Activation of the Contact System of Coagulation Does Not Contribute to the Hemostatic Imbalance in Hypertriglyceridemia

M.C. Minnema, M.E. Wittekoek, N. Schoonenboom, J.J.P. Kastelein, C.E. Hack, H. ten Cate

Abstract—In vitro, triglyceride-rich lipoproteins may act as a surface to initiate the contact system of coagulation. Therefore, we studied the activation of factor XII (FXII), prekallikrein, and FXI and the generation of thrombin in 52 hypertriglyceridemic patients before and after 12 weeks of triglyceride-lowering treatment with gemfibrozil or n-3 polyunsaturated fatty acids. Thrombin generation was assessed by measuring the levels of prothrombin fragment F1 + 2 and thrombin-antithrombin (TAT) complexes. Contact activation was assessed by measuring FXIIa, kallikrein, and FXIa in complex with their major inhibitor, C1 inhibitor, and FXIa was also determined as part of a complex with α2-antitrypsin. Triglyceride and cholesterol levels decreased equally in both treatment groups. In the gemfibrozil group, there was a significant decrease in F1 + 2, while TAT complexes did not change. FXIIa- and kallikrein-C1 inhibitor complexes were elevated in 13% and 9% of the patients before treatment, respectively, and no changes were observed on triglyceride-lowering therapy. Also, no significant changes in regard to FXIa–C1 inhibitor and FXIa–α2-antitrypsin complexes were seen. FXIa–α2-antitrypsin complexes were present in 70% of the patients before therapy and were positively correlated with the level of TAT complexes. In conclusion, we did not detect an effect on activation markers of the contact coagulation system in hypertriglyceridemic patients after triglyceride-lowering therapy. Therefore, contact activation is not likely to contribute to the hypercoagulability seen in these patients. (Arterioscler Thromb Vasc Biol. 1999;19:2548-2553.)

Key Words: hypertriglyceridemia ■ contact system ■ blood coagulation

The contact system of coagulation consists of the serine proteases factor XII (FXII), factor XI (FXI), prekallikrein (PK), and the cofactor high-molecular-weight kininogen. The role of this system in the initiation of coagulation has been questioned because deficiencies in FXII or PK are not associated with an increased bleeding tendency.1 In addition, the physiological activator or surface for the (auto)activation of FXII is unknown.2 Long-chain, saturated fatty acids have been suggested to provide a potent surface for contact activation.3–5 These fatty acids are present in most lipoproteins and are elevated in patients with hypertriglyceridemia. Little doubt exists that triglycerides are related to atherosclerotic disease. Triglycerides themselves do not accumulate in atherosclerotic lesions, so their atherogenicity must be related to the associated adverse quantitative and qualitative changes in circulating lipoproteins.6

It was demonstrated in several cross-sectional studies that levels of triglycerides are associated with levels of factor VII (FVII) as measured in a clotting assay (FVII-c).7,8 Furthermore, the Northwick Park Heart Study demonstrated that high levels of FVII-c constitute an independent risk factor for acute coronary events.9 Others have suggested that these high levels of FVII-c represent enhanced levels of activated FVII (FVIIa).10 Because FVIIa in complex with tissue factor activates prothrombin, this could explain the hypercoagulability seen in patients with hypertriglyceridemia and thus, the associated higher risk of coronary artery disease.11,12

Because activated FXII (FXIIa) can directly activate FVII in vitro, this activation route has been postulated to explain elevated FVII-c levels in patients with hypertriglyceridemia.13–15 FXIIa also activates FXI and thereby, the intrinsic coagulation pathway, leading to the downstream formation of thrombin and fibrin.16 To address the hypothesis that hypertriglyceridemia causes activation of the contact system, we analyzed markers of contact activation in a cohort of males with severe hypertriglyceridemia. Furthermore, we studied the effect on these markers of 2 different triglyceride-lowering interventions in our patients.

