REGULAR ARTICLE

Molecular weight dependent tissue factor pathway inhibitor release by heparin and heparin oligosaccharides

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Abstract   Heparin and low molecular weight heparins exert their vascular effects by mobilizing tissue factor pathway inhibitor (TFPI) from the vascular endothelium into the blood circulation. We compared the influence of molecular weight on the TFPI release by heparin and its fractions in a non-human primate model. Primates were treated with unfractionated heparin, a low molecular weight heparin (gammaparin), or a heparin-derived oligosaccharide mixture (C3). Endothelial TFPI release was determined using both immunologic and functional assays. After intravenous administration, all agents significantly increased TFPI levels ($p < 0.05$) in a dose dependent manner. The increase produced by unfractionated heparin and gammaparin was greater than that by C3 at an equal dosage ($p < 0.05$). With subcutaneous injection, all agents produced less TFPI release. Repeated administration of heparin-derived oligosaccharides gradually increased TFPI release. A 1.89 fold increase in TFPI levels was observed 4 days after C3 treatment (2.5 mg/ kg). Our findings indicated that TFPI release is dependent on the molecular weight of heparin and its derivatives. Heparin oligosaccharides exert their

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vascular effects through increased TFPI release after long-term repeated administration.

Introduction

Tissue factor pathway inhibitor (TFPI) formerly known as lipoprotein-associated coagulation inhibitor or extrinsic pathway inhibitor, is a molecule identified as a natural anticoagulant [1,2]. TFPI, mainly localized on the surface of the endothelial cells, is capable of turning off the extrinsic pathway by the direct inhibition of factor Xa and indirect neutralization of tissue factor.

TFPI contains three tandem Kunitz proteinase inhibitor (KPI) domains, which mediate its anticoagulant effects and the inhibition of fibrin clot formation via two mechanisms. Direct inhibition of factor Xa takes place when factor Xa binds to the second KPI domain of TFPI. The second mechanism involves binding of the tissue factor/factor VIIa complex to the first amino terminus KPI domain of the TFPI/factor Xa complex forming a quaternary complex, in which the tissue factor/factor VIIa cannot activate factors Xa and IXa [3]. Thus, as soon as tissue factor initiates the coagulation cascade, its activity is rapidly neutralized by TFPI, preventing further activation of cascade. Since the tissue factor pathway plays a predominant role in coagulation, it has been proposed that TFPI may possess more 'house-keeping' functions than antithrombin and activated protein C.

As an important natural anticoagulant, TFPI is known to contribute to the anticoagulant/antithrombotic action of heparin and low molecular weight heparins (LMWHs) [1]. Administration of heparin displaces TFPI from the endothelium, which raises anticoagulant activity of the blood as a result of heparin-mediated elevation and stimulation of TFPI activity [4]. Heparin exerts its anticoagulant effects through both antithrombin and TFPI. The antithrombin effect is mediated through the inhibition of thrombin and factor Xa, and the TFPI effect is mediated through the inhibition of the tissue factor pathway [5].

The differential effects of heparin and LMWHs on TFPI release have been extensively studied. Sandset demonstrated that TFPI levels increased markedly after intravenous heparin administration, whereas subcutaneous administration gave lower but prolonged increases in TFPI activity [6]. Subcutaneous administration of a LMWH (enoxaparin) resulted in larger increases in circulating TFPI levels than heparin [7]. In addition, plasma free TFPI antigen and heparin releasable TFPI were depleted during repeated or continuous treatment with heparin, but not during subcutaneous treatment with LMWHs [8,9,11]. In a recent study [10] with two different LMWHs, a clear dose–response relationship has been noted for dalteparin and enoxaparin on the release of endogenous TFPI. These results imply that LMWHs may have a different mode of action in releasing TFPI from heparin, in particular, after subcutaneous administration. The differential effect of heparin and LMWH on intravascular pools of TFPI may contribute to the understanding of the apparent superior efficacy of LMWHs in the treatment of thrombosis. However, limited data currently are available on the interactions between heparin oligosaccharides and TFPI release.

In the present study, the effects of heparin and its fractions, gammaparin and C3, on TFPI release from endothelium were investigated in a non-human primate model. Both gammaparin and C3 were depolymerized from heparin by gamma irradiation with mean molecular weights of 5500 and 2400 Da, respectively. These agents have similar physiochemical characteristics such as same end groups. While gammaparin represents LMWHs, C3 is considered as a model compound for heparin-derived oligosaccharides [12]. This study clearly demonstrated the molecular weight dependence of TFPI release by heparin and its fractions after intravenous and subcutaneous administration.

