Cellular Effects of Heparin on the Production and Release of Tissue Factor Pathway Inhibitor in Human Endothelial Cells in Culture

Cristina Lupu, Emma Poulsen, Sylvie Roquefeuil, Andrew D. Westmuckett, Vijay V. Kakkar, Florea Lupu

Abstract—Tissue factor pathway inhibitor (TFPI), the major downregulator of procoagulant activity of the tissue factor–factor VIIa complex (TF·FVIIa), is synthesized and constitutively secreted by endothelial cells (ECs). Here we describe the in vitro effects of heparin on the cellular localization, gene expression, and release of TFPI in human ECs in culture. Both unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH; Fragmin) time-dependently induced a significant enhanced secretion of TFPI, paralleled by a redistribution and increase of TFPI on the cell surface and a decrease of intracellular TFPI. Immunogold electron microscopy showed the presence of clusters of TFPI, both on the plasmalemma proper and within cell-surface opened caveolae/enlarged caveolar profiles. Activation of FX by TF·FVIIa on ECs treated with endotoxin was inhibited by both heparins but to a higher extent by LMWH. Long-term incubation (48 hours) resulted in a time-dependent enhanced production of TFPI. After the first 4 to 8 hours, depletion of intracellular TFPI was observed, more significantly with UFH. Northern blot analysis of TFPI mRNA also showed a decrease of the 1.4-kb transcript after 4 hours of incubation with UFH, followed by recovery and an increase over the control level after 24 hours. Incubation of ECs with phorbol ester (PMA) significantly enhanced the secretion of TFPI and increased its activity on the cell surface, probably by preventing invagination of caveolae. Heparin-stimulated release of TFPI decreased significantly in the presence of PMA to a level that was 2.4 times lower than the expected additive value for PMA and UFH separately. Our results suggest that the heparin-induced release of TFPI might involve a more specific mechanism(s) than the previously hypothesized simple displacement of TFPI from the cell surface glycocalyx. We assume that the increased secretion and redistribution of cellular TFPI induced by heparins in ECs in culture can play an important role in the modulation of the anticoagulant properties of the endothelium. (Arterioscler Thromb Vasc Biol. 1999;19:2251-2262.)

Key Words: tissue factor pathway inhibitor ■ human endothelial cells ■ unfractionated heparin ■ low-molecular-weight heparin ■ caveolae

Endothelial cells (ECs) play a central role in the regulation of hemostasis by ensuring the cellular control of both procoagulant and anticoagulant mechanisms. The anticoagulant and profibrinolytic functions predominate in the quiescent state of the endothelium, thus maintaining blood fluidity (for a review, see References 1 and 2). Blood coagulation is initiated when factor VII/VIIa (FVII/VIIa) in plasma gains access to tissue factor (TF) at sites of blood vessel injury, and the resulting TF·FVIIa complex activates FX to Xa and FIX to IXa, leading to thrombin generation and the formation of a fibrin clot.3

Although healthy ECs do not express TF constitutively, marked expression of this protein occurs both in vitro after perturbation of ECs with different agonists4–6 and in vivo, during sepsis7 or within the tumor vasculature.8 The most physiologically significant inhibitor of the TF·FVIIa complex is the Kunitz-type tissue factor pathway inhibitor (TFPI),9 whose effect becomes manifest with the generation of limited quantities of FXa.10 TFPI uses the tandem Kunitz-type domains in its structure to form a quaternary complex with FXa bound to TF·FVIIa11 and thus prevents further production of FXa and FIXa through the TF-dependent pathway. Recent studies with transgenic mice have shown that TFPI(K1–2/2) mice do not survive the neonatal stage, probably owing to unregulated TF·FVIIa hyperactivity, with the consequent consumptive coagulopathy and bleeding, and suggest that human TFPI–deficient embryos may suffer a similar fate.12 The concentration of TFPI in plasma is low (≈2 nmol/L). The majority of the circulating TFPI is associated with...
lipoproteins\(^{13}\) and represents several carboxy-terminal-truncated forms, whereas only a minor proportion of TFPI is the full-length, free form of the molecule.\(^{14,15}\) ECs express and produce TFPI constitutively and probably contain the major pool of TFPI, residing as uniform clusters both on the cell surface and within the apical cytoplasm.\(^{16–19}\) After acute stimulation with thrombin, the anticoagulant potency of the ECs toward the TF \cdot \text{FVIIa} complex increases significantly due to TFPI redistribution and enhanced exposure on the plasma membrane.\(^{18}\) In resting endothelium, TFPI is exposed on the cell surface via a glycosylphosphatidylinositol (GPI) link and is targeted by apical vectorial delivery to restricted plasmalemma microdomains (caveolae) of particular proteolipid composition and function.\(^{20}\) Because the caveolae plasma membrane is devoid of anionic binding sites,\(^{21}\) particularly heparan sulfate and/or heparin,\(^{22}\) it is likely that there is at least 1 population of TFPI, the caveolar fraction, which is not weakly bound to sulfated glycosaminoglycans (GAGs) on the cell surface glyocalyx after secretion, as has been inferred until now. Sevinsky et al\(^{23}\) recently described the involvement of cellular TFPI in the assembly of the TF \cdot \text{FVIIa} complex in caveolae in stimulated ECs; therefore, we can assume that cell-associated TFPI plays a more important role than the fluid-phase form of the inhibitor in the maintenance of hemostatic balance.

