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Blocking Inhibition of Prothrombinase by Tissue Factor Pathway Inhibitor alpha: A Procoagulant Property of Heparins

Jeremy P. Wood¹, Lisa M. Baumann Kreuziger^{1,2}, Umesh R. Desai³, and Alan E. Mast^{1,4}

¹Blood Research Institute, Blood Center of Wisconsin, Milwaukee, WI

²Department of Medicine, Hematology and Oncology, Medical College of Wisconsin, Milwaukee, WI

³Department of Medicinal Chemistry and Institute for Structural Biology, Drug Discovery and Development, Virginia Commonwealth University, Richmond, VA

⁴Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, WI

Summary

Unfractionated heparin (UFH) has procoagulant activity in antithrombin/heparin cofactor II (HCII)-depleted plasma. UFH prevents tissue factor pathway inhibitor alpha (TFPI α) from inhibiting the procoagulant enzyme complex, prothrombinase, providing a possible mechanism for its procoagulant activity. The procoagulant potential of UFH and various low molecular weight heparins (LMWHs) were characterized for TFPI α dependence, using thrombin generation assays performed with antithrombin/HCIIDepleted plasma. UFH, the LMWHs enoxaparin and dalteparin, and the low anticoagulant LMWH 2-O, 3-O desulphated heparin (ODSH) all promoted thrombin generation, but fondaparinux did not, and this activity was blocked by a TFPI α antibody. UFH, enoxaparin, and dalteparin were anticoagulant in reactions containing 1–2% normal plasma. In prothrombinase activity assays, UFH, enoxaparin, dalteparin and ODSH blocked prothrombinase inhibition by TFPI α , while again fondaparinux did not. In both the plasma and purified assays, LMWHs displayed greater procoagulant potential than UFH, even when normalized to saccharide concentration. These biochemical data reveal that UFH and LMWHs, but not fondaparinux, block prothrombinase inhibition by TFPI α , thereby producing their paradoxical procoagulant activity observed in the absence of antithrombin/HCIIDepleted plasma. The findings may help to understand the complex pathophysiology and treatment of patients that are simultaneously bleeding and clotting, such as those with disseminated intravascular coagulation.

Correspondence to: Alan E. Mast, Blood Research Institute, BloodCenter of Wisconsin, PO Box 2178, Milwaukee, WI 53201, alan.mast@bcw.edu, 414-937-6310, 414-937-6283 (FAX).

Author Contributions

J.P.W. performed experiments, analysed data, and wrote the manuscript. L.M.B.K. and U.R.D. analysed data and edited the manuscript. A.E.M. designed the research, analysed data and edited the manuscript.

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Conflicts of Interest

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Keywords

Heparin; TFPI; Prothrombinase; Antithrombin

Introduction

Unfractionated heparin (UFH) is a heterogeneous mixture of sulphated polysaccharides with well-characterized anticoagulant function mediated by promoting the inhibition of the coagulation serine proteases thrombin and factor Xa (FXa) by antithrombin (Rosenberg 1989). Antithrombin binds with high affinity to a specific pentasaccharide sequence found in ~33% of UFH chains (Andersson, *et al* 1976, Hook, *et al* 1976, Lam, *et al* 1976). Only chains that contain the antithrombin binding sequence possess significant anticoagulant activity. UFH exhibits paradoxical procoagulant activity in human plasma, in the absence of antithrombin and heparin cofactor II (HCII) (Smith and Morrissey 2008).

The biochemical mechanism underlying this procoagulant activity of UFH is not fully understood. One possible explanation is the inhibition of tissue factor pathway inhibitor (TFPI) α anticoagulant activity, a procoagulant activity of heparin first observed more than 15 years ago (Mast and Broze 1996) and more recently biochemically characterized (Wood, *et al* 2013). TFPI α is a trivalent Kunitz-type protease inhibitor that dampens the initiation phase of clot formation by inhibiting tissue factor/factor VIIa (TF-FVIIa), the catalytic complex that initiates blood clotting, in a FXa-dependent manner (Baugh, *et al* 1998, Girard, *et al* 1989) and by inhibiting early forms of prothrombinase (Wood, *et al* 2013), the catalytic complex of FXa and factor Va (FVa) that produces thrombin (Barton, *et al* 1967, Nesheim, *et al* 1979, Rosing, *et al* 1980). TFPI α inhibits prothrombinase by interacting with both FXa and FVa. The second Kunitz-type inhibitor domain (K2) of TFPI α directly binds and blocks the FXa active site (Girard, *et al* 1989). When assembled in the prothrombinase complex, FXa is protected from inhibition by the K2 domain (Franssen, *et al* 1997, Mast and Broze 1996). TFPI α overcomes this protection via an exosite interaction between its C-terminus and an acidic region of the B-domain present in early forms of FVa (Wood, *et al* 2013), which are secreted by activated platelets (Monkovic and Tracy 1990a, Viskup, *et al* 1987) or produced through limited proteolysis by FXa (Monkovic and Tracy 1990b, Schuijt, *et al* 2013). Once thrombin is generated, it rapidly removes this acidic region from FVa (Monkovic and Tracy 1990b). Thus, TFPI α inhibits prothrombinase only during the initiation phase of blood clotting, before sufficient thrombin has been produced to remove the FVa acidic region (Mast and Broze 1996, Wood, *et al* 2013).

UFH blocks the interaction between the FVa B-domain acidic region and the TFPI α C-terminal basic region, in a manner that promotes thrombin generation in assays using purified proteins (Wood, *et al* 2013). Consistent with these findings describing the interactions between TFPI α , prothrombinase and UFH, the procoagulant activity of UFH described in antithrombin/HCIII-depleted plasma was not observed if either thrombin or thrombin-activated FVa had been added to the reaction mixtures (Smith and Morrissey 2008). We hypothesized that the procoagulant activity of UFH, observed in the absence of

antithrombin/HCI_{II}, is mediated by the ability of UFH to block prothrombinase inhibition by TFPI α and thereby promote the initiation phase of thrombin generation.

