Hypocoagulant and Lipid-Lowering Effects of Dietary n-3 Polyunsaturated Fatty Acids With Unchanged Platelet Activation in Rats

Cécile M.A. Nieuwenhuys, Suzette Béguin, René F.G. Offermans, Jef J. Emeis, Gerard Hornstra, Johan W.M. Heemskerk

Abstract—We investigated the effects of dietary polyunsaturated fatty acids (PUFAs) on blood lipids and processes that determine hemostatic potential: platelet activation, coagulation, and fibrinolysis. For 8 to 10 weeks, Wistar rats were fed a high-fat diet containing various amounts (2% to 16%) of n-3 PUFAs derived from fish oil (FO) or a diet enriched in n-6 PUFAs from sunflower seed oil (SO). Only the FO diets caused a reduction in mean platelet volume, platelet arachidonate level, and formation of thromboxane B2 by activated platelets, but neither of the diets had a measurable effect on platelet activation. The FO-rich diets decreased the plasma concentrations of triglycerides and cholesterol, whereas the SO diet reduced triglycerides only. Parameters of fibrinolysis and standard coagulation times, ie, activated partial thromboplastin time and prothrombin time, were only marginally influenced by these diets. In contrast, dietary FO, but not SO, led to decreased levels of the vitamin K–dependent coagulation factors prothrombin and factor VII, while the level of antithrombin III was unchanged. The endogenous thrombin potential (ETP) was measured with an assay developed to detect the hypocoagulable state of plasma. After activation with tissue factor and phospholipids, the ETP was reduced by 23% or more in plasma from animals fed a diet with 4% FO. No significant effect of the SO diet on ETP was observed. Control experiments with plasma from warfarin-treated rats indicated that the ETP was more sensitive to changes in prothrombin concentration than in factor VII concentration. Taken together, these results indicate that in rats, prolonged administration of n-3 but not n-6 PUFAs can lead to a hypocoagulable state of plasma through a reduced capacity of vitamin K–dependent thrombin generation, with unchanged thrombin inactivation by antithrombin III. (Arterioscler Thromb Vasc Biol. 1998;18:1480-1489.)

Key Words: fish oil • coagulation • n-3 polyunsaturated fatty acids • thrombin • platelets

In the early 1970s, it had already been noted that Greenland Eskimos often showed a tendency for prolonged bleeding and suffered relatively little from ischemic heart disease. Initial epidemiological studies suggested that the putative cardioprotective factor was of dietary origin and, in particular, resulted from the consumption of large amounts of marine oils by these Eskimos.1,2 Although nowadays the relationship between fish consumption and cardiovascular health is well documented, the mechanism responsible for the protective effect is still debated.3,4

An initial hypothesis was that dietary marine oils prolong bleeding as a consequence of their fatty acid–modulating effects on platelets. Consumption of fish oil (FO), rich in n-3 polyunsaturated fatty acids (PUFAs), causes replacement of n-6 PUFAs by n-3 PUFAs in the platelet phospholipids; ie, arachidonic acid is replaced mainly by eicosapentaenoic and docosahexaenoic acids. During platelet activation, arachidonic acid is released from the phospholipids and then converted into prostaglandin H2 and thromboxane A2, which strongly potentiate the activation process. Once released, however, eicosapentaenoate is converted into the metabolites prostaglandin H3 and thromboxane A3, both of which also support platelet activation but in a much less effective manner.5 On the other hand, both the arachidonate- and eicosapentaenoate-derived vessel wall products, prostaglandins I2 and I3 (prostacyclins), are considered to be equally potent in inhibiting platelet activation. Accordingly, the original explanation for the advantageous effect of dietary n-3 PUFAs was that they suppressed the platelet-stimulating effect of thromboxane production while leaving unchanged the platelet-inhibiting effect of vessel wall–derived prostacyclins.6,7 Dietary intervention studies have indeed demonstrated that n-3 PUFAs (ie, FO) effectively decrease the release of thromboxane A2 by platelets. However, in only some of such studies was the reduced thromboxane formation accompanied by a decreased activation tendency of the platelets, whereas in other reports platelet activation was unchanged or even increased.7,8

More recently, other mechanisms have been put forward to explain the health-protecting effect of FO-rich diets. Various
authors have shown that FO reduces the plasma concentrations of cholesterol and triglycerides. Others have stated that dietary FO may decrease the levels of specific clotting factors in plasma, although global coagulation measurements usually failed to detect diet-induced effects on the coagulation process.

