Inhibition of the prothrombinase complex on red blood cells by heparin and covalent antithrombin–heparin complex

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The role of red blood cells (RBCs) in coagulation is not well understood. Overt exposure of phosphatidylserine on surfaces of RBCs provide docking sites for formation of the prothrombinase complex, which further aids in amplification of coagulation leading to subsequent thrombosis. No studies to date have evaluated heparin inhibition of the RBC-prothrombinase system. Therefore, this study examines the ability of heparin and a covalent antithrombin–heparin complex (ATH) to inhibit the RBC-prothrombinase system. Discontinuous inhibition assays were performed to obtain k_2 values for inhibition of free or prothrombinase-bound Xa by antithrombin and unfractionated heparin (AT + UFH) versus ATH. In addition, components of the complex (prothrombin, RBCs or Va) were excluded prior to reaction with inhibitors to investigate potential mechanisms involved. Inhibition of thrombin generation, fibrinogen conversion and plasma clotting by the RBC-prothrombinase system was also examined. Protection of Xa was observed for AT + UFH and not for ATH reactions. Inhibition rates for ATH were significantly faster when compared with AT + UFH results. The greatest impact on Xa inhibition was observed from factor Va omission for both inhibitors. ATH inhibited thrombin generation, fibrinogen conversion and plasma clotting better compared with AT + UFH. This study determined potential control of coagulation contributed by RBCs. Moreover, greater control of coagulation is achieved by covalently linking heparin to AT.

Keywords: covalent antithrombin–heparin/heparin/prothrombinase complex/red blood cells.

Abbreviations: AT, antithrombin; ATH, covalent antithrombin–heparin complex; II, prothrombin; RBCs, red blood cells; UFH, unfractionated heparin.

Erythrocytes constitute the majority of cell components in blood. For a long while it has been thought that red blood cells (RBCs) were inert with minimal function in the body, outside of their involvement with gas exchange. However, evidence exists to show that RBCs may have other important functions, such as their role in immunity and vasodilation for example (1, 2). More recently, RBCs have been receiving much attention for their participation in thrombosis (3); however, the exact mechanism is not completely understood.

RBCs from patients with various haematological conditions, such as sickle cell anaemia, beta-thalassemia or preeclampsia, for example, have been linked with having increased procoagulant activity (4–7). Evidence suggests that alterations in RBC membrane, particularly, overt exposure of phosphatidylserine (PS) on the outer leaflet or production of microvesicles rich in PS, may be a potential mechanism to explain their involvement in thrombosis (8). PS exposure on the outer surface of the membrane may provide docking sites for the formation of large macromolecular enzyme complexes (tenase or prothrombinase) that help to amplify and propagate coagulation (9, 10). The prothrombinase complex [which is composed of factor Xa (Xa, the catalytically active component), its cofactor Va (Va), a phospholipid surface (PCPS) and Ca^{2+} ions] is the enzyme complex responsible for converting the substrate prothrombin (II) to thrombin (11–13). Thus, overt prothrombinase activity resulting from increased PS exposure on the surface of RBCs may lead to subsequent thromboembolic complications.

In patients with acute thromboembolism, heparin (UFH) may be used as the first line of treatment in these patients (14, 15). Heparin interacts with an *in vivo* serine protease inhibitor called antithrombin (AT), to assist reaction with many serine proteases in the coagulation cascade (16, 17). However, heparin contains numerous clinical limitations, such as reduced ability to inhibit thrombin and Xa bound to surfaces (18–20). Thus, to overcome these limitations, Chan *et al.* have developed a covalent antithrombin–heparin complex (ATH) with high anticoagulant activity compared with heparin (21, 22). Recently, we have investigated comparison of non-covalent AT + UFH versus ATH to inhibit Xa in prothrombinase composed of synthetic vesicles (23) or platelets (24) and have noticed greater inhibition of Xa by the ATH. Much of the work with prothrombinase and use of anticoagulants has been performed on the native platelet system or synthetic vesicles (25, 26). However, very little is

known regarding heparin's efficiency to pacify the pro-coagulant RBC-prothrombinase system. Considering the vast amount of evidence to show participation of RBCs in coagulation, understanding the ability of heparin or other anticoagulants to pacify coagulant complexes on the surface of RBCs is pertinent. Therefore, this study examines the ability of AT+UFH, compared with ATH, to inhibit the RBC-prothrombinase system.