Methods

Patients
Male patients from outpatient lipid clinics from the Academic Medical Center and the Slotervaart Hospital in Amsterdam, Nether-
lands, aged 18 to 70 years with severe hypertriglyceridemia, were asked to participate in the study. This study was part of a double-blind treatment study evaluating the effect on serum trypsigns of n-3 polyunsaturated fatty acids (n-3 PUFAs) compared with gemfibrozil. Patients using Coumadin were excluded from analysis. After written, informed consent was obtained, patients followed a diet during a run-in period of 6 weeks, according to the National Guidelines for Healthy Nutrition in the Netherlands. Patients who had a serum triglyceride level of 4.5 mmol/L or higher after this period were randomized to receive either oral therapy with n-3 PUFAs (Omacor), 4 g daily, or gemfibrozil (Lopid), 1200 mg daily, for 12 weeks. All other medications used by the patients were recorded and continued during the trial.

Clinical cardiovascular disease was considered to be present if subjects met at least 1 of the following criteria: (1) if a diagnosis of clinically documented angina pectoris had been made; (2) if subjects had developed a myocardial infarction or if an intervention by either coronary bypass surgery or balloon angioplasty had been performed; (3) if the patients had suffered an ischemic stroke; or (4) if a history of intermittent claudication was present.

Blood Sampling and Assays

After 12 hours, fasting blood samples were taken by venipuncture into siliconized tubes (with 0.34 mmol/L EDTA; Vacutainer, Becton Dickinson) to assess cholesterol and triglyceride levels and into siliconized, citrated tubes (with 0.105 mmol/L sodium citrate; Vacutainer, Becton Dickinson) for detection of thrombin-antithrombin (TAT) complexes and prothrombin fragment 1+2 (F1+2). Blood samples for the measurement of FXI, FXII, and PK activation were collected in siliconized tubes to which a solution of 0.1 mmol/L EDTA, 0.1 mg/mL soybean trypsin inhibitor (type I-S from Sigma Chemical Co), and 20 mmol/L benzamidine (Acros; all final concentrations) was added to prevent any ex vivo activation of the clotting system. Platelet-poor plasma was obtained by centrifugation at 2000g for 20 minutes at room temperature. Plasma samples were stored at −70°C until assayed.

Total cholesterol in plasma was determined by an enzymatic colorimetric procedure with cholesterol esterase.17 Tryglycerides were quantified by an enzymatic colorimetric procedure with lipase, glycerokinase, and glycerol-3-phosphate.18 Prothrombin F1+2 and TAT complexes were assayed using commercially available kits according to the manufacturer’s instructions (Enzygnost F1+2 and Enzyngnost TAT, respectively, Behringwerke). Complexes between FXIIa and kallikrein with C1 inhibitor were assayed with ELISAs that had been modified from radioimmunoassays. In short, microtiter plates were coated with a monoclonal antibody (MAb) directed against complexed C1 inhibitor and incubated with the plasma samples to be tested. Bound complexes were detected with biotinylated MAb directed against FXII or kallikrein, respectively. As an in-house standard, dextran sulfate–activated ELPK/plasma was used, which was calibrated against purified FXIIa or kallikrein-C1 inhibitor complexes. Normal values were <30 pmol/L for FXIIa-C1 inhibitor complexes and <350 pmol/L for the kallikrein–C1 inhibitor complexes, as established in 30 healthy volunteers.

FXII antigen was measured in an ELISA with anti-human FXII MAb B7C920 (kindly provided by Dr R. Pixley, Temple University, Philadelphia, Pa) as a capture antibody and MAb F3, directed against the light-chain region of FXII, for detection.21 PK antigen was also measured in an ELISA with anti-human PK MAb K15 as the capture antibody and MAB 13G1122 (kindly provided by Dr R.W. Colman, Temple University, Philadelphia, Pa) as the detecting antibody. Pooled plasma obtained from 30 healthy volunteers was used as the standard for the antigen ELISAs. The interassay variation of these ELISAs was <10%.