Supplementation of FO diets rich in n-3 PUFAs to laboratory rats results in effects that, to a certain degree, resemble those of human nutritional studies. Lipid-lowering effects of FO-rich diets have often been documented in rats. In earlier work from our laboratories, it has been shown that FO-fed rats have an increased bleeding time and that their blood contains platelets with consistently reduced levels of arachidonic acid. However, we and others have also found that the reduced production of thromboxane by these platelets was not accompanied by a decreased platelet activation tendency or by a diminished transduction of activating signals. This suggests that the prolonged bleeding effect may be due to factors other than reduced platelet activation. In the present article, we report on a study with rats in which we investigated the effects of various doses of FO-derived n-3 PUFAs on a variety of factors involved in normal and abnormal hemostasis: blood lipids, platelet activation, coagulation, and fibrinolysis. As a comparison, we used a diet derived from sunflower seed oil (SO) that was highly enriched in n-6 PUFAs. While both types of diet appeared to be lipid lowering and did no more than marginally influence platelet activation, the FO-containing diets were unique in decreasing the coagulant state of the plasma, as detected by a sensitive assay measuring endogenous thrombin potential (ETP). This hypocoagulant effect was most likely due to an FO-induced lowering of the levels of vitamin K–dependent coagulation factors.

Methods

Materials

Beef tallow, hydrogenated coconut oil, and olive oil came from Chempri, a pelleted stock diet was from Hope Farms, and FO fatty acid ethyl esters were obtained from Hoffmann-La Roche. SO (Trisun) and the remaining dietary components were supplied from Unilever. Recombinant disulfatidihorin (variant 1 Revase) was a gift from Ciba-Geigy, Basel, Switzerland. Warfarin and kaolin were bought from Sigma Chemical Co, prothrombin- and factor VII–deficient plasmas and thromborel S came from Behring, and recombinant tissue factor was from Dade. Human factor VII was purchased from Kordia. Human prothrombin was a kind gift of Dr J. Rosing, Maastricht, The Netherlands. Chromogenic substrate for thrombin, S2238, and test kits for antithrombin III were obtained from Maastricht, The Netherlands. Chromogenic substrate for thrombin, S2238, and test kits for antithrombin III were obtained from Chromogenix. Kits for determination of thromboxane B_2 came from Cayman. The origin of other chemicals was as described before.

Animals and Diets

Three-week-old, male Wistar rats (Charles River, Sulzfeld, Germany) were individually housed in stainless steel cages at 23°C and fed the pelleted stock diet. After 2 weeks, the rats were randomly divided into 6 groups of 24 animals each and fed 1 of the 6 high-fat diets for a period of 8 to 10 weeks. All diets had equal energy content of 19.5 kcal/g and contained 50% of digestible energy as fat, 23 energy % as casein, and 27 energy % as cornstarch. Other dietary components (minerals, vitamins, and fiber) were added as before. Cholesterol was equally low in all diets, ie, 50 mg/g. To prevent lipid peroxidation, the diets were prepared weekly and stored at −20°C under N₂ until use. Water and food were available to the animals ad libitum. The studies were approved by the Institutional Animal Care Committee.

Rats from the control group were fed a control diet composed of saturated fatty acids, monounsaturated fatty acids, and n-6 PUFAs in a ratio of 2:3:1 (wt/wt/wt). This diet was prepared by mixing equivalent amounts of SO, beef tallow, hydrogenated coconut oil, and olive oil. Other rats received a diet in which 2%, 4%, 8%, or 16% (wt/wt) of the fatty acids were replaced by n-3 PUFAs from an FO preparation containing 19.9% ethyl eicosapentaenoate and 36.9% ethyl docosahexaenoate (2% to 16% FO groups). Rats from the sixth group were fed an SO-rich diet composed of saturated fatty acids: monounsaturated fatty acids, and n-6 PUFAs in a ratio of 1:2:5 (wt/wt/wt) (SO group). Mean daily food intake and weight gain were similar for animals of each diet group.

Control experiments also were performed with rats fed the pelleted stock food. Where indicated, the animals were injected intraperitoneally with 1 mg of dissolved warfarin, and blood was collected at 4, 11, or 27 hours after injection.

Blood Sampling and Blood Cell Characteristics

The rats were fasted overnight, anesthetized with diethyl ether, and subjected to abdominal aortic puncture. From 12 animals per diet group, 1 mL of blood was collected into dry EDTA (5 μmol), and 10.8 mL of blood was collected into 1.2 mL of 80 mmol/L trisodium citrate. From the other rats, 2 mL of blood was collected into trisodium citrate solution (80 mmol/L) and the remaining 10 mL into 2 mL of citrate–citric acid–glucose (80 mmol/L trisodium citrate, 52 mmol/L citric acid, and 180 mmol/L glucose). EDTA-anticoagulated blood was used to count blood cells and to determine mean cell volumes with the use of a Technicon H-1 analyzing system (Bayer).