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, ethylenediaminetetraacetic 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). Phosphatidic acid (PA; 1,2-dioleoyl-*sn*-glycero-3-phosphate) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Human factor Xa, II and thrombin were purchased from Enzyme Research Laboratories Ltd (South Bend, IN, USA). Human factor Va was obtained from Haematologic Technologies Inc. (Essex Junction, VT, USA). Purified fibrinogen was purchased from American Diagnostica Inc. (Stamford, CT, USA). Substrates for Xa (S-2222; *N*-benzoyl-isoleucyl-glutamyl-glycylarginyl-*p*-nitroanilide-hydrochloride and its methyl ester) and thrombin (S-2238; *H*-*D*-phenylalanyl-pipecolyl-arginyl-*p*-nitroanilide-dihydrochloride) were from DiaPharma Group Inc. (Westchester, OH, USA). AT and adult pooled plasma were purchased from Affinity Biologicals (Ancaster, ON, Canada). Unfractionated sodium heparin was obtained from Sigma (St. Louis, MO, USA). Thrombin-specific inhibitor, Pefabloc-TH [$\text{N}\alpha$ -(2-naphthylsulfonylglycyl)-4-amidino-(*D,L*)-phenylalanine piperidide acetate (NAPAP)] was purchased from Centerchem (Norwalk, CT, USA).

Preparation of ATH

ATH was synthesized as described by Chan *et al.* (21). In brief, 1.052 mg of human AT and 64 g of UFH were separately dialysed against 2 M NaCl followed by phosphate buffered saline (PBS) and mixed to a volume of 900 ml, followed by incubation at 40°C for 14 days. The reaction mixture was then mixed with 0.5 M NaBH_3CN reducing agent and incubated for another 5 h at 37°C. A two-step purification process was used, which involved Butyl-Sepharose hydrophobic chromatography (Amersham, Uppsala, Sweden) and DEAE-Sepharose anion exchange chromatography (Amersham) for the removal of any unbound heparin or AT, respectively. ATH was analysed for purity using SDS-PAGE under reducing conditions and was found to be >95% pure (21). 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 (27).

Preparation of RBCs

With approval from the Hamilton Health Sciences/McMaster Research Ethics Board, 10 ml of human blood was drawn from the antecubital vein of healthy donors using 10% acid-citrate/dextrose anticoagulant solution (0.085 M sodium citrate + 0.079 M citric acid + 0.18 M glucose) in a syringe and a 19G butterfly needle (Venisystems, Hospira Inc., Lake Forest, IL, USA) on the day of each experiment. The blood was then transferred to a 15-ml round bottom polypropylene tube and centrifuged at $150 \times g$ for 15 min at 22°C. The platelet rich plasma and buffy coat layers were removed after spinning. The RBCs (taken from the middle of the packed RBCs) were then transferred to another 15 ml round bottom polypropylene tube, resuspended with PBS (1 mM KH_2PO_4 , 154 mM NaCl and 3 mM Na_2HPO_4 ; pH 7.4) and washed three times, twice with PBS and once with Tris buffer (15 mM Tris-HCl, 150 mM NaCl, 5 mM KCl and 1 mM MgCl_2 ; pH 7.4). RBCs were resuspended to 1.0×10^8 RBCs/ml in Tris buffer for use in experiments

within a 6-h time period. The final concentration of RBCs used in all experimental reactions was 1.0×10^6 RBCs/ml.

Determination of second-order rate constants (k_2 values)

