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Mechanism of inhibition of the prothrombinase complex by a covalent antithrombin–heparin complex

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Factor-Xa assembly into the prothrombinase complex decreases its availability for inhibition by antithrombin + unfractionated heparin (AT + UFH). We have developed a novel covalent antithrombin–heparin complex (ATH), with enhanced anticoagulant actions compared with AT + UFH. The present study was performed to extend understanding of the anticoagulant mechanisms of ATH by determining its inhibition of Xa within the critical prothrombinase. Discontinuous inhibition assays were performed to determine final k_2 values for inhibition of Xa. Fluorescent microscopy was conducted to evaluate inhibitor–prothrombinase interactions. The k_2 for inhibition of prothrombinase versus free Xa by AT + UFH was lower, whereas for ATH were much higher. Relative to intact prothrombinase, rates for Xa inhibition by AT + UFH in complexes devoid of prothrombin/vesicles/factor-Va were higher. For ATH, exclusion of prothrombin decreased k_2, removal of vesicles increased k_2 and exclusion of factor-Va gave no effect. While UFH may displace Xa from prothrombinase, Xa is detained within prothrombinase during ATH reactions. We confirm prothrombinase hinders inhibitory action of AT + UFH, whereas ATH is less affected with prothrombin being a key component in the complex responsible for the opposing effects. Overall, the results suggest that covalent linkage between AT-heparin assists access and neutralization of complexed Xa, with concomitant inhibition of prothrombinase function compared with conventional non-conjugated heparin.

Keywords: antithrombin/covalent antithrombin–heparin complex/heparin/prothrombinase complex.

Abbreviations: AT, antithrombin; ATH, covalent antithrombin–heparin complex; PCPS, phoshphatidylcholine/serine phospholipids; II, prothrombin; UFH, unfractionated heparin.

The coagulation cascade is a series of enzymatic reactions involving conversion of proenzymes to activated coagulation factors. Some of these factors combine to form macromolecular complexes responsible for generating the key enzymes of the cascade. The prothrombinase complex, which is composed of factor Xa (Xa), its cofactor Va (Va), phospholipid surface (PCPS), calcium ions and prothrombin (II), is the enzymatic complex responsible for converting II to thrombin (*1–3*). The catalytically active component of the prothrombinase is Xa, which requires Va in a minimum ratio of 1:1 for enhanced II to thrombin conversion (*1*, *4*, *5*). Formation of the full prothrombinase complex may enhance the conversion of II to thrombin by 3.0×10^5-fold (*6*, *7*). Several studies have looked at omitting components of the prothrombinase complex on thrombin generation, and it has been observed that absence of any components from the prothrombinase significantly reduces its capability to generate thrombin (*8–10*).

The cascade is a fine balance between pro- and anticoagulant activity and a net shift in this activity may lead to development of thrombotic complications or severe haemorrhaging (*11–15*). Inhibitors of the coagulation cascade are important and are required to adequately regulate the cascade's activity. Antithrombin (AT) is the major *in vivo* serine protease inhibitor (serpin) that is variously capable of inhibiting thrombin (*16*), factors VIIa (*17*), IXa (*18*), Xa (*19*), XIa (*20*) and XIIa (*21*). Inhibition of the serine proteases by AT is significantly accelerated by the presence of unfractionated heparin (UFH) (*22*). UFH is part of the glycosaminoglycan family and UFH interacts with AT through a high-affinity pentasaccharide sequence (*23–25*). Reportedly, UFH catalytically increases the rate of AT reaction with thrombin or Xa in excess of 1,000-fold (*26*).

Clinical use of heparin has yielded many of its limitations, including a short dose-dependent intravenous half life and unpredictable anticoagulant response (*27–29*). Complications from UFH may arise, such as heparin-induced thrombocytopenia or severe bleeding (*30*). Also, only one-third of UFH molecules contain the high-affinity pentasaccharide sequence (*31*, *32*). In addition, several studies have shown that assembly of Xa in the prothrombinase complex results in protection from inhibition by AT + UFH (*33–35*). The extent of this protection is a matter of debate. Reports range from complete protection (*10*, *36*) to <5- to 10-fold differences (*33*, *37–39*).

To overcome some of these limitations, we have employed a covalent complex of AT and UFH (ATH) to study its effect on blood coagulation. Over the past decade, we have determined that the covalent conjugate results in a higher intravenous half-life (*40*), increased inhibition of fibrin-bound thrombin (*41*), reduced interaction of its heparin chains with endothelial surfaces (*42*), more potent antithrombotic activity

(*43*), predictable anticoagulant response (*44*) and higher rates for inhibition of coagulation factors in isolation (*45*). However, interaction of ATH with surface-based enzyme complexes has not been studied before. Therefore, we investigated inhibition of the prothrombinase complex by ATH versus AT + UFH and determined potential mechanisms involved in the enhanced anticoagulant actions of ATH.