Materials and methods

Heparin, gammaparin and C3

Porcine mucosal heparin (lot RB21055) was obtained from Sanofi, Choay Institute, Paris, France. The average molecular weight of this batch was specified by the manufacturer to be 14,000 Da. Gammaparin (lot G 0101) and C3 (lot 1997c.2.) were obtained from CORCON, and Laboratori Derivati Organici, Milan, Italy. Both agents were manufactured from heparin by means of a controlled de-polymerization induced by γ-irradiation. The weight average molecular weights of these agents were specified by the manufacturer to be within 4700 to 5700 Da and 2300 to 2500 Da, respectively.

Animals

Four adult male or female Rhesus monkeys (*Maccaca mulatta*) in each group (two males and two females) weighing 4.0 to 16.0 kg were used for this study. The animals were fed with an open formula (extruded, non-human primate diet) ad libitum and were group housed in accordance with the Guide for the Care and Use of Laboratory Animals, National Research Council (National Academy Press, Washington, DC, 1996). Blood samples were drawn through a catheter placed in the femoral vein contralateral to the site of drug injection. Each agent was tested on the separate group of four primates.

Comparative investigation of heparin and its derivatives

All agents were dissolved in 0.9% physiological saline, and the drug solution was sterilized through a 0.22 mm filter. Four primates were anesthetized with ketamine (20 mg/kg, im) before drug administration. Each agent (heparin: 0.6 and 1.0 mg/kg; gammaparin: 1.0 and 2.5 mg/kg; C3: 1.0 and 2.5 mg/kg) was administered iv or sc. The dosage selection was initially based on anti-factor Xa activity. The mean potency was 165, 100 and 40 anti-Xa unit/mg for heparin, gammaparin and C3, respectively. As a result, heparin was given at doses of 100 and 165 unit/kg; gammaparin at doses of 100 and 250 unit/kg; and C3 at doses 40 and 100 unit/kg. Comparisons of TFPI releasing acitivities were based upon gravimetric (1 mg/kg) and anti-Xa activity (100 unit/kg) equivalence. Blood samples were obtained before and after iv administration at 5 min, 0.25, 0.5, 1, 2, 4, 6 and 24 h, and 0.25, 0.5, 1, 2, 3, 4, 6, and 24 h after sc administration. Animals were evaluated for signs of bleeding for 24 h and monitored with a complete blood count 6 h after the injection. Plasma was separated immediately by centrifugation of citrated blood (4.5 mL of blood mixed with 0.5 mL of 3.8% sodium citrate) at 3000 rpm for 15 min and frozen at −70 °C for future analysis.

Investigation of C3 after repeated administration

C3 was dissolved in 0.9% physiological saline and the solution was sterilized through a 0.22 mm filter. Four primates were anesthetized with ketamine (20 mg/kg, im) before drug administration. C3 was administered at doses of 1.0 and 2.5 mg/kg subcutaneously for 7 and 4 days (once daily), respectively. Blood samples were obtained before and after sc administration at 0.25, 0.5, 1, 2, 3, 4, and 6 h on day 1. From day 2 to day 7, baseline and 3 h blood samples were collected. Animals were evaluated for signs of bleeding with inspection of injection sites and monitored with multiple complete blood counts 6 h after each injection during the entire course of repeated administration. Plasma was separated immediately by centrifugation of citrated blood at 3000 rpm for 15 min and frozen at −70 °C for future analysis.

TFPI assay

Levels of TFPI activity in primate plasma samples were measured using an amidolytic assay based on that of Sandset et al. [6]. In this assay, 300 to 400 μL of test plasma was heated in a plastic test tube at 56 °C in a water bath for 15 min to inactivate endogenous coagulation enzymes and to precipitate fibrinogen. Following heating, the samples were cooled for 1 min on ice and then centrifuged for 10 min at 3000 rpm. The supernatant plasma was diluted 1 to 20 in TFPI buffer (0.1 M NaCl, 0.05 M Tris−HCl, 0.01 M trisodium dicitrate; pH = 8.0). These dilutions were then kept on ice until assayed. The following reagents were placed in plastic test tubes: 50 μL of 0.025 U/mL human factor VII, 50 μL of Thromboplastin C Plus diluted 1 to 20 in TFPI buffer, 50 μL of 0.025 U/mL human factor X, and 50 μL of 0.075 M $CaCl_2$. Each tube was incubated at 37 °C for 5 min. 50 μL of the plasma dilution was then added to each tube. The tubes were incubated for 40 min at 37 °C. 50 μL of 0.4 U/mL factor X was added to each tube. Following pulse vortexing, the tubes were incubated for 20 min at 37 °C. 50 μL of 3.25 mM Spectrozyme Xa was added to each tube. Following a 15-min incubation period, 200 μL of 50% acetic acid to each tube was added to stop the reaction. 200 μL of each sample was added onto a microtiter plate and the optical density was read at 410 nm. Percent inhibition was measured relative to the baseline sample.

