Fluid Flow Induces Upregulation of Synthesis and Release of Tissue Factor Pathway Inhibitor In Vitro

Andrew D. Westmuckett, Cristina Lupu, Sylvie Roquefeuil, Thomas Krausz, Vijay V. Kakkar, Florea Lupu

Abstract—Fluid flow modulates the synthesis and secretion by endothelial cells (ECs) of several proteins that control the hemostatic properties of the vessel wall. Tissue factor pathway inhibitor (TFPI), also synthesized by ECs, is the main downregulator of tissue factor–dependent procoagulant activity. In the present study, we investigated the effect of physiological shear stress on the expression, distribution, and release of TFPI in cultured ECs. The EA.hy926 cell line was grown in a hollow-fiber perfusion system and exposed for variable times to different shear values: 0.27 dyne/cm² (minimal flow), 4.1 dyne/cm² (venous flow), and 19 dyne/cm² (moderate arterial flow). Step increase of the shear stress from 0.27 to 19 dyne/cm² induced a sharp increase of TFPI released into the medium and a parallel decrease and redistribution of cell-associated TFPI, which suggests that an acute release of TFPI occurred from the cellular pools. During 24 hours of high shear stress, cell-associated TFPI antigen and mRNA increased time-dependently. Subjecting ECs to steady shear stress for 72 hours also upregulated the expression and production of TFPI, in direct correlation with the degree of the shear. The secretion of TFPI was enhanced 1.9-fold under venous flow and 2.4-fold under arterial flow compared with minimal flow. Equally, cell-associated TFPI antigen and cell surface TFPI activity increased proportionally with the shear stress. The expression of TFPI mRNA, as determined by Northern blotting, increased up to 2-fold in ECs under venous flow and up to 3-fold under arterial flow. These results suggest that shear forces regulate TFPI by modulating its release and gene expression in ECs in vitro. (Arterioscler Thromb Vasc Biol. 2000;20:2474-2482.)

Key Words: tissue factor pathway inhibitor  ■  shear stress  ■  flow  ■  endothelial cells

Endothelial cells (ECs) serve as a functional barrier between blood and the vessel wall and control the fluidity of the blood by distinct mechanisms. Accordingly, ECs produce antiplatelet aggregants and vasoactive compounds such as prostacyclin (PGI₂) and NO; antithrombotic proteins such as thrombomodulin, tissue factor (TF) pathway inhibitor (TFPI), and heparan sulfate proteoglycans; and fibrinolytic proteins such as tissue plasminogen activator (tPA) (for a review, see Reference 1). Because of their strategic location, ECs interface with fluid shear forces, which in turn affect their function and gene expression. Examples include the reorganization of the cytoskeletal components, ² regulation of gene expression for proteins that play key roles in the maintenance of homeostasis, ³,⁴ cell migration, ⁵ and cell growth. ⁶ Hemodynamic forces also regulate several of the hemostatic proteins produced by ECs. Fluid shear stress induces significant increases in the release of PGI₂ and NO, ⁸,⁹ as well as enhanced synthesis of regulators that inactivate certain clotting enzymes or cofactors. Recent studies showed that shear stress regulates the generation of thrombomodulin, ¹⁰,¹¹ which interacts with protein C and protein S to inactivate factor (F)V and FVIII. Shear stress also stimulates the production and release of tPA, ³,⁴ the major activator of vascular fibrinolysis.

The effect of shear stress on the expression of TFPI, however, has not been addressed so far. TFPI is the most physiologically significant inhibitor of the TF-FVIIa complex (for a review, see Reference 12). TFPI uses the tandem Kunitz type domains in its structure to form a quaternary complex with FXa bound on TF-FVIIa and thus prevents further production of FXa and FIXa through the TF-dependent pathway.

The main purpose of the present study was to investigate the effect of physiological levels of fluid shear stress on the acute release of TFPI as well as the long-term effect on the gene expression and synthesis of TFPI in human ECs in culture. Using an in vitro hollow-fiber perfusion system, ¹³ we demonstrated both acute and chronic alterations in TFPI secretion, storage, and expression in ECs in response to shear stress.

