Association of Vitamin K–Dependent Coagulation Proteins and C4b Binding Protein With Triglyceride-Rich Lipoproteins of Human Plasma

Ning Xu, Björn Dahlbäck, Ann-Kristin Öhlin, Åke Nilsson

Abstract—The triglyceride (TG) concentration in plasma is an independent risk factor for coronary heart disease. There is evidence that TG-rich lipoprotein (TGRLP), ie, chylomicrons (CMs), chylomicron remnants (CMRs), and VLDLs associate with factor VII and prothrombin and that the association enhances a platelet factor Xα–mediated prothrombin activation when the CM-prothrombin complex is exposed to platelets. In this study, we examined the association of the vitamin K–dependent coagulation factors VII, IX, X, and prothrombin, as well as the anticoagulation protein C and its cofactor protein S, in plasma lipoproteins obtained from human fasting and postprandial plasma. We also analyzed some other proteins that are related to the coagulation system but not to vitamin K–dependent proteins, including factor V, serum amyloid P component (SAP), C4b binding protein (C4BP), and thrombomodulin (TM), and as a control, Ig G. Human TGRLP (d<1.006 kg/L), LDL (d=1.006 to 1.063 kg/L), and HDL (d=1.063 to 1.210 kg/L) were separated from normal subjects both in fasting and 2 to 3 hours after the ingestion of a meal containing 100 g fat. The different coagulation proteins, SAP, C4BP, TM, and Ig G were determined by SDS-polyacrylamide gel electrophoresis combined with Western blotting, using specific polyclonal or monoclonal antibodies, and were visualized by peroxidase staining. All the vitamin K–dependent proteins associate with TGRLP in both fasting and postprandial plasma, but not with LDL or HDL. Factor V, SAP, TM, and Ig G were not found in any lipoprotein classes. C4BP, which is a regulatory protein of the classic pathway of the complement system and which binds protein S in vivo to regulate blood coagulation, was present in TGRLP, especially postprandial, but not in LDL or HDL. The amounts of prothrombin, protein S, and C4BP in postprandial TGRLP were larger than those in fasting TGRLP. Vitamin K–dependent procoagulation and anticoagulation proteins, as well as C4BP, could be associated with TGRLP in vivo. If the association enhances prothrombin activation, this effect may thus be counteracted by simultaneous binding of protein S. (Arterioscler Thromb Vasc Biol. 1998;18:33-39.)

Key Words: triglyceride ▪ lipoproteins ▪ blood coagulation ▪ atherosclerosis

Despite more than 30 years of epidemiological and clinical research, the relations between hypertriglyceridemia, TGRLP, and CHD remain obscure and much debated.1–7 TGRLPs, including CMs, CMRs, and VLDLs, are a heterogeneous population of particles varying in origin, structure, and interactions with cellular receptors. More than a decade ago, Zilversmit8 advanced the hypothesis that postprandial lipoproteins are important in atherosclerosis in humans. As summarized by Patsch et al,4 this possibility was discussed also in earlier studies.9–13 Since there are only a few clinical studies of postprandial lipoproteins in subjects with premature CHD, this hypothesis has not yet been definitely confirmed.12–14 However, decreased clearance of CMs and CMRs after a fat load has been indicated to be associated with the presence of CHD in studies using semiquantitative determination of apo-lipoprotein B–4813 or quantification of the lipid moiety only.13,14 In an angiographic study on survivors of a myocardial infarction, Karpe et al15 demonstrated that the number of small CMRs during the postprandial phase was related to the progression of coronary artery diseases.