TFPI antigen levels were determined using Imubind® TFPI ELISA kits (American Diagnostica, Greenwich, CT). This was a sandwich, enzyme-linked immunoassay which utilized a murine anti-TFPI monoclonal as the capture antibody and a biotinylated antibody/streptavidin conjugated horseradish peroxidase complex for detection of the captured TFPI. The detection limit of the kit was stated as 200 pg TFPI/mL. Six TFPI standards ranging in concentration from 0 to 10 ng/mL diluted in deionized water were used to construct a calibration curve. A full-length recombinant TFPI that was expressed in *E. coli* was used as the calibration standard. This rTFPI contained a full carboxy tail, but was lacking the native glycosyla-

tion. Optical density readings and standard curve calculations were made using a Dynatech MR7000 system (Dynatech Laboratories, Chantilly, VA).

Statistical analysis

GraphPad Prism version 3.00 for Windows (Graphpad Software, San Diego, CA) was used for statistical analysis. The data were analyzed by using Student t-test and one-way ANOVA. $p < 0.05$ was considered statistically significant.

Results

Intravenous studies

The release of TFPI following intravenous administration of heparin, gammaparin and C3 to primates was determined using both the functional and immunologic assays. In Fig. 1, the functional TFPI levels expressed as the unit activity per milliliter at various time points after heparin administration are shown in panel A. At 5 min post-administration, a functional TFPI level of 1.3 and 2.4 U/mL was observed for 0.6 and 1.0 mg/kg doses, respectively. The levels decreased to 0.8 and 1.8 U/mL by 1 h and
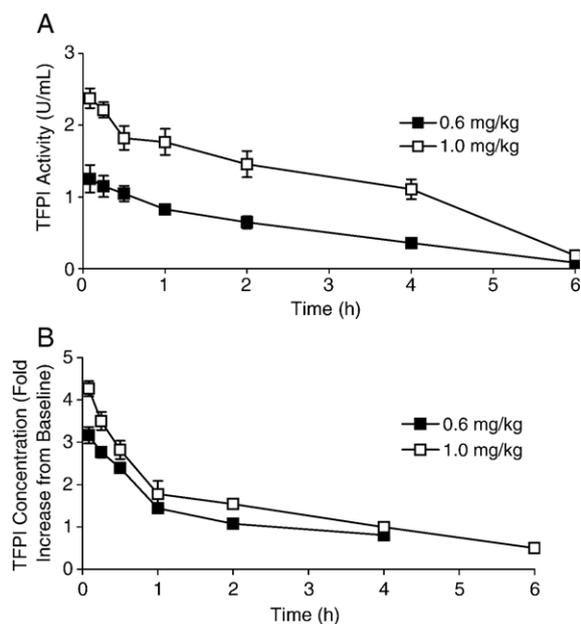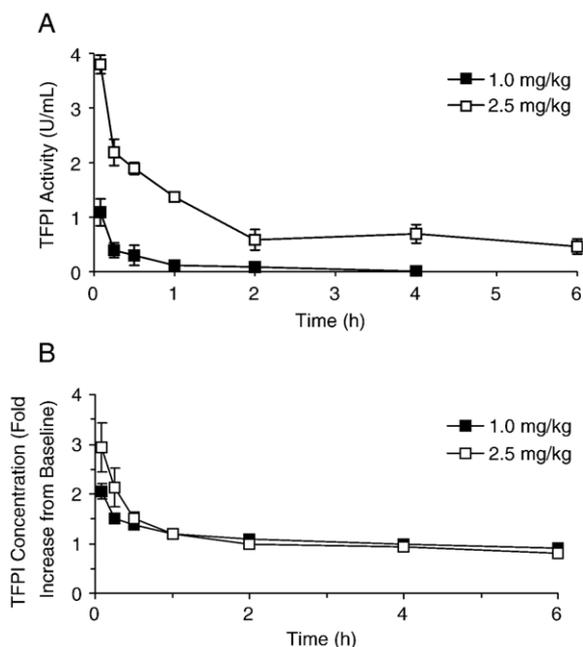

