Phosphatidylethanolamine Participates in the Stimulation of the Contact System of Coagulation by Very-Low-Density Lipoproteins

Sonja Klein, Michael Spannagl, Bernd Engelmann

Abstract—We have analyzed the influence of plasma lipoproteins on the activation of the contact pathway of blood coagulation in platelet-rich plasma (PRP). The formation of thrombin in PRP incubated in vitro was abolished by the factor XIIa antagonist corn trypsin inhibitor and by severe factor XII deficiency, indicating mediation by the contact system. Addition of VLDL to the PRP shortened the lag period and increased the generation of thrombin. There was no effect of HDL and LDL. In whole blood, VLDL accelerated the rate of fibrin formation, the procoagulant effect being prevented by factor XII deficiency and by corn trypsin inhibitor. The thrombin formation in the PRP was strongly increased by microemulsions of the VLDL lipids while it was reduced by the aqueous phase of the particles. Separation of the VLDL lipids indicated the phospholipid component as the major activating principle. Vesicles supplemented with all VLDL phospholipids but lacking specifically the fraction containing phosphatidylethanolamine (PE) prolonged the lag time. The PE containing fraction alone as well as vesicles enriched with egg PE shortened the lag period. In summary, VLDL stimulates the contact pathway of blood coagulation, ethanolamine phospholipids being the most active components of the particles. (Arterioscler Thromb Vasc Biol. 2001;21:1695-1700.)

Key Words: contact pathway ■ phosphatidylethanolamine ■ very-low-density lipoproteins ■ factor XII ■ corn trypsin inhibitor

The physiological role of the contact system in coagulation is still a matter of debate. The absence of bleeding abnormalities in patients with deficiencies in factor XII, high-molecular-weight kininogen, and prekallikrein has been interpreted to indicate that the system is not involved in the activation of coagulation under in vivo conditions. In line with this view, factor XI is formed more efficiently on the platelet surface by thrombin (previously generated by the tissue factor pathway) than by factor XIIa.1 However, in several recent studies, an association between certain types of hyperlipidemias and increased plasma contents of factor XIIa has been noted.2–5 Earlier work suggested that factor XII activation might contribute to the elevation of factor VIIa activity.6,7 Moreover, factor VIIa antigen levels were positively correlated with plasma triglyceride contents.8,9 In particular, it was proposed that the concentrations of triglyceride-rich lipoproteins in postprandial plasma were responsible for the increased factor XIIa formation. Somewhat later it became evident that the augmented factor VIIa activity was independent of the presence of factor XIIa.10,11 Because hyperlipidemias predispose for atherosclerotic diseases and their thrombotic complications, it was analyzed whether there was a relation between the plasma factor XIIa concentration and the severity of atherosclerotic damage. Indeed, a strong relation of the factor XIIa concentration to the extent of coronary atherosclerosis has been established.2–5

In view of these associations, we have evaluated in the present study whether the activity of the contact system of coagulation was affected by the different types of (isolated) plasma lipoproteins. The activity of the contact pathway was analyzed in platelet-rich plasma (PRP) and in whole blood under in vitro conditions. We found that VLDLs specifically stimulated the contact system in the PRP and in whole blood. This led us to analyze the nature of the component(s) within the particles mediating the activation. After several separation procedures, we identified phosphatidylethanolamine (PE), a quantitatively minor VLDL-associated phospholipid, as the lipoprotein component mainly responsible for the stimulation of the contact pathway.

Methods

Materials
Egg phosphatidylcholine (PC), egg PE, 1-palmitoyl, 2-palmitoyl-PC (dipalmitoyl-PC), 1-palmitoyl, 2-lyso-PC (lysoPC), α-thrombin, and phospholipid standards were obtained from Sigma. Substrate S-2238 and CTI were purchased from Hemochrom Diagnostica.

