Further Insight Into the Heparin-Releasable and Glycosylphosphatidylinositol-Lipid–Anchored Forms of Tissue Factor Pathway Inhibitor

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The release of tissue factor pathway inhibitor (TFPI) from human umbilical vein endothelial cells (HUVECs) was investigated using heparin and phospholipase C. The experiment included incubating HUVECs with 0, 1, or 10 U/mL heparin diluted in Dulbecco Modified Eagle’s Medium plus 5% fetal calf serum for 1 or 24 hours. A statistically significant increase in TFPI activity levels was seen at 1 hour, but not at 24 hours. A 20-fold increase in the release of TFPI after phospholipase C treatment of HUVECs was demonstrated, confirming that it is glycosylphosphatidylinositol-lipid (GPI) anchored. Sequential treatment of HUVECs with phospholipase C and heparin was performed, and a trend was observed where GPI-anchored TFPI levels were increased after 1 hour of pretreatment with heparin but were decreased after 24 hours. Serum is a requirement for the heparin-dependent release of TFPI from HUVECs. Heparin pretreatment of HUVECs may affect levels of GPI anchored TFPI in a time and dose-dependent manner.

Keywords: glycosylphosphatidylinositol-lipid anchor; heparin; tissue factor pathway inhibitor
Approximately 75% of cell-surface-bound TFPIα is released by phosphatidylinositol-specific phospholipase C (PLC; Figure 1C), with the remainder released by sequential PLC then heparin treatment (Figure 1D). Tissue factor pathway inhibitor-β is an alternate splice variant of the TFPI gene that lacks the third Kunitz domain and C-terminus of TFPIα. It is anchored directly to the endothelial cell surface by a GPI anchor and is released entirely by PLC in vitro (Figure 1C).
The aims of this study were to:

1. validate the conditions required to demonstrate the heparin-dependent release of TFPI from human umbilical vein endothelial cells (HUVECs);
2. determine the effect of PLC on heparin-releasable TFPI in HUVECs, and
3. evaluate the effect of heparin on GPI-anchored TFPI in HUVECs.

Materials and Methods

Materials

Factor VII was purchased from Enzyme Research Laboratories (South Bend, Ind). Factor X was purchased from Sigma Chemical Company (St Louis, Mo). Innovin was from Dade Behring (Marburg, Germany). S-2222, a chromogenic substrate specific for FXa, was obtained from Chromogenix (Milano, Italy).

Tissue culture flasks (25 cm²) were purchased from Orange Scientific (Braine-l’Alleud, Belgium) and 35-mm culture dishes from Becton Dickinson (Franklin Lakes, NJ). All culture media components (fetal calf serum, penicillin/streptomycin, amphotericin B, L-glutamine, sodium bicarbonate) and culture reagents (trypsin/ethylenediaminetetraacetic acid, Hanks Balanced Salt Solution, and Dulbecco Modified Eagle’s Medium [DMEM]) were purchased from TRACE Scientific (Mulgrave, VIC, Australia), and MCDB 131 culture media was purchased from Invitrogen (Mulgrave, VIC, Australia).

Unfractionated heparin (UFH) and low-molecular-weight heparin (LMWH; Fragmin) were from Pharmacia and Upjohn (Peapack, NJ). Phosphatidylinositol-specific PLC (P-6466) was purchased from Invitrogen. Albumin (Albumex) was from CSL (Parkville, VIC, Australia). All laboratory grade reagents were from Merck (Kilsyth, Victoria, Australia), Sigma Chemical Company (St Louis, Mo), or Ajax Chemicals (Auburn, NSW, Australia).

Methods

Study Approval

Ethics approval for umbilical cord collection for this study was obtained from the Human Research Ethics Committee, Curtin University of Technology (Approval No HR 62/2002) and the North Metropolitan Health Services Board. Informed written consent was obtained from all participants in the study before collection.

Cell Culture

A previously published method was used to isolate HUVECs from umbilical cords.²² Cells were grown in 1% gelatin-coated 25 cm² tissue culture flasks at 37°C in 5% carbon dioxide. The culture medium consisted of MCDB 131 basal medium, 15% fetal calf serum (FCS), 100 μg/mL streptomycin, 100 U/mL penicillin, 2.5 μg/mL amphotericin B, 4 mM L-glutamine, and 2.2% sodium bicarbonate.

