Effect of Heparin on the Inactivation Rate of Human Activated Factor XII by Antithrombin III

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Human antithrombin III (ATIII) is a plasma inhibitor of several serine proteases of the blood coagulation system. Previous investigations have reported that the presence of heparin has a multifaceted accelerating effect on the inactivation of factor Xla and XIIa, the active species derived from factor XII. Recent studies from our laboratories have confirmed that ATIII inactivates factor Xla and factor XIIa, but only contributes 2% to 3% to the inhibition of activated factor X species in plasma. The major inhibitor is C1 inhibitor. Therefore, we have reexamined the heparin effect on the rate of inhibition of factor Xla and factor XIIa in purified systems. We also have studied the effect of heparin on the inactivation of both factor XII-derived active species by various plasmas. Using purified factor Xla and ATIII, we found that heparin (0.7 to 34.0 U/mL) increased the rate of inhibition of Factor Xla. However, when heparin concentrations usually achieved during anticoagulant therapy (0.7 U/mL), the inhibition accelerated only fourfold. This implies only a 6% contribution to the inhibitory effect of this plasma. This suggestion was confirmed by the observation that heparin (1.5 U/mL) added to factor XII-deficient plasma and reconstituted with factor XIIa did not produce a detectable enhancement of the rate of inhibition of factor Xla. Furthermore, using purified factor XIIa and antithrombin III, heparin (3.6 to 57.2 U/mL) increased the inactivation rate constant of factor XIIa by 1.6 to 14.0 times. This small effect was confirmed by the observation that heparin at a concentration greater than that sufficient for anticoagulation (1.4 U/mL) did not modify the inactivation rate of factor XIIa by prekallikrein-deficient plasma, and thus C1 inhibitor remains the major inhibitor even in the presence of heparin. From this study and our previous investigations on the effect of heparin on the inhibition of kallikrein and factor Xla, we conclude that heparin does not significantly affect the protease activity of purified contact activation factors or the activities expressed by these proteases in plasma.

Antithrombin III (ATIII) is a major inhibitor of the proteases of the coagulation cascade. ATIII has been shown to inhibit thrombin, factor IXa, factor Xa, factor Xla, factor XIIa, and factor XIIa inhibitor and plasma kallikrein. Heparin is a catalytic cofactor that markedly accelerates the action of ATIII to inactivate these enzymes, particularly factor IXa, factor Xa, and thrombin. Heparin contains a specific carbohydrate sequence that facilitates binding to ATIII, resulting in a conformational change in the structure of the inhibitor to form a more efficient enzyme inactivator. Rosenberg and Damus demonstrated that ATIII neutralizes human thrombin by formation of a covalent enzyme–inhibitor complex involving the active site serine of the enzyme and a specific arginine group of the inhibitor. Heparin dramatically accelerates the rate of this reaction. Stead, Kaplan, and Rosenberge extended these observations and examined the interaction of ATIII with activated factor XII. They found that factor Xla and factor XIIa are progressively neutralized by ATIII and that heparin “dramatically accelerates” the rate of the interaction.

ATIII is not a major inhibitor of either factor Xla or plasma kallikrein. Furthermore, we have shown that heparin at concentrations used in clinical practice show only a minimal effect on the rate of inactivation of both these enzymes by various plasma mixtures. We recently reported that ATIII is only a minor inhibitor of factor XIIa and factor XIIa inhibitor. Therefore, we have reexamined the question of the action of heparin on the rate of inhibition of factor Xla and factor XIIa by ATIII using different assays for measuring residual activated factor XII.