The dependence of the paradoxical procoagulant activity of UFH on TFPI α was characterized and compared to the procoagulant potential of the low molecular weight heparins (LMWHs) enoxaparin and dalteparin, the antithrombin-binding pentasaccharide fondaparinux, and the low anticoagulant LMWH, 2-O, 3-O desulphated heparin (ODSH).

Methods

Materials

UFH (MW ~12000–16000Da) was from Sigma Aldrich (St. Louis, MO), fondaparinux (MW = 1728Da) from Apotex (Weston, FL), enoxaparin (MW ~5000Da) from Sandoz (Princeton, NJ) and dalteparin (MW ~5000Da) from Eisai (Woodcliff Lake, NJ). ODSH (Fryer, *et al* 1997) was a gift from J.A. Voynow (Virginia Commonwealth University). FV810^{QQ}, an altered form of FVa in which the B-domain residues 811–1490 are absent and the thrombin cleavage sites at Arg709 and Arg1545 have been mutated to Gln (Bos and Camire 2012), was a gift from R.M. Camire (University of Pennsylvania). Human FXa and prothrombin were from Enzyme Research Laboratories (South Bend, IN). Thrombin, the thrombin inhibitor dansylarginine N-(3-ethyl-1,5-pentanediy)amine (DAPA) and corn trypsin inhibitor (CTI) were from Haematologic Technologies (Essex Junction, VT). Recombinant TFPI α was as described (Lockett and Mast 2002), and an inhibitory monoclonal antibody directed against the second Kunitz domain of TFPI α (anti-K2) was a gift from Novo Nordisk (Bagsvaerd, Denmark). TF (Dade Innovin) was from Siemens (Washington, DC). Thrombin and FXa chromogenic substrates (Spectrozyme TH and Spectrozyme FXa, respectively) were from Sekisui Diagnostics (Lexington, MA).

Plasma

Experiments using human plasma samples were approved by the BloodCenter of Wisconsin Institutional Review Board, and informed consent was obtained from all study participants. Blood was collected from healthy adult donors into a mixture of citrate and CTI (final concentration 50 μ g/ml) and plasma was prepared by centrifugation (700 \times g, 15 min). Plasma was subsequently centrifuged (5000 \times g, 10 min) to remove any residual platelets. Antithrombin/heparin cofactor II (HCI_{II})-depleted plasma was from Affinity Biologicals (Ancaster, ON, Canada). For some experiments, the antithrombin/HCI_{II}-depleted and normal plasma were mixed at the indicated ratios. The plasma was then diluted 1:4 with 20 mM HEPES, 150 mM NaCl, pH 7.4 (HEPES buffered saline, HBS), containing 0.1% albumin (HBSA). Dilution was necessary because the amount of non-inhibited thrombin generated in undiluted antithrombin/HCI_{II}-depleted plasma was too high to accurately quantify. Thrombin generation in normal plasma was measured without dilution.

Phospholipid vesicles

Phosphatidylcholine (PC, cat #840051), phosphatidylethanolamine (PE, cat #840026), and phosphatidylserine (PS, cat #840032), were from Avanti Polar Lipids (Alabaster, AL).

Phospholipid vesicles containing PC, PE and PS at a 40:40:20 ratio (PC:PE:PS) were prepared according to the protocol of Morrissey (2001).

Calibrated automated thrombography

Plasma thrombin generation assays were performed as described (Wood, *et al* 2013). Briefly, 10 μ l of a 6x activation mixture (either 0.6 nM FXa or 6 pM TF, and 24 μ M PC:PE:PS in HBSA) was incubated with 40 μ l of plasma, containing the indicated concentration of UFH, dalteparin, enoxaparin, fondaparinux or ODSH, and reactions were initiated by addition of 10 μ l of a mixture of fluorogenic thrombin substrate and calcium (FluCa; Diagnostica Stago, Parsippany, NJ). Fluorescence was monitored using a Fluoroskan Ascent fluorometer (ThermoScientific, Waltham, MA.), and data were analysed using Thrombinoscope v5.0 software (Thrombinoscope, Maastricht, The Netherlands). The lag time, as calculated by the Thrombinoscope software, is the time corresponding to 6% of the peak thrombin.

Purified prothrombinase assays

Prothrombinase inhibition assays were as described (Wood, *et al* 2013). Briefly, FV810^{QQ} (0.5 nM) was incubated with PC:PE:PS (20 μ M), the thrombin inhibitor DAPA (3 μ M), and the indicated concentrations of TFPI α and heparins in 20 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) containing 5 mM CaCl₂ and 0.1% polyethylene glycol (PEG)-8000. Reactions were initiated by addition of prothrombin (1.4 μ M) and FXa (5 nM). Aliquots were removed at timed intervals and quenched by addition to HBS containing EDTA (33.3 mM final concentration) and 0.1% PEG-8000. Thrombin concentration at each time point was measured on a SpectraMax Plus 384 microplate reader by adding 20 μ l of quenched sample to 80 μ l of 0.4 mM thrombin chromogenic substrate, measuring the absorbance change at 405nm, and comparison to a thrombin standard curve. TFPI α :heparin complex concentrations were calculated using a quadratic binding equation and published K_d values (Xu, *et al* 2002).

FXa activity assays

FXa (0.2 nM) was incubated with phospholipid vesicles (20 μ M), in the presence or absence of TFPI α (0.5 nM) and UFH, enoxaparin, dalteparin, or fondaparinux (1 u/ml), and cleavage of a FXa chromogenic substrate (0.5 mM) was monitored at 405 nm.