Plasma levels of TFPI increase severalfold after in vivo infusion of unfractionated heparin (UFH) or low-molecular-weight heparin (LMWH)\(^{24,25}\) owing to the release of TFPI from readily available endothelial stores.\(^{26}\) Heparin has wide clinical use as an anticoagulant in the initial treatment of acute venous thromboembolism and unstable angina,\(^ {26,27}\) it is likely that there is at least 1 population of TFPI, the caveolar fraction, which is not weakly bound to sulfated glycosaminoglycans (GAGs) on the cell surface glyocalyx after secretion, as has been inferred until now. Sevinsky et al\(^ {23}\) recently described the involvement of cellular TFPI in the assembly of the TF \cdot \text{FVIIa} complex in caveolae in stimulated ECs; therefore, we can assume that cell-associated TFPI plays a more important role than the fluid-phase form of the inhibitor in the maintenance of hemostatic balance.

There is compelling experimental evidence showing that TFPI plays an important role in the antithrombotic effect of heparin. The heparin-releasable TFPI is mainly the free, full-length form of the molecule, with higher inhibitory activity toward FXa, enhanced to a greater extent by heparin, than the truncated forms of TFPI that normally circulate in plasma.\(^ {30,31}\) Although the heparin-mediated increase of TFPI has been largely documented, most of the results have come from clinical studies and refer mainly to the plasma levels of TFPI. Therefore, the precise mechanisms behind the heparin-induced release of TFPI are still largely unknown. The main purpose of our study was to try to establish the molecular basis of the effect that different heparin preparations may have on the gene expression, synthesis, constitutive secretion, and induced release of TFPI in human ECs in culture.

Methods

Materials

Antibodies and suppliers were as follows. Rabbit anti–recombinant (r) TFPI\(^ {16–16}\) IgG was developed in our laboratory, immunoaffinity purified, and tested for specificity (50 \(\mu\)g IgG per mL, produced 95% inhibition of TFPI activity in normal human plasma, and the concentration of IgG that still produced 50% inhibition of TFPI activity was \(\approx0.6 \mu\)g/mL; by immunofluorescence, the experiments of competition, eg, staining in the presence of rTFPI, were entirely negative). Monoclonal anti-caveolin IgG (No. C13620; clone C60 raised against an 11.1-kDa N-terminal fragment of human caveolin) was from Transduction Laboratories; monoclonal anti-human TF IgG (No. 4509) was from Alpha Labs. Secondary antibodies conjugated to FITC and Vectashield mounting medium were from Vector Laboratories Inc. Secondary antibodies conjugated to 5- or 10-nm colloidal gold were from BioCell Research Labs; protein A coupled to 10-nm gold was from the Department of Cell Biology, University of Utrecht, The Netherlands; and all other reagents used for electron microscopy were from TAAB Laboratory Equipment Ltd. Unfractionated heparin (UFH; \(\mathrm{M}_{\text{r}} \approx12\text{kDa}, 160\ \text{IU/mg}\)) was from Chromogenex AB, and low-molecular-weight heparin (LMWH), Fragmin\(^{\text{TM}}\) \(\mathrm{M}_{\text{r}} \approx5.6\text{kDa}, 160\ \text{anti-FXa units, 68 anti-thrombin U/mg}\) was from Pharmacia. Octyl-\(\beta\)-d-glucopyranoside was from Calbiochem-Novabiochem Ltd. Human coagulation FVIIa, FX, and FXa were purchased from Enzyme Research Labs, Ltd, and chromogenic substrate S-2337 (\(\text{N}-\text{benzoyl-L-isoleucyl-L-glutamyl-(piperidyl)-glycyl-L-arginine-nitroanilide hydrochloride}\)) was from Quadtech. TRizol was purchased from Life Technologies Ltd, Rapid-Hyb hybridization buffer was from Amersham Life Science Ltd, Gene Screen nucleic acid transfer membrane was from NEN Research Products, and polyvinylidene difluoride protein transfer membrane was from Bio-Rad. De–N-sulfated heparin (deNSH), rabbit brain thromboplastin, cycloheximide (CHX), phorbol 12-myristate 13-acetate (PMA), as well as secondary antibodies conjugated with biotin, cell culture media and supplements, endotoxin (LPS, a lipopolysaccharide extract from \textit{Escherichia coli} serotype 0128:B12), PMSF, HEPES, Tris, BSA, ovalbumin, sodium orthovanadate, and all other reagents were purchased from Sigma Chemical Co Ltd unless otherwise stated.