k_2 values for inhibition of Xa $\pm$ RBC-prothrombinase complex by AT + UFH versus ATH were determined by performing discontinuous second-order rate constant assays under pseudo first-order conditions, as described earlier (23). All rate experiments were performed at physiological temperature (37°C) and a minimum inhibitor:enzyme ratio of 10:1 was maintained for all reactions. In addition, we have previously determined that maximal inhibition of Xa by AT occurs in the presence of 10-fold mole ratio of heparin to AT, or 30 nM AT + 300 nM UFH (23). For rate experiments involving the inhibition of free-Xa by AT + UFH and ATH, the reaction protocol was as follows: First, 10 μl aliquots of TSP buffer (0.02 M Tris HCl + 0.15 M NaCl + 0.6% polyethylene glycol, pH 7.4) was added to six separate wells of a 96-well flat bottom microtiter plate (Fisher, Nepean, ON, Canada), followed by the addition of 10 μl aliquots containing 24 nM Xa, 32 mM CaCl_2 and 320 μM Pefabloc-TH in TSP buffer to all the wells and incubated for 3 min. Next, 20 μl of 2.75 μM II in TSP was simultaneously added to all six wells with a multichannel pipette and allowed to incubate for another 3 min. To well number 6, 40 μl of TSP buffer was added to represent the control reaction for the experiment. At time intervals ranging from 2 to 5 s, a 40- μl aliquot of 60 nM AT + 600 nM UFH or 60 nM ATH was added in wells one to five. Following another 2–5 s, the reactions were neutralized by the simultaneous addition of 120 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.

For RBC-prothrombinase reactions, the protocol was as follows: freshly isolated RBCs were activated with 20 μM PA and 4 mM Ca^{2+} for 15 min before subsequent reactions. Ten microlitre of the activated RBCs were added to six wells in a 96-well plate, followed by the addition of 10 μl of solution containing 24 nM Xa, 76.8 nM Va, 32 mM CaCl_2 and 320 μM Pefabloc-TH in TSP buffer and incubated for 3 min. Next, 20 μl of 2.75 μM II was added to all six wells with a multichannel pipette and allowed for further incubation. Inhibitors were added and the reactions terminated by the developing solution in the manner as described earlier.

After terminating the inhibition reactions, residual Xa activity for each well was determined by continuous absorbance measurement at 405 nm (SpectraMax Plus 384 plate reader) over a period of time (10–30 min). To calculate the pseudo first-order rate constant k_1 , plots of $\ln V_t/V_0$ (where V_t represents the enzyme activity at time t and V_0 is the initial enzyme activity in well number 6) versus time and the slope were determined. The negative value of the slope gave k_1 ($k_1 = -\text{slope}$). 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.

To investigate the roles of individual components of the RBC-prothrombinase complex on the anticoagulant effects of ATH versus AT + UFH, additional experiments were performed where components of the complex (II, activated RBCs or Va) were excluded before reaction with the inhibitors.

Thrombin generation and inhibition of thrombin generation

Thrombin-specific substrate S-2238 was used to assess the effect of inhibitors on thrombin generation by the RBC-prothrombinase system. Inhibition of RBC-prothrombinase activity by 30 nM AT + 300 nM UFH versus 30 nM ATH was assessed. The concentration of the prothrombinase components was the same as above. Briefly, 10 μl of activated RBCs were added to seven wells of a 96-well plate, followed by the addition of 10 μl of the solution mixture containing 24 nM Xa, 76.8 nM Va and 32 mM CaCl_2 in TSP buffer and incubated for 3 min. Sixty microlitre of a mixture containing 1.6 μM II + 40 nM AT + 400 nM UFH or 1.6 μM II + 40 nM ATH in TSP buffer was then added to the seven wells, one at a time, at 10 s intervals. Ten seconds after addition of the last II/inhibitor mixture, 120 μl of developing solution containing thrombin-specific substrate S-2238 was simultaneously added to all wells, and immediately read in the plate reader at 405 nm for 10 min. Linear portions

of the $V_{\max}$ curves were 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.

Fibrinogen turbidometric analysis

Conversion of purified fibrinogen to fibrin by the RBC-prothrombinase system in the presence of inhibitors was assessed by a clotting turbidity assay at $A_{350\text{nm}}$. Briefly, 10 μl of activated RBCs were added to a well of a 96-well plate, followed by the addition of 10 μl of solution containing 24 nM Xa, 76.8 nM Va, 32 mM CaCl_2 in TSP-2 buffer (0.02 M Tris HCl + 0.15 M NaCl + 0.1% polyethylene glycol, pH 7.4) and incubated for 3 min. Next, 60 μl of solution containing 1.6 μM II, 4 μM fibrinogen, 6.67 mM CaCl_2 , 40 nM AT + 400 nM UFH or 40 nM ATH in TSP-2 buffer was added and immediately read in the plate reader for 60 min at $A_{350\text{nm}}$. The time to half max was then compared for each condition.