Materials and Methods

The chromogenic substrates, S-2302 (H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride) and S-2238 (H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide) were purchased from Kabi Diagnostica, Stockholm. Kaolin was from Fisher Scientific, Pittsburg, Penn, and Inosithin from Associated Concentrates, Long Island City, NY. Polystyrene and polypropylene tubes were from Sarstedt Inc, Princeton, NJ; Polybrene (hexadimethrine bromide), bovine serum albumin (BSA), QAE Sepharose and SP Sephadex were obtained from Sigma Chemical Corp, St Louis. Enzymes and inhibitors used in this study were of human origin except where noted. Purified human ATIII used in the inhibition studies with factor Xla was a gift from Dr M. Wickerhauser of the American Red Cross National Foundation Center, Bethesda, Md. ATIII was 95% homogeneous, mol wt 58,000, on a reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and had a specific activity of 6 U/mg. ATIII used for factor XIIa inhibition studies was purchased from Kabi Diagnostica with a specific activity of 5 U/mL. The concentration of active ATIII was established by measuring its reaction with thrombin and with plasma kallikrein. Concentrations of ATIII in experiments were based on U/mL with 1 unit of ATIII defined as the amount of ATIII present in 1 mL of pooled normal human plasma and equivalent to 125 μg/mL or 2.5 μmol/L. Human thrombin with a specific activity of 4012 U/mg was a gift from Dr John Fenton of State University of New York at Albany, NY. Bovine lung heparin with a

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specific activity of 162 U/mg was purchased from Upjohn and was used in the factor XIIa studies. Porcine intestinal mucosa heparin (150 U/mg) (Liquemin) used in the factor XIIIf studies was purchased from Hoffman-La Roche, Basel, Switzerland.

Pooled normal plasma, a mixture of fresh frozen plasma obtained from 20 carefully screened normal donors, was purchased from George King Biomedicals Inc, Overland Park, Kan. Factor XII-deficient plasma and prekallikrein-deficient plasma were from individuals congenitally lacking the activities and were kindly supplied by Dr Margaret Johnson, Wilmington, Del, and by Dr Charles Abligaard, University of California at Davis, Sacramento, Calif, respectively.

Human factor XII. Human factor XII was purified as described by Fujikawa and Davie, except that an SP Sephadex column was substituted for a CM Cellulose column in the last ion exchange chromatography step. The factor XII appeared as a single band of mol wt 80,000 under both reduced and nonreduced conditions by SDS-PAGE. The specific activity was determined to be 62 U/mg when measured by a coagulant assay (see later). Protein determination was by the Lowry method using IgG as a standard. One unit of factor XII was defined as the amount in 1 mL of pooled normal plasma.

Factor XII was converted to factor XIIa in a similar manner as described in Silverberg et al. Kallikrein was incubated with factor XII in a 1:6 molar ratio in a buffer (0.02 mol/L Tris, 0.02 mol/L NaCl, 0.1% polyethylene glycol [PEG] 8000, pH 7.5) at 37°C for 40 to 60 minutes in a polypropylene test tube. Kallikrein was separated from factor XIIa and XIIf on a 2-mL QAE Sepharose column equilibrated with the incubation buffer. Kallikrein was completely removed by elution with 0.1 mol/L NaCl in the above buffer. Factor XIIa and XIIf were consecutively eluted with a 24-ml gradient of 0.1 to 0.6 mol/L NaCl. The factor XIIa had an 80,000 mol wt under nonreducing conditions and a 50,000 and 32,000 mol wt under reducing conditions on SDS-PAGE gels.

Chromogenic assay for factor XIIa. Factor XIIa activity was assayed by its amidolytic activity on the chromogenic substrate S-2302. Two hundred microliters of assay buffer (0.05 mol/L Tris, 0.14 mol/L NaCl, 1 mmol/L EDTA, pH 7.8) was added to a glass microwell plate along with 30 mL of S-2302 stock (4 mmol/L) to give a final substrate concentration of 0.5 mmol/L. The cuvette was placed in a thermostated cuvette chamber of a Cary 210 spectrophotometer (Varian Instruments, Palo Alto, Calif) for approximately two minutes to reach the equilibrium temperature of 37°C. Ten microliters of sample to be assayed was then added to the cuvette and quickly mixed with a plastic stirrer. The absorbance change at 405 nm was recorded from two to five minutes to determine the initial velocity of the reaction. In all cases, the increase in absorbance was linear over time. Under the stated conditions, 1 unit of purified factor XIIa (as measured by a coagulation assay) gave a change of absorption per minute of 0.057.