Methods

Reagents

The polyclonal anti–recombinant TFPI ¹⁴ IgG developed in our laboratory was immunoaffinity-purified and tested for specificity (50

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Flow Rate, mL/min | Shear Stress, dyn/cm² | Pulse Hydrostatic Pressure, mm Hg | M/m | Δ (M–m)
--- | --- | --- | --- | ---
Minimal flow | 0.3 | 0.27 | 16/14 | 2
Venous flow | 4.5 | 4.1 | 28/16 | 12
Arterial flow | 19.7 | 19 | 54/24 | 30

M indicates maximal pressure; m, minimal pressure.

μg/mL produced 95% inhibition of TFPI activity in normal human plasma; by immunofluorescence, the competition experiments, eg, staining in the presence of recombinant TFPI, were negative). Mouse monoclonal antibodies against caveolin were from Transduction Laboratories, FITC and Texas red–conjugated secondary antibodies and Vectashield mounting medium were from Vector Laboratories Inc. Secondary antibodies conjugated to 10-nm colloidal gold were from BioCell Research Labs, and all the reagents used for electron microscopy were from TAAB Laboratory Equipment Ltd. Octyl β-D-glucopyranoside (OGP) was from Calbiochem-Novabiochem Ltd. Human coagulation FVIIa, FX, and Fxa were purchased from Enzyme Research Labs Ltd, and N-benzoyl-L-iso-leucyl-L-glutamyl-(piperidyl)-glycyl-L-arginine-p-nitroanilide hydrochloride (chromogenic substrate S-2337) was from Quadtach. TRIZol was from Life Technologies Ltd, Rapid-Hyb hybridization buffer and [α-32P]dCTP were from Amersham Life Science Ltd, and Gene Screen transfer membranes were from NEN Research Products. Cell culture media and supplements were from Gibco unless stated otherwise. Phenylmethylsulfonyl fluoride, HEPES, Triton X-100, the glucose concentration assay kit, and all other reagents were purchased from Sigma Chemical Co unless stated otherwise.

Cell Culture and the Flow System
We used the immortalized endothelial cell line EA.hy926, kindly provided by C.J.S. Edgell (University of North Carolina, Chapel Hill). This cell line has been previously characterized with regard to the expression and release of TFPI and other endothelium-dependent parameters, such as von Willebrand factor, thrombomodulin, tPA, and plasminogen activator inhibitor-1. It has been found to have very much like human umbilical vein endothelial cells, from which it was initially derived. The cells were grown on T-75 flasks in DMEM containing 4 mM/L glucose, 15 mM/L HEPES, 100 mM/L penicillin, 0.1 mg/mL streptomycin, and 1:10 (vol/vol) heat-inactivated FCS (Harlan Seralab). After reaching confluence under static conditions, ECs were trypsinized from the culture dishes, washed with PBS, and incubated for 30 minutes at 37°C in a microplate reader (Molecular Devices THERMOmax, Alpha Labs, Ltd) by use of the dual kinetic mode measuring the enzyme activity of FXa.

Northern Blotting
Total RNA was extracted from ECs by an acid phenol method with the use of TRIZol reagent according to the manufacturer’s instructions. Electrophoresis and transfer of the total RNA, prehybridization, and hybridization were performed as described. The probes used were a 601-bp fragment from EcoRI/ClaI digestion of human TFPI (see Figure 7).

Transgenic Mice
Fluorescence-activated cell sorting (FACS) analysis was performed as previously described. After centrifugation, the supernatants were removed, as described above, at defined time intervals (at 30 seconds, 5, at 10, 15, and 30 minutes, and at 1 and 2 hours). In another experiment, the period of time during which the ECs were kept under arteiral flow was extended up to 24 hours.

Steady Shear Stress
After reaching confluence under minimal flow rate, ECs were either maintained at minimal flow or subjected to a gradual increase of the flow over 2 days, up to shear stress levels of 4.1 or 19 dyne/cm², after which these flow rates were kept constant for 72 hours. The medium was then discarded and replaced with a fresh medium for a further 6 hours under the same flow rates, at which time the medium was collected for further assay. Finally, ECs were harvested by mechanical backflushing with 5 mmol/L EDTA in PBS.

Total Cell Lysate Preparation
Cells were centrifuged at 3000 rpm for 10 minutes, and the pellets were washed twice in ice-cold Tris-buffered saline (TBS, consisting of 0.1 mol/L Tris-HCl and 0.15 mol/L NaCl), pH 7.8, containing a cocktail of inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L aprotinin, 1 mmol/L sodium orthovanadate, and 10 mmol/L EDTA). After centrifugation, cell pellets were resuspended in ice-cold lysis buffer (TBS containing 10 g/L Triton X-100, 60 mmol/L OGP, and the inhibitors mentioned above) and incubated for 30 minutes at 37°C with vigorous vortexing, after which the insoluble proteins were removed by centrifugation, and the supernatants representing the total cellular lysates were stored at −70°C until the assay.

