Factors Influencing the Acceleration of Human Factor XIa Inactivation by Antithrombin III

By Cheryl F. Scott and Robert W. Colman

Controversy exists in the literature concerning the potentiating effect of heparin on the inactivation rate of factor XIa by antithrombin III (AT III) in both purified systems and in plasma. We have analyzed the factors that could influence this reaction and found that ionic strength is the medium, as well as the type and concentration of the heparin preparations accounted for the major discrepancies in the literature. At I = 0.43 N, a preparation of bovine lung heparin at 1 U/mL did not augment the inactivation rate of factor XIa by inhibitors in plasma or by purified AT III. However, when ionic strength was decreased, a progressive increase in the potentiating effect was observed, reaching 6.5-fold at 1 = 0.15 N. At saturating concentrations of heparin, which results in the formation of 100% AT III-heparin complex, (~ten-fold molar excess over AT III) in purified systems, all heparin preparations (porcine, bovine, low molecular weight [LMW], and high affinity) yielded an ~30-fold augmentation of the factor XIa inactivation rate. However, when heparin was less than saturating, we observed that various heparin preparations affected the AT III-induced inactivation of factor XIa to different degrees even though they exhibited the same inhibitory activity (1 U/mL) against thrombin. This variation resulted from differences in the number of AT III binding sites in each heparin preparation, despite a similar Kd for each. Addition of high molecular weight kininogen (HK) to AT III-heparin complexes did not enhance their ability to inhibit factor XIa, and high concentrations of HK decreased the inactivation rate. A high therapeutic dose of heparin only permits the formation of 2.5% to 16.5% of the AT III-heparin complexes that can be achieved at saturation. We observed that 1 U/mL heparin (bovine lung heparin) (high therapeutic concentration) in virtually undiluted plasma only accelerated the inactivation rate of factor XIa (in the absence of other active enzymes) less than two-fold. These new observations further support our previous conclusion that therapeutic levels of heparin have little to no influence on the inactivation rate of factor XIa in plasma.

We have analyzed the factors responsible for the apparently discrepant results and in this report, we provide new data that should resolve most of the previous difficulties in understanding the influence of AT III-heparin complexes on the inactivation of factor XIa in plasma when heparin is present at therapeutic concentrations.

MATERIALS AND METHODS

Heparins

Bovine lung heparin (140 U/mg) was purchased from Sigma Chemical Co (St Louis). USP Reference Standard heparin was obtained from U.S.P.C., Inc (Rockville, MD). Low molecular weight heparin (Fragmin) and AT III were gifts of KablVitrum (Malmö, Sweden). Fragmin contains 68 factor Xa U/mg and 142 factor Xa Xa U/mg. AT III (affinity purified on Heparin-Agarose) has a specific activity > 51 U/mg protein (KablVitrum). High affinity heparin that has been highly characterized was generously provided by Dr Robin A. Pixley (Temple University School of Medicine, Philadelphia).

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Factor XI and HK

Factor XI was prepared by the method of Scott et al., and was activated with human factor XIa at a molar ratio of 1:100. The reaction was terminated by neutralization of factor XIa with corn trypsin inhibitor. The resultant factor XIa had a specific activity of 200 U/mg (1 unit is defined as the amount in 1 mL normal, pooled plasma). HK was purified as described and had a specific activity of 12 coagulant U/mg.

Other Reagents

The chromogenic substrate < Glu-Pro-Arg-paranitroanilide (S-2366) was kindly provided by KabiVitrum (Mölndal, Sweden) and its United States distributor, Helena Laboratories (Beaumont, TX). Tris base and polyethylene glycol 8000 (PEG) were purchased from Sigma Chemical Co. Microplates were Falcon Plastics Micro Test III 96-well tissue culture-treated plates (no. 3075), purchased from Becton Dickinson Labware (Lincoln Park, NJ). The microplate reader was a Bio-Rad 2550 EIA Reader (Bio-Rad Laboratories, Richmond, CA). All other reagents used were the best grade available.