Materials and Methods

Chemicals

All chemical reagents were of analytical grade. Sodium chloride and Tris [tris (hydroxymethyl) amino-methane] were purchased from Bioshop (Burlington, ON, Canada). Polyethylene glycol 8000, ethylene-diamine tetraacetic acid-disodium salt (Na_2EDTA) and calcium chloride dihydrate ($CaCl_2$) were from BDH Inc. (Toronto, ON, Canada). Hexadimethrine bromide (polybrene) was obtained from Aldrich Chemical Company Inc. (Milwaukee, WI, USA) and sucrose was purchased from ACP Chemicals Inc. (Montreal, QC, Canada). Human factor Xa, II and thrombin were purchased from Enzyme Research Laboratories Ltd. (South Bend, IN, USA). Human factor Va and fluorescein FPR-chloromethylketone (FPPAck) was obtained from Haematologic Technologies Inc. (Essex Junction, VT, USA). Substrates for Xa (S-2222; *N*-benzoyl-isoleucyl-glutamyl-glycylarginyl-*p*-nitroanili de-hydrochloride and its methyl ester) and thrombin (S-2238; H-D-phenylalanylpipecolyl-arginyl-*p*-nitroanilide-dihydrochloride) were purchased from DiaPh arma Group Inc. (Westchester, OH, USA). AT was purchased from Affinity Biologicals (Ancaster, ON, Canada). Unfractionated sodium heparin (UFH) was obtained from Sigma (St Louis, MO, USA). Thrombin inhibitor, Pefabloc-TH [Nα-(2-Naphthylsulfonylglycyl)-4-amidino-(D,L)-phenylalanine piperidide acetate (NAPAP)] was purchased from Pentapharm Ltd. (Engelgasse, Switzerland). L-α-phosphatidylcholine type XVI-E (PC), L-α-phosphatidyl-L-serine (PS) phospholipids and fluorescein isothiocyanate (FITC) were purchased from Sigma Aldrich Canada Ltd. (Oakville, ON, Canada). FPR-chloromethylketone dihydrochloride (PPAck) was obtained from Calbiochem (La Jolla, CA, USA).

Preparation of ATH

ATH was synthesized as described by Chan *et al.* (*40*). Briefly, 1,052 mg of human AT and 64 g of UFH was separately dialysed against 2 M NaCl followed by phosphate-buffered saline and mixed to a volume of 900 ml. The reagents were incubated at 40°C for 16 days. Following incubation, 0.5 M $NaBH_3CN$ reducing agent was added to the mixture and incubated for another 5 h at 37°C. ATH was purified using a two-method extraction process involving butyl-sepharose hydrophobic chromatography (Amersham, Uppsala, Sweden) and DEAE-sepharose anion exchange chromatography (Amersham, Uppsala, Sweden) for the removal of any unbound heparin or AT, respectively. ATH was analysed for purity using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under non-reducing conditions and was found to be >95% pure (*40*). We have previously shown that the AT content (amino acid analysis) and heparin content (by three different mass analysis methods) of ATH preparations are in a mole ratio of 1:1 (*46*).

Preparation of phosphatidylcholine/serine (PCPS) vesicles

PCPS vesicles in a 3:1 (PC:PS) concentration ratio were made by taking 60 µl of 250 mg/ml of PC and mixing with 100 µl of a 50 mg/ml solution of PS, followed by dissolution in two parts chloroform to one part methanol in a KIMAX borosilicate glass round bottomed tube (Daigger, Vernon Hills, IL, USA). The mixture was then dried under a light stream of argon gas. To remove any remaining organic solvents, the product was lyophilized for ~1 h. After the lipids were completely dried, they were re-suspended in 5 ml of 20 mM Tris, 0.15 M NaCl, pH of 7.4 buffer and sealed under an argon gas atmosphere. Tubes were then sonicated at 4°C for ~2 h or until the cloudy solution turned clear. The sonicated vesicles were transferred to Beckman Clear Ultracentrifuge tubes (Beckman Coulter, Brea, CA, USA) and centrifuged at 40,000 rpm in a SW55 rotor (Beckman Centrifuge) for 3 h. Resultant vesicle solution (4 ml) was then mixed with 0.4 g of sucrose and aliquots were stored at −80°C. Vesicle solution (1 ml) was used for quantification by phosphate analysis.

The PCPS vesicles were analysed for concentration by phosphate analysis. Thirty microlitre of magnesium nitrate solution [1 g of $Mg(NO_3)_2{\cdot}6H_2O$ per 10 ml of 95% ethanol] was added to 5 µl of the sample. The solution was heated over a Bunsen burner flame in a fume hood until brown fumes were produced by the solution. After cooling, 0.3 ml of 0.5 M HCl was added to hydrolyse any pyrophosphate that may have been formed. The tubes were then capped with a marble and put in a boiling water bath for 15 min. After cooling, 700 µl of one part 10% ascorbic acid to six parts 0.42% ammonium molybdate·$4H_2O$ in 0.5 M H_2SO_4 was added and incubated for 20 min at 45°C. After cooling for 15 min, 200 µl of solution was transferred to a 96-well round bottom well plate. Absorbance readings at 820 nm were taken using a SpectraMax Plate Reader for the samples and reference phosphate standards. A standard curve was produced from readings of 0, 5, 10, 15, 25, 50 and 70 µl of 1 mM phosphate standards. The PCPS vesicle concentration was determined by comparing the 820 nm absorbance readings from the samples against the standard curve.

Determination of second-order rate constants (k_2 values)

k_2 values for inhibition of Xa ± prothrombinase complex by AT + UFH or ATH were determined by performing discontinuous second-order rate constant assays under pseudo first-order conditions, as described previously (*45*). Briefly, rate experiments were performed at physiological temperature (37°C) and a minimum inhibitor:enzyme ratio of 10:1 was maintained for all reactions. To determine the