Plasma turbidometric analysis

Plasma clotting was assessed using a similar turbidity protocol outlined earlier. Briefly, 25 μl of activated RBCs were added to a well of a 96-well plate, followed by the addition of 25 μl of 40 mM CaCl_2 containing 120 nM AT + 1,200 nM UFH or 120 nM ATH. The control contained no inhibitors. Addition of 50 μl of adult pooled platelet poor plasma (3.2% v/v sodium citrate) started the reaction, and the reaction absorbance at 350 nm was immediately read in the plate reader for 3 h. The time to half max was then compared for each condition.

Data analysis

The inhibition rate experiments were performed at $n=5$, fibrinogen and plasma turbidometric analyses were at $n=5$ and at least $n=3$,

Fig. 1 Thrombin generation. A single time point comparison of thrombin generation by non-activated red blood cells (nRBCs) to those activated with PA and Ca^{2+} (aRBC) for 15 min prior to reaction with prothrombinase components. These data suggests that aRBCs contained enhanced prothrombinase activity compared with nRBCs; * $P < 0.006$.

respectively, as previous work using these assays showed this number of replicates is sufficient to show statistical significance between groups. Statistical analysis for multiple groups was performed using ANOVA. In the case of comparison between groups, a student t -test was used. Values with a $P < 0.05$ were considered significant.

Results

Thrombin generation by the RBC-prothrombinase system

Results from Noh *et al.* were recapitulated using our thrombin generation method, thus confirming the functionality of the PA-induced RBC-prothrombinase system (Fig. 1).

Comparison of k_2 values for inhibition of Xa $\pm$ RBC-prothrombinase complex

Discontinuous second-order rate constant assays (28) were performed to determine the effect of RBC-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 RBC-prothrombinase complex. Inhibition of both free Xa and Xa within the RBC-prothrombinase by ATH resulted in significantly higher values compared with those of AT + UFH. Protection of Xa by the components of the RBC-prothrombinase complex was not observed for ATH, whereas a moderate protection of $\sim 60\%$ for free Xa was observed for AT + UFH (Fig. 2). Overall, there was a >15 -fold protective effect for AT + UFH when compared with ATH.

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

To examine the roles of prothrombinase components on mechanisms of Xa inhibition by AT + UFH versus ATH, discontinuous inhibition assays were also performed to compare the inhibition of the intact RBC-prothrombinase to a prothrombinase where various components were combined or omitted before reaction with inhibitors (Table I). For AT + UFH reactions, relative to the intact prothrombinase, there was a significant increase in Xa inhibition when the substrate II was added to the system, a drastic increase almost to the level of free Xa when activated RBCs were omitted, and a further decrease in Xa inhibition

Table I. Inhibition of Xa within the prothrombinase complex by AT + UFH versus ATH.

Condition	AT + UFH (k_2 values $\pm$ SD)	ATH (k_2 values $\pm$ SD)
Free Xa	$3.14 \times 10^8 \pm 0.27 \times 10^8^{**}$	$5.12 \times 10^8 \pm 0.60 \times 10^8$
Prothrombinase	$1.40 \times 10^8 \pm 0.19 \times 10^8$	$4.47 \times 10^8 \pm 1.09 \times 10^8$
Prothrombinase + II	$2.10 \times 10^8 \pm 0.57 \times 10^8^*$	$4.85 \times 10^8 \pm 0.53 \times 10^8$
Xa + Va + Ca^{2+} + II	$2.95 \times 10^8 \pm 0.59 \times 10^8^{**}$	$4.99 \times 10^8 \pm 1.01 \times 10^8$
Activated RBCs + Xa + Ca^{2+} + II	$4.63 \times 10^7 \pm 0.82 \times 10^7^*$	$2.86 \times 10^8 \pm 0.32 \times 10^8^*$

* $P < 0.05$, ** $P < 0.001$ relative to prothrombinase.

Fig. 2 Protection of Xa by the prothrombinase complex for AT + UFH and ATH. Inhibition of Xa decreased by ~60% for AT + UFH when Xa was assembled in the prothrombinase complex, whereas an insignificant decrease of 4% was observed for ATH. An overall >15-fold change in the protective effect was noticed between the two inhibitors; * $P < 0.0001$.

upon Va exclusion. As for ATH reactions, a decrease in Xa inhibition was observed only for Va omission, whereas no change was observed for the other conditions.