Estimation of Molar Concentration of Heparins

Bovine lung and porcine intestinal. Assuming an average molecular weight of 15,000 and a specific activity of 140 IIa U/mg (manufacturer), 1 U/mL = 0.5 µmol/L.

Low molecular weight. Fragmin has an average molecular weight of 6,000 and contains 68 IIa U/mg (KabiVitrum). Therefore, 1 U/mL = 2.4 µmol/L.

High Affinity. The average molecular weight of high affinity heparin was 22,000 and specific activity was 870 IIa U/mg. Therefore, 1 U/mL = 0.05 µmol/L.

Determination of the Inactivation Rate of Factor XIa by AT III or Plasma in the Presence or Absence of Heparin

All inactivation rates were determined at 23°C by a two-stage reaction. Previous studies of FXIa inhibition indicate that the rate of inhibition at 37°C is 3.3-fold that at 23°C. The buffer used in the present experiments was 20 mmol/L Tris-Cl, pH 7.4, containing 0.15 mol/L NaCl, 2 mmol/L EDTA, and 0.1% PEG. Factor XIa was added to polypropylene centrifuge tubes containing either plasma or purified AT III. At various times, a portion was transferred to a microplate containing the chromogenic substrate, S-2366 (1.9 mmol/L, final concentration). After a fixed hydrolysis time, the reaction was quenched with 50% acetic acid. The plate was read at 405 nm and a blank (all reagents added to acetic acid) was subtracted from each value.

Validation of the Microplate Assay for Determining the k' for Factor XIa and AT III

Factor XIa (50 nmol/L) was incubated with AT III ranging in concentration from 0.52 µmol/L to 2.6 µmol/L. At all concentrations tested, we observed pseudo-first-order inactivation rates (Fig 1). The k' (pseudo-first-order rate constant) was calculated from the half-life (t½) at each concentration and was plotted as a function of the AT III concentration (Fig 1, inset). We observed a linear relationship between k' and AT III concentration with a k" (second-order-rate constant) of 1.1 x 10⁴ (mol/L)⁻¹ min⁻¹, assuming 1 U/mL AT III equals 2.6 µmol/L.

RESULTS

Effect of Ionic Strength on the Acceleration of Factor XIa Inactivation Rate by AT III

We previously used a phosphate buffer of high ionic strength (I = 0.43 N) as compared with the ionic strength of plasma (I = 0.15 N). Because the interaction of heparin with AT III is primarily due to electrostatic forces, we postulated that the different buffers used in various studies could explain some discrepancies. We, therefore, examined the effect of increasing ionic strength on the heparin-induced acceleration. Although in our earlier report (using porcine intestinal mucosal heparin) we failed to observe differences...
in the inactivation rate with heparin, we did observe a slight effect with the bovine lung heparin using the high ionic strength phosphate buffer (data not shown). However, we observed a more pronounced acceleration of ~6.5-fold using a Tris buffer at I = 0.15 N when bovine lung heparin was included at a concentration of 1.0 U/mL in the incubation mixture (Fig 2). When the ionic strength was increased to 0.22 keeping the buffer species constant, the potentiation decreased to 2.2-fold. At I = 0.32, the observed potentiation was only 1.3-fold, and no potentiation was observed at I = 0.43. Therefore, ionic strength and heparin type both affect the heparin-induced acceleration of factor XIa inactivation by AT III.

