Natural inhibitors of fibrinolysis

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There are two main types of inhibitors of fibrinolysis: those which inhibit plasmin (plasmin inhibitors or antiplasmins) and those which inhibit the activation of plasminogen (anti-activators). Many substances derived from biological fluids, tissues, plants, or micro-organisms inhibit fibrinolysis. Some active site titrants of serine proteases are potent inhibitors of fibrinolytic enzymes and certain amino-acids such as lysine, 6-aminohexanoic acid, or tranexamic acid inhibit the digestion of fibrin. This review will deal mainly with the natural inhibitors of fibrinolysis occurring in human blood. The properties of the other inhibitors are reviewed elsewhere.¹

Inhibitors of plasmin occurring in human blood

Human plasma exerts a very important inhibitory action on plasmin. There are at least five well-defined proteins which inhibit plasmin in a purified system—namely, α_2 -macroglobulin, α_1 -antitrypsin, inter- α -trypsin inhibitor, antithrombin III-heparin complex, and C1-esterase inhibitor. The physiological inhibitor of plasmin formed in blood, however, is a relatively recently described plasma protein called α_2 -antiplasmin.² This inhibitor was independently identified by three groups³⁻⁵ and most probably also by a fourth.⁶ Upon activation of plasminogen in plasma the formed plasmin is first preferentially bound to α_2 -antiplasmin. Only upon complete activation of plasminogen (concentration in plasma approximately 1.5 μ mol/l), resulting in saturation of the inhibitor (concentration 1 μ mol/l), is the excess plasmin neutralised by α_2 -macroglobulin. In the presence of normal concentrations of these two inhibitors the other plasma protease inhibitors do not play a role in the inactivation of plasmin.

α_2 -ANTIPLASMIN (THE PHYSIOLOGICAL PLASMIN INHIBITOR)

α_2 -Antiplasmin is a single chain glycoprotein with a molecular weight of 70 000 containing approximately 13% carbohydrate.^{4,7} The inhibitor is immunochemically different from the other known plasma protease inhibitors. α_2 -Antiplasmin forms a very stable 1:1 stoichiometric complex with plasmin,

which is devoid of protease or esterase activity.^{4,5,7} Complex formation occurs by strong interaction between the light-(B)-chain of plasmin and the inhibitor. The physiological role of α_2 -antiplasmin as an inhibitor of proteases other than plasmin seems negligible.^{8,9}

The reaction between plasmin and α_2 -antiplasmin proceeds in at least two steps—a very fast reversible second order reaction followed by a slower irreversible first order reaction^{10,11} and may be represented by

The rate constant k_1 at pH 7.5 is $3.8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ and $1.8 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ for the two plasmin forms which have different affinities for lysine-Sepharose.¹¹ This reaction rate is one of the fastest so far described for protein-protein interactions and is one order of magnitude higher than the reaction rate of trypsin with its inhibitors. The dissociation constant of the reversible step is approximately $2 \times 10^{-10} \text{ M}$ and the rate constant of the second step $4 \times 10^{-3} \text{ s}^{-1}$.¹¹ Plasmin which has 6-aminohexanoic acid¹¹ or lysine¹⁰ bound to its lysine-binding sites or substrate bound to its active site¹¹ reacts only very slowly with α_2 -antiplasmin. These findings indicate that free lysine-binding sites and a free active site in the plasmin molecule are of great importance for the rate of its reaction with α_2 -antiplasmin. As discussed further, these interactions are probably of great importance for the regulation of fibrinolysis *in vivo*.

A structural analysis of the plasmin- α_2 -antiplasmin complex suggested that the stable complex is formed by a plasmic attack at a specific leucyl-methionyl peptide bond in the COOH-terminal portion of the inhibitor. A strong, probably covalent bond is formed between the active site seryl residue in plasmin and the carbonyl group of this specific leucyl residue in the inhibitor.¹²

The turnover of ¹²⁵I-labelled α_2 -antiplasmin was studied in control subjects and in patients during thrombolytic therapy.¹³ In the control group α_2 -antiplasmin had a plasma half life of 2.64 ± 0.32 days and a fractional catabolic rate of 0.53 ± 0.09 of

the plasma pool per day. During thrombolytic therapy the half life shortened to approximately 0.5 days as a result of formation of plasmin- α_2 -antiplasmin complex. The long half life of the plasmin- α_2 -antiplasmin complex was confirmed by studying the turnover of the purified complex both before and during thrombolytic therapy in patients with thrombotic disease.