Cell Cultures

We used the immortalized human EC line EA.hy926,\(^ {32}\) kindly donated by Dr. Cora-Jean S. Edgell (Department of Pathology, University of North Carolina, Chapel Hill). The cells were grown on Petri dishes (30-mm diameter), T-75 flasks, or glass coverslips coated with gelatin, in Dulbecco’s modified Eagle’s medium containing 4 mmol/L \(\text{L}-\text{glutamine}, 15 \text{mmol/L HEPES, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 1:10-diluted, heat-inactivated FBS. Cells were used at } \approx 90\% \text{ morphological confluence, and the assays were carried out in serum-free medium supplemented with 1 g/L BSA and 5 mmol/L CaCl}_2.\)

Cell Treatments

ECs were incubated with the heparin preparations described above, using different concentrations (1 or 10 U/mL for UFH and LMWH or 160 \(\mu\)g/mL for deNSH) and for different periods of time: up to 1 hour for a short-time effect or up to 48 hours for the long-term effect. In the experiments designed to study the dependence of the heparin-induced TFPI release on protein synthesis, ECs were preincubated for 1 hour with CHX (10 \(\mu\)g/mL), followed by either medium only (control cells), UFH, or LMWH (1 U/mL each) for 30 minutes at 37°C. In other experiments, ECs were either preincubated with PMA (0.1 \(\mu\)mol/L) for 1 hour to activate protein kinase C (PKC),\(^ {33}\) followed by heparin for 30 minutes, or coincubated with PMA and heparin for 1 hour at 37°C. When the net increase in TFPI secretion was calculated, the level of TFPI measured in the control culture medium was subtracted from that obtained after agonist treatment. The “expected additive effect” of 2 different agonists was determined by adding the net amount of TFPI secreted in cultures treated with each agonist individually. In experiments in which sequential treatments were done, the effect of the primary agonist on TFPI secretion was determined using medium alone for the secondary treatment. The residual response was used as a correction factor when interpreting the effect of the second agonist during sequential incubations.

For all of the conditions described, supernatants were harvested after incubations and assayed for TFPI antigen and activity, whereas cell monolayers were processed as follows: (1) Cells grown on glass coverslips were rinsed with 0.1 mol/L PBS, pH 7.4, fixed for 1 hour at room temperature with 3% \(\text{wt/vol}\) paraformaldehyde in PBS, and used for immunofluorescence studies. (2) Cells grown in small Petri dishes were used for determination of TFPI antigen or activity on the intact monolayers, as previously described.\(^ {18}\) (3) Cells grown in T-75 flasks were used either for extraction of total RNA or for cellular lysate preparation.
Immunofluorescence Studies
We used the indirect immunofluorescence procedure previously described. In brief, fixed cells were incubated with the anti-TFPI IgG for 1 hour at room temperature, either directly or after permeabilization with 0.2% saponin. All samples were rinsed, incubated with goat anti-rabbit IgG/FITC, and mounted with Vectashield on glass slides. Fluorescence microscopy and digital image collection were performed with a Bio-Rad MRC 600 confocal laser unit attached to a Nikon Diaphot inverted microscope (Bio-Rad Microscience Ltd). Samples were analyzed by optical sectioning through the z axis of the cells, followed by computer-assisted reconstruction of the images. To allow a semiquantitative comparison between control and treated cells, the confocal parameters were kept unchanged during the digital image collection (neutral density filter No. 2 and diaphragm opening 1/3), images were processed by computer-assisted pseudocolor banding, and the scale of signal intensities was kept constant throughout all of the experiments.

Cellular Lysates
After the appropriate incubation, cell monolayers were placed on ice, rinsed with ice-cold PBS, and scrapped off the dishes in ice-cold Tris-buffered saline (0.1 mol/L Tris-HCl and 0.15 mol/L NaCl), pH 7.8, containing 1 mmol/L PMSE, 10 g/L aprotinin, 1 mmol/L sodium orthovanadate, and 10 mmol/L EDTA. After centrifugation, cell pellets were resuspended in ice-cold lysis buffer (Tris-buffered saline containing 10 g/L Triton X-100, 60 mmol/L octyl-β-D-glucopyranoside, and the cocktail of inhibitors), vigorously vortexed, and incubated for 30 minutes at 37°C with occasional vortexing. After this step, the insoluble proteins were removed by centrifugation; the supernatants, representing total cellular lysates, were collected and frozen.

Western Blotting
Cell supernatants collected after different treatments were concentrated in Ultrafree-CL filters, and protease inhibitors were added. From each sample, aliquots containing equivalent concentrations of total protein (200 ng) were precipitated with 72% (wt/vol) trichloroacetic acid. Precipitates were washed with cold acetone, resuspended in Laemmli sample buffer, boiled, and subjected to nonreducing SDS–polyacrylamide gel electrophoresis (5% to 15% acrylamide gradient). Proteins on gels were electrotransferred onto polyvinylidene difluoride membranes, and TFPI was detected with the anti-TFPI IgG, followed by biotin-conjugated anti-rabbit IgG, streptavidin coupled to horseradish peroxidase, and peroxidative reaction with diaminobenzidine.