The vitamin K–dependent procoagulation and anticoagulation proteins have been considered to be related to arteriosclerosis and thrombosis.16–19 It has been suggested that all four vitamin K–dependent coagulation factors are related to a higher risk of ischemic heart disease. Burns et al16 analyzed factor IX and X activities, as well as concentrations of prothrombin and factor VII, and found general increases of all four vitamin K–dependent coagulation factors in high-risk CHD subjects. Increased factor VII activity has been identified as a risk factor for ischemic heart disease.17,18,20–22 A general increase in all four vitamin K–dependent coagulation factors could produce a hypercoagulable state much greater than that predicted for an isolated increase in factor VII. There is also a strong correlation between factor VII and plasma TG level, suggesting that there is a probable interaction of factor VII with...
TGRLP in vivo. Deficiencies of vitamin K–dependent anticoagulation proteins, ie, protein C and its cofactor protein S, are also related to the initiation and progression of deep venous thrombosis.\textsuperscript{23–25} It is still unknown whether vitamin K–dependent proteins interact with TGRLP in vivo, although prothrombin and factor Xα may bind to VLDLs in vitro, partially through a calcium–dependent association.\textsuperscript{26,27} Carvalho de Sousa et al\textsuperscript{28} found that coagulation factors VII and X could associate with isolated plasma CMs and VLDLs by using immunoenzyme assay. The associations of factor VII and factor X were stronger on CM and VLDL fractions than on LDL fraction, and no such association was found on HDLs. Furthermore, we have demonstrated that CMs bind prothrombin in vitro in a calcium–dependent manner.\textsuperscript{29–31} The question was therefore raised whether the TGRLPs bind vitamin K–dependent proteins and other coagulation proteins in vivo. In this study, we examined the association in vivo with human fasting and postprandial TGRLP of prothrombin, factors VII, IX, and X, protein C, and protein S and found that all these vitamin K–dependent proteins, but not SAP, factor V, TM, or Ig G, associated with the lipoproteins. We also analyzed C4BP, which is a regulatory protein of the classical pathway of the complement system and which can also bind protein S in vivo to regulate blood coagulation.\textsuperscript{32–34} This protein was earlier shown to bind to a soybean TG–egg phospholipid emulsion (Intralipid) and was also demonstrated in human plasma CMs (then called proline–rich protein).\textsuperscript{35} The present report confirms its association with TGRLP and shows a postprandial increase in this association.

Methods

Materials

Highly purified human plasma–derived prothrombin, human coagulation factor VII, and polyclonal sheep antibody against human factor VII were obtained from Enzyme Research Laboratories. Polyclonal rabbit antibodies against human protein C, protein S, coagulation factors V, IX, and X, SAP, and Ig G were from DAKO A/S. Purified human protein C, protein S, factors V, IX, and X, C4BP, and mouse antibody against human protein S (MK 54) were available in the Department of Clinical Chemistry, University Hospital of Malmo, Sweden. Polyclonal rabbit antibody against human TM was a kind gift from Dr J. Morser, Berlex Biosciences. Rabbit anti–human prothrombin antiserum and monoclonal mouse antibodies against human factor IX (MK 13) and prothrombin (MK 10) were kind gifts from Prof Johan Stenflo, Department of Clinical Chemistry, University Hospital of Malmo, Sweden. Monoclonal mouse antibody against human factor V (MK 30) was described earlier.\textsuperscript{36} Rabbit antiserum against C4BP and monoclonal mouse antibody against C4BP (MK 104) were characterized earlier.\textsuperscript{36,37} Rainbow–colored protein molecular-weight standards of 14.3 to 220 kD and 4 to 250 kD were from Amersham Life Science and Novel Experimental Technology, respectively. Hirudin was purchased from Sigma, and PPACK from Calbiochem–Nonabiochem Co.

Volunteers and Blood Samples

Nine healthy volunteers, aged 27 to 55 years (four men and five women; mean±SD 40.9±10.8 years), were included in the present study, which was approved by the local ethics commission. No medication was used within 2 weeks before the examination. Blood samples were obtained from each volunteer after fasting overnight and 2 to 3 hours after eating a meal containing 100 g fat in total as butter, cheese, and cream, together with strawberry. K,EDTA (4 mmol/L) or the mixture of hirun (2 U/ml blood), benzamidine (10 mmol/L), and PPACK (1 μmol/L) was used as anticoagulant. Thimerosal (sodium ethylmercurithiosalicylate), purchased from Sigma, was immediately added to the blood to a final concentration of 25 μmol/L to inhibit proteolytic activity.