The normal concentration of α_2 -antiplasmin is between 80% and 120% (mean $\pm$ 2 SD) of the value obtained for pooled normal plasma.¹⁴⁻¹⁶ The concentration of α_2 -antiplasmin in pooled normal plasma is approximately 1 $\mu\text{mol/l}$.^{4,5,7} The concentration may decrease to below 30% in severe cases of liver disease or intravascular coagulation¹⁴⁻¹⁶ but is normal in patients with cardiovascular, renal, or malignant disease. The inhibitor is temporarily exhausted during thrombolytic therapy with streptokinase,^{15,17} when measured enzymatically. Residual antigen may, however, be found immunologically representing complexed or degraded inhibitor, or both.¹⁷ α_2 -Antiplasmin is a weak acute phase reactant.¹⁵ Possibly some of the α_2 -antiplasmin in plasma is inactive.⁵

α_2 -ANTIPLASMIN AND REGULATION OF FIBRINOLYSIS

Physiological fibrinolysis appears to be regulated and controlled by specific molecular interactions between plasminogen activator and fibrin, between plasminogen and fibrin, and between plasmin and α_2 -antiplasmin.

An important property of the blood-vascular-tissue type of plasminogen activator is its high affinity for fibrin,^{18,19} which has been used for its isolation.²⁰ Tissue activator is a relatively poor plasminogen activator in pure systems but fibrin strikingly stimulates the activation.^{20,21}

Human plasminogen and plasmin contain structures, known as lysine-binding sites, which are responsible for their interaction not only with amino-acids such as lysine or 6-aminohexanoic acid²²⁻²⁴ but also with fibrin²⁵ and with antiplasmin.^{4,7} In purified systems native plasminogen (with NH_2 -terminal glutamic acid) has a weak affinity for fibrin whereas the partially degraded form of plasminogen (with NH_2 -terminal lysine) has a much stronger affinity.²⁶ When whole plasma is clotted approximately 4% of the plasminogen is specifically bound to fibrin through its lysine-binding sites.²⁷

The rapidity of the plasmin- α_2 -antiplasmin reaction is strongly dependent on the availability of free lysine-binding sites and active site in the enzyme. At a concentration of 1 $\mu\text{mol/l}$ of α_2 -antiplasmin (the normal plasma concentration) the half life of small amounts of free plasmin can be calculated to be

about 100 ms. Plasmin with occupied lysine-binding sites and active site, however, would react at least 100 times slower and have a half life of the order of magnitude of 10 s.²⁸

Thus fibrinolysis *in vivo* seems to be regulated at two levels: (1) efficient activation at the fibrin surface of fibrin-bound plasminogen by fibrin-bound plasminogen activator, and (2) degradation of fibrin by fibrin-bound plasmin which is protected from rapid inactivation by α_2 -antiplasmin since it has both its lysine-binding sites and active site occupied. Since the fibrin-bound plasmin has an estimated half life of about 10 s effective clot dissolution *in vivo* would also seem to require a continuous replacement at the fibrin surface of inactivated plasmin molecules (complexed with α_2 -antiplasmin) by plasminogen molecules.²⁸⁻²⁹

Several lines of evidence indicate that this model for the regulation of fibrinolysis, which was constructed mainly on the basis of molecular interactions which were demonstrated in purified systems, is also operative *in vivo*.³⁰

α_2 -MACROGLOBULIN

α_2 -Macroglobulin represents the slower reacting plasmin inhibitor of plasma. Its role seems to be to inactivate plasmin formed in excess of the inhibitory capacity of α_2 -antiplasmin.^{3,5} Indeed, when the plasma plasminogen (concentration approximately 1.5 $\mu\text{mol/l}$) is activated the formed plasmin is initially primarily bound to α_2 -antiplasmin (concentration approximately 1 $\mu\text{mol/l}$) until after its saturation. Excess plasmin is neutralised by α_2 -macroglobulin. A number of reviews on the physiology and biochemistry of α_2 -macroglobulin have recently appeared.³¹⁻³³

OTHER PLASMA PROTEASE INHIBITORS

There are at least three plasma protease inhibitors in addition to α_2 -antiplasmin and α_2 -macroglobulin that inhibit plasmin in purified systems. These are α_1 -antitrypsin, antithrombin III, and C1-inactivator.³⁴ Inter- α -trypsin inhibitor is a polyvalent inhibitor and probably also reacts to some extent with plasmin.³⁴ In the presence of normal concentrations of α_2 -antiplasmin and α_2 -macroglobulin, however, none of these inhibitors play a role in the neutralisation of plasmin formed in the blood.^{3,35}

Purified antithrombin III is a progressive, time-dependent inhibitor of purified plasmin. Heparin accelerates the rate of this reaction 50 to 100 fold.³⁶ On the basis of these findings it has been suggested that antithrombin-heparin complex may be a major inhibitor of *in-vivo* fibrinolysis. The role of antithrombin III-heparin complex as an inhibitor of plasmin formed in plasma has been re-evaluated

in vitro and *in vivo*.^{37 38} In the absence of heparin and after complete activation of the plasma plasminogen about 1% of the formed plasmin binds to antithrombin III. In the presence of therapeutic concentrations of heparin (1-2 IU/ml plasma) this value increased to 2-5% both *in vitro* and *in vivo*. These data suggest that the antithrombin III-heparin complex plays a very limited role in the inactivation of plasmin.