To prepare standard plasma, blood from 10 rats fed the pelleted stock diet was collected into 80 mmol/L trisodium citrate and then centrifuged first at 1200g for 15 minutes and subsequently at 18 000g for 5 minutes, after which the platelet-free plasma fractions were pooled.

Preparation of Platelet-Rich Plasma and Washed Platelets

Blood collected in citrate was used to prepare platelet-rich plasma by centrifugation at 2800g for 10 seconds. Platelet-free plasma was prepared by centrifugation of a portion of the platelet-rich plasma at 18 000g for 5 minutes. After dilution with platelet-free plasma to 2×10¹¹ platelets/mL, the resulting platelet suspension was used for aggregation studies. Blood collected on citrate–citric acid–glucose was used to prepare washed platelets. In this case, the platelets were finally suspended in HEPES buffer, pH 7.4, consisting of 136 mmol/L NaCl, 5.6 mmol/L glucose, 5 mmol/L HEPES, 2.7 mmol/L KCl, 2 mmol/L MgCl₂, 0.42 mmol/L NaH₂PO₄, and 0.1% (wt/vol) BSA.

Platelet Activation and Aggregation

Platelets shape change and aggregation were measured with platelet-rich plasma (2×10² platelets/mL) in an automated aggrerometer while being stirred at 37°C. Coagulation was blocked by adding 1 μmol/L recombinant hirudin, after which 5 mmol/L CaCl₂ was added to measure platelet activation under Ca²⁺-rich conditions. Aggregation of washed platelets was determined in a similar way, except that hirudin was absent and CaCl₂ was added to a concentration of 1 mmol/L. After 10 minutes of activation, samples were taken from the platelet suspensions and centrifuged at 18 000g for 5 minutes. The supernatants were collected for later determination of thromboxane B₂. Aggregation rate (% T/min), time of onset of aggregation (s), shape change (% T), and maximal aggregation (% T) were determined from the optical transmission curves with computer-assisted analyses.

For measurements of cytosolic [Ca²⁺], washed platelets suspended in HEPES buffer, pH 7.4 (5×10⁴/mL), were incubated with fluorescent fura 2-AM in the presence of pluronic F-127 as de-
scribed. After loading, the cells were resuspended at $1 \times 10^8$ platelets/mL in the same buffer.

### Lipid Analyses

Total phospholipids were isolated from suspensions of washed platelets by chloroform-methanol extraction, as described elsewhere. Concentrations of total cholesterol, cholesterol in HDLs, and triglycerides were determined in diluted plasma samples with an automated Cobas-Bio analyzer (Roche).

### Fibrinolysis

Blood collected in citrate was immediately centrifuged at 1200 g for 15 minutes and subsequently at 18 000 g for 5 minutes to obtain platelet-free plasma. This was frozen in LN$_2$ and stored at $-80^\circ$C. Plasminogen activator inhibitor and tissue-type plasminogen activator were quantified, as described before, for the rat fibrinolytic system.

### Coagulation Parameters

Activated partial thromboplastin time (APTT) and prothrombin time (PT) were determined in platelet-free plasma activated with kaolin/phospholipids and rat brain thromboplastin, respectively, as described elsewhere. Fibrinogen in plasma was determined according to Clauss. Prothrombin was measured in plasma samples diluted 1:50 to 1:80 (vol/vol) with a buffer composed of 28.5 mmol/L sodium barbital and 126 mmol/L NaCl (pH 7.35) in a coagulation assay with prothrombin-deficient plasma and thromborel S. Factor VII levels were measured under similar conditions but with factor VII–deficient plasma. Antithrombin III levels were determined on a Boehringer coagulation apparatus with a test kit from Chromogenix and by following the manufacturer’s instructions.

### ETP

Formation and degradation of thrombin were measured in rat plasma basically as described for the human plasma system, although several adjustments were made. In brief, citrated platelet-free plasma was diluted 4:5 (vol/vol) with a pH 7.35 buffer consisting of 50 mmol/L Tris-HCl, 100 mmol/L NaCl, and 0.05% (wt/vol) BSA. After 1 μmol/L of sonicated phospholipid vesicles (phosphatidylcholine/phosphatidylserine, 80:20, mol/mol) was added, the mixture was equilibrated at 37°C, and coagulation was started by adding 16.7 mmol/L CaCl$_2$ and recombinant human tissue factor diluted to give a clotting time of 25 seconds. At fixed time intervals after the start, 10-μL aliquots were sampled into cuvettes containing 490 μL of a prewarmed (37°C) mixture of 100 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.9), 20 mmol/L EDTA, 0.2 mmol/L S2238 (chromogenic substrate), and 0.05% (wt/vol) BSA. In each cuvette, color development was stopped after 2 minutes by adding 300 μL of 1 mol/L citric acid. Precise sampling and stopping times were recorded by a personal computer connected to pushbutton-equipped pipettes. The absorption value of each cuvette was read at 405 nm, and the amidolytic activity was calculated and converted to the thrombin concentration of the coagulation mixture by reference to a calibration curve for human α-thrombin. Fibrin clots were manually removed from the coagulation mixture immediately after their formation (~45 seconds).