Results

UFH, enoxaparin, and dalteparin are procoagulant in antithrombin/HCIi-depleted plasma, but fondaparinux is not

The potential procoagulant activity of the LMWHs enoxaparin and dalteparin and the pentasaccharide fondaparinux was measured using TF-initiated thrombin generation in antithrombin/HCIi-depleted plasma (Fig 1A–C). UFH, dalteparin, and enoxaparin (5 u/ml of each) promoted thrombin generation, primarily by reducing the lag time for thrombin generation, while fondaparinux had no effect. Neither UFH nor enoxaparin expressed procoagulant activity when assays were performed in the presence of a monoclonal antibody against K2, while dalteparin expressed greatly reduced procoagulant activity, indicating that the procoagulant activity is dependent on TFPI anticoagulant activity. When reactions were

initiated with FXa, the effect of TFPI on heparin procoagulant activity was more enticing (Fig 1D–H). In these reactions, UFH decreased the lag time for thrombin generation by 1.8 ± 0.9 min at 5 u/ml, while the LMWHs exhibited much more pronounced procoagulant activity, with 5 u/ml enoxaparin or dalteparin decreasing the lag time by 5.1 ± 1.3 min or 5.9 ± 1.2 min, respectively. The procoagulant activity of UFH showed a dose response with procoagulant activity apparent at 1 u/ml, while both enoxaparin and dalteparin had full procoagulant activity at the lowest concentration tested (0.5 u/ml) (Fig 1H). By contrast, fondaparinux had minimal effect at any concentration tested (Fig 1H). As observed in the TF-initiated reactions, the procoagulant activity of the heparins in FXa-initiated reactions was dependent on the presence of plasma TFPI, with no procoagulant activity observed for any heparin when TFPI was neutralized with anti-K2 (Fig 1I).

Heparin anticoagulant activity is restored at low antithrombin/HCI concentrations

The minimum concentration of antithrombin and HCI necessary to restore anticoagulant function to the heparins was determined by mixing antithrombin/HCI-depleted plasma with normal plasma and measuring FXa-initiated thrombin generation (Fig 2). Addition of normal plasma dose-dependently prolonged the lag time for thrombin generation in the absence of heparin. Therefore, all experiments performed with heparin were compared to equivalent mixed control plasmas in the absence of heparin. In comparison to control, UFH (5 u/ml) exhibited anticoagulant function in 0.5% normal plasma and completely blocked thrombin generation in 5% normal plasma. Enoxaparin and dalteparin required greater amounts of normal plasma to display anticoagulant activity. Dalteparin was anticoagulant in 1% normal plasma and enoxaparin in 2% (Fig 2G, expanded graph). Both completely blocked thrombin generation in 20% normal plasma (Fig 2F–G).

UFH, enoxaparin and dalteparin block TFPI α -mediated prothrombinase inhibition, but fondaparinux does not

TFPI α inhibits prothrombinase only during the initiation of thrombin generation (Mast and Broze 1996, Wood, *et al* 2013), because once thrombin is produced, it rapidly removes the TFPI α -binding acidic region from FVa (Monkovic and Tracy 1990b). Therefore, blocking TFPI α inhibition of prothrombinase results in a decrease in the lag time of FXa-initiated thrombin generation (Wood, *et al* 2013), consistent with the procoagulant effect of UFH, enoxaparin and dalteparin (Fig 1). The ability of therapeutic concentrations of UFH, dalteparin, enoxaparin and fondaparinux to block TFPI α was measured in prothrombinase activity assays utilizing FV810^{QQ}, a recombinant form of FVa that contains the acidic region that binds TFPI α and lacks the primary thrombin cleavage sites (Bos and Camire 2012). The resistance of FV810^{QQ} to thrombin proteolysis produces an assay system, in which thrombin generated during the experiment cannot remove the FVa acidic region and thereby alter the inhibitory kinetics. In this system, TFPI α inhibited thrombin generation with 50% inhibitory concentration (IC₅₀) of 6.8 nM in the absence of heparin (Fig 3). UFH (1 u/ml) blocked TFPI α inhibitory activity, shifting the IC₅₀ to 14.9 nM. Consistent with their increased effect on the lag time in antithrombin/HCI-depleted plasma, enoxaparin (0.8 u/ml, IC₅₀=30.3 nM) and dalteparin (1 u/ml, IC₅₀=29.7 nM) were more effective at blocking prothrombinase inhibition by TFPI α than was UFH. Fondaparinux (0.8 u/ml, IC₅₀=8.4 nM) did not

significantly alter the ability of TFPI α to inhibit prothrombinase. Similar results were obtained when all heparins were used at 0.5 u/ml (data not shown).

Several aetiologies for the enhanced activity of enoxaparin and dalteparin were considered. First, in addition to blocking the interaction between the TFPI α C-terminus and FVa, UFH promotes the interaction of the K2 domain of TFPI α with the FXa active site in assays performed without FVa (Huang, *et al* 1993, Jesty, *et al* 1994, Mast and Broze 1996). The ability of each of the heparins to enhance the direct inhibition of FXa was measured in a chromogenic assay (Fig 4A). Under the conditions of this assay, TFPI α slowly inhibited FXa in the absence of heparin, but rapidly inhibited in the presence of 1 u/ml UFH. FXa was also rapidly inhibited in the presence of 1 u/ml enoxaparin or dalteparin, while fondaparinux had minimal effect. Thus, the increased procoagulant activity of enoxaparin and dalteparin is not explained by decreased ability to enhance direct FXa inhibition.

Second, given that 1 u/ml UFH has a different total saccharide concentration than 1 u/ml enoxaparin or fondaparinux, the effect of saccharide concentration on prothrombinase inhibition was examined. Prothrombinase inhibition assays were performed at constant TFPI α concentration (10 nM) and varying heparin concentrations (in μ g/ml) (Fig 4B). Increasing UFH or enoxaparin concentration progressively blocked TFPI α inhibitory activity. However, the effect plateaued and then decreased at high concentrations (100–10,000 μ g/ml). Fondaparinux required 100- to 1000-fold higher concentrations than UFH or enoxaparin to impede the inhibitory activity of TFPI α (Fig 4B). Published binding affinities (Xu, *et al* 2002) were used to normalize titration curves to the TFPI α :heparin complex concentration. The differences in the ability of the heparins to block TFPI α inhibitory activity were greatly reduced following normalization for their respective binding affinities (Fig 4C). Therefore, while higher concentrations of LMWHs than UFH are necessary to inhibit TFPI α , the LMWHs possess a greater procoagulant potential, which is not explained by differences in saccharide concentration.

Third, the potential for UFH to directly inhibit prothrombinase, independent of TFPI α , was evaluated as a potential explanation for the decreased apparent procoagulant activity of UFH compared to enoxaparin and dalteparin. However, in the absence of TFPI α , only high concentrations of UFH (> 1000 μ g/ml) directly inhibited prothrombinase (Fig 4D), suggesting that TFPI α -independent prothrombinase inhibition does not impact experiments performed at lower concentrations.