Figure 1   TFPI activity and concentration following intravenous administration of heparin in primates. Primates were administered with intravenous doses of 0.6 and 1.0 mg/kg of heparin via the femoral vein (n = 4). TFPI activity and concentration were determined using functional (panel A) and immunologic (panel B) assays, respectively. The baseline plasma concentrations of TFPI were approximately 80—85 ng/mL.
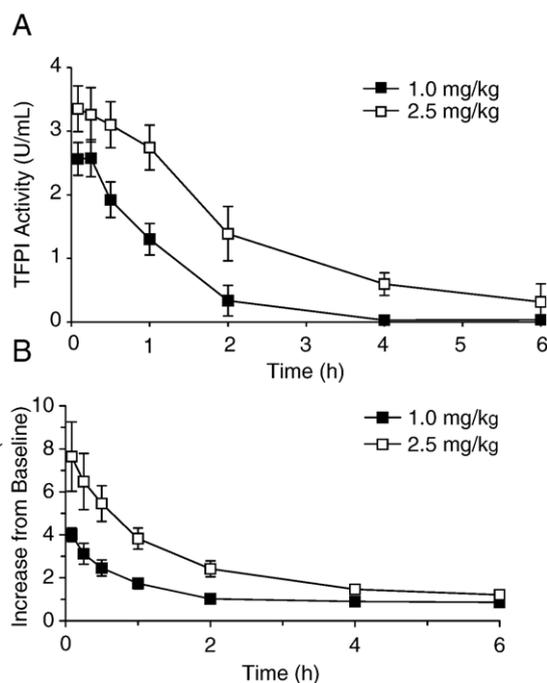
Figure 2   TFPI activity and concentration following intravenous administration of gammaparin in primates. Primates were administered with intravenous doses of 1.0 and 2.5 mg/kg of gammaparin via the femoral vein (n = 4). TFPI activity and concentration were determined using functional (panel A) and immunologic (panel B) assays, respectively. The baseline plasma concentrations of TFPI were approximately 55—60 ng/mL.

then progressively declined with time through 6 h. TFPI levels were significantly elevated till 2 h post-administration for both doses ($p < 0.05$). At the higher dose (1.0 mg/kg), TFPI activity was still elevated by 4 h (1.1 U/mL). In panel B, using the immunologic assay, TFPI levels were 3.2 and 4.3 fold from baseline values at 5 min post-administration for 0.6 and 1.0 mg/kg, respectively. Thereafter, TFPI levels reached baseline level by 4 h post-administration for both doses, which was relatively faster than its activity.

Fig. 2 illustrates the time course of TFPI levels after gammaparin administration. As shown in panel A, at 5 min post-administration, functional TFPI levels expressed as 2.6 and 3.4 U/mL were observed for 1.0 and 2.5 mg/kg, respectively. The TFPI levels decreased to baseline by 4 h for the low dose group, whereas progressively declined with time through 6 h for the high dose group. A significant difference between TFPI levels was noted at 6 h post-administration ($p < 0.05$). In panel B, the immunologic TFPI levels were 4.0 and 7.6 fold of baseline values at 5 min post-administration for 1.0 and 2.5 mg/kg treatment groups, respectively. However, TFPI levels reached baseline level by 2 h post-administration, which was faster than its activity.

Figure 3 TFPI activity and concentration following intravenous administration of C3 in primates. Primates were administered with intravenous doses of 1.0 and 2.5 mg/kg of C3 via the femoral vein (n=4). TFPI activity and concentration were determined using functional (panel A) and immunologic (panel B) assays, respectively. The baseline plasma concentrations of TFPI were approximately 55—60 ng/mL.

Fig. 3 illustrates the time course of the TFPI levels after C3 administration. As shown in panel A, at 5 min post-administration, functional TFPI levels expressed as 1.1 and 3.8 U/mL were observed. The TFPI level decreased to the baseline level by 1 h after 1.0 mg/kg administration, whereas a more progressive decline was observed with 2.5 mg/kg through 6 h. A significant difference between TFPI levels was noted at 6 h post-administration ($p < 0.05$). In panel B, the immunologic TFPI levels were 2.1 and 2.9 fold in relation to the baseline values at 5 min post-administration after 1.0 and

2.5 mg/kg, respectively. TFPI levels reached the baseline level rapidly by 1 h post-administration, which was significantly faster than its plasma activity. The modulatory effects of heparin, gammaparin and C3 on endogenous TFPI release are compared in Tables 1 and 2.

Subcutaneous studies

Figs. 4—6 depict the time response curves of the plasma TFPI levels following heparin, gammaparin and C3 administration, respectively. In Fig. 4, the functional TFPI levels after heparin administration are shown in panel A. At 3 h post-administration, a peak of 1.1 U/mL was observed for 1.0 mg/kg treatment group, whereas no significant elevation of plasma TFPI activity was noted after 0.6 mg/kg administration (below 0.5 U/mL). The TFPI level progressively decreased to 0.26 U/mL by 8 h. In panel B, the immunologic TFPI levels were observed to peak 1.6 fold in relation to the baseline values at 1 h post-administration for 1.0 mg/kg treatment group, whereas no significant elevation of plasma TFPI levels was noted after 0.6 mg/kg injection. Both functional and immunologic TFPI levels after subcutaneous administration were significantly lower than those obtained after intravenous injection ($p < 0.05$).