Differential Detergent Extraction
Differential detergent extraction of ECs with Triton X-100 and OGP was performed as previously described.

TFPI Antigen Assay
The measurement of TFPI antigen in supernatants and cell lysates was performed by ELISA as described. Estimation of the total protein concentration was performed by use of the bicinchoninic acid assay kit (Pierce & Warriner Ltd).

TFPI Activity Assay
The quantification of the inhibitory activity of TFPI against TF-FVIIa was performed as previously described. The 2-stage amidolytic chromogenic assay. In brief, the cells were fixed in suspension with 1% (wt/vol) paraformaldehyde in PBS for 10 minutes, washed with PBS, and incubated for 30 minutes at 37°C with 600 μL of a combined reagent containing (all final concentrations) 2.5 mg/L FVIIa, 5 U/L FXa, 1:80 diluted rabbit brain thromboplastin (from one vial reconstituted with 2 mL distilled water), and 15 mmol/L CaCl2. After centrifugation, the supernatants were transferred into the wells of a 96-well microtiter plate, to which a mixture of 0.4 U/mL FX and 0.3 mmol/L chromogenic substrate S-2337 was added. The rate of substrate cleavage was monitored over 25 minutes at 37°C in a microplate reader (Molecular Devices THERMOmax, Alpha Labs, Ltd) by use of the dual kinetic mode (Lmax − Lmin, where L is the excitation wavelength [nm]).

TFPI activity was extrapolated from a standard curve constructed with serial dilutions of normal human plasma, which was assigned a TFPI functional potency of 1 U/mL.

Northern Blotting
Total RNA was extracted from ECs by an acid phenol method with the use of TRIZol reagent according to the manufacturer’s instructions. Electrophoresis and transfer of the total RNA, prehybridization, and hybridization were performed as described. The probes used were a 601-bp fragment from EcoRI/ClaI digestion of human TFPI.
full-length TFPI cDNA (gift from Dr G. Broze, Washington University, St. Louis, Mo) and a 526 full-length cDNA housekeeping gene,20 both radiolabeled by random priming with [α-32P]dCTP. Densitometry and comparisons between the intensity of the bands were performed by using the public domain NIH Image software.

**Immunofluorescence**

Randomly selected fibers were removed from cartridges, gently flushed with PBS at 37°C, fixed with 4% (wt/vol) paraformaldehyde in PBS at room temperature, cryoprotected with sucrose, embedded in Tissue-Tek OCT compound (Raymond A. Lamb Ltd), and frozen in liquid nitrogen-cooled isopentane. The cellular localization of TFPI was studied on cryosections (6-μm thickness) by indirect immunofluorescence as previously described.14,15 For double-labeling procedures, the sections were incubated with cocktails containing a polyclonal IgG anti-TFPI and monoclonal IgG anti-caveolin. As detection antibodies, a mixture of goat anti-rabbit Texas red and goat anti-mouse FITC was applied. Samples were examined with a Bio-Rad MRC 600 confocal laser scanning unit attached to a Nikon Diaphot inverted microscope (Bio-Rad Microscience Ltd). To show elongated cytoplasm projections that either formed the capillary matrix (Figure 1c [round inset] and 1f). This feature was much more prominent with the cells grown under arterial levels of flow. The adjacent cells were always closely adherent to one another (Figure 1c), connected at the lateral borders through cell junctions, often with the characteristics of tight junctions (Figure 1c [oval inset]). ECs exhibited abundant organelles, vesicles, and microfilaments. A well-developed protein synthesis machinery consisting of rough endoplasmic reticulum (Figure 1c) and Golgi apparatus (Figure 1c) represented common features of the cells subjected to the arterial level of flow.

**Flow-Mediated Acute Release of TFPI**

To test the effect of the arterial flow on TFPI in ECs, confluent monolayers grown in the capillaries at 0.27 dyne/cm² were subjected to a step increase of the stress to 19 dyne/cm². The time course of the secretion of TFPI in the cell medium over the following 2 hours (Figure 2a) showed a sharp increase in the first 30 seconds after the onset of the arterial flow. The amount of released TFPI decreased after 60 minutes but maintained an ≈60% increase over minimal flow values during the following 60 minutes.

