Acceleration of Fibrinolysis by the N-Terminal Peptide of α2-Plasmin Inhibitor

By Shigeru Kimura, Taro Tamaki, and Nobuo Aoki

When blood plasma containing the NH2-terminal 12-residue peptide (N-peptide) of α2-plasmin inhibitor (α2PI; α2-antiplasmin) was clotted in the presence of calcium ions, the N-peptide and α2PI were cross-linked to fibrin by activated coagulation factor XII. The amount of N-peptide cross-linked to fibrin was proportional to the concentration of N-peptide present in plasma. On the other hand, the amount of α2PI cross-linked to fibrin was decreased by the presence of N-peptide, and the decrease was in reverse relationship to the increase of cross-linking of N-peptide. Spontaneous fibrinolysis or fibrinolysis induced by tissue plasminogen activator was accelerated by the presence of N-peptide, and the acceleration was dependent on the concentrations of N-peptide and directly proportional to inhibition of α2PI cross-linking exerted by N-peptide. The acceleration was more pronounced when the clot was compacted by platelet-mediated clot retraction or by a squeeze. Fibrinolysis of an α2PI-deficient or a factor XIII-deficient plasma clot was not accelerated by N-peptide. These findings were substantiated in a purified system and support the previous proposal that α2PI is cross-linked to fibrin at the glutamine residue that is next to the NH2-terminus of α2PI, and this factor XIII-mediated cross-linking of α2PI is significant in inhibition of physiologically occurring endogenous fibrinolysis.

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PLASMIN-CATALYZED fibrinolysis is efficiently inhibited by a plasma proteinase inhibitor called α2-plasmin inhibitor (α2PI) or α2-antiplasmin. When blood coagulation takes place, approximately 20% of the α2PI present in plasma is cross-linked to fibrin by activated blood coagulation factor XIII (XIIIa). The α2PI thus cross-linked to fibrin serves only as a glutamine substrate for XIIIa in the cross-linking reaction, and the cross-linking occurs between lysine residues of fibrin α-chains and a glutamine residue of the α2PI molecule that is the second residue from the NH2-terminal. This is further evidenced by the finding that a synthesized 12-residue peptide of the NH2-terminal region of α2PI (N-peptide) is incorporated rapidly into fibrin by XIIIa and thereby inhibits the cross-linking of α2PI with fibrin in a purified system. In the present study, the presence of the N-peptide in plasma is shown to inhibit the cross-linking of α2PI with fibrin when plasma is clotted and accelerates the subsequent fibrinolysis.

MATERIALS AND METHODS

Plasma. Blood was withdrawn from a normal individual or a patient with congenital deficiency of α2PI into 0.1 vol of 3.8% sodium citrate. The blood was then centrifuged to prepare platelet-rich or poor plasma.

Purified proteins. α2PI was purified from human plasma by the method described previously. The concentration was determined spectrophotometrically using A 1%/1 cm = 7.03 at 280 nm. Human fraction I-4, prepared according to the method of Blombäck and Blombäck, was used as the fibrinogen preparation after removing contaminating plasminogen and plasma fibrinectin from the preparation with lysine-Sepharose and gelatin-Sepharose, respectively.

The concentration was determined spectrophotometrically using A 1%/1 cm = 15.1 at 280 nm, and the coagulable protein was >95%. The fibrinogen preparation contained factor XIII in a concentration of 0.2 U/mL as a contaminant. Native Glu-plasminogen was prepared from fresh plasma in the presence of aprtinin (10 kallikrein inhibitor units [KIU]/mL) (Mochida Pharmaceuticals Co, Tokyo) by affinity chromatography on lysine-Sepharose followed by diethylaminoethyl (DEAE)-Sephadex chromatography. Purified streptokinase was prepared from a bovine streptokinase preparation (Mochida Pharmaceuticals) according to the method of Lundblad.

N-peptide. The NH2-terminal 12-residue peptide of α2PI, Asn-Gln-Glu-Gin-Val-Ser-Pro-Leu-Thr-Gly-Leu-Lys-NH2, was kindly supplied by Dr H. Tani, Tokyo Research Institute, Kowa Co, Tokyo.

Plasminogen activator. Tissue plasminogen activator (t-PA) was purified from the culture media of human melanoma cell line according to the method described by Rijken and Collen and kindly supplied by Dr H. Tani, Tokyo Research Institute, Kowa Co. The t-PA activity was assayed by the clot lysis method using a calibration curve constructed with the WHO standard preparation kindly supplied by Dr P.J. Gaffney, National Institute for Biological Standards and Control, London.

