

The mechanism of action of heparin in purified systems and in plasma

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Summary

The work presented in this thesis comprise a number of studies concerning the action of the antithrombin III/heparin system at several levels of the coagulation cascade. Our goal was to gain a better insight into the interactions of activated coagulation factors with the plasma inhibitor antithrombin III and the modulation of this action by heparin.

Heparin is a mucopolysaccharide that greatly accelerates the reaction between antithrombin III and activated coagulation factors. In the clinic, heparin is used in a variety of ways to treat or prevent thrombosis. Its mechanism of action in the patient, however, is not very well understood, because little is known about the effect of heparin on the complex reaction systems that govern blood coagulation *in vivo*.

Therefore it was our aim to obtain more information on the mechanism of action of heparin in (complex) purified systems and in plasma.

After a general introduction concerning the coagulation process, its initiation and inhibition, it is demonstrated in Chapter II that the prothrombin activation peptides, that are released during the generation of thrombin, are able to neutralize heparin. These results show a possible role for these fragments in the regulation of blood coagulation.

Chapter III describes the effect of antithrombin III and heparin during the enzymatic conversion of factor X into factor X_a by factor IX_a in the presence of phospholipids and calcium. The situation is a complicated one because both the enzyme (factor IX_a) and the generated product (factor X_a) are inactivated by antithrombin III/heparin. Under these circumstances, that start to approach the complexities of plasma, important differences become apparent with the effect of antithrombin III and heparin on isolated activated coagulation factors.

Chapters II and III clearly show that in more complex systems the activated coagulation factors behave quite different from what would be predicted from studies performed on isolated factors. In order to approach the *in vivo* situation still further, the investigations were continued in whole (platelet free) plasma.

To assess the effect of heparin and heparin-analogues on activated coagulation factors in plasma, sensitive and specific bio-assays were developed that can monitor the generation of activated coagulation factors in clotting plasma.

In Chapter IV it is demonstrated that not factor X_a inhibition, but rather the inhibition of (thrombin-induced) factor V_a generation by heparin explains the inhibition of coagulation in thromboplastin-activated plasma.

Using an assay for factor IX_a , the kinetics of inactivation (by antithrombin III/heparin) of the factor XI_a -catalyzed factor IX_a generation in plasma were studied (Chapter V). It was found that the inactivation of factor XI_a in plasma was fast, and independent of the presence of heparin or heparin analogues. Factor IX_a , generated in plasma by factor XI_a , was not significantly inactivated. However, heparin and pentasaccharide clearly caused inactivation of this factor IX_a .

In Chapter VI, the activation of the non enzymatic protein cofactors VIII:C and V was studied. It was demonstrated that in thromboplastin-triggered plasma, *in situ* generated thrombin, but not factor X_a is responsible for the activation of factors VIII:C and V.

From these studies, it can be concluded that (1) inhibition of activated coagulation factors by antithrombin III/heparin in complex reaction systems is quite different from inhibition of these factors in isolated systems, and (2) thrombin is the enzyme that plays the key role in plasma coagulation. It therefore should be the prime target of antithrombotic therapy by anticoagulation.

Samenvatting

In dit proefschrift worden een aantal studies beschreven met betrekking tot de werking van antitrombine III en heparine op verschillende niveaus van de stollcascade. Ons doel was een beter inzicht te verkrijgen in de interacties van geactiveerde stoffactoren met de plasma remmer antitrombine III en heparine.

Heparine is een mucopolysaccharide dat in staat is de reactie tussen antitrombine III en geactiveerde stoffactoren drastisch te versnellen. In de kliniek wordt heparine gebruikt ter behandeling of voorkoming van trombose. Het werkingsmechanisme van heparine in de patiënt is echter slecht begrepen, omdat weinig bekend is over het effect van heparine op de meer complexe reactie systemen die deel uitmaken van de bloedstolling *in vivo*.

Vandaar dat wij ons ten doel gesteld hebben meer informatie te verkrijgen over het werkingsmechanisme van heparine in (complexe) gezuiverde systemen en in plasma.

Na een algemene inleiding betreffende het bloedstollingsproces, de initiatie en de remming van dit proces, wordt in hoofdstuk II aangetoond dat de protrombine activeringspeptiden die gevormd worden tijdens de trombine generatie, in staat zijn om heparine te neutraliseren. Deze resultaten tonen een mogelijke rol aan voor deze fragmenten in de regulatie van de bloedstolling.

Hoofdstuk III beschrijft het effect van antitrombine III en heparine gedurende de enzymatische omzetting van factor X in factor X_a door factor IX_a in aanwezigheid van fosfolipiden en calcium. Deze situatie is gecompliceerd omdat zowel het enzym (factor IX_a) als het gevormd product (factor X_a) geïnactiveerd worden door antitrombine III/heparine. Onder deze omstandigheden, die de complexiteit van het plasma benaderen, worden belangrijke verschillen duidelijk met het effect van antitrombine III en heparine op geïsoleerde stoffactoren.

De hoofdstukken II en III laten zien dat in complexe systemen geactiveerde stoffactoren zich geheel anders gedragen als voorspeld zou worden op basis van studies uitgevoerd met geïsoleerde factoren.

Om de *in vivo* situatie nog meer te benaderen, werden de studies voortgezet in vol (plaatjesvrij) plasma.

Om het effect van heparine en heparine analoga op geactiveerde stoffactoren in plasma te bestuderen, werden gevoelige en specifieke bepalingen ontwikkeld om de generatie van geactiveerde stoffactoren in plasma te kunnen volgen.

In hoofdstuk IV wordt aangetoond dat niet de remming van factor X_a , maar veeleer de remming van de (trombine-afhankelijke) factor V_a generatie door heparine de remming van de stolling in tromboplastine-geactiveerd plasma verklaard.

Met behulp van een factor IX_a bepaling, werd de kinetiek van de remming (door antitrombine III/heparine) van de factor XI_a gecatalyseerde factor IX_a generatie in plasma bestudeerd (hoofdstuk V). De resultaten laten zien, dat factor XI_a in plasma snel wordt geremd, en dat deze remming onafhankelijk is van de toevoeging van heparine of heparine analoga. Factor IX_a , gegenereerd in plasma door factor XI_a , werd niet significant geïnactiveerd. Toevoeging van heparine of pentasaccharide veroorzaakte echter een duidelijke remming van factor IX_a .

In hoofdstuk VI werd de activering van de niet enzymatische eiwitcofactoren VIII:C en V bestudeerd. De resultaten tonen aan, dat in tromboplastine-geactiveerd plasma, *in situ* gegenereerd trombine, en niet factor X_a , verantwoordelijk is voor de activering van factor VIII:C en V.

Uit de studies die beschreven worden in dit proefschrift, kan geconcludeerd worden dat (1) inactivatie van geactiveerde stoffactoren door antitrombine III en heparine in complexe reactie systemen grote verschillen vertoont met de remming van deze factoren in geïsoleerde systemen, en (2) dat trombine het enzym is dat een sleutelrol speelt in de stolling van plasma. Vandaar dat trombine de belangrijkste rol zou kunnen spelen als doelwit enzym bij antistolling.