The FXIIa–C1 inhibitor and FXIIa-α1-antitrypsin complexes were assayed as described.23 In short, microtiter plates were coated overnight at 4°C with the MAb XI-3, directed against the heavy chain of FXI. After incubation of the plasma samples, FXIIa–C1 inhibitor or FXIIa–α1-antitrypsin complexes were detected with biotinylated MAb directed against C1 inhibitor or α1-antitrypsin, respectively. The detection limit is 10 pmol/L for both assays, and in 17 healthy volunteers, FXIIa–C1 inhibitor or FXIIa–α1-antitrypsin complexes were not detectable. FXII antigen was measured with MAb XI-5 on the solid phase and biotinylated MAb XI-3 for detection.

Statistical Analysis

Results are presented as mean±SD; coagulation activation markers are presented as median and range.

Age, triglycerides, cholesterol, FXII, PK, and FXII antigen are presented as mean±SD; coagulation activation markers are presented as median and range.

Table 1. Patient Characteristics and Laboratory Parameters Before Treatment

<table>
<thead>
<tr>
<th></th>
<th>n-3 PUFAs</th>
<th>Gemfibrozil</th>
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</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>50.2±7.7</td>
<td>49.8±8.9</td>
</tr>
<tr>
<td>Medication, %</td>
<td>53.1</td>
<td>67</td>
</tr>
<tr>
<td>Atherosclerosis, %</td>
<td>21.9</td>
<td>13.8</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>13.1±8.6</td>
<td>10.6±5.8</td>
</tr>
<tr>
<td>Cholesterol, mmol/L</td>
<td>8.7±2.5</td>
<td>7.8±1.7</td>
</tr>
<tr>
<td>F1+2, nmol/L</td>
<td>1.12 (0.68–1.82)</td>
<td>1.03 (0.37–1.7)</td>
</tr>
<tr>
<td>TAT, µg/L</td>
<td>3.8 (1.2–12.5)</td>
<td>3.3 (0.8–9.6)</td>
</tr>
<tr>
<td>FXII antigen, nmol/L</td>
<td>560±170</td>
<td>540±170</td>
</tr>
<tr>
<td>FXIIa–Cl inhibitor, pmol/L</td>
<td>&lt;80 (&lt;80–800)</td>
<td>&lt;80 (&lt;80–210)</td>
</tr>
<tr>
<td>PK, nmol/L</td>
<td>620±200</td>
<td>540±120</td>
</tr>
<tr>
<td>Kallikrein–Cl inhibitor, pmol/L</td>
<td>&lt;80 (&lt;80–830)</td>
<td>102 (&lt;80–733)</td>
</tr>
<tr>
<td>FXI, nmol/L</td>
<td>36±9.8</td>
<td>32.9±5.7</td>
</tr>
<tr>
<td>FXIIa–α1-antitrypsin, pmol/L</td>
<td>&lt;10 (&lt;10–57.7)</td>
<td>&lt;10 (&lt;10–301)</td>
</tr>
</tbody>
</table>

Age, male, female, race, smoking status, family history of cardiovascular disease, diabetes, hypertension, and hyperlipidemia were not significantly different between the two treatment groups. The baseline triglyceride level was higher in the gemfibrozil group (2.5±7.8 mmol/L) compared with the n-3 PUFAs group (1.7±5.5 mmol/L) (P=0.004).

