We used a fluorescent assay similar to that developed by Connor and Schrof22 and that was recently applied to calcium-promoted transbilayer diffusion of phospholipids.8 Briefly, freshly prepared human erythrocytes were washed three times in HBS by centrifugation. NBD-PS was dissolved in chloroform and methanol (2:1), and 75 μg was deposited as a film after evaporation of the solvent under a stream of nitrogen and drying in a vacuum desiccator for 2 hours. The film was suspended by vigorous vortexing with 1.5 mL HBS, and 0.5 mL of packed erythrocytes was added to this suspension. This corresponds to a ratio of approximately 1:50 of NBD-PS to erythrocyte phospholipid. The cells were then incubated at 37°C for 1 hour to allow internalization of the NBD-PS. After this incubation, the cells were placed on ice for 5 minutes. NBD-PS that was not internalized was removed by washing the cells nine times with ice-cold 2% fatty acid-free BSA in HBS, followed by three additional washes with ice-cold HBS alone. After the final wash, the volume of the suspension was adjusted to 2.0 mL. The rate of exposure of NBD-PS by transbilayer diffusion was measured by incubating 40 μL of the labeled erythrocyte suspension in a total volume of 100 μL in the presence or absence of apoA-I and/or 0.5 μmol/L A23187 with 1 mmol/L CaCl2 for 20 minutes at 37°C. Control experiments demonstrated that apoA-I at the highest concentration used (0.2 mg/mL) did not extract any NBD-PS from the membrane. At the end of the incubation period, 70 μL of the cell suspension was removed to presedimented Eppendorf tubes containing 10 μL of 120 mg/mL BSA in HBS. The tubes were incubated on ice for 3 minutes to allow for the extraction of any NBD-PS that had undergone transbilayer diffusion. The tubes were then centrifuged for 2 minutes in an Eppendorf microfuge. Forty microliters of the supernatant was diluted with 2.0 mL of 2% Triton in HBS. Total cell-incorporated NBD-PS was also determined using an aliquot from the cell suspension in BSA before centrifugation. The relative concentration of NBD-PS were determined using an SLM-Aminco Series II spectrofluorimeter with the excitation wavelength set at 460 nm and the emission monochrometer at 534 nm.

Reconstitution of HDL

ApoA-I was codissolved in cholate micelles with or without cholesterol and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) in various proportions. Equal molar amounts of POPC and cholate were used. In the case of dialyzed apoA-I, the molar ratio of cholate to protein was 80:1. The cholate was removed by dialysis to produce both vesicular and discoidal reconstituted HDL (rHDL) as previously described.23,24 Controls were made also by dialyzing cholate solutions of apoA-I alone or of lipid alone.

Protein Concentration

Protein concentration was determined by the method of Lowry et al.25

Phospholipid Concentration

Phospholipid concentration was determined by phosphate analysis after the sample was digested using the method of Ames.26

Results

Incubation of erythrocytes at 37°C in the presence of 1.0 μmol/L A23187 and 1.0 mmol/L CaCl2 causes an increase in procoagulant activity that is linear with time over 20 minutes. The generation of this procoagulant activity is strongly inhibited by apoA-I (Fig 1). ApoA-I has no effect in the absence of Ca2+ and A23187.
Neither Ca²⁺ alone nor A23187 alone are potent promoters of procoagulant activity (Fig 1). The high degree of inhibition of procoagulant activity is not shown by other peptides and proteins with class A helices. The apolipoprotein A-II (apoA-II) at a concentration of 60 μg/mL (about twice the molar concentration of apoA-I) exhibits only 10% inhibition of procoagulant activity (results not presented in the figures). The peptides 18A and Me-18A, which have some, but weaker, effects in raising the bilayer-to-hexagonal phase transition temperature, also show only 10% or less inhibition of procoagulant activity up to a peptide concentration of 50 μg/mL (20 μmol/L). (Also see Reference 3 for the structure of these peptides.) The former peptides contain only a single α-helical segment. ApoA-I contains several helical segments separated by a few amino acid residues, usually including Pro. A synthetic model amphipathic helical peptide that more closely resembles apoA-I in this respect is the peptide 37pA (called 18A-Pro-18A in Reference 27). This peptide contains two amphipathic α-helical segments, each corresponding to the 18A sequence, joined by a Pro residue, and it has the sequence DWLKAFYDKVAEKLKEAFLPD-WLKAFYDKVAEKLKEAF. This peptide has a higher lipid affinity than 18A²⁷ and can even activate the plasma enzyme lecithin:cholesterol acyltransferase at least as well as apoA-I.²⁸ This peptide, 37pA, is also capable of inhibiting procoagulant activity (Fig 2), although it is much less potent than apoA-I in this activity.