PLASMIN INHIBITORS FROM PLATELETS

Johnson and Schneider³⁹ reported on an antiplasmin activity of bovine platelets which accounted for a major part of the antiplasmin of bovine blood. In human blood, however, the platelets contributed only 1-3% of the antiplasmin activity.^{40 41} The platelet contribution to the total antiplasmin activity of whole human blood is therefore probably small.

Antiplasmin materials in platelets have, however, been characterised to some extent. McDonagh *et al.*⁴² found two antiplasmin activities, one of which was platelet factor XIII and the other one was eluted earlier on Sephadex G-200. Ganguly *et al.*^{43 44} described low molecular weight antiplasmins, which were dialysable in extracts of human platelets. All these plasmin inhibitors are at present poorly characterised.

Inhibitors of plasminogen activation in human blood

The existence in blood of physiological inhibitors to the activation of plasminogen has been much disputed. The demonstration of activation inhibitors in plasma is indeed hampered by two obstacles. Firstly, it is very difficult to measure antiactivator activity in the presence of antiplasmins. Antiactivator activity may, however, be demonstrated in the following ways: complex formation between activator and antiactivator, inhibition of activator-catalysed hydrolysis of low molecular weight substrates, and inhibition of cleavage of the internal peptide bond in plasminogen.⁴⁵ Secondly, the various protease inhibitors in plasma may each have some affinity for plasminogen activators and in concert confer some inhibitory activity to the plasma without actually being specific inhibitors. Despite these difficulties it seems, however, that plasma contains components which act or may act as inhibitors of plasminogen activation. But the physiological role of most of these inhibitors is unknown.

Plasminogen activation may occur by three different pathways: an intrinsic or humoral pathway in which all participating components are present in precursor form in the blood, an extrinsic pathway in which the activator originates from tissues or from

the vessel wall and is released into the blood by certain stimuli or trauma, and an exogenous pathway in which the activating substances streptokinase or urokinase may be infused for therapeutic purposes. All plasminogen activators studied so far exert their action through hydrolysis of the Arg 560-Val 561 bond in plasminogen.

Intrinsic activation of plasminogen may occur by one or more pathways involving factor XII (Hageman factor), prekallikrein (Fletcher factor), high molecular weight kininogen (Fitzgerald factor), and possibly other components. However, the exact mechanism of this activation as well as its biological role remain unknown.

The plasminogen activator found in blood represents released vascular plasminogen activator, and these activators are similar or identical to the tissue activator but different from urokinase.

The physiological importance of extrinsic plasminogen activators has been inferred from the association between low blood fibrinolytic activity and thrombotic or atherosclerotic disease.

INHIBITORS OF INTRINSIC PLASMINOGEN ACTIVATION

Several inhibitors of intrinsic plasminogen activation occur in human plasma, C1-inactivator,⁴⁶ an inhibitor of factor XIIa-induced fibrinolysis,^{47 48} heparin-antithrombin III complex,⁴⁹ and α_2 -macroglobulin.⁵⁰ Since the physiological role of the intrinsic fibrinolytic pathway is not established the role of these inhibitors in the regulation and control of fibrinolysis thus remains entirely speculative.

INHIBITORS OF EXTRINSIC PLASMINOGEN ACTIVATION

The presence in plasma of inhibitors of extrinsic plasminogen activators forming a complex which dissociates in the presence of fibrin has been postulated already in the 1950s¹⁹ and also in more recent studies.⁵¹ The formation of a reversible activator-activator inhibitor complex which dissociates in the presence of fibrin has been invoked to explain the rapid lysis of fibrin in plasma and the resistance of fibrinogen to degradation by plasmin.⁵¹ The enhancing effect of fibrin on the plasminogen activation may be explained by adsorption of activator and plasminogen to its surface facilitating activation.^{20 21 28} The evidence for the existence of a specific inhibitor of extrinsic plasminogen activators in plasma, forming a reversible complex, can thus at best be regarded as preliminary.⁵²

There is good evidence that a significant amount of extrinsic plasminogen activator released in the blood is cleared *in vivo* by mechanisms other than neutralisation by plasmatic inhibitors. Indeed, whereas

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plasminogen activator released in the plasma by nicotinic acid injection in normal subjects has a half life *in vivo* of approximately 15 min^{53 54} its half life in plasma *in vitro* is approximately 75 min,⁵³ as measured with clot lysis assays.