The low anticoagulant LMWH ODSH has procoagulant activity at higher normal plasma concentrations than anticoagulant LMWHs

Low anticoagulant LMWHs lack the high affinity antithrombin-binding pentasaccharide, and have been proposed as treatments for a variety of conditions, including sepsis (Wildhagen, *et al* 2014), cancer (Pisano, *et al* 2005, Ritchie, *et al* 2011), sickle cell disease (Alshaiban, *et al* 2016) and heparin-induced thrombocytopenia (Rao, *et al* 2010). We hypothesized that low anticoagulant LMWHs would retain procoagulant activity at higher antithrombin/HCI concentrations than enoxaparin and dalteparin. The potential procoagulant activity of low anticoagulant LMWHs was examined using ODSH (Fryer, *et al* 1997). Similar to the activity of the other LMWHs, ODSH displayed procoagulant activity in

antithrombin/HCI1-depleted plasma initiated with either TF or FXa, but not following addition of anti-TFPI-K2 to the plasma (Fig 5A). ODSH (5 u/ml) decreased the lag time by 5.9 ± 0.9 min in reactions initiated with FXa (Fig 5B), similar to the effect of 5 u/ml enoxaparin or dalteparin (Fig 1H). In contrast to enoxaparin and dalteparin, ODSH did not exhibit maximal procoagulant activity at 0.5 u/ml, but instead displayed a dose response with increasing concentration (Fig 5B). In prothrombinase activity assays, ODSH blocked TFPI α -mediated prothrombinase inhibition in a dose-dependent manner (Fig 5C) that was indistinguishable from enoxaparin (Fig 4B). In contrast to UFH, enoxaparin and dalteparin, ODSH retained partial procoagulant activity in 20% normal plasma (Fig 6A–C). ODSH exhibited anticoagulant activity in 100% normal plasma, as has been previously described (Fig 6D) (Fryer, *et al* 1997).

Discussion

UFH has paradoxical procoagulant activity in thrombin generation assays performed in plasma lacking antithrombin and HCI1. The experiments presented here demonstrate that LMWHs, but not the pentasaccharide fondaparinux, produce a similar procoagulant activity, as measured by a decrease in lag time for thrombin generation in TF- or FXa-initiated assays. Procoagulant activity was not observed when plasma TFPI was blocked with an anti-K2 antibody, revealing that the procoagulant activity of the heparins was mediated by their ability to obstruct the anticoagulant properties of TFPI. This finding was confirmed in prothrombinase assays using purified proteins, which demonstrated that the heparins effectively obstruct TFPI α anticoagulant activity. In addition to prothrombinase, UFH has been shown to impede TF-FVIIa inhibition by TFPI α (Jesty, *et al* 1994), which may have partially contributed to the procoagulant activity observed in TF-initiated assays. However, the promotion of prothrombinase activity is probably the primary procoagulant function of heparins, because the procoagulant activity is eliminated when antithrombin/HCI1-depleted plasma is supplemented with either thrombin or thrombin-activated FVa (Smith and Morrissey 2008), both of which would prevent TFPI α from inhibiting prothrombinase but would not impact TF-FVIIa inhibition.

Heparins block TFPI α prothrombinase inhibitory activity by interfering with the charge-dependent interaction between the basic C-terminus of TFPI α and an acidic region in FVa (Wood, *et al* 2013). This effect is reliant on charge density, but does not require the specific pentasaccharide sequence necessary for antithrombin binding. Therefore, ODSH, a LMWH lacking the pentasaccharide sequence, had procoagulant activity indistinguishable from enoxaparin or dalteparin, LMWHs containing the pentasaccharide sequence. However, charge-dependent interactions do not adequately explain the differences in the procoagulant activities of UFH, LMWHs and fondaparinux. Enoxaparin and dalteparin are produced by chemical depolymerization of UFH (Hoppensteadt, *et al* 2003). Therefore, differences in the average glycan polymer length are likely to contribute to the differences in procoagulant activity observed between UFH, LMWHs and fondaparinux. This assertion is supported by the progressively increasing amounts (in ng/ml) of heparin polymer required, as polymer length decreased, for inhibition of TFPI α in prothrombinase activity assays. Exhibited in Fig 4B, it took 10 ng/ml UFH, 50 ng/ml enoxaparin and 10,000 ng/ml fondaparinux to reverse 40% of the TFPI α activity. This size-dependent effect could be attributed largely to the

affinity of the heparins for TFPI α , as correction for differences in binding affinity for TFPI α (Xu, *et al* 2002) accounted for over 90% of the differences between fondaparinux and the other heparins (Fig 4C). For example, 1000 times more fondaparinux than UFH (by ng/ml) was required to achieve 40% inhibition of TFPI α , but after correcting for binding affinity, only 23 times more was necessary (by calculated TFPI α :heparin complex).

The three LMWHs promoted thrombin generation in antithrombin/HCII-depleted plasma and increased prothrombinase activity by blocking TFPI α in assays using purified proteins, to a greater extent than did UFH when examined at therapeutically relevant concentrations. For example, in prothrombinase activity assays, enoxaparin, fondaparinux and ODSH reversed 80–95% of TFPI α inhibitory activity, while UFH reversed only about 50% (Figs 4 and 5). This was unexpected for a reaction that is primarily dependent on size and charge density. Possible explanations included a difference in the ability of each heparin to promote direct FXa inhibition by TFPI α or a TFPI α -independent effect of UFH on prothrombinase activity. However, both of these explanations were disproved. First, enoxaparin and dalteparin promoted direct FXa inhibition to a similar extent as UFH. Second, while UFH did directly inhibit prothrombinase activity, it only did so at concentrations substantially higher than those used in the plasma-based assays (1000 μ g/ml). Thus, the reason for the increased procoagulant activity of LMWHs remains uncertain but may relate to microscopic differences between full-length and low molecular weight heparins.