ApoA-I is a major component of HDL. We therefore determined the effect of intact HDL on the appearance of procoagulant activity. This intact lipoprotein appeared at least as potent as the apoA-I in inhibiting procoagulant activity (Fig 3). Concentrations of HDL and rHDL (below) are given in terms of the concentration of their protein component. We compared apoA-I and HDL in the same assay at the same protein concentrations and confirmed that HDL, at an equivalent protein concentration, was a more potent inhibitor of procoagulant activity than was apoA-I (Fig 4).

To further evaluate the cause of the increased potency of HDL over apoA-I, we prepared a series of rHDL at various lipid-to-protein molar ratios. We found that all of the rHDLs were potent inhibitors of procoagulant activity, as was the dialedyzed lipid alone (Fig 5). This suggested that both the apolipoprotein(s) and lipid contribute to the inhibition of procoagulant activity in HDL. We noted that solutions of apoA-I gradually lost their potency in inhibiting procoagulant activity after storage for several days at 4°C. To more accurately compare the potency of apoA-I, lipid vesicles, and rHDL, we prepared these fresh and assayed their potency in inhibiting procoagulant activity (Fig 6). In this assay, 30 μg rHDL (1 nmol apoA-I) also contains 100 nmol phospholipid. The relative molar ratio of POPC:cholesterol:apoA-I in this rHDL is 80:8:1. Therefore, the inhibitory effects of rHDL likely has contributions from both the protein and the lipid.

We compared the potency of different normal human plasma lipoprotein fractions with regard to their ability to inhibit procoagulant activity. It is somewhat arbitrary.
on what basis the lipoprotein fractions are compared. We have chosen to compare them on the basis of equal concentrations of lipoprotein particles. However, the ratio of the different lipoproteins can vary widely among individuals and also between postprandial and fasting levels. We have compensated for the different protein contents of the lipoproteins used so that, for example, a higher protein concentration of HDL was used to give a concentration of HDL comparable to the other lipoprotein particles. The concentrations indicated in Fig 7 refer to the protein component of the lipoprotein particle. On a per-lipoprotein-particle basis, HDL was the most effective inhibitor and, at certain concentrations, was the only one of the three lipoprotein fractions to inhibit procoagulant activity (Fig 7).

A possible mechanism for the inhibitory effects on procoagulant activity is that the agents sequester the A23187 ionophore and inhibit the influx of Ca\(^{2+}\) into the erythrocytes. This possibility was made less likely by our finding that the results were independent of the order of addition. We obtained the same extent of inhibition of procoagulant activity whether the A23187 and Ca\(^{2+}\) were added before the apoA-I, HDL, rHDL, or dialyzed lipid. Adding the A23187 first would allow Ca\(^{2+}\) entry into the cells before the possible extraction of A23187 by the hydrophobic agents. If the A23187 and Ca\(^{2+}\) were added subsequently, this would make Ca\(^{2+}\) entry less likely if the ionophore bound these inhibitors. Because procoagulant activity is independent of the order of addition, it is less likely that the inhibitors are acting by extracting the ionophore. We further demonstrated that incubation of the erythrocytes at 0°C in the presence of A23187 and Ca\(^{2+}\) did not lead to the generation of procoagulant activity over a period of up to 2 hours. When the cells that were incubated with Ca\(^{2+}\) and A23187 for 20 minutes at 0°C were subsequently washed with HBS containing 1 mmol/L Ca\(^{2+}\) and were then maintained at 37°C for 20 minutes, procoagulant activity developed. This activity was still inhibited by rHDL, lipid, or apoA-I (Fig 8). However, the washing procedure led to some loss of procoagulant activity (Fig 8), possibly because some Ca\(^{2+}\) is washed out of the cells before removal of the A23187 or as a result of incomplete removal of the ionophore by the buffer wash. In any case, most of the procoagulant activity is retained despite the buffer wash. We further tested the inhibitory action of HDL and apoA-I in erythrocytes that have been loaded with Ca\(^{2+}\) by incubation for 20 minutes at 0°C followed by washing with Ca\(^{2+}\)-free buffer in the absence of ionophore. Even in these washed cells, in which extraction of the A23187 by the inhibitory agents could not lower the intracellular Ca\(^{2+}\) levels, HDL and apoA-I inhibited procoagulant activity (Fig 9).

