Plasma Lipoproteins Support Prothrombinase and Other Procoagulant Enzymatic Complexes

Martin P. Moyer, Russell P. Tracy, Paula B. Tracy, Cornelis van’t Veer, Charles E. Sparks, Kenneth G. Mann

Abstract—The prothrombinase complex (factor [F]Xa, FVa, calcium ions, and lipid membrane) converts prothrombin to thrombin (FIIa). To determine whether plasma lipoproteins could provide a physiologically relevant surface, we determined the rates of FIIa production by using purified human coagulation factors, and isolated fasting plasma lipoproteins from healthy donors. In the presence of 5 nmol/L FVa, 5 nmol/L FXa, and 1.4 μmol/L prothrombin, physiological levels of very low density lipoprotein (VLDL) (0.45 to 0.9 nmol/L triglyceride, or 100 to 200 μmol/L phospholipid) yielded rates of 2 to 8 nmol FIIa·L⁻¹·s⁻¹ in a donor-dependent manner. Low density lipoprotein (LDL) and high density lipoprotein (HDL) also supported prothrombinase but at much lower rates (≤1.0 nmol FIIa·L⁻¹·s⁻¹). For comparison, VLDL at 2 nmol/L triglyceride yielded ≈50% the activity of 2×10⁸ thrombin-activated platelets per milliliter. Although the FIIa production rate was slower on VLDL than on synthetic phosphatidylcholine/phosphatidylserine vesicles (≈50 nmol FIIa·L⁻¹·s⁻¹), the prothrombin Kᵣ values were similar, 0.8 and 0.5 μmol/L, respectively. Extracted VLDL lipids supported rates approaching those of phosphatidylcholine/phosphatidylserine vesicles, indicating the importance of the intact VLDL conformation. However, the presence of VLDL-associated, factor-specific inhibitors was ruled out by titration experiments, suggesting a key role for lipid organization. VLDL also supported FIIa generation in an assay system comprising 0.1 nmol/L FVIIa; 0.55 nmol/L tissue factor; physiological levels of FV, FVIII, FIX, and FX; and prothrombin (3 nmol/L FIIa·L⁻¹·s⁻¹). These results indicate that isolated human VLDL can support all the components of the extrinsic coagulation pathway, yielding physiologically relevant rates of thrombin generation in a donor-dependent manner. This support is dependent on the intact lipoprotein structure and does not appear to be regulated by specific VLDL-associated inhibitors. Further studies are needed to determine the extent of this activity in vivo.


Key Words: lipoproteins ■ blood coagulation ■ prothrombinase ■ thrombin

The coagulation cascade is a complex set of enzymatic reactions that results in the activation of prothrombin to thrombin. For the enzymatic reactions in this cascade to occur at physiologically relevant rates, the components must be localized to an appropriate surface.¹ This surface has been provided by PL vesicles in vitro and is presumed to be supplied by platelets³ and, to a lesser degree, by mononuclear cells⁵ in vivo. The PL component of these biological surfaces is important in complex assembly and function, although specific receptors have not been ruled out.⁶ Lipoproteins are also a source of plasma PLs and may provide another surface that will support coagulation reactions.

Plasma lipoproteins have been shown to be risk factors for CHD.⁸ High levels of LDL-C are associated with CHD, whereas high levels of HDL-C are inversely associated with CHD. The relationship of VLDL-C, as estimated by fasting TG levels, to CHD is less clear,⁹–¹³ but some recent data suggest that these larger lipoproteins also may be associated with the atherothrombotic process, especially in postprandial states.¹⁴–¹⁸ There is evidence from population studies that plasma levels of vitamin K–dependent coagulation factors (prothrombin; FVII, FIX, and FX; and proteins C and S) are correlated with fasting cholesterol and TG levels, suggesting some form of coordinate regulation.¹⁹,²⁰ If lipoproteins were shown to support coagulation reactions under physiologically relevant conditions, then this would support the hypothesis that they are thrombotic risk factors as well as atherogenic risk factors and would provide a possible mechanism, through direct binding, for the epidemiological association of lipoprotein levels with factor levels.

