Pergamon

Thrombosis Research, Vol. 80, No. 1, pp. 35–46, 1995

0049-3848/95 $9.50 + .00

0049-3848(95)00148-4

BINDING OF PROTHROMBIN TO CHYLE CHYLOMICRONS: EFFECTS OF TEMPERATURE AND CALCIUM IONS, AND ROLE OF SURFACE PHOSPHOLIPIDS

[1]Ning Xu, [2]Ann-Kristin Öhlin, [1]Li Zhou, and [1]Åke Nilsson
[1]Department of Medicine and [2]Department of Clinical Chemistry, University Hospital of Lund, S-221 85 Lund, Sweden

(Received 19 June 1995 by Editor V. Rasi, revised/accepted 24 July 1995)

ABSTRACT

The ability of chyle chylomicrons to bind prothrombin has been studied. Rat chyle chylomicrons were incubated with human ^{125}I-prothrombin and binding was examined by separating the chylomicrons from free ^{125}I-prothrombin by density-gradient ultracentrifugation, and by gel filtration on Sepharose CL-2B. A significant binding of prothrombin to chyle chylomicrons occurred. The complex formation was calcium dependent, and decreased markedly when the temperature was lowered from 37 °C to 20 °C and when pH was raised above 8. The time course for the binding at 37 °C in presence of 2 mmol/L $CaCl_2$ exhibited an initial lag phase at about 10 minutes. Thereafter most of the binding occurred within 30 minutes. Bound prothrombin could not be removed from chyle chylomicrons by treatment with EDTA, suggesting that this binding is not a simple Ca^{2+} dependent association between prothrombin and chyle chylomicrons. Inclusion of 1% purified human serum albumin caused a 50% decrease in binding, half of which was reversed by increasing the Ca^{2+} ion concentration. Addition of pancreatic phospholipase A_2 (PLA_2) in doses sufficient to hydrolyze more than 95% of the phosphatidylethanolamine (PE) and 37% of the phosphatidylcholine (PC) decreased the binding by 50%. Doses of PLA_2 that hydrolyzed 60-80% of PE and 4-10% of the PC decreased the binding by only 7-15%. It is suggested that the binding of prothrombin to chyle chylomicrons is in part mediated by negatively charged phospholipids of the chylomicron surface, although a specific role of the PE could not be demonstrated.

Key words: blood coagulation factors, lipoproteins, phospholipids, hypertriglyceridemia, atherosclerosis.

Corresponding author: Dr. Åke Nilsson, Department of Medicine, University Hospital of Lund, S-221 85, Lund, Sweden.

Hypertriglyceridemia is a common lipoprotein abnormality in patients with increased risk of ischemic heart disease (IHD). Moreover, evidence suggests that hypercoagulability is associated with elevated triglycerides and this may promote myocardial infarction in the hypertriglyceridemic subjects (1-3). An increase in factor VII has also been found to be a risk factor for IHD (4-7). Recent observations indicated that all four of the vitamin K-dependent blood coagulation factors, i.e., factor II, VII, IX and X are higher in high-risk IHD subjects when compared with low-risk subjects (8). Persistent hypertriglyceridemia is associated not only with an increase in factor VII activity, but also with increases in the concentration of factor VII, IX, X and prothrombin (4). The basis of these associations is not yet clear.

Previous work from our laboratory suggested that rat chyle chylomicrons that had been preincubated with rat or human plasma or purified human prothrombin, i.e., chylomicron-prothrombin complexes, can induce platelet aggregation (9). Bajaj et al demonstrated that VLDL phospholipids supported the conversion of prothrombin to thrombin by factor Xa (10). Simpson et al found an increase in postprandial triglycerides in patients with coronary heart disease compared to controls (11). Treatment of hypertriglyceridemic patients that leads to a lowering of triglycerides accompanied by a lowering of factor VII also reduced prothrombin activation peptide concentration by 25% (12). It has been shown that prothrombin and factor Xa may bind to very low-density lipoproteins (VLDL), partially through a Ca^{2+} dependent association (13,14). The detailed mechanism of the binding of vitamin K-dependent blood coagulation factors to triglyceride-rich lipoproteins is unknown. However, prothrombin has been demonstrated to be bound to artificial phospholipid vesicles (15-17), the binding to vesicles containing both phosphytidylcholine (PC) and phosphatidylserine (PS) or phosphatidic acid (PA) being more efficient than the binding to pure phosphatidylcholine (PC) liposomes (18). Binding of prothrombin to membrane surfaces is essential for biological activation, but the role of plasma lipoprotein phospholipids in binding and activation of prothrombin and other vitamin K-dependent blood coagulation factors is not known.

In the present study, we used rat chyle chylomicrons which contain more PE than circulating plasma lipoproteins (19) and small amounts of PA and PS as well, to examine the interaction between triglyceride-rich lipoproteins and prothrombin. The effects of varying Ca^{2+} concentration, incubation time, temperature, pH, and albumin concentration on the binding of prothrombin to chyle chylomicrons were determined. Furthermore the effect of exposure of chyle chylomicrons to human pancreatic phospholipase A_2 was studied.