Similar to the heparins, polyphosphate has procoagulant activity in human plasma that is mediated, at least partially, through obstruction of prothrombinase inhibition by TFPI α (Smith, *et al* 2006, Wood, *et al* 2013). Polyphosphates display increasing ability to inhibit TFPI α with increasing phosphate chain length, suggesting that they have size-dependent procoagulant properties similar to the heparins (Smith, *et al* 2010). Fucoidan, a sulphated polysaccharide derived from seaweed (Liu, *et al* 2006) that blocks the interaction of TFPI α with FVa (Wood, *et al* 2013) and decreases bleeding in dogs with hemophilia A (Prasad, *et al* 2008), also exhibits a size-dependent capability to block TFPI anticoagulant activity in plasma (Zhang, *et al* 2014).

The data presented here further our understanding of the effects of heparin-based molecules on the coagulation system. Given that UFH, enoxaparin and dalteparin are mixtures of molecules, of which only ~33% contain the high affinity antithrombin binding sequence (Andersson, *et al* 1976, Hook, *et al* 1976, Lam, *et al* 1976), the majority of heparin chains are not anticoagulant. However, all heparin chains appear to block TFPI α from interacting with FVa and, therefore, have procoagulant activity.

Any circumstances where the procoagulant effect of heparins may be clinically relevant remain speculative. As the plasma antithrombin concentration is several orders of magnitude higher than that of TFPI α (Maclean and Tait 2007, Novotny, *et al* 1991), the heparins are anticoagulant under most conditions. In fact, anticoagulant activity of UFH, enoxaparin and dalteparin was restored by reconstitution of antithrombin/HCII-depleted plasma with small amounts of normal plasma (2%), suggesting that a severe antithrombin deficiency would be required for these drugs to have a procoagulant effect *in vivo*. The amount of normal plasma necessary to avoid procoagulant effects was higher with ODSH (20%), which is consistent

with its weak affinity for antithrombin (Richard, *et al* 2009). The inhibition of TFPI α anticoagulant activity by UFH and LMWHs may become clinically relevant in severely ill patients with acquired antithrombin deficiency, such as sepsis and disseminated intravascular coagulation, or treatment with L-asparaginase; These patients can simultaneously bleed and clot and, as a result, are difficult to treat (Maclean and Tait 2007). Treatment with fondaparinux to anticoagulate these patients is an option for clinicians to consider, as it does not alter TFPI α activity at clinically effective concentrations.

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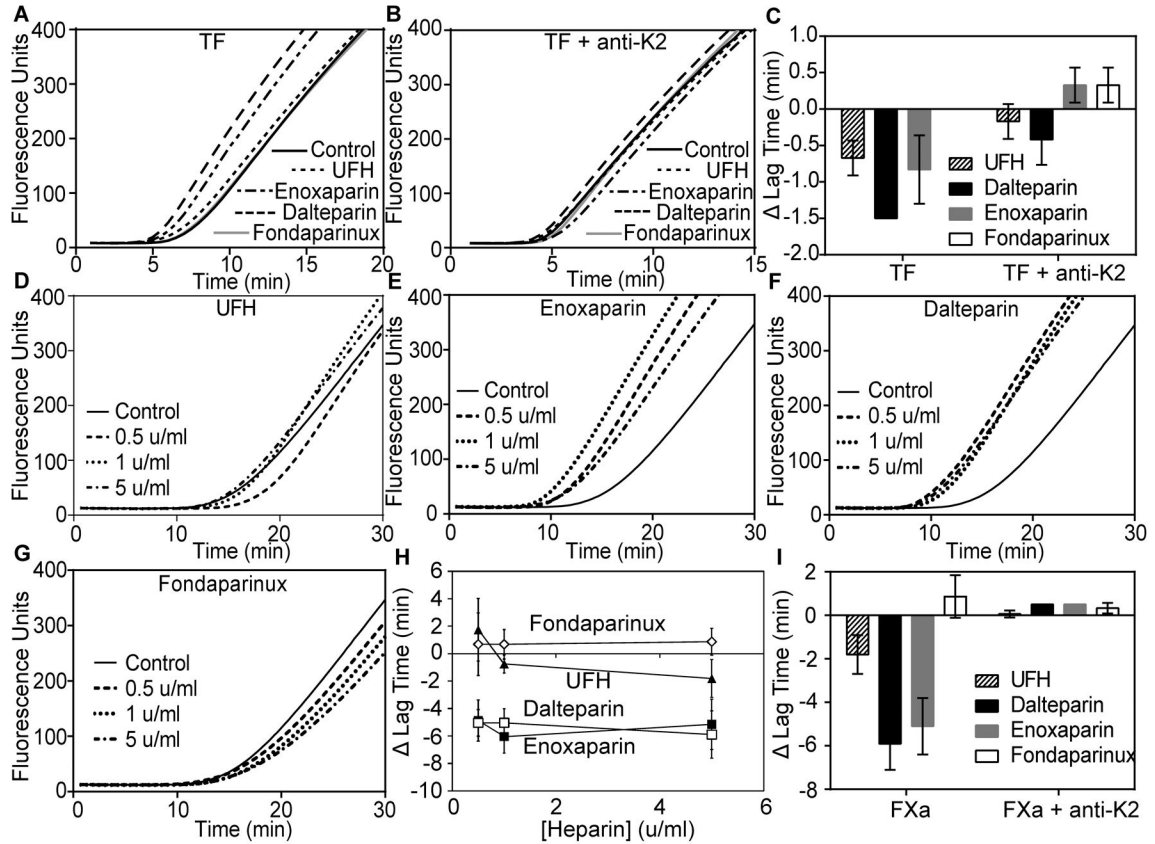


Fig 1. UFH, enoxaparin and dalteparin, but not fondaparinux, are procoagulant in antithrombin/HCI-depleted plasma

Thrombin generation assays initiated by addition of a mixture of phospholipid vesicles (4 μ M) and either TF (1pM) or factor Xa (0.1 nM) to antithrombin/HCI-depleted plasma that had been diluted 1:4 in HEPES-buffered saline containing 0.1% bovine serum albumin (A–B) Average fluorescence curves of reactions initiated with TF in the absence (A) or presence (B) of anti-K2 (50 nM) and 5 u/ml UFH, enoxaparin, dalteparin or fondaparinux (n=3). Note that in (A and B) the fondaparinux and control lines are nearly identical. (C) Average changes in lag time, relative to control, from the curves in (A–B) (mean \pm SD). (D–G) Average fluorescence curves of reactions initiated with FXa in the absence (black) or presence of 0.5 u/ml, 1 u/ml, or 5 u/ml UFH (D), enoxaparin (E), dalteparin (F), or fondaparinux (G) (n=3). (H) Average changes in lag time, relative to control, from the curves in (D–G) (mean \pm SD). (I) Average changes in lag time, relative to control, from reactions initiated with FXa in the absence or presence of anti-K2 and the different heparins (5 u/ml) (mean \pm SD; n=3). The lag times in the absence of heparin are reproduced from panel H for display purposes. UFH, unfractionated heparin; HCI, heparin cofactor II; TF, tissue factor; SD, standard deviation.

