

Covalent antithrombin-heparin effect on thrombin-thrombomodulin and activated protein C reaction with factor V/Va

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Summary

Thrombomodulin (TM), which variably contains a chondroitin sulfate (\pm CS), forms an anticoagulant complex with thrombin (IIa). IIa-TM(\pm CS) converts protein C (PC) into activated PC (APC), which then inactivates activated factors V (FVa) and VIII (FVIIIa). This reduces prothrombinase and tenase complexes that generate IIa. Heparin (H) increases the rate of IIa-TM inhibition by antithrombin (AT) and enhances FV cleavage by APC. Our novel covalent AT-H (ATH) product, has superior anticoagulant activity compared to AT + unfractionated H (UFH). We studied mechanisms by which ATH versus AT + UFH inhibits IIa-TM(\pm CS), and ATH influences on APC cleavage of FV/FVa compared to UFH. Findings would determine how these reactions moderate ATH's overall effects as an anticoagulant. Discontinuous second order rate inhibition assays of IIa-TM(\pm CS) inhibition by AT + UFH or ATH were performed in presence or absence of human umbilical vein endothelial

cells (HUVECs). FV/FVa cleavage by APC in the presence of UFH or ATH was analysed by Western blots. ATH increased IIa-TM(\pm CS) inhibition to a greater degree than AT + UFH, both on plastic and HUVEC surfaces. Unlike UFH, ATH did not accelerate FV cleavage by APC, but ATH did enhance FVa cleavage relative to UFH. Increased IIa-TM inhibition by ATH downregulates PC activation. However, ATH does accelerate downstream inactivation of FVa, which increases its potency for IIa generation inhibition compared to UFH. This trend holds true in the presence of APC's cofactor, protein S. Overall, ATH may have a balanced function towards inhibiting or accelerating PC pathway activities.

Keywords

Antithrombin-heparin complex, thrombomodulin, factor V/Va, anticoagulant, heparin

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Introduction

Thrombomodulin (TM) is an integral membrane protein composed of an amino-terminal domain, six epidermal growth factor-like (EGF-like) repeats, an O-linked sugar domain, a transmembrane domain, and a cytoplasmic tail (1). TM's last two EGF-like repeats interact with the thrombin (IIa) anion exosite 1 (2) to form a 1:1 complex (3). Formation of the IIa-TM complex converts IIa from a procoagulant to an anticoagulant (4). TM exists with or without a chondroitin sulfate (CS) bound to it (5–8). Mechanistically, the presence of CS enhances TM's affinity for IIa, through interaction with exosite 2 (5, 9). IIa-TM readily converts protein C (PC) to activated PC (APC) since TM causes a conformational change in IIa that makes IIa more reactive towards PC (6, 10). Moreover, the activation of PC by IIa-TM is enhanced when PC is bound to the membrane-associated endothelial PC receptor (EPCR) (11). Previous work in our laboratory of APC generation on endothelial cells has shown that activation by IIa-TM and inhibition of the PC pathway is significantly different in new-

born compared to adult plasmas (12). The differences observed due to the presence of cells could not be explained solely by variation in protein levels and were suggestive of different developmental protein subtypes (glycoforms) for PC pathway components. Prior to assessing age-related qualitative differences in the PC plasma proteins, it is necessary to characterise the control of IIa activity by different glycoforms of TM. Additionally, APC functions by inactivating factor V (FV) and activated FV (FVa), thus preventing their participation in the prothrombinase complex and ultimately reducing IIa generation (4, 13, 14). Hence, mechanisms that affect the PC pathway would strongly impact control of IIa generation.

Inhibition of IIa generation has been studied extensively in the prevention or inhibition of thrombotic complications (15–17). Heparin (H) is a common and well-known anticoagulant therapeutic drug (18). H acts as a catalyst in the inhibition of IIa and several other coagulation factors by AT (19–23), which reduces IIa generation to yield potent anticoagulant activity. Additionally, H can assist in IIa-TM inhibition by AT (24) and influence inacti-

vation of FV/FVa by APC (25, 26). However, H has a number of limitations. These include: a short half-life, inability to inhibit fibrin-bound Ila, and non-specific binding to plasma and endothelial proteins (27–35). To overcome many of these limitations, Chan et al. developed a covalent antithrombin (AT)-H complex (ATH) (36). ATH is shown to be an efficient inhibitor of coagulation. Compared to unfractionated H (UFH), ATH has been demonstrated to have a longer half-life, increased Ila inhibition, the ability to inhibit fibrin-bound Ila, and reduced endothelial cell and plasma protein binding (36–39). Furthermore, immobilising ATH on surfaces of stents and catheters inserted *in vivo*, provides improved anticoagulant activity and reduced fibrin accretion, compared to UFH (40–42). Recently, detailed studies have been conducted to elucidate anticoagulant mechanisms of ATH in whole blood (43) and the effect of the AT moiety of ATH on biodistribution *in vivo* (44). Also, ATH can be sequestered in the lung, due to its large size, and thus may prevent intra-alveolar coagulation during respiratory distress syndrome (RDS) (36, 45). Collectively, ATH acts as a superior anticoagulant through enhanced inhibition of procoagulant Ila activity. However, inhibition of Ila-TM or catalysis of APC reactions with FV/FVa by ATH could moderate the overall effects of this agent. Given the global importance of the PC pathway, it is critical to determine the impact of the novel ATH drug on PC reactions. This report studies mechanisms involved in ATH versus AT + UFH inhibition of Ila-TM(\pm CS), and the influence of ATH on APC reaction with FV/FVa.

Materials and methods

Materials

All reagents used were of analytical grade or higher. Hexadimethrine bromide (Polybrene) and Alcian Blue GX were obtained from Sigma (Mississauga, ON, Canada). Heparin (with average M.W. of 15 kDa) isolated from porcine intestinal mucosa, was obtained from Sigma (St. Louis, MO, USA). Human alpha Ila, PC and APC were obtained from Enzyme Research Laboratories (South Bend, IN, USA). The Ila substrate S-2238 (H-D-Phenylalanyl-L-pipecoyl-L-arginine-p-nitroaniline dihydrochloride) and APC substrate S-2366 (L-pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline hydrochloride) were purchased from Diapharma (West Chester, OH, USA). Recombinant human TM (CD141) without a CS ($A_{280} = 0.88$ for 1 mg/mL) was from American Diagnostica Inc. (Stamford, CT, USA). The TM is a recombinant carboxyl-terminal truncated form which lacks the transmembrane and cytoplasmic domains. The recombinant soluble TM with a CS ($A_{280} = 1.0$ for 1 mg/ml) was a generous gift from Dr. Charles Esmon and colleagues. Human FV and FVa were obtained from Haematologic Technologies Inc. (Essex Junction, VT, USA). The primary antibody (sheep anti-factor V affinity-purified IgG) and secondary antibody (anti-sheep IgG peroxidase conjugate antibody [raised in donkey]) were from Affinity Biologicals (Ancaster, ON, USA) and Sigma (Mississauga, ON, Canada), respectively. Culture media and human umbilical

vein endothelial cells (HUVECs) were obtained from Lonza (Basel, Switzerland). Pefabloc (a very selective thrombin inhibitor) was from CenterChem (Norwalk, CT, USA). ATH (Affinity ATH010914) was produced by Paul Chindemi in Dr. Anthony Chan's laboratory using a previously described method (36).