MATERIALS AND METHODS

Materials: Highly purified human plasma-derived prothrombin was obtained from DIA-Service, Göteborg, Sweden, or from Enzyme Research Laboratories, South Bend, USA. [1,2-^{14}C]-Ethanolamine hydrochloride (1 mCi /mmol, NEC-038) and ^{125}iodine (17 Ci / mg, NEZ-033) were from Du Pont NEN Research Products, USA. Iodo-Gen iodinating reagent was from Pierce Europe, Holland. Sephadex G-25 column PD-10 was from Pharmacia, Uppsala, Sweden. Intralipid (20%, w/v) was from Kabi Vitrum AB, Stockholm, Sweden. Triglycerides GPO-PAP kit and total cholesterol CHOL kit were obtained from Boehringer Mannheim Test-combination, Germany, and phospholipase D-cholineoxidase-PAP kit from Wako Chemicals GmbH, Germany. Sepharose CL-2B and Pharmacia Column C 16/20 were from Pharmacia, Uppsala, Sweden. Human pancreatic phospholipase A_2 (PLA_2) purified to homogeneity from human pancreas (20) was a kind gift from Dr. B. Sternby, Dept. of Medicine, University

Hospital of Lund, Sweden. Highly purified human serum albumin was from Kabi Pharmacia AB, Lund, Sweden. Other chemicals and reagents were from Sigma. Male Sprague-Dawley rats (Mφllegaard Ltd, Skensved, Denmark), weighing 250-280 g, were housed in mesh stainless-steel cages at a constant room temperature (22 °C) with a 12-hours light-dark cycle and provided standard rodent chow (ALTROMIN NR. 324, Altromin Spezialfutter-werke, GmbH, Germany) and water ad libitum. All the animal work was conducted in compliance with the recommendations of "the Guide for the Care and Use of Laboratory Animals" and approved by the Lund University Medical Faculty Animal Care Committee.

Preparation of rat chyle chylomicrons: The mesenteric lymph duct of rats which had been fasted for 24 hours was cannulated and a gastric fistula was inserted (21). Twenty four hours after surgery, 3 mL of Intralipid were infused through the gastric fistula during one hour. The chyle was collected for 6 hours on ice in the presence of Na_2EDTA (final concentration was 2 mmol/L). Chyle chylomicrons were floated by density-gradient ultracentrifugation at 25,000 rpm for 2 hours at 4 °C using a Beckman SW 40 Ti Swing out rotor after the chyle was adjusted to 1.006 kg /L with 188 mmol/L NaCl solution containing 1 mmol/L Na_2EDTA. The chyle chylomicrons were extensively dialyzed against phosphate buffered saline (PBS, 2.7 mmol/L KCl, 137 mmol/L NaCl, 8.1 mmol/L Na_2HPO_4, 1.5 mmol/L KH_2PO_4, pH 7.4) which was used in all experiments except the buffer mentioned. Triacylglycerol (TG), total cholesterol and total phospholipids in chyle chylomicrons were determined by enzymatic kit methods according to the manufacturer's protocols. Phospholipid classes in chyle chylomicrons were separated by high performance liquid chromatography (HPLC) as described below. ^{14}C-PE labelled chylomicrons were prepared by the same procedure except that ^{14}C-ethanolamine hydrochloride (50 μCi) was added to the Intralipid meal infused, and chyle was collected for 4 hours. 2-5 % of the given ^{14}C was incorporated into chyle PE.

Procedures for iodination of prothrombin and incubation with chyle chylomicrons: Human prothrombin was iodinated with ^{125}iodine by the Iodo-Gen method according to the manufacturer's protocol. Iodinated prothrombin was separated from free iodine by gel filtration on a Sephadex G-25 column PD-10 and was then dialyzed against PBS for 24 hours. The specific radioactivity of iodinated prothrombin was 600-1000 cpm / ng. The purity of the ^{125}I-prothrombin was determined by SDS-PAGE with silver staining demonstrating only one visible band on SDS-PAGE. Autoradiography of the SDS gels demonstrated radioactivity only in the prothrombin containing band (9). Generally, 1 mL of chyle chylomicrons containing 10 mg TG was incubated with 1 μg of ^{125}I-prothrombin at the temperature, pH, incubation time and $CaCl_2$ concentrations given in the figures, using ultracentrifuge tubes (Polyallomer, 14x95 mm, Beckman). After incubation, 1 mL of a stock solution of KBr-NaCl (d=1.35) was added and the mixture was layered under 188 mmol/L NaCl (d=1.006) containing 1 mmol/L Na_2EDTA, and the tubes were then ultracentrifuged for 3 hours at 25,000 rpm in Beckman L5-65 ultracentrifuge. The top layer (about 2 mL) containing more than 95 % of the added chyle chylomicrons was collected. Radioactivity was determined in a gamma counter (1272 Clinigamma, Automatic Gamma Counter, Wallac Service, Finland) and the percentage binding of ^{125}I-prothrombin to chyle chylomicrons was calculated. This procedure was used to study the time course (figure 2), the effect of different concentrations of $CaCl_2$ (figure 3), of temperature (figure 5), of pH (figure 6) and of different concentrations of purified human serum albumin. The specific binding of ^{125}I-prothrombin to chyle chylomicrons was examined by incubation of different amounts of ^{125}I-prothrombin (figure 7) with chyle chylomicrons, or 1 μg ^{125}I-prothrombin with chyle chylomicrons in presence of different amounts of unlabelled

prothrombin (figure 8) at 37 °C for 30 minutes. To test whether the binding of ^{125}I-prothrombin to chylomicrons was stable during washing of chylomicron-prothrombin complexes by density ultracentrifugation in presence of EDTA, 1 mL of chylomicron-prothrombin complexes containing 8 mg TG which was obtained as described above and aliquots were mixed with different amounts of Na_2EDTA, for 5 minutes at room temperature with gentle shaking. 1 mL of NaBr-NaCl solution (d=1.35) was then added. The mixtures were layered under 188 mmol/L NaCl containing 1 mmol/L Na_2EDTA or no Na_2EDTA in case of the controls, and were then ultracentrifuged for 3 hours as described above. The radioactivity of the added amount of chylomicron-prothrombin complexes was considered 100 %. The recovery of the radioactivity in the chylomicron fraction after washing was compared with the control and was expressed as percentage of control.