To explore this relationship, we characterized the support of surface–dependent coagulation reactions by lipoproteins in a purified human system. We determined the degree of support provided by VLDL, LDL, and HDL for the prothrombinase complex and determined the kinetic parameters of the prothrombinase reaction on VLDL and LDL. We also determined...
the ability of lipoproteins to support thrombin generation in a complete vitamin K–dependent protein procoagulant assay system.

Methods

Materials and Reagents

Trizma (Tris base), Tris HCl, PS (from bovine brain), PC (from egg yolk), EDTA, benzamidine, NaBr, HEPES, NaCl, and chloroform were purchased from Sigma Chemical Co. NaN₃ and diethyl ether were purchased from Krackler. BSA was purchased from GIBCO BRL. The α-thrombin inhibitor DAPA was purchased from Haematologic Technologies Inc. PL vesicles composed of 75% (wt/wt) PC and 25% (wt/wt) PS were prepared as described previously. 21,22 The concentration of the PL vesicles was determined by a phosphorus assay. 23 The chromogenic thrombin substrate S-2238 was purchased from Pierce Chemical Co. Isolated lipoproteins were used within 5 days.

Proteins

Proteins were purified from human, fresh frozen plasma. FV was isolated by immunoaffinity chromatography as described and was activated to FVa with 2 NIH U/mL of recombinant FVIIa as described elsewhere. 35 Samples were analyzed for forward- and right-angle scatter and for green fluorescence with a Coulter Elite fluorescence activated cell sorter using platelet and microparticle gates as described. 36

Prothrombinase Activity on Lipoproteins

To perform the prothrombinase experiments, FVs and DAPA were incubated with a solution of lipoproteins or PCPS vesicles for 1 minute. The reactions were carried out in HBS (pH 7.4) with 5 mmol/L CaCl₂ and 0.1% BSA at room temperature. DAPA was required to inhibit thrombin from converting prothrombin to thrombin 1, a species that is not a suitable substrate for prothrombinase. Following that incubation, prothrombin was added and incubated with the reaction mixture for 1 minute. The reaction was initiated by adding 25 μL of FXa to 175 μL of PCPS. The concentrations of PCPS were titrated. The final concentrations of the reactants (other than PL) were 5 mmol/L FXa, 5 mmol/L FXa, 1.4 μmol/L prothrombin, and 5 μmol/L DAPA. In some cases, the concentrations of FVa, FXa, and prothrombin were varied. The concentrations of PCPS typically ranged from 1 to 100 μmol/L phosphatidylethanolamine, and the concentrations of lipoprotein, though dependent on the concentration of the isolated sample, typically ranged from 0 to 500 μmol/L PL (≈0 to 2.25 mmol/L TG) for VLDL, 1 to 1000 μmol/L PL (≈0 to 500 μmol/L TG) for LDL, and 5 to 1000 μmol/L PL (≈0 to 2500 μmol/L TG) for HDL.

Isolation and Characterization of Lipoproteins

Blood for lipoprotein isolation, collected under institutionally approved protocols for the use of human subjects, was collected after an overnight fast into a preservative cocktail of EDTA, benzamidine, and NaN₃, so that the final concentrations of each were 5 mmol/L, 1 mmol/L, and 1.5 mmol/L, respectively. Donors were healthy, young adult, laboratory personnel. Lipoproteins were isolated from fresh human plasma by sequential flotation ultracentrifugation as described 30 using an SW-41 rotor from Beckman. In one experiment, lipoproteins were isolated by gradient centrifugation using Iodixanol (Accurate Chemical) according to the manufacturer’s recommendations.

After isolation, lipoproteins were dialyzed into HBS, pH 7.4, and stored at 4°C. After sequential flotation, the HDL preparation was purified further by magnesium/phosphotungstic acid precipitation 34 to remove contaminating dense LDL and lipoprotein(a). The quality and purity of isolated lipoproteins were determined by agarose electrophoresis. Purity and quality were also assessed by 3% to 10% SDS–polyacrylamide gel electrophoresis after delipidation of the VLDL and LDL samples by ethanol/ether as described in “Methods.” The bands at 97 and 68 KDa in lane 4 are from the molecular weight standards in the adjacent lane. Lanes 5 to 7, 5% to 15% SDS–polyacrylamide gel electrophoresis of prothrombin (5 μg, lane 5), FXa (2 μg, lane 6), and FV (0.5 μg, lane 7) isolated as described in “Methods.”
5.15 mmol/L cholesterol) for LDL, and 0 to 650 μmol/L PL (≈0 to 1.5 mmol/L cholesterol) for HDL. After initiation of the reactions, 25-μL aliquots were quenched at various times in 75 μL of HBS with 50 mmol/L EDTA and then assayed for thrombin concentration by assessing the rate of S-2238 (0.4 mmol/L) conversion by thrombin in a Spectra Max 250 spectrophotometer from Molecular Devices. Thrombin generation was calculated from a standard curve prepared by using various concentrations of purified α-thrombin.