Second order rate constant inhibition assay of Ila and Ila-TM(\pm CS)

Second order rate constants (k_2) were determined for Ila, and Ila-TM(\pm CS) inhibition by AT + UFH or ATH at 37°C using a similar method as described by Patel et al. (46). The UFH concentrations used ranged from 0 to 6,000 nM. AT and ATH were at consistent concentrations of 20 nM and 10 nM, respectively. Moreover, Ila concentrations were either 1 or 2 nM. To make Ila-TM(\pm CS), Ila (1 or 2 nM) was incubated with a 2.5-fold higher concentration of TM(\pm CS) (i.e. 2.5 nM or 5 nM) at 37°C for 30 seconds (sec). The Ila-TM ratio of 1:2.5 was consistent with that previously used by Bourin et al. (5).

In six wells of a 96-well flat-bottom microtiter plate, 10 μ l of Ila or Ila-TM(\pm CS) were added. After 5 minutes (min), in the sixth well, 10 μ l of 0.02 M Tris-HCl 0.15 M NaCl, 0.6% polyethylene glycol 8000 (PEG) pH 7.4 (TSP) buffer and 2.5 mM CaCl₂ were added. At either 3 or 5 sec intervals AT + UFH or ATH was added to wells 1 through 5. After another 3 or 5 sec the reactions were terminated through the simultaneous addition of 80 μ l of 1.25 mg polybrene/mL TSP buffer and 0.5 mM chromogenic substrate, S-2238. Residual enzyme activity was measured by reading the absorbance (at 405 nm) for three consecutive 10-min time intervals using the SpectraMax Plus 384 plate reader (Molecular DEVICES, Sunnyvale, CA, USA). The pseudo-first order rate constant (k_1) was determined from the negative slope of the plot $\ln(V_t/V_0)$ versus inhibition time length, where V_t is the remaining enzyme activity at time t , and V_0 is the initial enzyme activity. The k_2 was determined by dividing k_1 by the inhibitor concentration.

A confirmatory two-stage assay was done using PC as a substrate to determine Ila-TM(-CS) inhibition by AT + UFH or ATH. The method used was similar to the one described above, with a few changes. Following the addition of 80 nM AT + 1870 nM UFH or 20 nM ATH at given time intervals, the inhibition reaction was terminated by addition (20 μ l) of a 2 mg/ml polybrene + PC (100 nM for AT + UFH or 25 nM for ATH) solution. Any uninhibited Ila (from starting concentrations of 8 nM for AT + UFH and 2 nM for ATH) bound to TM(-CS) (20 nM for AT + UFH, and 5 nM for ATH) was allowed to react with the polybrene + PC solution for 10 min. The PC reaction was terminated with a 2 mg/ml polybrene + 0.58 μ g/ μ l Pefabloc + 0.725 mM S-2366 substrate solution. Pefabloc was added to prevent any further Ila reaction with PC or the APC substrate. The APC generated was measured by reading absorbance at 405 nm (as described above).

Determination of presence or absence of a CS on the two forms of TM

To confirm that the two forms of TM do or do not contain a CS, both forms of TM were analysed. Rate experiments, as described above, were performed for IIa-TM(\pm CS) in the presence of 0.001 U chondroitin ABC lyase (ABCCase). Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) both before and after treatment with ABCase was also done. An Alcian blue stain, followed by a silver stain was performed. This method, as described previously, sensitively stains negatively charged glycosaminoglycans (GAGs) (47, 48).

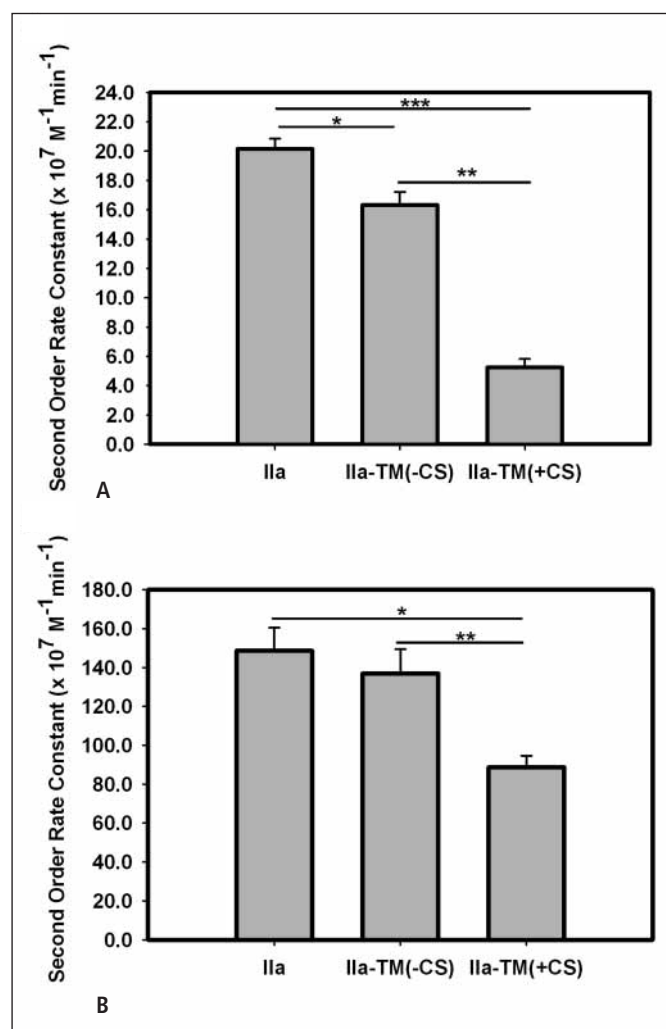


Figure 1: Maximal second order rate constants for IIa and IIa-TM(\pm CS) inhibition by AT + UFH (A) or ATH (B). A) IIa [2 nM] or IIa-TM(\pm CS) [2 nM for IIa, 5 nM for TM] was inhibited by AT [20 nM] + UFH [468 nM and 1,000 nM, for IIa-TM(-CS) and IIa-TM(+CS), respectively]. Statistical significance is by Student t-test (* $P=0.012$, ** $P<0.001$; $n=5$). B) IIa [1 nM] and IIa-TM(\pm CS) [1 nM] was inhibited by ATH [10 nM]. Statistical significance was by Student t-test (* $P=0.001$, ** $P=0.006$; $n=5$).