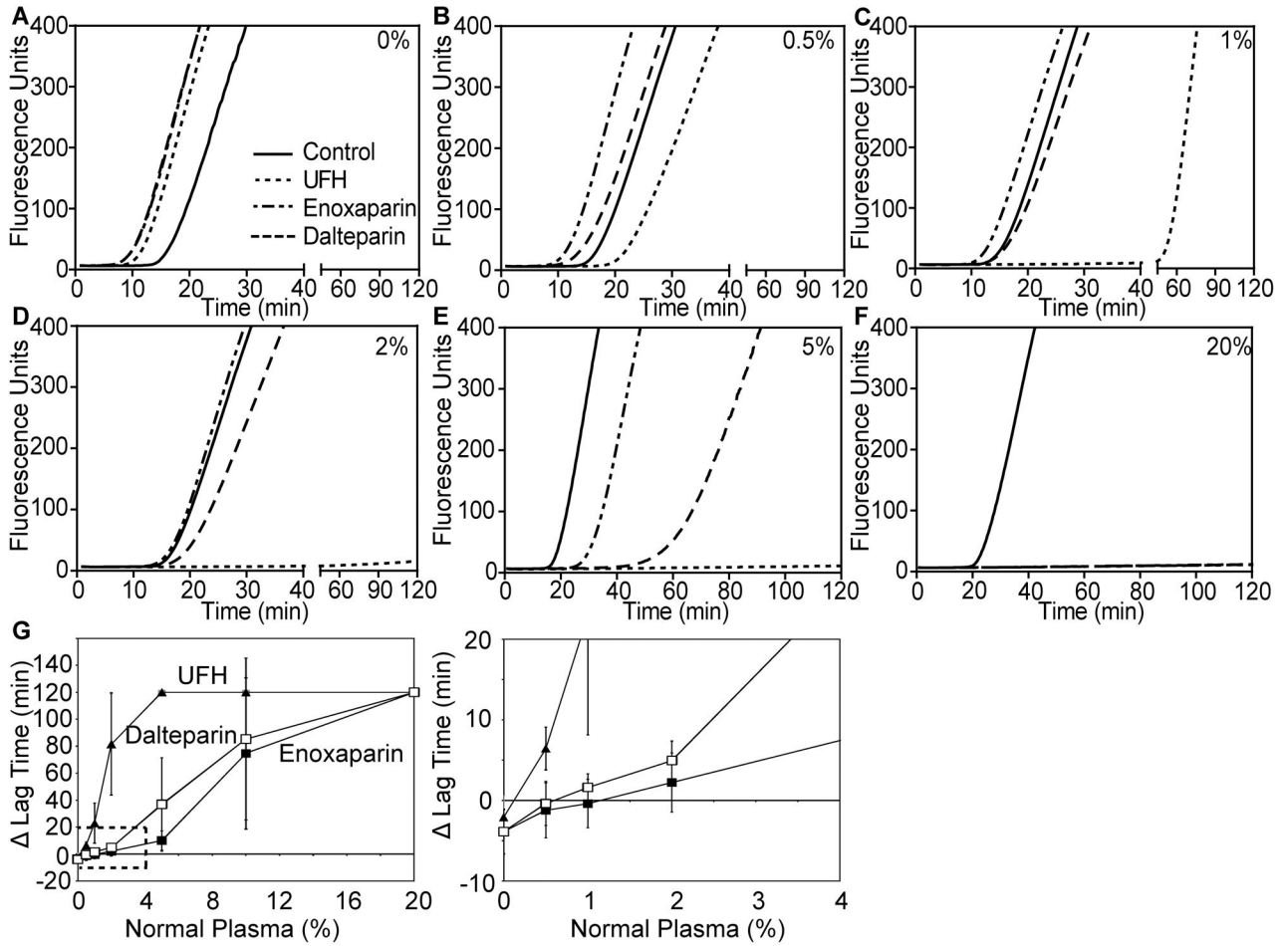


Fig 2. The anticoagulant activity of UFH, enoxaparin and dalteparin is restored at low antithrombin/HCI concentrations

Antithrombin/HCI-depleted plasma was mixed with various percentages of normal plasma (upper right corner of A–F) prior to dilution and factor Xa-initiated thrombin generation assays as described in Fig 1. Assays were performed in the absence (solid line) or presence of 5 u/ml UFH (dotted line), enoxaparin (dashed/dotted line), or dalteparin (dashed line). Shown are curves from one of three sets of experiments (A–F) and the average change in lag time, compared to control, as a function of normal plasma concentration, from all three experiments (G, left panel; mean ± S.D). The right panel in (G) is an expanded view of the region indicated by dashed lines included to visualize the pro- and anti-coagulant activities of enoxaparin and dalteparin in 0.5%–2% normal plasma in comparison to UFH. UFH, unfractionated heparin; HCI, heparin cofactor II.

