Effect of Unfractionated Heparin on TFPI Elimination

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ABSTRACT

Background: Tissue factor pathway inhibitor (TFPI) plays an important role for the anticoagulant effect of heparin. Depletion of intravascular TFPI by treatment with unfractionated heparin (UFH), and not by low molecular weight heparin (LMWH), has been suggested to explain the superiority of LMWH in treatment of both arterial and venous thrombosis. The present study was undertaken to investigate the impact of UFH on clearance kinetics, and organs and cells responsible for the clearance of recombinant human full length TFPI purified from baby hamster kidney cells ($TFPI^{BHK}$) and from *E.Coli* ($TFPI^{E.Coli}$).

Methods: Male Sprague-Dawley rats were used as research animals. $TFPI^{BHK}$ and $TFPI^{E.Coli}$ were labelled with ^{125}I, and used to study clearance *in vivo*.

Results: Surface Plasmon Resonance (SPR) analysis revealed that both types of TFPI bound to UFH *in vitro*, but $TFPI^{E.Coli}$ exhibited a faster association rate and a much slow dissociation rate. Intravenous administration of 100 IU/kg UFH immediately prior to TFPI decreased the circulatory survival ($t_{1/2}\alpha$) of $TFPI^{BHK}$ from 1.99 ± 0.10 min to 1.17 ± 0.13 min ($p<0.001$) without affecting the fast clearance of $TFPI^{E.Coli}$. Presence of UFH significantly increased the circulatory survival during the slow $t_{1/2}\beta$ phase of $TFPI^{E.Coli}$ from 27.44 ± 1.91 min to 36.88 ± 1.87 min ($p<0.05$) without affecting the $t_{1/2}\beta$ of $TFPI^{BHK}$. Hepatocellular distribution of radiolabeled ligands showed that both forms of TFPI were mainly taken up by PCs in the absence of UFH (≥ 90%). UFH administration switched the hepatocellular distribution of $TFPI^{E.Coli}$ from PCs towards LSECs, without affecting the distribution of $TFPI^{BHK}$.

Conclusions: Our findings revealed a specific increase in the elimination of $TFPI^{BHK}$ during UFH treatment. This observation may represent the underlying mechanism for depletion of endogenous TFPI in humans during UFH treatment.

INTRODUCTION

Tissue factor pathway inhibitor (TFPI) is an endogenous serine protease inhibitor of tissue factor (TF)-induced blood coagulation [1]. It exerts its function by neutralizing the catalytic activity of factor Xa (FXa) by forming a TFPI-FXa complex, and by feedback inhibition of the factor VIIa-TF complex in the presence of FXa [2, 3]. TFPI contains three Kunitz-type domains in which the first and second domains are responsible for binding of FVIIa and FXa respectively [3]. The third and C-terminally located domain contains the heparin binding sites [4, 5], is involved in the association with lipoproteins [6], is also mandatory for the anticoagulant function of TFPI in TF-induced coagulation *in vitro* [7], and is required for binding to the cell surface [8].

The vascular endothelium is the primary site of TFPI synthesis [9] and 50-80% of intravascular TFPI is located in association with the endothelial cells, residing both in intracellular store, bound to glycosaminoglycans (GAGs) [10, 11] and glycosylphosphatidylinositol (GPI) anchored binding sites at the endothelial surface [12, 13].

TFPI plays an important role for the anticoagulant effect of heparin by a prompt mobilization of TFPI from the endothelium into the circulation [14-16], and by enhancing the inhibition of FXa due to increase molecular interaction promoted by simultaneous binding of FXa and TFPI to the same heparin molecule [17, 18]. Despite up-regulation of the synthesis and release of TFPI by heparins in endothelial cells *in vitro* [19, 20], prolonged treatment of humans with unfractionated heparin (UFH) causes partial depletion of intravascular TFPI [8, 21]. Urinary loss of TFPI has been suggested to explain the selective depletion of intravascular TFPI during continuous UFH treatment. However, recently we showed that only

trace amounts of endogenous TFPI is detected in the urine under basal conditions, and that even less TFPI was excreted in the urine during heparin treatment [22].