Fig. 5 illustrates the time course of the TFPI levels after gammaparin administration. TFPI levels were significantly elevated from 1 to 4 h post-administration in a dose-dependent manner ($p < 0.05$). As shown in panel A, maximal functional TFPI levels expressed as 1.0 and 2.1 U/mL was observed at 1 h post-administration for 1.0 and 2.5 mg/kg, respectively. The TFPI levels decreased to approximately 0.5 U/mL by 6 h for both doses. In panel B, a similar pattern of immunologic TFPI levels was observed that they were observed to peak 1.6 and 4.2 fold of baseline values 1 h post-administration for 1.0 and 2.5 mg/kg treatment

Table 1 Comparative functional TFPI levels after heparin, gammaparin and C3 administration

Dose (mg/kg)	Heparin		Gammaparin		C3	
	0.6	1.0	1.0	2.5	1.0	2.5
Intravenous						
Emax (U/mL)	1.3 ± 0.2	2.4 ± 0.1*	2.6 ± 0.3	3.4 ± 0.6	1.1 ± 0.2	3.8 ± 0.2*
AUC (U/mL h)	3.1 ± 0.2	7.3 ± 0.5*	3.0 ± 0.3	7.8 ± 0.6*	2.5 ± 0.2	5.3 ± 0.5*
ke (1/h)	0.426	0.372	0.849	0.423	1.194	0.874
Subcutaneous						
Emax (U/mL)	0.4 ± 0.2	1.1 ± 0.1*	1.0 ± 0.1	2.2 ± 0.1*	0.5 ± 0.2	1.2 ± 0.1*
Tmax (h)	2.1 ± 0.6	2.4 ± 0.3	3.3 ± 1.1	1.8 ± 0.3	2.1 ± 0.6	2.0 ± 0.0
AUC (U/mL h)	1.7 ± 0.1	4.1 ± 0.3*	4.7 ± 0.4	7.8 ± 0.6*	2.0 ± 0.2	4.5 ± 0.4*

Data are calculated based on functional TFPI assay and presented as mean ± S.E.M. from 4 primates. *$p < 0.05$, vs. the lower dose. Emax: maximal functional TFPI levels; AUC: area under functional TFPI level—time curve; Tmax: time to reach maximal functional TFPI levels; ke: mean elimination constant.

Table 2 Comparative TFPI concentrations after heparin, gammaparin and C3 administration

Dose (mg/kg)	Heparin		Gammaparin		C3	
	0.6	1.0	1.0	2.5	1.0	2.5
Intravenous						
Emax (fold increase)	3.2 ± 0.2	4.3 ± 0.2	4.0 ± 0.3	$7.6 \pm 1.6^*$	2.1 ± 0.2	2.9 ± 0.5
AUC (Fold h)	2.0 ± 0.2	$3.6 \pm 0.4^*$	4.1 ± 0.5	$9.4 \pm 0.8^*$	1.1 ± 0.4	$1.9 \pm 0.2^*$
ke (1/h)	0.345	0.345	0.679	0.591	0.900	0.925
Subcutaneous						
Emax (fold increase)	1.3 ± 0.7	1.6 ± 0.1	1.6 ± 0.1	4.2 ± 0.8	1.0 ± 0.1	1.3 ± 0.2
Tmax (h)	0.5 ± 0.2	1.2 ± 0.2	1.3 ± 0.3	1.1 ± 0.3	0.5 ± 0.2	0.7 ± 0.2
AUC (fold h)	0.7 ± 0.1	$2.2 \pm 0.2^*$	3.3 ± 0.3	$10.8 \pm 0.9^*$	0.8 ± 0.1	1.3 ± 0.1

Data are calculated based on an immunologic TFPI assay and presented as mean $\pm$ S.E.M. from 4 primates. $^*p < 0.05$, vs. the lower dose.

Emax: maximal fold increase of TFPI concentrations; AUC: area under fold increase of TFPI concentraton—time curve; Tmax: time to reach maximal fold increase of TFPI concentrations, ke: elimination constant.

groups. A significant difference between these two doses was noted ($p < 0.05$). TFPI levels reached 1.2 and 1.5 fold by 6 h, respectively.

Fig. 6 illustrates the time course of the TFPI levels after C3 administration. TFPI levels were significantly elevated from 0.25 to 4 h post-administration in a dose-dependent manner ($p < 0.05$). As shown in panel A, at 2 h post-administration, a maximal functional TFPI level expressed as 1.2 U/mL was observed for 2.5 mg/kg treatment group, whereas a 0.5 U/mL peak was seen at 3 h for

1.0 mg/kg. The TFPI levels decreased to 0.2 and 0.5 U/mL by 6 h, respectively. A wide variation in the inhibition of functional TFPI activity was observed in the low dose treatment group. In panel B, no elevation of the immunologic TFPI levels was observed except a 1.3 fold peak was seen at 1 h after the administration of 2.5 mg/kg ($p < 0.05$).