Immunoelectron Microscopy
We performed both preembedding and postembedding immunogold localization of TFPI in ECs. For preembedding, after treatment with heparin or PMA, cell monolayers were fixed for 30 minutes at room temperature with a mixture of 2% paraformaldehyde and 0.05% glutaraldehyde in electron microscopy buffer (EMB; 0.1 mol/L sodium phosphate buffer supplemented with 3 mmol/L KCl and 3 mmol/L MgCl₂), pH 7.6; rinsed with EMB; quenched with 0.1 mol/L glycine in EMB; blocked for 30 minutes at 37°C with 10 g/L BSA in EMB; and incubated for 1 hour at 37°C with anti-TFPI IgG (50 μg/mL) followed by protein A coupled to 10-nm gold particles (diluted 1:40) in blocking solution. After being washed, monolayers were fixed for 30 minutes with 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate–HCl buffer, pH 7.4; postfixed for 10 minutes with 1% OsO₄ in the same buffer; scraped off the dishes; washed; dehydrated as pellets in a graded series of ethanol; and embedded in epoxy resin.

For postembedding, ECs were fixed for 90 minutes at room temperature with a mixture of 3% paraformaldehyde and 0.05% glutaraldehyde in PBS, dehydrated in an ascending series of ethanol while the temperature was progressively lowered, and embedded in Lowicryl K4M as described. Thin sectioning was performed on a Reichert Ultracut microtome (Reichert-Jung Optische Werke), and sections placed on Formvar-coated, 200-mesh nickel grids were immunogold labeled, as described, by using a mixture of rabbit anti-TFPI IgG and mouse anti-caveolin IgG, followed by a mixture of secondary antibodies (goat anti-rabbit IgG coupled to 10-nm gold and goat anti-mouse IgG adsorbed to 5-nm gold).

After being counterstained with 1% OsO₄, 2.5% uranyl acetate, and lead citrate, the sections were examined with a Philips 201 EM. Controls included omission of the first antibodies or their replacement with nonimmune IgGs.

Northern Blotting
Total RNA was extracted from ECs by an acid-phenol method by using TRIZol reagent according to the manufacturer’s instructions. Total RNA (30 μg per lane) was size fractionated by gel electrophoresis in 1.2% agarose–6% formaldehyde gels and transferred onto a Gene Screen membrane by the capillary blot method. Prehybridization was performed in Rapid-Hyb buffer for 2 hours at 65°C under agitation. The membrane was then hybridized under the same conditions in buffer containing 1 to 5 ng/mL 32P-labeled probe. The cDNA probes used were a 601-bp fragment resulting from EcoRI/ClaI digestion of human full-length TFPI cDNA (a kind gift of Dr G. Broze, Washington University, St Louis, Mo) and an S26 full-length cDNA as the housekeeping gene, both radiolabeled by random priming with [α-32P]dCTP. The blots were washed under increased stringency at 65°C and exposed to x-ray film at −70°C. Densitometry and comparison between the intensity of the bands were performed by using the public domain NIH (National Institutes of Health, Bethesda, Md).

TF-Dependent Activation of FX
For measurement of the proteolytic activity of the TF · FVIIa complex toward FX, we used a 2-stage chromogenic assay. ECs were stimulated with endotoxin (10 μg/mL) in complete medium for 4 hours to express TF, then UFH or LMWH was added, and incubation was continued for 30 minutes at 37°C. ECs were rinsed with 10 mmol/L Tris-buffered saline, pH 7.8, containing 1 g/L ovalbumin; incubated for 10 minutes at 37°C with 10 mmol/L EDTA in the same buffer to remove endogenous and serum-derived coagulation factors; rinsed with buffer A (10 mmol/L HEPES, 137 mmol/L NaCl, 4 mmol/L KCl, and 11 mmol/L-L-glucose), pH 7.45; and incubated for 30 minutes at 37°C with 10 mmol/L FVIIa in buffer A containing 10 mmol/L CaCl₂ and 5 g/L ovalbumin. Activation was initiated by adding 200 mmol/L FX to each dish, and the incubation was continued for 20 minutes at 37°C with shaking. At defined intervals, subsamples of 20 μL (duplicates) were withdrawn from the cell supernatants and transferred into microplate wells containing 20 μL of 50 mmol/L Tris-buffered saline, pH 8.2, and 10 mmol/L EDTA. After collection of all samples, chromogenic substrate S-2337 was added (final concentration 0.3 mmol/L), and the initial rate of substrate cleavage at 405 nm (mOD U/min) was measured for 15 minutes at 37°C using a Molecular Devices THERMOMax microplate reader (Alpha Laboratories Ltd). The amount of FXa generated was extrapolated from a standard curve prepared with serial dilutions of human FXa (5 to 50 fmol per well).

In antibody-blocking experiments, cells were preincubated with anti-TFPI IgG (50 μg/mL) or anti-TF IgG (100 μg/mL) for 30 minutes at room temperature before the addition of FVIIa, and the antibodies were also kept in the medium during the incubation with FVIIa. In control experiments, similar incubations of the cells with normal rabbit or mouse IgG fractions did not have any effect on the activation of FX.