Recombinant human TFPI purified from *E.Coli* ($TFPI^{E.Coli}$) is rapidly cleared from the circulation with a plasma half-life of less than 1 minute in rats [23]. The clearance is prompted by an initial association with heparan sulfate proteoglycans (HSPGs) at the cell surface with subsequent LDL-receptor related protein-1 (LRP-1) mediated endocytosis in hepatoma cell lines [24-26]. Administration of UFH to mice 10 min following the administration of $TFPI^{E.Coli}$ resulted in a rapid rise in the plasma TFPI, to a level which was maintained for over 30 min [24]. The effect was attributed to release of TFPI from HSPGs on the vascular endothelial cells. Blockade of HSPGs with protamine resulted in prolonged plasma clearance of $TFPI^{E.Coli}$ in mice [24], inhibited binding to rat hepatoma MH_1C_1 cells [24], and increased degradation of $TFPI^{E.Coli}$ by LRP-positive cells (MH_1C_1 cells and mouse embryonic fibroblasts heterozygous PEA10 cells) [24, 27]. In contrast, recombinant human TFPI purified from mammalian cells characterized by post-translational glycosylation, i.e. expressed in mouse C127 fibroblasts ($TFPI^{C127}$), does not bind to HSPGs at the cell surface and is degraded independently of LRP-1-mediated endocytsis [28].

However, the mechanism beyond a selective depletion of intravascular TFPI by UFH remains a puzzle. In this study we aimed to investigate the mechanism of TFPI depletion during UFH treatment by investigating the effect of UFH *in vivo* and *in vitro* on the clearance of a recombinant full length glycosylated TFPI purified from baby hamster kidney cells ($TFPI^{BHK}$) as compared to recombinant full length non-glycosylated TFPI purified from *E.Coli* ($TFPI^{E.Coli}$).

MATERIALS AND METHODS

Materials

Human recombinant full length TFPI, 42 kDa, isolated from baby hamster kidney cell line ($TFPI^{BHK}$) [29] was from Novo Nordisk (Måløv, Denmark). Human recombinant full length TFPI, 35 kDa, isolated from *E.Coli* ($TFPI^{E.Coli}$) was obtained from American Diagnostica Inc (Greenwich, CT, USA). Unfractionated heparin (UFH) (5000 IU/ml) was from Nycomed Pharma AS (Oslo, Norway). Carrier free $Na^{125}I$ was from Perkin-Elmer Norge AS (Oslo, Norway), and 1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril (Iodogen) was from Pierce Chemical Co. (Rockford, IL, USA). Collagenase P type II was from Worthington Biochemical Corporation (Lakewood, NJ, USA). Human serum albumin (HSA) was from Octapharma (Ziegelbrucke, Switzerland). Culture medium RPMI 1640, supplemented with 20 mM sodium bicarbonate, 0.006% (w/v) penicillin and 0.01% (w/v) streptomycin, was from Gibco BRL (Roskilde, Denmark). Phosphotungstic acid (PTA) was from Merck (Darmstadt, Germany). Bovine serum albumin (BSA), fraction 5 was from ICN Bichemicals Inc., CA, USA. Human fibronectin was a kind gift from Dr. Peter McCourt, University of Tromsø, Norway. Collagen was from Cohesion, Pablo Alto, CA, USA. Sephadex G-25 (PD-10 columns) and Percoll were from Amersham Biotech (Uppsala, Sweden). Formaldehyde treated serum albumin (FSA) was prepared as described [30].

Binding of $TFPI^{E.Coli}$ and $TFPI^{BHK}$ to UFH

Binding of $TFPI^{E.Coli}$ and $TFPI^{BHK}$ to immobilized haparin were analyzed by Surface Plasmon Resonance (SPR) in a Biacore 3000 Biosensor instrument (GE Healthcare). Biotinylated heparin (Merck KGaA, Darmstadt, Germany) was reconstituted in 20 mM HEPES, 150 mM NaCl, 5 mM $CaCl_2$ and 0.05% Tween-20, pH 7.4 to a concentration of 100 μg/ml, which was immobilized to a streptavidin chip (Biacore, GE healthcare) at a flow rate of 10 μl/min for 5

min. Kinetic analysis was performed in running buffer HBS-P (10 mM HEPES, 150 mM NaCl, 0.005% Tween-20) supplemented with $CaCl_2$ (5 mM) at a flow rate of 30 µl/min. The concentrations of TFPIs used for the analysis were 100, 50, 25, 12.5 and 6.25 nM. The dissociation phase lasted 10 min and regeneration was performed with a 3-min pulse of 50 mM EDTA, 1 M NaCl in HBS-P buffer. SPR data were fitted to 1:1 Langmuir binding model (supplied by the software) using T100 and BIA 4.1 evaluation software, respectively. The enzyme PNGAse (N-Glycosidase) cleaving between the innermost GlcNAc and asparagine residues from N-linked glycoproteins was supplied by New England Biolabs (Hertz, UK) and buffers, reagents and procedures supplied with the kit was employed for TFPI deglycosylation.