PC activation by HUVEC surface-bound IIa in the presence of AT+UFH or ATH

Similar to a previously described method by Mewhort-Buist et al. (49), HUVECs were cultured in 96-well flat-bottom microtiter plates (Primaria-treated, Becton Dickinson, Franklin Lakes, NJ, USA) to confluency (30,000–50,000 cells/well). Media was aspirated from the wells, and wells were washed with minimum essential medium (MEM) + 0.1% PEG + 10 mM HEPES pH= 7.4 (MEMPH). IIa (150 nM) was added to the wells to bind to any cell surface IIa-receptors (i.e. TM). Excess unbound IIa was washed off. Cells were incubated with 6 nM AT + 140.25 nM UFH + 25 nM PC or 6 nM ATH + 25 nM PC for 0, 10, 20, 30, 45, and 60 min. Reactions were stopped through the simultaneous addition of 2.0 mg/mL polybrene (to neutralise the heparin) + 0.58 μ g/ μ l Pefabloc (to inhibit the IIa) + 0.725 mM S-2366 substrate solution. APC activity was measured at 405 nm using the SpectraMax Plus 384 plate reader and V_{max} versus time was plotted.

SDS-PAGE analysis of FV and FVa cleavage by APC

SDS-PAGE analysis of FV and FVa cleavage by APC in the presence or absence of UFH or ATH was performed. FV or FVa was incubated with APC \pm UFH or ATH for 0, 1, 5, 10, 20, or 60 min. These reactions were in the presence of PC/PS (3:1) vesicles (40 μ M) and 2.5 mM Ca^{2+} . The samples were reduced in SDS sample buffer containing 2-mercaptoethanol, and were heated to 100°C for 3 min. The samples were loaded onto a discontinuous gel, and run at 200 V for approximately 30 min. The samples were electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was probed with sheep anti-factor V affinity-purified IgG (8.0 x 10⁻⁴ mg/ml), followed by donkey anti-sheep IgG peroxidase conjugate secondary antibody (3.3 x 10⁻⁴ mg/ml). Enhanced Chemiluminescence (ECL) Plus Western Blotting Detection Kit from GE Healthcare (Piscataway, NJ, USA) was used for the detection of the bands. The bands were analysed by densitometry using both the Typhoon 9410 (GE Healthcare, Baie d'Urfe, QC, Canada) and ECL detection by exposure to film. Specifically, the band intensities were quantified as a ratio to a consistent internal reference band found in the unreacted FV or FVa. The experiments for FVa cleavage in the presence of UFH or ATH were repeated in the presence of protein S (15 nM).

Statistical analysis

Statistical significance between and within study groups was determined using analysis of variance (ANOVA). Statistical difference between two sample means was done using a Student t-test. $P<0.05$ was considered significantly different. Results were presented as the mean \pm SE.

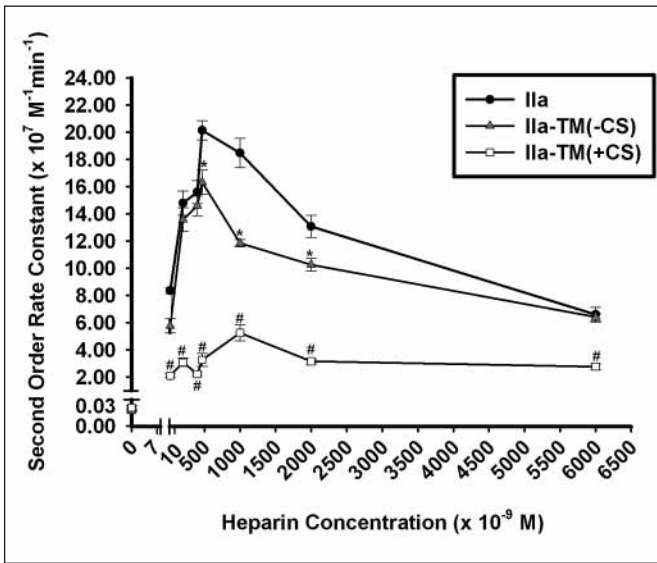


Figure 2: Maximal second order rate constants for Ila [2 nM] and Ila-TM(\pm CS) [2 nM for Ila, 5 nM for TM] inhibition by AT [20 nM] and varying concentrations of UFH [0–6,000 nM]. Statistical significance was determined between Ila and Ila-TM(\pm CS) using GLMANOVA ($P \leq 0.001$; $n=5$). Point comparison relative to the Ila curve was done using Student t-tests ($*P < 0.05$, and $\#P \leq 0.001$; $n=5$).

Results

Ila and Ila-TM(\pm CS) inhibition by AT + UFH and ATH

Ila-TM(-CS) and Ila-TM(+CS) inhibition by AT alone is slow ($k_2 = 2.2 \times 10^5 \pm 1.7 \times 10^4$ and $2.8 \times 10^5 \pm 2.6 \times 10^4 \text{ M}^{-1} \text{ min}^{-1}$, respectively). These results are consistent with findings by Bourin (24) indicating that the CS assists in Ila-TM inhibition by AT. Very different results are found upon addition of UFH or ATH. In the presence of AT + UFH the rate of Ila inhibition is significantly increased compared to AT alone ($P < 0.001$, data not shown). Moreover, ATH further increases the rate of Ila inhibition relative to AT + saturating UFH ($P < 0.001$). There is a reduced rate of Ila inhibition by both AT + UFH and ATH in the presence of TM(+CS) compared to TM(-CS) ($P < 0.001$ and $P = 0.006$, respectively) (► Fig. 1). Further, there is a significant reduction in the rate of inhibition of Ila-TM(-CS), compared to Ila, by AT + UFH ($P = 0.012$) (Fig. 1A). However, no significant change is seen for inhibition by ATH ($P = 0.456$) (Fig. 1B). Pilot experiments at increased TM:Ila molar ratios (10:1) showed similarly decreased inhibition rates for Ila-TM(-CS) by AT + UFH relative to ATH, giving supporting evidence that reactions were with Ila bound to TM. For all cases, Ila, Ila-TM(-CS) and Ila-TM(+CS), inhibition was significantly faster by ATH relative to AT + UFH ($P < 0.001$). Overall, there is a seven- to eight-fold increase in rate for inhibition of Ila, nine-fold increase for Ila-TM(-CS) inhibition rate, and a 17-fold increase for Ila-TM(+CS) inhibition rate by ATH, compared to AT + UFH. The two-stage APC generation assay further