Clotting assay for factor XIIa. Factor XIIa was assayed for the correction of clotting time of factor XIIIf-deficient plasma. The assay was carried out as follows: in polystyrene clotting tubes (10 × 75 mm); 0.1 mL of kaolin (5 mg/mL in buffer); 0.1 mL of inosithin (0.2% in buffer); 0.1 mL of buffer (0.02 mol/L Tris, 0.15 mol/L NaCl, pH 7.4, with and without 18 μg/mL polybrene); and 0.1 mL of factor XIIIf-deficient plasma were mixed. One to twenty microliters of factor XIIa sample was added to individual clotting tubes. After addition, the tubes were sequentially incubated at 37°C for exactly eight minutes. At that point, 0.1 mL CaCl₂ (0.03 mol/L in H₂O) was added, mixed, and a clotting end point was determined by tip-tube method. The effect of heparin on the clotting assay was examined by adding heparin to pooled normal plasma. The heparinized plasma was tested for correction of clotting time of factor XIIIf-deficient plasma. Polybrene at a concentration of 4.5 μg/mL was included in the clotting mixture. Dilutions of normal plasma (factor XII, 0.01 to 0.20 U/mL) containing equivalent concentrations of heparin were assayed to generate standard curves for each condition.

Factor XIIIf. Factor XIIIf was purified by a previously described method with recent modifications. Factor XIIIf was a single band, mol wt 32,000, on nonreduced SDS-PAGE. Upon reduction, factor XIIIf was a single band of mol wt 162,000 and had a specific activity of 17.9 μmol/min/mg with S-2302 as the substrate under the conditions described below.

Factor XIIIf activity was measured by amidolytic activity on the chromogenic substrate S-2302. A 0.6 mmol/L solution of the substrate was prepared in 85 mmol/L sodium phosphate buffer, pH 7.6, containing 127 mmol/L NaCl. Ten microliters of the solution to be tested was added to 330 μL of substrate at 37°C, and absorbance change at 405 nm was continuously recorded.

For kinetic studies, factor XIIIf was incubated with various reagents in freshly silicone-coated glass vessels at 23°C. Factor XIIIf residual activity was measured by its amidolytic activity as described above.

Kinetic studies. The kinetics of inhibition were determined under pseudo-first-order conditions with inhibitor in excess. Under these conditions, the equation ln(E/E₀) = -kt, where E₀ is the initial concentration of factor XIIa or factor XIIIf, and E is the concentration of factor XIIa or factor XIIIf remaining at time t, was utilized to determine k, the pseudo-first-order rate constant for the reaction.

RESULTS

The concentrations of heparin used in the experiments described below were found to not interfere with the enzyme–chromogenic substrate reactions in the assays using S-2302 and S-2238. To test the functional activity of the purified ATIII and heparin used in these studies, we examined these components for their inhibitory activity against thrombin. Thrombin (0.072 μmol/L) was incubated with antithrombin III (0.610 μmol/L) in the absence and presence of 1.20 U/mL heparin in 0.02 mol/L Tris, 0.15 mol/L NaCl, containing 1 mg/mLBSA, pH 7.5 at 24°C. The loss of thrombin activity was followed over time by removing an aliquot of the incubation mixture at time intervals and assaying for residual activity by an initial velocity method using S-2302. In the absence of heparin, ATIII inhibited thrombin with a second-order rate constant of 1.2 × 10⁻¹ minute⁻¹. In the presence of heparin, the second-order rate constant was too fast to be determined by this methodology, but a minimum estimate is 1.2 × 10⁻¹ minute⁻¹. Similar results were obtained with the porcine mucosa heparin employed for the factor XIIIf studies.