concentration of UFH that yields maximal inhibition of Xa by AT, a range of UFH concentrations were tested (Fig. 1). It was determined that maximal inhibition of Xa was achieved with a 10-fold mole ratio of UFH to AT or 300 nM UFH. For rate experiments involving the inhibition of free-Xa by AT + UFH or ATH, the reaction protocol was as follows: 10 µl aliquots of 6 nM Xa (3 nM final) containing 8 mM $CaCl_2$ (4 mM final) in TSP buffer (0.02 M Tris–HCl + 0.15 M NaCl + 0.6% polyethylene glycol, pH = 7.4) were incubated in seven separate wells of a 96-well flat bottom microtitre plate (Fisher, Nepean, ON, Canada) for 5 min. To well number 7, 10 µl of TSP buffer was added to represent the control reaction of the experiment. At time intervals ranging from 2 to 5 s, a 10 µl volume of inhibitor (30 nM AT + 300 nM UFH or 30 nM ATH final) was added to the remaining wells (one through six). At 2–5 s following addition of inhibitor to well 6, the reactions were neutralized by the addition of 80 µl of developing solution containing 1.25 mg/ml of polybrene + 0.53 mM Xa-specific substrate S-2222 + 1.14 mM of Na_2EDTA in TSP buffer. After terminating the inhibition reactions, residual Xa activity for each well was determined by measuring the absorbance at 405 nm (SpectraMax Plus 384 plate reader) for a specific period of time (10–30 min). For prothrombinase reactions, 5 µl of the solution (containing 9.6 nM Va, 48 µM PCPS, 1.2 µM II, 4 mM $CaCl_2$ and 40 µM Pefabloc-TH, final) were first incubated in seven wells for 2 min, followed by simultaneous addition of 5 µl of enzyme (3 nM Xa final) to all wells. Inhibitors were then added as described above. To calculate the pseudo first-order constant k_1, plots of $\ln V_t/V_o$ (V_t represents the enzyme activity at time t and V_o is the initial enzyme activity in well number 7) versus time were generated in order to determine the slope. The negative value of the slope gave k_1 ($k_1 = -$slope). The k_2 value was then calculated by dividing k_1 by the concentration of inhibitor [AT(H)] used ($k_2 = k_1/[\text{AT(H)}]$). Averages from at least five experiments were used to generate the final k_2 value.

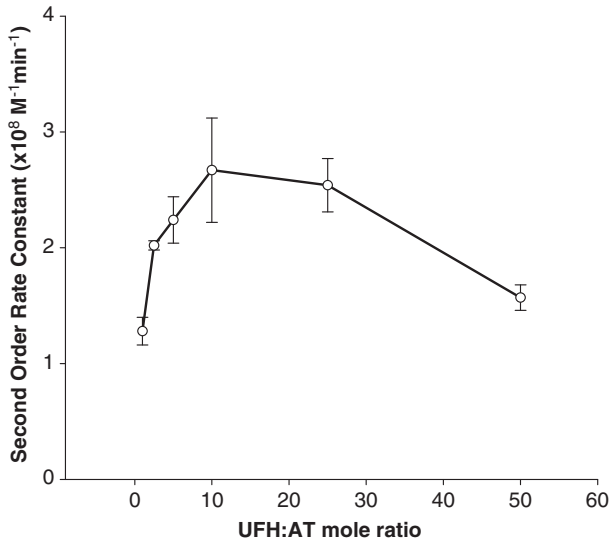

Fig. 1 Rate of Xa inhibition by AT and varying amounts of UFH. Xa (3 nM) was reacted with 30 nM AT + 30–3,000 nM UFH and the k_2 values determined. Plots of maximal k_2 values versus relative concentration of UFH (with respect to 30 nM AT) was produced and it was determined that a 10-fold mole ratio of UFH:AT (300 nM UFH) yielded the highest Xa inhibition by AT.

To investigate the roles of individual components of the prothrombinase complex on the anticoagulant effects of ATH versus AT + UFH, additional experiments were performed where components of the complex (II, PCPS or Va) were excluded prior to reaction with the inhibitors. To further understand the mechanisms, additional rate experiments were performed to evaluate the effect of mixing 300 nM UFH with 30 nM ATH on inhibition of prothrombinase-based Xa, with additional experiments repeated in the absence of II.

Thrombin generation

Thrombin-specific substrate S-2238 was used to assess the effect of inhibitors on thrombin generation by the prothrombinase complex. Varying levels of II (20–1,200 nM) were utilized to refine examination of inhibition by 30 nM AT + 300 nM UFH versus 30 nM ATH on prothrombinase inhibition. The concentration of the prothrombinase components were similar to those used above. Briefly, 10 µl of the prothrombinase mixture was added to each of the seven wells of a 96-well flat bottom plate. In well one, 10 µl of TSP buffer was added as a control and in wells two to seven 10 µl of the appropriate inhibitor was added at 5 s intervals. Five seconds after addition of inhibitor to the last well, 80 µl of 0.53 mM solution containing thrombin-specific substrate S-2238 in TSP buffer was added simultaneously to all wells, and immediately read in the plate reader at 405 nm, for 10 min. Linear portions of the V_{max} curves were utilized and compared against a standard curve to determine the concentration of thrombin being generated. Plots of thrombin concentration versus time were produced to compare the effect of inhibitors on thrombin generation by the prothrombinase complex, and the area under the curve was used to determine thrombin potential for the system.

Fluorescent microscopy

Xa was labelled with Fluorescein FPR-chloromethylketone (FPPAck-Xa) and experiments were performed to determine the binding interactions of active site-blocked Xa to the vesicle surface in combination with various components of the prothrombinase complex (PCPS, Va or II). Use of fluorescent, active-site blocked FPPAck-Xa allowed us to observe direct interaction of inhibitors with stable/static prothrombinase complexes since II remained in the complex without being cleaved.