Thrombin Generation In Vitro
The formation of thrombin under in vitro conditions was determined essentially as previously described.12 Venous blood was obtained from healthy fasting volunteers (age 20 to 35 years) and from a person with severe factor XII deficiency (factor XII activity below...
chosen within the time interval where the slopes of most of the
lag time value of thrombin formation was defined. The value was
containing 90
mL. The generation of thrombin was assessed by using a chro-
mogenic substrate. The lag periods of thrombin generation (cal-
mation of the samples was determined by using a standard curve with
ELISA-Reader (Dynatech MR7000). The free thrombin concentra-
to those membranes. Phase-contrast microscopy was used to con-
confirm the migration of lipoprotein contents, contamination of the lipoprotein suspensions by cell
derived microparticles is unlikely. The amount of lipoprotein-
associated protein was determined by using the Bradford
procedure.13

**Whole Blood Coagulometry**

Whole blood coagulation was estimated mechanically by ROTEG
(Pentapharm).13 Blood (citrated, 0.3 mL) was pipetted into a plastic
cup that had been prewarmed to 37°C. Either isolated lipoproteins or
vehicle was added. The coagulation process was started by the
addition of 60 μL of a 100-mmol/L CaCl2 solution (suspended in
10-mmol/L HEPES, pH 7.4). The results were registered under
standardized conditions as established by the manufacturer. The
following parameters were calculated: the fibrin formation time \( r \)
(the distance between the start of the recalcification and the point
where the amplitude of the tracing reaches 2 mm) and the clot
formation time \( k \) (the distance between \( r \) and the point where
the amplitude of the tracing reaches 30 mm).

**Lipoprotein Isolation**

VLDL, LDL, and HDL were prepared from healthy donors by
ultracentrifugation,14 dialyzed against argon-bubbled PBS contain-
ing 0.3 mmol/L EDTA, and stored under argon at 4°C. In view of the
typical migration pattern (agarose gel electrophoresis) and the
characteristic phospholipid composition (e.g., high PC and low PE
contents), contamination of the lipoprotein suspensions by cell
derived microparticles is unlikely. The amount of lipoprotein-
associated protein was determined by using the Bradford
procedure.13

**Preparation of Microemulsions and of Lipid Vesicles**

The lipoproteins (50 μg protein) were divided into aqueous and lipid
phases according to the Bligh and Dyer procedure.16 The chloroform
used for the lipid extractions was supplemented with butylated
hydroxytoluene (50 mg/L). The aqueous fractions were directly
tested for their effects on the formation of thrombin in the PRP. The
organic phase was evaporated under \( \mathrm{N}_2 \), and an isotonic NaCl buffer
was added. A sonic probe (Branson sonifier model W 185, at 40
W) was immersed into the suspension, and microemulsions were
prepared by three sonication steps (3 minutes each, with 1-minute
breaks in between) at 4°C under a constant stream of \( \mathrm{N}_2 \). Thereafter,
the suspensions were centrifuged in the presence of \( \mathrm{N}_2 \) to remove
large vesicles. The microemulsions were added to the PRP. To
prepare the lipid mixtures from VLDL deficient in specific lipid
components, the lipid phases were first resuspended in 500
μL of
CHCl3/CH3OH (2:1). Then, 10 identical portions of the suspensions
were applied in parallel onto thin layer chromatography (TLC) plates
(Silica G60, Merck), and the samples developed in CHCl3/CH3OH/
H2O (90:40:12.2). The spots of the lanes were visualized with
diphenylhexatriene spray. The following areas were scraped from
the plates: (1) the complete lane between the origin and the top;
(2) the complete lanes lacking one specific spot (which was left on
the plate); and (3) a portion of the plate to which no material had
been applied having the same length and width as the lane mentioned
under 1 (blank). In other experiments, the individual lipid spots
containing the different lipid fractions were recovered and further
processed as described below. Using standards for the main phos-
pholipids of the VLDL, we found that fraction A-D comigrated with the
phospholipids indicated:

Fraction A: lyso PC
Fraction B: sphingomyelin (SM)
Fraction C: PC, phosphatidylinositol (PI), phosphatidylserine (PS)
Fraction D: PE

