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Effect of endothelium on the anticoagulant activity of a covalent antithrombin-heparin complex

Helen M. Atkinson^{1,2}, Ivan Stevic³, Leslie R. Berry^{1,2} & Anthony K. C. Chan^{1,2,4} 

We developed a covalent antithrombin-heparin complex (ATH) with superior *In vivo* anticoagulant efficacy compared to non-covalent antithrombin (AT) + unfractionated heparin (H). Previous *in vitro* studies of ATH, investigating the mechanisms behind its efficacy, were done in the absence of endothelium. Since the endothelial surface modulates hemostasis, we investigated its impact on the *in vitro* anticoagulant properties of ATH and AT+H. Discontinuous second order rate constant enzyme inhibition assays, fibrin formation, and plasma clot generation were performed in the presence of ATH or AT+H, with and without endothelium present. ATH had an increased rate of direct inhibition of IIa and Xa, and increased inhibition of IIa-induced fibrin formation, compared to AT+H. When compared at equal anti-Xa levels, ATH was less effective than AT+H at catalyzing inhibition of plasma clot generation. These results were found in both the presence and absence of endothelium. Endothelium decreased the rate of IIa inhibition, and reduced clot time in IIa-induced fibrin formation and plasma clot generation assays, for both ATH and AT+H. Endothelium did not impact the activity of ATH differently to AT+H. This supports the growing body of evidence suggesting ATH may be a beneficial anticoagulant for potential clinical use.

Heparinoid anticoagulants are commonly used to treat and prevent thrombotic disease. Heparinoids function by catalyzing the anticoagulant activity of the plasma protease inhibitor antithrombin (AT). Unfractionated heparin (H) suffers from a number of shortcomings, including inconvenient IV administration requiring hospitalization, a short half-life, variable anticoagulant response, requirement for frequent monitoring, and limited effectiveness at inhibiting clot-bound IIa. Heparin also increases the risk of complications such as adverse bleeding or heparin-induced thrombocytopenia^{1,2}. A number of additional anticoagulants have been developed in an attempt to address these shortcomings, such as orally administered anticoagulants (Vitamin K antagonists, direct oral anticoagulants (DOACs)), direct thrombin inhibitors (bivalirudin, argatroban), and heparin derivatives (low molecular weight heparins (LMWH's), fondaparinux). While many of these molecules have become the new clinical standard for prophylaxis or treatment of a variety of specific thrombotic pathologies, there remain certain clinical indications, such as cardiopulmonary bypass (CPB), continuous renal replacement therapy (CRRT), or hemodialysis, where unfractionated heparin anticoagulation, despite its limitations, is still the standard of care^{3,4}.

We have developed a covalent antithrombin-heparin complex (ATH) which has potential as an improved heparin-based therapeutic agent. *In vivo*, ATH had an increased half-life⁵, and displayed increased antithrombotic efficacy in animal models of venous⁶ and arterial⁷ thrombosis without increased risk of bleeding. Administration of ATH in a pig CPB model reduced the occurrence of brain microemboli compared to H alone, and had comparable or reduced risk of bleeding⁸. *In vitro*, ATH directly inhibits several coagulation factors with significantly increased rates of inhibition compared to non-covalent AT and H mixtures (AT+H)⁹. ATH has decreased non-specific binding to plasma proteins and endothelial cells¹⁰, and is more effective at inhibiting clot-bound IIa compared to AT+H¹¹.

In vivo, the endothelial surface can modulate hemostasis in various ways, for example by providing protein receptor binding sites for thrombin (e.g. thrombomodulin (TM)) which alter its activity; or by expressing

¹Thrombosis and Atherosclerosis Research Institute, McMaster University, Hamilton, ON, Canada. ²Department of Pediatrics, McMaster University, Hamilton, ON, Canada. ³Pathology and Laboratory Medicine, London Health Sciences Centre and Western University, London, ON, Canada. ⁴Thrombosis and Atherosclerosis Research Institute (TaARI), DBCVSRI, Hamilton General Hospital, 237 Barton St. E., Hamilton, ON L8L 2X2, Canada.  email: akchan@mcmaster.ca

anticoagulant glycosaminoglycan (GAG) molecules (e.g. heparan sulfate (HS)) on its surface which helps with anticoagulation through heparin-like antithrombin cofactor activity^{12,13}. The in vitro investigations of ATH anticoagulant properties have primarily been carried out in plasma or buffer solutions containing purified proteins, in the absence of endothelium. Thus, the effects the endothelium may have on ATH's anticoagulant mechanisms have not been studied before. Therefore, the objective of this study was to assess the influence of an endothelial surface on the anticoagulant mechanisms of ATH and AT+H. This will lend further physiological relevance to our mechanistic studies, and help to bridge the gap in knowledge between our previous *In vivo* and *in vitro* data. This will be particularly important in our clinical development of ATH as an anticoagulant and determining potential dosing, which is presently underway for CPB.

