Acceleration of Fibrinolysis by the N-Terminal Peptide of $\alpha_2$-Plasmin Inhibitor

By Shigeru Kimura, Taro Tamaki, and Nobuo Aoki

When blood plasma containing the NH$_2$-terminal 12-residue peptide (N-peptide) of $\alpha_2$-plasmin inhibitor ($\alpha_2$PI; $\alpha_2$-antiplasmin) was clotted in the presence of calcium ions, the N-peptide and $\alpha_2$PI were cross-linked to fibrin by activated coagulation factor XIII. 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 $\alpha_2$PI 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 $\alpha_2$PI 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 $\alpha_2$PI-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 $\alpha_2$PI is cross-linked to fibrin at the glutamine residue that is next to the NH$_2$-terminus of $\alpha_2$PI, and this factor XIII-mediated cross-linking of $\alpha_2$PI is significant in inhibition of physiologically occurring endogenous fibrinolysis.

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Materials and Methods

Plasmin-catalyzed fibrinolysis is efficiently inhibited by a plasma proteinase inhibitor called $\alpha_2$-plasmin inhibitor ($\alpha_2$PI) or $\alpha_2$-antiplasmin. When blood coagulation takes place, approximately 20% of the $\alpha_2$PI present in plasma is cross-linked to fibrin by activated blood coagulation factor XIII (XIIIa). The $\alpha_2$PI thus cross-linked renders the fibrin clot more resistant to the fibrinolytic process that occurs subsequently to fibrin formation and is caused by fibrin-associated plasminogen activation. $\alpha_2$PI serves only as a glutamine substrate for XIIIa in the cross-linking reaction, and the cross-linking occurs between lysine residues of fibrin $\alpha$-chains and a glutamine residue of the $\alpha_2$PI molecule that is the second residue from the NH$_2$-terminal. This is further evidenced by the finding that a synthesized 12-residue peptide of the NH$_2$-terminal region of $\alpha_2$PI (N-peptide) is incorporated rapidly into fibrin by XIIIa and thereby inhibits the cross-linking of $\alpha_2$PI 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 $\alpha_2$PI with fibrin when plasma is clotted and accelerates the subsequent fibrinolysis.

Plasma. Blood was withdrawn from a normal individual or a patient with congenital deficiency of $\alpha_2$PI into 0.1 vol of 3.8% sodium citrate. The blood was then centrifuged to prepare platelet-rich or poor plasma.

Purified proteins. $\alpha_2$PI 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 fibronectin 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 aprotinin (10 kallikrein inhibitor units [KIU]/mL) (Mochida Pharmaceuticals Co, Tokyo) by affinity chromatography on lysine-Sepharose followed by diethylaminoethyl (DEAE)-Sephadex chromatography. Purified thrombin was prepared from a bovine thrombin preparation (Mochida Pharmaceuticals) according to the method of Lundblad.

N-peptide. The NH$_2$-terminal 12-residue peptide of $\alpha_2$PI, Asn-Gln-Glu-Gln-Val-Ser-Pro-Leu-Thr-Gly-Leu-Lys-NH$_2$ - AcOH, 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 medium of a 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. $\alpha_2$PI and fibrinogen were radioiodinated using solid-state lactoperoxidase-glucose oxidase (Enzymobead, Bio-Rad Laboratories, Richmond, Calif) and Na$_2$[125I]Iodide (17 Ci/mg) (New England Nuclear, Boston). The labeled $\alpha_2$PI and fibrinogen preparations had specific activities of 1 x 10$^6$ cpm/ug and 1.2 x 10$^6$ cpm/ug, 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/\mu mol) (Radiochemical Center, Amersham, England). The radioiodinated N-peptide had a specific activity of 1.4 x 10$^6$ cpm/ug. 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 $\mu$L of normal platelet-rich plasma, and the plasma was clotted by the addition of 10 $\mu$L of 250 mmol/L CaCl$_2$ at 37°C. For estimation of the extent of cross-linking of N-peptide or $\alpha_2$PI, radioactively labeled N-peptide or a trace amount of radioactive $\alpha_2$PI 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...
shaking it in 4-mL aliquots of Tris-buffered saline (0.10 mol/L NaCl, 0.05 mol/L Tris–HCl buffer, pH 7.4), containing antiprotein (10 KIU/mL), EDTA (2 mmol/L), iodoacetamide (1 mmol/L), and 2% bovine serum albumin (Miles Laboratories, Inc, Elkhart, Ind), to wash out unbound material, for five minutes at room temperature each time. EDTA and iodoacetamide were used to stop the XIIIa-catalyzed reaction, and antiprotein was used to inhibit the fibrinolytic reaction, if any, occurring during the washing procedures. Albumin was used to prevent nonspecific binding of α2PI or N-peptide to fibrin. Practically complete removal of unbound materials from the washed fibrin by these procedures was previously confirmed. After washing, the fibrin was counted for radioactivity. The amount of α2PI or N-peptide cross-linked to fibrin was calculated from the radioactivity remaining in the washed fibrin, the original radioactivity present in the clotting plasma, and the specific radioactivity of the α2PI preparation or N-peptide used in the experiments.