We studied the effect of heparin on the inhibitory reaction between purified factor XIIa and ATIII (Fig 1). ATIII was incubated in 107-fold excess (16 μmol/L) over the concentration of factor XIIa to enhance the rate of reaction, since it was found that ATIII at physiologic concentration (2.5 μmol/L) is a poor inhibitor of factor XIIa. At the indicated enzyme and inhibitor concentration, heparin at 0.7 U/mL increased the pseudo-first-order rate constant from 0.035 to

†The concentration values in parentheses are the initial concentrations in the reaction mixture.
factor XII-deficient plasma. A clotting assay for factor XIIa in the presence of heparin was developed (Fig 3), based on the assay described by Lamme et al.\textsuperscript{27} in which polybrene is utilized to neutralize the heparin present in the plasma. As expected (Fig 3), heparin (1.5 U/mL) greatly prolonged the coagulation times in the factor XII assay, presumably due to its effect on factor Xa and thrombin. The presence of polybrene at a concentration of 4.5 mg/mL in the assay of normal plasma in the absence of heparin had little or no effect on the clotting time. Normal plasma containing heparin (1.5 U/mL) assayed in the presence of polybrene (4.5 mg/mL) had a slightly prolonged clotting time but was still linear on a log-log plot and had a slope parallel to the control (Fig 3). Thus, this method was found to be valid to specifically measure residual factor XIIa activity in plasma containing heparin.

Purified factor XIIa was reconstituted in factor XII-deficient plasma containing heparin, incubated at 37 °C, and assayed at various times for residual factor XIIa activity by the above clotting assay. The results of this experiment indicate that the rate of factor XIIa inactivation was not accelerated by heparin concentrations ranging from 0.6 to 3.6 U/mL (Fig 4).

Since ATIII accounts for 2% of the inhibitory effect on factor XIIa in plasma, the reaction with purified ATIII and factor XIIa predicted that the presence of heparin (1.5 U/mL) in plasma would contribute at most 6% of the inhibitor effect and therefore have little or no effect on the expression of factor XIIa activity. Studies were undertaken to determine if the presence of heparin would affect the inactivation of exogenously added factor XIIa in plasma.

The concentration of ATIII in plasma is reported to range from 2.5 to 4.7 μmol/L,\textsuperscript{28,29} which is 6.4 to 3.4 times less than the concentration of ATIII used in the experiment in Fig 1. When the concentration of ATIII is near physiologic (3.1 μmol/L) and heparin is in a concentration that may occur during anticoagulation (1.2 U/mL), the presence of heparin increased the pseudo–first-order rate constant from 0.007 to 0.029 minute\textsuperscript{−1}, only a fourfold increase in the inhibition rate (Fig 2).

Since ATIII accounts for 2% of the inhibitory effect on factor XIIa in plasma,\textsuperscript{10} results from the reaction with purified ATIII and factor XIIa predicted that the presence of heparin (1.5 U/mL) in plasma would contribute at most 6% of the inhibitor effect and therefore have little or no effect on the expression of factor XIIa activity. Studies were undertaken to determine if the presence of heparin would affect the inactivation of exogenously added factor XIIa in plasma.
ANTITHROMBIN INACTIVATION OF FACTOR XIIa

Fig 4. The inhibition of factor XIIa in plasma containing heparin. Fifty microliters of water or a solution of heparin (for final heparin concentrations) was added to 195 μL of factor XII-deficient plasma. Fifty microliters of factor XIIa was added (for a final concentration 0.15 μmol/L or 0.4 U/mL) to 200 μL of this plasma and incubated at 37°C. At intervals, 20 μL of the incubation mix was removed and assayed for residual factor XIIa activity.

Factor XIIa activity was measured as described in Materials and Methods using the appropriate standard curve from Fig 3. (a) no heparin, N = 4; (b) 0.6 U/mL heparin, N = 4; (c) 1.2 U/mL, N = 4; (d) 3.6 U/mL heparin, N = 2.