Fig. 7 illustrates the immunologic TFPI levels after repeated C3 administration. The TFPI levels were significantly elevated from baseline after
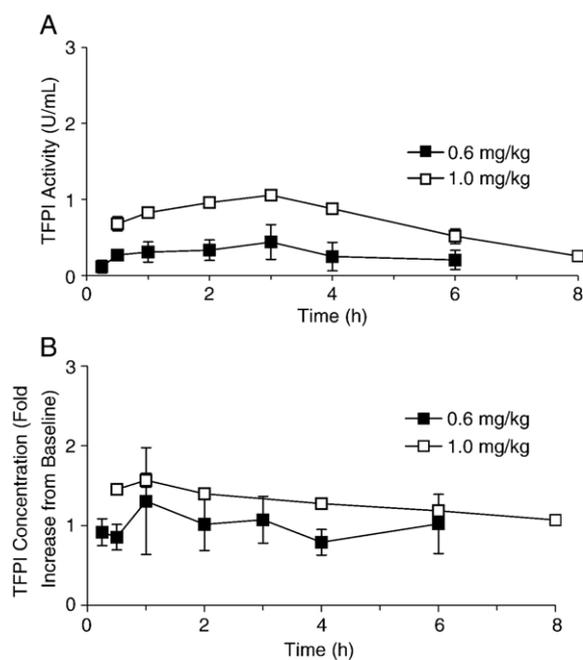
Figure 4 TFPI activity and concentration following subcutaneous administration of heparin in primates. Primates were administered with subcutaneous doses of 0.6 and 1.0 mg/kg of heparin in the abdominal area (n=4). TFPI activity and concentration were determined using functional (panel A) and immunologic (panel B) assays, respectively. The baseline plasma concentrations of TFPI were approximately 60—70 ng/mL.
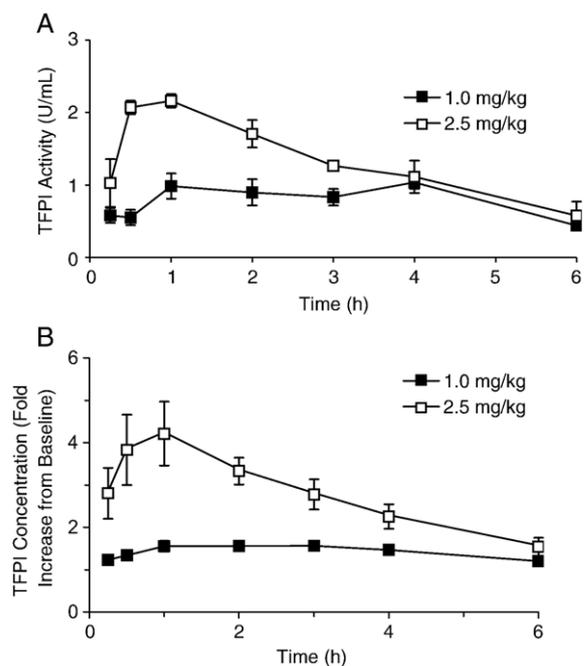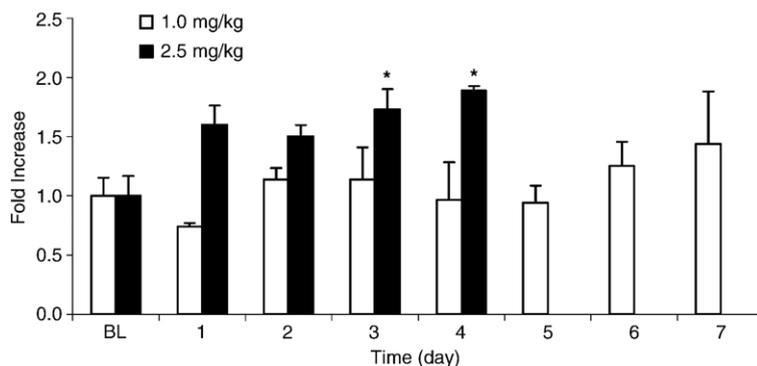

