Increased Tissue Factor Pathway Inhibitor Activity in IDDM Patients With Nephropathy

HIROKI YOKOYAMA, MD
BJARNE MYRUP, MD
PETER ROSSING, MD
PER B. ØSTERGAARD, PHD

OBJECTIVE — Tissue factor pathway inhibitor (TFPI) is bound to vascular endothelium (presumably to heparan sulfate) and circulates in complex with plasma lipoproteins. It directly binds and inhibits factor Xa. The purpose of the study is to investigate whether plasma TFPI activity is altered in IDDM and nephropathy and to evaluate the possible determinants of the alteration.

RESEARCH DESIGN AND METHODS — We assessed plasma concentration of TFPI (total, truncated, and domain 3 TFPI) and plasma activity of factor Xa inhibition in nondiabetic control subjects (n = 22) and in IDDM patients with normoalbuminuria (urinary albumin excretion rate [UAE] <30 mg/24 h, n = 17), incipient nephropathy (UAE 30–300 mg/24 h, n = 17), and clinical nephropathy (UAE >300 mg/24 h, n = 25).

RESULTS — Total, truncated, and domain 3 TFPI concentrations were increased in IDDM patients compared with those in control subjects and were more pronounced in IDDM patients with nephropathy. Plasma activity of factor Xa inhibition measured by the HEPTEST (Haemachem, St. Louis, MO) assay was increased in IDDM patients, especially in those with nephropathy. TFPI-dependent factor Xa inhibition, obtained as the difference in clotting time with and without adding activity-neutralizing anti-TFPI antibody to samples, was increased in IDDM patients with nephropathy. This was, however, not sufficient to inhibit the biological activity of factor Xa as demonstrated by increased levels of prothrombin fragment 1 + 2. LDL cholesterol and HbA_{1c} were independently correlated to plasma TFPI.

CONCLUSIONS — Inhibition of factor Xa activity is increased in IDDM patients with nephropathy, mainly because of increased plasma TFPI activity. The increased plasma TFPI activity in these patients may be associated with and regulated by LDL in plasma and metabolic control. The anticoagulant activity of TFPI may attenuate the hypercoagulable state in diabetes but does not seem to be able to normalize hemostasis.

The pathogenesis of vascular disease in diabetes involves changes to the hemostatic mechanism. These changes include alterations to platelet function, endothelial cell function, and the hemostatic balance, particularly to plasma proteins involved in coagulation. Tissue factor pathway inhibitor (TFPI) is the factor Xa–dependent inhibitor of the factor VIIa/tissue factor complex (1,2). It has been recently isolated and identified as a glycoprotein of 276 amino acids, and it is essential in maintaining a normal hemostatic balance (3,4). The TFPI molecule contains an acidic amino terminus followed by three tandem Kunitz-type proteinase inhibitory domains (domains 1, 2, and 3) and a basic COOH-terminal (3,4). It is the Kunitz-type domain 2 that mediates its binding and inhibition of factor Xa, whereas domain 1 is required for its inhibition of the factor VIIa/tissue factor catalytic complex (4). The TFPI is present in vivo in three separate pools: 50–90% is on the endothelium, 10–50% is in plasma, and <2.5% is in platelets (5). The positively charged COOH-terminal of TFPI is considered to be bound to heparan sulfate on the endothelium (1); and in plasma TFPI, it is mainly associated with lipoproteins and only 5% is free (5). Plasma TFPI is heterogeneous in size. Only a fraction of the inhibitor circulates in the form of the full-length molecule; the remainder consists of variably COOH-terminal truncated forms (6). The TFPI complexed to lipoproteins seems to lack a substantial portion of the COOH-terminal, including at least a portion of the Kunitz-type domain 3 (6).

Little is known about plasma TFPI activity in IDDM patients with nephropathy. A slight increase of plasma TFPI was shown only in IDDM patients without complications (7). Diabetic nephropathy is associated with impaired endothelial function (8), decreased synthesis of heparan sulfate (9), and lipid abnormalities (10). These may affect plasma TFPI activity and hemostatic balance. The purpose of the study is, therefore, to investigate whether plasma TFPI activity is altered in IDDM and nephropathy and to evaluate the possible determinants of the alteration.