Determination of TFPI Antigen and Anticoagulant Activity
TFPI antigen and activity were determined in cell supernatants, cellular lysates, and on the cell surface, essentially as previously described: the antigen was measured by both direct ELISA (on cell monolayers) and indirect competitive ELISA (supernatants and lysates); the activity was determined with a 2-stage chromogenic assay, based on the ability of TFPI to inhibit activation of FX by TF · FVIIa in the presence of FXa. Protein estimation was done using the bicinchoninic acid assay kit (Pierce & Warriner [UK] Ltd).
**Statistical Analysis**

All of the experiments described were repeated at least 3 times. Three or 4 cell-culture dishes were used for each experimental condition (time point, different concentration of agonists, simultaneous or consecutive treatments, etc), and the optical readings made in duplicate or triplicate were averaged separately for each dish. Results from replicate experiments were grouped for each experimental condition, and data within groups were statistically compared between each other by the unpaired *t* test and expressed as mean ± SD. The mean differences between groups illustrate comparisons either between different cell treatments at the same point or at various points within 1 individual treatment and were considered significant when the *P* value was ≤ 0.01.

**Results**

**Effect of Heparin on TFPI After Short-Time Incubation**

*Increased Release of TFPI*

ECs incubated with UFH or LMWH (1 or 10 U/mL) for up to 1 hour released 2 to 3 times more TFPI in the supernatants, measured as either antigen or activity, than did control cells (Figures 1a and 1b). For each time point, *P* was ≤ 0.0001 for the mean differences between control and heparin-treated cells. For UFH, the release of TFPI was both time- and concentration-dependent (Figure 1a), with *P* ≤ 0.0001 for the differences between 2 consecutive time points and *P* ≤ 0.01 for the differences between 1 and 10 U/mL. For LMWH, the mean differences between time points and different concentrations were less significant than for UFH.

The activity of TFPI expressed on the EC surface did not decrease after incubation with heparin but was enhanced by '20% for UFH and by 25% for LMWH (Figure 1c).

**Ultrastructural Distribution of TFPI**

Resting ECs exhibited small clusters of gold-immunolabeled TFPI dispersed both on the plasmalemma proper and within or nearby small, uncoated membrane invaginations, which were positively identified as caveolae by postembedding double immunolabeling for TFPI and caveolin (Figure 3a). TFPI was also found in vesicles/caveolae within the apical cytoplasm (same panel, arrowheads).

Preembedding immunostaining for TFPI on UFH- or LMWH-treated ECs consistently revealed gold labeling on the cell surface (Figures 3b and 3d), with frequent patches of gold particles on the plasma membrane (illustrated for LMWH in the inset). The postembedding double immunolabeling for TFPI and caveolin proved that TFPI was not displaced from caveolae after treatment with UFH or LMWH (Figures 3c and 3e). Rows of cell-surface, opened caveolae or enlarged caveolar profiles underneath the plasma membrane exhibiting strong immunostaining for TFPI were observed in both UFH- and LMWH-treated ECs (Figures 3e and 3f).

**Activation of FX on EC Monolayers**

ECs stimulated with endotoxin for 4 hours promoted activation of FX by FVIIa in a time-dependent manner (Figure 4). Formation of FXa did not occur on resting cells, and LPS-treated ECs supported activation of FX as a consequence of expression of TF, as demonstrated by the lack of FX activation on cells preincubated with anti-TF IgG (not shown). The rate of FXa formation decreased after 5 to 10 minutes on control cells but continued on ECs preincubated with anti-TFPI IgG, thus excluding the possibility of substrate depletion. For UFH-treated cells, the amount of FXa detected in the cellular medium after 5 minutes decreased by 30% (*P* < 0.0001) compared with control cells. The rate of activation was significantly slower and the activation stopped earlier than on control cells. LMWH produced a similar pattern of FX activation, although failure to detect the initial
increase in FXa accumulation observed for UFH during the first 5 minutes might suggest that LMWH is more efficient in reducing FX activation. Accordingly, the mean differences between UFH and LMWH were significant for each time-point (P≤0.0098 for all values). The generation of FXa proceeded on heparin-treated cells when TFPI was blocked with the anti-TFPI IgG, which suggests that TFPI directly inhibits the accumulation of FXa in the cellular medium.

Effect of Heparin on TFPI After Long-Time Incubation

Secretion of TFPI

Figure 5a illustrates the time-dependent enhancement of TFPI secretion from ECs during their incubation with UFH, LMWH, or deNSH for up to 48 hours. The mean differences between control and heparin-treated ECs were highly significant (P<0.002), and so were the differences between 2 consecutive time points (P<0.004) for each heparin preparation tested.

TFPI in cellular lysates (Figure 5b) decreased after 4 to 8 hours of incubation with UFH (P=0.0075), after which TFPI gradually increased and exceeded its concentration in control cells after 48 hours (P=0.015 for the mean difference). The rate of TFPI secretion was relatively constant for control cells (<5 ng TFPI·mL⁻¹·h⁻¹), whereas for UFH-treated cells, the production increased ~3-fold during the first hour of incubation, then decreased for the next 8 hours, and increased again after 24 hours (Figure 5c). After 4 hours of incubation with UFH, ECs failed to respond to a second challenge with UFH (10 U/mL) or calcium ionophore A23187 (5 μmol/L) to the same extent as did control cells. As illustrated in Figure 5d, the release of TFPI was reduced by 56% for UFH and 47% for A23187, which suggests depletion of intracellular stores rather than desensitization to the homologous agent.