Comparison of Various Heparins on the Inactivation Rate of Factor XIa by AT III

Because of the differences between bovine lung heparin (Fig 2) and porcine intestinal mucosa heparin, we compared the effect of various heparin preparations on the acceleration of AT III-induced inactivation of factor XIa. AT III (0.24 μmol/L) was incubated with saturating concentrations of heparin (72 to 83-fold molar excess over AT III) before incubation with factor XIa. The inactivation kinetics were compared with those of factor XIa with 7.4 μmol/L AT III in the absence of heparin. The inactivation followed pseudo-first-order inactivation kinetics in all cases (Fig 3). Using either bovine lung, porcine intestinal, or low molecular weight heparin (Fig 3), the inactivation rate in the presence of each heparin preparation was similar. Furthermore, each was similar to the inactivation rate with 7.4 μmol/L AT III in the absence of heparin, which indicates that the maximal observable potentiation was ~30-fold, in agreement with the report by Soons et al. Even high affinity heparin, which had a specific activity of 840 U/mg, at saturating concentrations, also exhibited a maximal, 30-fold acceleration of AT III-induced inactivation of factor XIa.

![Figure 2](image1.png)

**Fig 2.** Effect of ionic strength on the acceleration of factor XIa by AT III. Factor XIa (50 nmol/L) was incubated with AT III (1.58 μmol/L) in 20 mmol/L Tris-Cl, pH 7.4, containing 0.1% PEG and 2 mmol/L EDTA containing various concentrations of NaCl (I = 0.15 – 0.43) in the presence of 1 U/mL bovine lung heparin. The inactivation rates were compared with factor XIa incubated with 1.58 μmol/L AT III, in the absence of heparin, in the above-mentioned buffer (I = 0.15) (□). The data are the average of duplicate experiments. • = 0.15, x = 0.22, ○ = 0.32, □ = 0.43.

![Figure 3](image2.png)

**Fig 3.** Saturation of AT III by various heparin preparations. Reference heparin (USPC) (porcine mucosal) was added to AT III before addition of factor XIa, achieving a concentration of 20 μmol/L heparin and 0.24 μmol/L AT III (□). Bovine lung heparin was added to AT III before the addition of factor XIa to achieve a concentration of 17.3 μmol/L heparin and 0.24 μmol/L AT III (○). Low molecular weight heparin was added to AT III before the addition of factor XIa to achieve a final concentration of 19.8 μmol/L heparin and 0.24 μmol/L AT III (×). Factor XIa was also incubated with 7.4 μmol/L AT III in the absence of heparin (●). The data represents the average of duplicate or triplicate determinations.
(data not shown). However, this did not explain the variation in the degree of augmentation of the inactivation rate produced by heparin preparations when used below saturation.

**Determination of the Number of AT III Binding Sites on Heparin From Various Sources**

To further probe the cause for the varying effectiveness of heparin preparations, we determined heparin binding sites by the method of Soons et al. Since each heparin preparation exhibited a maximal potentiation of an ~30-fold (Fig 3), the fraction of heparin bound could be determined by the ratio of the acceleration by heparin concentrations, which were less than saturating, to the inactivation rate of factor XIa by AT III in the absence of heparin. A plot of the reciprocal of the bound heparin ν the reciprocal of the free heparin allows calculation of the Kd and the number of binding sites. These values are stated in Table 1. We found the Kd to be similar for each preparation but the number of binding sites differed considerably. Bovine lung heparin contained 1.2 binding sites/mol, reference heparin (U.S.P.C.) 0.66 sites/mol, low molecular weight kininogen (Fragmin) 0.12 sites/mol, and high-affinity heparin 2.2 sites/mol. These values compare with 0.42 sites/mol observed with the heparin preparation used by Soons et al. It therefore appears that, at saturating concentrations of heparin, any preparation will potentiate the AT III-induced inactivation of factor XIa ~30-fold. However, on a molar basis, or more importantly, when concentrations are compared in factor IIa U/mL, which is the International Reference Standard, major differences in heparin preparations can be observed, especially at therapeutic heparin levels (0.15 to 0.35 μmol/L) which are far below the level required to saturate AT III in the patient’s plasma.