Once confluent, cells were passaged using 0.05% trypsin/0.025% EDTA and seeded into 1% gelatin-coated 35-mm tissue culture dishes at a density of approximately 10 000 cells/cm². They were grown to confluence in DMEM basal medium, 15% FCS, 100 μg/mL streptomycin, 100 U/mL penicillin, 2.5 μg/mL amphotericin B, 4 mM L-glutamine, and 2.2% sodium bicarbonate.

Heparin Stimulation of HUVECs

Once confluence was achieved, the culture medium was removed and replaced with DMEM containing 5% FCS, and the cells were incubated for 24 hours. This medium was removed and the cells washed twice with phenol-red free Hanks Balanced Salt Solution (HBSS). To this was added 1 mL of heparin at a concentration of 0 (control), 1, or 10 U/mL (diluted in either phenol red free DMEM or phenol red free DMEM containing 5% FCS), and the cells were incubated at 37°C for 1 hour or 24 hours. The supernatants were collected and assayed for TFPI activity.

Phospholipase C Treatment of HUVECs

After the HUVECs grew to confluence, the medium was removed, and the cells were serum starved in 5% FCS for 24 hours. This medium was removed, and the cells were washed twice with Tris-buffered saline (TBS; 50 mM Tris, 150 mM sodium chloride, pH 7.4) and incubated with 0.5 mL of 0.4 U/mL PLC (diluted in TBS) or TBS (control) for 2 hours at 37°C. The supernatants were collected and assayed for TFPI activity.

Heparin, Then Phospholipase C Treatment of HUVECs

Once the HUVECs had grown to confluence, the growth medium was removed, and the cells were serum starved (in 5% FCS) for 24 hours. This medium was removed, and the cells were washed twice with HBSS and incubated with 1 mL of heparin (0, 1, or
10 U/mL; diluted in phenol red free DMEM containing 5% FCS) at 37°C for 1 hour or 24 hours, after which the supernatants were collected. The cells were washed twice in TBS and incubated with 0.5 mLs of 0.4 U/mL PLC (diluted in TBS) or TBS (control) for 2 hours at 37°C. The supernatants were collected and assayed for TFPI activity.

**Phospholipase C, Then Heparin Treatment of HUVECs**

The HUVECs were grown to confluence as previously stated. The growth medium was removed, and the cells were serum starved (in 5% FCS) for 24 hours. This medium was removed, and the cells were washed twice in TBS. The cells were incubated with 0.5 mL of 0.4 U/mL PLC (diluted in TBS) or TBS (control) for 2 hours at 37°C, after which the supernatants were collected. The cells were washed twice with HBSS and incubated with 1 mL of heparin (0, 1, or 10 U/mL; diluted in phenol red free DMEM containing 5% FCS) at 37°C for 1 hour or 24 hours. The supernatants were collected and all supernatants were assayed for TFPI activity.

**Tissue Factor Pathway Inhibitor Activity Assay**

The TFPI activity assay was based on a previously published method, with slight modifications as previously published. Briefly, 25 µL of the test sample (pooled normal plasma or endothelial cell supernatant) was incubated with 100 µL of a reaction mixture (containing final concentrations of 0.005 U/mL FX, 0.0075 U/mL FVII, 18.75 mM calcium chloride and 1/160 Innovin) at 37°C for 20 minutes. Then 50 µL of a 0.2 U/mL FX and 1.0 mM S-2222 mixture was added and incubated at 37°C for 30 minutes. The reaction was stopped with 50 µL of 5% acetic acid, and the absorbance was read at 405 nm using a Labsystems Multiskan Ascent plate reader (Thermo Scientific, Waltham, Mass).

Samples were compared with a standard curve to determine TFPI activity. The standard curve was generated using dilutions of pooled normal plasma (neat to 1/128) obtained from 20 healthy male donors and designated to contain 1 U/mL TFPI. All samples were heated for 15 minutes at 56°C, placed on ice for 2 minutes, and then centrifuged at 1500g for 15 minutes before assay to remove FVII, FX, and fibrinogen. All dilutions were made in a modified TBS (50 mM Tris, 150 mM sodium chloride, 10 mM trisodium citrate, 2 mg/mL albumin, and 2 µg/mL Polybrene, pH 8.0). The intraassay and interassay coefficients of variation were 1.7% and 5.0%, respectively.