Inhibition of thrombin generation

Thrombin generation was performed to examine the effect of AT + UFH versus ATH on functionality of the intact RBC-prothrombinase system using physiological levels of II (Fig. 3). Both inhibitors decreased thrombin generation compared to the control, with ATH having a greater effect (Fig. 3A). When the results were converted to inhibition of thrombin potential (area under the curve), ATH significantly reduced thrombin potential compared to AT + UFH (Fig. 3B).

Inhibition of fibrinogen conversion by the RBC-prothrombinase

A turbidity assay was performed to determine the effect of the RBC-prothrombinase system on fibrinogen conversion in the presence or absence of inhibitors (Fig. 4). Both AT + UFH and ATH significantly increased the time to half max (a measure of time to clot) compared to a control assay with no inhibitors. Moreover, fibrinogen conversion by the RBC-prothrombinase was inhibited much better to ATH compared to AT + UFH.

Inhibition of plasma clotting

Addition of activated RBCs to plasma increased the time at half max compared to a system with no activated RBCs (data not shown). The effect of inhibitors was tested in this activated RBC/plasma clotting system. Relative to a control containing activated RBCs in plasma with no added inhibitor, the time to half max was significantly prolonged for ATH but not for AT + UFH. These data suggested that ATH may be a more efficient inhibitor of plasma clotting (Fig. 5), consistent with results for purified fibrinogen.

Discussion

Heparin, heparin derivatives and other anticoagulants are clinically used for the treatment and prophylaxis of thromboembolic diseases (15). In the past few decades, much of the work on heparin's ability to inhibit the prothrombinase complex has been performed on systems comprised of vesicles and platelets (25, 26). This is understandable because platelets are the primary cells in the body that participate in clotting and formation of coagulant complexes. However, as synthetic vesicles readily reproduce platelet results, are easier to maintain and are facile to make, they are very useful platform for studying coagulation.

Various investigations have reported that RBC counts are closely related to bleeding time and occurrence of thrombosis (29). For example, transfusion of RBCs abolishes bleeding in patients with prolonged haemorrhagic complications (30). Conversely, individuals with polycythemia vera (having abnormally high blood cell counts) often experience thrombotic complications (31, 32). In both these examples, RBC levels were a predictive marker for thrombosis (8). Furthermore, although venous thrombi contain a large proportion of fibrin-entrapped RBCs with relatively few platelets (33), venous thrombi have a high occurrence rate of thromboembolic events. Even though RBCs constitute the highest proportion of the cell component in blood, they have been regarded as inert with respect to thrombosis and hemostasis (8). While the exact mechanism for RBCs participation in thrombosis is still under investigation, a large body of evidence suggests that PS exposure on the outer surface of RBCs may be a major contributor, as PS allows for formation of enzyme complexes (such as prothrombinase) that aid with amplification and propagation of the coagulation cascade (7). To better understand treatment and prophylaxis of thromboembolic events, it is important to examine inhibition of surface-complexed enzymes when the surface is contributed by a major cell type, such as RBCs.

Previous investigations have revealed that intracellular signalling through an increase in Ca^{2+} , Ca^{2+} -dependent activation of phosphokinase C- α (PKC- α) (34) and ATP depletion (35) can mediate PS exposure in RBCs. In addition, other signalling factors, such as arachadonic acid (3), prostaglandins (36) and platelet-activating factor (37), can induce PS exposure on RBCs. However, the extent of this exposure is minute, ranging from 3 to 5% of the total lipids on the surface (9). As demonstrated by a recent study, RBCs treated with PA responded quickly by decreasing intracellular ATP levels, increasing Ca^{2+} and activating PKC- α , which further resulted in activation of the scramblase and attenuation of flippase enzymes (9). Cumulatively, this caused exposure of PS on the surface of RBCs and accelerated thrombin generation through enhanced prothrombinase activity (9). We modified the protocol from Noh *et al.* and adapted it with our well-established discontinuous inhibition assays (28) to examine inhibition of prothrombinase on the RBC surface. Before starting the discontinuous inhibition assays, we wanted to prove through reproduction of