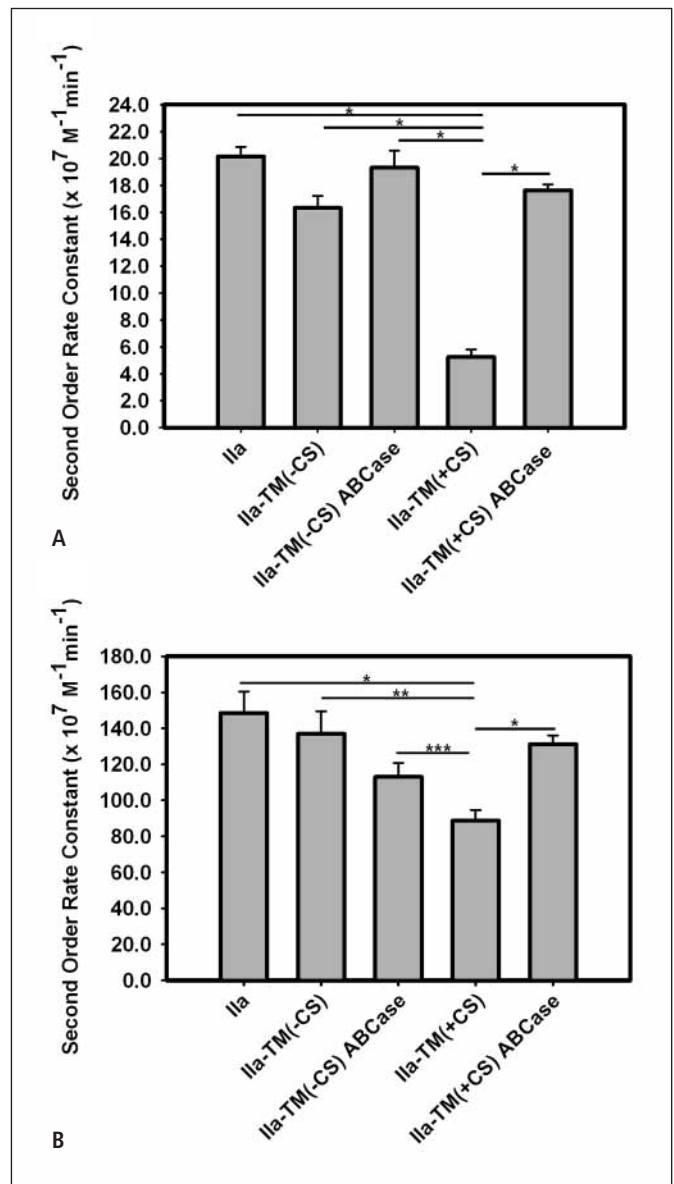


Figure 3: Maximal second order rate constants for Ila and Ila-TM(\pm CS) inhibition by AT + UFH (A) or ATH (B) in the presence or absence of ABCase. Statistical significance was by Student t-test ($*P \leq 0.001$, $**P = 0.006$, $***P = 0.042$; $n=5$).

confirmed that ATH is a significantly faster inhibitor of Ila-TM(-CS) compared to AT + UFH ($P=0.001$; $k_2 = 1.3 \times 10^9 \pm 1.7 \times 10^8$ and $5.9 \times 10^7 \pm 5.2 \times 10^6 \text{ M}^{-1} \text{ min}^{-1}$, respectively). The non-inhibited fraction of Ila bound to TM was able to activate PC, and APC generation by this remaining Ila was significantly lower in the presence of ATH compared to AT + UFH.

Observation of the curves for the inhibition of Ila and Ila-TM(\pm CS) by AT + varying concentrations of UFH shows that the maximum k_2 value for Ila-TM(+CS) occurs at a higher UFH concentration compared to Ila and Ila-TM(-CS) (► Fig. 2). The maximum k_2 is the fastest reaction rate that is determined by varying

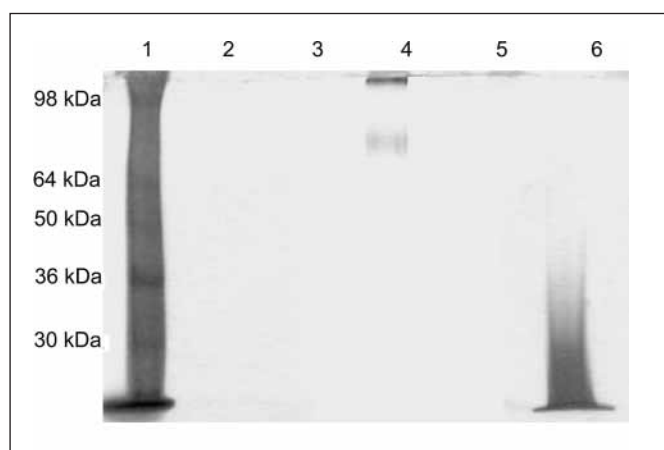


Figure 4: SDS-PAGE of TM(\pm CS) in the presence or absence of ABCase. On a 7.5% separating gel samples were run under reducing conditions (2-mercaptoethanol) and stained using an Alcian Blue-Silver staining technique. Lane #1 contains a SeeBlue Pre-stained standard (M.W. markers, Invitrogen, Burlington, ON, Canada). Lane 2 contains 0.1 μ g TM(-CS) and lane 3 contains 0.1 μ g TM(-CS) + 0.001 U ABCase. Similarly, lane 4 contains 0.1 μ g TM(+CS), and lane 5 contains 0.1 μ g of TM(+CS) + 0.001 U ABCase. As a positive glycosaminoglycan staining control, lane 6 contains 0.1 μ g of UFH.

UFH concentration, and at which the high affinity pentasaccharide binding sites are in vast excess to AT. Thus, more UFH is required to force the inhibition reaction when TM contains CS chains.

To confirm the presence or absence of a CS on the two forms of TM, several experiments were performed in the presence of ABCase. As expected, there is no significant difference in the rate of inhibition of the Ila-TM(-CS) compared to the Ila-TM(-CS) + ABCase (\blacktriangleright Fig. 3; $P = 0.096$ and $P = 0.063$ for AT + UFH and ATH, respectively).