Effect of pretreatment of chyle chylomicrons with PLA_2: One mL of chyle chylomicrons containing 10 mg TG was preincubated with different amounts of PLA_2 at 37 °C for 30 minutes in the presence of 2 mmol/L $CaCl_2$ in PBS. After preincubation 1 μg ^{125}I-prothrombin was added and the chylomicrons were then incubated for another 30 minutes at 37 °C. In another experimental series the chyle chylomicrons were preincubated with different amounts of PLA_2 at 37 °C for 30 minutes in presence of 2 mmol/L of $CaCl_2$ in PBS. The preincubated chylomicrons were separated by ultracentrifugation and were then incubated with 1 μg ^{125}I-prothrombin at 37 °C for 30 minutes in presence of 2 mmol/L of $CaCl_2$. The ^{125}I-prothrombin-chylomicron complexes were separated by density-gradient ultracentrifugation as described above. There was no change in pH in the incubation after added PLA_2. For determining the hydrolysis of chylomicron phosphatidylethanolamine (PE) which is a preferred substrate for PLA_2 (22) and phosphatidylcholine (PC), 1 mL of chyle chylomicrons (labelled with ^{14}C-ethanolamine in the PE portion) was incubated without (control) or with different amounts of PLA_2 at 37 °C for 30 minutes in presence of 2 mmol/L $CaCl_2$. After incubation the lipids were extracted by chloroform / methanol (1:2) containing 0.005% butylated hydroxytoluene. The extract was treated as described earlier (23). Phospholipid classes were separated by HPLC using a Spectra Physics SP 8750 system with Shimadzu SPD-2A UV detector, equipped with a Nucleosil 50-5, 250-mm silica column (inner diameter 4.6 mm)(Genetec). The mobile phase was water / sodium phosphate buffer (1 mol/L, pH 7.0) / acetic acid / 2-propanol / hexane / ethanol (55:1.2:0.6:485:370:100, v/v), and the flow rate 1.0 mL / minute (24). The masses of PE and PC were calculated and the degree of hydrolysis of PE and PC estimated as percent decrease in peak area compared to chylomicrons that had not been exposed to PLA_2. Furthermore hydrolysis of PE was estimated from measuring ^{14}C in PE and lyso-PE by using continuous flow liquid scintillation counting using a Radiomatic Flow-One β- radioactivity detector as described earlier (25).

Separation of chylomicron-^{125}I-prothrombin complexes by gel filtration: Sepharose CL-2B was packed in a Pharmacia Column C 16/20 and equilibrated with PBS. One mL of chyle chylomicrons containing 10 mg TG was incubated with 1 μg of ^{125}I-prothrombin at 37 °C, pH 7.4, in presence of 2 mmol/L $CaCl_2$ in PBS for 30 minutes. After incubation the mixture was applied to Sepharose CL-2B column and eluted by PBS at 4 °C. Two mL fractions were collected, and the radioactivity and TG concentration were measured. The chylomicron-^{125}I-prothrombin complexes and free chyle chylomicrons were eluted in the void volume of the column.

Statistical analysis: Values are reported as the mean ± standard error (SEM). Data were analyzed by using a statistical software package in a IBM personal computer. One-way analysis of variance (ANOVA) followed by unpaired Student's *t*-test was employed for statistical analysis. A p value < 0.05 in a two-tailed test was considered significant.

RESULTS

Lipid composition of chyle chylomicrons and separation of chylomicron-prothrombin complexes: The lipid composition of the chyle chylomicrons was 83-86 % of TG, 4-6 % of cholesterol and 8-10 % of phospholipids. Of the phospholipids about 80% was PC and 12% PE, the rest was PA, PI, PS and lyso-PC (Table I). As reported previously (9), chylomicron-prothrombin complexes and free prothrombin could be separated by density-gradient ultracentrifugation at 25,000 rpm for 3 hours at 4 °C using a Beckman SW 40 Ti swing bucket rotor at d=1.006 (188 mmol/L NaCl with 1 mmol/L Na_2EDTA). There was not any difference in the percentage binding whether the incubation mixtures were layered under 188 mmol/L NaCl with 1 mmol/L Na_2EDTA or without Na_2EDTA. More than 95% of added chylomicrons were recovered in the top layer (about 2 mL) as estimated by the determination of TG and the free prothrombin in the infranatant. During gel filtration on Sepharose CL-2B to separate the chylomicron-bound and free prothrombin at pH 7.4, the chylomicron-prothrombin complexes and free chyle chylomicrons were eluted with the void volume, which also contained virtually all of the TG (Figure 1). The percentage binding of ^{125}I-prothrombin to chyle chylomicrons separated by gel filtration did not differ from that seen after separation by ultracentrifugation, when the same incubation conditions were used (data not shown). We therefore regularly used the density gradient ultracentrifugation.