Plotting of the amidolytic activity values of the samples as a function of time resulted in curves of thrombin formation and degradation. However, for the conversion of amidolytic activity to concentration of active thrombin (ie, the thrombin not bound to protease inhibitors), a correction was made for the accumulation of thrombin that was capable of hydrolyzing S2238 but inactive in coagulation by using a numerical fitting procedure. In rat plasma, unlike in human plasma, this concerns mainly thrombin that is bound by...
TABLE 2. Effects of Diets on Platelet Aggregation and Ca\(^{2+}\) Signaling

<table>
<thead>
<tr>
<th>Activation Condition</th>
<th>Type of Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recalculated platelet-rich plasma</td>
<td>Control</td>
</tr>
<tr>
<td>ADP</td>
<td></td>
</tr>
<tr>
<td>Shape change, %T</td>
<td>13.9±1.6</td>
</tr>
<tr>
<td>Aggregation, %T</td>
<td>47.7±6.3</td>
</tr>
<tr>
<td>Collagen</td>
<td></td>
</tr>
<tr>
<td>Shape change, %T</td>
<td>16.3±2.8</td>
</tr>
<tr>
<td>Aggregation, %T</td>
<td>76.6±10.0</td>
</tr>
<tr>
<td>Washed platelets</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td></td>
</tr>
<tr>
<td>Shape change, %T</td>
<td>8.7±1.2</td>
</tr>
<tr>
<td>Aggregation, %T</td>
<td>48.5±5.7</td>
</tr>
<tr>
<td>(\Delta[Ca^{2+}], \text{nmol/L})</td>
<td>830±122</td>
</tr>
<tr>
<td>Collagen</td>
<td></td>
</tr>
<tr>
<td>Shape change, %T</td>
<td>7.5±1.2</td>
</tr>
<tr>
<td>Aggregation, %T</td>
<td>23.3±2.5</td>
</tr>
<tr>
<td>(\Delta[Ca^{2+}], \text{nmol/L})</td>
<td>197±56</td>
</tr>
</tbody>
</table>

ND indicates not determined. Platelet-rich plasma (2×10\(^8\) platelets/mL) was supplemented with 1 \(\mu\text{mol/L}\) hirudin and recalculated with 5 mmol/L Ca\(_{2+}\). Platelet activation was started by addition of 0.5 \(\mu\text{mol/L}\) ADP or 5 \(\mu\text{g/mL}\) collagen (n = 6 to 10). Washed platelets (2×10\(^8\)/ml) were activated with 2 nmol/L thrombin (n = 11 to 14) or 5 \(\mu\text{g/mL}\) collagen (n = 6 to 8) in the presence of 1 mmol/L CaCl\(_2\). Shape change and maximal aggregation of platelets were determined from changes in light transmission (%T). Rises in \([Ca^{2+}]\) were measured in fura 2–loaded platelets by ratio fluorometry. Values are mean±SEM.

*ANOVA.

Figure 2. Effects of diets on platelet arachidonate content and thromboxane formation in activated platelets. Arachidonic acid was measured in total platelet phospholipids. Thromboxane B\(_2\) was determined after activation of washed platelets with collagen (squares) or thrombin (circles) under conditions indicated in Table 2. Numbers refer to various diet groups: 1, control group; 2 to 5, 2%, 4%, 8%, and 16% FO groups, respectively; and 6, SO group. Data are mean±SEM (n = 7 to 13). Calculated Pearson correlation coefficient (\(R\)) and level of significance (\(P\)) are for collagen-activated platelets, \(R = 0.95\) and \(P = 0.043\), and for thrombin-activated platelets, \(R = 0.83\) and \(P = 0.013\).