Figure 5 TFPI activity and concentration following subcutaneous administration of gammaparin in primates. Primates were administered with subcutaneous doses of 1.0 and 2.5 mg/kg of gammaparin in the abdominal area (n=4). TFPI activity and concentration were determined using functional (panel A) and immunologic (panel B) assays, respectively. The baseline plasma concentrations of TFPI were approximately 55—60 ng/mL.
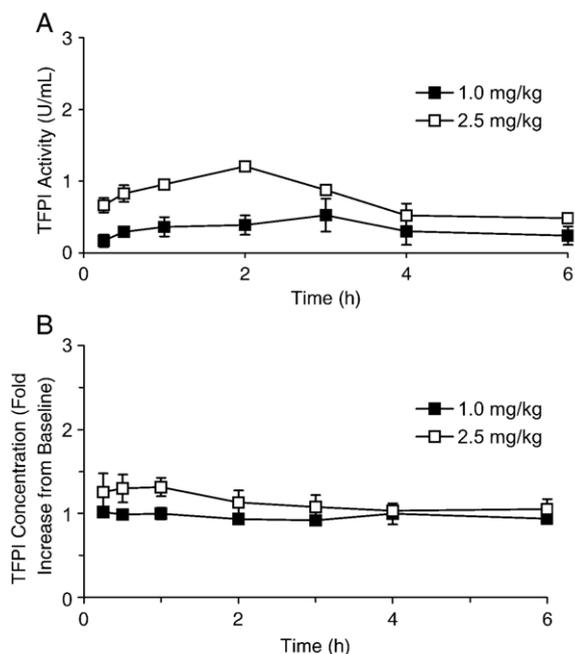
Figure 6 TFPI activity and concentration following subcutaneous administration of C3 in primates. Primates were administered with subcutaneous doses of 1.0 and 2.5 mg/kg of C3 in the abdominal area (n=4). TFPI activity and concentration were determined using functional (panel A) and immunologic (panel B) assays, respectively. The baseline plasma concentrations of TFPI were approximately 55—60 ng/mL.

each dose of C3 in 2.5 mg/kg treatment group (1.89 fold increase on day 4, $p<0.05$). At 3 h post-administration on day 1, immunologic TFPI levels expressed as 1.60-fold increase from baseline were observed. These levels gradually increased to 1.89-fold during 4-day repeated administration of C3 ($p<0.05$). No significant elevation of TFPI levels was observed during 7-day repeated 1.0 mg/kg C3 administration until the last day that 1.44-fold increase from baseline was seen.

Discussion

The anticoagulant effect of heparin and LMWHs is mediated largely through its direct interaction with antithrombin and heparin cofactor II [13]. Amounting evidence indicates that the release of TFPI from the endothelium contributes to the overall anticoagulant effect of both heparin and LMWHs. The main purpose of the present studies was to investigate the influence of molecular weight of heparin on the TFPI release in a primate model. This study for the first time demonstrated that heparin oligosaccharides with ultra low molecular weight are capable of gradually releasing TFPI from endothelium after repeated administration.

TFPI is a Kunitz-type serine protease inhibitor, which may play an important role in mediating the actions of heparins [15]. Both heparin and LMWHs are known to release TFPI from vascular endothelium by displacing the TFPI from endothelial cell surface glycosaminoglycans with subsequent release into the circulation [6,14,16]. In addition, it has been reported that heparin and LMWHs are capable of binding to TFPI to form heparin—TFPI complexes and promoting a synergistic anticoagulant action [16]. All these studies indicate that heparin-releasable TFPI contributes significantly to the anticoagulant effects [17].

In the present studies, a primate model was used to compare the effects of heparin, gammaparin and C3 on TFPI release because of the molecular homology of TFPI in these animals to the humans. Both functional and immunologic TFPI levels were measured using chromogenic and ELISA assays. The functional assay measures non-truncated form of TFPI present in plasma through evaluating its potent antiprotease activity. Carboxy truncation of the TFPI molecule results in a significantly reduced antiprotease activity [18,19]. The ELISA

Figure 7 TFPI concentration following repeated subcutaneous administration of subeparin (C3) in primates. Primates were administered once daily with subcutaneous doses of 1.0 and 2.5 mg/kg of C3 in the abdominal area for 7 and 4 days, respectively (n=4). TFPI concentration was determined using immunologic assays. The baseline plasma concentrations of TFPI were approximately 55—60 ng/mL. * $p<0.05$ compared to baseline (BL).

assay measures the total TFPI antigen in the plasma sample, in which TFPI is detected by a monoclonal antibody directed against the first Kunitz domain [20].