For the elution of the lipids, CHCl3/CH3OH (1:4) was added to the
silica, the procedure being repeated twice. Subsequently, the lipids
were resuspended in a biphasic system consisting of 5 vol of
CHCl3/CH3OH (2:1) and 2 vol of CH3OH/H2O (1:2) at 4°C to
remove the residual silica. After centrifugation at 4°C, the lower
phase was saved. Dipalmitoyl-PC (100 nmol) was added to the
purified organic phase. After evaporation, the lipids were dispersed
by sonication in a buffer composed of 145 mmol/L NaCl, 10 mmol/L
HEPES, 5 mmol/L KCl, 1 mmol/L MgCl2, 5 mmol/L glucose (pH
7.4) (resuspension buffer). The dispersions were centrifuged for
10 minutes at 4°C and 1200g, and the upper 80% of the supernatant
was used as vesicle solution. Lipid vesicles were also directly prepared

**Figure 1.** Formation of thrombin in PRP incubated in vitro. a,
Control. b, Addition of isolated VLDL (0.3 mg protein/mL).
Because of the addition of the VLDL, the total triglyceride con-
ten was elevated from 103±27 (control) to 164±36 mg/100
mL. The generation of thrombin was assessed by using a chro-
mogenic substrate. The lag periods of thrombin generation (cal-
culated as described in Methods) were 38.9 (without added
VLDL) and 32.0 minutes (added VLDL). A value of 100
mU/mL of thrombin concentration. Circles, control; triangles, severe factor XII deficiency; squares,
presence of CTI (32 μg/mL). Mean±SD on PRP from 10 to 14
different donors (control, CTI) and one individual (factor XII defi-
ciency). *P<0.05 versus same time point in control [a), presence
of CTI; b) factor XII deficiency].
from dipalmitoyl-PC (95 mol%) and enriched with 5 mol% of egg PC, 1-palmitoyl, 2-lyso-PC, and egg PE. The phospholipids were dispersed in the resuspension buffer as described above for the VLDL-extracted lipids. Subsequently, the vesicle suspensions were added to the PRP.

Statistics
Statistical analysis was performed by one-way ANOVA for multiple comparisons. $P < 0.05$ was considered significant. All mean values are given ±SD

Results
Addition of calcium to PRP isolated from citrated blood results in the slow formation of thrombin in vitro.12,17 In accordance with the earlier data, in PRP obtained from healthy donors, a small rise in the concentration of thrombin was noted within the first 30 minutes of incubation (Figure 1a). Thereafter, the thrombin concentrations started to increase up to the longest time interval analyzed (70 minutes). The time dependence of the thrombin formation could be fitted by an exponential curve (see Methods). At a predefined steepness of the curve, a lag time value was defined falling into the time period where the thrombin concentrations started to rapidly increase (see legend for Figure 1a). When the lag time values obtained from the PRP of 12 different donors were compared with the maximum values of thrombin formation as measured after the 70-minute incubation period, a negative relation between the two indices was obtained ($r = -0.65, P < 0.05$). To evaluate whether the thrombin generated in vitro in the PRP was caused by the activation of the contact pathway as initiated by the activated factor XII, CTI, a specific inhibitor of factor XIIa (20 μg/mL), was added to the PRP. As a consequence, the generation of thrombin was completely prevented during the whole incubation period (Figure 1a). Furthermore, no thrombin generation was seen with recalcified PRP obtained from an individual with severe factor XII deficiency. Together, these data suggested that the thrombin formation observed under our experimental conditions was elicited by the activated factor XII.

To analyze the influences of the major lipoproteins on the activation of the contact pathway, isolated VLDL was tested first. The addition of VLDL (0.3 mg protein/mL) to the PRP resulted in a shortening of the lag time of thrombin generation by 18% (see legend for Figure 1). Concomitantly, the amount of thrombin formed was higher in the VLDL-supplemented samples compared with the control (within the time interval between 40 and 70 minutes; Figure 1b versus Figure 1a). In the presence of CTI, in vitro supplementation with VLDL did not elicit the formation of thrombin in the PRP (Figure 1b). Moreover, in PRP prepared from the donor lacking factor XII, VLDL was unable to trigger the thrombin synthesis. Together, these data indicated that the stimulation of thrombin formation by VLDL was mediated by the activated factor XII.