TABLE I
Lipid composition of chyle chylomicrons (Mean ± SEM, n=3-5)

Triglycerides:	11.38 ± 0.12 mmol/L
Total cholesterol:	0.91 ± 0.04 mmol/L
Total phospholipids:	1.21 ± 0.03 mmol/L
Phospholipid classes in chyle chylomicrons (%)	
Phosphatidic acid (PA):	2.09 ± 0.85
Phosphatidylethanomine (PE):	11.92 ± 0.45
Phosphatidylinositol (PI):	0.82 ± 0.14
Phosphatidylserine (PS):	3.97 ± 0.12
Phosphatidylcholine (PC):	80.96 ± 0.45
Lyso-phosphatidylcholine (Lyso-PC):	0.35 ± 0.14

Time course for the binding of prothrombin to chyle chylomicrons: The time course for the binding is shown in figure 2. After 30-60 minutes incubation the main part of the added ^{125}I-prothrombin was bound indicating that the binding of prothrombin to chyle chylomicrons occurred with a high affinity. It exhibited an initial lag phase of about 10 minutes during which little binding occurred. More than 70 % of the maximal binding then occurred between 10 and 30 minutes, and thereafter there was only a limited increase in binding up to 60 minutes. Thirty

minutes was therefore used as the incubation time in the studies of other characteristics of the binding.

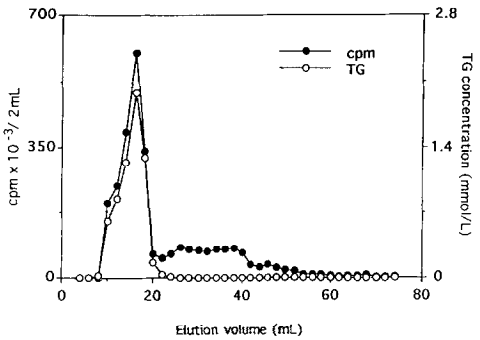

FIG. 1.

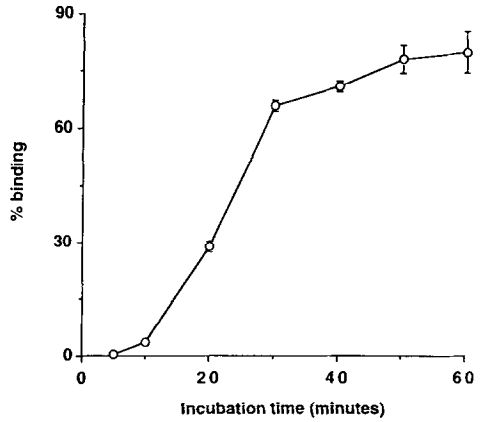

FIG. 2.

Fig. 1 shows gel chromatography of chyle chylomicrons that were incubated with ^{125}I-prothrombin. Two mL fractions were collected for determining the radioactivity (●—●) and the concentration of triglycerides (○—○). The data shown are from one of three similar experiments.

Fig. 2 shows time course for the binding of ^{125}I-prothrombin to chyle chylomicrons. One mL of chyle chylomicrons was incubated with 1 μg ^{125}I-prothrombin at 37 °C, pH 7.4, in the presence of 2 mmol/L $CaCl_2$. Data are expressed as the mean ± SEM (n=3-6).

Effects of calcium ions and albumin: In the first series of experiments, ^{125}-I radiolabelled prothrombin was incubated with chyle chylomicrons at different concentrations of $CaCl_2$. In the absence of $CaCl_2$ the percentage binding was very low. Concentrations below 1 mmol/L Ca^{2+} had little effect on the binding, whereas an increase in binding occurred when the Ca^{2+} concentration was increased to 1.5 mmol/L, and reach a plateau when calcium was 2 to 8 mmol /L (Figure 3). When the $CaCl_2$ concentration was increased to more than 8 mmol/L, there were some aggregates formed in the incubation solution leading to a decrease of the binding. Although EDTA prevented the Ca^{2+}-dependent binding, when present during the incubation, repeated washing in saline containing 2 mmol/L Na_2EDTA did not removed the ^{125}I-prothrombin from the chylomicrons, once it had been bound during incubation in presence of Ca^{2+}. Washing in presence of up to 10 mmol/L Na_2EDTA decreased the binding by only about 20% or less (data not shown). Albumin also influence the binding. There was a decrease in binding when the concentration of albumin was increased in presence of 2 mmol/L $CaCl_2$. 1% albumin decreased the binding by 50%. There was a significant correlation between albumin concentration and the percentage binding ($r=0.97$, $p<0.0001$). As shown in figure 4, increasing the concentration of $CaCl_2$ in the presence of 1% albumin increased the binding, but did not fully restore it to the level seen with 2 mmol/L $CaCl_2$ without albumin present.

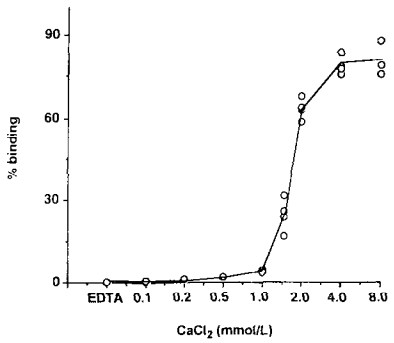

FIG. 3.