Lipoprotein Separation and Delipidation

TGRLPs (d<1.006 kg/L) including VLDLs, CMs, and CMRs; LDL (d=1.006 to 1.063 kg/L); and HDL (d=1.063 to 1.210 kg/L) were isolated by sequential flotation.\textsuperscript{38,39} Briefly, 10 to 13 mL of plasma obtained from each volunteer both in fasting and 2 to 3 hours after fat meals were adjusted to d=1.006 kg/L with EDTA saline (188 mmol/L NaCl in 1 mmol/L Na2EDTA, d=1.006 kg/L) and ultracentrifuged at 37 000 rpm for 18 hours at 10°C in a Beckman L5 65 ultracentrifuge with SW 40 Tl swing-out rotor. The top layer containing TGRLP was collected. LDL was obtained after adjusting the infranatant to d=1.063 kg/L and ultracentrifuged at 38 000 rpm for 24 hours at 10°C. After the LDL fraction was removed and adjusted to d=1.210 kg/L by KBr, the HDL was separated by ultracentrifugation at 38 000 rpm for 48 hours at 10°C. Each lipoprotein fraction was washed twice by ultracentrifugation at the designated density in the presence of 1 mmol/L Na2EDTA. In the case of the hirudin, benzamidine, and PPACK mixture as anticoagulant, the lipoprotein fractions were separated as described above but without Na2EDTA. The samples were also washed once by buffer containing the mixture of hirudin, benzamidine, and PPACK. The three lipoprotein fractions were delipidated with ethanol: diethyl ether (3:1, vol/vol) at 4°C for 24 hours.\textsuperscript{40} The delipidation procedure was repeated three times, and the protein precipitate was washed twice with cold diethyl ether and dried under nitrogen. The protein precipitate was resolubilized in 30 mmol/L Tris–acetic acid buffer (pH 7.5) in presence of 0.01% Triton X–100 and then passed through 0.2–μm Millipore filters. The final concentration of protein mass was adjusted to 1.0 mg/mL.

Electrophoresis and Immunological Determinations of Proteins

Proteins were separated by 10% or 12% polyacrylamide or 4% to 15% polyacrylamide gradient slab gel electrophoresis in the presence of SDS, and Western blotting analysis was performed according to the manufacturer’s instructions, using the Bio–Rad system (Bio–Rad Laboratories). Aliquots of delipidated samples containing 30 μg of total protein were applied to each well of the SDS–polyacrylamide slab gels. Purified specific proteins were used as standards, as indicated in the figure legends.

Determination of Factor VII Activity

Factor VII activity in plasma and in different lipoprotein fractions that had been delipidized was determined by a photometric determination kit according to the manufacturer’s protocol (Chromogenix AB). The mixture of human normal plasma obtained from 31 volunteers was used as control plasma. The factor VII activity in the control plasma was considered as 100%.

Proportion of Prothrombin, Factors IX and X, Protein C, Protein S, and C4BP in Fasting and Postprandial TGRLP

The separation of the proteins and the Western blotting analysis were carried out as described above. Different amounts of purified specific

Selected Abbreviations and Acronyms

\begin{itemize}
\item C4BP = C4b binding protein
\item CHD = coronary heart disease
\item CM = chylomicron
\item CMR = CM remnant
\item PPACK = d-Phe-Pro-Arg chloromethyl ketone
\item SAP = serum amyloid P
\item TG = triglyceride
\item TGRLP = TG-rich lipoprotein
\item TM = thrombomodulin
\end{itemize}
proteins were used to make a standard curve that was subjected to linear regression analysis after the membranes were scanned by a Bio-Rad imaging densitometer (model GS-670) combined with a software for image analysis system, version 2.0 (Hercules).

**Chemical Determinations**

The protein contents of the lipid-free lipoprotein fractions were determined with the method of Lowry et al., using bovine serum albumin as the standard. The concentrations of TG, total cholesterol, and choline-containing phospholipids in plasma were determined by the respective enzymatic assay methods, using Boehringer test-combination kits according to the manufacturer’s protocols.

**Statistical Analysis**

Values are reported as mean±SEM. Data were analyzed by using an IBM personal computer statistical software package. One-way ANOVA followed by unpaired Student’s t test was employed for statistical analysis. A value of \( P<.05 \) in a two-tailed test was considered significant.

**Results**

### Plasma Lipids in Fasting and Postprandial States

As shown in Table 1, the plasma TG concentrations of fasting and postprandial plasma were 195.3±9.9 and 218.0±8.4 mg/dL, respectively. Total cholesterol concentrations of the plasma were 105.6±17.1 and 211.6±12.7 mg/dL. The choline-containing phospholipid concentrations of the samples, determined by a phospholipase C choline oxidase kit, were 255.4±16.6 mg/dL.