Experimental Animals

Male albino rats, Sprague-Dawley (mean body weight 250 g), purchased from Scanbur BK, AB (Sollentuna, Sweden) were kept under controlled animal room conditions at 21°C, relative humidity 55±10% and 12:12 light-darkness cycle (8.00-20.00 light), and fed a standard chow (Scanbur BK, Nittedal, Norway) *ad libitum*. For *in vivo* experiments, anesthesia was induced with 4% Isofluran (Abbott Scandinavia AB, Solna, Sweden) and maintained at 2.1%. For *in vitro* studies, rats were anesthetized by subcutaneous injection of a mixture of 0.4 mg/kg Domitor® (Orion Pharma, Espoo, Finland) and 60 mg/kg Ketalar (Pfizer AS, Lysaker, Norway). All experimental protocols were approved by the Norwegian Animal Research Authority in accordance with the Norwegian Animal Experimental and Scientific Purposes Act of 1986.

Preparation of ^{125}I-labelled TFPI

$TFPI^{E.Coli}$ and $TFPI^{BHK}$ in PBS were labelled with carrier-free $Na^{125}I$ in a direct reaction employing Iodogen as oxidizing agent [31]. The ligands and activated ^{125}I were allowed to

react for 30 min and the reaction was stopped by the addition of $Na_2S_2O_5$ and excess amount of KI. Radiolabelled ligands and free iodine were separated by gel filtration on a PD-10 column equilibrated with 1% HSA in PBS. Fractions of 0.5 ml were collected with PBS as eluting buffer. Radioactivity was measured using a gamma-counter (Cobra II, Packard, New York, NY, USA). The resulting specific radioactivities were 3.2 - 4.4 x 10^7 cpm/μg.

Distribution studies

Circulatory survival and organ distribution of intravenously administered labeled TFPI were determined in rats as described [32]. Under Isofluran anaesthesia, ^{125}I-TFPI$^{E.Coli}$ and ^{125}I-TFPI^{BHK} (0.1 nM) were injected through the tail vein alone or just after the intravenous injection of 100IU/kg UFH. Immediately thereafter, blood samples of 25 μl were collected from the tip of the tail into calibrated capillary tubes containing 0.5 ml water and then mixed with 0.75 ml of 4°C 20% TCA and 0.5% PTA. Blood collection was done every 10 seconds during the first 5 min, followed by one sample per minute during the interval 5-20 min and one sample every 5th minute up to 1h. Radioactivity in the supernatant after centrifugation (acid-soluble radioactivity) was taken as degraded TFPI. Radioactivity in blood 1 min after injection was taken as 100%. At 10 and 20 min after injection of and ^{125}I-TFPI^{BHK} with or without pre-injection of 100IU/kg UFH, the organs were washed free of blood by systemic perfusion through the heart with physiological saline, removed and analyzed for radioactivity.

Hepatocellular Distribution

Fifteen min after intravenous administration of ^{125}I-TFPI$^{E.Coli}$ and ^{125}I-TFPI^{BHK} (0.1 nM) alone or just after the intravenous injection of 100 IU/kg UFH, a cannula was inserted into the portal vein. Collagenase perfusion and purification of liver cells were carried out as described elsewhere [33]. The distribution of radiolabelled ligands in different liver cell populations was assessed by quantifying the amount of radioactivity per million cells of parenchymal cells (PCs) and

non-parenchymal cells (NPCs). The NPC fraction consisted mainly of LSECs and KCs and was essentially devoid of PCs, red blood cells, stellate cells and debris. Further purification of LSECs from KCs requires an at least 30 min incubation at 37°C of NPCs seeded on culture dishes coated with glutaraldehyde treated BSA. This step was omitted in order to avoid TFPI degradation with a subsequent escape of the radioactivity from the cells. Cell numbers were assessed by visual counting in a phase contrast microscope. The uptake per cell was calculated based on the fact that the ratio between KCs, LSECs and PCs in rat liver is 1:2.5:7.7 [34]. The method for determining the hepatocellular distribution of different ligands has previously been used by us and others [32, 35, 36].