RESEARCH DESIGN AND METHODS

— A group of nondiabetic subjects recruited from the medical staff and surroundings of the hospital served as a control group (group 1). A total of 59 IDDM patients (onset before the age of 31 years) were recruited from the outpatient clinic at the Steno Diabetes Center. Patients were enrolled in the study in an order determined by birth date. Only patients in the 21- to 57-year age range were included. The patients were allocated to three groups based on the level of urinary albumin excretion (UAE) rate. The goal was to obtain three groups (n >15): one with UAE <30 mg/24 h (normoalbuminuria, group 2), one in the range 30–300

From the Steno Diabetes Center (H.Y., B.M., P.R.) and Vascular Wall Biology (P.B.Ø.), Novo Nordisk A/S, Gentofte, Denmark.

Address correspondence and reprint requests to Hiroki Yokoyama, MD, PhD, Diabetes Center, Tokyo Women's Medical College, 8–1 Kawada-cho, Shinjuku-ku, 162, Tokyo, Japan.

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ELISA, enzyme-linked immunosorbent assay; F1 + 2, prothrombin fragment 1 + 2; TFPI, tissue factor pathway inhibitor; UAE, urinary albumin excretion.

mg/24 h (microalbuminuria, group 3), one and >300 mg/24 h (diabetic nephropathy, group 4). Normoalbuminuric patients were only included if they had a diabetes duration of 10–30 years in an attempt to cover the range of diabetes duration in patients with microalbuminuria and diabetic nephropathy. Patients who were receiving any medical treatment other than insulin or who had nondiabetic kidney disease were not included. All patients and nondiabetic control subjects participated after giving informed consent, and the study protocol was approved by the local ethical committee. Numbers and clinical information on patients and nondiabetic control subjects are given in Table 1.

Blood samples were obtained in the fasting state after 30 min of supine rest. After minimal stasis, a 17-gauge needle was placed in an antecubital vein. The blood in the first tube was used for determination of serum total cholesterol, HDL cholesterol, and triglyceride. EDTA-plasma samples (0.054 ml [0.134 mol/l] to 4.5 ml blood) were prepared for the analysis of TFPI and prothrombin fragment 1 + 2 (F1 + 2). The samples were placed on ice immediately after sampling and remained on ice for 30 min. After centrifugation at 2,000*g* for 20 min at 4°C, specimens of plasma were stored in polypropylene tubes at −40°C until analysis.

UAE was measured by an enzyme-linked immunosorbent assay (ELISA) technique (11), and the level was expressed as the median UAE in three 24-h urine collections taken at home during normal physical activity. HbA_{1c} was measured by high-performance liquid chromatography (Bio Rad, DIAMAT, Richmond, CA; normal range 4.1–6.4%) and blood glucose by a One-Touch apparatus (Life Scan). Total cholesterol and triglyceride levels were determined enzymatically by the cholesterol oxidase–peroxidase-aminopyrine and glycerol phosphate–oxidase-peroxidase-aminopyrine methods (Boehringer, Mannheim, Germany). HDL cholesterol was determined after precipitation of VLDL and LDL cholesterol with phosphotungstic acid (Boehringer Mannheim). LDL cholesterol was calculated according to the Friedewald formulation by subtracting HDL cholesterol and 45% of triglyceride (as VLDL cholesterol) from total cholesterol (12).

F1 + 2 was measured by ELISA method (Enzygnost F1 + 2, Behringwerke AG, Marburg, Germany) (13). In this assay, F1 + 2 was bound to wells in a microtiter plate precoated with a rabbit antibody against a synthetic peptide mimicking a sequence in the negatively charged region of F1 + 2. Tagged F1 + 2 was determined by a prothrombin-antibody/POD (*o*-phenylendiamine-dihydrochloride) conjugate, and the enzyme activity was measured. The results are expressed in nanomoles per liter according to a calibrator delivered from the manufacture.