Methods

Materials

Human umbilical vein endothelial cells (HUVEC), EBM-2 media and EGM-2MV bullet kits were purchased from Lonza (Walkersville, MD, USA). Minimal Essential Media (MEM) was purchased from Invitrogen. IIa and Xa were from Enzyme Research Laboratories (South Bend, IN, USA). Human normal pooled plasma and purified human AT were purchased from Affinity Biologicals (Ancaster, ON, CA). H, hexadimethrine bromide (polybrene), heparan sulfate and gelatin were purchased from Sigma (Mississauga, ON, CA). Fibrinogen (plasminogen, fibronectin, FXIII-depleted) and recombinant human thrombomodulin were from American Diagnostica Inc. (Stamford, CT, USA). S-2238 and S-2222 were from Diapharma (West Chester, OH, USA). ATH was produced as previously described⁵. All other reagents were of reagent grade quality. We have previously determined that Sigma H has an average molecular weight of 15 kDa, and the heparin moiety of ATH has an average molecular weight of 18 kDa^{5,14}. Catalytic Anti-Xa activities of H and ATH were determined as previously described using a Stachrom heparin colorimetric assay from Diagnostica Stago, with comparison to an unfractionated heparin standard curve^{5,15}. Assays were done in the presence of exogenously added excess AT and thus measured the catalytic activity of the H chains. Catalytic anti-Xa values were 9.604 U/nmol for ATH and 3.135 U/nmol for H.

Cell culture

HUVEC were cultured on tissue culture treated plasticware (Primaria, BD, Mississauga, ON, CA) coated with 2% gelatin. For experiments, cells were seeded in 96-well plates at 20000-40000 cells/mL and grown to confluence in EBM-2 media supplemented with EGM-2MV Bullet kit, in a humidified air/5% CO₂ atmosphere. Cells were used between passages 2 and 5. There were 35000 \pm 13800 (mean \pm s.d.) cells in the wells at confluence.

Rate experiments

Second order rate constant (k_2) values for ATH and non-covalent AT+H inhibition of IIa and Xa were measured at 37 °C by a discontinuous assay under pseudo-first order conditions (inhibitor:enzyme ratio = 10:1) using a method similar to Patel et al.⁹. IIa, Xa, AT+H and ATH were diluted in Minimal Essential Media (MEM) containing 10mM HEPES pH 7.4 and 0.1% (w/v) PEG8000 (MEMPH). IIa or Xa was incubated with AT+H or ATH in wells of a 96-well plate containing confluent HUVEC monolayers. Endothelial cell monolayers were washed with MEMPH before addition of reaction components. The molar ratios of H:AT in the non-covalent AT+H mixtures were 23:1 and 10:1 for reactions with IIa and Xa, respectively. These H:AT ratios were previously found to produce maximal k_2 values for inhibition in the absence of HUVEC by determining k_2 values across a large range of H concentrations^{9,16}. After incubating for various time intervals, reactions were stopped by addition of 1.25 mg/mL polybrene in TSP buffer (20mM Tris-Cl, 150mM NaCl, 0.6% (w/v) PEG8000) containing 0.4 mg/mL of the appropriate chromogenic substrate (S-2238 for IIa or S-2222 for Xa). Residual enzyme activities in each well were measured as the change in absorbance at 405 nm (A_{405}) over time using a SpectraMax Plus 384 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The k_2 values were calculated from these results as previously described⁹. Identical assays were also performed in wells without HUVEC for comparison, using untreated Falcon Pro-Bind Flat Bottom 96-well plates (BD, Franklin Lakes, NJ, USA).

Fibrin formation assay

Wells containing confluent HUVEC monolayers were washed with MEMPH, then 20 μ L of 2nM IIa (diluted in MEMPH) was added to wells. Following a 3 min incubation at 37 °C, 80 μ L of MEMPH containing 1 mg/mL human fibrinogen and varying concentrations (0–0.75nM) of AT+H or ATH were added simultaneously to the wells. Fibrin clot formation at 37°C was monitored turbidimetrically by measuring optical density at 350 nm (OD_{350}) using a SpectraMax Plus 384 spectrophotometer, similarly to previously published studies^{17,18}. The time to clot formation (clot time) was defined as the time for the OD_{350} to reach the midpoint of the clear to maximum turbidity transition¹⁷. Assays were also performed in wells without HUVEC for comparison. In some assays, 2nM IIa was mixed with 20nM thrombomodulin (TM) or 5 μ M heparan sulfate (HS) prior to the 3 min incubation step in wells without HUVEC.

Inhibition of cell-associated IIa was also assessed by this method, with the following modifications: 40 μ L of 150nM IIa was incubated with HUVEC monolayers for 3 min at 37°C. The supernatant containing non-associated IIa was then removed and cells were washed once with MEMPH. 100 μ L of 0.8mg/mL fibrinogen solutions containing various concentrations of ATH or AT+H was then added simultaneously to each well and clot formation monitored, as above.