Radioiodination of protein. α2PI and fibrinogen were radioiodinated using solid-state lactoperoxidase-glucose oxidase (Enzymobead, Bio-Rad Laboratories, Richmond, Calif) and Na125I (17 Ci/mg) (New England Nuclear, Boston). The labeled α2PI and fibrinogen preparations had specific activities of 1 x 106 cpm/μg and 1.2 x 106 cpm/μg, respectively. The N-peptide was radioiodinated as described by Bolton and Hunter using N-succinimidyl 3-(4-hydroxy-5-[125I]iodophenyl) propionate (1.8 Ci/μmol) (Radiochemical Center, Amersham, England). The radioiodinated N-peptide had a specific activity of 1.4 x 106 cpm/μg. Free unconjugated radiolabeled compounds were removed by gel filtration using Sephadex G-10. The radioiodinated N-peptide was indicated to behave in a similar fashion to the unlabeled molecule in the cross-linking reaction with fibrin, since increasing amounts of the unlabeled peptide inhibited the incorporation of the labeled peptide into the plasma clot (Fig 1).

Measurement of the cross-linking to fibrin. Ten microliters of N-peptide in various concentrations was added to 80 μL of normal platelet-rich plasma, and the plasma was clotted by the addition of 10 μL of 250 mmol/L CaCl2 at 37 °C. For estimation of the extent of cross-linking of N-peptide or α2PI, radioactively labeled N-peptide or a trace amount of radioactive α2PI was added to the plasma, respectively. After 30-minute incubation, the clot was squeezed with a bamboo stick against the wall of the tube to express as much fluid as possible. The fibrin was then washed three times by soaking and
Measurement of fibrinolysis. Two milliliters of platelet-rich plasma was mixed with 2 μL of radiolabeled fibrinogen. A 180-μL aliquot of this mixture was mixed and incubated at 37 °C with 10 μL of calcium chloride (0.5 mol/L) containing various concentrations of N-peptide and 10 μL of t-PA (787 U/mL) in a glass test tube. The clot soon formed and underwent retraction. After incubation for 30 minutes, the clot was suspended by adding 800 μL normal platelet-poor plasma containing various amounts of N-peptide, a small fixed amount of t-PA and a trace amount of radiolabeled fibrinogen was clotted in the presence of calcium ions. After 30 minutes, the clot formed was suspended in platelet-poor normal plasma. Fibrinolysis was followed by the release of radioactivity from radiolabeled fibrin into the suspending plasma milieu. The presence of N-peptide in the clot accelerated fibrinolysis. The acceleration was dependent on the concentration of N-peptide and became more pronounced when a higher concentration of N-peptide was used (Fig 3). The extent of fibrinolysis was found to be linearly proportional to the degree of inhibition of αsPI cross-linking exerted by N-peptide (Fig 4). When t-PA was not added, the progress of fibrinolysis was very slow, but still the effect of N-peptide on fibrinolysis could be observed. When platelet-poor plasma instead of platelet-rich plasma was clotted, acceleration of fibrinolysis by N-peptide was less remarkable.

When αsPI-deficient or factor XIII-deficient plasma was formed was compacted by squeezing. To measure fibrinolysis, 5-μL aliquots of the supernatant were removed at intervals for counting of radioactivity. Results were expressed as described above.

RESULTS

Cross-linking of N-peptide and αsPI. Platelet-rich plasma was clotted by adding calcium ions in the presence of various concentrations of N-peptide, and the extent of cross-linking of N-peptide or αsPI was measured. When the N-peptide concentration was increased, the cross-linking of N-peptide increased parabolically (Fig 2). It was calculated that 1 mol N-peptide was cross-linked with approximately 1.5 mol fibrin (monomer) at the concentration of 1 mmol/L N-peptide.

In the absence of N-peptide, the cross-linking of αsPI was 1:20 mol fibrin (monomer). The cross-linking was decreased by the presence of N-peptide. The decrease was reversely related to the increase of cross-linking of N-peptide (Fig 1), and the 50% reduction of αsPI cross-linking was achieved by approximately 130 μmol/L of N-peptide.

Acceleration of fibrinolysis by N-peptide. Platelet-rich normal plasma containing various amounts of N-peptide, a small fixed amount of t-PA and a trace amount of radiolabeled fibrinogen was clotted in the presence of calcium ions. After 30 minutes, the clot formed was suspended in platelet-poor normal plasma. Fibrinolysis was followed by the release of radioactivity from radiolabeled fibrin into the suspending plasma milieu. The presence of N-peptide in the clot accelerated fibrinolysis. The acceleration was dependent on the concentration of N-peptide and became more pronounced when a higher concentration of N-peptide was used (Fig 3). The extent of fibrinolysis was found to be linearly proportional to the degree of inhibition of αsPI cross-linking exerted by N-peptide (Fig 4). When t-PA was not added, the progress of fibrinolysis was very slow, but still the effect of N-peptide on fibrinolysis could be observed. When platelet-poor plasma instead of platelet-rich plasma was clotted, acceleration of fibrinolysis by N-peptide was less remarkable.

When αsPI-deficient or factor XIII-deficient plasma was
The cross-linking of $\alpha_2$PI significantly enhances the $\alpha_2$PI cross-linking exerted by N-peptide (Fig 5). When $\alpha_2$PI, calcium ions. The acceleration was dependent on the concentration of N-peptide, and the extent of fibrinolysis was again observed in the presence of N-peptide.