These findings were consistent for both inhibition by AT + UFH and ATH. However, after the addition of ABCase to Ila-TM(+CS), the rate of inhibition was increased back up to that of Ila-TM(-CS) (Fig. 3; $P=0.001$). It was possible that the presence of CS on TM interacted with Ila and allosterically altered Ila reaction with its substrates and inhibitors. However, similar to previous findings (24), pilot experiments showed that APC generation by Ila-TM(+CS) was unaffected by removal of the CS with ABCase ($P>0.05$). A gel, run under reducing conditions and using an Alcian Blue-Silver staining technique confirmed the presence (TM[+CS]) or absence (TM[-CS]) of CS in the two TM subforms (\blacktriangleright Fig. 4).

PC activation by Ila-TM on HUVEC surfaces in the presence of AT + UFH or ATH

A more physiological system, wherein HUVECs were incubated with PC \pm the anticoagulant over a 60 min time course, showed reduced APC generation in the presence of AT + UFH compared to no anticoagulant addition (\blacktriangleright Fig. 5). APC generation was considerably lower still for ATH compared to AT + UFH (Fig. 5). Thus, from these findings it is evident that in the presence of HUVECs ATH is a faster inhibitor of Ila bound to the cell surface molecule TM, compared to AT + UFH.

FV and FVa cleavage by APC in the presence or absence of UFH or ATH

Cleavage of FV by APC occurs primarily at Arg306, followed by Arg506, Arg679, and Lys944 (13). Cleavage results in 200 kDa, 70

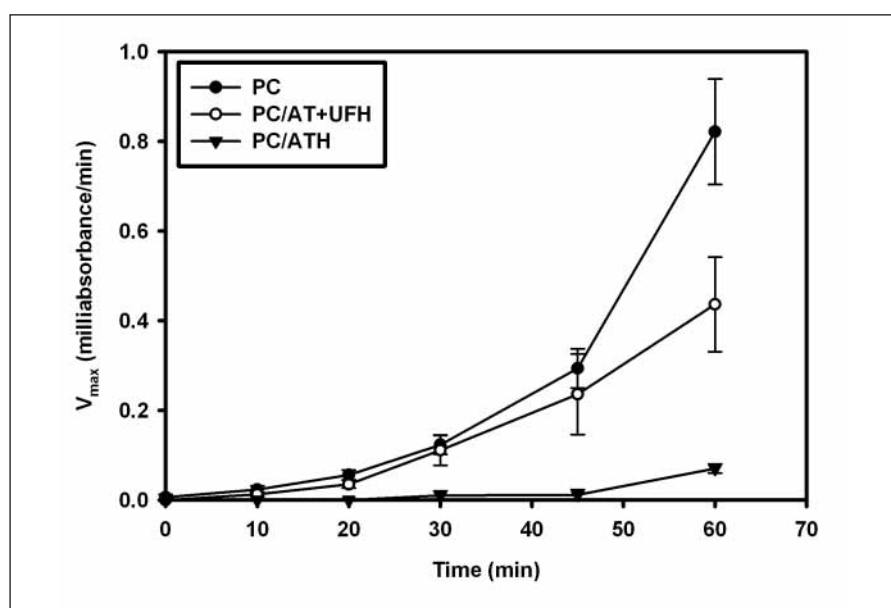


Figure 5: PC activation by Ila-TM on HUVEC surface in the presence of AT + UFH or ATH. HUVEC surface bound Ila reacted with 25 nM PC (closed circle), 25 nM PC + 6 nM AT + 140.25 nM UFH (open circle), or 25 nM PC + 6 nM ATH (closed triangle) for 0, 10, 20, 30, 45, and 60 min. APC generation over the given time course is presented in units of milliabsorbance/min.

kDa, 45 kDa, 30 kDa, and 20/22 kDa cleavage products (13). FVa cleavage by APC occurs primarily at the Arg506, followed by Arg306, and Arg679 producing the 45 kDa, 30 kDa, 26 kDa, 28 kDa, and 20/22 kDa cleavage products (13, 25). Due to a clear appearance over time of the 26 kDa, 28 kDa, and 30 kDa bands, these are the products analysed and quantified. Thus, these bands represent the cleavages that occurred.

Consistent with previous findings (25), there is a trend towards increased cleavage of FV by APC in the presence of H, compared to cleavage by APC alone (► Fig. 6). However, this trend is not found for ATH (Fig. 6). Over time there is increased cleavage of FV by APC ± UFH or ATH, as indicated by the increased appearance of the 30 kDa band product over the 60 min time course (Fig. 6).

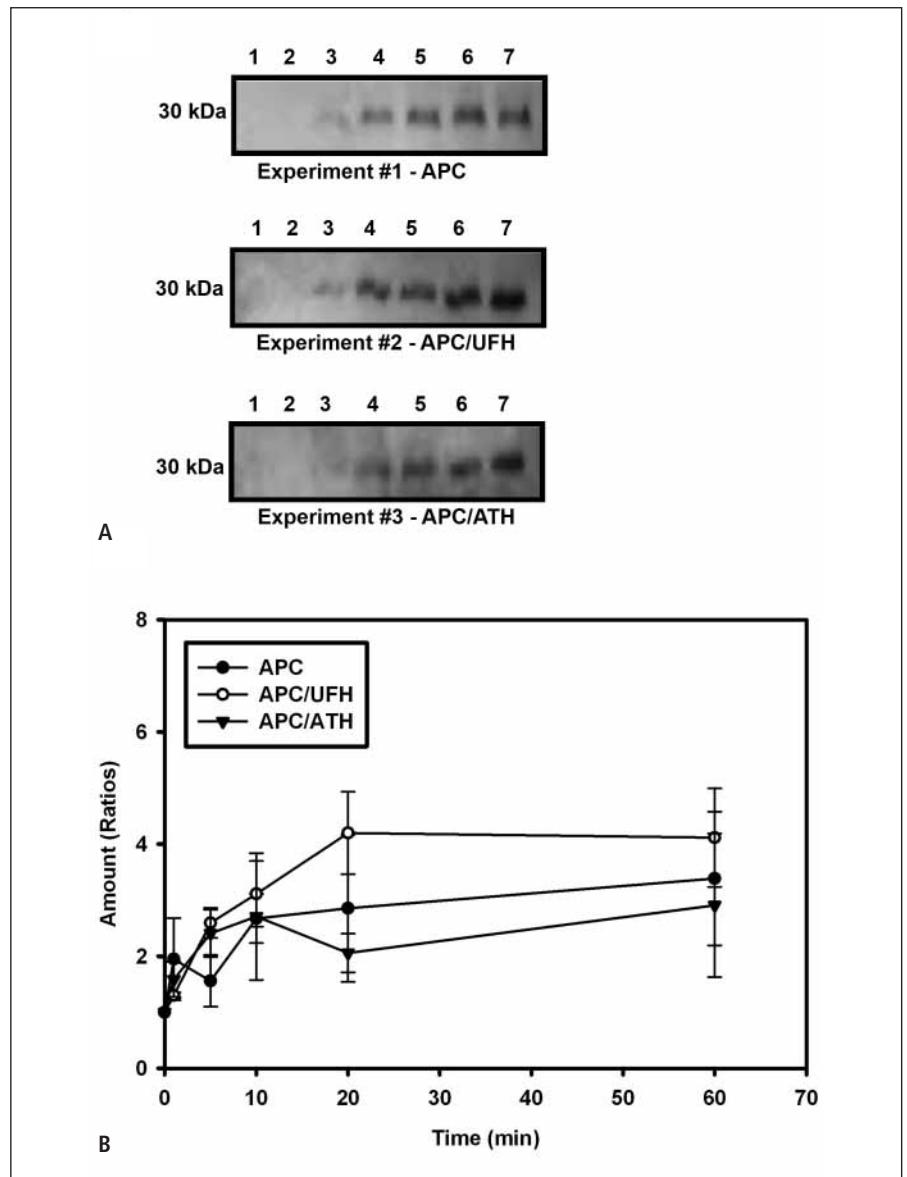
For FVa, reverse effects were found. Consistent with previous findings, the presence of H did not enhance FVa cleavage by APC (► Fig. 7). However, ATH did enhance cleavage, with increasing in-