Concentrations of the prothrombinase components in the fluorescence experiments were as follows: FPPAck-Xa was 8 nM, Va was 9.6 nM, PCPS was 48 µM, II was 1,200 nM and $CaCl_2$ was 4 mM. The concentrations of AT(H) were 30–300 nM and UFH were 300–2,000 nM. To avidly view vesicles containing prothrombinase under the microscope, only the large vesicles were studied in the reactions. All experiments were performed in the dark, under the same conditions

and experimental protocols, with systematic addition of $CaCl_2$, PCPS vesicles, Va and FPPAck-Xa to TSP buffer, followed by addition of appropriate concentrations of inhibitors. To determine if the inhibitors play a role on FPPAck-Xa assembly within or displacement from the prothrombinase complex, inhibitors were added in the reaction mixture either before or after FPPAck-Xa. The reaction mix was transferred to a glass microscope slide and viewed under the Leica DMIL inverted microscope (Leica Microsystems, Wetzler, Germany) using white light, from 40 to 400× magnification field. The 40× magnification field was used to locate the general area containing vesicles, whereas higher magnifications were used to hone in on the individual vesicle. The 400× magnification field was used to observe all vesicles in this study and all digital photographs were taken at 12× digital zoom using a Canon Power Shot: S31S digital camera attached to the #54150 assembly unit (Martin Microscope Company, SC, USA). Once vesicles were located under the 400× magnification field, a picture was acquired for a reference micrograph under white light. Immediately, the filter was changed to blue/UV light (Leica EL6000 External UV Assembly, Leica Microsystems, Wetzler, Germany) and another photograph was taken capturing fluorescence of the vesicle harboring the prothrombinase complex, which was used for fluorescence analysis.

Additional experiments were performed to validate that fluorescence was indeed occurring as a result of a full prothrombinase complex being constructed on the vesicle surface. These results were confirmed functionally by comparing thrombin generation of a system in which the conversion of II to thrombin occurred in the presence of all of the components of the prothrombinase complex compared to the conversion of II to thrombin by Xa alone (data not shown). Fluorescence intensity was determined by converting all photographs from JPEG to TIFF followed by conversion of the micrographs from color to 16-bit grey scale. Photographs were uploaded onto a Molecular Imager® GelDoc XR System (BioRad Laboratories Inc, Hercules, CA, USA) and vesicles appearing on the grey-scale micrograph were outlined by the manufacturer's image software. The software determined the intensity within the highlighted area and simultaneously converted the results to intensity per square millimetre (Int/mm^2) in order to normalize for any discrepancies in vesicle size.

To further understand the mechanism of ATH inhibition, additional fluorescence was conducted utilizing a functional prothrombinase system, by labelling amino groups on the Xa with FITC (F-Xa). The concentration of F-Xa was 8 nM and concentrations of the prothrombinase complex components were the same as above. Fluorescence experiments for the functional prothrombinase system were performed as described above, with the use of F-Xa instead of FPPAck-Xa in the presence of various concentrations of inhibitors (10–30 nM AT + 100–300 nM UFH or 10–30 nM ATH). Once vesicles harbouring the prothrombinase complex were located on the slide, digital micrographs were immediately taken at 15 s intervals for 75 s and analysed for fluorescence intensity utilizing the procedure described above. Plots of fluorescence intensity per unit area (Int/mm^2) versus time (s) were produced.

Experiments utilizing inhibitors labelled with FITC (F-UFH and F-ATH) were also carried out to examine interactions of the inhibitors with prothrombinase complex containing active site inhibited factor Xa (PPAck-Xa). Additionally, various prothrombinase components were excluded from the active-site blocked prothrombinase prior to interaction with fluorescent inhibitors and measurement of fluorescent intensity on the vesicles were determined.

Data analysis

The inhibition rate experiments were performed at $n = 5$, since previous work showed this number of replicates is sufficient to show statistical significance between groups and the number of replicates for the fluorescence experiments were conducted at a minimum of $n = 3$. Statistical analysis for multiple groups was performed using ANOVA. In the case of comparison between groups, a Student's *t*-test was used. Values with a $P < 0.05$ were considered significant.

Results

Comparison of k_2 values for inhibition of Xa ± prothrombinase complex

Discontinuous second-order rate constant assays were performed to determine the effect of prothrombinase complexation on k_2 values for inhibition of Xa by AT + UFH versus ATH and the underlying mechanisms involved. Results for inhibition of Xa alone or Xa within the prothrombinase complex are shown in Table I. Maximal k_2 values for non-conjugated AT + UFH occurred when reacted with Xa alone, whereas the k_2 value significantly decreased when Xa was incorporated into the prothrombinase complex ($P < 0.001$). Inhibition of free Xa by ATH resulted in

Table I. Inhibition of free Xa and prothrombinase complex by AT + UFH versus ATH.

Condition	AT + UFH (k_2 values ± SD)	ATH (k_2 values ± SD)
Free Xa	$1.47 \times 10^8 \pm 0.18 \times 10^8$	$2.83 \times 10^8 \pm 0.42 \times 10^8$
Prothrombinase complex	$0.67 \times 10^8 \pm 0.11 \times 10^8$	$2.11 \times 10^8 \pm 0.85 \times 10^8$
Prothrombinase devoid of II	$1.24 \times 10^8 \pm 0.08 \times 10^8$	$1.57 \times 10^8 \pm 0.08 \times 10^8$
Prothrombinase devoid of PCPS	$1.49 \times 10^8 \pm 0.18 \times 10^8$	$2.79 \times 10^8 \pm 0.32 \times 10^8$
Prothrombinase devoid of Va	$1.44 \times 10^8 \pm 0.18 \times 10^8$	$2.09 \times 10^8 \pm 0.19 \times 10^8$
Prothrombinase + UFH	–	$0.62 \times 10^8 \pm 0.07 \times 10^8$
Prothrombinase devoid of II + UFH	–	$1.8 \times 10^8 \pm 0.04 \times 10^8$

Table II. Inhibition of thrombin potential by AT + UFH versus ATH.