Fig. 3 shows the effect of calcium ion concentration on the binding of ^{125}I-prothrombin to chyle chylomicrons. Data are expressed as the percentage binding (n=2-5).

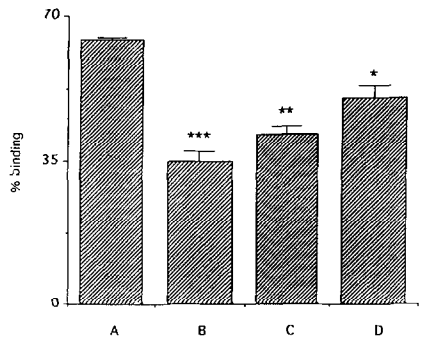

FIG. 4.

Fig. 4 shows the effect of calcium ion concentration on the binding of prothrombin to chyle chylomicrons in the presence of 1% human serum albumin. A, without albumin and with 2 mmol/L $CaCl_2$; B-D, with 1% albumin and different amounts of $CaCl_2$ (B, 2 mmol/L; C, 5 mmol/L; D, 10 mmol/L). Data are expressed as means ± SEM (n=3-4). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. A).

Effects of temperature and pH: The binding also exhibited a strong temperature dependence. Lowering the temperature from 37 °C to 20 °C or 4 °C decreased the binding by more than 90% (Figure 5). Also pH influenced the binding, which was similar at neutral and slightly acidic pH, but decreased at alkaline pH (Figure 6). There was thus no change in the binding of prothrombin to chylomicrons when the pH was varied between 6.0 and 7.8 (data not shown).

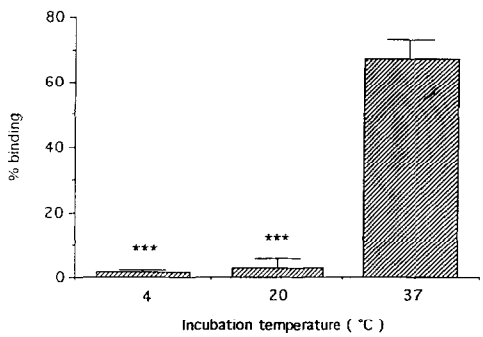

FIG. 5.

Fig. 5 shows the temperature influence on the binding of ^{125}I-prothrombin to chyle chylomicrons. One mL of chyle chylomicrons containing 10 mg TG was incubated with 1 µg ^{125}I-prothrombin at 4 °C, 20 °C or 37 °C, pH 7.4, in presence of 2 mmol/L $CaCl_2$ for 30 minutes. Data are expressed as means ± SEM (n=4). (*** $p < 0.0001$, vs. 37 °C)

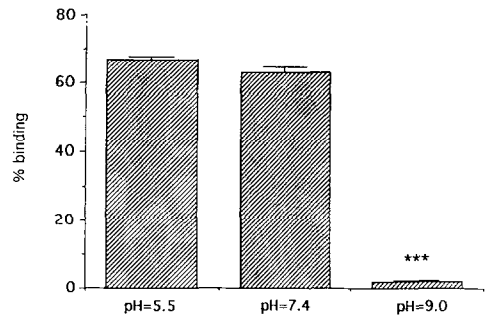

FIG. 6.

Fig. 6 shows the effect of pH on the binding of ^{125}I-prothrombin to chyle chylomicrons. At pH 5.5 KH_2PO_4-NaOH buffer (100 mmol/L KH_2PO_4, 73 mmol/L NaOH) was used and at pH 9.0 Tris-HCl buffer (100 mmol/L Tris, 11.4 mmol/L HCl). Data are expressed as means ± SEM (n=4). (*** $p < 0.0001$, vs. pH=7.4)

Specific binding of ^{125}I-prothrombin to chyle chylomicrons: As shown in figure 7, the percent binding of ^{125}I-prothrombin to chyle chylomicrons were decreased when the amounts of ^{125}I-prothrombin were increased. The unlabelled prothrombin could significantly inhibit the binding of ^{125}I-prothrombin to chyle chylomicrons (figure 8).

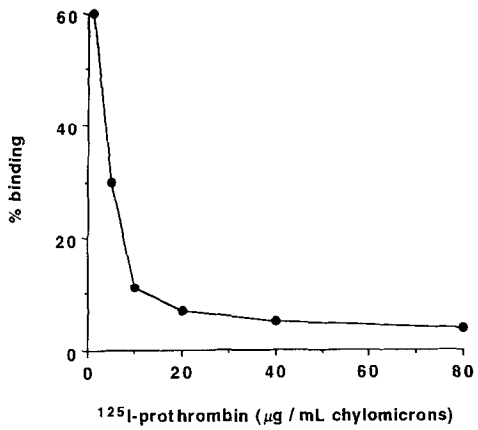

FIG. 7.

Fig. 7 shows percent binding of ^{125}I-prothrombin to chyle chylomicrons in different amounts. Data are from one of two similar experiments.

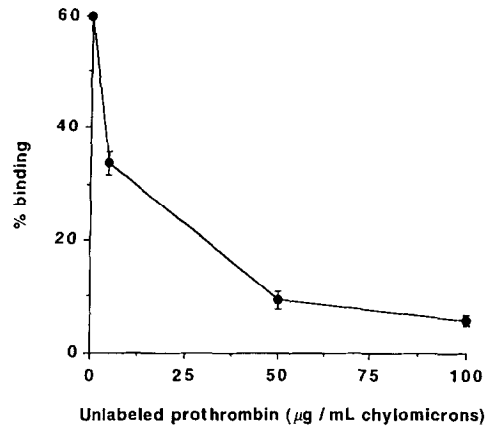

FIG. 8.