Experiments to determine kinetic parameters were done using prothrombin concentrations from 0.067 to 3.195 nmol/L. Data were analyzed using curve-fitting software to determine $K_m$ and $V_{max}$ (Enzfitter, Elsevier-Biosoft). For experiments using rabbit IgG, the lipoprotein samples were incubated for 30 minutes at room temperature with 0.15 mg/mL rabbit anti-TFPI IgG, 0.15 mg/mL control rabbit IgG, or diluent. After the incubation was complete, the prothrombinase reaction was carried out as described above.

**FVIIa/TF-Dependent Procoagulant Assay System**

TF, 0.55 nmol/L, was incubated with lipoproteins or PCPS vesicles for 30 minutes at 37°C in HBS (pH 7.4) with 2 mmol/L CaCl$_2$, FVIIa was then added at 0.1 nmol/L and incubated for 20 minutes to allow complex formation. To initiate the reaction, 50 μL of the lipoprotein/TF/FVIIa solution was mixed with 50 μL of a solution of FV, FVIII, FIX, FX, and prothrombin. The final concentrations were 20 nmol/L FV, 0.7 nmol/L FVIII, 90 nmol/L FIX, 170 nmol/L FX, and 1.4 μmol/L prothrombin, which were chosen to reflect typical plasma concentrations. After initiation of the reaction, 5-μL aliquots were quenched in 100 μL of HBS with 20 mmol/L EDTA, and the concentration of thrombin was determined as described above.

**Platelet Isolation**

Platelets were isolated by the method of Mustard et al using venous blood from healthy, nonmedicated individuals as described above. Modifications to this procedure included omission of apyrase from all washing steps and the use of 5 mmol/L HEPES/Tyrode’s solution, pH 7.4, as the final platelet suspension buffer. Platelets were counted on a Coulter counter (Coulter Electronics). Platelet activation was accomplished by incubation of platelets with 20 nmol/L thrombin (2 NIH units) for 30 minutes at 37°C in HBS (pH 7.4) with 2 mmol/L CaCl$_2$. FVIIa, 0.55 nmol/L, was incubated with lipoproteins or PCPS vesicles. After initiation of the reaction, 5-μL aliquots were quenched in 100 μL of HBS with 20 mmol/L EDTA, and the concentration of thrombin was determined as described above.

**Results**

**Prothrombinase Activity on Lipoproteins and PCPS Vesicles**

The relationships between the rate of thrombin generation and the amount of PL (as either lipoprotein or synthetic vesicle PL) used are shown in Fig 2. We observed that although all three lipoprotein classes supported prothrombinase activity, VLDL consistently supported the highest rate. In all cases the maximum rates observed were lower than those obtained with PCPS vesicles. We observed a range in rate with different VLDL samples, 2 to 10 nmol FIIa·L$^{-1}$·s$^{-1}$. The range we observed with different samples of LDL was between 0.4 and 1.0 nmol FIIa·L$^{-1}$·s$^{-1}$. HDL supported prothrombinase activity at very slow rates, consistently <0.1 nmol FIIa·L$^{-1}$·s$^{-1}$.

Using flow cytometry, we determined that there were 4×10$^5$ microparticles per milliliter of plasma as prepared for lipoprotein isolation. If one assumes that all microparticles were concentrated in the VLDL preparation (unlikely, since even mild centrifugation pellets the majority of microparticles), this would yield ∼4×10$^5$ microparticles per milliliter of VLDL. Since ∼40% of the prothrombinase activity of activated platelets is in the microparticle fraction and approximately one or two microparticles are formed for every two thrombin–activated platelets (our observations and Reference 40), then on the basis of our observed thrombin generation rates for platelets (eg, Fig 6), the maximum contribution of microparticles to the rate of thrombin generation would be ≈0.08 nmol FIIa·L$^{-1}$·s$^{-1}$, or <3% of the typical rates we observed with VLDL. We conclude that platelet microparticles cannot be a major contributor to prothrombinase rates under the conditions described here.