The amount of cell-associated TFPI was also determined at different time points during 24 hours of high shear stress. The partition of TFPI between the Triton extract and the OGP extract was determined in ECs kept under minimal flow and compared with that in cells subjected to arterial levels of flow for 2, 6, 12, and 24 hours (Figure 2b). A significant decrease of cellular TFPI was observed after 2 hours, which occurred equivalently in both fractions (P<0.01). After 6 hours of subsequent arterial flow, the OGP-soluble pool of TFPI was restored and remained at approximately the same level for 24 hours. In contrast, the amount of TFPI in the Triton-soluble fraction was still very small after 6 hours (P=0.008 for the difference between time points); however, this pool was also replenished after 12 hours and slightly increased after 24 hours compared with the starting point (P=0.1).

We also studied the expression of TFPI mRNA in ECs subjected to arterial flow for up to 6 hours. By Northern blotting (Figure 2c), we identified the 2 transcripts of 4 kb and 1.4 kb, originally described by Girard et al.21 Subjecting the cells to a sudden increase of shear stress led to an increase of TFPI expression, which was manifested by both transcripts and apparently time dependent (Figure 2c). The densitometry of the blots confirmed that the intensity of both TFPI bands was strongly enhanced after 6 hours of arterial flow (Figure 2d).

The quantitative data were paralleled by the immunocytochemical localization of TFPI in ECs subjected to different shear stress values. The cell surface–located TFPI was visualized in double labeling with caveolin, the major protein resident of the caveolae (Figure 3a through 3c). ECs under minimal flow displayed the characteristic immunostaining for TFPI that was previously observed in human umbilical vein endothelial cells and EA.hy926 grown under static conditions,14 with uniform distribution of TFPI over the cell surface (Figure 3b and 3d). Pseudo–color-banding analysis revealed that after 1 hour of exposure to arterial flow, ECs exhibited a decrease in the intensity of fluorescence (Figure 3e) compared with that in ECs grown under minimal flow (Figure 3d).
Effects of Steady Fluid Flow

ECs grown in capillary cartridges were subjected to different flow rates for 3 days, after which time the medium was changed and collected after 6 hours. The amount of TFPI released into the medium and that of cellular TFPI were determined at the end of this incubation time and compared for different shear values. As illustrated in Figure 4a, the production of TFPI by ECs was positively correlated with the level of shear stress (correlation coefficient 0.968). Cells under venous or arterial flow secreted significantly more TFPI than did the cells maintained under minimal flow (∼1.9-fold increase for the venous flow and 2.4-fold increase for the arterial flow, *P*<0.0001 for both). The difference between the arterial and venous flow with regard to the TFPI secretion was also significant (*P*=0.01). The distribution of TFPI in total cell lysates matched the TFPI released into the medium. Accordingly, total lysates prepared from ECs kept under venous or arterial flow contained more TFPI (50% increase for venous shear stress and ∼100% increase for arterial shear stress, *P*<0.05 and *P*<0.001, respectively) than did the equivalent lysates of cells maintained under minimal levels of flow (Figure 4b). Interestingly, the cell surface-
exposed TFPI displayed considerably higher inhibitory activity toward TF-FVIIa after exposure to shear stress (Figure 4c, exposed TFPI displayed considerably higher inhibitory activity toward TF-FVIIa after exposure to shear stress (Figure 4c), indicating time points after the onset of arterial flow was analyzed by Northern blotting with use of radiolabeled specific TFPI and S26 (as housekeeping gene) cDNA probes. A representative Northern blot is shown (c), displaying the characteristic TFPI transcript bands was stronger in cells subjected to arterial flow (Figure 3j). The latter showed increased levels showed redistribution of TFPI in a manner similarly

By immunofluorescence, ECs subjected to chronic venous flow (Figure 3i) displayed less staining for TFPI than did cells under arterial flow (Figure 3j). The latter showed clusters of TFPI distributed all over the plasma membrane (Figure 3j), also colocalizing with caveolin (Figure 3f through 3h). A strong accumulation of TFPI occurred at the apical and lateral front of the cells and on the cell projections entering the capillary wall (arrowheads, Figure 3f through 3j).