Results

Effects of FO and SO Diets on Blood Cells

To evaluate the long-term effects of dietary n-3 and n-6 PUFAs on crucial hemostatic functions, Wistar rats were fed for 8 to 10 weeks with 1 of 6 high-fat diets (Table 1): a control diet enriched in monounsaturated fatty acids (mainly oleate); a diet in which 2%, 4%, 8%, or 16% (wt/wt) of the fatty acids were replaced by n-3 PUFAs (mainly eicosapentaenoate and docosahexaenoate) originating from FO (2%, 4%, 8%, and 16% FO groups, respectively); and a diet containing 60% n-6 PUFAs, mainly linoleate, derived from SO (SO group). All diets were low in arachidonate content (≤0.5%). At the end of the feeding period, numbers of leukocytes, erythrocytes and platelets in blood samples were counted to evaluate possible dietary effects on blood cell formation or turnover. However, no diet-dependent effects were observed, except that mean platelet volume was decreased significantly and dose dependently (\(R = 0.49\), the ETP value of pooled plasma from rats maintained on commercial stock food.

Statistical Analyses

To evaluate the significance of differences between the various diet groups, a 1-way ANOVA was applied by using Statview 4.1 (Abacus Concepts, 1992 to 1994). When the ANOVA test showed statistical significance, a \(t\) test was performed with adjustment of the \(\alpha\) level with a factor of 0.2 according to the Bonferroni method for multiple comparisons to determine which of the groups differed from the control group. Dose dependence of the effects of increasing amounts of FO in the diet was disclosed by linear regression analysis according to Pearson.33

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P = 0.0008) in rats from the FO groups. For the control group, this parameter amounted to 7.1 ± 0.1 fl, whereas for the 16% FO group, it was 6.6 ± 0.1 fl (mean ± SEM, n = 8 to 9, P = 0.01).

**Effects of Diets on Membrane Composition and Activation of Platelets**

Platelets from the diet-modified rats were analyzed for their membrane fatty acid composition. As expected, increasing amounts of FO caused a gradual replacement of n-6 PUFAs by n-3 PUFAs in the platelet phospholipids (Figure 1). Notably, the levels of eicosapentaenoate (20:5 n-3), docosahexaenoate (22:6 n-3), and docosapentaenoate (22:5 n-3), and arachidonate (20:4 n-6) were increased at the expense of arachidonate (20:4 n-6) and adrenate (22:4 n-6). Platelets from the SO group differed from the control group only in slightly increased levels of linoleate (18:2 n-6) at the expense of arachidonate.

Platelet function was studied by measuring shape change and aggregation tendency in the presence of recalciﬁed plasma with hirudin as the anticoagulant, after stimulation with submaximal concentrations of ADP or collagen. Aggregation studies were also carried out with washed platelets (in the absence of plasma) on stimulation with submaximal doses of ADP or collagen. Aggregation studies were also carried out with washed platelets (in the absence of plasma) on stimulation with submaximal concentrations of thrombin or collagen. These agonists were chosen because in suspensions of rat platelets, collagen acts predominantly through the generation of thromboxane B2 (a stable breakdown product of thromboxane A2). When the diet groups were compared, a good correlation was obtained between arachidonate level in the platelet phospholipids and thromboxane formation after activation with either thrombin or collagen (Figure 2). Thus, as reported before, dietary effects on platelet arachidonate concentration were reﬂected by altered eicosanoid production without leading to a measurably changed activation tendency of the cells.

**Effects of Diets on Blood Lipids, Fibrinolysis, and Global Coagulation**

In plasma from rats fed the control diet, the triglyceride level was relatively high, 0.42 mmol/L. This level gradually decreased with the dose of dietary FO, to 0.12 mmol/L for the 16% FO group (Table 3). Rats from the SO group had a similar, reduced level of plasma triglycerides. In contrast to the SO diet, the FO diets also caused a considerable reduction in plasma cholesterol concentration. Because most of the rat cholesterol was collected as HDL, this type of cholesterol was also decreased in the FO groups (Table 3).

### Table 3. Effects of Diets on Triglycerides and Cholesterol in Plasma

<table>
<thead>
<tr>
<th>Plasma Concentration, mmol/L</th>
<th>Type of Diet</th>
<th>Statistics</th>
<th>P*</th>
<th>R†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triglycerides</td>
<td>Control</td>
<td>2% FO</td>
<td>4% FO</td>
<td>8% FO</td>
</tr>
<tr>
<td></td>
<td>0.42±0.07</td>
<td>0.44±0.10</td>
<td>0.34±0.04</td>
<td>0.16±0.03‡</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>2.12±0.09</td>
<td>1.78±0.10</td>
<td>1.71±0.13‡</td>
<td>1.18±0.06¶</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>1.75±0.08</td>
<td>1.58±0.11</td>
<td>1.46±0.13</td>
<td>0.89±0.06¶</td>
</tr>
</tbody>
</table>

Plasma levels of triglycerides, total cholesterol, and cholesterol in HDL were determined. Values are mean ± SEM (n = 10).