**Prediction of k’ in Plasma With and Without Heparin**

Plasma deficient in kininogens (HK-LK-deficient) was used to avoid contact activation, which would otherwise occur on incubation in polystyrene, as well as to avoid protection of factor XIa from inactivation by HK. The k’ of plasma was calculated from the expected concentrations of inhibitors in plasma, as previously assayed. Plasma was diluted with Tris buffer to maintain ν 0.15. Factor XIa was then added and an aliquot, at various times, was transferred to a microplate well containing < Glu-Pro-Arg-pNA (S-2366). After incubation at 23°C, with the substrate, the hydrolysis was quenched with acetic acid. The k’ was calculated from the reciprocal of the free heparin. The k’ value that was predicted from data derived in purified systems was calculated k’ and the observed k’ for each concentration plasma as well as a subtended angle of 45°, indicating close correspondence between the theory and observations. We then added bovine lung heparin (0.5 μmol/L) to either a dilution containing 12.5% plasma (−0.31 μmol/L, AT III) or 6.25% plasma (−0.156 μmol/L, AT III) in order to change the ratio of heparin to AT III in the sample. These conditions resulted in the degree of potentiation that was predicted from the acceleration of factor XIa inactivation by the AT III-heparin complex. For example, in the 12.5% plasma sample using 0.5 μmol/L bovine lung heparin that contains 1.2 binding sites/mol, we expected that the k’ would increase from 0.032 min⁻¹ to 0.18 min⁻¹, since all of the AT III in the sample would be complexes with heparin. We observed an increase from 0.036 min⁻¹ to 0.19 min⁻¹. Similarly, for the 6.25% plasma sample, we calculated an increase in the k’ from 0.016 min⁻¹ to 0.09 min⁻¹ and observed an increase from 0.016 min⁻¹ to 0.11 min⁻¹. Therefore, the effect of heparin can be estimated by knowing its molarity and the number of binding sites for AT III.

If, however, concentrated bovine lung heparin was added to plasma to achieve a concentration of 1 U/mL (0.5 μmol/L) before diluting the plasma for assay, little effect was observed because the heparin was diluted as well. With plasma at a concentration of 98% (Fig 5A), an approximate two-fold potentiation was observed, consistent with a calculated 1.8-fold potentiation. Using a 10% plasma dilution (Fig 4). We observed a linear relationship between the calculated k’ and the observed k’ for each concentration plasma as well as a subtended angle of 45°, indicating close correspondence between the theory and observations.

**Table 1. AT III-Factor XIa Binding Sites on Heparins**

<table>
<thead>
<tr>
<th>Type of Heparin</th>
<th>Sites/mol Heparin</th>
<th>Kd (μmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMW</td>
<td>0.12</td>
<td>145</td>
</tr>
<tr>
<td>Reference</td>
<td>0.66</td>
<td>111</td>
</tr>
<tr>
<td>Bovine Lung</td>
<td>1.2</td>
<td>143</td>
</tr>
<tr>
<td>High Affinity</td>
<td>2.2</td>
<td>125</td>
</tr>
</tbody>
</table>

The number of binding sites and the Kd were determined according to Soons et al.
Fig 5. Effect of a high therapeutic level of heparin on the inactivation rate of factor Xla by plasma. (A) Bovine lung heparin was added to plasma before addition of factor Xla (98% plasma). (B) Plasma containing heparin from A was diluted to achieve a final concentration of 10% plasma. Samples were assayed as in Materials and Methods. The data represent duplicate determinations. • = no heparin; + = 1 U/mL heparin.

5B) (where heparin had been added prior to plasma dilution), we also observed about a two-fold potentiation.

Effect of High Molecular Weight Kininogen on the Inactivation Rate of Factor Xla by AT III

Because of the recent report of HK containing a binding site for heparin, we queried whether HK could modulate the influence of heparin on the inactivation rate of factor Xla by AT III. In the presence of 4.7 μmol/L AT III, 0.37 U/mL HK, and 0.5 μmol/L bovine lung heparin, the inactivation rate of factor Xla was indistinguishable from the inactivation rate in the absence of HK (Fig 6). At 0.94 U/mL HK, there was a slight decrease in the inactivation rate. A high concentration of HK (1.88 IU/mL) slowed the inactivation rate almost to the rate observed in the absence of heparin.