Plasma clot generation assay

Wells containing HUVEC monolayers were washed with HBS (20mM HEPES, 150mM NaCl, pH 7.4), before incubating for 3 min at 37 °C with a mixture of 50 μ L of normal human platelet poor plasma and 25 μ L of AT+H

or ATH at varying concentrations (0–25nM AT or ATH). The concentration of H in the AT+H mixture gave an anti-Xa level equal to ATH, resulting in an H:AT molar ratio of 3:1. Coagulation was initiated by simultaneous addition of 25 μ L of 40mM CaCl_2 to the wells. Clot formation at 37 °C was monitored turbidimetrically, by measuring OD_{350} using a SpectraMax Plus 384 spectrophotometer. The clot time was defined as the time for the OD_{350} to reach the midpoint of the clear to maximum turbidity transition. If the OD_{350} did not reach 0.0500 by 90 min, the clot time was assessed as “no clot”. Clot generation was also performed in wells without HUVEC for comparison. Replicate ($n = 6$) clot generation reactions were performed at each dose of anticoagulant on each surface. Where 4 or more replicate reactions resulted in a clot with a measurable clot time, the results are reported as mean \pm SEM. Otherwise, the results are denoted as “no clot” along with the number of reactions out of 6 where this occurred.

Statistics

Results are expressed as mean \pm SEM. Analysis of statistical significance was performed using two-way ANOVA (Figs. 1, 3 and 4) or three-way ANOVA (Fig. 2, Table 1). The data was log transformed to stabilize the variance. ANOVAs were performed using GraphPad Prism 10.2.3. In all tests $p < 0.05$ was considered significant. For comparison of fibrin formation in the absence of anticoagulants initiated by IIa-TM versus IIa, or IIa-HS versus

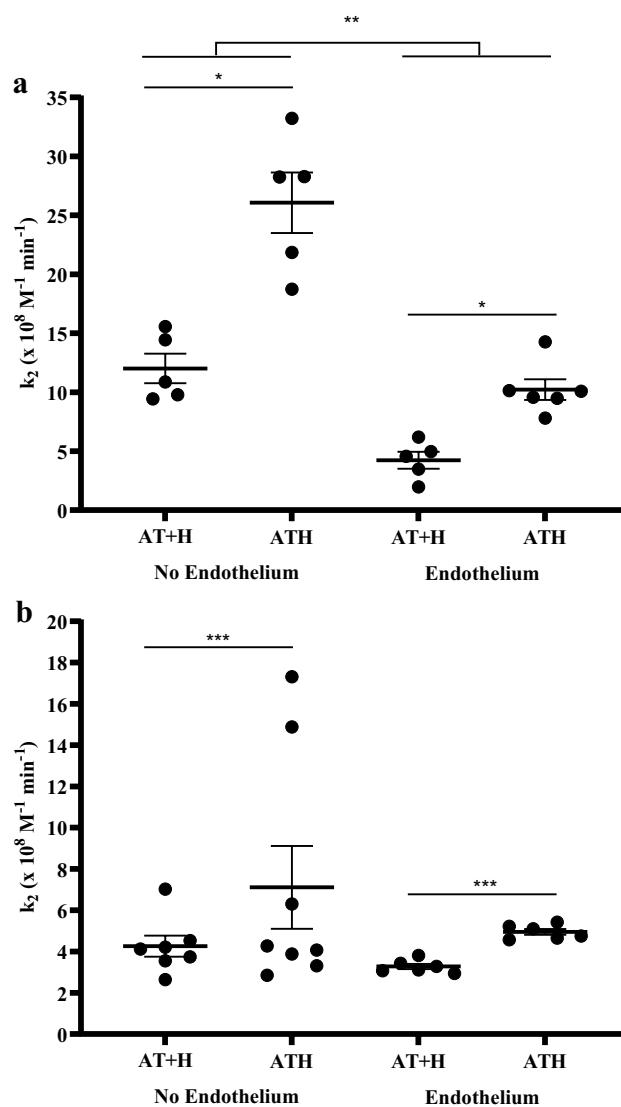


Fig. 1. Rate of inhibition of coagulation factors by ATH and AT+H in the presence of endothelium. K_2 values were determined for inhibition of IIa (a) and Xa (b) by ATH and AT+H in the presence or absence of a HUVEC monolayer. K_2 values were measured under pseudo first order conditions using a discontinuous method. Circles represent individual data points, and horizontal lines and error bars represent mean \pm SEM ($n \geq 5$). P values are for the indicated comparisons as performed by two-way ANOVA, reflecting the significant main effects of surface and anticoagulant. $^*p(\text{ATH vs. AT+H}) < 0.0001$. $^{**}p(\text{endothelium vs. no endothelium}) < 0.0001$. $^{***}p(\text{ATH vs. AT+H})=0.0299$.