*ANOVA.

†Pearson correlation coefficient; P values of post hoc t test analyses compared with control group: §P = 0.01, ¶P = 0.002, and #P = 0.0002.

ND indicates not determined. Levels of tissue-type plasminogen activator antigen (t-PA, n = 9 to 10), plasminogen activator inhibitor-1 (PAI-1, n = 9 to 10), fibrinogen (n = 22 to 24), and prothrombin (% of standard rat plasma, n = 7 to 12) were measured in plasma samples as described in Methods. Standard coagulation times, APTT, and PT were measured in 20×-diluted samples (n = 22 to 24). Values are mean ± SEM.

*ANOVA.

†Pearson correlation coefficient, with ||P = 0.006, P values of post hoc t test analyses compared with control group with †P = 0.002, §P = 0.0002, and #P = 0.00079 (comparing FO groups).
of (circles). Hatched area indicates amidolysis due to accumulation of amidolytic activity (circles). Samples from coagulation mixture were taken to measure amidolytic activity (Table 4). Plasma activation with thromboplastin prepared from rat brain resulted in a high amidolytic activity that reached a maximum after ~2 minutes and then decreased rapidly (Figure 3). Conversion of amidolytic activity to concentration of free thrombin was carried out by correcting for the thrombin that was inactivated by binding to a1-macroglobulin but that still contributes to the chromogenic assay. Similarly as described for the human system,31 the stable end level of the amidolytic activity was used to determine the accumulation of such inactivated thrombin. After this correction, the area under the thrombin concentration–time curve reached a value of 445±38 nmol/L · min (mean±SEM, n=4), which, by definition, is the ETP of the activated plasma (Figure 3). Plasma activation with thromboplastin prepared from rat brain resulted in a similar ETP value of 434±21 nmol/L · min (n=5). Because these preparations of thromboplastin appeared to be rather unstable, however, they were not used for further experiments.

**Effects of Diets on ETP and Vitamin K–Dependent Coagulation Factors**

In human clotting plasma, the ETP determines the total enzymatic capacity of thrombin during the coagulation process as a whole, taking into account both the rise and subsequent fall of thrombin concentration. The result of the ETP assay is therefore sensitive for conditions influencing either prothrombin activation or thrombin inactivation.31,36 With rats that were fed a commercial stock diet, initial experiments showed that the ETP was reduced in the presence of low doses of heparin and of decreased levels of vitamin K–dependent coagulation factors, indicating that this assay can detect a hypocoagulable state of rat plasma (C.M.A.N. et al, unpublished observations, 1997). Triggering of the rat extrinsic coagulation system with phospholipids, Ca21, and recombinant tissue factor resulted in a high amidolytic activity that reached a maximum after ~2 minutes and then decreased rapidly (Figure 3). Conversion of amidolytic activity to concentration of free thrombin was carried out by correcting for the thrombin that was inactivated by binding to a1-macroglobulin but that still contributes to the chromogenic assay. Similarly as described for the human system,31 the stable end level of the amidolytic activity was used to determine the accumulation of such inactivated thrombin. After this correction, the area under the thrombin concentration–time curve reached a value of 445±38 nmol/L · min (mean±SEM, n=4), which, by definition, is the ETP of the activated plasma (Figure 3). Plasma activation with thromboplastin prepared from rat brain resulted in a similar ETP value of 434±21 nmol/L · min (n=5). Because these preparations of thromboplastin appeared to be rather unstable, however, they were not used for further experiments.

**Figure 3.** Kinetics of thrombin formation and inactivation in coagulating rat plasma. Changes in amidolytically active thrombin were determined in platelet-free plasma from rats maintained on stock food. Plasma was activated with Ca21 phopholipids, and tissue factor (see Methods). Samples from coagulation mixture were taken to measure amidolytic activity (Table 4). Shown are representative curves obtained with plasma from rats fed control diet (●), 8% FO diet (▲), or SO diet (■). Significant differences were calculated for α1-macroglobulin-bound thrombin (see Figure 3). Plasma from modified diet-fed rats was activated with Ca21, phopholipids, and tissue factor. A, Curves of thrombin formation and inactivation were determined and corrected for α1-macroglobulin-bound thrombin (see Figure 3). Shown are representative curves obtained with plasma from rats fed control diet (●), 8% FO diet (▲), or SO diet (■). B, Bars represent ETP values calculated from these curves per diet group (mean±SEM, n=7 to 9). Statistics: ANOVA with *P=0.016; † test analysis with †P=0.029, †P=0.005 compared with control group.
Application of this assay to plasma from rats fed the n-3 or n-6 PUFA diets gave consistent, diet-induced effects. For rats fed >2% FO, the ETP of tissue factor–activated plasma was ≈22% lower than that for rats from the control group (Figure 4). In contrast, feeding the rats with SO did not significantly influence the ETP. Using a limited number of remaining plasma samples, we investigated whether the relatively low ETP in the FO groups was a consequence of either decreased thrombin formation or increased thrombin inactivation. In plasma of rats from the control group and the 8% and 16% FO groups, we thus measured the levels of antithrombin III (a major thrombin inactivator) and factor VII (rate limiting in extrinsic coagulation). When compared with the control group, the antithrombin III concentration remained at 99 ± 3.0% and 100 ± 4.6% (mean ± SEM, n = 7 to 11) in the 8% and 16% FO groups, respectively. On the other hand, factor VII was significantly reduced in the FO groups, to 74 ± 9.0% and 74 ± 8.7%, respectively (mean ± SEM, n = 8 to 10, P < 0.05).