The titration curve for PCPS vesicles reached a maximum and then declined because the maximum represents the point at which all components (FVa, FXa, and prothrombin) are optimally bound to the surface; additional surface “dilutes” the components and reduces the rate. In this experiment, VLDL (and HDL) had titration profiles similar to that of PCPS vesicles: a maximum followed by a decline. Although LDL did not reach a maximum in this experiment, we have performed this experiment five times with generally similar results and have seen LDL reach a maximum in other experiments.

**Kinetic Parameters of Prothrombinase on VLDL, LDL, and PCPS Vesicles**

We determined the $K_m$ values for prothrombin by using two different concentrations of VLDL, two different concentrations of LDL, and PCPS vesicles (the Table). The concentrations of lipid were chosen to represent points on the ascending side of the curves shown in Fig 2. The $K_m$ values obtained with

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Phospholipid, μmol/L</th>
<th>$K_m$, μmol/L</th>
<th>$V_{max}$, nmol·L$^{-1}$·s$^{-1}$</th>
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</thead>
<tbody>
<tr>
<td>VLDL</td>
<td>119</td>
<td>0.96</td>
<td>3.67</td>
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<tr>
<td>VLDL</td>
<td>60</td>
<td>0.802</td>
<td>2.77</td>
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<tr>
<td>LDL</td>
<td>767</td>
<td>0.739</td>
<td>0.534</td>
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<tr>
<td>LDL</td>
<td>384</td>
<td>0.79</td>
<td>0.386</td>
</tr>
<tr>
<td>PCPS</td>
<td>5</td>
<td>0.5</td>
<td>40.0</td>
</tr>
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</table>

The $K_m$ and $V_{max}$ values for prothrombin were determined as described in “Methods.” Prothrombin concentrations ranged from 0.067 to 3.195 μmol/L. Data were analyzed using curve-fitting software.
lipoproteins (0.74 to 0.96 μmol/L) were similar to those obtained with PCPS vesicles (0.5 μmol/L), though consistently slightly higher. The $V_{\text{max}}$ values observed with lipoproteins reflected the lower rates that we observed in the titration experiments.

Variability in Prothrombinase Activity With VLDL From Different Donors

As mentioned previously, we have observed a range of rates for prothrombinase on VLDL between plasma from different donors. Fig 3 is an example of what we have typically seen, illustrating data for six different donors, with lipoprotein preparations and enzyme assays done simultaneously. The maximum rates from these donors varied from 1.8 to 6.0 nmol FIIa\cdot $L^{-1} \cdot s^{-1}$. Because of our concern about variability due to phlebotomy or sample preparation, we obtained two blood samples simultaneously from 1 donor, one from each arm, and treated these samples separately. We performed this experiment twice on 2 separate days. The thrombin generation rates observed with 0.55 mmol/L VLDL TG were as follows: day 1, 4.5 and 4.2 nmol FIIa\cdot $L^{-1} \cdot s^{-1}$; and day 2, 4.3 and 4.3 nmol FIIa\cdot $L^{-1} \cdot s^{-1}$. These results indicate excellent reproducibility for the method.

Effect of TFPI in the Prothrombinase Assay System

TFPI is known to inhibit prothrombinase, but very slowly. We determined the rate of prothrombinase on lipoproteins in the presence and absence of an inhibitory polyclonal anti-TFPI IgG. VLDL-supported prothrombinase rates (1 mmol/L VLDL TG) were 4.98 nmol FIIa\cdot $L^{-1} \cdot s^{-1}$ without IgG, 5.31 nmol FIIa\cdot $L^{-1} \cdot s^{-1}$ with 0.15 mg/mL rabbit anti-TFPI IgG, and 5.51 nmol FIIa\cdot $L^{-1} \cdot s^{-1}$ with 0.15 mg/mL control rabbit IgG. LDL-supported prothrombinase rates (1 mmol/L LDL-C) were 0.96 nmol FIIa\cdot $L^{-1} \cdot s^{-1}$ without IgG, 0.96 nmol FIIa\cdot $L^{-1} \cdot s^{-1}$ with 0.15 mg/mL rabbit anti-TFPI IgG, and 1.18 nmol FIIa\cdot $L^{-1} \cdot s^{-1}$ with 0.15 mg/mL of control rabbit IgG. Because this anti-TFPI is known to inhibit TFPI activity in systems using PL vesicles (R.P. Tracey et al, unpublished data, 1997), we think that these data are consistent with TFPI’s having no effect in our assays.

