Response of Plasma Tissue Factor Pathway Inhibitor to Diet-Induced Hypercholesterolemia in Crab-Eating Monkeys

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Abstract Tissue factor pathway inhibitor (TFPI), a protease inhibitor associated with lipoproteins in plasma and endothelial cells, can inhibit the initial reactions of the tissue factor-mediated coagulation pathway. A positive relationship between TFPI and cholesterol has been demonstrated in human plasma. To investigate this relation in more detail, in the present study we measured TFPI in the plasma of monkeys on a high-cholesterol diet. After diet treatment, cholesterol levels and TFPI activity were increased 3- and 1.5-fold, respectively. Three forms of TFPI, low-density lipoprotein (LDL)/very-low-density lipoprotein (VLDL)–associated TFPI, high-density lipoprotein (HDL)–associated TFPI, and free TFPI, were measured after gel filtration of plasma. In hypercholesterolemic monkeys, levels of TFPI activity and antigen in the LDL/VLDL fraction were increased to about three times those of normal monkeys. Changes in HDL-associated TFPI and free TFPI were not significant compared with the change in LDL/VLDL-associated TFPI. After the monkeys received heparin infusions TFPI was increased about fivefold, but there was no significant difference in these increases between normal and hypercholesterolemic monkeys. The increase in TFPI after heparin infusion is discussed in terms of the relationship between lipoprotein-associated TFPI in plasma and endothelial cell–associated TFPI.

Key Words • tissue factor pathway inhibitor • monkeys • hypercholesterolemia • heparin

The extrinsic blood coagulation pathway is triggered by the interaction between tissue factor (TF) and factor VII, which leads to activation of the precursors of the serine proteases, factor VII, factor IX, factor X, and prothrombin, resulting in the generation of thrombin. (For a review see References 1 and 2.) These reactions are regulated by several different mechanisms, eg, inactivation of cofactors by activated protein C and inactivation of activated proteases by protease inhibitors such as antithrombin III. TF pathway inhibitor (TFPI), a protease inhibitor with three tandem Kunitz-type inhibitory domains, inhibits factor VIIa and factor Xa in the presence of TF, so that TFPI is an inhibitor of the initial reaction of the extrinsic pathway. (For reviews see References 3 through 7.)

The most striking characteristics of TFPI are its association with lipoproteins in plasma and endothelial cells. It has been demonstrated that three forms of TFPI, low-density lipoprotein (LDL)/very-low-density lipoprotein (VLDL)–associated TFPI, high-density lipoprotein (HDL)–associated TFPI, and the free form of TFPI, can be detected by gel filtration of human plasma; it has also been shown that heparin infusion markedly increases the free form of TFPI, which is thought to be derived from endothelial cell–associated TFPI.7,8 These findings tempted us to speculate that TFPI participates in the regulation of thrombosis and atherosclerosis. Indeed, the relationship between cholesterol and TFPI in human plasma has been investigated. Sandset et al9 have demonstrated that TFPI is significantly correlated with age-related cholesterol levels in a normal population and that the increases in TFPI that are observed in hypercholesterolemia can be reduced by treatment with hydroxymethylglutaryl–coenzyme A reductase inhibitors.10 On the other hand, low levels of plasma TFPI have been found in abetalipoproteinemia and hypolipoproteinemia.11 To analyze the role of TFPI in thrombosis and atherosclerosis in more detail, we selected the monkey as an animal model and measured TFPI activity and antigen levels in monkey plasma after administration of a high-cholesterol diet. In an earlier experiment we cloned monkey TFPI cDNA, determined the amino acid sequence of monkey TFPI, and prepared recombinant monkey TFPI (rTFPI) and its polyclonal and monoclonal antibodies.12 In the present study we demonstrate that LDL/VLDL–associated TFPI is increased in crab-eating monkeys fed a high-cholesterol diet. We also discuss the changes in TFPI levels after injection of heparin into these monkeys.