The negative controls, which consisted of the omission of the primary antibody, did not show notable staining (Figure 3k and 3l).

Figure 2. Acute shear stress–mediated release and upregulation of TFPI. ECs grown under minimal flow (0.27 dyne/cm²) were acutely exposed to 19 dyne/cm² (moderate arterial flow), and the flow was continued for up to 24 hours. a, Time course of release of TFPI into the medium showed substantial increase in the first minute after the onset of arterial flow, followed by sharp decrease during the next hour. b, The amount of TFPI in the Triton-soluble and OGP-soluble cell fractions decreased equivalently after 2 hours and was restored within the OGP extract after 6 hours and within the Triton extract after 24 hours. Values are mean±SD. c and d, Total RNA extracted from ECs at the indicated time points after the onset of arterial flow was analyzed by Northern blotting with use of radiolabeled specific TFPI and S26 (as housekeeping gene) cDNA probes. A representative Northern blot is shown (c), displaying the characteristic TFPI transcripts of 1.4 and 4 kb. The intensities of the bands corresponding to TFPI and S26 (as housekeeping gene) cDNA probes. A representative Northern blot is shown (c), displaying the characteristic TFPI transcripts of 1.4 and 4 kb. The intensities of the bands corresponding to TFPI and S26 transcripts were quantified by densitometry and expressed as the ratio between TFPI and S26 (d).

The immunogold labeling of TFPI in ECs subjected to minimal flow showed mainly single gold particles that uniformly decorate the plasma membrane (Figure 3m), whereas the cells that were chronically subjected to arterial flow also exhibited clusters of gold particles located in the caveolae (Figure 3p). Moreover, these cells displayed a higher intracellular labeling for TFPI in the endoplasmic reticulum (Figure 3q) compared with that in the cells exposed to minimal flow (Figure 3n), suggesting an increase synthesis of this protein. The negative controls, which consisted of secondary antibodies or protein A conjugated to colloidal gold particles, showed a low background staining (Figure 3o).

Discussion

We have used the perfusion system developed by Redmond et al23 and commercialized by Cellco Inc to study the effect of hemodynamic forces on the TFPI production and release by ECs in vitro. The model consisted of an enclosed perfused bundle of semipermeable polypropylene capillaries that permitted acute or chronic exposure of ECs to pulsatile flow within the range of physiological shear stress. To date, most of the experimental studies on the effects of flow on ECs have used simple systems, such as the conic plate viscometer or the parallel-plate flow chamber, to generate uniform laminar shear. In these cases, the shear force was being applied to static cultures and therefore represented an abrupt transition in the biomechanical loading of the system.22 In contrast, the system used by us offered the possibility to evaluate the response of ECs subjected continuously to flow. The system permitted the generation of temporal fluctuations of the flow parameters as well as the combination of shear stress and hydrostatic pressure in the same in vitro system, which generated a more physiological biomechanical environment for the ECs. The levels of shear used in the present study were within the physiological range of the vascular system.23

The electron microscopic data in the present study showed that cells grown under flow displayed typical ultrastructural characteristics of endothelium (eg, caveolae, tight junctions, and basement membrane). The cells chronically exposed to flow exhibited pseudopodia-like projections that penetrated into the pores of the polypropylene tubes. These may represent adaptive changes to increased mechanical forces, inasmuch as similar cell protrusions were described in an in vivo model of flow-induced arterial remodeling.24 The increased presence of TFPI on these projections may indicate a role in cell adhesion to the matrix, as was suggested by Ruf’s group (Fischer et al25).

Our main findings were that a step increase of the shear stress induces the acute release of TFPI from ECs, whereas steady fluid flow upregulates the production of TFPI at the transcriptional level. The acute release occurred rapidly within minutes after the step increase of the shear stress from 0.27 to 19 dyne/cm² and probably involved the redistribution of preexisting TFPI molecules. We have shown that in resting ECs in culture, TFPI is located as uniform clusters on the cell adhesion to the matrix, as was suggested by Ruf’s group (Fischer et al25).