The Western blot analysis of the TFPI released into the cell medium showed the presence of a major band (sometimes visible as a doublet) with an M_r of 40 to 45 kDa (Figure 6a, lane 1). The intensity of TFPI bands increased time-dependently in samples from heparin-incubated ECs (lanes 2, 3, and 5) compared with the TFPI constitutively secreted by control ECs (lanes 1 and 4).

Expression of TFPI Message

As illustrated in Figure 6b, we identified by Northern blot, for TFPI mRNA in ECs, the 2 transcripts of 4.0 and 1.4 kb originally described by Girard et al. The expression of TFPI was analyzed at different time points during incubation of ECs with UFH or LMWH for 24 hours. After densitometric scanning of the gels, the ratio between the band intensity for each TFPI transcript and S26 was calculated and represented as a percentage of the appropriate value obtained for control cells at the same time point. Both preparations of heparin induced a similar pattern of response (illustrated for UFH in Figure 6c). The 4.0-kb TFPI transcript remained essentially unchanged for the period of time tested. In contrast, the intensity of the 1.4-kb band was reduced after 4 hours (~50% for UFH and 30% for LMWH), after which it showed complete recovery, even increased after 8 hours (90% over control for UFH and 50% for LMWH), and remained at the same high level after 24 hours.

Cellular Distribution of TFPI

The quantitative data presented above correlate well with the immunofluorescence studies (Figure 2). After 8 hours of incubation with UFH or LMWH, the distribution of TFPI was
Figure 3. Immunogold staining for TFPI in ECs in culture. a, Postembedding double immunogold labeling for TFPI (anti-TFPI IgG followed by anti-rabbit IgG/10-nm gold) and caveolin (anti-caveolin IgG followed by anti-mouse IgG/5-nm gold) in resting ECs shows colocalization in surface opened caveolae (arrows) and subapical vesicles (arrowheads). Preembedding immunostaining for TFPI (anti-TFPI IgG followed by protein A/10-nm gold) on ECs treated with UFH (b) or LMWH (d) reveals gold labeling on the cell surface and in caveolae, with frequent formation of patches (d, inset). Postembedding double staining for TFPI and caveolin (as above) proves that TFPI is not displaced from caveolae after incubation with UFH (c) or LMWH (e). Rows of opened caveolae are strongly labeled for TFPI (f, UFH-treated ECs, tangential section underneath the plasmalemma, en face view). ECs treated with PMA exhibit strong immunolabeling for TFPI over rows of cell-surface, opened caveolae (g). Bars = 100 nm.
Effect of CHX on TFPI Release

The effect of CHX, as a general protein synthesis inhibitor, on the secretion of TFPI and its induced release was studied in experiments in which ECs were preincubated for 1 hour with medium only (control) or CHX (10 mg/L) and then treated for 30 minutes at 37°C with medium alone, UFH, or LMWH (Figure 7a). As expected, the constitutive secretion of TFPI decreased by 35% in cells preincubated with anti-TFPI IgG. The quantity of FXa detected in the cell medium is represented time-dependently for ECs treated with UFH, LMWH (Fragmin), or UFH and anti-TFPI IgG. All values are expressed as mean±SD.

Effect of PMA on Heparin-Induced TFPI Release

To gain further insights into the mechanism(s) of TFPI release induced by heparin, the possible role of PKC was investigated. Treatment of ECs with PMA for 1 hour causes activation of PKC\(^{33}\) and inhibits the internalization of caveolae, leading to their enhanced exposure on the cell surface.\(^{58}\) An electron photomicrograph illustrating the effect of PMA on the distribution of TFPI in ECs after a 1-hour incubation is presented in Figure 3g, which shows strong immunogold staining for TFPI over continuous rows of cell-surface opened caveolae. Similar to thrombin,\(^{18}\) PMA also enhanced the release of TFPI in the cell medium (≈3 times over the constitutive secretion), decreased the antigen in cellular lysates, and induced an ≈40% increase of TFPI activity on the cell surface (Figures 8b and 8c). Pretreatment of ECs with PMA inhibited the subsequent heparin-induced release of TFPI, by ≈60% for UFH and completely for deNSH (Figure 8a).

When ECs were coincubated with PMA and heparin for 1 hour, the net release of TFPI in the PMA-and-heparin–treated sample was 2.4 times lower than the expected additive value for UFH and PMA tested individually (Figure 8b). The cell surface activity measured for TFPI was ≈2 times lower than the expected additive value for PMA and heparin and 1.3 times lower for PMA and LMWH (Figure 8c). The \(P\) value was \(≤0.001\) for all of the comparisons described in this paragraph.

Discussion

This study analyzed the possible mechanism(s) through which UFH, an LMWH preparation (Fragmin), and deNSH act on the human immortalized EC line EA.hy926 to induce an increased release of TFPI, alteration of the cellular expression and distribution of the inhibitor, and modification of the anticoagulant activity of the cells.