Acceleration of fibrinolysis by N-peptide was also seen in a purified system, in which fibrinogen solution containing factor XIII, $\alpha_2$PI, plasminogen, t-PA, and various concentrations of N-peptide was clotted by thrombin in the presence of calcium ions. The acceleration was dependent on the concentration of N-peptide, and the extent of fibrinolysis was again found to be directly proportional to the degree of inhibition of $\alpha_2$PI cross-linking exerted by N-peptide (Fig 5). When $\alpha_2$PI was missing in the system, no acceleration of fibrinolysis by N-peptide was observed.

**DISCUSSION**

When blood coagulation takes place, part of the $\alpha_2$PI present in plasma is rapidly cross-linked to fibrin at the glutamine residue that is next to the NH2-terminus of $\alpha_2$PI.1,2 The cross-linking of $\alpha_2$PI significantly enhances the resistance of fibrin to naturally occurring fibrinolytic activity, thus stabilizing hemostatic plugs.3 In a purified system, the NH2-terminal 12-residue peptide of $\alpha_2$PI (N-peptide) was also found to be cross-linked to fibrin and to competitively inhibit the cross-linking of $\alpha_2$PI to fibrin.4 From these observations, it is conceivable that the N-peptide may inhibit the cross-linking of $\alpha_2$PI to fibrin when plasma is clotted, thus accelerating the subsequent fibrinolytic process. This view was tested in the present study.

In the present study, the N-peptide present in plasma was found to be cross-linked to fibrin when plasma was clotted (Fig 2). It was calculated that one molecule of N-peptide was cross-linked to one or two molecules of fibrin (monomer) at 1 mmol/L concentration of N-peptide. The value is less than 1:20 of the value obtained in a purified system in which roughly 20 molecules were cross-linked to each molecule of fibrin (monomer) at 1 mmol/L concentration of N-peptide.5 However, inhibition of $\alpha_2$PI cross-linking achieved by N-peptide was more pronounced in the plasma clot than in a purified system; 50% reduction of $\alpha_2$PI cross-linking was achieved by $\sim$130 $\mu$mol/L of N-peptide in the plasma clot, whereas $\sim$350 $\mu$mol/L of N-peptide was needed to achieve the 50% reduction in the purified system.6 These differences between the plasma clot and the purified system might have been caused partly by plasma fibronectin that was absent in the purified system. Plasma fibronectin is known to be cross-linked to fibrin by activated factor XIII.7 In the absence of fibronectin, fibronectin cross-linking sites on fibrin molecules may be more available for cross-linking of N-peptide. N-peptide may also be cross-linked randomly to other various sites on fibrin molecules, and these rather unspecific cross-linkings may be prevented in plasma by plasma proteins which may have high affinity for N-peptide. From these or other mechanisms, more N-peptide may be available for $\alpha_2$PI cross-linking sites on fibrin molecules in plasma than in a purified system, thus resulting in a more efficient inhibition of $\alpha_2$PI cross-linking by N-peptide in plasma.
Fibrinolysis was accelerated by the presence of N-peptide (Fig 3), and the degree of acceleration was proportional to the inhibition of cross-linking of $\alpha_2$PI exerted by N-peptide (Figs 4 and 5). When platelet-poor plasma instead of platelet-rich plasma was used, so that no clot retraction took place, the acceleration was less prominent. No acceleration was observed when $\alpha_2$PI-deficient or factor XIII-deficient plasma was used, indicating that acceleration of fibrinolysis was mediated by inhibition of $\alpha_2$PI cross-linking. These findings were substantiated in a purified system (Fig 5) and support the previously made proposal that cross-linking of $\alpha_2$PI to fibrin plays a significant role in inhibition of naturally occurring fibrinolysis that is caused by activation of fibrin-bound plasminogen by fibrin-bound activators (t-PA), particularly when the clot is compacted by platelet-mediated clot retraction.7

Interesting is the fact that more marked acceleration of fibrinolysis by N-peptide was seen when the clot retraction took place. This enhancement of fibrinolysis was also seen when the clot was compacted by squeezing, suggesting that the effect of clot retraction is produced simply by a mechanical contraction of the clot. Carroll et al17 observed that clot retraction facilitates spontaneous lysis of clots prepared from diluted plasma.17 Previously we demonstrated that the $\alpha_2$PI which has lost cross-linking capacity has significantly less inhibitory activity on fibrinolysis when clot is compacted.5 These observations made previously and in the present study suggest that inhibition of fibrinolysis may become more dependent on fibrin-bound $\alpha_2$PI than on free unbound $\alpha_2$PI when the clot is retracted, because most of the $\alpha_2$PI not bound to fibrin is squeezed out from the clot. Therefore, reduction of the amount of fibrin-bound $\alpha_2$PI by N-peptide may have made the clot more susceptible to the fibrinolytic process when the clot was retracted.

REFERENCES

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