Methods

Materials

Superdex 200HR was obtained from Pharmacia LKB Biotechnology; multiwell plates (type H) from Sumitomo Bakelite; Z-Pyr-Arg-methylcoumarin amide from the Peptide Institute; bovine serum albumin (BSA) from Boehringer Mannheim Yamanouchi; rabbit brain thromboplastin from Kokusai Siyaku; peroxidase-labeled goat anti-rabbit immunoglobulin G antibody from American Qualex; and Novo-hepa-
Enzyme Immunoassay (EIA) of TFPI Antigen

Addition of TFPI antigen was measured by a sandwich EIA method with polyclonal and monoclonal antibodies to monkey rTFPI. Each well of the microplate was coated with 50 µL of monoclonal anti-mouse recombinant TFPI antibody (13.6 µg/mL in TBS). After overnight incubation at 4°C the plates were washed with 200 µL TBS containing 1% BSA and were left overnight at 4°C. Samples or rTFPI standard were diluted with TBS containing 0.01% BSA and then mixed with Triton X-100 (final concentration, 1%). After addition of 50 µL of the sample or the rTFPI standard to each well, the plate was incubated for 2 hours at room temperature. The plates were then washed with TBS-Tween (20 mmol/L Tris-HCl, pH 8.0, containing 0.5 mol/L NaCl and 0.05% Tween 20), and 50 µL of polyclonal anti-mouse recombinant TFPI antibody (27.9 µg/mL in TBS-Tween) was dispensed into each well. After a 2-hour incubation at room temperature, the plates were washed with TBS-Tween and incubated with 100 µL of peroxidase-labeled goat anti-rabbit immunoglobulin G antibody (diluted 3000-fold in TBS-Tween) per well for 2 hours at room temperature. After further washing with TBS-Tween, 200 µL of 0.1 mol/L sodium citrate buffer, pH 4.5, containing o-phenylenediamine (0.4 mg/mL) and H2O2 (0.004%) was dispensed into each well. After 10 minutes the enzyme reaction was stopped by adding 3 mol/L H2SO4. Absorbance at 492 nm was measured with an ELISA reader (model 2550, Bio-Rad Laboratories). The amounts of TFPI antigen were calculated from a standard curve obtained with the serially diluted rTFPI.

Gel Filtration of Plasma

Five hundred microliters of plasma were applied to a column (1.6x60 cm) of Superdex 200 equilibrated with TBS containing 0.05% NaN3. Gel filtration was performed at a flow rate of 1 mL/min at room temperature, and 1-mL fractions were collected. The column was calibrated with gel filtration standards from Bio-Rad Laboratories.

Results

Cholesterol and TFPI Activity Levels in Plasma

Blood collection was performed before (-10 and 0 weeks) and after (4, 12, 16, and 20 weeks) the animals began the high-cholesterol diet. The mean plasma cholesterol level of six monkeys before initiation of the high-cholesterol diet was 4.57±0.67 mmol/L. Four weeks after beginning the high-cholesterol diet, the mean value rose to 14.04±1.96 mmol/L and remained virtually constant until 20 weeks (Fig 1A). The mean plasma TFPI activity of the monkeys before initiation of the high-cholesterol diet was 0.89±0.08 U/mL. Four weeks after beginning the high-cholesterol diet, TFPI activity rose to 1.33±0.11 U/mL and decreased slightly thereafter until 20 weeks (Fig 1B). That is, maximum levels of cholesterol and TFPI activity in the plasma of these monkeys were increased 3- and 1.5-fold, respectively, in response to the high-cholesterol diet.
Increase in Lipoprotein-Associated TFPI in Response to Diet-Induced Hypercholesterolemia

When plasma from two monkeys (at 0, 4, and 20 weeks) was gel filtered as described in “Methods,” cholesterol was found as a large peak in the void-volume fraction and as a small peak in the lower-molecular-weight (MW) fraction (Fig 2A). When each fraction was analyzed for TFPI activity, one large TFPI peak was detected in the void-volume fraction, and two small peaks at MWS of $3.0 \times 10^5$ and $1.6 \times 10^5$ were detected, as shown in Fig 2B. Analysis of lipoproteins from rhesus monkeys has shown that the MW of LDL is $3.01 \times 10^6$ in normal monkeys and $3.52 \times 10^6$ in hyperlipidemic monkeys,13 and the MW of two species of HDL has been shown to be $3.90 \times 10^5$ and $1.97 \times 10^5$.18 In our present experiment LDL and VLDL could not be separated on the column. Therefore, we assumed that the large TFPI peak in the void-volume fraction was LDL/VLDL-associated TFPI and that the two small peaks were TFPI associated with two different species of HDL. Although this profile of TFPI activity was similar to that of human plasma, it was different, in that HDL-associated TFPI was separated into two separate peaks.

Fig 2 also shows that the cholesterol level in the LDL/VLDL fraction increased at 4 and 20 weeks after diet treatment, whereas the cholesterol level in the HDL fractions decreased. TFPI activity in the LDL/VLDL fraction also increased after diet treatment. On the other hand, TFPI activity in the HDL fractions behaved differently. High-MW HDL-associated TFPI activity was reduced, while low-MW HDL-associated TFPI activity was increased by the treatment. Changes in the free form of TFPI were not significant.