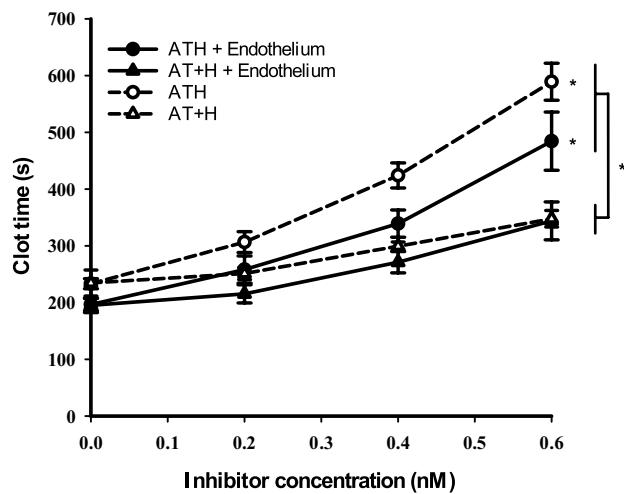


Fig. 2. Inhibition of IIa-induced fibrin formation by ATH and AT+H in the presence of endothelium. IIa was incubated in the presence or absence of a HUVEC monolayer, before addition of a mixture of purified fibrinogen, CaCl_2 and AT+H or ATH. Final inhibitor (AT or ATH) concentrations are indicated in the X-axis, and the molar ratio of H:AT in the AT+H mixture was 23:1. Fibrin formation was monitored turbidimetrically, and the clot time represents the time to reach the midpoint of the clear to maximally turbid transition. Data represent mean \pm SEM (n=5). The P values are for the indicated comparisons performed by three-way ANOVA, reflecting the significant main effects of anticoagulant and surface. *p(ATH vs. AT+H)<0.0001. **p(endothelium vs. no endothelium) < 0.0001.

Concentration (U/mL)	ATH		AT+H	
	Endothelium	No Endothelium	Endothelium	No Endothelium
0	16.3 \pm 1.4	24.3 \pm 0.9	17.6 \pm 1.3	21.8 \pm 1.0
0.024	17.7 \pm 1.8	25.5 \pm 2.1	18.4 \pm 1.3	32.7 \pm 2.2
0.06	18.2 \pm 1.2	32.4 \pm 4.1	26.4 \pm 2.6 (n=5), no clot (1/6)	48.7 \pm 2.3 (n=5), no clot (1/6)
0.12	27.3 \pm 1.1	39.7 \pm 5.6	no clot (5/6)	45.8 \pm 12.5 (n=4), no clot (2/6)
0.18	40.3 \pm 7.9 (n=4), no clot (2/6)	No clot (4/6)	No clot (5/6)	No clot (4/6)
0.24	No clot (5/6)	No clot (4/6)	No clot (6/6)	No clot (5/6)

Table 1. Clot times (min) of plasma clot generation reactions. Data are mean \pm SEM (n = 6, except where indicated). “No clot” indicates no measurable clot time within 90 min, and the number of reactions out of 6 where this occurred is shown in brackets. At anticoagulant concentrations up to 0.06 U/mL $p(\text{ATH vs. AT+H}) = 0.0002$ and $p(\text{endothelium vs. no endothelium}) < 0.0001$.

IIa, Student’s t tests were performed using GraphPad Prism 10.2.3. Data in Figure 1 was assessed for the presence of outliers using the Rout test in GraphPad Prism, with Q = 1%.

Results

Effect of endothelial surface on rate of inhibition of IIa and Xa

To assess the effect of endothelium on inhibition of IIa and Xa by non-covalent AT+H or ATH, we determined k_2 values for the rates of inhibition of these proteases in the absence or presence of an endothelial monolayer. The results for inhibition of IIa can be seen in Fig. 1a. The k_2 value of ATH was significantly higher than the k_2 value of AT+H, regardless of which surface was present ($p < 0.0001$). However, the rate of inhibition by both anticoagulants was significantly decreased in the presence of endothelium ($p < 0.0001$). The interaction term of the two-way ANOVA for relative effects of the surfaces was non-significant ($p = 0.5304$), meaning ATH displayed a similar increase in IIa inhibition compared to AT+H regardless of surface. In the case of Xa inhibition (Fig. 1b), the rate of inhibition of Xa by ATH was significantly higher than by AT+H in the presence of either surface ($p = 0.0299$), although the magnitude of the increase was small. There was no difference in the k_2 values of the anticoagulants in the presence of endothelium versus its absence ($p = 0.2683$). As for IIa, ATH had a similar effect on inhibition of Xa compared to AT+H regardless of surface ($p = 0.7691$). It was noted that there was a fair degree of variability of the k_2 value for ATH in the absence of endothelium, particularly in Fig. 1b. The data was analysed for the presence of outliers using the Rout test, and no outliers were detected.