Tests for Specific Inhibitory Activity of FVa or FXa by VLDL

To further characterize the support of prothrombinase by VLDL, we investigated prothrombinase activity on extracted VLDL lipid (ŒŒ) from one donor and PCPS vesicles (ŒŒ) were used in a prothrombinase assay. Results are expressed as in Fig 2.

VLDL lipid reconstituted in aqueous buffer. This procedure (1) removed any protein from the lipid particles and (2) allowed for major reorganization of the lipid components. Fig 4 shows that VLDL lipid supported prothrombinase activity to a greater degree than did isolated VLDL particles, with maximal rates of thrombin generation approaching those observed with PCPS vesicles.

To determine whether or not the variability in VLDL-dependent thrombin generation was due to an inhibitory activity toward either FVa or FXa, we titrated PCPS vesicles, intact VLDL, and extracted VLDL lipid from a single donor in prothrombinase assays using three different combinations of FVa and FXa concentrations. The three combinations used were as follows: (1) 5 nmol/L FVa, 5 nmol/L FXa; (2) 0.5 nmol/L FVa, 5 nmol/L FXa; and, (3) 5 nmol/L FVa, 0.5 nmol/L FXa. If intact VLDL contains specific inhibitory activity toward one of the components (FVa or FXa) when the concentration of that component is decreased, then we would expect to see a greater decline in activity compared with the decline observed with PCPS vesicles, where there is no specific inhibitory activity. Fig 5 shows the results of this experiment. There were no differences in activity changes seen in any of the experiments when compared with PCPS vesicles. The maximum rates observed with 0.5 nmol/L FVa were $\approx 10\%$ of those seen with 5 nmol/L FVa in all cases, and the maximum rates observed with 0.5 nmol/L FXa were $\approx 20\%$ of those seen with 5 nmol/L FXa in all cases. This observation is consistent with the idea that there is no specific inhibitory activity of either FVa or FXa associated with intact VLDL.

Prothrombinase on Platelets Compared With Isolated VLDL

Activated platelets are known to be a major source of procoagulant surfaces in vivo. To test the physiological significance of VLDL-dependent prothrombinase rates, we determined the rate of thrombin generation on VLDL and activated platelets from the same donor. The blood utilized for VLDL isolation was drawn 1 day before platelet isolation so that all prothrombinase assays could be performed on the same day. Fig 6 shows that VLDL at 2 mmol/L (175 mg/dL) TG gave rates of thrombin generation that were at least one half of those seen with $2\times 10^5$ platelets per milliliter from these same donors.
FVIIa/TF-Dependent Procoagulant Assays Using Lipoproteins and PCPS Vesicles

To determine whether isolated lipoproteins could support other surface-dependent coagulation reactions besides prothrombinase, we used a more complex procoagulant assay system. Physiological levels of FV, FVIII, FIX, FX, and prothrombin were combined with lipoproteins, TF, and FVIIa, and the rate of thrombin generation was measured over time (Fig 7). In this experiment PCPS vesicles were used at 200 μmol/L phosphate, whereas VLDL from three different donors and one sample of LDL were used at their fasting levels. As in the simpler prothrombinase assay system, VLDL was more effective a surface than LDL but less so than PCPS vesicles. Donor variability in VLDL-dependent rates was reflected in differences in the observed lag time, not in the actual rate of thrombin generation, which was ≈3 nmoL FIIa · L⁻¹ · s⁻¹ in each case.

Discussion

In this article we present the first kinetic data, using exclusively human components, which demonstrate plasma lipoprotein support for prothrombinase complex activity and, in fact, all reactions of the tissue factor pathway. The maximal VLDL-dependent rates were similar to those seen using physiological levels of activated platelets, suggesting that under certain conditions, lipoproteins, especially VLDL, might play a significant role supporting thrombin generation in vivo.