To quantitatively compare the peak values of TFPI activity in each lipoprotein fraction, they were defined as follows: LDL/VLDL, fractions 38 through 49; high-MW HDL, fractions 50 through 57; and low-MW HDL, fractions 58 through 65. When values at 4 and 20 weeks after diet treatment were averaged and compared with those before treatment (week 0), TFPI activity had increased about twofold in the low-MW HDL fraction, had been reduced to half in the high-MW HDL fraction, and had increased about twofold in the low-MW HDL fraction. The distribution of the three forms of lipoprotein-associated TFPI activity in plasma was calculated to be 44% of the LDL/VLDL-associated form, 32% of the high-MW HDL-associated form, and 25% of the low-MW HDL-associated form before treatment and 65%, 11%, and 25%, respectively, after treatment.

Relationship Between TFPI Activity and Antigen and the Effects of Heparin on TFPI in Hypercholesterolemia

As described in the previous section, LDL/VLDL-associated TFPI activity increased in the high-cholesterol diet-fed monkeys. To determine whether the increased TFPI activity was due to an increase in TFPI protein, we developed an EIA method for measuring TFPI antigen as described in “Methods.” We measured TFPI activity and antigen in three monkeys once before and three times after (2, 4, and 8 weeks) they had begun the diet. TFPI antigen and activity were significantly correlated in each plasma sample ($r=0.837$). We also measured TFPI in plasma after the heparin infusion in normal and hypercholesterolemic monkeys; this form of TFPI was assumed to be derived from endothelial cell-associated TFPI. When all values were analyzed together, the correlation was found to be highly significant ($r=0.974$). These results indicate that increases in TFPI activity during hypercholesterolemia and after heparin injection are mainly due to increases in TFPI protein, although an increase in the relative activity of TFPI cannot be completely excluded. Fig 3 shows the profiles of TFPI activity and antigen after gel filtration of plasma before and after diet treatment. As shown in the previous section, TFPI activity in the LDL/VLDL and low-MW HDL fraction increased after treatment, whereas it was reduced in the high-MW HDL fraction (Fig 3A). TFPI antigen in the LDL/VLDL fraction also increased, but there was no increase in TFPI in the low-MW HDL fraction (Fig 3B). This result indicates that the antibody used in this experiment did not recognize low-MW HDL-associated TFPI.

After heparin infusion, TFPI activity increased about fivefold in both normal and hypercholesterolemic plasma, as shown in Fig 4. No significant difference was found in TFPI activity levels between groups. When the postheparin plasma sample from a normal monkey was analyzed by gel filtration immediately after blood collection, we found that TFPI activity in the LDL/VLDL, high-MW HDL, and free-form fractions was increased compared with the activity in preheparin plasma (Fig 5). However, after the postheparin plasma was left for 24 hours at 4°C, TFPI in the LDL/VLDL fraction was reduced while TFPI in the HDL fraction was increased. In the analysis of antigen level by EIA, the same tendency was observed except for poor detection of TFPI in the low-MW HDL fraction (data not shown). These results indicate that TFPIs released by heparin are first present as the LDL/VLDL-associated, HDL-associated, and free forms and are transported by...
unknown mechanisms to the HDL fraction by leaving the plasma at 4°C.

**Discussion**

In the present study, we demonstrated that TFPI activity in the plasma of crab-eating monkeys increased in response to a high-cholesterol diet. Our findings indicated that a high-cholesterol diet produced an increase in TFPI protein that was mainly associated with LDL/VLDL, in parallel with increases of the lipoprotein. The behavior of HDL-associated TFPI in response to a high-cholesterol diet is rather complicated. As reported by Fless et al., hypercholesterolemia in monkeys reduced the MW of HDL particles after the loss of phospholipid, unesterified cholesterol, and triglyceride. In the present study, we found that high-MW HDL-associated TFPI activity decreased, but low-MW HDL-associated TFPI activity increased in response to a high-cholesterol diet. This change is possibly due to the compositional change of HDL particles. However, we could not confirm that changes in HDL-associated TFPI activity corresponded to changes in TFPI protein because we did not detect increases in low-MW HDL-associated TFPI protein. We speculated that the poor detection resulted from reactivity with the monoclonal antibody. Why the monoclonal antibody did not cross-react with TFPI in the low-MW HDL fraction is not known. It is possible that the epitope of TFPI may have been "buried" after binding with low-MW HDL particles. We tried to improve the EIA method to detect low-MW HDL-associated TFPI by using different antibodies or by adding detergents. However, our attempts were unsuccessful. Another possible explanation for the discrepancy between TFPI activity and antigen in the HDL fraction should also be taken into consideration; i.e., the inhibitory activity in this fraction may not be due to TFPI. The inability to detect TFPI antigen may suggest that the functional assay reflects the inhibitory activity of apolipoprotein A-II, as described by Carson and Gramzinski et al.