Fig. 8 shows the effect of unlabelled prothrombin on the binding of ^{125}I-prothrombin to chyle chylomicrons. Data expressed as means ± SEM (n=3).

Effect of PLA_2 on the binding of prothrombin to chyle chylomicrons: After pre-treatment of chyle chylomicrons with PLA_2 the percentage binding of prothrombin to the chylomicrons was decreased in a dose dependent manner. When the PLA_2 concentration was 40 µg / mL, the binding was 50 % of the binding observed in control incubations without PLA_2 (Figure 9). Figure 10 shows the hydrolysis of PE and PC by PLA_2. There was a decrease of PE and PC when increasing the concentration of PLA_2. At all concentrations of PLA_2 the degree of hydrolysis of PE exceeded , however, that of PC. With a PLA_2 concentration of 40 µg / mL more than 95 % of PE, but only 37% of the PC was hydrolyzed. Even with 1.6 and 8.0 µg PLA_2 / mL a major part of the PE but only 4 and 10% of the PC was hydrolyzed (figure 10).

Despite the extensive PE hydrolysis the degree of inhibition of the prothrombin binding with these PLA_2 concentrations was modest (figure 9). Values for the degree of PE hydrolysis obtained by measuring ^{14}C in PE and lyso-PE by HPLC were very similar to these calculated from the decrease in UV absorbance of the PE peak after PLA_2 exposure (data not shown). The degree of hydrolysis of PS and PA could not be accurately determined due to the low levels, and because lyso-PE formed overlapped the PS peak.

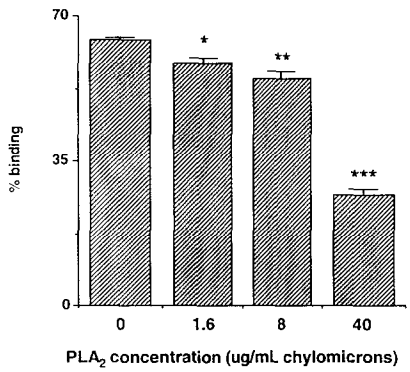

FIG. 9.

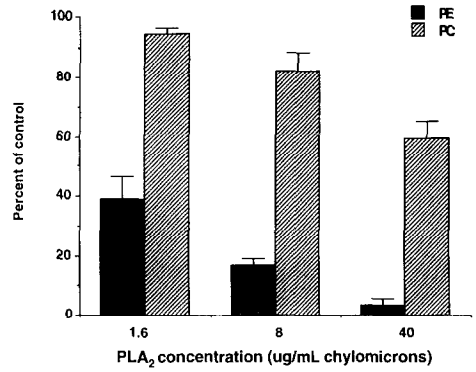

FIG. 10.

Fig. 9 shows the effect of pretreatment of chyle chylomicrons with PLA_2 on the binding of prothrombin to chyle chylomicrons. Data are expressed as means ± SE (n=4-6). (* $p < 0.05$; ** $p < 0.01$; *** $p = 0.002$, vs. no PLA_2).

Fig. 10 shows the hydrolysis of PE and PC of chyle chylomicrons by PLA_2. Data (means ± SE, n=5) are expressed as percentage decrease in PE or PC levels compared to the levels of control chylomicrons not exposed to PLA_2 which were set to 100 %.

DISCUSSION

Factor VII, IX, X and prothrombin, as well as protein C and its co-factor protein S are synthesized in the liver. They show considerable sequence homology and all undergo a post-translational vitamin K-dependent step to acquire γ-carboxyglutamic acid residues which forms medium affinity calcium binding sites (17,26). In some hypertriglyceridemic subjects, not only factor VII but also the other procoagulant vitamin K-dependent proteins are increased in plasma (27). It has been reported that the increased vitamin K-dependent blood coagulation factors co-circulate with triglyceride-rich lipoproteins. Bradley and Gianturco who used Western blotting and a competitive radioimmunoassay specific for prothrombin demonstrated that large VLDL of hypertriglyceridemic patients contained prothrombin (13). They also demonstrated that prothrombin can bind to plasma VLDL in vitro at physiologic concentration of VLDL, prothrombin and Ca^{2+}, whereas binding to LDL and HDL was negligible (13,14). In this study we examined binding of prothrombin to native chyle chylomicrons, which contain more phospholipids and a higher proportion of PE in the polar surface coat (28), than particles that have been exposed to plasma and lipoprotein lipase.

