EFFECT OF ANTITHROMBIN III AND HEPARIN ON FACTOR X ACTIVATION BY FACTOR IXa.

T. Lindhout (1), G. Willems (2), G.C. Henker (1) and T. Linnemann (3). (1) Louisian State University Medical Center, Shreeveport, LA, USA, and (2) The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA.

Identification of lysyl residue(s) in human plasma antithrombin III required for binding of heparin: Identification was accomplished using chemical modification with the amino-group reagent, pyridoxal-5'-phosphate. Modification of antithrombin (AT) with this reagent caused a 50-60% loss of anticoagulant activity. Upon sequencing of the unique peptide maps of these two modified ATs indicated that eight amino acid residues were protected from the chemical modification reaction, and that the modification reaction products observed upon gelelectrophoresis are best explained assuming a mechanism of inactivation in which the two AT species, the proteins were reductively denatured, and stained either for glycoprotein or for AT III using antibodies against AT III.

The heparin-catalyzed inactivation of activated coagulation factors by antithrombin III (AT III) has mostly been studied for isolated serum proteases. However, we decided to study the interaction of the factor IXa under more physiological conditions, i.e. during the activation of factor X by factor IXa in the presence of phospholipid and calcium. Thereby we made use of a methodical approach described previously. We found that the heparin-catalyzed inactivation of factor X by factor IXa, phospholipid and calcium in the presence of AT III and heparin. Fitting the experimental factor X activation data to the pseudo-first-order rate constants of factor Xa and factor IXa. In a first approach we examined the effect of AT III alone on factor X activation. We found that the pseudo-first-order rate constant of inhibition of formed factor Xa was 2 x 10⁻⁵ M⁻¹s⁻¹, whereas that of factor Xa in solution was 5 x 10⁻⁵ M⁻¹s⁻¹, indicating that phospholipid-bound factor X competes with AT III for factor Xa. The second order rate constant of inhibition of formed factor Xa was 8 x 10⁻³ M⁻¹s⁻¹. Unfractionated heparin (168 USP units/mg) was found to stimulate the inhibition of generated factor Xa by AT III (200 μM) with 0.15 min⁻¹ per μM of UFP, and a synthetic peatassacharide (PS; 4000 anti-Xa units/mg) stimulated this inhibition with only 0.03 min⁻¹ per μM. Due to the presence of phospholipid-bound factor X this stimulation was 4-fold lower when compared with factor Xa in solution. AT III concentrations higher than 3 μM, and PS concentrations higher than 10 μM (PS; 4000 anti-Xa units/mg) did not affect the activity of factor Xa generation could be measured because of the rapid inactivation of factor Xa whereas factor IXa was not inhibited. Using a factor IXa assay we found that PS, even at relatively high concentrations, had no effect on factor IXa inactivation by AT III (200 μM), both in the presence and absence of accessory components. The inactivation of factor IXa by AT III (200 μM) during factor X activation was stimulated by UFP with 1.5 x 10⁻³ M⁻¹s⁻¹ per μM of UFP. Surprisingly, this was 4-fold more when compared with factor IXa in solution. Inhibitory experiments with AT III established that calcium stimulates the heparin-dependent inhibition of factor IXa.

LYSINE-125 IS ESSENTIAL FOR HEPARIN BINDING TO ANTITHROMBIN. G.C. Henker (1), S.L. Peterson (2), I.C. Sorensen (2), J.M. Pecon (1), F.L. Church (2), and M.R. Blackburn (1). (1)Louisian State University Medical Center, Shreeveport, LA, USA, and (2)The University of North Carolina at Chapel Hill, Chapel Hill, NC, USA.