Condition (nM)	Control (nM • sec ± SD)	AT + UFH (nM • sec ± SD)	ATH (nM • sec ± SD)
20 nM II	$1.15 \times 10^3 \pm 0.34 \times 10^3$	$0.40 \times 10^3 \pm 0.07 \times 10^3$	$0.25 \times 10^3 \pm 0.06 \times 10^3$
300 nM II	$2.19 \times 10^4 \pm 0.58 \times 10^4$	$1.82 \times 10^4 \pm 0.21 \times 10^4$	$1.41 \times 10^4 \pm 0.16 \times 10^4$

significantly higher k_2 values, compared to those of AT + UFH ($P < 0.01$). In addition, the k_2 values for ATH inhibition of Xa within the prothrombinase was also significantly higher than either of the AT + UFH results ($P < 0.001$). Protection of Xa by the components of the complex was less observed for ATH compared to AT + UFH ($P < 0.0001$).

Inhibition of thrombin generation

Thrombin generation was performed to examine the effect of AT + UFH versus ATH on functionality of the intact prothrombinase complex with varying levels of II in the reaction mixture (Table II). When 20 and 300 nM of II was used to construct the prothrombinase, there was enhanced inhibition of thrombin generation by ATH compared with AT + UFH. Both inhibitors significantly decreased the thrombin potential ($P < 0.0001$), with ATH having a greater effect ($P < 0.001$).

Comparison of k_2 values for the inhibition of Xa by combining/excluding prothrombinase components

To examine the roles of prothrombinase components on mechanisms of Xa inhibition by AT + UFH versus ATH, discontinuous second-order rate constant assays were performed comparing the inhibition of the full prothrombinase to a prothrombinase where various components were omitted prior to reaction with inhibitors (Table I). When II was excluded from the complex, the k_2 values for inhibition of Xa by AT + UFH increased ($P < 0.001$), relative to intact prothrombinase. Similar results were observed for reactions devoid of PCPS or Va ($P < 0.001$). In contrast, exclusion of II from prothrombinase significantly decreased the k_2 for Xa inhibition by ATH ($P < 0.05$). Although removal of PCPS from the system resulted in a higher k_2 ($P < 0.01$), exclusion of Va resulted in no significant difference when compared with the intact prothrombinase for ATH reactions ($P < 0.80$).

Differences in effects of II exclusion on Xa inhibition by AT + UFH versus ATH were studied more closely. We posited that mechanisms behind these opposing results might lie in the interactions of free UFH not permanently bound to AT. Thus, experiments were carried out to examine the impact of excess UFH on inhibition of Xa by ATH, with or without II in the prothrombinase complex (Table I). The k_2 values for inhibition of Xa within an intact prothrombinase significantly decreased when excess UFH was added to reactions containing ATH ($P < 0.001$). To test whether excess UFH may prevent non-specific interactions with the vesicle surface by the conjugate, we examined the rate of inhibition by ATH + UFH of Xa in prothrombinase devoid of II. For this experimental reaction, the k_2 values significantly increased when compared with reactions without excess UFH ($P < 0.001$), suggesting that free UFH may compete with ATH for sites at the vesicle surface which keep ATH from reacting with Xa.

Table III. Fluorescence intensity of the prothrombinase complex and its components.

Condition	Fluorescence (Int/mm^2)
Full prothrombinase	$4.25 \times 10^3 \pm 0.98 \times 10^3$
Vesicles + FPPAck-Xa	$0.204 \times 10^3 \pm 0.068 \times 10^3$
Vesicles + Ca^{2+} + FPPAck-Xa	$1.04 \times 10^3 \pm 0.15 \times 10^3$
Vesicles + Ca^{2+} + Va + FPPAck-Xa	$1.66 \times 10^3 \pm 0.27 \times 10^3$
Vesicles + Ca^{2+} + II + FPPAck-Xa	$1.13 \times 10^3 \pm 0.096 \times 10^3$

Fluorescence microscopy and formation of the prothrombinase complex

Fluorescence microscopy was performed to determine the structural construction of the prothrombinase complex on the vesicle surface and to examine the effects of the inhibitors on Xa stability within the prothrombinase. Table III shows numerical values for fluorescent intensity of a prothrombinase complex with or without various components. Systematic exclusion of prothrombinase components from the reaction mixture results in a significant loss of overall fluorescence from the system. Furthermore, it is evident that high intensity of fluorescence is achieved only when all of the components of the prothrombinase are present ($P < 0.05$). Additional functional experiments (chromogenic thrombin generation activity) were performed to validate these results (data not shown).

Effect of UFH, AT + UFH and ATH on fluorescence intensity of a static prothrombinase complex

Labelling Xa with FPPAck at its active-site inhibits the serine hydroxyl of the protease, which creates a static and non-reactive prothrombinase complex. Fluorescence intensity for reactions of inhibitor with the fluorescently tagged prothrombinase is listed in Table IV. Interacting AT + UFH or UFH alone with the prothrombinase complex resulted in diminished fluorescence intensity ($P < 0.01$), which was not evident with ATH. UFH reactions with the static prothrombinase complex reduced FPPAck-Xa fluorescence intensity in a dose-dependent manner, albeit statistically not significant. Fluorescence intensity was similar whether UFH was added before or after FPPAck-Xa. Similar results were observed when AT was included with UFH during interactions with the prothrombinase; however, the dose-dependent effect was not evident.

Table IV. Effect of UFH on FPPAck-Xa binding within the prothrombinase complex.