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 formed was suspeded in 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 susceptible 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 α2PI 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 α2PI-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 α2PI. 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 α2PI 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 α2PI 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 α2PI 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 susceptible 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 α2PI 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 α2PI-deficient or factor XIII-deficient plasma was

Fig 1. Decrease of cross-linking of the radiolabeled N-peptide with fibrin by increasing amounts of the unlabeled peptide. The fixed amount of the radiolabeled N-peptide (2.17 × 10^6 cpm) was added to plasma containing antiprotein (10 U/mL plasma) and various concentrations of the unlabeled peptide (shown by a logarithmic scale on the abscissa). The plasma was clotted by the addition of calcium chloride, and the cross-linking of the labeled peptide to fibrin was measured as described in Materials and Methods.

Fig 2. Decrease of cross-linking of α2PI to fibrin with increasing concentrations of N-peptide in a plasma clot. Platelet-rich plasma was clotted by adding calcium ions in the presence of various concentrations of N-peptide. After 30-minute incubation at 37 °C, α2PI (0) or N-peptide (0) cross-linked to fibrin was measured as described in Materials and Methods.
The cross-linking of α2PI significantly enhances the cross-linking exerted by N-peptide (Fig 5). When α2PI was missing in the system, no acceleration of fibrinolysis by N-peptide was observed.

Discussion

When blood coagulation takes place, part of the α2PI present in plasma is rapidly cross-linked to fibrin at the glutamine residue that is next to the NH2-terminus of α2PI. The cross-linking of α2PI significantly enhances the resistance of fibrin to naturally occurring fibrinolytic activity, thus stabilizing hemostatic plugs. In a purified system, the NH2-terminal 12-residue peptide of α2PI (N-peptide) was also found to be cross-linked to fibrin and to competitively inhibit the cross-linking of α2PI to fibrin. From these observations, it is conceivable that the N-peptide may inhibit the cross-linking of α2PI 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. However, inhibition of α2PI cross-linking achieved by N-peptide was more pronounced in the plasma clot than in a purified system; 50% reduction of α2PI cross-linking was achieved by ~130 μmol/L of N-peptide in the plasma clot, whereas ~350 μmol/L of N-peptide was needed to achieve the 50% reduction in the purified system. 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. 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 α2PI cross-linking sites on fibrin molecules in plasma than in a purified system, thus resulting in a more efficient inhibition of α2PI 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 α2PI 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 α2PI-deficient or factor XIII-deficient plasma was used, indicating that acceleration of fibrinolysis was mediated by inhibition of α2PI cross-linking. These findings were substantiated in a purified system (Fig 5) and support the previously made proposal that cross-linking of α2PI 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.2

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 dilute plasma.17 Previously we demonstrated that the α2PI which has lost cross-linking capacity has significantly less inhibitory activity on fibrinolysis when clot is compacted.3 These observations made previously and in the present study suggest that inhibition of fibrinolysis may become more dependent on fibrin-bound α2PI than on free unbound α2PI when the clot is retracted, because most of the α2PI not bound to fibrin is squeezed out from the clot. Therefore, reduction of the amount of fibrin-bound α2PI by N-peptide may have made the clot more susceptible to the fibrinolytic process when the clot was retracted.

REFERENCES

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S Kimura, T Tamaki and N Aoki