The several-fold increase in plasma TFPI levels after in vivo infusion of heparin has been largely documented,\(^{24,25}\) but the mechanism by which this process occurs has never been deciphered. Based on circumstantial evidence, it was widely accepted that part of the TFPI secreted from ECs became reattached to sulfated proteoglycans in the cell surface glycolalyx through electrostatic bonds between the positively charged C-terminus of TFPI and the negatively charged sulfate groups in GAGs.\(^{39,40}\) Heparin was simply thought to displace TFPI, from its binding sites/GAGs on the EC surface and into the bloodstream, in the form of heparin-TFPI complexes.\(^{41}\) Recently, more pieces of evidence have been gathered to point out a possibly greater significance of the endogenous, cell-associated TFPI than of the circulating form of the inhibitor in maintaining the anticoagulant properties of the endothelium. First, TFPI is located constitutively in ECs within specific glycolipid microdomains/caveolae,\(^{20}\) which represent highly specialized plasmalemma domains performing specific functions: endocytosis and transcytosis,\(^{42,43}\) photocytosis,\(^{44}\) regulation of cell surface–associated proteolysis,\(^{45,46}\) calcium regulation, and signal transduction.\(^{44,46}\) Second, TFPI is exposed on the cell surface through a specific link, the GPI anchor,\(^{20,23}\) and mediates the formation and translocation of TF-FVIIa-FXa complexes in caveolae in activated ECs.\(^{23}\) Third, removal of heparan sulfate from the EC surface with heparitinase or treatment with sodium chlorate to inhibit GAG sulfation does not affect the exposure...
of TFPI on the cell surface or its anticoagulant activity, nor do they prevent the binding of exogenously added rTFPI to the cells. Last, in a recent study of immunolocalization of TFPI in ECs, Hansen et al detected cellular TFPI on the cell surface and in plasmalemma vesicles, as well as within the endocytic compartment, thus confirming the presence of at least 1 pool of cellular TFPI, probably GPI anchored in the membrane, which can undergo recycling and degradation. On the basis of these considerations and the biochemical and morphological evidence we present herein, we propose that the heparin-induced release of TFPI, at least from ECs in culture, involves more complicated and specific cellular mechanisms than a simple displacement of TFPI allegedly bound to sulfate residues in the cell surface glycocalyx.

As expected, short-time incubation of ECs with heparin resulted in enhanced release of TFPI. The process is both time and concentration dependent and is more significant for UFH than for LMWH, at least at high concentrations (10 U/mL).

Inhibition of protein synthesis in ECs does not reduce the effect of heparin, which suggests that the release occurs mostly from preformed TFPI pools, likely to be located intracellularly. Immunofluorescence images also showed that TFPI decreases intracellularly after treatment with heparin and redistributes over the cell surface by unclustering within large patches. We suggest that this patchy redistribution might support the increased TFPI activity and antigen determined by quantitative measurements on intact monolayers. Ultrastructurally, clusters of immunogold-labeled TFPI were frequently found on the plasmalemma surface and in caveolae in ECs treated with heparin, as proved by the postembedding double staining for TFPI and caveolin.

The significance of membrane-bound TFPI is reinforced by results from the experiments in which the TF-FVIIa complex–dependent formation of FXa on LPS-stimulated ECs was studied. The amount of FXa measured in the medium of control cells decreased after 5 to 10 minutes, but...
the activation reaction continued when ECs were preincubated with anti-TFPI IgG. The ability of heparin-treated ECs to support FX activation is very poor, and after a slight but significant increase of FXa in the medium during the first 5 minutes, the accumulation of FXa stops. It is known that heparin potentiates the inhibition of FXa by TFPI by increasing both the affinity and the rate of reaction, probably through direct acceleration of the interaction between TFPI and FXa, which leads to rapid inhibition of TF·FVIIa by the TFPI-FXa complex.48 For ECs, where free FXa is not efficiently bound by cellular TFPI,23 downregulation of TF·FVIIa activity on the cell surface is dependent on the formation of the quaternary complex with FXa and TFPI and its translocation in caveolae, whose particular composition, notably the lack of anionic binding sites, inefficiently supports the proteolytic function of the TF·FVIIa complex.23 Our data showing that the amount of FXa in the medium of heparin-treated cells decreases below starting point levels, though puzzling at first,
can be explained if we consider that the FXa generated initially is used for the formation of the quaternary complex by binding to TFPI in caveolae. From this stage, the complex might undergo endocytosis on the route followed by TFPI, which would lead to downregulation of the TF · FVIIa activity on the EC surface. Alongside the morphological evidence, results from FX activation experiments reinforce the idea that caveolar TFPI can play an important role in heparin-treated ECs. Accordingly, TFPI in caveolae/enlarged caveolar profiles, which are devoid of anionic sites, is probably not displaced by heparin, which, on the contrary, seems to induce an enhancement of the number of opened caveolae. We suggest that this can provide ECs with a protected reservoir of potent anticoagulant molecules, stabilized by heparin and probably ready to become functionally active when the ECs are induced to express TF after stimulation.