We also directly measured the effect of apoA-I on the uptake of \(^{45}\)Ca\(^{2+}\) by erythrocytes in the presence of A23187. The difficulty with this direct measurement is that any procedure to remove extracellular \(^{45}\)Ca\(^{2+}\) can...
also result in efflux of this ion from inside the cell, since the ionophore A23187 will still be incorporated into the cell membrane. We used a method of centrifuging the cells through oil that very rapidly stops the flux of Ca\(^{2+}\) across the cell membrane.\(^{17}\) This experiment directly demonstrated that apoA-I was not affecting the ability of A23187 to increase Ca\(^{2+}\) accumulation by the erythrocytes and that HDL had only a relatively small inhibitory effect (Table 1). Higher concentrations of apoA-I actually slightly increased Ca\(^{2+}\) accumulation. This may have been a result of the inhibition of calcium loss through microvesiculation by apoA-I (see below). In any case, the primary conclusion of this experiment is that apoA-I inhibits a step in the formation of the procoagulant complex subsequent to calcium uptake.

We also evaluated the role of microvesiculation in the observed inhibition of procoagulant activity by apoA-I and HDL. We found that 30 \(\mu\)g/mL HDL completely suppressed A23187- and Ca\(^{2+}\)-promoted microvesiculation using Na\(^+\)-containing buffers (Table 2). When K\(^+\) replaced Na\(^+\), no microvesiculation was observed either in the presence or in the absence of HDL or apoA-I. Despite the absence of microvesiculation in the presence of K\(^+\)-containing buffers, apoA-I and HDL still maintained a comparable inhibitory effect on the formation of procoagulant activity (not shown) as observed when microvesiculation occurred (Fig 4). This confirms that microvesiculation, which is not required for phospholipid transbilayer diffusion,\(^{29}\) is also not related to the inhibition of procoagulant activity by apoA-I or HDL. In addition, the ability of HDL to suppress microvesiculation may be another indication of its bilayer stabilizing effects.

ApoA-I binds to anionic lipid more readily than to zwitterionic lipid in the liquid crystalline phase.\(^{29}\) It is thus possible that apoA-I and HDL inhibit procoagulant activity by binding to exposed anionic lipid. However, when erythrocytes were incubated for 20 minutes at 37°C with Ca\(^{2+}\) and A23187 alone and then transferred into a procoagulant assembly incubation with prothrombin, Ca\(^{2+}\), and factors Xa and Va, no inhibition of procoagulant activity was observed with either apoA-I, rHDL, or dialyzed lipid at the highest concentrations that would be transferred from the initial incubation of these agents with erythrocytes using the standard assay procedures. However, if the concentration of rHDL or dialyzed lipid required to inhibit procoagulant activity when added to the erythrocytes incubated with A23187 and Ca\(^{2+}\) is instead added directly to the procoagulant assay with prothrombin, Ca\(^{2+}\), and factors Xa and Va, then an approximately fourfold increase in procoagulant activity is observed. We have no explanation for this phenomenon; however, it is not likely to be related to changes in the rate of initiation of

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**Fig 6.** Bar graph shows comparison of the inhibitory potencies of reconstituted high-density lipoprotein (rHDL), apolipoprotein A-I (apoA-I), and dialyzed lipid. The rHDL and lipid were prepared by dialysis from a solution containing 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC):cholesterol:apoA-I etiolate at a molar ratio of 80:8:1 and 80:8:0, respectively. Dialyzed POPC (100 nmol) was added to the erythrocytes, which corresponds to the amount of lipid in rHDL containing 30 \(\mu\)g apoA-I. Procoagulant activity of 100% corresponds to 14.5 nmol/L thrombin; mean of 3 determinations ± SD.

**Fig 7.** Bar graph shows comparison of the inhibitory potencies of different plasma lipoprotein fractions. Procoagulant activity was generated with A23187 and Ca\(^{2+}\) at 37°C. Concentrations given are in terms of protein content, and the relative amounts of the three fractions used have been adjusted to give similar concentrations of lipoproteins. Procoagulant activity of 100% corresponds to 7.8 nmol/L thrombin; mean of 2 determinations ± range. VLDL indicates very-low-density lipoprotein; LDL, low-density lipoprotein; and HDL, high-density lipoprotein.
% Procoagulant Activity

0 5 10 15 20 25 30

Control
A23187 + Ca^{2+}(+,-)
A23187 + Ca^{2+}(+,-) + 30μg HDL
A23187 + Ca^{2+}(+,-) + 30μg apo A-I
A23187 + Ca^{2+}(+,-) + 50μg apo A-I