### Association of Vitamin K–Dependent Proteins With TGRLP

As shown in Fig 1, prothrombin and factors IX and X are found in the TGRLP in both fasting and postprandial samples, but not in LDL and HDL fractions. The results were the same in the determinations with polyclonal or monoclonal antibodies (data not shown). Also, protein C and protein S are found in the TGRLP, but not in LDL and HDL (Fig 2). Again, the results were the same when using polyclonal or monoclonal antibodies (data not shown). No distinct immunoreaction of factor VII in the TGRLP was observed in the Western blotting examination, possibly due to limitation of the sensitivity of the method (data not shown); nor was factor VII found in LDL and HDL. We therefore also determined factor VII activity in both the plasma and delipidized lipoprotein fractions; the factor VII activity was increased by 30% to 35% compared with the control plasma in the postprandial plasma. Factor VII activity was also found in TGRLP, although it was only about 4% of the plasma factor VII activity, but was not found in LDL and HDL. There was no obvious change of factor VII activity between fasting and postprandial TGRLP.

### Determination of SAP, TM, Factor V, C4BP, and Ig G

No SAP, TM, factor V, or Ig G could be demonstrated in TGRLP, LDL, or HDL (data not shown). In contrast, C4BP was found in the TGRLP (Fig 2C), and the amount was increased in postprandial samples (Table 2). Only two of nine volunteers were found to have C4BP in their fasting TGRLP samples, but all of the postprandial TGRLP samples have C4BP. No positive reaction for C4BP was found in the LDL or HDL fractions in either fasting or postprandial samples.

### Comparison of Fasting and Postprandial States

The proportions of the vitamin K–dependent proteins and C4BP in both fasting and postprandial TGRLP are shown in Table 2 and Fig 3. There were increases of prothrombin, protein S, and C4BP per mass of delipidized protein in postprandial samples compared with fasting samples, whereas factor IX, factor X, and protein C were not increased in the TGRLP after fat meals. The proportion of these proteins associated with the TGRLP is about 1% of the total delipidized protein mass of the lipoproteins. There was no statistical difference in the association of these proteins with TGRLP by using the mixture of hirudin, benzamidine, and PPACK or the K<sub>3</sub>EDTA (data not shown).

**Discussion**

Factors VII, IX, X, and prothrombin, as well as protein C and its cofactor protein S, are synthesized in the liver. They show considerable sequence homology and all undergo a posttranslational vitamin K–dependent step to acquire γ-carboxyglutamic acid residues, which form medium-affinity calcium-binding sites. The modification of glutamic acid residues endows these proteins with the property of binding to negatively charged phospholipid membranes at physiological calcium concentration. The calcium binding is characterized by an initial positive cooperativity and induces two conformational transitions in the molecule. The first of these is cation nonspecific, whereas the second transition, necessary for membrane binding, is specifically dependent on calcium ions. In some hypertriglyceridemic subjects, not only factor VII but also the other procoagulant vitamin K–dependent proteins are increased in plasma. Bradley and Gianturco reported that the large VLDLs that were separated from hypertriglyceridemic patients contained prothrombin, but not those of normal subjects; they also demonstrated that prothrombin can bind to plasma VLDLs in vitro at physiological concentrations of VLDLs, prothrombin, and calcium, whereas binding to LDLs and HDLs was negligible. In our previous studies, we examined binding of prothrombin to native chyle CMs, which contain more phospholipids and a higher proportion of phosphatidylethanolamine in the polar surface coat than the particles that have been exposed to plasma or lipoprotein lipase. We found that human plasma-derived prothrombin can bind to rat chyle CMs with a high affinity and in a calcium–dependent manner. The data thus indicate that prothrombin binding is not due to a simple electrostatic association but may rather be related to a conformational change of the prothrombin during the interaction with calcium ions and CM phospholipids.
The data in the present study indicate that all vitamin K–dependent procoagulation and anticoagulation proteins could associate with TGRLP in vivo, but not with LDLs or HDLs. There was a potential loss of proteins during a long procedure of the separation. EDTA was used as anticoagulant.