**Statistical Analysis**

Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, San Diego, Calif). Results are reported as the mean of at least 2 experiments and were compared using the 1-way analysis of variance (ANOVA). Two-way ANOVA was used for the heparin/PLC experiments. A value of $P < .05$ was considered to be statistically significant.

**Results**

**Assessment of Heparin-Releasable TFPI in the Absence of 5% FCS**

The activity levels of TFPI in the HUVEC supernatants after 1 hour of incubation with 0, 1, or 10 U/mL UFH diluted in DMEM were 8.0, 6.5, and 10.0 mU/mL, respectively (Figure 2A). After 24 hours, TFPI activity levels were 101.5, 124.5, and 104.2 mU/mL using 0, 1, or 10 U/mL UFH (Figure 2B). No significant increase in TFPI activity was observed using 1 or 10 U/mL of UFH at 1 hour ($P > .05$) or 24 hours ($P > .05$) compared with the 0 U/mL control.

**Assessment of Heparin Releasable TFPI in the Presence of 5% FCS**

Tissue factor pathway inhibitor activity levels in HUVEC supernatants after 1 hour of incubation with 0, 1, or 10 U/mL UFH diluted in DMEM plus 5% FCS were 9.0, 6.5, and 10.0 mU/mL, respectively (Figure 2A). After 24 hours, TFPI activity levels were 101.5, 124.5, and 104.2 mU/mL using 0, 1, or 10 U/mL UFH (Figure 2B). No significant increase in TFPI activity was observed using 1 or 10 U/mL of UFH at 1 hour ($P > .05$) or 24 hours ($P > .05$) compared with the 0 U/mL control.

Tissue factor pathway inhibitor activity levels in HUVEC supernatants after 1 hour of incubation with 0, 1, or 10 U/mL LMWH diluted in DMEM were 7.5, 8.9, and 12.5 mU/mL, respectively (Figure 3A). After 24 hours, TFPI activity levels were 97.9, 118.4, and 151.7 mU/mL using 0, 1, or 10 U/mL LMWH (Figure 3B). There were increases in TFPI activity at 1 hour and 24 hours using LMWH, although these were not statistically significant ($P > .05$ for both time points).

**Assessment of Heparin Releasable TFPI in the Presence of 5% FCS**

Tissue factor pathway inhibitor activity levels in HUVEC supernatants after 1 hour incubation with 0, 1, or 10 U/mL UFH diluted in DMEM plus 5% FCS were 9.0, 18.3, and 18.4 mU/mL, respectively (Figure 4A). After 24 hours, TFPI activity levels were 93.6, 131.8, and 121.2 mU/mL using 0, 1, and
A statistically significant increase in TFPI activity among all UFH concentrations was observed at 1 hour ($P < .0001$) but not at 24 hours ($P > .05$).

Tissue factor pathway inhibitor activity levels in HUVEC supernatants after 1 hour incubation with 0, 1, or 10 U/mL LMWH diluted in DMEM plus 5% FCS were 2.23, 7.01, and 7.01 mU/mL in control cells and 4.58, 6.03, and 8.68 mU/mL in PLC-treated cells, respectively (Figure 5A). Heparin-releasable TFPI activity levels in HUVEC supernatants after 24 hours' UFH treatment (0, 1, or 10 U/mL) were 75.49, 102.12, and 100.48 mU/mL in control cells and 79.90, 96.07, and 90.66 mU/mL in PLC-treated cells, respectively (Figure 7B). There was no statistically significant difference between PLC-treated and control cells in heparin-releasable TFPI activity levels after subsequent treatment with UFH after 1 hour ($P > .05$) or 24 hours ($P > .05$).

Heparin-releasable TFPI activity levels in HUVEC supernatants after 1 hour of LMWH treatment (0, 1, or 10 U/mL) were 2.95, 3.83, and 3.98 mU/mL in control cells and 4.29, 3.10, and 4.34 mU/mL in PLC-treated cells, respectively (Figure 8A). Heparin-releasable TFPI activity levels in HUVEC supernatants after 24 hours' LMWH treatment (0, 1, or 10 U/mL) were 38.22, 45.88, and 46.17 mU/mL in control cells and 29.68, 37.44, and 43.54 mU/mL in PLC-treated cells, respectively (Figure 8B). There was no statistically significant difference between PLC-treated and control cells in heparin-releasable TFPI activity levels after subsequent treatment with LMWH after 1 hour ($P > .05$) or 24 hours ($P > .05$).