The addition of VLDL (0.3 mg protein/mL) or vehicle to PPP did not result in the formation of thrombin within the whole incubation period of 70 minutes (data not shown), indicating that the presence of the platelets was required for the stimulation of thrombin generation by VLDL. The concentration dependence of the stimulation of thrombin generation by VLDL is shown in Figure 2a. The thrombin synthesis steeply increased between 0 and 0.3 mg/mL of added VLDL, a somewhat less pronounced elevation of the thrombin formation being observed between 0.3 mg/mL and 0.6 mg/mL of VLDL. On adding 0.9 mg VLDL/mL, no further increase of the thrombin generation was noted (not shown). The total triglyceride concentrations of the PRP achieved an increase of the thrombin generation was noted (not shown). The total triglyceride concentrations of the PRP achieved after inclusion of 0.6 mg VLDL/mL were similar to those encountered in mild hypertriglyceridemia (see legend for Figure 2a). Subsequent to the addition of the same amount of LDL and HDL (0.6 mg protein/mL), the lag time values of thrombin formation were similar to those of the control (Figure 2b). Thus, the stimulation of the activity of the contact system was specific for VLDL.

In further experiments, we analyzed whether VLDL also stimulated the contact system in whole blood. The rates of fibrin formation and of clot growth in the recalcified blood were estimated by whole blood coagulometry (see Methods). Control experiments verified that under these conditions the activation of coagulation was triggered by factor XIIa. After the addition of VLDL (0.3 mg protein/mL) to the whole blood, the rate of fibrin formation was accelerated (Figure 3a and 3b), and the $r$ value decreased by 16.7±12.5% (mean±SD on blood from 4 different donors). No activation of coagulation could be elicited by VLDL in whole blood taken from the individual with factor XII deficiency (Figure 3c and 3d). Furthermore, in the presence of CTI, the effect of VLDL on the fibrin formation was prevented (not shown). After the addition of LDL (0.6 mg protein/mL) to the PRP, the fibrin generation rate tended to be prolonged (147±30% of the control value [100%], respectively; mean±SD on whole blood from 4 different donors).

To identify the component(s) of the VLDL particles causing the stimulation of the factor XIIa formation, we first separated the lipoproteins into aqueous and lipidic phases. The lipoprotein lipids were recovered and added to the PRP as microemulsions. The thrombin generation as determined
after 70 minutes was strongly stimulated (5.9- and 6.0-fold compared with the control in PRP of 2 different donors). In contrast, the addition of the aqueous phases to the PRP reduced the thrombin generation at this time point (0.51±0.20-fold versus control; mean±SD on PRP from 5 different donors). To further differentiate between the two main lipid fractions of the lipoproteins, namely neutral lipids and phospholipids, preparative TLC was used. Supplementation of dipalmitoyl-PC vesicles with all (recovered) lipids resulted in a 33% decreased lag period of thrombin formation (Figure 4a). The addition of PC vesicles supplemented with the VLDL phospholipids reduced the lag period by 37%, indicating activation of the contact system. Vesicles enriched with the VLDL neutral lipids did not affect the lag time of thrombin formation (Figure 4a).

These results led us to search for the active component within the VLDL phospholipids. We separated the different phospholipid classes by preparative TLC. The phospholipids of the lanes were re-isolated in a way that only one, respectively different fraction was left on the plate. Subsequently, the phospholipid fractions were mixed with dipalmitoyl-PC and tested for their influence on the activation of the contact system. Vesicles made from the total VLDL phospholipids lacking specifically phospholipid fraction A, selective removal of fraction B, and the absence of fraction C did not affect the lag time (Figure 4b). In contrast, in the presence of the VLDL phospholipid portion specifically deficient in fraction D, the lag time was prolonged by 31% (Figure 4b). As this result pointed to fraction D as major stimulator of the contact activation, in further experiments, fraction D consisting of PE (Methods) was isolated from the VLDL particles and incorporated into PC vesicles. The vesicles thus prepared shortened the lag period of thrombin formation by 41% (Figure 4b). To further substantiate the role of PE for the stimulation of the contact system of coagulation, vesicles were generated containing commercially obtained phospholipids. Vesicles composed of pure egg PC did not alter the lag period (Figure 5). The addition of vesicles containing 95 mol% egg PC plus 5 mol% lysoPC tended to