Fig 8. Bar graph shows inhibitory effects of reconstituted high-density lipoprotein, apolipoprotein A-I, and dialyzed lipid on procoagulant activity in erythrocytes that had been preloaded with Ca^{2+} at 0°C, followed by a buffer wash to remove the A23187 (see text for details). Weight concentrations and molar ratios have the same meaning as in Fig 5. The two + and - symbols in parentheses refer to the presence or absence of A23187 in the preincubation of the erythrocytes at 0°C and in the subsequent incubation of the erythrocytes with the dialyzed samples at 37°C, respectively. Procoagulant activity of 100% corresponds to 9.0 nmoVL thrombin; mean of 2 determinations±range.

% Procoagulant Activity

0 5 10 15 20

Control
A23187 + Ca^{2+}(+,-)
A23187 + Ca^{2+}(+,-) + 10μg HDL
A23187 + Ca^{2+}(+,-) + 50μg apo A-I

Fig 9. Bar graph shows inhibitory effects of high-density lipoprotein (HDL) on procoagulant activity of erythrocytes that had been preloaded with Ca^{2+} at 0°C, followed by a buffer wash and incubation of the cells at 37°C in the absence of Ca^{2+} (in contrast to results presented in Fig 8) and in the absence of A23187. HDL or apolipoprotein A-I (apoA-I) was added just before the 37°C incubation. Procoagulant activity of 100% for cells incubated at 37°C with Ca^{2+} and A23187) is 14 nmoVL thrombin; mean of 2 determinations±range.

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TABLE 1. \textsuperscript{44}Ca^{2+} Uptake by Erythrocytes

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Cell-Associated \textsuperscript{44}Ca^{2+}, \textit{dpm} \times 10^6</th>
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<tbody>
<tr>
<td>No addition</td>
<td>1.2±0.6</td>
</tr>
<tr>
<td>10 μmol/L A23187</td>
<td>32±1</td>
</tr>
<tr>
<td>10 μmol/L A23187+25 μg/mL apoA-I</td>
<td>32±4</td>
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<tr>
<td>10 μmol/L A23187+50 μg/mL apoA-I</td>
<td>35.7±0.5</td>
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<tr>
<td>10 μmol/L A23187+25 μg/mL HDL</td>
<td>26±2</td>
</tr>
<tr>
<td>10 μmol/L A23187+50 μg/mL HDL</td>
<td>24±1</td>
</tr>
</tbody>
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\textit{dpm} indicates disintegrations per minute; apoA-I, apolipoprotein A-I; and HDL, high-density lipoprotein.

prothrombin to thrombin or its activity against synthetic substrates.

We also tested whether apoA-I, rHDL or dialyzed lipid could be extracting something from the erythrocyte membrane to inhibit procoagulant activity. Erythrocytes were incubated for 20 minutes at 3°C with 30 μg/mL rHDL, 50 μg/mL apoA-I, 100 nmol phosphate per milliliter of dialyzed phospholipid/cholesterol, or buffer control. The cells were then centrifuged and resuspended in buffer with Ca^{2+} and A23187. Procoagulant activity generated by Ca^{2+} and A23187 in cells that had been preincubated with any of the three inhibitory agents was no lower than that of the buffer control. This result demonstrates that the agents were not inhibiting procoagulant activity by extracting something from the erythrocyte.

A more direct measure of transbilayer diffusion than measurement of procoagulant activity is the measurement of the transbilayer asymmetry of labeled phospholipids. This is done by measuring the fraction of labeled lipid that is in the outer monolayer and is extractable with BSA. The presence of A23187 and Ca^{2+} accelerates the rate of transbilayer diffusion (Fig 10). This is similar to the findings reported by Devaux and his colleagues (Williamson et al).\textsuperscript{8} The presence of apoA-I inhibits calcium-promoted transbilayer diffusion of NBD-PS, but this protein has little effect on the basal rate of transbilayer diffusion.