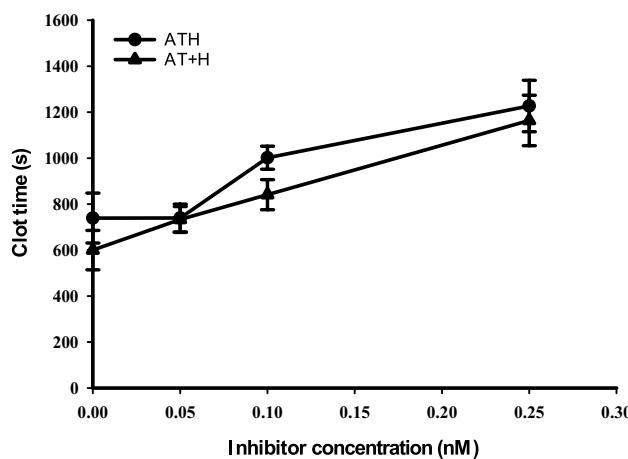


Fig. 3. Inhibition of fibrin formation induced by endothelial-bound IIa by ATH and AT+H. IIa was incubated in HUVEC-coated wells, followed by removal of excess unbound IIa. A mixture of purified fibrinogen, CaCl_2 and AT+H or ATH was added and fibrin formation monitored as in Fig. 2. Final inhibitor (AT or ATH) concentrations are indicated in the X-axis, and the molar ratio of H:AT in the AT+H mixture was 23:1. Data represent mean \pm SEM ($n=5$).

Effect of endothelial surface on inhibition of fibrinogen cleavage by IIa

Inhibition of IIa was further investigated by a fibrin formation assay. Fig. 2 illustrates that ATH was more effective than AT+H at preventing fibrin formation ($p < 0.0001$). Additionally, clot times were lower in the presence of endothelium ($p < 0.0001$). The interaction term of the ANOVA between anticoagulant and surface showed no significant effect of surface on anticoagulant ($p = 0.2237$).

To study the population of IIa molecules bound to the endothelial surface, we performed similar fibrin formation experiments but with removal of IIa not bound to the endothelial surface, followed by exposure to fibrinogen and the anticoagulants. There was no significant difference between ATH and AT+H for inhibition of endothelium-bound IIa and fibrin formation ($p = 0.2438$, Fig. 3).

Two molecules which constitute major IIa binding sites on the endothelial surface are HS and TM¹². To determine if IIa binding to either of these molecules affected anticoagulant activity, soluble HS or TM were pre-incubated with IIa and fibrin formation was assessed in the absence or presence of ATH or AT+H. HS did not affect IIa-induced fibrin formation in the absence of anticoagulants (clot time = 327.7 ± 14.0 s vs. 325.7 ± 14.2 s in the presence or absence of HS respectively, $p = 0.919$, $n = 9$). The presence of TM significantly delayed fibrin formation, with a clot time of 400.7 ± 9.3 s, compared to 344.7 ± 19.3 s in the absence of TM ($p = 0.024$, $n \geq 8$). ATH significantly delayed fibrin formation compared to AT+H across all doses in the presence of both HS (Fig. 4a, $p = 0.0023$) and TM (Fig. 4b, $p = 0.0103$).

Effect of endothelial surface on inhibition of plasma clot generation

To compare performance of ATH versus AT+H in a more physiologically-relevant plasma-based clot assay, plasma in the presence or absence of endothelium was activated with calcium to initiate the coagulation cascade, and thus clot formation. Unlike our previous assays in which we compared the anticoagulants at equivalent molar concentrations, inhibition of plasma clot formation by ATH and AT+H was assessed by comparison of the anticoagulants at equal catalytic anti-Xa levels, which emphasizes the ability of ATH or H to catalyze endogenous plasma AT inhibition of coagulation. We have summarized the results in Table 1. The data included both quantitative (mean clot times) and qualitative (“no clot”) data, which limited our ability to undertake a full statistical analysis. We conducted statistical analysis on the data at anticoagulant doses up to 0.06 U/mL, where the majority of reactions clotted. We have included a descriptive summary of the higher doses at which most of the “no clot” data was recorded. There was an increase in clot time as concentration increased for both anticoagulants, eventually leading to an absence of clotting in the majority of the reactions. At doses up to 0.06 U/mL clot times were significantly longer in the presence of AT+H compared to ATH ($p = 0.0002$) regardless of surface, and clot times were significantly decreased in the presence of endothelium, for both anticoagulants ($p < 0.0001$). However, there was no significant differential effect of surface on anticoagulant at these concentrations ($p = 0.6987$). Complete failure to clot occurred in a majority of reactions exposed to 0.12 U/mL AT+H in the presence of endothelium and 0.18 U/mL in the absence of endothelium. Some reactions failed to clot at concentrations as low as 0.06 U/mL AT+H. By contrast, it required 0.24 U/mL ATH to prevent clotting in the majority of reactions in the presence of endothelium, and 0.18 U/mL ATH in its absence. All reactions exposed to 0.12 U/mL or lower concentrations of ATH clotted with measurable clot times.