Figure 5. Lipoprotein-dependent prothrombinase activity with reduced enzyme or cofactor levels. The concentrations of FVa or FXa were decreased 10-fold and the effect on the rate of thrombin generation measured for PCPS-dependent (top), VLDL-dependent (middle), and extracted VLDL lipid-dependent (bottom) prothrombinase reactions. Concentrations used in the assays were 5 nmol/L FVa, 5 nmol/L FXa ( ), 5 nmol/L FVa, 0.5 nmol/L FXa ( ), 0.5 nmol/L FVa, and 5 nmol/L FXa ( ). Results are expressed as in Fig 2.

Figure 6. Comparison of platelet-dependent and VLDL-dependent prothrombinase activity from 2 healthy, nonmedicated donors. Blood was drawn for VLDL isolation (donor 1 (); donor 2 ( )); 1 day before the blood draw for platelet isolation so that all prothrombinase assays could be performed on the day of platelet isolation. Results are expressed as in Fig 2, with the exception that VLDL concentrations are given as VLDL TG to emphasize physiological relevance. For VLDL, 100 μmol/L PL<202>0.45 mmol/L TG.

Figure 7. Thrombin generation over time using the procoagulant assay system initiated with TF/FVIIa. PCPS vesicles ( ), VLDL isolated from 3 healthy, fasting donors (donor 1 (); donor 2 () ); donor 3 ( )); and fasting LDL isolated from donor 3 ( ) were used in the assay system described in “Methods,” initiated with TF/FVIIa, and comprising FIX, FX, V, and prothrombin. Results are expressed as micromoles of FIIa assessed at different time points during the reactions. The concentrations of lipoproteins corresponded to the fasting levels from these donors are as follows: donor 1 VLDL, 0.6 mmol/L TG (~133 μmol/L PL); donor 2 VLDL, 0.9 mmol/L TG (~200 μmol/L PL); donor 3 VLDL, 2.3 mmol/L TG (~511 μmol/L PL); and donor 3 LDL, 5.2 mmol/L cholesterol. PCPS vesicles were used at 200 μmol/L PL.
Purified VLDL particles have the ability to support prothrombinase at a level near that of activated human platelets. These rates are achieved with physiological levels of VLDL, 0.3 to 1.7 mmol/L TG. The results from the procoagulant assay system also indicate that VLDL can support generation of thrombin in a reaction initiated with FVIIa/TF at a significant rate. This further supports the concept that lipoproteins may provide a suitable surface for coagulation reactions and do not require activation, as in the case of platelets.

Because lipoproteins accumulate in atherosclerotic plaque,52–46 their presence may have significance for plaque-associated thrombin generation, especially in relatively minor situations as proposed by Harker et al,47 wherein small amounts of thrombin generation have implications for plaque progression. Regarding venous thrombosis and thromboembolic disease, conditions of blood stasis may be particularly relevant, where the components may have the opportunity to form complexes, at least transiently. It has also been suggested that postprandial LDL increases may be associated with a hypercoagulable state.14,48 Our observations suggest that, if this is true, VLDL-mediated procoagulant complex assembly may be a significant contributor to this process.

Our observed $K_z$ values for prothrombin experiments using lipoproteins are similar to those determined with PCPS vesicles. They also agree with previous reports of prothrombinase kinetics on PL surfaces.49 This is consistent with the notion that prothrombinase has the same mechanism of action on lipoproteins as on PCPS vesicles. The lower $V_{max}$ values suggest that substrate delivery and incorporation are not significantly affected on the lipoprotein surface when compared with PCPS vesicles but that there is an effect on the turnover rate of the enzyme.

We observed donor variability in VLDL-dependent thrombin generation. In the prothrombinase assay, different individuals exhibited consistently different lipid titration profiles, regarding both the level of lipoprotein required for the maximum rate and the maximum rate achieved. Reproducibility studies indicated that donor variability was most likely not a result of sample preparation artifacts.

We considered the concentration of negatively charged PLs on the surface of the VLDL particles from different donors as a source of variability. It is known that the concentration of negatively charged phosphate groups on a surface will influence the degree of protein binding and the reaction rate.50 Because lipoprotein particles consist of $\approx 5\%$ negatively charged PLs,52 whereas PCPS vesicles as we prepared them are $\approx 25\%$ negatively charged PLs, this is a possible explanation for the difference between the rates on PCPS vesicles and the rates on lipoproteins. However, because the VLDL–dependent rates could be dramatically increased to approximately the same values as the PCPS–dependent rates by extracting the lipids, this suggests that rate differences cannot be explained simply by the different percentages of negatively charged PLs and that lipoprotein conformation has a major influence on prothrombinase rates.