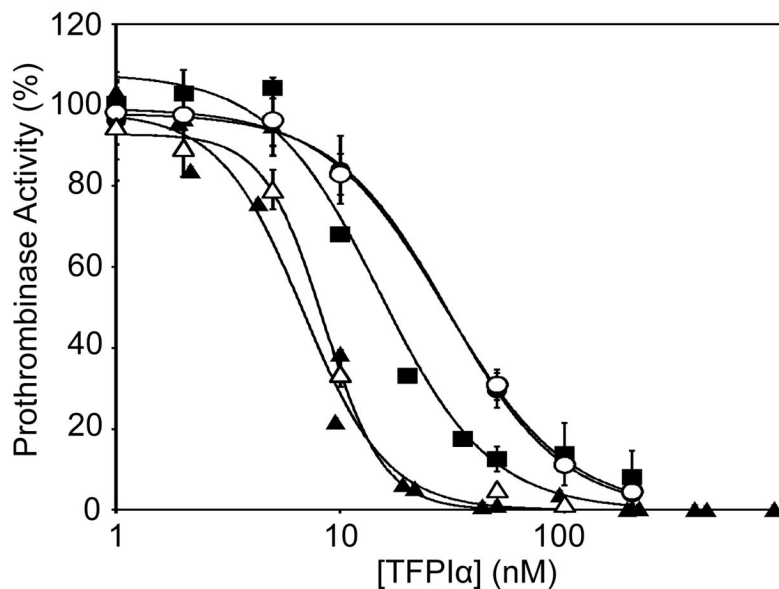


Fig 3. UFH, enoxaparin and dalteparin block TFPI α inhibition of prothrombinase, but fondaparinux does not

Prothrombinase activity assays were performed by incubating FV810^{QQ} (0.5 nM) with phospholipid vesicles (20 μ M), prothrombin (1.4 μ M), the thrombin inhibitor dansylarginine N-(3-ethyl-1,5-pentanediy)amine (DAPA; 3 μ M) and tissue factor pathway inhibitor alpha (TFPI α), in the absence (\blacktriangle) or presence of unfractionated heparin (UFH; (u/ml, \blacksquare), enoxaparin (0.8 u/ml, \bullet), dalteparin (1 u/ml, \circ) or fondaparinux (0.8 u/ml, \triangle), followed by the addition of FXa (5 nM) to initiate the reaction. The heparin concentrations were chosen to mimic the average recommended therapeutic concentrations of each. Shown are the initial rates of thrombin generation, expressed as the percentage of a control experiment performed in the absence of TFPI α (mean \pm standard error of the mean, n 3). Note that the dalteparin and enoxaparin curves are very similar.

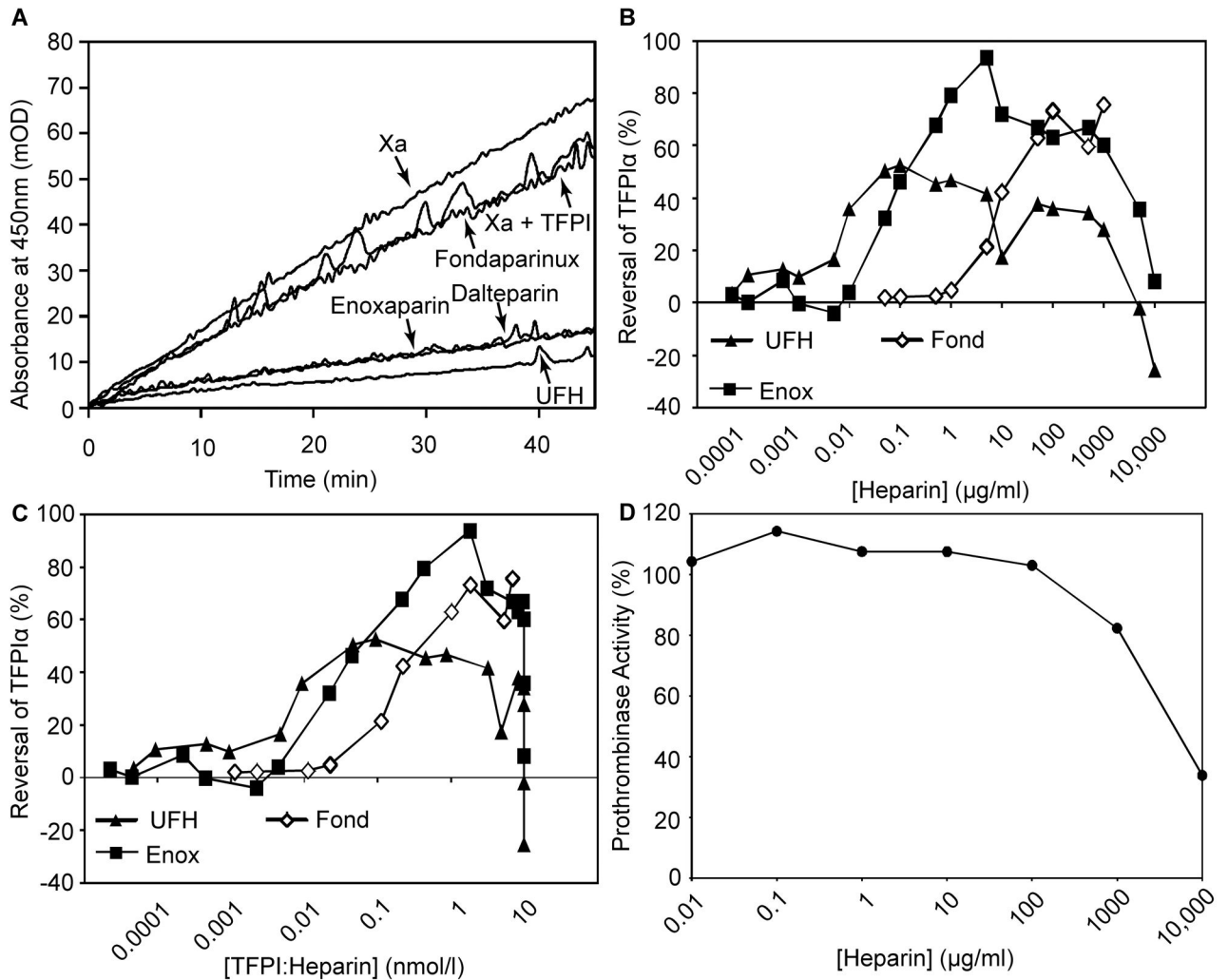


Fig 4. The enhanced procoagulant activity of LMWHs is not explained by differences in FXa inhibition or saccharide concentration