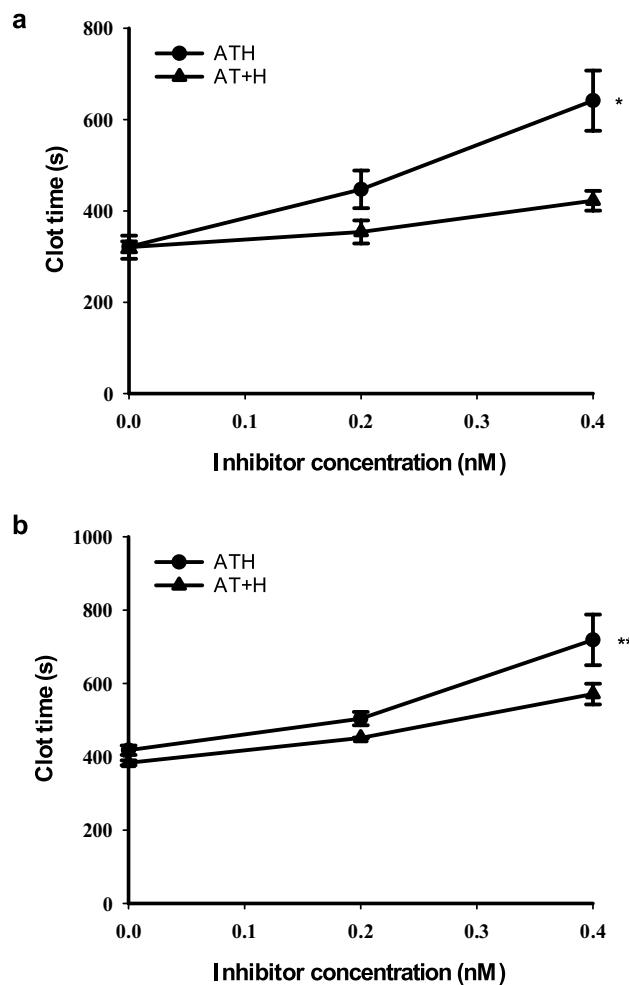


Fig. 4. Inhibition of fibrin formation induced by IIa bound to HS or TM by ATH and AT+H. IIa was mixed with excess HS (a) or TM (b) before fibrin formation assays were conducted as described in Fig. 2, but in the absence of endothelium. Final inhibitor (AT or ATH) concentrations are indicated in the X-axis, and the molar ratio of H:AT in the AT+H mixture was 23:1. Data represent mean \pm SEM ($n=4$). The P values are for the indicated comparisons performed by two-way ANOVA, reflecting the significant main effect of anticoagulant. * p (ATH vs. AT+H) = 0.0023. ** p (ATH vs. AT+H) = 0.0103.

Discussion

The endothelial cell surface plays a number of roles in modulating hemostasis. On its own, endothelium is generally considered an antithrombogenic surface in its non-activated state. Anticoagulant glycosaminoglycans (GAGs) such as HS can bind both procoagulant IIa and its inhibitor AT, promoting inactivation of coagulation¹². TM, a cell surface receptor, also binds IIa thus initiating generation of activated protein C which cleaves procoagulant factors VIIIa and Va, limiting further IIa formation¹⁹. The endothelium has also been shown to participate in procoagulant reactions in vitro. Exposure of endothelial cells to endotoxins, viral infections, hypoxia, and other stimulants has led to the expression of procoagulant activity^{20–23}. Prothrombinase and the intrinsic tenase complexes are also able to assemble on the endothelial surface^{24,25}. In addition to its presence in cell surface prothrombinase, Xa has also been shown to bind endothelial protein C receptor and effector protease receptor 1, and is involved in cell signaling^{26,27}. However, the effect of endothelium on the anticoagulant activities of heparinoids, and in particular ATH, in the surrounding fluid phase is largely unknown. While ATH has been shown to have significantly improved anticoagulant efficacy compared to other heparinoids in various animal models of thrombosis and bleeding, the previous in vitro mechanistic studies of its anticoagulant activities (inhibition rates of various coagulation factors, inhibition of plasma thrombin generation, etc.) have been conducted in the absence of the endothelium. Given the multiple ways in which endothelium can impact thrombosis, the objective of this study was therefore to measure the anticoagulant activities of ATH and AT+H in these in vitro mechanistic assays in the presence of an endothelial cell surface, and thus provide a stronger link between our in vivo and in vitro data.

K_2 values measured in this study in the absence of endothelium were very similar to those previously reported for reaction of ATH and AT+H with IIa and Xa^0 ⁹. The presence of endothelium had a mild, non-significant reduction of the K_2 for Xa, however it reduced the K_2 values for the reaction with IIa by an average of 62% (Fig. 1),

resulting in a statistically significant effect of endothelium on the rate of inhibition of IIa. We have previously shown that binding of IIa to soluble TM decreases the rate of inhibition by both ATH and AT+H¹⁶, with the degree of decrease depending on the absence or presence of a chondroitin sulfate moiety on TM. The chondroitin sulfate reduces the rate of heparin-enhanced inhibition of TM-bound IIa by steric repulsion, and HUVEC are known to contain approximately 18% TM with an attached chondroitin sulfate²⁸.