TFPI was measured by a Imubind Total TFPI ELISA Kit, Product 850, from American Diagnostica (Greenwich, CT). The kit is designed to quantitate both the total concentration of TFPI in plasma and the concentration of truncated TFPI lacking the COOH-terminal and at least a part of the Kunitz-type domain 3. A traditional "sandwich" ELISA uses a rabbit anti-human TFPI polyclonal antibody as the capture antibody and two different biotinylated monoclonal antibodies specific for the Kunitz-type domains 1 and 3 of TFPI, respectively, as the detecting antibody. Subsequent binding of streptavidin-conjugated horseradish peroxidase completes the formation of the antibody enzyme–detecting complex. The addition of tetramethylbenzidine substrate and its subsequent reaction with horseradish peroxidase provides a blue color. Sulfuric acid (0.5 mol/l), which yields a yellow color, was used to stop the reaction and to increase the sensitivity. TFPI levels were determined by measuring sample solution absorbance at 450 nm, and comparison against those of a standard curve were developed using full-length TFPI. The detecting antibody directed against domain 1 binds full-length, complexed, and truncated TFPI and measures the total TFPI concentration. The other detecting antibody directed against domain 3 measures only full-length or domain 3 TFPI. The difference between the TFPI concentrations determined by the two antibodies represents the concentration of truncated TFPI. Thus, by this ELISA kit, three different populations of TFPI molecules can be measured: total TFPI, including full-length TFPI; truncated forms of TFPI; and complexed TFPI, domain 3

Table 1—*Clinical characteristics of subjects*

	Nondiabetic control subjects (group 1)	IDDM patients		
		UAE <30 mg/24 h (group 2)	UAE 30–300 mg/24 h (group 3)	UAE >300 mg/24 h (group 4)
Men/women	12/10	6/11	12/5	16/9
Age (years)	39 ± 7	41 ± 8	41 ± 9	37 ± 10
Diabetes duration (years)	—	20 ± 6	24 ± 9	23 ± 7
UAE (mg/24 h)	7 (3–14)	12 (4–30)	130 (32–274)	632 (336–7025)
Glomerular filtration rate (ml · min^{-1} · $1.73m^{-2}$)	—	108 ± 18	109 ± 19	106 ± 23
HbA_{1c} (%)	5.4 ± 0.4	8.1 ± 1.2	9.0 ± 1.2*	9.1 ± 1.1*
Fasting blood glucose (mmol/l)	4.5 ± 0.4	10.3 ± 4.2	9.1 ± 4.3	10.8 ± 5.0
Total cholesterol (mmol/l)	5.1 ± 0.9	4.6 ± 0.5	5.4 ± 0.8*	5.3 ± 1.1*
Triglyceride (mmol/l)	0.9 (0.4–4.1)	0.8 (0.5–1.1)	0.8 (0.5–2.4)	0.9 (0.5–3.8)*
HDL cholesterol (mmol/l)	1.5 ± 0.5	1.5 ± 0.4	1.5 ± 0.5	1.4 ± 0.4
LDL cholesterol (mmol/l)	3.1 ± 0.8	2.8 ± 0.6	3.4 ± 0.9*	3.5 ± 1.0*

Data are absolute numbers, means ± SD, or median (range). *$P < 0.05$ vs. group 2.