Assessment of the Release of GPI-Anchored TFPI

As shown in Figure 6, treatment of HUVECs with PLC resulted in a significantly greater release of TFPI compared with control (16.87 mU/mL versus 0.68 mU/mL, $P < .0001$).

Assessment of Heparin-Releasable TFPIα After Phospholipase C Treatment of Endothelial Cells

Heparin-releasable TFPI activity levels in HUVEC supernatants after 1 hour of UFH exposure (0, 1, or 10 U/mL) were 2.23, 7.01, and 7.01 mU/mL in control cells and 4.58, 6.03, and 8.68 mU/mL in PLC-treated cells, respectively (Figure 7A). Heparin-releasable TFPI activity levels in HUVEC supernatants after 24 hours' UFH treatment (0, 1, or 10 U/mL) were 75.49, 102.12, and 100.48 mU/mL in control cells and 79.90, 96.07, and 90.66 mU/mL in PLC-treated cells, respectively (Figure 7B). There was no statistically significant difference between PLC-treated and control cells in heparin-releasable TFPI activity levels after subsequent treatment with UFH after 1 hour ($P > .05$) or 24 hours ($P > .05$).

Heparin-releasable TFPI activity levels in HUVEC supernatants after 1 hour of LMWH treatment (0, 1, or 10 U/mL) were 2.95, 3.83, and 3.98 mU/mL in control cells and 4.29, 3.10, and 4.34 mU/mL in PLC-treated cells, respectively (Figure 8A). Heparin-releasable TFPI activity levels in HUVEC supernatants after 24 hours' LMWH treatment (0, 1, or 10 U/mL) were 38.22, 45.88, and 46.17 mU/mL in control cells and 29.68, 37.44, and 43.54 mU/mL in PLC-treated cells, respectively (Figure 8B). There was no statistically significant difference between PLC-treated and control cells in heparin-releasable TFPI activity levels after subsequent treatment with LMWH after 1 hour ($P > .05$) or 24 hours ($P > .05$).

Assessment of GPI-Anchored TFPI After Heparin Treatment of Endothelial Cells

Glycosylphosphatidylinositol–lipid–anchored TFPI activity levels in HUVEC supernatants of cells treated for 1 hour with UFH (0, 1, and 10 U/mL) were 13.09, 14.71, and 15.58 mU/mL, respectively (Figure 9A), and after 24 hours of treatment with
UFH (0, 1 and 10 U/mL) were 11.43, 8.53 and 9.63 mU/mL, respectively (Figure 9, B). Although there was no statistically significant difference in GPI-anchored TFPI levels in HUVECs that were treated with different UFH concentrations for 1 hour (P > .05) or 24 hours (P > .05), there was a trend where GPI-anchored levels were increased by UFH pre-treatment after 1 hour and decreased after 24 hours.

Glycosylphosphatidylinositol-lipid–anchored activity levels in HUVEC supernatants of cells treated for 1 hour with LMWH (0, 1 and 10 U/mL) were 19.49, 20.90, and 24.54 mU/mL, respectively (Figure 10A), and for 24 hours with LMWH (0, 1, and 10 U/mL) were 24.29, 15.84, and 16.94 mU/mL, respectively (Figure 10B). Although there was no statistically significant difference in GPI-anchored TFPI levels in HUVECs that were treated with different LMWH concentrations for 1 hour (P > .05) or 24 hours (P > .05), there was a trend where GPI-anchored levels were increased by

Figure 3. Effect of low-molecular-weight heparin (LMWH) on the release of tissue factor pathway inhibitor (TFPI) from human umbilical vein endothelial cells. Cells were stimulated with LMWH (diluted in Dulbecco Modified Eagle’s Medium only) for (A) 1 hour or (B) 24 hours. Columns represent mean heparin-releasable TFPI activity (mU/mL), and the error bars show 1 standard deviation.