Long-time incubation of ECs with heparin also induced enhanced secretion of TFPI and a decrease of the level of the inhibitor in cellular lysates. However, the rate of secretion decreased after 4 to 8 hours of incubation with heparin and was enhanced again at 24 hours. The amount of TFPI in cellular lysates also decreased during the first 4 to 8 hours of incubation, suggesting that the intracellular pools of TFPI had become depleted. In addition, ECs treated with heparin for 4 hours failed to respond to a second challenge with heparin or calcium ionophore A23187, with the same release of TFPI as from control cells. In an in vivo study reporting the effect of repeated or continuous intravenous infusion of heparin on the release of TFPI in plasma, demonstrated depletion of intravascular pools of TFPI, with the major part of the decrease observed after 4 to 8 hours, and suggested that the constitutive synthesis of TFPI was overwhelmed by enhanced secretion of the inhibitor. From our analysis of the expression of TFPI message during the incubation of ECs with UFH or LMWH, we can suggest that the observed depletion might also be due to the decrease of TFPI mRNA, significantly manifested for the 1.4-kb transcript after 4 hours of heparin treatment.

The recovery and increase of intracellular TFPI observed at longer times might be due to cell-mediated degradation of heparin, although the enhanced rate of TFPI release may indicate more specific intracellular effects involving protein synthesis. A study of the time course of heparin binding to human umbilical vein ECs showed that at least 3 hours were necessary to reach saturation at 37°C, then part of the bound heparin underwent endocytosis, and ~30% of the specifically bound heparin remained associated with the cells as an internalized pool. Although the intracellular location of heparin has not been identified in ECs, some of the internalized heparin is known to proceed to the nucleus of HeLa cells and hepatocytes; and additionally, a putative heparin receptor has been described in ECs. Other specific cellular effects of heparin include the selective inhibition of the mitogenic stimulation of smooth muscle cells by phorbol esters and serum, the suggested mechanism being that heparin selectively represses phorbol ester–inducible activator protein-1–mediated gene expression by interfering with the binding of the heterodimeric Fos-Jun/activator protein-1 transcription factor complex to activator protein-1–like promoter elements. Whether or not heparin can also affect TFPI expression by an interference with the activator protein-1–like consensus sequences present in the 5′ upstream region of the TFPI gene is a matter of speculation at this moment and requires further investigation. Comparison between UFH, LMWH, and deNSH showed that, at least for cultured ECs, there is no major difference regarding their effect on TFPI. This suggests that the electric charges and the M, of heparin are not major determinants of TFPI release in vitro.

To better understand the mechanism(s) by which heparin induces the observed modifications of TFPI in ECs, we addressed the question of whether heparin can interfere with some of the signal transduction pathways whose machinery is found in caveolae. PKC-α and a protein phosphatase are probably the key regulatory enzymes that control the internalization cycle of caveolae. PKC-α is highly concentrated in caveolae in unstimulated cells and is constitutively active in this location. Treatment of cells with phorbol...
esters or agonists that raise the concentration of diacylglycerol in the cells (thrombin or histamine) further activates PKC-α and inhibits internalization of caveolae. In our experience, pretreatment of ECs with PMA for a short time inhibits subsequent heparin-induced secretion of TFPI. The coincubation of ECs with PMA and heparin also shows strong antagonism and suggests that the effects of heparin on endogenous TFPI in ECs might involve specific cellular mechanism(s). It is known, for example, that heparin inhibits the PKC-α-dependent pathway of mitogenesis in smooth muscle cells and fibroblasts, but because it does not directly affect PKC activity or the phosphorylation step, it is believed that the heparin block is distal to the activation of PKC. On the other hand, there are other cellular responses elicited by PMA, such as stimulation of phospholipase D, which can occur independently of PKC. Therefore, further work is needed to elucidate the nature of the mechanism(s) involved in the release and redistribution of TFPI in ECs in culture.

The enhanced secretion of TFPI induced by heparin and the modifications of cellular TFPI observed in human ECs in culture, if proven to take place in vivo also, may represent an important mechanism designed to mobilize TFPI from the endothelium to sites of TF exposure with ongoing thrombosis (eg, a damaged vessel wall or TF expressed on monocytes or in atherosclerotic plaque rupture areas) or, probably more important, to confer highly regulatory anticoagulant properties to the intact endothelium.

Acknowledgments
This work was supported by stipends from Pharmacia Upjohn (C.L.), the British Heart Foundation (C.L.), (grant PG/97045), Stanley Thomas Johnson Foundation (F.L.), The Royal Society (C.L., F.L.), and the Thrombos Research Trust. We thank Dr Cora-Jean S. Edgell (Department of Pathology, University of North Carolina, Chapel Hill) for kindly providing us with the EC line EA.hy926, as well as Dr George Broze, Jr. (Washington University, St Louis, Mo) for the human TFPI cDNA. We highly acknowledge the skilful help of Sally Mill (cell culture).

References
32. Levin EG, Marotti KR, Samuell L, Protein kinase C and the stimulation of tissue plasminogen activator release from human endothelial cells:
Cellular Effects of Heparin on the Production and Release of Tissue Factor Pathway Inhibitor in Human Endothelial Cells in Culture
Cristina Lupu, Emma Poulsen, Sylvie Roquefeuil, Andrew D. Westmuckett, Vijay V. Kakkar and Florea Lupu

doi: 10.1161/01.ATV.19.9.2251

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/19/9/2251

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/