**Figure 1.** Association of vitamin K–dependent coagulation factors with the different lipoprotein fractions. Association of prothrombin (A), factor IX (B), and factor X (C) with lipoproteins is shown. In A, lanes 1 and 10 show molecular-weight standards; lanes 2 and 9, purified prothrombin as positive reaction; lanes 3 and 4, TGRLP from fasting and postprandial samples, respectively; lanes 5 and 6, LDLs from fasting and postprandial samples, respectively; and lanes 7 and 8, HDLs from fasting and postprandial samples, respectively. In B and C, lanes 2 and 9 show purified factors IX and X, respectively; all other lanes show the same substances as in A. The protein mass of different lipoprotein fractions applied to each well of the gel was 30 μg; experimental conditions were described in “Methods.” Standards used during the determinations were prothrombin (300 ng), factor IX (45 ng), and factor X (45 ng). Data are from one of nine volunteers.

**Figure 2.** Association of vitamin K–dependent anticoagulation proteins and C4BP with different lipoprotein fractions. Association of protein S (A), protein C (B), and C4BP (C) with lipoproteins, as determined by Western blotting, is shown. Purified protein C, protein S, and C4BP were used as standards in lanes 2 and 9. Specific standards used were protein S (80 ng), protein C, (27.2 ng), and C4BP (40 ng). Other conditions are the same as described in Fig 1. Data are from one of nine volunteers.

The data in the present study indicate that all vitamin K–dependent procoagulation and anticoagulation proteins could associate with TGRLP in vivo, but not with LDLs or HDLs. There was a potential loss of proteins during a long procedure of the separation. EDTA was used as anticoagulant.
TABLE 2. Proportions of the Vitamin K–Dependent Proteins Associated With the TGRLP (n=7–9, mean±SEM)

<table>
<thead>
<tr>
<th></th>
<th>Fasting Samples, ng/30 μg protein mass</th>
<th>Postprandial Samples, ng/30 μg protein mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prothrombin</td>
<td>25.5±8.1</td>
<td>50.0±11.4*</td>
</tr>
<tr>
<td>Factor IX</td>
<td>15.2±2.3</td>
<td>12.1±2.3</td>
</tr>
<tr>
<td>Factor X</td>
<td>4.5±0.9</td>
<td>5.0±1.0</td>
</tr>
<tr>
<td>Protein S</td>
<td>33.6±8.7</td>
<td>58.6±9.3*</td>
</tr>
<tr>
<td>Protein C</td>
<td>19.7±2.5</td>
<td>17.2±2.9</td>
</tr>
<tr>
<td>C4BP</td>
<td>6.0±4.3†</td>
<td>44.1±9.6†</td>
</tr>
</tbody>
</table>

*P<.05; †P<.01 vs fasting samples.
‡C4BP were found in only two of nine fasting samples.

Figure 3. Determination of proportion of prothrombin in fasting and postprandial TGRLP. The concentration regression of the image of prothrombin (A) and the proportion of prothrombin in fasting and postprandial TGRLP (B) are shown. Lanes 1 and 10 show molecular-weight standards, and lanes 2 and 3 are delipidized TGRLP (30 μg protein) from fasting and postprandial plasma, respectively. Purified prothrombin (from 10 to 320 ng) was applied to lanes 4 through 9, respectively. The membrane was scanned by a densitometer, and the concentration of prothrombin in the TGRLP was estimated by matching with the standard curve under a linear regression. Other conditions are the same as described in Fig 1. Data are from one of nine volunteers. Determination of other proteins, including factors IX and X, protein S, protein C, and C4BP, were the same as described in Figs 1 and 2.

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lipid accumulation, creating a foam cell morphology, in murine peritoneal macrophages and P388D1 macrophages. Other studies show that human monocyte-derived macrophages also have specific high-affinity TGRLP binding sites, producing a saturable uptake of TGRLP. This uptake was apolipoprotein E and LPL independent. The accumulation of lipids in the cells leads to foam cell formation. Two membrane-binding activities were identified as receptor candidates for this uptake. The effect of bound coagulation factors on the process of foam cell formation and on the normal receptor-mediated catabolism of remnants formed from TGRLP is another interesting area for further studies. In addition, the binding of the different vitamin K–dependent factors to TGRLP in different hypertriglyceridemic states should be examined.

Acknowledgments

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References


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