Figure 3. VLDL increases whole blood coagulation in vitro. Fibrin formation in whole blood (r value) was assessed by whole blood coagulometry. a and b, Control blood from a healthy donor. c and d, Blood from the patient with factor XII deficiency. b and d, Addition of VLDL (0.3 mg protein/mL). The r value shown in panel a corresponds to 930 s. Representative experiments of a total of 5 experiments (4 different donors; control) and 2 experiments (one donor; factor XII deficiency).

Figure 4. Effect of VLDL lipid fractions on thrombin generation by the contact system of coagulation. a, Lag time of thrombin synthesis in the PRP subsequent to addition of the indicated lipid fractions enriched in dipalmitoyl-PC vesicles. b, VLDL phospholipid preparations selectively deficient in the single phospholipid fractions indicated (A-D) were enriched in dipalmitoyl-PC vesicles and given to the PRP. Column denoted with D indicates addition of the isolated fraction D. Mean values of 4 experiments on PRP from 3 to 4 different donors. *P<0.05 versus control.

Figure 5. Activation of the contact system by PE-containing phospholipid vesicles. The indicated phospholipid vesicles consisted of 95 mol% egg PC plus 5 mol% egg PC (100% PC), 5 mol% lysoPC, or 5 mol% egg PE. Mean±SD on PRP of 3 to 5 different donors. *P<0.05 versus control.
prolong the generation of thrombin as evidenced by the prolonged lag time (Figure 5). In contrast, vesicles consisting of 95 mol% egg PC plus 5 mol% egg PE led to a 30% reduction of the lag period. Together, these results indicated that the lipoprotein associated ethanolamine phospholipids were mainly responsible for the activation of the contact system by VLDL.

Discussion

Hypertriglyceridemia is a well established risk factor for the development of atherosclerosis. Plasma triglyceride concentrations were earlier shown to be strongly associated with increased plasma concentrations of factor XIIa, the major initiator of the contact system. Importantly, the factor XIIa concentrations were also positively related to the extent of coronary sclerosis. Moreover, the plasma factor XIIa contents were also positively correlated to the plasma cholesterol concentration. In view of these findings we investigated the influence of the main plasma lipoproteins on the activation of the contact system in the PRP and in whole blood. After addition of the triglyceride-rich VLDL, the generation of thrombin in the PRP was enhanced, and the fibrin formation rate of whole blood was shortened. When LDL and HDL were added to the PRP, no effect was seen. In the presence of CTI, a specific inhibitor of factor XIIa, and with blood obtained from an individual lacking factor XII, the stimulation of thrombin and fibrin generation by VLDL was completely abolished. This demonstrated that VLDL augmented the activity of the contact system by increasing the factor XIIa activity. The results suggest that the previously observed positive associations of the plasma triglycerides to the factor XIIa concentration are of causal nature.

The triglyceride concentrations required to activate the contact system encompass the upper range of normal triglyceride concentrations and those encountered in mild hypertriglyceridemia. In line with these findings, factor XIIa levels were recently found to strongly correlate with plasma triglyceride concentrations not only in patients with hyperlipidemias but also within a control population. However, previous results indicate no increase in plasma factor XIIa antigen levels after an oral fat load leading to augmented plasma concentrations of triglyceride-rich lipoproteins. Furthermore, alimentary lipemia was found to augment the factor VII activity in individuals deficient in factor VII. However, the situation encountered during alimentary lipemia in vivo cannot be compared with the specific in vitro enrichment of plasma with VLDL particles as performed in the present study. Indeed, the oral fat load is expected to modify a great variety of different parameters (e.g., increased concentrations of chylomicrons, altered hormone levels) which might differentially affect the factor XIIa antigen levels. In addition, the increased formation of thrombin as observed in the present study after activation of the contact system by VLDL might not involve the activation of factor VHa, but rather proceed through alternate pathways.