Statistics

All data are presented as the means ± SEM unless otherwise indicated. Statistical analyses were assessed by GraphPad Prism 4 (GraphPad Software Inc, San Diego, CA, USA). Two-sided p values less than 0.05 were considered statistically significant. Clearance kinetics were analyzed as described previously [37].

RESULTS

Surface Plasmon Resonance (SPR) assay of interactions between TFPI and heparin

Binding of TFPI to heparin was investigated by SPR analysis (Figure 1). Both types of TFPI bound to UFH *in vitro*, but with different binding profiles. Although similar concentrations of $TFPI^{E.Coli}$ and $TFPI^{BHK}$ were added to the heparin chip, the sensorgrams gave rise to higher response levels for $TFPI^{E.Coli}$ as compared to $TFPI^{BHK}$. The binding appeared to be dose-dependent for both types of TFPI. $TFPI^{E.Coli}$ displayed an almost 1:1 profile of binding interaction with UFH, while $TFPI^{BHK}$ did not; indicating that only one heparin site on $TFPI^{E.Coli}$ is available for heparin binding. This could indicate that only one heparin site is available per $TFPI^{E.Coli}$ molecule. The $TFPI^{E.Coli}$-UFH profile exhibited a slow dissociation rate (Figure 1A), while the $TFPI^{BHK}$-UFH interaction resulted in a fast dissociation rate (Figure 1B). PNGase treatment of $TFPI^{BHK}$ drastically changed the dissociation rate to a very slow dissociation rate similar to $TFPI^{E.Coli}$ (data not shown).

Effect of heparin on blood clearance of TFPI

Intravenous administration of 100 IU/kg UFH prior to TFPI decreased the circulatory survival of $TFPI^{BHK}$ during the α-phase ($t_{1/2}\alpha$) from 1.99 ± 0.10 min to 1.17 ± 0.13 min ($p<0.001$) without affecting the clearance of $TFPI^{E.Coli}$ (Table 1). The presence of UFH significantly increased the circulatory survival during the slow $t_{1/2}\beta$ phase of $TFPI^{E.Coli}$ from 27.44 ± 1.91 min to 36.88 ± 1.87 min ($p<0.05$) without affecting the $t_{1/2}\beta$ of $TFPI^{BHK}$.

Effect of heparin on anatomical distribution of $TFPI^{BHK}$

Anatomical distribution of ^{125}I-$TFPI^{BHK}$ was assessed without and with simultaneous administration of UFH 10 min and 20 min after injection. The radioactivity in the primary organs of uptake (liver and kidneys) was not significantly affected by the presence of UFH (Figure 2).

Effect of heparin on hepatocellular distribution of TFPI

At 15 min after intravenous injection, the hepatocellular distribution of both types of TFPI was assessed with and without pre-administration of UFH. The uptake of ^{125}I-TFPI$^{E.Coli}$ in parenchymal cells (PCs) was reduced from 81% in absence of UFH to 46% in the presence of UFH (p<0.01), and the distribution within the non-parenchymal cells (NPCs) was increased from 19 to 54% (p<0.01), respectively (Figure 3A). In contrast, pre-administration of UFH did not change the distribution for TFPI^{BHK}. PCs were the main site for uptake of ^{125}I-TFPI^{BHK} administered without or with UFH (Figure 3B).

DISCUSSIONS

This study was carried out with the aim to investigate the mechanism of TFPI depletion during UFH treatment by studying the effect of UFH *in vivo* and *in vitro* on the clearance of human recombinant full length $TFPI^{BHK}$ as compared to $TFPI^{E.Coli}$. We found that $TFPI^{BHK}$ binds weaker to heparin compared to $TFPI^{E.Coli}$, and that intravenous administration of UFH immediately prior to TFPI significantly decreased the circulatory survival of $TFPI^{BHK}$ during the alpha-phase of elimination, while the circulatory survival of $TFPI^{E.Coli}$ during the beta-phase of elimination was significantly increased. Administration of UFH did not affect the organ distribution of $TFPI^{BHK}$. Hepatocellular distribution of $TFPI^{BHK}$ was not affected by the presence of UFH, while the uptake of $TFPI^{E.Coli}$ was switched from PCs towards NPCs.