The results show that human plasma derived prothrombin bind to rat chyle chylomicrons with a high affinity, saturable, and in a calcium dependent manner. The binding was influenced by temperature and pH, a very low binding observed when the temperature was decreased to 20 °C (Figure 5) or pH raised till 9 (Figure 6). Binding studies with both different concentrations of ^{125}I-prothrombin, and with a constant amount of ^{125}I-prothrombin and varying amounts of unlabelled prothrombin, showed that the binding of prothrombin to chyle chylomicrons is saturable and competed by addition of cold prothrombin (Figure 8 and 9). The binding was decreased by purified human serum albumin, which could only in part be attributed to a decrease of free calcium ions. Increasing the Ca^{2+} ion concentration in presence of albumin thus did not fully restore the binding of prothrombin to the level observed without albumin

present (Figure 4). It was previously reported that the binding of prothrombin to phospholipid vesicles needs a Ca^{2+} dependent two-step conformational change of the prothrombin (15) and that the prothrombin-phospholipid complexes were tightly bound (18). In this study we found that most of the binding of prothrombin to chyle chylomicrons was not dissociated when washed in up to 10 mmol/L EDTA. The data thus indicate that it is not due to a simple electrostatic association, but may rather be related to a conformational change of prothrombin during the interaction with calcium ions and phospholipids of the chyle chylomicrons (15). Such a mechanism might also explain the strong temperature dependence. Bull et al reported that prothrombin was tighter bound to PS-PA vesicles than to PS-PC vesicles when examined by treatment with EDTA (18). Although PC is the major phospholipid in chyle chylomicrons the surface coat of these particles contain both PA and PS (Table 1) and also a high proportion of PE compared to the plasma lipoproteins (19). Furthermore the phospholipid / apolipoprotein ratio of the chylomicrons surface coat is significantly higher than in the circulating high-density lipoproteins to which most of surface material is transferred during the metabolism of the chylomicrons (29). In our PLA_2 experiments there was a decrease in the binding after hydrolysis of some of the chyle chylomicron phospholipids. At a high concentration of PLA_2 more than 95 % of PE were hydrolyzed, whereas the binding was only decreased by 50 %. At lower PLA_2 concentrations 60-80% of the PE but only 4-10% of PC were hydrolyzed (figure 10), which caused only 7-15 % decrease in binding (figure 9). Although the data demonstrate that the binding of prothrombin to chyle chylomicrons may in part be mediated by the negatively charged phospholipids which form a hydrophilic surface of the chyle chylomicrons a specific role of PE could not be demonstrated.

The evidence obtained in another study (30) demonstrated that the chylomicron-prothrombin complexes that were formed during incubation of native chyle chylomicrons with human prothrombin may cause platelet activation in vitro including platelet aggregation, serotonin release, arachidonic acid release and increase of cytosolic free calcium. Furthermore this effect could be inhibited by a chemical compound that is a specific inhibitor (TenStop) of blood coagulation factor Xa (31,32). The data thus strongly suggest that in vitro the binding of prothrombin to chyle chylomicrons may accelerate a factor Xa dependent prothrombin activation and platelet aggregation.

In summary, the data demonstrate that chylomicrons are able to bind prothrombin in vitro in a Ca^{2+} dependent manner. The chylomicron bound prothrombin could be activated by the platelet factor Xa (30). However, it is, at the present stage, hard to evaluate the physiological importance of this binding. Although the data indicate that native chyle lipoprotein can bind prothrombin strongly, there was a lag phase of about 10 minutes at 37 °C before significant binding occurred, i.e. a time sufficient to metabolize most chylomicrons in vivo. Further studies focused on the question how the initiation of the lipolysis and the transfer of chylomicron surface material influences prothrombin binding during the initial phase of the chylomicron metabolism are therefore warranted.

ACKNOWLEDGMENTS

This research was supported by grants from the Swedish Medical Research Council (Nr -3969), the Nutrition Foundation of the Swedish Margarine Industry, the Anna and Sven-Erik Lundgren Foundation, the Albert Påhlsson Foundation, the Crafoord Foundation, and the Medical Faculty, University of Lund.