DISCUSSION

The importance of heparin as an accelerator of AT III-induced inactivation of factor Xla has been controversial. Several reasons exist that could possibly explain the conflicting data and varying conclusions. First, when working with a heparin concentration that is below saturation of the AT III, various types of heparin will yield different degrees of acceleration of the inhibition of factor Xla. Second, most conclusions regarding the effectiveness of heparin were based on the observation that the AT III-heparin complex inactivates factor Xla ~30-fold faster than does free AT III. However, therapeutic concentrations of heparin in plasma are far below saturation and, therefore, would only allow the formation of 2.5% to 16.5% of the potential AT III-heparin complexes. Third, this formation would critically depend not only on heparin concentration, but also on the type of heparin used. The concentration of AT III-heparin complex occurring at therapeutic heparin doses would only produce a 10% to 70% increase in the total plasma inhibitory capacity toward factor Xla, as opposed to the 3,000% increase that can be achieved in vitro where heparin is present at concentrations exceeding a 10-fold molar excess over AT III and assuming that factor Xla as the only active enzyme in the plasma sample.

We observed no augmentation of the inactivation rate in the presence of 1 U/mL bovine lung heparin when factor Xla and AT III were incubated in a buffer at I = 0.43. However, a progressive decrease of ionic strength from I = 0.43 to I = 0.15 increased the degree of heparin-mediated acceleration of the AT III-induced inactivation of factor Xla (Fig 2), which is consistent with predominantly electrostatic interaction.
tions. This observation explains the difference in the results that we previously obtained when a high ionic strength buffer was used. At saturating heparin concentrations, however, all heparins, regardless of their specific activity (as measured in factor IIa units), produced a potentiation of ~30-fold (Fig 3), which most likely reflects the achievement of a ten-fold molar excess of “active heparin” over AT III. This observation agrees well with that of Soons et al for their heparin preparation.

The number of AT III binding sites differed for each preparation of heparin tested (Table 1), which is consistent with varying amounts of the biologically active oligosaccharide required to form the ternary complex of factor XIa, AT III, and heparin. Danielsson et al reported that a minimum of 18 monosaccharide units are necessary to bind AT III with thrombin on a heparin molecule, whereas an oligosaccharide unit containing the “specific pentasaccharide region” is sufficient to bind factor Xa with AT III. The inactivation of factor IXa (mol wt = 55,000) is accelerated 10,200-fold by heparin while the inactivation of thrombin (mol wt = 35,000) is accelerated 4,000-fold.

The k’ in plasma for factor XIa can be predicted in the presence and absence of heparin (Fig 4), based on the rate constants derived in purified systems. We observed excellent agreement between calculated and observed rate constants (Fig 4), which indicated a 1.8-fold potentiation of the inactivation rate of factor XIa by plasma containing 1 U/mL bovine lung heparin (high therapeutic dose) as compared with nonheparinized plasma. Although heparin can interact with a wide variety of plasma proteins, the ability to predict the interaction from experiments in purified systems probably stems from the fact that the affinity and/or concentration of such binding proteins is less than AT III, and thus, the effective heparin concentration is not decreased by these interactions.

The effect of HK on the inactivation rate of factor XIa by AT III ± heparin was assessed (Fig 6). HK, at a concentration of 0.37 U/mL did not influence the reaction. This behavior contrasts with the marked acceleration of the inactivation rate of plasma kallikrein by AT III in the presence of heparin (Steven T. Olson, personal communication, November 1988). At an HK concentration of 0.94 U/mL, we observed a slight decrease in the inactivation rate of factor XIa (Fig 6, O—O), and a substantial decrease in the inactivation rate at an HK concentration of 1.88 U/mL (O—O), which may be the combined effect of HK protection of factor XIa inactivation and heparin binding to HK.