SPR analysis of the interaction between TFPI and heparin showed that $TFPI^{E.Coli}$-UFH interactions are much stronger than the $TFPI^{BHK}$-UFH interactions. Human recombinant full-length TFPI expressed in mammalian cells and bacteria differ in molecular weight (42 kDa and 35 kDa, respectively) most probably due to N-linked glycosylation at three potential sites; Asn 117, Asn 167, Asn 228 in the mammalian variant of TFPI [38-41]. Removal of the N-glycosylation sites on the $TFPI^{BHK}$ molecule by PNGase treatment significantly decreased the dissociation of $TFPI^{BHK}$ from $TFPI^{BHK}$-UFH complex, suggesting that the glycosylation sites may be the reason for the different binding profiles observed for the two types of TFPI. Another obvious difference between the two sensorgrams is the level of response (RU); the same TFPI concentrations employed gave rise to a higher response level for $TFPI^{E.Coli}$ as compared to $TFPI^{BHK}$. The differences may be explained by the possible conformational changes that occur during glycosylation of $TFPI^{BHK}$. Carbohydrate branches added during post-translational modifications may affect protein folding or may partially mask the binding

sites for heparin located on the third Kunitz-type domain and at the C-terminal region of the TFPI molecule [4, 5].

Plasma carrier-free TFPI and heparin releasable TFPI were shown to be depleted during repeated intravenous injections and during continuous intravenous infusion of UFH [8, 21]. A previous study showed that the recovery of $TFPI^{BHK}$ in the absence of LMWH in rabbits was very low and the authors suggested a very rapid distribution phase (18). We sought to look further into the alpha-phase of elimination and found that the half-life ($t_{1/2}\alpha$) of $TFPI^{BHK}$ in rats was 2 min and decreased by 41% by pre-administration of UFH. We observed no effect of UFH on the circulatory survival of $TFPI^{BHK}$ during the beta-phase of elimination, whilst a slight effect of LMWH was previously demonstrated by Bregengaard et al. [42]. However, both studies suggest that the beta-phase of clearance may be independent of heparin binding. The non-mammalian type of TFPI, $TFPI^{E.Coli}$, was cleared significantly slower during the beta-phase in the presence of UFH as compared to its clearance in the absence of UFH. Together with strong $TFPI^{E.Coli}$-UFH interactions assessed by SPR analysis, the slower clearance of $TFPI^{E.Coli}$ during the beta-phase suggest that UFH inhibited the ability of $TFPI^{E.Coli}$ to interact with the heparan sulfate proteoglycans (HSPGs) at the surface of vascular endothelial cells [10, 23].

Anatomical distribution study showed that while $TFPI^{E.Coli}$ is mainly found in the liver of rats [23], $TFPI^{C127}$ and $TFPI^{SK}$ are found both in liver and in kidneys of rabbits [43]. Similarly, we found that the liver and the kidneys are the main site of uptake of intravenously injected ^{125}I-$TFPI^{BHK}$ in rats. It was suggested that the reticuloendothelial system in the liver may promote an enhanced clearance of the TFPI-heparin complexes [8]. Our finding showing that the hepatocellular distribution of $TFPI^{E.Coli}$ within the liver cells was switched from the parenchymal cells (PCs) toward the non-parenchymal cells (NPCs) supports this suggestion.

The NPC fraction obtained upon liver cell separation consists mainly of liver sinusoidal endothelial cells (LSECs) and Kupffer cells (KCs), both members of the reticuloendothelial system, with KCs eliminating particles (> 200 nm) from the circulation via phagocytosis, and LSECs removing colloids and soluble macromolecules (< 200 nm) via non-phagocytic receptor-mediated endocytosis [44]. Experimental studies *in vivo* and *in vitro* provide strong evidence that LSECs are the principal site for binding and uptake of UFH via a yet unknown scavenger-like receptor [45]. Furthermore, binding of $TFPI^{E.Coli}$ to rat hepatoma MH_1C_1 cells was previously shown to be significantly inhibited in the presence of UFH [23]. Based on this knowledge, we assume that in our study the uptake of $TFPI^{E.Coli}$ following administration of UFH was switched from PCs towards LSECs, rather than KCs. This result suggests that in the presence of heparin, the scavenger receptor on LSECs may have higher affinity for binding of $TFPI^{E.Coli}$-UFH complexes than the HSPGs and/or LRP-1 receptor on PCs and/or LSECs for binding of $TFPI^{E.Coli}$ alone.