tensity of bands at 26, 28, and 30 kDa (Fig. 7). In the presence of PS, ATH gave increased cleavage of FVa compared to UFH. Thus, relative trends for APC cleavage with ATH versus UFH remained similar to results without protein S (► Fig. 8). Overall for both FV and FVa (± protein S) cleavage, the initial cleavage rate is rapid, followed by a slow rate of cleavage after 10 min.

Discussion

ATH has been shown to increase inhibition of several coagulation factors. Compared to AT + UFH, ATH had increased rates for inhibition of FIXa, FXIa, IIa, and FVIIa-TF, and had similar rates of inhibition of FXa, and FXIIa (46). As a result, ATH is a more potent anticoagulant for reduction of IIa generation compared to AT +

Figure 6: Western blot analysis of FV cleavage. FV [100 nM] cleavage by APC [15 nM], APC + UFH [100 nM], and APC + ATH [100 nM] was performed in the presence of PC/PS vesicles [40 µM] and calcium [2.5 mM] (n=3). A) The 30 kDa cleavage product of FV (Exp. 1, 2, 3) released by APC in the presence or absence of UFH or ATH are shown. Lane 1 is a control and contains only FV. Lanes 2–7 contain FV in the presence of the inhibitor over a time-course of 0, 1, 5, 10, 20, and 60 min, respectively. B) For the above mentioned reactions, densitometry was used to analyse bands of the Western blots. The 30 kDa fragments were quantified relative to a consistent internal reference band found in the unreacted FV. The 30 kDa fragment was not found for FV alone, and gave background values similar to the zero time.