INHIBITORS OF EXOGENOUS PLASMINOGEN ACTIVATION

Human plasma contains antibodies directed against streptokinase, which most probably result from previous infections with beta-haemolytic streptococci. The amount of streptokinase antibodies varies over a wide range among individuals. Verstraete *et al.*⁵⁵ found that 352 000 units of streptokinase were required to neutralise the circulating antibodies in 95% of a healthy population, but that the individual requirements ranged between 25 000 and 3 000 000 units. Since streptokinase reacts with antibodies and is thereby rendered biochemically inert, sufficient streptokinase must be infused to neutralise the antibodies before fibrinolytic activation is obtained.⁵⁶ A few days after streptokinase injection the antistreptokinase titre rises rapidly to 50 to 100 times the preinfusion value and remains high for 4 to 6 months, during which renewed treatment is impracticable. Administration of corticosteroids commonly is used as adjuvant to streptokinase to prevent allergic side reactions. The streptokinase-plasmin(ogen) activator complex is virtually unreactive towards α_2 -antiplasmin.⁵⁷

The mechanism of urokinase inhibition in blood is poorly known. Urokinase-inhibitor assays based on clot lysis are strongly dependent on the presence of plasmin inhibitors and are therefore in no way specific. Assayed with clot lysis methods the half life of urokinase *in vivo* is 9 to 16 min but *in vitro* 27 to 61 min,⁵⁸ suggesting that clearing of the enzyme from the blood plays an important role. α_2 -Macroglobulin,⁵⁹ α_1 -antitrypsin,⁶⁰ antithrombin III,⁶¹ and α_2 -antiplasmin⁴ all inhibit urokinase slowly.

INHIBITORS OF PLASMINOGEN ACTIVATION FROM PLATELETS

Platelets contain inhibitors which have been claimed primarily to inhibit fibrinolysis induced by activators.⁶²⁻⁶⁵ Washed platelets, at a concentration which does not inhibit plasmin, are capable of inhibiting plasminogen activators.⁶³ Murray *et al.*⁶⁴ partially purified an inhibitor of urokinase and tissue activator from human platelets with an estimated molecular weight of 45 000. Moore *et al.*⁶⁵ isolated a fraction possessing antiurokinase activity but no antiplasmin activity, in addition to a fraction containing antiplasmin activity.

The physiological significance of these inhibitors is uncertain. They may have a role in the protection of thrombi from premature lysis.

Synthetic inhibitors of fibrinolysis used for clinical application

Certain amino-acids such as 6-aminohexanoic acid (epsilon-aminocaproic acid, EACA), trans-4-aminomethylcyclohexane-1-carboxylic acid (AMCHA, tranexamic acid), and p-aminomethylbenzoic acid (PAMBA) inhibit fibrinolysis both *in vitro* and *in vivo*.⁶⁶ The relation between the structure of these substances and their antifibrinolytic effect has been studied in detail.⁶⁶ Their clinical usefulness has recently been reviewed.⁶⁷ In purified systems, however, these agents greatly accelerate the activation of native plasminogen.^{68 69} This paradox has recently been elucidated.^{20 70}

Native plasminogen (with NH₂-terminal glutamic acid) is slowly activated by urokinase, whereas partially degraded plasminogen (with NH₂-terminal lysine, valine, or methionine) is rapidly activated.^{68 69} In the presence of EACA, however, the activation rate of native plasminogen is greatly increased and approaches that of the partially degraded plasminogen. This acceleration has been ascribed to conformational changes in the plasminogen molecule induced by the presence of EACA or removal of the NH₂-terminal part of the molecule. The conformational change is probably a result of the dissociation between a site in the NH₂-terminal part (residues⁴⁵⁻⁵¹) and a structure in the plasminogen molecule which binds the antifibrinolytic amino acids ('lysine-binding site').^{71 72} The plasminogen molecule contains at least two such lysine-binding sites.^{24 73 74}

The antifibrinolytic effect of EACA and analogues appears to result from their interference with the binding of plasmin(ogen) to fibrin.²⁵⁻²⁷ When whole plasma is clotted, approximately 4% of the plasminogen is specifically bound to fibrin through its lysine-binding sites, and this interaction is abolished by EACA.²⁷ The specific affinity between plasminogen and fibrin seems to play an important role in the activation of plasminogen with tissue activator which is also specifically adsorbed to fibrin.^{20 75} Indeed, fibrin markedly potentiates the activation of plasminogen, but this stimulating effect is completely lost in the presence of EACA.^{20 70} EACA and its more active analogue tranexamic acid have been successfully used to reduce blood loss in essential menorrhagia, prostatectomy, and dental extraction in haemophiliacs.

Physiopathological conditions associated with inhibition of fibrinolysis

A number of physiopathological changes in the blood are associated with a delayed lysis of euglobulin clots or dilute blood clots. These include

pregnancy, hyperlipidemia, smoking, wine, and beer drinking and possibly atherosclerosis. The molecular mechanisms of inhibition of fibrinolysis by these factors is in most instances unknown.

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