Intravenous administration of heparin and gammaparin to primates was observed to rapidly increase the levels of functional TFPI activity. The TFPI antigen levels measured in the plasma correlated highly with the functional TFPI activity ($r>0.9$) suggesting that full-length, functional TFPI is released upon heparin and gammaparin administration. No significant differences between these two agents were noted based on equal gravimetric dosing regiments. Administration of C3 at doses up to 2.5 mg/kg markedly elevated the functional TFPI activity and plasma TFPI antigen levels, even though they were significantly lower than that of heparin or gammaparin. These results demonstrate that heparin oligosaccharides are capable of releasing TFPI from the vascular endothelium independent of anti-Xa activity as 0.6 mg of heparin, 1.0 mg of gammaparin and 2.5 mg of C3 have an equivalent anti-Xa activity of 100 units. It has been recently postulated that heparin-mediated TFPI release is induced by displacement of TFPI from the endothelial surface and/or by a release from intravascular stores [21]. However, it is still unknown what specific chemical features of heparin contribute to this effect. Valentin et al. [16] proposed that heparin displacing TFPI from endothelium might depend on not only the total sulfate content, but also the localization of the charged groups. Additionally, the size of the heparin molecules plays an important role in their ability to release TFPI [22]. Based on results from the present studies, it appears that high molecular weight components may contribute more to the TFPI release than the low molecular weight ones as C3 has very low molecular weight and lower capacity of TFPI release. This hypothesis is supported by the observations from the subcutaneous studies, in which despite its low bioavailability, heparin showed significantly more potent effects on TFPI release than C3 that has a high bioavailability [23]. The structural biology study has indicated that heparin increases the local concentration of TFPI on the cell surface and stabilizes the initial complex that forms through heparin-binding of the third Kunitz domain of TFPI [27]. Results obtained from in vitro studies demonstrate heparin fractions with lower molecular weights have less interaction with TFPI [28]. These findings are consistent to our result that higher molecular weight is associated with smaller TFPI elimination constant (ke, Fig. 8) after intravenous administration, suggesting high molecular weight compo-

nents bind to TFPI forming a complex with low clearance.

Recent studies have revealed that TFPI antigen and heparin-releasable TFPI are depleted during repeated and continuous intravenous infusion of heparin, but not during treatment with LMWHs [9,24]. Additionally, treatment with heparin by the subcutaneous route was also associated with depletion of TFPI but not with LMWHs [11]. The effects of C3 on plasma TFPI levels were investigated after repeated subcutaneous administration. In the present study, C3 was administered daily for a period of 7 and 4 days at doses of 1.0 and 2.5 mg/kg, respectively. It was observed that C3 increased plasma TFPI antigen levels within 15 min after subcutaneous administration and peak levels were achieved 30 to 40 min post-administration. The TFPI antigen levels were observed to correlate with anticoagulant activity measured by the APTT and the Heptest®. Repeated administration of C3 did not deplete endogenous TFPI stores following 7 or 4 days of treatment. Rather, a trend toward increased TFPI release was noted. It has been suggested that the deletion of TFPI by heparin is due to its inability to maintain the pools of both circulating TFPI and heparin-releasable TFPI [24]. This hypothesis is supported by the recent study showing that the maximum TFPI release was achieved at low heparin doses [25]. In the case of C3, a gradually increased TFPI release might be due to its containing fewer high molecular weight components, which contribute to the depletion of TFPI, and relatively long half-life.

The significance of the heparin releasable TFPI to the pharmacological effects of heparin remains largely unknown, despite being investigated in a number of studies. Recent evidence has indicated that enhancement of TFPI activity may contribute
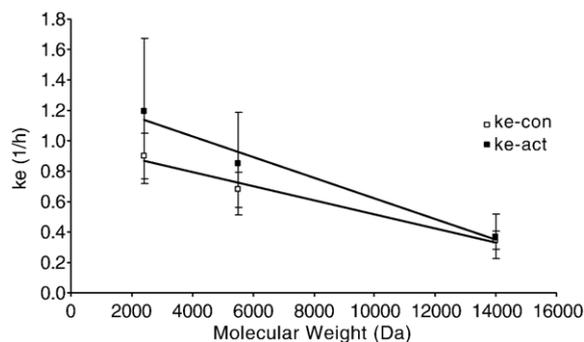
Figure 8 Elimination constant values of TFPI in relation to molecular weight of heparins (1.0 mg/kg). Mean molecular weights for heparin, gammaparin and C3 are 14,000, 5500 and 2400 dalton. ke-con: elimination constant of TFPI concentration; ke-act: elimination constant of TFPI activity.

to the antithrombotic effect of both LMWHs and heparin [6]. Additionally, TFPI may be involved in the non-anticoagulant actions of heparins, such as its anti-inflammatory effect and modulation of arteriosclerosis [26]. However, it is still unknown as to how much TFPI contributes to these heparin effects, as TFPI *per se* possesses both anticoagulant and anti-inflammatory effects. To date, no specific TFPI antagonist has been available. Further investigations on correlation between heparin oligosaccharides, TFPI release, anticoagulation and anti-inflammation are warranted.

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