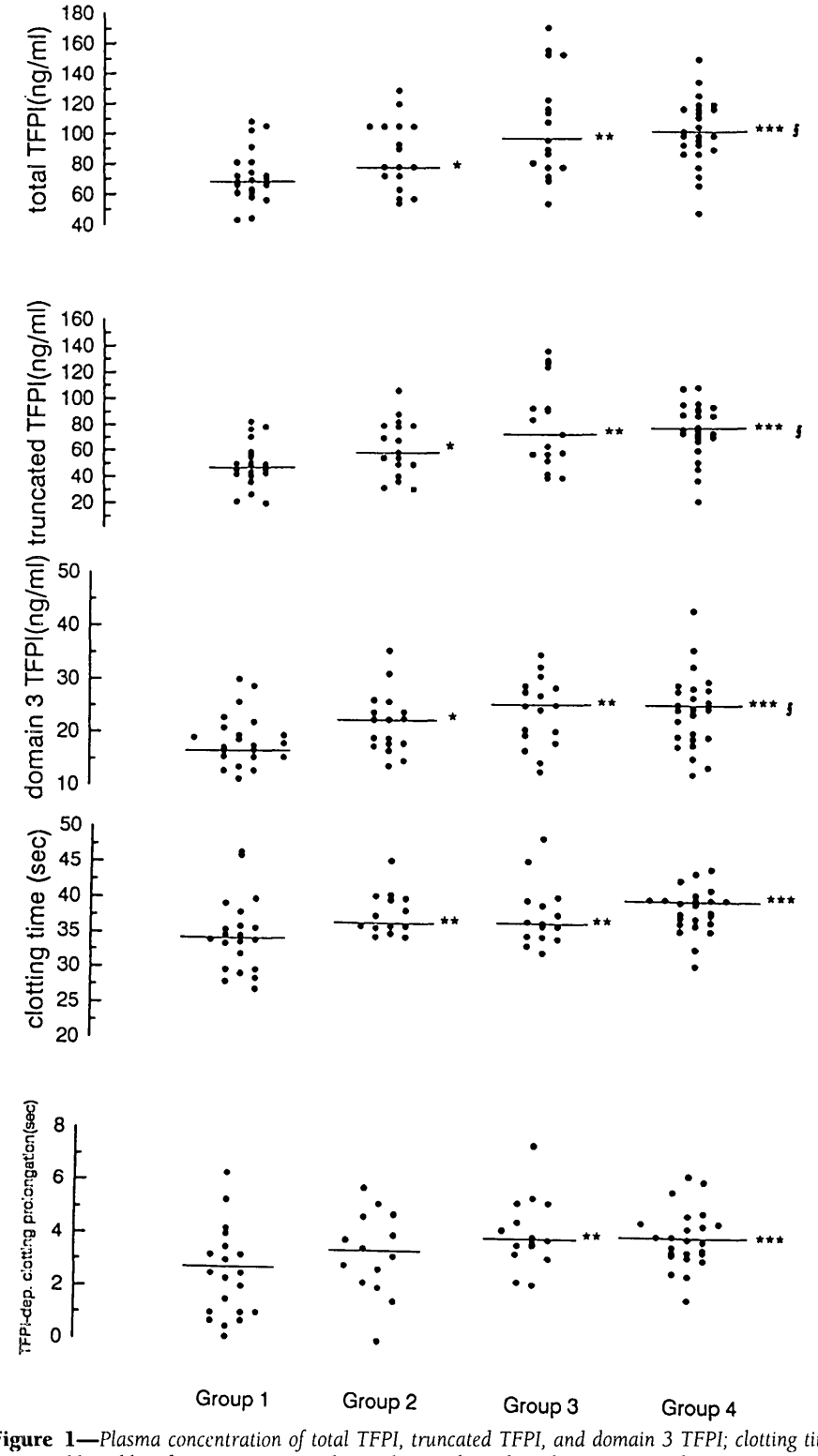

Figure 1—*Plasma concentration of total TFPI, truncated TFPI, and domain 3 TFPI; clotting time measured by adding factor Xa into samples; and TFPI-dependent clotting time prolongation in nondiabetic control subjects (group 1), IDDM patients with albumin excretion rates <30 mg/24 h (group 2), in the range 30–300 mg/24 h (group 3), and >300 mg/24 h (group 4). Horizontal bar represents median value. *P < 0.05 vs. group 1, **P < 0.01 vs. group 1, ***P < 0.001 vs. group 1, §P < 0.05 vs. group 2.*

TFPI, and truncated TFPI, which is the difference between total TFPI and domain 3 TFPI.