Discussion

Our results demonstrate that intact HDL and rHDL are potent inhibitors of A23187-mediated Ca^{2+}-induced procoagulant activity. HDL may thus provide an effective mechanism of inhibition of unregulated thrombosis. Both the lipid and apolipoprotein components of HDL appear to contribute to this inhibition. The principal protein, apoA-I, is probably an effective inhibitor because it contains segments of class A helixes that are bilayer stabilizers.\textsuperscript{3} This is supported by the finding that

TABLE 2. Inhibition of Microvesiculation by HDL

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Microvesiculation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A23187</td>
<td>100 (by definition)</td>
</tr>
<tr>
<td>No addition</td>
<td>18±1</td>
</tr>
<tr>
<td>A23187+30 μg/mL HDL protein</td>
<td>10±4</td>
</tr>
<tr>
<td>A23187, K+ replacing Na+</td>
<td>19±1</td>
</tr>
</tbody>
</table>

HDL indicates high-density lipoprotein.
the more potent model amphipathic helical peptide,^{27,28} 37pA, can also inhibit procoagulant activity to some extent, although the smaller model peptide 18A has little effect. Neither of these model peptides is as inhibitory as apoA-I, which has several segments of class A amphipathic helices and is a good bilayer stabilizer.^{3} However, on a weight basis or on the basis of the number of amphipathic helical segments, 37pA has an activity comparable to that of apoA-I. In contrast, apoA-II has a weak effect on the generation of procoagulant activity. Although both the apoA-I and apoA-II proteins of HDL have amphipathic helices, the two have opposite effects on the activity of lecithin:cholesterol acyltransferase, and the motifs of their amphipathic helical domains can be distinguished.^{30} The difference in inhibitory potency of these two apolipoproteins suggests that there is some specificity for the inhibition of procoagulant activity and that the inhibition is related to the marked bilayer stabilizing effects of apoA-I.

Because the agents used in this work inhibit the generation of procoagulant activity but do not affect procoagulant activity after cells have been incubated with Ca^{2+} and A23187, we conclude that apoA-I is inhibiting transbilayer diffusion of phosphatidylserine. In addition, changes in the rates of transbilayer diffusion can be measured more directly with the use of the fluorescently labeled phospholipid, NBD-PS. This type of assay requires the use of a phospholipid with a short acyl chain that can be extracted from the membrane with BSA. This property, as well as the presence of the fluorescent probe, likely allows NBD-PS to undergo somewhat more rapid transbilayer diffusion than endogenous phosphatidylserine. We used half the concentration of A23187 to monitor the redistribution of NBD-PS compared with the concentration used for the procoagulant assay. Higher concentrations of apoA-I were required to inhibit the transbilayer diffusion of NBD-PS compared with the concentration required to inhibit procoagulant activity. This may be a consequence of the fact that the short chain NBD-PS undergoes more facile transbilayer diffusion, which is more difficult to inhibit. In addition, one would not expect an exact correlation between the extent of inhibition of transbilayer diffusion and the inhibition of procoagulant activity. This is a consequence of the formation of the procoagulant complex not being linearly proportional to the molar fraction of exposed anionic lipid.^{31} The fact that apoA-I inhibits the externalization of NBD-PS provides further evidence that the inhibition of the rate of transbilayer diffusion of phosphatidylserine contributes to lowering the procoagulant activity.

We have identified an important mechanism by which bilayer-stabilizing class A amphipathic helices inhibit the generation of procoagulant activity by slowing the rate of phospholipid transbilayer diffusion. A major protein component of HDL, apoA-I, is composed of multiple segments of such class A helices. Furthermore, it is likely that some apoA-I becomes incorporated into the plasma membrane of blood cells.^{32} ApoA-I is known to undergo more rapid transfer from lipoprotein particles to cell membranes than do other serum apolipoproteins.^{30} The presence of this apolipoprotein in cell membranes will inhibit the spontaneous generation of procoagulant activity.

In addition to apoA-I, however, all of the lipoprotein fractions as well as dialyzed lipid also inhibit procoagulant activity, suggesting that there are other mechanisms by which this inhibition can occur. This could include sequestering of A23187 by hydrophobic substances. We have shown that a higher concentration of 50 μg/mL HDL can partially suppress Ca^{2+} uptake (Table 1). However, at 25 μg/mL HDL this inhibition is only marginal, and only 2.5 μg/mL HDL is required to inhibit about 50% of the procoagulant activity (Fig 3). Therefore, sequestration of A23187 by HDL is not a major mechanism in its inhibition of procoagulant activity, although it may contribute to the inhibition observed with dialyzed lipid or with the other lipoprotein fractions. In addition, there could be direct inhibition of protein components of the coagulation cascade. Lipoproteins have been shown to neutralize factor Xa, although most of this activity requires prior adsorption of lipoprotein components by Al(OH)_3.^{33} However, this independent mechanism of inhibiting coagulation may contribute to the increased potency of HDL over that of apoA-I. HDL has been suggested to have a protective effect against arteriosclerosis. This epidemiological correlation may be a consequence of the ability of this lipoprotein fraction to inhibit procoagulant activity in cells with damaged or unstable membranes.

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