Because the vitamin K–dependent coagulations factors prothrombin and factor VII were both decreased in the FO-fed animals, it was of interest to know which of these changes might explain the lowering effect of the FO diet on coagulation potency (ETP). To investigate this, rats maintained on commercial stock food were treated with the vitamin K antagonist warfarin during a variable time period of 4 to 27 hours. Because the half-life of factor VII in rats is much shorter than that of prothrombin, ie, 2.6 versus 9.0 hours,23 this resulted in plasmas with reduced prothrombin and even more severely decreased factor VII concentrations. Adequate combinations of these plasma preparations resulted in mixtures that were variable in factor VII or prothrombin level while the other factor was kept constant. Plasma mixtures in which the prothrombin concentration was reduced to ≈55% gave a proportional decrease in tissue factor–induced ETP, independently of whether factor VII was decreased to 25% or 51% of its original concentration (Figure 5A). Similarly, in plasma mixtures wherein the prothrombin level was reduced to ≈35%, the EPT was reduced correspondingly, irrespective of a decrease in factor VII concentration to 43% or 8% of its original value (Figure 5B). The main effect of a low factor VII level here was an increased delay in thrombin formation.

To confirm that the ETP is determined more by the prothrombin than the factor VII level, we measured the effects of addition of purified prothrombin and factor VII on the coagulation potential of a plasma mixture with 50% reduced concentrations of these factors. Because pure rat coagulation factors could not be obtained in sufficiently high amounts, these experiments were performed with human factors. Prothrombin and factor VII were added in concentrations to give normal clotting times (with the use of the factor-deficient plasma concerned). As shown in Figure 5C, supplementation of purified factor VII had no effect on the tissue factor–induced ETP, whereas addition of prothrombin, either alone or in combination with factor VII, had a strongly increasing effect on the ETP. Similar results were obtained with plasma preparations that contained normal levels of these factors or that were even more severely depleted in prothrombin/factor VII concentrations (data not shown).

### Discussion

**Dietary FO and SO Reduce Plasma Lipids but Have No More Than Marginal Effects on Platelet Activation or Fibrinolysis**

In the current study with Wistar rats, we determined the effects on hemostatic functions of high-fat diets containing...
various amounts of n-3 PUFAs (derived from FO) or n-6 PUFAs (originating from SO) in comparison with a control diet containing 50% monounsaturated fatty acids. After a dietary period of 8 to 10 weeks, the FO-derived fatty acids had well accumulated in the platelet phospholipids at the expense of mainly arachidonic acid. This replacement was already detectable at the lowest dose of FO (2%) and increased with increasing FO content in the diet (Figure 1). The production of thromboxane by activated platelets decreased proportionally with the reduction in platelet arachidonate level (Figure 2), which confirms our earlier conclusion that the substrate level of arachidonate esterified in the phospholipids is a major determinant of the rate of eicosanoid production.\textsuperscript{9,21} However, in spite of the greatly reduced capacity of thromboxane production by platelets from the higher FO groups, no dietary effects could be measured on platelet activation with either washed platelets or platelets in autologous plasma, with the use of a variety of agonists (Table 2). The SO diet was of minor influence on platelet fatty acid composition, thromboxane production, and activation tendency when compared with the control group. Taken together, these no more than marginal effects of dietary n-3 and n-6 PUFAs on platelet activation tendency confirm the findings of earlier feeding studies with rats.\textsuperscript{21–24} It should be noted here, however, that the hemostatic role of the arachidonic acid pathway in rats is less well defined and may be less important than in humans. For instance, whereas in rat thrombosis models aspirin appears to have little effect on fibrinolysis in rats. In the current study, we measured 2 other clotting factors or coagulation potency in rats are scarce, making it difficult to compare our data with the literature. We found a consistent, dose-dependent reduction in the prothrombin level of animals fed the FO-containing diets (Table 4). In addition, we detected a reduced level of factor VII in the plasma from rats fed either 8% or 16% FO. This apparently is of significance, because both clotting factors are of crucial importance in hemostasis: factor VII and its activated form as coagulation initiators and prothrombin as the immediate precursor of thrombin, which amplifies the coagulation cascade and causes the formation of fibrin clots. Intriguingly, this reduction in coagulation factors was not reflected by an increase in (standard) coagulation times; ie, neither the PT nor the APTT was changed, although the APTT is moderately sensitive for hypocoagulable states, such as occur in hemophilia or during heparin treatment. The reduction in prothrombin level stimulated us to make use of an assay with much higher sensitivity for hypocoagulable conditions, ie, the ETP assay, which determines the thrombin concentration–time integral of coagulating plasma. The result of this assay is an estimation of the total enzymatic capacity of thrombin during the time period of the clotting process and, as such, is a linear parameter of the coagulation potential of plasma.\textsuperscript{36} Application of this assay to plasma from Wistar rats fed the stock diet showed a high sensitivity to conditions interfering with either thrombin formation (reduction of vitamin K–dependent coagulation factors) or thrombin inactivation (heparin) (C.M.A.N. et al, unpublished observations, 1997).