Condition	Fluorescence (Int/mm^2)
Full Prothrombinase	$4.25 \times 10^3 \pm 0.98 \times 10^3$
Prothrombinase + 300 nM UFH[a]	$1.46 \times 10^3 \pm 0.35 \times 10^3$
Prothrombinase + 2,000 nM UFH[a]	$1.05 \times 10^3 \pm 0.13 \times 10^3$
Prothrombinase + 300 nM UFH[b]	$1.41 \times 10^3 \pm 0.015 \times 10^3$
Prothrombinase + 2,000 nM UFH[b]	$1.30 \times 10^3 \pm 0.086 \times 10^3$
Prothrombinase + 30 nM AT + 300 nM UFH[a]	$1.28 \times 10^3 \pm 0.25 \times 10^3$
Prothrombinase + 30 nM AT + 2,000 nM UFH[a]	$2.84 \times 10^3 \pm 0.68 \times 10^3$
Prothrombinase + 30 nM AT + 300 nM UFH[b]	$1.16 \times 10^3 \pm 0.12 \times 10^3$
Prothrombinase + 30 nM AT + 2,000 nM UFH[b]	$1.23 \times 10^3 \pm 0.18 \times 10^3$
Prothrombinase + 300 nM ATH[a]	$3.24 \times 10^3 \pm 0.33 \times 10^3$
Prothrombinase + 2,000 nM ATH[a]	$3.22 \times 10^3 \pm 0.80 \times 10^3$
Prothrombinase + 300 nM ATH[b]	$3.99 \times 10^3 \pm 0.64 \times 10^3$
Prothrombinase + 2,000 nM ATH[b]	$3.388 \times 10^3 \pm 0.49 \times 10^3$

[a](AT) UFH or ATH added 'before' FPPAck-Xa during prothrombinase assembly.
[b](AT) UFH or ATH added 'after' FPPAck-Xa during prothrombinase assembly.

In contrast, addition of ATH to the prothrombinase minimally reduced fluorescence intensity and lack of impact by the conjugate was dose- or order independent.

Effect of UFH, AT + UFH and ATH on fluorescence intensity of a functional prothrombinase complex

A functional prothrombinase complex was produced by developing an F-Xa containing prothrombinase complex. The lysl groups of Xa were reacted with FITC, thus leaving the serine hydroxyl group of Xa unaffected and capable of reacting with II or AT. Functional assessment of F-Xa revealed no compromise in Xa function (results not shown). Figure 2A depicts the fluorescence intensity of the functional prothrombinase complex over a period of 75 s, while reacting with either 300 nM UFH, 30 nM AT + 300 nM UFH or 30 nM ATH. The fluorescence of the functional prothrombinase without any inhibitors decreased somewhat linearly over the time course due to loss of prothrombinase complexes as II was consumed. However, adding UFH to the prothrombinase caused a relative drop in fluorescence, suggesting that UFH may be slightly disrupting Xa within the prothrombinase and not allowing it to concentrate on the vesicle surface. These results are similar to the ones observed in the static prothrombinase complex. When AT was mixed with UFH, fluorescence was knocked down to a background level over the time course. Similar results were observed for ATH reactions, although the background fluorescence appeared slightly higher compared with the AT + UFH.

We then reacted lower concentrations of AT + UFH (10 nM AT + 100 nM) and ATH (10 nM) with the functional prothrombinase to better differentiate effects of these two inhibitors on F-Xa fluorescence (Fig. 2B). Lower concentrations of AT + UFH strongly increased the initial fluorescence by $\sim 1.50 \times 10^3$ Int/mm^2, but intensity declined over the experimental time period to the same degree as the higher levels of inhibitors. Similarly, when ATH was reacted with the prothrombinase, there was an apparent increase in initial fluorescence by 2.80×10^3 Int/mm^2 with a steady decline over the period of time. Reacting the two concentrations of ATH with the functional prothrombinase seemed to have retained fluorescence intensity, relative to AT + UFH. These strong trends suggest that, although ATH more readily inhibits Xa within the prothrombinase, the Xa (bound to ATH inhibitor) may not be displaced from the prothrombinase to the same degree as AT + UFH, consistent with results from the static prothrombinase.