The presence of endothelium also significantly reduced the inhibition of IIa-mediated fibrinogen cleavage by the anticoagulants (Fig. 2). It should be noted that in these assays the molecules (enzymes and inhibitors) were present in two states: fluid-phase and surface-bound. This may have limited the contribution of HUVEC-bound IIa to the overall rate of fibrin formation by all IIa present. To address this issue, we also attempted to remove fluid-phase IIa and study inhibition of only the surface-bound IIa population. In contrast to the previous experiments, AT+H and ATH inhibited surface-bound IIa with equal efficacy across all doses (Fig. 3). It should be noted that under these conditions there may still be release of the IIa from the HUVEC surface to the fluid-phase, and also binding to the fibrin strands of the forming clot. Additionally, ATH may be less able to access IIa bound to the surface beneath the fibrin clot compared to AT+H, as ATH has been shown to bind strongly to fibrin via its H chain¹¹. In contrast, AT does not bind to fibrin¹¹ and may be more able to migrate to the surface-bound IIa and inhibit it, catalyzed by any excess H chains which have escaped binding to fibrin. However, ATH inhibits fibrin-bound IIa more efficiently than AT+H, since ATH cannot dissociate to form non-productive IIa-H-fibrin ternary complexes¹¹. The net effect of these various reactions may result in similar overall inhibition of the HUVEC-bound/fibrin-bound IIa populations by AT+H and ATH.

The reason why HUVEC-bound IIa yields similar ATH inhibition of fibrin formation compared to AT+H does not appear to be related to any binding of IIa by its major endothelial receptors HS or TM (Fig. 4). Binding of IIa to soluble TM did decrease the ability of IIa to promote fibrin formation in the absence of any inhibitor. This was expected as binding of IIa to TM decreases its affinity for fibrin and promotes activation of Protein C²⁹. The increased inhibition of IIa-TM induced fibrin formation by ATH compared to AT+H is in agreement with the work of Van Walderveen et al.¹⁶, as mentioned above. The increased inhibition of IIa-HS by ATH versus AT+H suggests that the heparin chain covalently bound to ATH can successfully compete with HS to interact with IIa. Since binding of IIa to glycosaminoglycans is primarily a charge-related phenomenon³⁰, the reduced concentration of sulfonate groups on HS³¹ may explain the inability of HS to prevent ATH inhibition of IIa. It should be noted that in these assays we did not definitively establish that all IIa was bound to TM or HS. However, we used a molar excess of both molecules (IIa:TM = 1:10 and IIa:HS = 1:2500) and we thus expect there to be minimal free IIa remaining under these conditions.

In order to study endothelial effects on heparinoid inhibition of coagulation in a more global assay, we also investigated the efficacy of ATH versus AT+H in a plasma-based clot generation system in the presence and absence of endothelium (Table 1). These experiments also tested effects on ATH's capability to catalyze inhibition of coagulation enzymes by the endogenous plasma AT. Thus, both anticoagulants were compared at equal catalytic anti-Xa activity concentrations. Anti-Xa levels are used clinically when monitoring and dosing patients receiving heparinoid anticoagulants, and allow for easier comparisons between different heparinoids, as, unlike anti-IIa activity, they are not affected by different chain lengths. AT+H was more effective than ATH in this system, as we observed complete inhibition of clotting at lower doses compared to ATH. As noted, we were unable to fully analyze the statistical significance of this difference across all doses, which tempers the conclusions that can be drawn from these results. It may seem counterintuitive that ATH appears less efficacious than AT+H in this system, however it should be remembered that ATH is being compared to H at equal anti-Xa activity levels in this particular experimental setup. The molar concentration of ATH is therefore approximately one third that of H, due to ATH's higher specific anti-Xa activity. Additionally, in calcium-initiated clot generation within plasma, multiple coagulation enzymes are being generated over time, and plasma contains a high concentration of endogenous AT (2–4 μ M). Therefore, in addition to direct inhibition of coagulant enzymes by the added ATH or AT+H, inhibition is also occurring via catalysis of the endogenous plasma AT by the H molecules present. It has been shown in multiple previous studies, and by many different methods, that despite the covalent linkage of H to AT in the ATH molecule, ATH is still able to catalyze inhibition of endogenous AT. ATH exhibits high specific anti-Xa activity in assays including excess AT, as reported in this study as well as previous work^{5,13,32}. ATH also binds to AT immobilized on Sepharose beads with high affinity³², and ATH can alter the fluorescence of exogenously added AT in a manner characteristic of AT binding to the H pentasaccharide¹⁴. AT/ATH bound complexes have also been detected by gel filtration¹⁴. ATH's catalytic activity can in part be explained by the fact that the H chains are longer on average than those found in commercial UFH preparations⁵, and approximately 30% of H chains in ATH contain two AT-binding pentasaccharide sequences¹⁴. Even in those molecules with only one pentasaccharide, some distance is required between the sequence and the H aldose terminus for covalent linkage with AT to occur³³. We have hypothesized that a displacement model may operate, whereby exchange at the pentasaccharide site occurs between covalently attached AT and free AT³². Catalytic inhibition is likely the dominant mechanism at low concentrations of anticoagulants, after the ATH has been consumed by direct inhibition to form enzyme-ATH covalent complexes. It was necessary to use low concentrations of anticoagulants in the assay reported here, as clot formation was initiated only by recalcification of the plasma. This design was chosen to keep the assay sensitive to any effects on clotting conferred by the presence of endothelium. It has previously been shown that the H chain of an enzyme-ATH covalent complex is less effective than free H at catalyzing the activity of endogenous plasma AT, likely due to steric hindrance from the bound enzyme¹⁵. In this previous study¹⁵, ATH also exhibited decreased efficacy compared to AT+H at low doses in a clot generation system, which supports the results in Table 1. It was also observed previously that ATH efficacy increased relative to AT+H as anticoagulant concentration increased, likely because more ATH was available for direct inhibition of coagulation¹⁵. In future translation to clinical usage, we anticipate having to conduct a variety of dosing studies to account for the variation in ATH's dose-response curve compared to H. Our previous research has compared