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Figure 3. Immunolocalization of TFPI in ECs exposed to different shear stress values for various times. a through c, Double labeling for caveolin (green, panel a) and TFPI (red, panel b) in ECs grown under minimal flow is shown. Colocalization of the 2 antigens is seen in yellow on the merged image (panel c). d and e, Acute effect of arterial shear on the distribution of TFPI in ECs is shown. Immunofluorescence labeling of TFPI was followed by confocal imaging and semiquantitative analysis of the fluorescence intensity by using pseudo-color banding. The gray-level scale of the black and white image was divided into 10 color-coded bands starting from black (coded in blue), which represents low (small L) fluorescence, to white, which represents high (H) fluorescence intensity. The cells grown under minimal flow display middle-scale staining (green, yellow, and orange bands) over the cell surface (panel d), whereas cells exposed to step arterial flow displayed a lower staining for TFPI after 1 hour (light blue and green bands, panel e). f through h, Double labeling for caveolin (green, panel f) and TFPI (red, panel g) reveals that the 2 proteins are highly expressed and colocalize on the apical and basolateral surfaces (yellow, panel h). Note that staining also followed the cell projections penetrating into the capillary wall (arrowheads). i and j, Chronic effect of steady shear stress (72 hours) on the distribution of TFPI is shown. ECs under venous flow displayed middle- to upper-scale (green, yellow, and orange bands) labeling for TFPI over the apical cell surface (panel i), whereas cells grown under arterial flow showed general stronger immunofluorescence (red, pink, and white bands), mainly in clusters on the luminal and lateral domains of the cells (panel j). k and l, The negative control for secondary antibody (goat anti-rabbit FITC) binding showed very low background staining (panel k) corresponding to low intensity bands (dark blue, panel l). m through q, Postembedding immunogold labeling for TFPI (rabbit anti-TFPI IgG followed by protein A/10-nm gold) on cells grown under minimal flow revealed single gold particles uniformly decorating mainly the plasmalemma proper (arrows, panel m) and in very low number in the endoplasmatic reticulum (ER) area (panel n), whereas the negative control for protein A binding showed no background labeling (panel o). ECs under steady arterial flow for 72 hours (panel p) displayed gold particles on the plasmalemma proper (arrows) and concentrated in caveolae (cv). TFPI-specific labeling was also found in relatively high amounts in the endoplasmatic reticulum area (panel q). CW indicates capillary wall; large L, lumen. Bars = 50 μm (a through l) and 0.2 μm (m through q).
described for thrombin.\textsuperscript{14} Because vascular endothelium responds to changes in shear stress by elevation of cytosolic Ca\textsuperscript{2+},\textsuperscript{26} and because of our observations that TFPI was rapidly released when [Ca\textsuperscript{2+}] was increased by treatment with Ca\textsuperscript{2+} ionophore,\textsuperscript{16} we speculate that a similar mechanism may be responsible for the acute release of TFPI induced by the onset of arterial flow.

Apart from inducing the acute release of TFPI, the high shear stress also enhanced TFPI gene expression. A variety of proteins are rapidly “activated” by shear stress in ECs, including certain cell surface potassium channels, members of the mitogen-activated and stress-activated protein kinase cascade, several transcription factors such as nuclear factor-kB, and subsets of receptor-associated G proteins.\textsuperscript{27–29} The mechanism(s) through which shear stress regulates TFPI expression remains to be explained. Recent data suggest that caveolae play a role in mechanotransduction.\textsuperscript{30} Caveolae are small plasmalemma invaginations rich in cholesterol and glycosphingolipids that can function as cell surface-organized signal transduction centers.\textsuperscript{31–32} It has been shown that the number of cell surface–opened caveolae is increased after EC exposure to shear stress.\textsuperscript{33} TFPI settles in caveolae in resting ECs\textsuperscript{33} and associates with glycosphingolipid microdomains, a feature that confers insolubility in Triton X-100.\textsuperscript{34} By using the differential detergent extraction technique, we established that TFPI is decreased in Triton-soluble and -insoluble fractions in ECs subjected to an acute increase in shear stress. The enhanced synthesis of TFPI probably led afterward to the replenishment of the Triton-insoluble pool, because the ultrastructural images confirmed that TFPI consistently immunolabeled the caveolae. After 6 hours of arterial flow, only the fraction of cellular TFPI solubile in Triton, which was probably responsible for the long-term sustained secretion of TFPI, was still variable. Inasmuch as the caveolar fraction of TFPI was depleted after 2 hours of arterial flow, we speculate that this process may have been mediated through shear stress signals transduced in caveolae (possibly Ca\textsuperscript{2+} transients). The events downstream from early signal transduction that may affect TFPI expression are not known either. For several of the shear-responsive promoters identified so far, the ability to respond to shear stress can be directly linked to the presence of specific cis-acting sequence elements, which have been named shear stress response elements.\textsuperscript{5,6,35} The promoter region of the TFPI gene contains the consensus sequence of the shear stress response element (104-GAGACC-109) that is responsible for shear regulation of the B chain of platelet-derived growth factor.\textsuperscript{3} We suggest that this element may control the shear dependence of TFPI expression as well.