Our present study also demonstrated that TFPI activity and antigen in plasma were significantly correlated. Although the EIA method did not detect the HDL-associated form of TFPI, the results indicate that the method is applicable for the analysis of TFPI antigen in plasma, since the majority of TFPI in plasma is in the LDL/VLDL-associated form.

The gel filtration profile of monkey plasma was quite similar to that of human plasma except that two forms of
HDL-associated TFPI were clearly separated in monkey plasma. Although we used plasma stored at ~80°C, the profile was not changed by freezing and thawing. Sandset et al have demonstrated that TFPI activity in familial hypercholesterolemia is higher than in normal subjects and can be correlated with LDL cholesterol. Our finding that LDL/VLDL-associated TFPI increases in response to a high-cholesterol diet is consistent with theirs. However, these findings are not consistent with those in rabbits, in which TFPI activity did not increase despite increases in cholesterol. The gel filtration profile of rabbit plasma was quite different from those of human and monkey plasmas. The major form of TFPI in rabbit plasma was the free form, not the lipoprotein-associated form. The homology of monkey TFPI to human TFPI is 94%. The different behavior of TFPI in rabbit and rat plasma (T. Abumiya et al, unpublished data) from that in human and monkey plasma may be due to differences in the amino acid sequence, posttranslational modification of TFPI, or species differences in the properties of lipoproteins. Many studies of the effects of TFPI have been performed with rabbits. However, our findings indicate that for studies of thrombosis and atherosclerosis, species differences should be taken into consideration if rabbits and rats rather than monkeys are the animal model.

It has been demonstrated that intravenous injections of heparin into normal human subjects induces twofold to fivefold increases in plasma TFPI within 5 to 10 minutes. In our study, heparin injections into monkeys produced about fivefold increases in plasma TFPI, but no significant differences in heparin-releasable TFPI levels were found between normal and hypercholesterolemic monkeys. This finding indicated that heparin-releasable TFPI in endothelial cells did not change during the 8-week study. If synthesis of TFPI in endothelial cells deteriorates in hypercholesterolemia, then heparin-releasable TFPI levels must decrease. Why was heparin-releasable TFPI not decreased in our study? One possible explanation may be that the induced hypercholesterolemia was mild and insufficient to damage endothelial cell functions related to TFPI. Otherwise, the main source of heparin-releasable TFPI may be endothelial cells in the extensive vascular bed except for arteries, i.e., capillaries and veins, so that damage to endothelial cells in arteries may not reflect a significant decrease in heparin-releasable TFPI.

The next question to answer is why did plasma TFPI increase in response to the high-cholesterol diet? Where did the increased TFPI come from? It is reasonable to assume that endothelial cell-associated TFPI, i.e., heparin-releasable TFPI, is the principal source of increased TFPI. Novotny et al have demonstrated that exogenously added 125I-labeled, heparin-releasable TFPI can bind LDL/VLDL and HDL in vivo. Furthermore, our data on postheparin plasma suggest that heparin-releasable TFPI can be transferred to the LDL/VLDL and HDL fractions. With all of the aforementioned considerations, we speculate that TFPI on the endothelial cell surface was transferred to LDL/VLDL in concert with increases in lipoproteins during monkey hypercholesterolemia. Other possibilities should be also taken into consideration, e.g., lowering of the catabolic rate of TFPI in plasma and the contribution of TFPI synthesized in other tissues, like platelets and macrophages.

The role of TFPI in the regulation of thrombosis and atherosclerosis remains to be established. Since LDLs accumulate in atherosomas where thrombosis is likely to occur, the increase in LDL-associated TFPI may play a role in the prevention of local thrombosis. However, because plasma TFPI occurs in small amounts, the increase in LDL/VLDL-associated TFPI in plasma may be less significant than changes in endothelial cell-associated TFPI. Therefore, we suspect that the increase in LDL/VLDL-associated TFPI reflects the decrease of TFPI on endothelial cells in atherosclerotic lesions, i.e., a lowering of local antithrombotic properties. Although we failed to detect significant changes in heparin-releasable TFPI, further studies of hypercholesterolemia in monkeys should provide one approach for clarifying these problems.

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