The results of this double-blind, randomized clinical trial show that n-3 PUFAs significantly decreased triglycerides and cholesterol levels compared with gemfibrozil. The triglyceride and cholesterol levels decreased to 4.8±1.2 mmol/L and 7.1±0.7 mmol/L, respectively, in the n-3 PUFAs group and to 6.5±1.9 mmol/L and 1.7±0.8 mmol/L, respectively, in the gemfibrozil group (P<0.01 for either group compared with baseline). The cholesterol levels decreased to 7.5±1.7 mmol/L and 6.7±1.9 mmol/L, respectively, in the n-3 PUFAs group and the gemfibrozil group (P<0.01 for either group compared with baseline). Although there were no differences in triglyceride, cholesterol, and contact activation levels at the start of treatment, the decreases in triglycerides and cholesterol were comparable in both treatment groups. Before and after treatment, the results show no significant differences in triglyceride and cholesterol levels between the two treatment groups. After 12 weeks of therapy and continuation of the prescribed diet, triglyceride and cholesterol levels had decreased significantly in both groups (Figure 1). In the n-3 PUFAs group, triglycerides decreased to 7.6±4.8 mmol/L and in the gemfibrozil group, to 5.6±7.1 mmol/L (P<0.0001 for either group compared with baseline levels). The cholesterol levels decreased to 7.5±1.7 mmol/L and 6.7±1.9 mmol/L, respectively, in the n-3 PUFAs group and the gemfibrozil group (P<0.01 for either group compared with baseline). Although there was a difference in triglyceride and cholesterol levels at the start of treatment, the decreases in triglycerides and cholesterol were comparable in both treatment groups. After 12 weeks of therapy and continuation of the prescribed diet, triglyceride and cholesterol levels had decreased significantly in both groups (Figure 1).
1.2 ± 1.9 and 1.1 ± 1.8 mmol/L, respectively (P=NS). Plasma samples for analysis of coagulation were not obtained in 9 patients, 5 in the n-3 PUFA treatment group and 4 in the gemfibrozil group.

FXIIa–C1 inhibitor and kallikrein–C1 inhibitor complexes were elevated in 13% and 9% of the patients before treatment, respectively. After therapy with n-3 PUFAs or gemfibrozil, FXIIa–C1 inhibitor and kallikrein–C1 inhibitor complexes were also elevated in 13% and 9% of the patients (Table 2, P=NS). FXIIa–C1 inhibitor complexes remained elevated in 2 patients; in 5 patients these complexes became normal, but they also increased in 5 patients. Kallikrein–C1 inhibitor levels were above the normal value of 350 pmol/L in 5 patients before therapy and remained elevated in 1. In 4 other patients, kallikrein–C1 inhibitor levels increased to >350 pmol/L after therapy. The FXII antigen and PK antigen levels did not significantly differ after n-3 PUFA therapy. In the gemfibrozil group, a significant increase from baselines values was observed for both FXII and PK antigen levels, to 600 ± 220 nmol/L (P = 0.02) and 630 ± 210 nmol/L (P = 0.01), respectively (Figure 2).

FXIIa–C1 inhibitor complexes were detectable in only 3 patients before treatment and remained elevated after 12 weeks of therapy in 1 patient receiving gemfibrozil and in another having n-3 PUFA treatment. FXIIa–α1-antitrypsin complexes were elevated in 71% of the patients before treatment and remained elevated after treatment in 60% (Table 2, P=NS). FXI antigen increased after therapy, from 34.4 ± 8 to 38.5 ± 9.2 nmol/L, which was highly significant (P < 0.0001). However, the increase in FXI antigen levels only occurred in the group receiving gemfibrozil, from 32.9 ± 5.7 to 41 ± 8 nmol/L (P < 0.0001), while antigen levels did not change after n-3 PUFA therapy (Figure 2).

A small but significant decrease occurred in F1+2 (P = 0.02), but TAT complexes were not affected by the triglyceride-lowering therapy (Table 3). The decrease in F1+2 levels occurred mainly in the gemfibrozil group (1.03 nmol/L; range from 0.37 to 1.7 to 0.87 nmol/L before; 0.87 nmol/L; range from 0.17 to 1.23 nmol/L after, P = 0.003), whereas F1+2 levels did not change significantly in the n-3 PUFA group.