Interestingly, the anatomical distribution and the hepatocellular distribution of $TFPI^{BHK}$ were not significantly affected by the presence of UFH. Moreover, in accordance with previous findings in humans [22], only traces of $TFPI^{BHK}$ were detected in the urine of rats with or without pre-administration of UFH. Furthermore, only slightly elevated levels of $TFPI^{BHK}$ were detected in the blood following UFH administration. These findings in addition to the rapid clearance of TFPI in the presence of UFH clearly demonstrate that the mechanism for depletion of TFPI is not the urinary loss. Several hypotheses may explain this mechanism: i) the PC receptor(s) for $TFPI^{BHK}$ has higher affinity for its ligand than the receptor for $TFPI^{E.Coli}$ (LRP) in the presence of UFH, ii) binding of $TFPI^{BHK}$ at the cellular site of uptake in liver and kidneys may be enhanced by the presence of UFH, and/or iii) other type of binding mechanism may exist for the fast clearance of $TFPI^{BHK}$ in the liver and kidneys when UFH is present in the circulation. The two latter possibilities are based on conformational changes in

the $TFPI^{BHK}$ molecule by UFH binding, enhancing the affinity of $TFPI^{BHK}$ for its receptor(s). Previously, we and others have shown that TFPI of mammalian origin, i.e. $TFPI^{BHK}$ and $TFPI^{C127}$ bind very weakly, or even fail to bind to HSPGs, and that their uptake is not mediated by the LRP-1 (unpublished data, [11, 28]). These findings along with the SPR analysis showing weaker $TFPI^{BHK}$-UFH interactions compared to $TFPI^{E.Coli}$-UFH interactions suggest that the faster clearance of $TFPI^{BHK}$ in the presence of UFH is caused by an enhanced binding affinity of the $TFPI^{BHK}$-UFH complexes to the yet unknown receptor(s) for $TFPI^{BHK}$ on PCs.

In conclusion, our study clearly demonstrates different mechanisms for clearance of $TFPI^{E.Coli}$ and $TFPI^{BHK}$ during heparin treatment. UFH forms stronger complexes with $TFPI^{E.Coli}$ than $TFPI^{BHK}$, and simultaneous administration of $TFPI^{E.Coli}$ and UFH switches hepatic binding of $TFPI^{E.Coli}$-UFH complexes from PCs during $TFPI^{E.Coli}$ administration alone towards LSECs, the primary site of UFH elimination. On the other hand, $TFPI^{BHK}$ which is glycosylated and thereby resembles endogenous TFPI, showed increased elimination during UFH treatment without affecting the target organ and specific cells responsible for binding and endocytosis. The latter findings may explain why prolonged treatment with UFH causes depletion of intravascular TFPI in humans. Further studies are needed to understand the impact of UFH on cell binding and degradation of $TFPI^{BHK}$.

Table 1. Effect of UFH on Clearance of $TFPI^{BHK}$ and to $TFPI^{E.Coli}$

Trace amounts of ^{125}I-$TFPI^{BHK}$ and ^{125}I-$TFPI^{E.Coli}$ (0.1 nM in 0.5 mL physiological saline) were injected into a lateral tail vein, either alone, or just after intravenous injection of 100 IU/kg UFH, and radioactivity was measured in blood samples over time. Radioactivity in blood 1 min after injection was taken as 100%. The clearance was fitted to two-phase exponential decay. The values are mean ± SEM from 9 separate experiments in each group.