TFPI is known to be associated with lipoproteins. TFPI has been shown to inhibit FXa in the prothrombinase complex.52 Mast and Broze53 observed a 50% drop in activity in an FXa-initiated assay when 8 nmol/L TFPI (approximately threefold the physiological concentration) was added to a reaction with 3 nmol/L FVa, 0.1 nmol/L FXa, and 1.4 $\mu$mol/L prothrombin.53 On the basis of their results, we would anticipate a minimal effect in our assays, as we used an $\approx 50\%$–fold higher enzyme concentration. Our immunological and titration–based data support this position.

VLDL contains apo B–100 as one of its apolipoprotein components. The effect of apo B–100 on prothrombinase activity is not well understood. Although our data with FVa and FXa titrations suggest that apo B–100 is not specifically inhibitory towards either FVa or FXa, the possibility that apo B–100 is inhibitory to the prothrombinase complex as a whole has not been ruled out. One interpretation of the data regarding extracted lipids might be that apo B does have some inhibitory effect, since its removal results in increased activity. However, since there are likely to be enormous changes in lipid organization during extraction, one must be cautious about the implications of the experiment regarding apo B–100. Nonetheless, it remains possible that apo B could have effects on the surface, including the masking of PL binding sites, alterations in fluidity of the PL surface, or direct protein–protein interactions with prothrombinase components. VLDL also contains exchangeable apolipoproteins of the C and E groups, which are absent on LDL. These apolipoproteins may play a role in the surface complexes involved in the procoagulant enzymatic activity by supporting protein–protein or protein–lipid interactions.

Another factor known to affect prothrombinase rates on surfaces is the composition of the fatty acid chains on the surface PLs. For example, vesicles composed of PLs containing predominately stearic acid (18:0) had rates that were $\approx 5\%$ of those containing oleic acid (18:1).54 Although we did not directly assess the fatty acid composition of our lipoprotein preparations, our data with extracted VLDL lipids again suggest that the fatty acid side-chain composition does not play an important role per se. However, we do note that although the extracted VLDL–dependent rates of thrombin generation approached those seen on PCPS vesicles, the level of PL needed to achieve these rates was higher. The reason for this is unclear at this time.

The presence of cholesterol in the lipoproteins might cause a loss of activity. However, vesicles composed of 60% PC, 20% PS, and 20% cholesterol have been shown to support prothrombinase to the same degree as vesicles composed of 80% PC and 20% PS.49 The experiments with the procoagulant assay system indicate that VLDL and LDL can support FVIIa/TF–initiated thrombin generation in an assay with physiological levels of FV, FVIII, FIX, FX, and prothrombin. VLDL–mediated reactions supported rates of 3 nmol FIIa $\cdot$ L$^{-1} \cdot$ s$^{-1}$ after a lag of $\approx 1$ minute, with little donor variability in the rate of thrombin generation after the initial lag. The initial lag time may represent the time that it takes for small amounts of thrombin to be formed and subsequently convert FV and FVIII to FVa and FVIIa, respectively. TFPI is known to be a potent inhibitor of the TF/FVIIa complex,31 and while we believe it is unlikely that TFPI inhibits FXa, we cannot rule out VLDL–associated inhibition of TF/FVIIa at this time.

Recently Ettelaie et al56 have reported inhibition of TF activity by apo B–100. However, it is difficult to apply their
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findings to our procoagulant assay system, since they used apo B-100 reconstituted in soybean PC vesicles and not native lipoprotein particles. Furthermore, they measured the inhibition of rabbit brain TF, and it is unclear whether apo B-100 would have the same effect on human TF reconstituted into purified lipoproteins or PCPS vesicles.

In conclusion, we have observed physiologically relevant, donor-dependent rates of VLDL-mediated thrombin generation in assay systems using appropriately isolated human, fasting lipoproteins and purified human coagulation factors. Lipid organization appears to be a key regulatory factor. We found no evidence for VLDL-mediated factor-specific inhibition.

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References


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