The ability of plasma to inhibit activated factor X was measured by a commercial kit HEPTEST (Haemachem). The measurement was performed essentially as described by the manufacturer using an ACL 300R Clottimer (Instrumentation, Milan, Italy) applying the activated partial thromboplastin time program. Each sample was divided into two subsamples to which either saline or activity-neutralizing anti-TFPI antibodies were added (one part per nine parts of plasma). The antibodies were a polyclonal rabbit anti-TFPI immunoglobulin containing 1.6 mg IgG/ml (14). The following procedure was used: 55 μl of plasma and 55 μl of factor Xa were incubated for 300 s at 37°C. Then 55 μl of Recalmix (calcium chloride and brain cephalin in bovine plasma) was added, and the clotting time was recorded. This procedure deviated from the one recommended by the manufacturer by a prolonged incubation time (300 s vs. the recommended 120 s). The difference between the clotting time of the subsamples without and with the anti-TFPI antibodies expressed the anti–factor Xa activity of TFPI.

Statistical analysis

Results are given as means ± SD, except for UAE and triglyceride, for which medians and ranges are given. Differences between groups were sought using the Mann-Whitney *U* test. The influences of various variables on TFPI were explored by Pearson's correlation analysis and multiple linear stepwise regression analysis. *P* values <5% (two-tailed) were considered to be of statistical significance.

RESULTS— The clinical features of the subjects are shown in Table 1. Micro- and macroalbuminuric patients were characterized by high levels of HbA_{1c}, total cholesterol, triglyceride, and LDL cholesterol.

Figure 1 shows plasma concentration of TFPI (total, truncated, and domain 3 TFPI), factor Xa–dependent clotting time in the HEPTEST assay, and the TFPI-dependent clotting time prolongation in each group. Plasma concentrations of total, truncated, and domain 3 TFPI were significantly higher in IDDM patients than in nondiabetic subjects. Furthermore, these levels were higher in group 4 than in group 2. The clotting time was longer in IDDM patients than in nondiabetic subjects. The TFPI-dependent clotting time was significantly prolonged in groups 3 and 4 compared with group 1.

Total TFPI and truncated TFPI

Table 2—*Correlation coefficients of total, truncated, and domain 3 TFPI to lipids and HbA_{1c} in all subjects*

	Total TFPI	Truncated TFPI	Domain 3 TFPI
LDL cholesterol	0.59*	0.57*	0.30†
Total cholesterol	0.51*	0.48*	0.34‡
HDL cholesterol	−0.25†	−0.26†	−0.03
Triglyceride	0.21	0.16	0.32‡
HbA_{1c}	0.48*	0.45*	0.36‡

*$P < 0.0001$, †$P < 0.05$, ‡$P < 0.01$.

significantly correlated to clotting time ($r = 0.46$ and $r = 0.46$, respectively; $P < 0.0001$) and TFPI-dependent clotting time prolongation ($r = 0.37$ and $r = 0.38$, respectively; $P < 0.001$), while domain 3 TFPI had a weaker correlation ($r = 0.19$ and $r = 0.14$, respectively; not significant). F1 + 2 was significantly higher in all diabetic subjects than in nondiabetic control subjects (1.12 ± 0.42 vs. 0.86 ± 0.36 nmol/l, $P < 0.05$), and there were no differences between diabetic groups. A close correlation was seen between F1 + 2 and TFPI-dependent clotting prolongation ($r = 0.32$, $P < 0.02$), which demonstrates the biological activities of factor Xa and TFPI, respectively.

Total and truncated TFPI showed closer correlations to LDL, total cholesterol, and HbA_{1c} than domain 3 TFPI in all subjects (Table 2). The correlations were similar when only IDDM patients were pooled in the analysis. TFPI had no correlations to fasting blood glucose or glomerular filtration rate. Multiple regression analysis indicated LDL and HbA_{1c} to have independent significant associations with total TFPI (partial correlation coefficient: 0.52 and 0.27; $P < 0.0001$ and $P < 0.05$, respectively).

CONCLUSIONS— The present study showed that plasma concentration of TFPI was elevated in IDDM patients with nephropathy compared with nondiabetic control subjects and normoalbuminuric IDDM patients. The fact that plasma TFPI in normoalbuminuric IDDM patients was higher than in nondiabetic control subjects was in agreement with the result obtained by Leurs et al. (7). TFPI directly binds factor Xa and inhibits factor Xa activity. We measured factor Xa inhibition activity by the clotting time after adding an equal volume of factor Xa into samples to initiate thrombin formation. It was higher in IDDM patients and was more pronounced in patients with nephropathy than in control subjects. It indicates that IDDM patients, especially those with nephropathy, demonstrate increased factor Xa inhibition, presumably because of increased TFPI activity.