In apparent contradiction to its anticoagulant activity, we have observed a previously undetected, and potentially opposing function for heparin: We have observed that heparin increases anticoagulantly-active heparin was also found to stimulate the rate of inactivation of antithrombin by the neutrophil enzyme. In the absence of heparin, or in the presence of the heparin antagonists platelet factor 4 or polybrene, little or no inactivation of antithrombin occurred. Catalytic amounts of heparin and elastase caused the complete inactivation of antithrombin (approximate molar ratio of 1:1:400 respectively) in 5-10 minutes. The loss of heparin binding affinity by the elastase-cleaved form of antithrombin permitted its separation from active antithrombin by heparin-Sepharose chromatography. The purified elastase-inactivated antithrombin was injected into rabbits for determination of its competitive clearance behavior. In contrast to intact, functional antithrombin (t 1/2 >30 hours) and the thrombin-antithrombin (T-AAT) complex (t 1/2 previously shown to be minutes), elastase-inactivated antithrombin circulated for approximately 13 hours. This prolonged clearance relative to the T-AAT complex may suggest an alternative explanation for the circulating, non-functional antithrombin observed in certain coagulopathic states. In summary, these results point to a potential and unexpected role for heparin in directing the inactivation of antithrombin and suggest a possible in vivo mechanism for inactivating the usually non-thromogenic nature of the vascular lining.

HEPARIN CATALYZED FACTOR XA INHIBITION BY ANTITHROMBIN III. M. Soosten, T. Janssens-Claeussen, E. Tan, and H.C. Henker.

University of Limburg, Maastricht, The Netherlands.

The inactivation of human factor Xa by human antithrombin III (AT III) was studied under pseudo-first order reaction conditions (excess AT III) both in the presence and absence of heparin. The time course of inhibition was followed using UNDA-PSA. After electrophoretic blotting onto nitrocellulose and stained either for glycoprotein or for AT III using antibodies against AT III. Concomitant with factor Xa inactivation two new slower migrating bands became visible on the blot. One of these, representing the intermediate complex consisting of one AT III complexed with one of the active sites present in factor Xa, appeared as a transient band. Complete inactivation resulted in a single band representing the complex of factor Xa with two AT III molecules. This indicates that inhibition of factor Xa by AT III can be described as: Xa + AT III → Xa - AT III.

Quantitative analysis of the time course of inactivation was accomplished by a comparison of the disappearance of factor Xa amidolytic activity towards the chromogenic substrate 2S366. Pseudo first order reaction kinetics were observed throughout. The time course of inactivation and the distribution of the reaction products observed upon gelelectrophoresis are best explained assuming a mechanism of inactivation in which the two active sites present in factor Xa are inhibited in random order (i.e., independent of each other) with the same rate constant of inhibition (k₁ + k₂). The rate constant of inactivation for the active sites in factor Xa was found to be 1,000 M⁻¹s⁻¹. In the presence of saturating amounts of heparin, from the kinetic data a binding constant Kₚ of 0.11 μM was inferred for the binding of AT III to heparin. Experiments with four well characterized heparin fractions indicate, that the actual magnitude of the rate enhancement of factor Xa inactivation is, however, not due to the binding of AT III to heparin but also depends on the type of heparin to which the AT III is bound.

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Heparin catalyzes the inactivation of antithrombin by elastase. S.R. Wray, J. Kilpatrick, R.M. Nelson, J.D. Neerhagen and R.A. Fournel, Cutter Biologicals, Miles Laboratories, Berkeley, CA, U.S.A.

Identification of lysyl residue(s) in human plasma antithrombin III required for binding of heparin: Identification was accomplished using chemical modification with the amino-group reagent, pyridoxal-5'-phosphate. Modification of antithrombin (AT) with this reagent caused a 50-60% loss of anticoagulant activity. Upon sequencing of the unique peptide maps of these two modified ATs indicated that eight amino acid residues were protected from the chemical modification reaction, and that the modification reaction products observed upon gelelectrophoresis are best explained assuming a mechanism of inactivation in which the two AT species, the proteins were reductively denatured, and stained either for glycoprotein or for AT III using antibodies against AT III. Concomitant with factor Xa inactivation two new slower migrating bands became visible on the blot. One of these, representing the intermediate complex consisting of one AT III complexed with one of the active sites present in factor Xa, appeared as a transient band. Complete inactivation resulted in a single band representing the complex of factor Xa with two AT III molecules. This indicates that inhibition of factor Xa by AT III can be described as: Xa + AT III → Xa - AT III.

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