At the beginning of the study, associations were found between triglyceride and cholesterol levels (r = 0.64, P < 0.0001), between TAT complexes and F1+2 (r = 0.33, P = 0.02), and between FXIIa–C1 inhibitor and kallikrein–C1 inhibitor complexes (r = 0.96, P < 0.001). Triglyceride or cholesterol levels were not correlated with F1+2, TAT complexes, or markers of contact activation. Significant correlations between TAT complexes and FXIIa–α1-antitrypsin complexes were found at the beginning of the study and after therapy (r = 0.47, P = 0.01, and r = 0.4, P = 0.04, respectively).

Eleven patients (18%) had a history of clinical cardiovascular disease. These patients had similar triglyceride and cholesterol levels. Markers of activation of coagulation in these patients were also comparable to those in the patients without cardiovascular disease (results not shown).

**Discussion**

In the present study, we have demonstrated that only a small portion of patients with severe hypertriglyceridemia have elevated complexes of factor XIIa and kallikrein with C1

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### Table 2. Markers of Contact Activation Before and After Treatment With n-3 PUFAs or Gemfibrozil

<table>
<thead>
<tr>
<th></th>
<th>Before</th>
<th>Detectable</th>
<th>After</th>
<th>Detectable</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXIIa–Cl inhibitor, pmol/L</td>
<td>&lt;80 (&lt;80–806)</td>
<td>13%</td>
<td>&lt;80 (&lt;80–309)</td>
<td>13%</td>
<td>0.4</td>
</tr>
<tr>
<td>Kallikrein–Cl inhibitor, pmol/L</td>
<td>102 (&lt;80–830)</td>
<td>9%</td>
<td>&lt;80 (&lt;80–565)</td>
<td>9%</td>
<td>0.9</td>
</tr>
<tr>
<td>FXIa–Cl inhibitor, pmol/L</td>
<td>&lt;10 (&lt;10–301)</td>
<td>6%</td>
<td>&lt;10 (&lt;10–205.1)</td>
<td>4%</td>
<td>0.4</td>
</tr>
<tr>
<td>FXIa–α1-antitrypsin, pmol/L</td>
<td>12 (&lt;10–45)</td>
<td>71%</td>
<td>12 (&lt;10–42)</td>
<td>60%</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Median, range, and percentage of patients with levels above normal values for the respective assays (see Methods) are given. Statistical analysis (Wilcoxon rank test) for differences between levels before and after treatment was performed on both treatment groups together.
inhibitor. Furthermore, lowering plasma triglycerides did not effect these activation markers. Because activation of the contact system, and especially FXII, by long-chain fatty acids is considered to be a potential mechanism for FVII activation and related cardiovascular risk in patients with hypertriglyceridemia, the present results are important for understanding the cardiovascular risk profile in these patients.

Baseline
Approximately 10% of the patients had evidence of contact activation; ie, in 13% and 9% of the patients, FXIIa– and kallikrein–C1 inhibitor complexes were above the normal values for these assays. However, plasma concentrations of these complexes were low. This result is in concordance with previous studies, demonstrating <0.05% of FXII activation in middle-aged men without clinical symptoms of atherosclerosis and in 21 men with combined hyperlipoproteinemia, suggesting the contact system to be slightly activated in nonacute situations in men with enhanced risk for vascular disease.25,26 Furthermore, the slightly increased FXII antigen levels compared with normal values (≈400 nmol/L) and the PK antigen levels comparable with given normal values (≈550 nmol/L) do not support a high level of activation of these contact factors.

We assessed activation of FXI by measuring FXIa in complex with C1 inhibitor or α1-antitrypsin, and the latter complex was detected in 71% of the patients. In particular, the elevation of FXIa–α1-antitrypsin complex in two thirds of the patients compared with the almost-undetectable levels of FXIa–C1 inhibitor complex is striking. Although C1 inhibitor is the predominant inhibitor of FXIa owing to a relatively long plasma half-life of inhibition, FXIa–α1-antitrypsin complexes may be a better parameter for establishing FXI activation in vivo.23,27 Moreover, the increased levels of FXIa–α1-antitrypsin complex in the presence of normal levels of FXIa–C1 inhibitor complex virtually exclude the possibility that these results were influenced by in vitro artifacts, since in that case, higher levels of FXIa–C1 inhibitor complexes would be expected.23 FXIa–α1-antitrypsin complexes have also been detected in patients with coronary artery disease and in patients with non–insulin-dependent diabetes mellitus,28,29 suggesting that these complexes are a sensitive marker for atherosclerotic vascular disease, although clinically evident vascular disease was present in only a small fraction of patients in our study. Enhanced activation of FXI was not reflected by a decrease in FXI antigen levels, which were comparable with normal values (≈31 nmol/L).