The influence of heparin on the inactivation of factor XIIa by purified ATIII was then assessed using the amidolytic assay (Fig 5). Increasing the concentration of heparin from 0 to 57.2 U/mL gave a linear increase in the pseudo-first-order rate constant, k, for factor XIIa inactivation by ATIII (inset, Fig 5). The pseudo-first-order rate constant for the inactivation of factor XIIa increased from 0.09 to 0.36 minute⁻¹, indicating that, at a heparin concentration of 57.2 U/mL, only a 14-fold increase in inactivation rate occurs. At lower concentrations of heparin (3.7 U/mL), still considerably above usual therapeutic concentrations, a pseudo-first-order rate constant of 0.07 minute⁻¹ was obtained, which represents only a 1.6-fold increase in the inactivation rate as compared with the absence of heparin. To assess the role of heparin on the inactivation of factor XIIa in plasma, we used plasma deficient in prekallikrein, to avoid the complications of formation of kallikrein catalyzed by factor XIIa. The minor role of heparin was confirmed by the observation that the inactivation rate constant of factor XIIa amidolytic activity by a 1:3.5 dilution of prekallikrein-deficient plasma (0.14 minute⁻¹) was not modified by the presence of heparin (final concentration, 1.4 U/mL). Increased concentrations of heparin induced a linear increase of the inactivation rate constant of factor XIIa by antithrombin III (Fig 5, insert), while in contrast, saturation of k was observed when factor XIIa was substituted for factor XIIa (Fig 1, insert). Since this difference could be dependent on a difference in the interactions of the enzymes and mucopolysaccharide, we examined whether insolubilized heparin was able to bind factor XII and factor XIIf. Freeze-dried factor XIIa (20 μg) was reconstituted in 100 μL of 10 mmol/L sodium phosphate buffer, pH 7.4, containing 0.14 mol/L NaCl. This mixture was applied to a heparin–agarose column (12 × 30 mm), which had been previously equilibrated in the same buffer. The same buffer was also used for washing the column. Factor XIIa was detected by its prekallikrein-activating activity of CHCl₃-treated plasma. Under these conditions, factor XIIa did not absorb to heparin agarose. In a similar experiment, factor XII (150 μg or 5 units) were dialyzed into the same buffer and applied to a heparin-agarose column (5 × 20 mm) equilibrated with the same buffer. The column was washed with the same buffer, then eluted with phosphate buffer, pH 7.5 first containing 0.4 mol/L NaCl, then 1.0 mol/L NaCl. Factor XII activity was measured by a coagulation assay using factor XII-deficient plasma. Trace amounts of factor XII activity was observed to leach in the first wash. The majority of factor XII activity was present in the 0.4 mol/L NaCl eluant, indicating there was absorption. No factor XII activity was found in the 1.0-molar eluant.

DISCUSSION

Stead et al. examined the interaction of ATIII with activated forms of factor XII and reported progressive inhibition of factor XIIa and factor XIIIf by ATIII. That report also described the formation of a 117,000 mol wt complex between factor XIIa and ATIII and formation of an 85,000 mol wt complex between factor XIIIf and ATIII on SDS-PAGE, thus indicating a 1:1 stoichiometry between the enzymes and inhibitor. Other studies have confirmed the observed inhibition of factor XIIa by ATIII. Recent studies from our laboratories have also demonstrated that ATIII inhibits factor XIIa and factor XIIIf with formation of 125,000- and 87,000-mol wt complexes, respectively. However, we found that ATIII contributes only a small percentage (2% to 3%) of both factor XII-active species' activity in plasma with Cl inhibitor serving as the major inactivator (92%).

Two studies indicated that heparin had little effect on the ATIII inhibition of the activated forms of factor XII. Stead et al. reported that heparin increases the rate of the interaction between factor XIIa and factor XIIIf by ATIII. In
that study, they measured residual factor XIIa activity by a coagulant assay using factor XII-deficient plasma. Since heparin interferes with this assay, it had to be removed from the factor XIIa–ATIII mixture prior to the assay. This task was accomplished by first incubating the mixture with heparinase and then absorbing the mixture with diethylaminoethyl (DEAE) cellulose to remove the residual heparin. The anion exchanger was then removed by centrifugation, and the supernatant was assayed for residual factor XIIa activity by another indirect method. They measured the inhibition of the release of bradykinin in plasma, which was generated by the activation of kallikrein by factor XII.

We have reexamined the question of heparin's action on the rate of inhibition of factor XIa and XIIa using a more direct system of assay. In purified systems, we determined the residual activity of factor XIa and XIIa directly by initial velocity measurements of the release of p-nitroaniline from the chromogenic substrate S-2302. We found that therapeutic concentrations of heparin (0.7 to 1.2 U/mL) only accelerated the rate of inhibition of factor XIa fourfold (Figs 1 and 2). Moreover, heparin, at three times the therapeutic concentration (3.6 U/mL) potentiated the inhibition of factor XIIa by ATIII less than twofold (Fig 5).