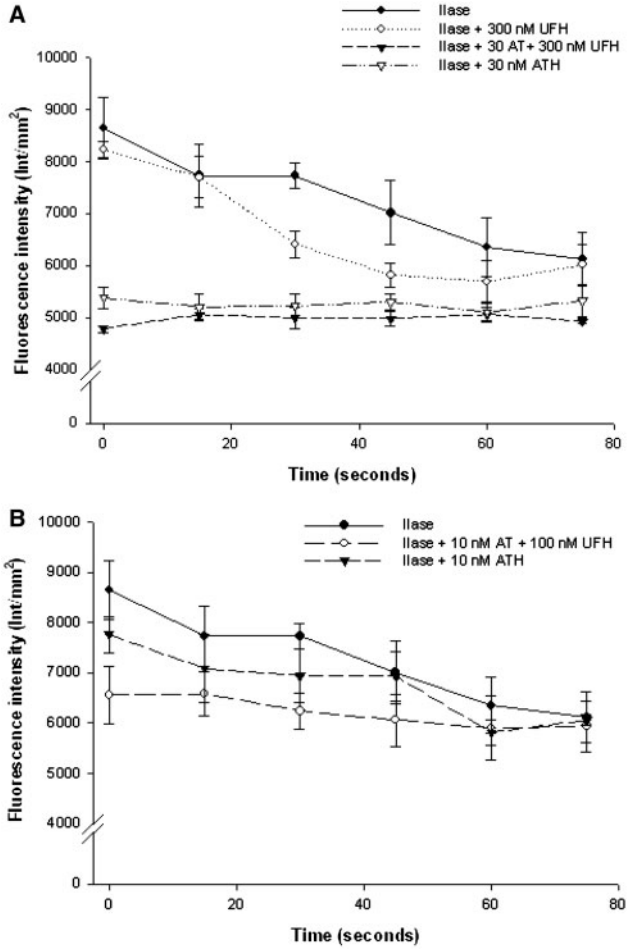

Fig. 2 Fluorescent microscopy illustrating the effect of UFH, AT + UFH and ATH on fluorescence intensity over time of a functional prothrombinase (IIase) complex. Using higher levels of inhibitors (A), the fluorescence intensity is quenched to a baseline reading over time. UFH alone decreases the fluorescence prematurely, suggesting fluorescent F-Xa displacement from the IIase complex in the presence of UFH. When lower levels of inhibitors were used (B), the fluorescence increased relative to the higher concentrations; however, the intensity was still lower than the control.

Dependence of various prothrombinase components on F-UFH and F-ATH binding to prothrombinase

In order to study a stable (static) prothrombinase complex with F-UFH and F-ATH, PPAck-Xa was used (Table V). The F-UFH bound to prothrombinase, prothrombinase without factor Va and prothrombinase without II, suggests that F-UFH may interact with variable combinations of components within the prothrombinase complex. Noteworthy, the F-UFH may also bind to PPAck-Xa, but addition of other components greatly enhanced F-UFH binding. In contrast, F-ATH showed no significant binding capabilities except for cases where the full prothrombinase or prothrombinase without II was present.

Discussion

Anticoagulation by UFH has been clinically used for the treatment and prophylaxis of thrombotic complications (*22*). Mechanistically, it is well established that a specific pentasaccharide sequence on UFH is involved in conformational activation of AT, which gives rise to a structure of AT that is more favourable for reacting with most serine proteases (*23*, *47*). A separate mechanism exists for thrombin inactivation called template-mediated inhibition, which additionally requires the UFH molecule to bridge AT and thrombin together (*48*, *49*). Inhibition of thrombin through the template-mediated reaction is chain length dependent, requiring the UFH molecule to contain 18 or more saccharides (*33*). AT interaction with UFH occurs through non-covalent means, which allows for UFH to dissociate from AT and may attenuate its catalysis of surface-bound enzyme inhibition (*50*, *51*). Other limitations have been discussed above; however, it is well demonstrated that Xa is protected from AT + UFH inhibition when it is incorporated in the prothrombinase complex (*33*, *34*). The degree to which this protection occurs has been variably reported due to the difficulty of measuring Xa activity in the presence of II, as AT binds both Xa and the reaction product thrombin (*34*, *52*). Several attempts have been made to measure Xa activity using a mutant AT that only binds Xa and not thrombin (*33*) or using a mutant II that generates a thrombin, which does not react with AT (*34*) (the latter potentially giving more accurate inhibition rates). However, these methods fail to use an assay containing a native prothrombinase complex, where none of the enzymes or components have been modified. In our study, we utilized an assay containing a native prothrombinase complex in the presence of a serine protease inhibitor called pefabloc-TH. Pefabloc-TH is highly reactive with thrombin, and we have verified that pefabloc-TH minimally cross-reacts with Xa (data not shown). Therefore, by using vast excess of pefabloc-TH in our assay, this ensured that any thrombin generated by prothrombinase was quickly quenched by the pefabloc-TH, allowing us to study only AT + UFH or ATH reaction with the Xa.

Regulating the prothrombinase complex is very important in individuals with thrombotic complications. Since we have previously demonstrated that ATH significantly accelerates the inhibition of clot-bound thrombin (*41*), we therefore wanted to determine whether ATH is similarly effective for inhibition of the prothrombinase complex and propose a mechanism of action for the anticoagulation by ATH in relation to the non-conjugated inhibitor + catalyst system.

Based on our findings, we have shown that the prothrombinase complex hinders the inhibitory action of AT + UFH on Xa (consistent with previous findings), whereas ATH is less affected. In fact, ATH inhibition of the prothrombinase complex was significantly higher than the inhibition rate for either free or prothrombinase-bound Xa by AT + UFH. Similar to inhibition of fibrin-bound thrombin (*41*), UFH in the non-covalent AT + UFH dissociates and binds to components or products of the prothrombinase complex, which leaves AT alone in the non-activated state. Furthermore, UFH complexation with prothrombinase or its products may act to repel any incoming AT + UFH to cause protection of Xa from inhibition. However, formation of these complexes on the prothrombinase cannot occur when AT is covalently linked to UFH, as in the case of ATH, thus allowing for enhanced inhibition of prothrombinase-bound Xa by ATH.

Previous studies have shown that in the presence of all the prothrombinase components, Xa was most protected from inhibition by pentasaccharide (fondaparinux), followed by LMWH (enoxaparin) and then regular heparin (*34*). In our study, we clearly showed that ATH overcame the protective effect and gave a modest 3.11-fold increase in the rate of Xa inhibition

Table V. Fluorescence intensity of F-UFH and F-ATH interactions with prothrombinase ± components of the complex.