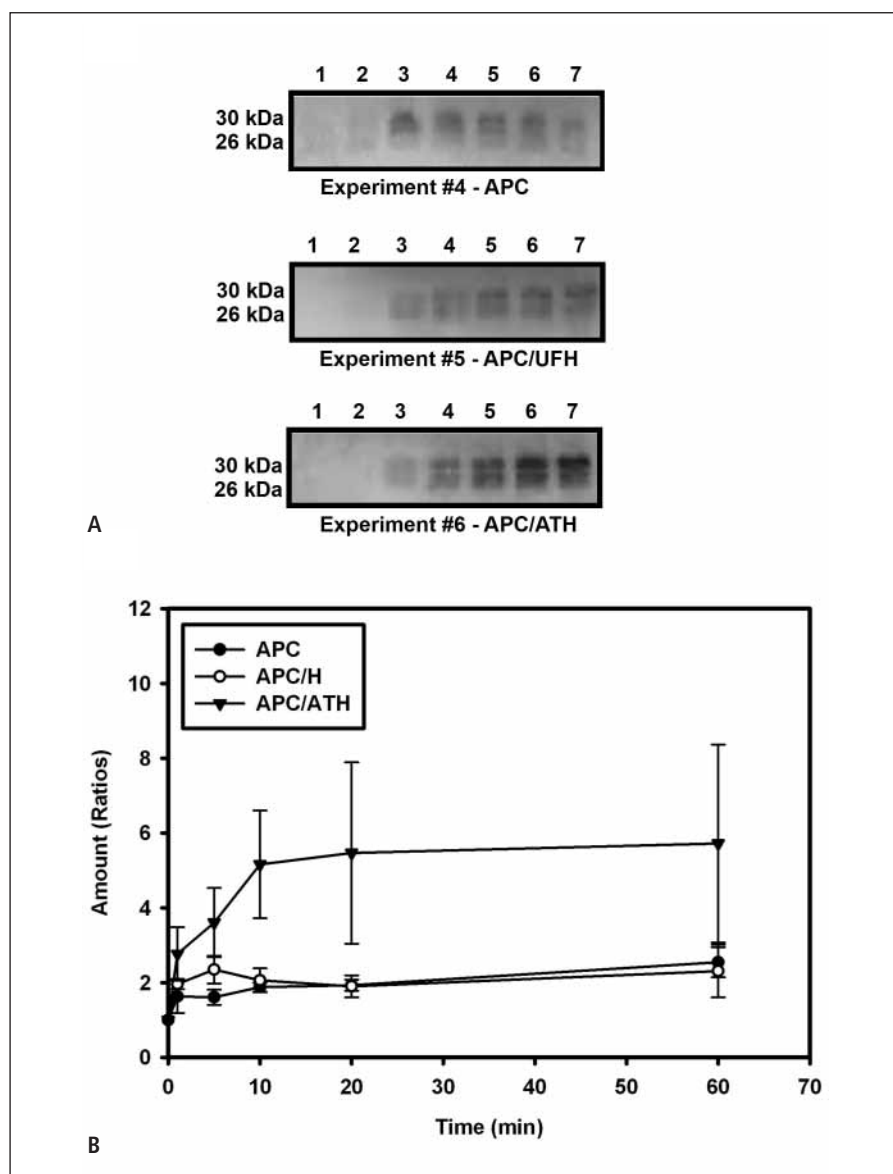


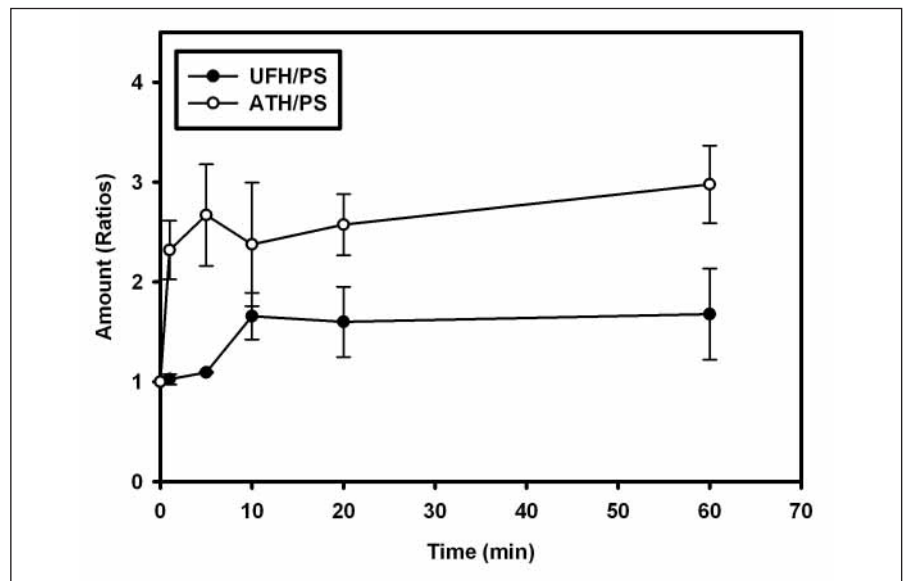
Figure 7: Western blot analysis of FVa cleavage. FVa [100 nM] cleavage by APC [15 nM], APC + UFH [100 nM], and APC + ATH [100 nM] was performed in the presence of PC/PS vesicles [40 μ M] and calcium [2.5 mM] ($n=3$). A) The 26, 28, and 30 kDa cleavage product of FV (Exp. 1, 2, 3) released by APC in the presence or absence of UFH or ATH are shown. Lane 1 is a control and contains only FVa. Lanes 2–7 contain FVa in the presence of the inhibitor over a time-course of 0, 1, 5, 10, 20, and 60 min, respectively. B) For the above mentioned reactions, densitometry was used to analyse bands of the Western blots. The 26, 28, and 30 kDa fragments were quantified relative to a consistent internal reference band found in the unreacted FVa. The 26, 28, and 30 kDa fragments were not found for FVa alone, and gave background values similar to the zero time.

UFH. The current project extends this work by focusing on coagulation factors within the PC pathway, specifically Ila-TM(\pm CS), FV, and FVa. Since Ila-TM can activate PC, which in turn inactivates FV and FVa, this ultimately leads to a reduced Ila generation. Effects by ATH that regulate this pathway may increase or decrease PC-associated reduction of Ila formation, depending on the stage at which ATH interacts with APC function and formation.

Ila-TM inhibition by AT + UFH or ATH may proceed through a similar mechanism as that previously described, by Berry et al., for fibrin-bound Ila inhibition (38). Fibrin-bound Ila is protected from inhibition by AT + UFH, due to the formation of a fibrin-H-Ila complex (38). The ternary complex prevents AT interaction with Ila. ATH is capable of inhibiting fibrin-bound Ila, presumably because H cannot dissociate and, thus, cannot form the above-mentioned ternary complex (38).

In the case of Ila-TM(-CS) inhibition by AT + UFH, UFH may dissociate and interact with the TM polypeptide. Although primary binding of H is through exosite 2 of Ila, there may however be minor secondary binding to the TM. The negatively charged CS binds to TM, and Ser474 is the primary site of attachment of CS to TM (50). We speculate that in the absence of CS these positively charged residues may also interact with UFH resulting in enhanced affinity for formation of a ternary Ila-TM-UFH complex. Regardless of how UFH binds to Ila-TM(-CS), AT bound to UFH cannot readily inhibit the Ila-TM(-CS) in this ternary complex. Similar consequences are observed when non-covalent Ila-UFH complexes form in AT + Ila reactions with vast excess of UFH (51). This Ila-TM(-CS)-UFH complex formation does not occur with ATH due to the covalent linkage between AT and H. The ternary complex formation may explain the significant decrease in the rate of in-

Figure 8: Western blot analysis of FVa cleavage in the presence of protein S. FVa [100 nM] cleavage by APC [15 nM] + protein S [15 nM] in the presence of UFH [100 nM] or ATH [100 nM] (n=3). The experimental system also contained PC/PS vesicles [40 μ M] and calcium [2.5 mM]. FVa was left to react with the inhibitors over a time-course of 0, 1, 5, 10, 20, and 60 min, respectively. The 26, 28, and 30 kDa cleavage fragments were quantified relative to a consistent internal reference band found in the unreacted FVa, and the quantities as a ratio are shown in this figure.



inhibition by AT + UFH found for Ila-TM(-CS) compared to Ila alone. Mutagenesis of the Ser474, should increase inhibition rates, and could confirm this speculation. Consistent with findings by Bourin et al., steric repulsion, when both CS and UFH compete for binding to exosite 2 of Ila, results in reduced rates of inhibition of Ila by AT in the non-covalent AT + UFH mixtures (24). The maximum reaction rate of Ila-TM(+CS), compared to Ila alone or Ila-TM(-CS) was at a higher concentration of UFH (Fig. 2). From this we can conclude that a higher UFH concentration is needed to force interaction with the Ila-TM complex when CS is present. This also suggests competitive binding of CS versus UFH to Ila. TM(+CS) has a higher affinity for Ila, as the CS can interact with exosite 2 of Ila (5, 9). This may affect Ila reaction with the anticoagulants AT + UFH and ATH. It has been shown that TM(+CS) can potentially bind two Ila molecules (52). Such heterogeneity in Ila binding was unlikely in the experimentation brought forth, as TM was in vast excess to Ila. More likely the TM(+CS) bound to both exosites of a single Ila molecule. Thus, upon addition, UFH will compete with CS for interaction at exosite 2.