(A) Factor Xa (0.2 nM) was incubated with phospholipid vesicles (20 μM) and cleavage of a chromogenic substrate (0.5 mM) was monitored at 405 nm. Experiments were performed in the presence of tissue factor pathway inhibitor alpha (TFPI; 0.5 nM) or TFPIα plus 1 u/ml fondaparinux (Fond), enoxaparin (Enox) dalteparin or unfractionated heparin (UFH). (B–C) Prothrombinase activity experiments were performed as in Fig 3 in the presence of 10 nM TFPIα and varying concentrations of UFH, enoxaparin or fondaparinux. On average, 60% of the prothrombinase activity was inhibited by TFPIα under these conditions in the absence of heparin. The results are displayed as per cent reversal of TFPIα inhibitory activity as a function of the heparin concentration (B) or the calculated concentration of heparin:TFPIα complex (C). Heparin:TFPIα complex concentrations were calculated using the following K_d values: UFH $-0.7 \mu\text{M}$; enoxaparin $-5 \mu\text{M}$; fondaparinux $-250 \mu\text{M}$ (Xu, *et al* 2002). (D) Experiments were performed as in panel B, in the absence of TFPIα, in the presence or absence of UFH and displayed as a percentage of prothrombinase control.

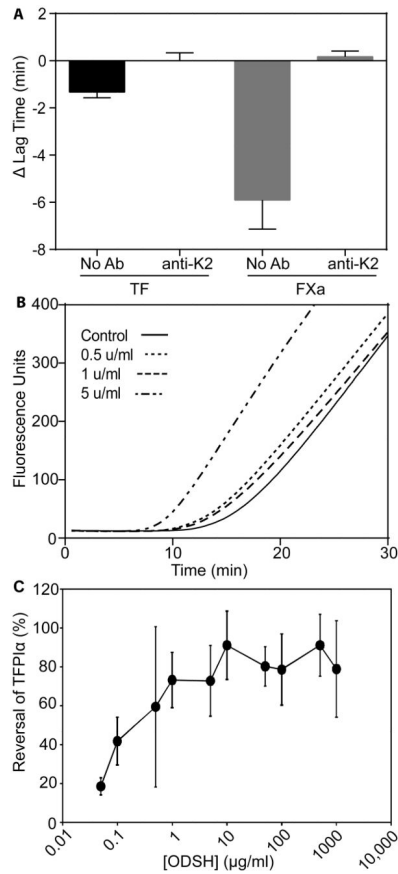


Fig 5. ODSH has procoagulant activity similar to enoxaparin and dalteparin in antithrombin/HCIi-depleted plasma and prothrombinase activity assays

(A) Thrombin generation assays were performed in antithrombin/heparin cofactor II (HCIi)-depleted plasma in the presence or absence of 2-O, 3-O desulphated heparin (ODSH; 5 u/ml) and anti-TFPI-K2 (50 nM) and were initiated with tissue factor (1pM) or factor Xa (0.1 nM). Shown are changes in lag time, relative to control (mean±SD; n=3). (B) Thrombin generation assays were performed in antithrombin/HCIi-depleted plasma as in Fig 1, in the presence of 0.5 (dotted line), 1 (dashed line), or 5 (dashed/dotted line) u/ml ODSH. Shown are the average fluorescence curves (mean±SD, n=3). (C) Prothrombinase activity assays in the presence of varying concentrations of ODSH were performed and are represented as described in Fig 4B. TFPI. TFPIα: tissue factor pathway inhibitor alpha. SD: standard deviation.

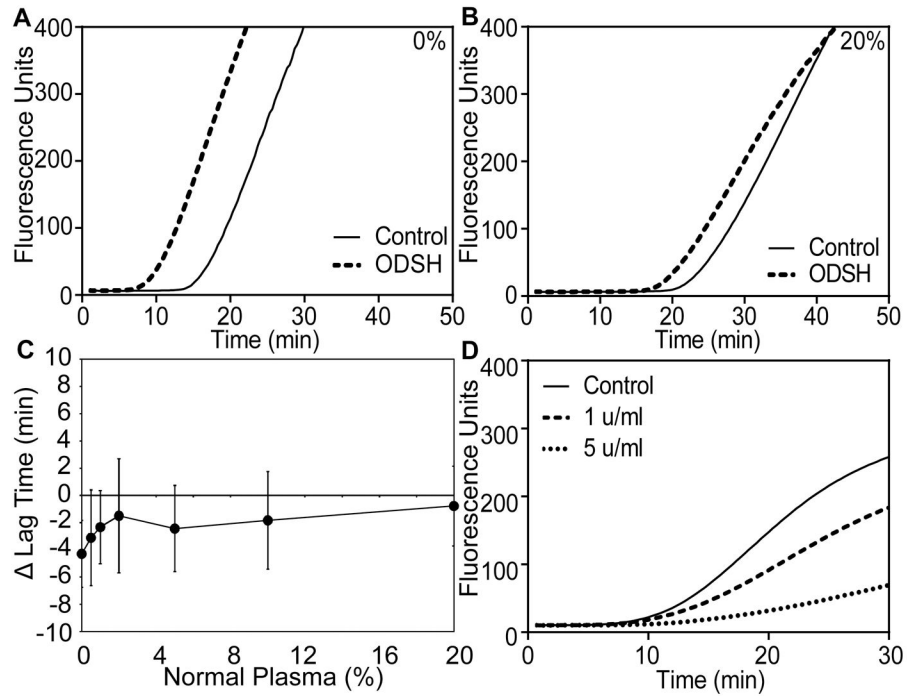


Fig 6. ODSH remains procoagulant in 20% normal plasma

(A–B) FXa-initiated (0.1 nM) thrombin generation assays were performed in the absence or presence of 2-O, 3-O desulphated heparin (ODSH; 5 u/ml) in antithrombin/HCIII-depleted plasma alone (A) or mixed with 20% normal plasma (B). Results are depicted as in Fig 2. (C) Average change in lag time (mean±standard deviation) as a function of the percentage of normal plasma. (D) FXa-initiated (0.1 nM) thrombin generation assays were performed in undiluted normal plasma in the absence or presence of 1 u/ml (dashed line) or 5 u/ml (dotted line) ODSH. Shown are the average curves from three sets of experiments.