Our results demonstrate for the first time that shear stress modulates the release and expression of TFPI, which may have significant pathophysiological roles in the regulation of hemostatic balance at the EC surface. Increasing shear levels from minimal to venous or arterial values of flow led to the enhancement of cellular TFPI antigen, cell surface activity against TF-FVIIa, and TFPI mRNA levels. Our results correlate well with previous reports showing that human umbilical vein endothelial cells stimulated with lipopolysaccharide and interleukin-1\beta developed a significantly greater TF-dependent procoagulant activity when perfused at a venous shear stress than at an arterial shear stress.\textsuperscript{36} This suggests that the cell-associated TF activity was inhibited to a greater extent under arterial shear stress. Likewise, Grabowski et al\textsuperscript{37} showed that ECs exposed to shear forces >0.68 dyne/cm\textsuperscript{2} exhibited a considerable increase of TF-dependent FXa generation when TFPI was neutralized with anti-TFPI antibodies. We found that the inhibitory potency of cell surface TFPI against TF-FVIIa activity increased to a higher extent than that of the cell-associated TFPI antigen in ECs exposed to flow. By correlating these data with the fact that flow increases the number of caveolae\textsuperscript{30} and with our findings that flow induces an increase of caveolar fraction of TFPI, one could speculate that the TFPI located in caveolae is more active than the TFPI located on the plasmalemma proper. These observations would endorse the data suggesting that the formation of the TF-FVIIa-FXa-TFPI quaternary complex takes place in glycosphingolipid-rich microdomains (caveolae).\textsuperscript{38}

More work remains to be carried out to establish whether the flow-induced changes in the release and expression of TFPI in vitro also reflect a heterogeneous in vivo expression of the inhibitor in ECs located in vascular beds exposed to different hydrodynamic forces. A comparative analysis of the expression of TFPI in vivo in the vascular system is currently under way in our laboratory. On the basis of previous results that showed that the responses induced by flow in vitro appeared to recapitulate many of the morphological features of the EC in vivo,\textsuperscript{2,39} we expect our in vitro data to mirror the in vivo situation.

Figure 4. Chronic shear stress–induced upregulation of TFPI. ECs were grown under minimal, venous, or arterial flow for 72 hours, after which the medium was replaced with a fresh medium and collected after 6 hours. a and b, Production of TFPI in the cell medium (panel a) and in cell lysates (panel b) increased significantly with the level of shear. c, TFPI activity on the cell surface increases after exposure to venous or arterial shear compared with minimal shear. All data are expressed as mean±SD. d, Total RNA extracted from ECs kept under different flow rates was analyzed by Northern blotting and densitometry (as for Figure 2). Cells under arterial flow displayed the strongest increase in TFPI mRNA vs cells grown under minimal flow, and the 1.4-kb band showed the highest augmentation.
The modulation of TFPI expression by shear stress may have a role in the pathogenesis of vascular disorders. Atherosclerotic lesions distribute focally at the bifurcations and curvatures of the arterial tree, where disturbed flow with low or fluctuating shear stress occurs,\(^\text{40,41}\) suggesting that such hemodynamic stimuli are critical regulators of the initiation and progression of the disease. Currently, there is clear evidence that areas of low shear stress are prone to atherosclerosis\(^\text{42}\) and that low shear stress promotes intimal hyperplasia.\(^\text{43}\)

Several lines of evidence indicate that the endothelial genes encoding products that can exert potent antithrombotic,\(^\text{10}\) antiadhesive,\(^\text{44}\) and anti-inflammatory\(^\text{45,46}\) effects in ECs are upregulated by high shear flow. These findings suggest that coordinated induction of these genes in ECs by the uniform shear stress may explain why the nonbranched linear regions of the vasculature are protected from atherosclerosis.\(^\text{47}\) We assume that the shear stress–enhanced production of TFPI in ECs may also contribute to the maintenance of the anticoagulant and nonatherogenic properties of the quiescent endothelium in vivo.

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**References**


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