The presence of a CS reduces the rate of inhibition, by both AT + UFH and ATH, as a consequence of steric repulsion. However, the significantly higher rate of Ila-TM(+CS) inhibition by ATH compared to AT + UFH suggests that the H moiety in the conjugate may repel the CS into a position that allows for ATH reaction with Ila.

Inhibition of free Ila by ATH has been reported to be at least four-fold faster than AT + UFH (37). Thus, efficient Ila neutralisation by ATH limits its availability to interact with TM, leading to significant reduction in the formation of Ila-TM complex, and consequently APC generation (as shown using a two-stage assay). As reported here, ATH more readily inhibits Ila-TM compared to UFH. Concurrent with free Ila inactivation, inhibition of Ila-TM may prevent excessive anticoagulant activity. Consequently, the in-

creased inhibition of Ila-TM by ATH, compared to UFH, might contribute to the reduced bleeding observed in rabbits at equivalent anti-Xa doses (53,54).

Compared to AT + UFH, ATH was demonstrated to be a better Ila-TM(\pm CS) inhibitor, both by detection of decreased chromogenic substrate activity, and by detection of a decrease in APC generation. To develop a deeper physiological understanding of the effect of ATH on the Ila-TM reaction system, similar rate experiments on HUVEC surfaces were performed. The findings, as seen in Figure 5, were consistent with those found *in vitro*. ATH was a faster inhibitor of Ila-TM and resulted in a greater reduction of APC generation compared to AT + UFH. Anticoagulation thus plays a critical role in APC generation as part of the PC pathway. The role of ATH or UFH extends well beyond coagulation, as it may be critical in the inflammatory response as well.

Ila is a potent activator of protease-activated receptor 1 (PAR-1), resulting in a pro-inflammatory response (55). However, Ila binding to TM inhibits Ila-dependent PAR signalling (56). APC can also induce the activation of PAR-1 (57, 58). The binding of APC to its cofactor EPCR and its activation of PAR-1 alters the signalling response from pro-inflammatory to anti-inflammatory (59). When EPCR is occupied by its APC ligand, PAR-1 activation by either Ila or APC initiates a protective response (59–61). The role and mechanism by which APC elicits a protective signalling response is still somewhat unclear. Bae et al. suggest APC-induced cleavage of PAR-1 leads to a G_i -protein coupled response which ultimately results in the protective signalling response (60). The presence of ATH or UFH could influence the PAR-1 response signalling at various levels. Firstly, ATH inhibits Ila-TM(\pm CS) to a greater degree than UFH, thus resulting in less APC generation. Consequently, the APC-induced cleavage of PAR-1 and resultant protective signalling response may be reduced, compared to UFH. Secondly, compared to UFH, ATH more significantly inhibits Ila

which may give minimal PAR-1 activation by Ila. Consequently, ATH may potentially cause a reduced pro-inflammatory response.

Apart from the roles of Ila and APC in PAR-1 inflammatory mechanisms, APC's primary anticoagulant action is through cofactor inactivation. In a key feature, Ila-TM activation of PC results in the inactivation of FV/FVa (4, 13). Since FXa, along with FVa, phospholipids and Ca²⁺, converts prothrombin (II) into Ila (14,62), inactivation of FV and FVa by APC down-regulates Ila generation. APC cleaves FV primarily at Arg306, followed by cleavage at Arg506, Arg679, or Lys944 (13). On the other hand, inactivation of FVa by APC involves an initial cleavage at Arg506, followed by a cleavage at Arg306, and/or Arg679 (13, 25). Loop 37/70 of APC binds to an electronegative area of FVa, an interaction needed for proper docking at Arg506 (25). H is capable of binding to the 37/70 loop of APC, thus interfering in the interaction with Arg506 and reducing FVa cleavage (25). For FV, H enhances cleavage, and docking at Arg306 is not hindered (25, 26). As indicated by Segers et al., residues Lys320, Arg321, and Arg400 are required for the heparin-mediated stimulation of cleavage at Arg306 by APC (63). These are positive residues with which H can interact (63) to assist in bridging FV and APC for cleavage enhancement, which is consistent with our results (25, 63).

ATH's ability to accelerate cleavage of FVa suggests a lack of interference with the proper docking at Arg506. One speculation to explain differences between UFH and ATH is that the AT moiety of ATH may interact with APC so that the H moiety can no longer interact with the 37/70 loop of APC. If mutagenesis to knock out H binding at the 37/70 loop results in no effect on cleavage of FVa by APC + ATH, this proposed mechanism would be substantiated. Similar to UFH enhancement of FV cleavage, the H moiety of ATH could interact with positive residues of FVa, resulting in accelerated cleavage. However, our data indicate that ATH does not facilitate FV cleavage. The APC-ATH complex may be too bulky to readily interact with Arg306. Since protein S is a cofactor for APC (64), we included a few studies with this agent to increase physiological relevance. Adding protein S gave similar trends for FVa cleavage by APC + UFH or ATH as those seen without protein S (Fig. 8). Thus, although inhibition rate was lower in the presence of protein S, this cofactor affected both UFH and ATH similarly.

Overall, the preliminary data indicate very different and unique results for cleavage of FV/FVa by APC in the presence of ATH compared to UFH. H does not reduce cleavage of FVa, thus allowing FVa to function within the prothrombinase complex. Once activated, FVa within the complex generates Ila (14). In contrast, ATH is capable of reducing the availability of FVa to act within the prothrombinase complex. FV needs to be activated for optimal prothrombinase activity (65). Although ATH does not as readily assist FV inactivation compared to UFH, it more effectively inactivates the FVa that is generated for the prothrombinase complex formation. ATH's enhanced downstream inactivation of the FV pathway may better limit the actual functional steps of the cofactor. Nicolaes et al. found the interference of UFH at the Arg506 site resulted in an inhibitory effect of APC inactivation of FVa, an effect that was concentration-dependent (25). Further experiments, using different concentrations of UFH and ATH may determine the optimal concentration for the collective inactivation of FV and FVa.

In conclusion, ATH has been shown to be a potent anticoagulant. The present *in vitro*-purified protein and HUVECs studies revealed that ATH increases the inhibition rate of coagulation factors controlling the amount of PC activation and efficiency of APC function. Assessing the influence of CS linkage and state of cofactor activation provided insight into the mechanisms by which ATH functions within the PC pathway. We concluded that ATH inhibits Ila and Ila-TM(\pm CS) at a faster rate than AT + UFH. However, ATH induces increased cleavage of FVa, but no effect on FV cleavage by APC. Collectively, these results indicate that ATH has a balanced effect on the PC pathway and continues to show promise as a treatment for thrombotic conditions.

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