Plasma TFPI circulates primarily in complex with lipoproteins. We confirmed a close association of TFPI with LDL, HDL, and triglyceride in IDDM patients. An influence of HbA_{1c} on plasma TFPI may also be explained by the close association between HbA_{1c} and lipoproteins. The strong association of truncated TFPI with LDL cholesterol was consistent with other studies that showed that truncated TFPI circulates predominantly bound to LDL in plasma (6) and that plasma TFPI is associated with and regulated by LDL in plasma from healthy volunteers and patients with hypercholesterolemia (15). Although truncated TFPI in vitro has a 50-fold lower anticoagulant activity than full-length TFPI (16), only a portion of TFPI is the full length, and our study suggested that truncated TFPI contributes to the anticoagulant activity in IDDM patients. This observation is consistent with a recent finding that LDL-associated TFPI has a potent anticoagulant activity (17).

Elevated concentrations of blood lipids have been shown to be correlated to increased factor VII coagulant activity (18, 19). The Rotterdam Study (20) has shown that factor VII coagulant activity increases with increasing cholesterol with the same strength in the elderly, while Mitropoulos et al. (18) found a strong association between factor VII and dietary fat, plasma triglycerides, and VLDL cholesterol (21). Thus, blood lipids may play a double role in the extrinsic coagulation pathway because they seem to display both procoagulant (factor VII activation) and anticoagulant (TFPI) activity. Whether or not lipoprotein-bound TFPI plays a physiological role counterbalancing the lipid-mediated factor VII activation is at present unknown.

The pool of TFPI bound to vascular endothelium must substantially exceed the pool of circulating plasma TFPI because an injection of heparin can raise plasma TFPI levels several-fold (22,23). Vascular endothelial cells synthesize TFPI and may be the principal source of plasma TFPI (24–26). On the endothelial cells, TFPI may be bound to heparan sulfate by charge interaction (1,5,27), though the binding protein of TFPI on the endothelium remains uncertain. This pool of TFPI must consist of full-length or at least the Kunitz-type domain 3 molecules because the binding of TFPI to heparin or heparan sulfate depends on the COOH-terminal and part of domain 3 (27). Thus, vascular endothelial damage may mobilize TFPI into the plasma pool. Experimental diabetes shows a general widespread reduction of the negative charges mostly by heparan sulfate (28), and endothelial synthesis of heparan sulfate is reduced in diabetes (29). Endothelial dysfunction in patients with diabetic nephropathy is seen by increased plasma concentration of von Willebrand factor (7). Increased plasma TFPI was observed in uremic patients, presumably because of renal impairment and endothelial damage (31). Therefore, the increase in the Kunitz-type domain 3 TFPI in IDDM patients with nephropathy may be in part due to the vascular endothelial damage. The concentration of TFPI and heparan sulfate on endothelium in relation to anticoagulant capacity needs to be clarified.

The difference of coagulation time with and without added anti-TFPI antibody reveals the anticoagulant activity by TFPI (14). This was significantly correlated to F1 + 2, which represents the biological activity of factor Xa activity. The elevation of F1 + 2 indicates that the increased anticoagulant activity exerted by TFPI in IDDM patients is not sufficient to counterbalance the hypercoagulable state described in diabetes (31,32).

In conclusion, inhibition of factor Xa activity is increased in IDDM patients with nephropathy, mainly due to increased plasma TFPI activity. The increased plasma TFPI activity in patients with nephropathy is essentially due to an increased concentration of truncated TFPI, which is probably directly associated with and regulated by the increased concentration of lipoproteins, especially LDL, and the poor metabolic control. The anticoagulant activity of TFPI may attenuate the hypercoagulable state in diabetes but is not able to normalize hemostasis.

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