Figure 4. Effect of unfractionated heparin (UFH) on the release of tissue factor pathway inhibitor (TFPI) from human umbilical vein endothelial cells. Cells were stimulated with UFH (diluted in Dulbecco Modified Eagle’s Medium plus 5% fetal calf serum) for (A) 1 hour or (B) 24 hours. Columns represent mean heparin-releasable TFPI activity (mU/mL), and the error bars show 1 standard deviation. **Represents a statistically significant result.
Discussion

Tissue factor pathway inhibitor is an important hemostatic regulator that contributes to the anticoagulant effect of heparins. The full-length form (43 kDa) has the greatest anticoagulant activity, is released from endothelial cells in response to UFH and LMWH, and is thus termed heparin-releasable TFPI. Previous investigations suggest that this is TFPIα. Studies on the effect of heparins on TFPI release from endothelial cells in vitro have demonstrated conflicting results, with a lack of consensus on the level and rate of TFPI release. More recent studies suggest that TFPIβ, an isoform of TFPI, is present in endothelial cells. Although not extensively studied, TFPIβ is not heparin-releasable but, rather, is GPI anchored and probably acts as a direct vessel wall anticoagulant. Therefore, the aims of this study were to (1) validate the conditions required to demonstrate the heparin-dependent release of TFPI from endothelial cells, (2) determine the effect of PLC on heparin-releasable TFPI in endothelial cells, and (3) evaluate the effect of heparin on GPI-anchored TFPI in endothelial cells.

A HUVEC model was used in this study because collection of the cord is noninvasive to the donor, isolation of cells is performed by well-defined methods, and the cells have physical and biochemical characteristics that are comparable with endothelial cells from other sites of the body. Previous studies have used the immortalized endothelial cell lines EA.hy926 and ECV304 cells, with recent evidence suggesting that the latter is not of endothelial origin. Variable sources of endothelial cells used to study the in vitro release of TFPI may contribute to the conflicting data in the literature.

In the present study, heparin-releasable TFPI activity levels were not significantly increased using 1 U/mL or 10 U/mL of UFH or LMWH after 1 hour or
24 hours of incubation with HUVECs when either heparin was diluted in DMEM (Figures 2 and 3). These findings are similar to recent reports in that a significant increase in TFPI levels after 1 hour of incubation with heparin was not demonstrated.\textsuperscript{17,18}

The effect of heparin diluted in DMEM containing 5% FCS on the release of heparin-releasable TFPI from HUVECs was then investigated. A statistically significant increase in heparin-releasable TFPI activity with both UFH (\(P < .001\)) and LMWH (\(P < .05\)) at 1 hour was demonstrated. No statistically significant increase in heparin-releasable TFPI activity was observed at 24 hours. Of interest was that the profile of TFPI release at 1 hour using the different heparins varied. Maximum TFPI release was achieved at a concentration of 1 U/mL when UFH was used (Figure 4A) but 10 U/mL when LMWH was used (Figure 5A). This suggests that at the same concentration, UFH is more effective than LMWH at inducing the in vitro release of TFPI. These findings agree with those of Li et al\textsuperscript{15} and suggest that a therapeutic dose of UFH (approximately 0.2-2 U/mL) induces maximum TFPI release from endothelial cells and depletes storage pools, whereas...
LMWH does not. These results thus offer further insight into the findings of Hansen et al\(^3\) and Bendz et al,\(^3\) who reported that UFH—but not LMWH—depletes heparin-releasable TFPI in vivo.

Our data clearly demonstrate that serum is required for the heparin-mediated release of TFPI from HUVECs. Previous studies that have reported a heparin-mediated release of TFPI from HUVECs used serum,\(^13,15,17\) although the significance of this was not discussed. Additional supporting evidence for our findings that serum is required for the heparin-dependent release of TFPI is provided by Perez-Ruiz et al,\(^18\) who demonstrated minimal TFPI release from HUVECs when using serum-free media. It is interesting to speculate what specific serum component(s) might be required for the heparin-dependent release of TFPI from HUVECs. It has recently been demonstrated that basic fibroblast growth factor, in conjunction with heparin, releases TFPI from smooth muscle cells.\(^38\) Neither of those components by themselves had any effect on TFPI levels, however. It could be that basic

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**Figure 9.** Effect of unfractionated heparin (UFH) on the phospholipase C (PLC)–dependent release of tissue factor pathway inhibitor (TFPI) from human umbilical vein endothelial cells. Cells were exposed to UFH (diluted in Dulbecco Modified Eagle's Medium plus 5% fetal calf serum) for (A) 1 hour or (B) 24 hours and then were treated with PLC. Columns represent mean glycosylphosphatidylinositol-lipid-anchored TFPI activity (mU/mL), and error bars show 1 standard deviation.