Ligand	$t_{½}$ α (min)	$t_{½}$ β (min)
$TFPI^{BHK}$	1.99 ± 0.10	25.08 ± 1.56
+ UFH	1.17 ± 0.13[1]	27.17 ± 1.80
$TFPI^{E.Coli}$	1.44 ± 0.08[2]	27.44 ± 1.91
+ UFH	1.67 ± 0.10	33.88 ± 1.87[3]

[1] $p<0.001$ for differences in $t_{1/2}$ α between $TFPI^{BHK}$ administered alone and with UFH

[2] $p<0.001$ for differences in $t_{1/2}$ α for $TFPI^{BHK}$ and $TFPI^{E.Coli}$

[3] $p<0.05$ for differences in $t_{1/2}$ β between $TFPI^{E.Coli}$ administered alone and with UFH

FIGURE LEGENDS

Figure 1 Binding of TFPI to immobilized UFH by SPR analysis

Sensorgrams of $TFPI^{E.Coli}$-UFH interaction (A), and $TFPI^{BHK}$-UFH interaction (B). Concentrations of TFPIs were 100, 50, 25, 12.5 and 6.25 nM.

Figure 2 Organ distribution of ^{125}I-$TFPI^{BHK}$ without and with administration of UFH

Trace amounts of ^{125}I-$TFPI^{BHK}$ and ^{125}I-$TFPI^{E.Coli}$ (0.1 nM in 0.5 mL physiological saline) were injected into a lateral tail vein, either alone, or just after intravenous injection of 100 IU/kg UFH. At 10 and 20 min after injection the organs were washed free of blood by systemic perfusion through the heart with physiological saline, removed and analyzed for radioactivity. The values are mean ± SD from 2 separate experiments in each group.

Figure 3 Effect of UFH on Hepatocellular distribution of ^{125}I-$TFPI^{E.Coli}$ and ^{125}I-$TFPI^{BHK}$

At 15 min after i.v. administration of ^{125}I-$TFPI^{E.Coli}$ (A) and ^{125}I-$TFPI^{BHK}$ (B) alone (dark bars) or just after 100 IU/kg UFH (white bars), the liver cells were dispersed by collagenase perfusion and the amount of radioactivity per million cells was measured in suspension of parenchymal cells (PCs) and non parencymal cells (NPCs). The uptake per cell in the total liver was calculated based on the knowledge that the ratio between KC, LSEC and PC in rat liver is 1:2.5:7.7 [34]. Bars are means ± SEM for three and six separate experiments for $TFPI^{E.Coli}$ and ^{125}I-$TFPI^{BHK}$, respectively.