Fig. 3 Inhibition of thrombin generation by the RBC-prothrombinase complex. Thrombin generation in the presence of inhibitors was assessed using a specific thrombin substrate S-2238 over a time course. It appears that both AT + UFH and ATH reduced thrombin generation compared to the control (no inhibitor) with ATH having a greater effect (A). However, when assessed for inhibition of thrombin potential, ATH significantly reduced the thrombin potential whereas AT + UFH did not, compared with the control (B). Final concentration of reactants: 3 nM Xa, 9.6 nM Va, 4 mM Ca^{2+} , 1.2 μM II, 30 nM AT, 300 nM UFH and 30 nM ATH; * $P < 0.005$.

the results by Noh *et al.*, that our protocol for pretreatment of RBCs with PA and Ca^{2+} also yielded elevated prothrombinase activity (Fig. 1).

Many studies on prothrombinase inhibition by AT + heparin, or heparin derivatives such as low molecular weight heparin (LMWH) and pentasaccharide (fondaparinux), have been performed using platelets and vesicles (18, 19, 23). In these previous studies, it was noticed that UFH yielded the highest inhibition rates, followed by LMWH then fondaparinux. In our study, we utilized activated RBCs instead and were able to show inhibition of RBC-prothrombinase

complex by AT + UFH versus ATH (Table I). We observed that ATH inhibited Xa in the prothrombinase complex far better than AT + UFH. We also observed a significant reduction in Xa inhibition when factor Xa was incorporated in the prothrombinase complex for AT + UFH reactions. This is consistent with previous findings using vesicle or platelet surfaces (18, 19, 23). The decrease in Xa inhibition is often referred to as the protective effect, where certain components of the complex protect the catalytically active enzyme from inhibition by anticoagulants (38). Conversely, protection of Xa was not observed for

Fig. 4 Inhibition of fibrinogen conversion by the RBC-prothrombinase system. Using a turbidity assay, the ability of thrombin (generated by the RBC-prothrombinase complex) to convert fibrinogen to fibrin in the presence or absence of inhibitors was assessed. Both inhibitors increased the time to half max (in essence time to clot) compared with the control. However, ATH caused a further increase in the time to half max compared with AT + UFH, suggesting that ATH suppressed fibrinogen conversion better than AT + UFH. Final concentration of reactants: 3 nM Xa, 9.6 nM Va, 4 mM Ca^{2+} , 1.2 μM II, 3 μM fibrinogen, 30 nM AT, 300 nM UFH and 30 nM ATH; * $P < 0.0001$, # $P < 0.05$.

ATH, as inhibition of both free Xa and Xa complexed within prothrombinase yielded similar k_2 values for all the ATH reactions. Moreover, AT + UFH displayed a >15-fold difference in the protective effect over ATH (Fig. 2). One simple explanation for ATH's enhanced inhibitory effect is that covalent linkage prevents dissociation of the heparin moiety and, thus, prohibits potentially non-productive interactions with prothrombinase components observed with the UFH from AT + UFH mixtures (23). Other detailed structural features may moderate these mechanisms. We have recently obtained preliminary data on inhibition of the RBC-prothrombinase system by LMWH and fondaparinux. Although protection of Xa inhibition by prothrombinase on activated RBCs was not observed, the measured inhibition rates were one and two orders of magnitude lower for LMWH and fondaparinux, respectively, relative to UFH and, especially, ATH. When we repeated this work with fondaparinux using synthetic vesicles in place of RBCs, protection of Xa was restored to levels reported in the literature (26). We intend to further investigate these findings using low molecular weight fractions of ATH and RBC membrane components to determine the heparin chain length dependence on anti-coagulant interactions of serpin-heparinoids with activated RBCs.