Condition	F-UFH (Int/mm^2)	F-ATH (Int/mm^2)
Full prothrombinase	$1.51 \times 10^4 \pm 0.16 \times 10^4$	$1.10 \times 10^4 \pm 0.22 \times 10^4$
Vesicles + Ca^{2+}	$3.41 \times 10^3 \pm 0.34 \times 10^3$	$4.34 \times 10^3 \pm 0.35 \times 10^3$
Vesicles + Ca^{2+} + Va	$4.54 \times 10^3 \pm 0.15 \times 10^3$	$3.86 \times 10^3 \pm 0.45 \times 10^3$
Vesicles + Ca^{2+} + II	$4.81 \times 10^3 \pm 0.67 \times 10^3$	$5.14 \times 10^3 \pm 2.04 \times 10^3$
Vesicles + Ca^{2+} + PPAck-Xa	$6.48 \times 10^3 \pm 1.21 \times 10^3$	$3.68 \times 10^3 \pm 0.78 \times 10^3$
Vesicles + Ca^{2+} + Va + II	$3.79 \times 10^3 \pm 0.92 \times 10^3$	$4.56 \times 10^3 \pm 0.13 \times 10^3$
Prothrombinase without II	$8.16 \times 10^3 \pm 1.99 \times 10^3$	$9.32 \times 10^3 \pm 2.91 \times 10^3$
Prothrombinase without Va	$9.13 \times 10^3 \pm 0.75 \times 10^3$	$5.33 \times 10^3 \pm 0.22 \times 10^3$

in the presence of all of the components of the complex, compared with heparin. Although a small protective effect by the prothrombinase was observed for Xa reaction with ATH, this difference was minor compared to the inhibition rates observed with AT + UFH. One possibility to explain this feature could be non-specific interaction of heparin chains from some ATH molecules with the prothrombinase complex. During the synthesis of ATH, the AT selects for relatively large UFH molecules that contain the high-affinity pentasaccharide sequence, with up to 30% of the ATH molecules containing two or more pentasaccharide sequences (*40*). The longer heparin chains in this small subpopulation of ATH molecules may interact non-specifically with the prothrombinase, giving a small hindrance in Xa inhibition. Our work also suggests that the calcium–phospholipid surface associating with prothrombinase components may be an important player responsible for deterring ATH from inhibiting Xa, particularly in absence of II. Whereas for AT + UFH, excess UFH molecules may neutralize exposed calcium–phospholipid that normally bind II so that incoming AT + UFH inhibits Xa and is not attracted to these sites when II is absent. However, with no free heparin chains to interrupt such binding site associations, ATH will tend towards these non-useful interactions. To confirm this clustering effect for ATH in the absence of II, we attempted to interrupt ATH interactions with any such binding sites by addition of excess UFH. This caused a moderate increase in Xa inhibition by the ATH. However, when experiments with added UFH were reproduced with a fully intact prothrombinase, the rate of Xa inhibition by ATH significantly decreased (suggesting bridging of free heparin with combinations of components in the full prothrombinase to repel binding of ATH through its heparin chain). Thus, the only aspects which might differentiate ATH's improved inhibition of Xa in prothrombinase are: covalent attachment of heparin to AT and absence of free non-pentasaccharide UFH within the reaction system.

Thrombin generation assays revealed strong trends to indicate that ATH is able to inhibit the function of the prothrombinase complex to a better degree than AT + UFH. When moderate to low levels of II were used (more indicative of local bursts of thrombin generation *in vivo*), ATH was capable of inhibiting thrombin generation better than AT + UFH. At the lower levels of II reaction, less thrombin was generated and not all of the inhibitors reacted with the thrombin, consequently the inhibitors could inhibit Xa to a better degree.

Our fluorescence results indicate that UFH alone may somewhat destabilize the prothrombinase, in part, by displacing the Xa from the complex. This is in sync with previously published work, which showed that heparin may displace Xa, and to a degree II, from the complex (*53*). In our work, we show that fluorescence intensity of the prothrombinase complex decreased in both the static and functional prothrombinase assays in the presence of UFH. We also found that the UFH in AT + UFH mixtures may displace the Xa. Non-covalent interactions of heparin with AT in AT + UFH enables the UFH to dissociate from AT, which provides free UFH molecules to destabilize the prothrombinase complex. However, if UFH interacts with prothrombinase to displace Xa, AT dissociated from its UFH will poorly react with any free Xa. In the static prothrombinase, the displacement of Xa was not observed with ATH and in the functional prothrombinase, the effect was not observed to the same degree as with AT + UFH or UFH alone. Although ATH reacts avidly with prothrombinase-incorporated Xa compared with AT + UFH, slower loss of F-Xa fluorescence suggests that Xa-ATH complexes may have affinity to assist retention in the prothrombinase. Cumulatively, these results imply that ATH may not be mechanistically acting in the same manner as UFH for inhibiting the prothrombinase complex.

Binding studies using the fluorescent inhibitors similarly demonstrate that F-UFH is capable of binding to the prothrombinase and, with varying affinities, to its component combinations. Prothrombinase-complexed UFH may prevent additional AT + UFH complexes from inhibiting Xa. Removal of II decreases these non-productive free UFH associations and allows better access/approach of AT + UFH to enhance Xa inhibition. F-ATH showed moderate binding only to full prothrombinase and, more importantly, to prothrombinase devoid of II. We propose that ATH may be interacting with sites in close proximity to Xa normally occupied by II, thus causing inappropriate interaction with Xa. Moreover, these associations may be occurring through the long heparin chain of ATH, since addition of excess UFH provided recovery of Xa inhibition.

Overall, these findings reveal important mechanisms involved in the enhanced anticoagulant action of ATH against surface-bound coagulation enzymes, particularly the prothrombinase complex. It is clear that the prothrombinase components hinder the ability of AT + conventional heparins to inhibit Xa. However, covalent linkage between AT and UFH assists access and neutralization of complexed Xa, with concomitant inhibition of prothrombinase function. Enhanced inhibition of serine proteases on surfaces and within supramolecular complexes encourages further investigation of ATH for application in thrombosis.

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Conflict of Interest

None declared.

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