REFERENCES

1. HAMSTEN, A., WALLDIUS, G., DAHLEN, G., JOHNSSON, B., and DE FAIRE, U. Serum lipoproteins and apolipoproteins in young male survivous of myocardial infarction. Atherosclerosis 59,223-235,1986.
2. STEINER, G. Triglyceride-rich lipoproteins and atherosclerosis from fast to feast. Ann Med 25,431-435,1993.
3. ELKELES, R.S., CHAKRABARTI, R., VICKERS, M., STIRLING, Y., and MEADE, T.W. Effect of treatment of hyperlipidaemia on haemostatic variables. Br Med J 281,973,1980.
4. MILLER, G.J. Hyperlipidaemia and hypercoagulability. Prog Lipid Res 23,61-67,1993.
5. SILVEIRA, A., KARPE, F., BLOMBLÄCK, M., STEINER, G., WALLDIUS, G., and HAMSTEN, A. Activation of coagulation factor VII during alimentary lipemia. Arterioscler Thromb 14,60-69,1994.
6. BALLEISEN, L., SCHULTE, H., ASSMANN, G., EPPING, P.H., and VAN DE LOO, J. Coagulation factors and the progress of coronary heart disease [letter]. Lancet 2,261,1987.
7 The ARIC investigators. The atherosclerosis risk in communites (ARIC) study: design and objectives. Am J Epidemiol 129,687-702,1989.
8. BUENS, P., HOFFMAN, C.J., KATZ, J.P., MILLER, R.H., LAWSON, W.E., and HULTIN, B.M. Vitamin K-dependent clotting factors are elevated in young adults who have close relatives with ischemic heart disease. J Lab Clin Med 122,720-727,1993.
9. XU, N., ÖHLIN, A-K., and NILSSON, Å. Chylomicron induced prothrombin activation and platelet aggregation. Arterioscler. Arterioscler Thromb 14,1014-1020,1994.
10. BAJAJ, S.P., HARMONY, J.A.K., MARTINEZ-CARRION, M., and CASTELLINO, F.J. Human plasma lipoproteins as accelerators of prothrombin activation. J Biol Chem 251,5233-5236,1976.
11. SIMPSON, H.S., WILLIAMSON, C.M., OLIVECRONA, T., PRINGLE, S., MACLEAN, J., LORIMER, A.R., BONNEFOUS, F., BOGAIEVSKY, Y., PACKARD, C.J., and SHEPHERD, J. Postprandial lipemia, fenofibrate and coronary artery disease. Atherosclerosis 85,193-202,1990.
12. WILKES, H.C., MEADE, T.W., BARZEGAR, S., FOLEY, A.J., HUGHES, L.O., BAUER, K.A. ROSENBERG, R.D., and MILLER, G.J. Gemfibrozil reduces plasma prothrombin fragment F1+2 concentration, a marker of coagulability, in patients with coronary heart disease. Thromb Haemost 67,503-506,1992.
13. BRADLEY, W.A., SONG, J.N., and GIANTURCO, S.H. Thrombin / prothrombin interactions with very low density lipoproteins. Ann NY Acad Sci 485,159-169,1986.
14. BRADLEY, W.A., and GIANTURCO, S.H. Vitamin K-dependent proteins bind to very low density lipoproteins. Semin Thromb Hemost 14,253-257,1988.
15. BOROWSKI, W., FURIE, B.C., BAUMINGER, S., and FURIE, B. Prothrombin requires two sequential metal-dependent conformational transitions to bind phospholipid. J Biol Chem 261,14969-14975,1980.
16. TENDIAN, S.W., and LENTZ, B.R. Evaluation of membrane phase behavior as a tool to detect extrinsic protein-induced domain formation: Binding of prothrombin to phosphatidylserine / phosphatidylcholine vesicles. Biochemistry 29,6720-6729,1990.
17 BOROWSKI, M., FURIE, B.C., GOLDSMITH, G.H., and FURIE, B. Metal and phospholipid binding properties of partially carboxylated human prothrombin varients. J Biol Chem 260,9258-9264,1985.

18. BULL, R.K., JEVONS, S., and BARTON, P.G. Complexes of prothrombin with calcium irons and phospholipids. J Biol Chem 247,2747-2754,1972.
19. LANDIN B., and NILSSON, Å. Metabolism of chylomicron phosphatidylethanolamine in the rat. Biochim Biophys Acta 793,105-113,1984.
20. VERHEIJ, H.M., WESTERMAN, J., STERNBY, B., and DE HAAS, G. The complete primary structure of phospholipase A2 from human pancreas. Biochim Biophys Acta 747,93-99,1983.
21. WARSHAW, A.L. A simplified method of cannulation the intestinal lymphatic of the rat. Gut 13,88-67,1972.
22. ANDERSSON, L., STERNBY, B., and NILSSON, Å. Hydrolysis of phosphatidylethanolamine by human pancreatic phosphalipse A2. Scand J Gastroenterol 29,182-187,1994.
23. FLORÉN, C-H., and NILSSON, Å. Binding, interiorization and degradation of cholesteryl ester labelled chylomicron-remnant particles by rat hepatocyte monolayers. Biochem J 168,483-494,1977.
24. PATTON, G.M., FASULO, J.M., and ROBBIN, S.J. Analysis of lipids by high performance liquid chromatography. J Nutr Biochem 1,493-500,1990.
25. CHEN, Q., and NILSSON, Å. Desaturation and chain elongation of n-3 and n-6 polyunsaturated fatty acids in the human CaCo-2 cell line. Biochim Biophys Acta 1166,193-201,1993.
26. STENFLO, J., FERNLUND, P., EGAN, W., and ROEPSTORFF, P. Vitamin K dependent modifications of glutamic acid residues in prothrombin. Proc Natl Acad Sci USA 71,2730-2733,1974.
27. CONSTANTINO, M., MERSKEY, C., KUDZMA, D.J., and ZUCKER, B.M. Increased activaty of vitamin K-dependent clotting factors in human hyperlipoproteinaemia- Association with cholesterol and triglyceride levels. Thromb Harmost 38,465-474,1977.
28. TALL, A.R., GREEN, P.H.R., GLICKMAN, R.M., and RILEY, J.W. Metabolic fate of chylomicron phospholipids and apoproteins in the rat. J Clin Invest 64,977-989,1979.
29. NILSSON, Å., LANDIN, B., and JOHANSSON, B. Plasma apolipoprotein A-I levels and bile lipid secretion during thoracic duct drainage in the rat. Biochim Biophys Acta 963,231-236,1988.
30. XU, N., ZHOU, L., ÖHLIN, A-K., and NILSSON, Å. Role of platelet factor Xa in chylomicron-prothrombin complexes induced platelet activation. Biochem Biophys Res Commun 208, 765-772, 1995.
31. STÜRZEBECHER, J., STÜRZEBECHER, U., VIEWEG, H., WAGNER, G., HAUPTMANN, J., and MARKWARDT, F. Synthetic inhibitors of bovine factor Xa and thrombin comparison of their anticoagulant efficiency. Thromb Res 54,245-252, 1989.
32. HAUPTMANN, J., KAISER, B., NOWAK, G., STÜRZEBECHER, J., and MARKWARDT, F. Comparison of the anticoagulant and antithrombotic effects of synthetic thrombin and factor Xa inhibitors. Thromb Haemost 63,220-223, 1990.