Figure 1

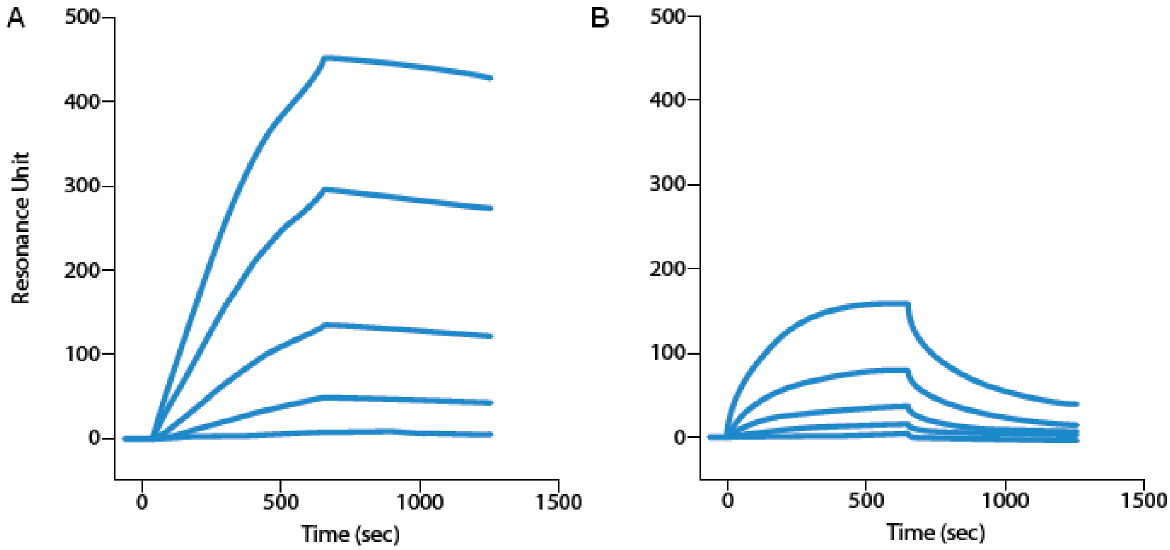


Figure 2

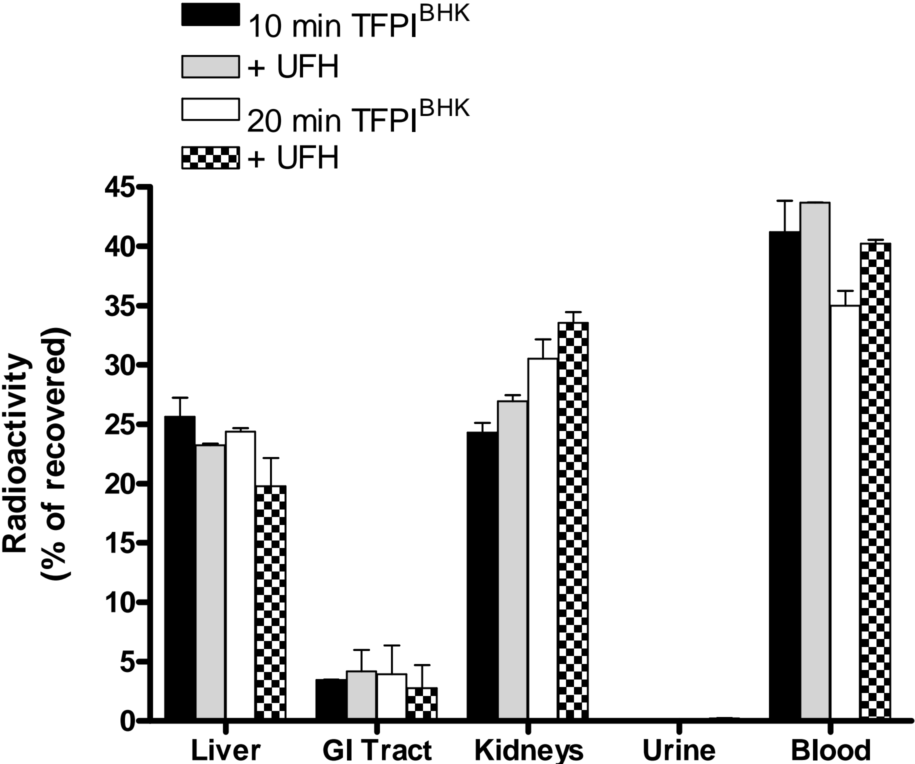
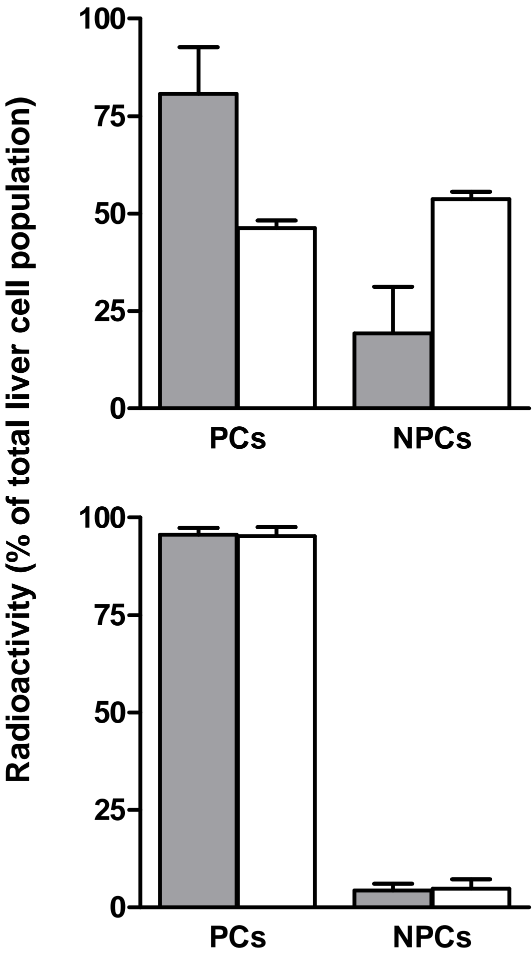


Figure 3



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