One question that had not been addressed in the past is the availability of the AT III-heparin complex to factor XIa in the presence of other active proteases, namely thrombin, factor Xa, or factor IXa. Based solely on kinetics, enzymes having a high kassoc would compete better for an inhibitor than an enzyme with a low kassoc. We recently compared the kassoc for AT III and coagulation proteases in the presence and absence of saturating heparin concentrations. The kassoc for thrombin and AT III-heparin complex was 3,000 times greater than kassoc for factor XIa with AT III-heparin complex. Moreover, there is 40 times more prothrombin in plasma than factor XI. Therefore, during pathologic processes where zymogens of plasma coagulation enzymes are activated, one may expect that if a small amount of thrombin were to form, it may compete more successfully than factor XIa for the available AT III-heparin complexes. However, a direct observation in a clinical situation would be required to critically test this hypothesis. Although the inactivation rate of factor IXa by AT III is markedly potentiated by heparin, factor IXa may also have difficulty competing with thrombin for AT III-heparin complex because the concentration of prothrombin is 30-fold greater than the concentration of factor IX in plasma, and the kassoc of thrombin and AT III is five times greater than the kassoc for factor IXa and AT III.

The prediction that factor XIa would not be significantly inactivated by AT III-heparin in contact activated plasma is supported by the findings of McNeely and Griffith who reported that plasma, whether or not it contained 1 U/mL heparin, after exposure to glass for 30 minutes, exhibited identical amounts of cleaved factor IX, the natural product resulting from proteolysis by factor XIa. They concluded that “the step in the intrinsic pathway of coagulation that is inhibited in the presence of heparin is at the level of factor X activation.” Thus, their results strongly argue against a role for the influence of therapeutic levels of heparin on plasma inhibition of factor XIa, an enzyme whose action precedes the step of factor X activation. It has not been reported in any pathologic condition that factor XI, alone, becomes activated. Should such an instance occur, one would expect heparin to have a modest (less than twofold) effect on the inactivation rate of factor XIa.

Beeler et al claim that because heparin-like molecules are synthesized by endothelial cells, these glycosaminoglycans may determine the rate of inhibition of factor XIa in the vascular system. This situation could only occur if sufficient heparin-like molecules were present to completely saturate the AT III in the circulation and there were preferential attachment of factor XIa to the AT III-glycosaminoglycan complex. The former assumption seems unlikely because the heparin-like molecule concentration attained would have to be well in excess of 2.5 μmol/L, and the latter neglects the possibility that both thrombin and factor Xa as well as factor IXa may successfully compete for the complex. In our study we added exogenous factor XIa to plasma and therefore, we did not see competition occurring from any other proteases, since contact proteins are not activated by factor XIa, and zymogens below factor XI (eg, factor IX, factor X, and thrombin) require calcium for activation to occur. Thus, our results represent a maximum estimate for the degree of potentiation by heparin that can be attained for the inactiva-
HEPARIN--AT III INACTIVATION OF FACTOR XIA

REFERENCES

5. Danielsson A, Raub E, Lindhal U, Björk I: Role of ternary complexes in which heparin binds both antithrombin and proteinase (10,000 to 20,000 U/d) while others did not. In all cases, the concentration of factor XIA-AT III complexes and heparin administration failed to change the concentration of factor XIA-AT III complex detectable in the patients' plasma. These investigators concluded that in vivo, $\alpha_2$-PI was the major inhibitor of factor XIA, in the presence or absence of heparin, in agreement with our in vitro studies. In light of both kinetic (in vitro) studies and clinical data, we again conclude that in plasma, therapeutic heparin does not significantly influence the inactivation rate of factor XIA. This finding is similar to the lack of effect of heparin on the inactivation rate of the two other contact system proteases, namely plasma kallikrein and activated factor XII.

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