ATH and AT+H at equimolar concentrations in rabbit venous thrombosis treatment and bleeding models⁶. It was found that ATH was significantly more effective at reducing clot weight and fibrin accretion in this model, and that despite considerably higher anti-Xa activity levels measured in plasma (due to the equimolar dosing), the degree of bleeding observed was comparable to AT+H. When evaluated in a rabbit arterial thrombosis model, ATH exhibited improved vessel patency, reduced clot accretion, and similar levels of blood loss and plasma anti-Xa levels despite being administered at considerably reduced dosages compared to H⁷. We believe that ATH may have enhanced utility over H in indications where high anti-Xa levels are required, such as cardiac bypass where extremely large doses of H are given. ATH is also likely to have value in cases of heparin resistance, caused by reduced endogenous plasma AT levels, since AT is also present in the complex and administered along with the H. We have previously shown a benefit of ATH over H in a pig CPB model⁸, and additional studies to further explore these possibilities are currently being designed.

In vivo, the endothelium in its non-activated state is considered to be non-thrombogenic, when compared to its activated or damaged state, or to the underlying extracellular matrix. In vitro, and when compared to a plastic or other non-endothelial artificial surface, the thrombogenicity of cultured endothelium can be affected by a variety of factors, such as endothelial cell type, passage number, flow versus static experimental conditions, confluence of the cells, surface to blood/plasma volume ratio, and the nature and endpoints of the assay system (reviewed in³⁴). In our plasma clot generation assay we observed a decreased clot time in the presence of endothelium versus its absence. A trend towards a procoagulant effect of HUVEC compared to a collagen surface in a plasma recalcification clot time assay has also been shown previously in the literature³⁵, although in this work the trend did not reach statistical significance. In our experiments we optimized the health of the endothelial cells by limiting the passage number to less than 5 and growing the cells under manufacturer recommended conditions. Cultured cells were inspected microscopically before and after the experiment and exhibited the typical morphology characteristic of HUVEC. Despite these precautions we cannot rule out the possibility that some proportion of cells may have been damaged while carrying out the experiment. This possibility may in fact lend further significance to our results, as it might be expected that in *in vivo* pathological thrombotic situations where ATH is employed the endothelial surface it encounters is likely to be activated. There is evidence to suggest that activated endothelium may play an important role in providing a membrane surface for assembly of prothrombinase to accelerate thrombin formation²⁵. We have previously shown that ATH was a stronger inhibitor of prothrombinase compared to AT+H, when measured on model phospholipid surfaces³⁶, as well as activated platelets³⁷ and red blood cells³⁸. With these results as a base, future planned studies will further investigate ATH anticoagulant activity in the presence of activated versus non-activated endothelium, and using assay systems incorporating whole blood and other blood components such as platelets and leukocytes.

In conclusion, heparin-based anticoagulants exhibit reduced inhibition of procoagulant enzymes in the vicinity of the endothelial surface, possibly through binding of these enzymes and/or binding of the anticoagulants themselves to the endothelium. However, endothelium did not differentially impact ATH compared to AT+H in any of the *in vitro* assays investigated in this study. Our investigation helps to better understand how the endothelium, which is found throughout the vasculature in the body, may interact with and impact the function of ATH or AT+H travelling throughout the circulation. This study provides additional information that further supports a growing body of evidence for the continued development of ATH as a clinical therapeutic agent, particularly in the areas of cardiopulmonary bypass or hemodialysis, where unfractionated heparin remains the standard of care despite its well-documented limitations.

Data availability

All data generated or analysed during this study are included in this published article.

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Author contributions

A.K.C. and L.R.B. conceived the project and supervised the experiments. H.M.A. and L.R.B. designed the experiments. H.M.A. performed all experiments, analyzed the data, and wrote the manuscript with input from all other authors. L.R.B. and I.S. contributed to data analysis and interpretation. The manuscript has been read and approved for submission by all authors.

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to A.K.C.C.

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