To identify the components of the VLDL responsible for the activation of the contact system, we separated the particles into their water-soluble and lipid-soluble components. Microemulsions prepared from the particle lipids strongly enhanced the thrombin formation as induced by the contact system whereas addition of the water phases was without effect. Because this indicated that the active components were located in the lipid phases, the VLDL lipids were further separated into neutral lipids and phospholipids. The fraction of the total neutral lipids did not affect the thrombin generation elicited by the contact system. In contrast, the VLDL phospholipids had a marked stimulatory influence. In view of these observations, we searched for the active components within the different types of phospholipids present in VLDL. The particle phospholipids were re-isolated in such a way that only one phospholipid fraction was removed at a time. After the selective removal of lysoPC from the total VLDL phospholipids, the thrombin generation as triggered by factor XIIa was enhanced. With VLDL phospholipids lacking either SM or PC plus PI, the contact system–dependent generation of thrombin was unaffected. Thus, SM, PC, and PI were unlikely to be the active components within the VLDL phospholipids. However, in vesicles selectively devoid of PE, the activation of the contact system was clearly reduced. The role of PE as the active principle of the particles was reinforced by experiments with pure phospholipid vesicles. Indeed, PC vesicles supplemented with PE stimulated the contact system dependent thrombin formation whereas PC alone was without effect.

Accordingly, PE was identified as the main activating component of the VLDL particles. VLDL is indeed known to be the particle with the highest percentage of PE among the main plasma lipoproteins. This may explain why LDL and HDL were unable to enhance the activity of the contact system. It is rather unlikely that PS contributed to the activation elicited by VLDL because the PS content of the VLDL particles is exceedingly low (<0.4%). Furthermore, the VLDL phospholipid fraction III containing (trace amounts of) PS did not alter the thrombin synthesis elicited by the contact system. In several recent studies, PE has been shown to stimulate the activity of proteases involved in blood coagulation. This holds for the protein C–mediated inactivation of factor Va and VIIIa, for the prothrombinase complex of the platelet surface, and for the activation of the tenase complex. Interestingly, high-molecular-weight kininogen and prekallikrein, main components of the contact system, were previously characterized as PE-binding proteins. Their PE-binding properties are responsible for recognition by certain anti-PE autoantibodies.

Stimulation of factor XII activation by VLDL has already previously been noted in investigations performed with the isolated lipoproteins and contact pathway components in the absence of plasma. Treatment of VLDL with lipoprotein lipase was found to be necessary to evoke the increased factor XII cleavage, and free fatty acids were proposed as active principles. In our study, the VLDL-neutral lipids containing the free fatty acids did not stimulate the activation of the contact system in PRP. However, this does not exclude free fatty acids as activating components because their stimulating effect might have been masked by other neutral lipids. Furthermore, different lipid species of the VLDL particles could be responsible for the stimulation of the factor XII cleavage in the presence and absence of plasma, respectively. This could indicate that additional plasma components are involved in the activation of the contact system as elicited by VLDL-associated PE. The failure of VLDL to promote the generation of thrombin in the absence of the platelets (see
Results) could indicate that interactions of components of the contact pathway with the platelet surface are relevant. Indeed, proteins of the contact system of coagulation were recently shown to bind to the platelet surface with considerable affinity.\(^{31,32}\) Alternatively, the results could be explained by assuming that inhibitors of the contact pathway of coagulation are active in PPP, but not in PRP. In conclusion, our results show that activation of blood coagulation by the contact system as determined in vitro in the PRP and in whole blood is enhanced by VLDL. We could identify the VLDL-associated PE as the main activator of the contact pathway. The results help explain the well known association of the factor XIIa concentration with the plasma concentration of triglyceride-rich lipoproteins.

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