Higher concentrations of heparin affected the rate of inhibition of factor XIa and XIIa differently. On increasing the concentration of heparin from 0 to 57 U/mL in the presence of factor XIIa and antithrombin III, there was a proportional linear increase in the accelerated rate of inhibition (Fig 5). Increasing the concentration of heparin from 0 to 34 U/mL in the presence of factor XIa and ATIII resulted in a saturation of the rate for the inhibition (Fig 1). Factor XIIa is an 80,000-mol wt protein composed of a heavy (50,000-mol wt) and light (32,000-mol wt) subunit. The light subunit contains the catalytic site of the enzyme. The heavy subunit contains a binding region to negatively charged surfaces, such as heparin. Factor XIIa is a 28,000-mol wt degradation product of factor XII and is similar to the factor XIIa light chain containing a catalytic region but is different because it lacks the heavy subunit containing a binding region. The observed difference in the rate saturation between factor XIa and factor XIIa was possibly due to a dependence of heparin binding to the heavy chain of factor XIa, which is lacking in factor XII. The observation is similar to what is seen for the effect of ATIII in the presence of heparin on the inhibition of thrombin when compared with factor Xa. Factor XIa behaves similarly to thrombin, and factor XIIa resembles factor Xa. Bovine factor XIa binds to a heparin-sepharose affinity column and is eluted at high salt concentration, and we have observed this binding with human factor XII (Results). In contrast, factor XIIa lacks the heavy chain of factor XIa, which contains the surface-binding region of the enzyme and does not bind to heparin. Thus, in the case of factor XIIa–ATIII interaction, heparin binds only to the inhibitor. Other explanations for the differences in the heparin concentration dependence of inhibition are the different types of heparin used, the ranges of heparin concentrations over which the effect is observed, and the different temperature used.

We also examined the effect of plasma containing heparin on the inhibition of factor XIa and found that heparin, at the concentrations tested, had no effect on the rate of factor XIIa inactivation. The presence of heparin in plasma was tested for its effect on the inhibition of factor XIa (Fig 4) and factor XIIa (not shown). We found that the inhibition of both factor XIa and factor XIIa by plasma proteolytic inhibitors was unaffected. The coagulant assay (Fig 3) depends on the action of factor XIa on two natural protein substrates, prekallikrein and factor XI. Thus, the finding that heparin failed to alter the rate of inhibition of factor XIIa coagulant activity indicates that our conclusions apply to protein as well as peptide substrates. This observation is not surprising when it is considered that CI inhibitor is the primary inhibitor of factors XIIa (contributing 92%) and XII (contributing 93%). ATIII is a minor contributor to both of these enzymes (2% for XIa, 4% for XIIa). A fourfold increase in the inactivation rate of the endogenous ATIII would be masked by the much greater reaction rate of both enzymes with CI inhibitor. As a result, the accelerating effect of heparin is not observable.

The importance of determining the role of heparin in the inhibition of plasma proteolytic enzymes is not just due to its pharmacologic use. Marcum et al provided evidence that proteoglycans derived from endothelial cells contain the appropriate monosaccharide sequences required for accelerating the action of antithrombin III. Their demonstration that anticoagulantly active heparin fragments and heparin sulfate-like species are present in close proximity to endothelial cells lends credence to the view that they may function to maintain the nonthrombogenic characteristics of blood vessels.

Heparin has been shown to have only a two- to fourfold accelerating effect on the ATIII inhibition of purified kallikrein and factor XIa and has little significance in the effect of regulating kallikrein and factor XIa in plasma, although there have been conflicting reports on both enzymes. From this present study on activated factor XII and previous studies of the heparin effect on kallikrein and factor XIa, we suggest that heparin and other sulfated proteoglycans do not significantly affect the ability of ATIII to regulate the contact-activation enzymes in plasma.

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