Previous investigations with vesicle and platelet surfaces determined that II is the key player protecting factor Xa from inhibition by AT + heparins (UFH, LMWH or fondaparinux) (18, 19, 23, 26). However, these systems utilized a prothrombinase where the substrate for the enzyme was part of the complex, which does not fall under the definition of prothrombinase in

Fig. 5 Inhibition of plasma clotting by AT + UFH versus ATH. Adult pooled plasma was reacted with activated RBCs (aRBCs) + CaCl_2 with or without inhibitors and analysed for the time to reach half max. ATH significantly attenuated plasma clotting compared with the control (plasma + aRBCs with no inhibitors added) and AT + UFH as shown by a significant prolongation of the time to half max. There was a trend for increased time to half max for AT + UFH relative to the control; however, it was not statistically significant; * $P < 0.01$, ** $P < 0.001$.

this study (11). For AT + UFH reactions, we determined that inhibition of prothrombinase in the absence of substrate II yielded a significant 60% reduction in the inhibition of complexed Xa compared with free Xa. However, when the substrate was included with the prothrombinase system, inhibition rates increased. This is contrary to previous findings with vesicle systems (23). In our previous study with synthetic phospholipid vesicles, when a prothrombinase without the presence of its substrate was reacted with AT + UFH or ATH, the rate of Xa inhibition increased relative to a prothrombinase in the presence of its substrate (although not reaching the rates observed for free Xa) (23). Furthermore, omission of Va from the RBC prothrombinase caused an even further drop in the k_2 values, which is also contrasts with previous findings on the vesicle system (23). Thus, surface-based dissimilarities between a heterogeneous RBC plasma membrane and purified PCPS bilayers may underlie these confounding results. Although consistent with previous observations, when the phospholipid surface (aRBCs) was removed, the k_2 values increased almost to the same level as free Xa reactions (Table I). As there is no membrane for the prothrombinase complex to form on, it makes sense to observe elevated k_2 values for these conditions. For ATH reactions, relative to the intact prothrombinase, addition of the substrate or exclusion of activated RBCs caused no net effect, but omission of Va reduced the k_2 values. Thus, in the absence of Va, the Xa/II/ Ca^{2+} /RBCs complex configuration resulted in a greater protection of Xa, which is opposite to results of previous studies involving different cell surfaces (23). Mechanistically, this suggests that the membrane surface required for coagulation complex interaction

on activated RBCs may be different compared with vesicles or platelets, and should be subjected to further investigations in the future. The methodology employed in this study is well-recognized and has been used in prior experiments with synthetic vesicles or platelets to acquire results consistent with that of other investigators (23).

Prothrombinase has been the target of past, present and novel anticoagulants, thus emphasizing the importance of targeting this enzyme for treatment and prevention of thromboembolism (33). Since prothrombinase is the enzyme complex responsible for converting II to thrombin, examining the ability of the RBC-prothrombinase to convert II to thrombin in the presence of inhibitors is important. In this study, it appeared that both AT+UFH and ATH may attenuate thrombin generation (Fig. 3A) and the thrombin potential (Fig. 3B); however, further analysis revealed that inhibition by ATH was statistically greater compared with AT+UFH.

Thrombin feeds back variously in the coagulation cascade, with one of the functions involving conversion of fibrinogen to fibrin (39). In this study, we attempted to show the capability of the RBC-prothrombinase-generated thrombin to convert fibrinogen to fibrin in the presence of inhibitors using a fibrinogen turbidity (clotting) assay (Fig. 4). We observed that both AT+UFH and ATH significantly increased the time to half max compared to the control with no inhibitors, suggesting overall suppression of fibrinogen conversion by both agents. Moreover, the time to half max was much greater for ATH compared with AT+UFH. Similar results were observed when a plasma turbidogenic assay was employed (Fig. 5). In the plasma experiments, ATH significantly prolonged the time to half max compared with AT+UFH, suggesting an overall enhanced antithrombotic effect for ATH compared with AT+UFH in this more native system.

This study sheds some light into potential control of coagulation occurring in samples containing RBC products. These data also reveal important findings involved in the anticoagulant effects of heparin and ATH against RBC-bound coagulation enzymes. It is clear that the prothrombinase components hinder the ability of conventional heparin to inhibit Xa. However, covalent linkage between AT and heparin assists access and neutralization of complexed Xa, with concomitant inhibition of prothrombinase function, as assessed through inhibition of thrombin generation and fibrinogen conversion. Enhanced inhibition of serine proteases on surfaces and within supramolecular complexes encourages further investigation of ATH for clinical application in thrombosis. Moreover, participation of the membrane surface on activated RBCs in coagulation complex formation may be different compared with conventional surfaces studied previously. This aspect further incites continued investigation into the prothrombotic properties of RBCs.

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Conflict of Interest

None declared.

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