In the revised model of coagulation, FXI is activated by thrombin instead of FXII.30 In this study, we found a weak but significant correlation between FXIa–α1-antitrypsin complexes and TAT complexes, indicating that activation of these clotting factors might be related. In 2 recent studies, it was shown that cell-free phospholipids, like atherogenic lipoproteins, can support the assembly of the prothrombinase complex and thrombin generation in vitro.31,32 We speculate that in these hypercholesterolemic patients, thrombin generated on atherogenic phospholipids might be responsible for FXI activation and thereby, the increased values of the FXIa–α1-antitrypsin complex. However, because elevated markers of contact activation were also present, we cannot exclude the

**TABLE 3.** Markers of Thrombin Generation Before and After Treatment With n-3 PUFAs or Gemfibrozil

<table>
<thead>
<tr>
<th></th>
<th>Before Treatment</th>
<th>After Treatment</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>TAT, μg/L</td>
<td>3.4 (0.8–12.5)</td>
<td>3.9 (1.4–24.2)</td>
<td>0.3</td>
</tr>
<tr>
<td>F1 + 2, nmol/L</td>
<td>1.04 (0.37–1.82)</td>
<td>0.97 (0.17–1.84)</td>
<td>0.02</td>
</tr>
<tr>
<td>F1 + 2, n-3 PUFAs, nmol/L</td>
<td>1.12 (0.66–1.82)</td>
<td>1.05 (0.56–1.84)</td>
<td>0.7</td>
</tr>
<tr>
<td>F1 + 2, gemfibrozil, nmol/L</td>
<td>1.03 (0.37–1.7)</td>
<td>0.87 (0.17–1.23)</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Median and range are given; statistical analysis (Wilcoxon rank test) for differences between levels before and after treatment were performed on both treatment groups together and for F1 + 2 for each treatment group separately.

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** A, FXII antigen (Ag) levels before (white bars) and after (shaded bars) treatment for 12 weeks with n-3 PUFAs or gemfibrozil. B, PK Ag levels before (white bars) and after (shaded bars) treatment for 12 weeks with n-3 PUFAs or gemfibrozil. C, FXI Ag levels before (white bars) and after (shaded bars) treatment for 12 weeks with n-3 PUFAs or gemfibrozil. *P < 0.05, **P < 0.001.
The observation that changes in the activation markers FXII or PK did not occur, whereas triglyceride levels decreased with ±50%, suggests that triglyceride levels did not influence contact activation. Our results are in accordance with those of Broijersen et al., who also did not detect an effect of FXII on activation in the plasma of hypertriglyceremic patients after treatment with gemfibrozil for 10 to 12 weeks. These combined results do not support the notion that FXII is activated by long-chain fatty acids present in triglycerides. Furthermore, not only in chronic hypertriglyceridemia but also after a high-fat meal are triglycerides elevated and positively associated with FVII-c.27,34 FXII could be excluded as a mediator in the postprandial activation of FVII by 2 recent studies, which demonstrated a clear FVIIa response after a high-fat meal in homozygous FXII-deficient patients.39,40 Moreover, in healthy individuals, no changes in FXIIa were measured after a high-fat meal.39

We therefore conclude that the contact system is not activated by triglycerides in vivo. Contact activation is not likely to contribute to the hypercoagulability observed in hypertriglyceridemic patients. However, FXI activation is frequently present and is positively correlated with the level of TAT complexes.

Acknowledgments

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References

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