**Figure 10.** Effect of low-molecular-weight heparin (LMWH) on the phospholipase C (PLC)–dependent release of tissue factor pathway inhibitor (TFPI) from human umbilical vein endothelial cells. Cells were exposed to LMWH (diluted in Dulbecco Modified Eagle's Medium plus 5% fetal calf serum) for (A) 1 hour or (B) 24 hours and then were treated with PLC. Columns represent mean glycosylphosphatidylinositol-lipid-anchored TFPI activity (mU/mL), and error bars show 1 standard deviation.
fibroblast growth factor or a related protein is an additional requirement for the heparin-mediated release of TFPI from HUVECs.

Treatment of HUVECs with PLC was used to investigate the release of GPI-anchored TFPI. An approximate 20-fold increase in TFPI activity in HUVEC supernatants was observed after treatment with PLC (Figure 6), confirming that a portion of endothelial cell surface-associated TFPI is GPI anchored. The amount of TFPI released by PLC in this study is significantly greater than that by Lupu et al (1997) and is probably accounted for by the higher concentration of PLC used in our study.

Sequential treatment of HUVECs with heparin and PLC was performed to determine if PLC affected heparin-releasable TFPI and if heparin influenced PLC-releasable TFPI. Treatment of HUVECs with PLC, then with heparin, for 1 or 24 hours resulted in no statistically significant difference in the amount of heparin-releasable TFPI compared with the control (Figures 7 and 8), suggesting that PLC has no effect on heparin-releasable TFPI. This contrasts with the 2002 findings of Mast et al, who observed an approximate 4-fold increase in the amount of heparin-releasable TFPI in minced placental samples after pretreatment with PLC. These results may be due to cells other than that of endothelial origin, for example, syncytiotrophoblasts, cytotrophoblasts, and extravillous trophoblasts of the placenta, which have been demonstrated to produce TFPI. Therefore, the differences observed between the studies are likely due to the different experimental models.

Although no statistically significant difference in the levels of GPI-anchored TFPI after treatment of HUVECs with heparin (UFH or LMWH for 1 or 24 hours) then PLC (Figures 9 and 10) was evident, 2 clear trends were observed. After a 1-hour incubation with either heparin, a dose-dependent increase in GPI-anchored TFPI was observed (Figures 9 and 10A). Compared with the control, the increase was 12% and 19% for 1 and 10 U/mL UFH and 7% and 26% for 1 and 10 U/mL LMWH. These results may be due to TFPI binding to a GPI-anchored protein on the cell surface (eg, glypican-3), after the initial release by heparin. After treatment with PLC, TFPI would then be cleaved from the cell surface, accounting for the heparin dose-dependent increase in GPI-anchored TFPI.

After 24 hours’ incubation with either heparin preparation, a decrease in GPI-anchored TFPI was observed (Figures 9 and 10B). At concentrations of 1 and 10 U/mL, the respective decreases compared with the control were 25% and 15% with UFH and 35% and 30% with LMWH. The reason for this decrease is not known, although it may be that binding of TFPI to a putative GPI-anchored protein promotes its internalization and degradation, resulting in the lesser amount of GPI-anchored TFPI. It has also been suggested that a small pool of TFPI exists on the cell surface that is resistant to heparin but is released after PLC treatment. However, the heparin incubation period used was just 20 minutes. A longer period of time may be required for heparin to dissociate TFPI from the GPI-anchored protein to which it binds, which would account for the decrease in GPI-anchored TFPI observed after 24 hours’ heparin treatment.

**Conclusion**

This study demonstrated that serum is a requirement for the heparin-dependent release of TFPI from HUVECs. Furthermore, a trend was observed where a 1-hour pretreatment of HUVECs with heparin dose dependently increased PLC-releasable TFPI, whereas a 24-hour pretreatment resulted in a dose-dependent decrease. Further studies are required to elucidate the exact serum components required for the heparin-dependent release of TFPI and to confirm the observed effect of heparin on GPI-anchored TFPI.

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