The tissue factor–induced ETP of plasma from the rats that received >2% FO was decreased in comparison with the control group (Figure 4). Because the level of anti-thrombin III (an important vitamin K–independent anticoagulant) remained unchanged, we can conclude that dietary FO leads to a hypocoagulable state in rat plasma by influencing (vitamin K–dependent) thrombin formation rather than thrombin inactivation. To demonstrate which of the changes in clotting factors was responsible for the hypocoagulant effect, control studies were performed with plasma from rats that were treated with warfarin for various times. Owing to the quite different half-lives of the various vitamin K–dependent coagulation factors, ie, prothrombin > factors IX and X > factor VII, these resulted in plasma preparations that were more depleted in factor VII than in prothrombin. By combining these plasmas, mixtures could be prepared containing variable amounts of either factor VII or prothrombin (and intermediate amounts of factors IX and X). Determination of the ETP in these plasmas strongly suggested that the prothrombin level is the major determinant of coagulation efficacy (Figure 5). Whereas the factor VII level may well influence the initial rate of thrombin formation (Figure 5B), it apparently is of less importance for coagulation potency. Essentially the same conclusion has been drawn in earlier studies with human plasma; ie, that primarily the variation in prothrombin level determines thrombin generation and hence, the antithrombotic effect of anticoagulant therapy.\textsuperscript{41}

It is tempting to speculate on the mechanism whereby vitamin K–dependent coagulation factors are reduced after consumption of an FO diet. Vitamin K is a highly lipophilic molecule, which needs to be transported through the blood in a lipid-bound way. It is possible that the strong reduction in lipoprotein-bound cholesterol and tri-

Dietary FO but Not SO Evokes a Hypocoagulable State in Rat Plasma

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Hypocoagulant Effect of Fish Oil in Rats

glycerides, also observed after feeding of an FO diet (Table 3), limits the transport of vitamin K and thereby the synthesis of vitamin K–dependent clotting factors in the liver. Recent studies have indeed pointed to a positive correlation between levels of blood lipids and factor VII. Typically, in our study with rats, the SO diet was essentially without hypocoagulant effect while causing a reduction in only triglycerides and not in cholesterol, which would imply that cholesterol plays a more important role in vitamin K delivery than do triglycerides.

There is little literature concerning effects of n-3 PUFA–supplemented diets on coagulation in humans, putatively because many investigators have used global assays (APTT or PT) that are rather insensitive in detecting hypocoagulable conditions. Of the individual coagulation factors, mainly factor VII has been studied, because it is considered a possible risk factor for cardiovascular disease. In a limited number of human nutrition studies, small decreasing effects of FO on factor VII activity have indeed been reported. However, none of these studies included sensitive coagulation measurements, eg, determination of the coagulation potential. It will therefore be important to see whether dietary FO (n-3 PUFAs) causes similar hypocoagulant effects in humans as reported here in rats.

Summarizing, we present evidence that n-3 PUFAs, when administered to rats as FO mixtures, cause decreased levels of plasma lipids and have no major effects on platelet activation or fibrinolysis. On the other hand, they appear to lead to a hypocoagulable state because of their reducing effect on the levels of vitamin K–dependent coagulation factors. Such a hypocoagulant effect is not seen with dietary n-6 PUFAs.

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References

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