P1 and P2' site mutations convert protease nexin-2 from a factor XIa inhibitor to a plasmin inhibitor

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The kunitz protease inhibitor domain of PN2 (PN2KPI) is a potent and specific inhibitor (K_i 0.5–2 nM) of factor XIa (FXIa) and inhibits cerebrovascular thrombosis in mice. To determine whether the antithrombotic properties of PN2KPI arise from its FXIa-inhibitory activity, we have now prepared mutant forms of PN2KPI. Mutations at the P1 (Arg¹⁵) site in combination with P2' (Met¹⁷) mutations profoundly affect inhibition of FXIa, plasmin, kallikrein, factor Xa and thrombin. The mutant proteins PN2KPI-R¹⁵K, -M¹⁷K, -R¹⁵K,M¹⁷K and -R¹⁵K,M¹⁷R lost inhibitory activity against FXIa (K_i 34, 94, 3081 and 707 nM, respectively) and kallikrein (no inhibition) and gained inhibitory activity against plasmin (K_i 108, 7, 8 and 8 nM, respectively). The intravenous administration of rPN2KPI into mice dramatically decreased thrombus formation in a murine model of FeCl₃-induced carotid injury, whereas rPN2KPI-R¹⁵K,M¹⁷K failed to inhibit thrombus formation. Molecular modeling studies showed that fine structural variations explain the observed functional differences in FXIa and plasmin inhibition. PN2KPI has potent antithrombotic activity due to its specific FXIa anticoagulant activity, whereas PN2KPI-R¹⁵K,M¹⁷K and PN2KPI-R¹⁵K,M¹⁷R have potent antifibrinolytic (antiplasmin) activity without anticoagulant or antithrombotic activity.

Keywords: Factor XIa/fibrinolysis/plasmin/protease nexin-2/thrombosis.

Abbreviations: AMI, acute myocardial infarction; BPTI, basic (or bovine) pancreatic trypsin inhibitor; DVT, deep venous thrombosis; FVIIa, factor VIIa; FX, factor X; FXI, Factor XI; FXII, factor XII; OPD, *O*-phenylenediamine; PCR, polymerase chain reaction; PK, prekallikrein; PN2, protease nexin 2; PN2KPI, KPI domain of PN2; TAFI, thrombin-activatable fibrinolysis inhibitor; TF, tissue factor.

Blood coagulation factor XI (FXI) plays an essential part in the maintenance of normal haemostasis and also in pre-disposing to the development of thromboembolic disorders (1). Current models of the initiation of blood coagulation *in vivo* suggest that, following vascular injury, the initial event is the formation of the enzyme complex of factor VIIa (FVIIa) on the transmembrane protein tissue factor (TF) present in many cell types resulting in the activation of factor X (FX) (2). However, FVIIa is rapidly inhibited when a complex is formed between factor Xa (FXa) and the tissue factor pathway inhibitor (3). As a consequence, the formation of thrombin through the TF or extrinsic pathway is transient, requiring the intrinsic pathway to generate adequate concentrations of thrombin to promote normal haemostasis (3). FXI, which is required for normal functioning of the intrinsic pathway, can be activated by four biologically relevant proteases: FXIIa (factor XIIa), FXIa (factor XIa), thrombin and meizothrombin (4–8). It participates in the contact phase of blood coagulation in the presence of anionic surfaces for optimal surface-mediated *in vitro* activation by FXIIa (9). However, as deficiencies of FXII, prekallikrein (PK) and high Mr kininogen are not associated with haemostatic abnormalities but FXI deficiency produces relatively mild, post-traumatic bleeding complications in ~50% of affected individuals (10), the more relevant pathway for activation of plasma FXI has been postulated to require feedback activation by thrombin or possibly autoactivation by FXIa (5, 6).

Elevated plasma FXI levels comprise a risk factor for deep venous thrombosis (DVT) (11) and cardiovascular disease in women (12). Although FXI deficiency was shown in one study not to protect against acute myocardial infarction (AMI) (13), patients with the acute coronary syndrome and coronary artery disease with a history of AMI have been shown to have increased levels of circulating FXIa that correlate with markers of coagulation and inflammation (14). Moreover, the risk of DVT among the 10% of individuals with the highest levels of FXI was >2-fold and for the remaining 90% of the population it was greater than that (11, 12, 15), and patients with severe FXI deficiency have been shown to have a reduced incidence of DVT (16). The incidence of ischaemic stroke is also reduced in patients with severe FXI deficiency (17). An increased risk of recurrence of venous thromboembolism, has been demonstrated among patients with increased levels of thrombin-activatable fibrinolysis inhibitor (TAFI) and FXI (18), whereas increased levels of TAFI, but not FXI, increased the risk of venous thromboembolism in patients who are

carriers of FV_{Leiden} (15). Consistent with these results is the demonstration of increased rabbit jugular vein thrombolysis by FXI antibody neutralization attributed to decreased activation of TAFI (19). Studies in FXI knockout mice showed protection from FeCl₃-induced carotid thrombosis (20) or from death due to thrombosis in protein-C-deficient animals (21). FXI has also been implicated in the growth of arterial thrombi in a rabbit model of arterial thrombosis (22), whereas another study in primates demonstrated the dependence on FXI levels of surface- and tissue-factor-initiated thrombus propagation (23). These studies provide compelling evidence that FXI/XIa levels in plasma constitute an important thrombogenic factor and that either FXI or FXIa constitutes a potential target for antithrombotic therapy (24).

Important control mechanisms exist for the regulation of FXIa, including the serine protease inhibitors, i.e. protease nexin 1 (25), antithrombin III (26), C1 inhibitor (27, 28), α -1-protease inhibitor (29, 30) and α -2-antiplasmin (31). Another class of proteins, the kunitz inhibitors, are also important regulators of coagulation protease function. Following platelet activation by physiological activators, protease nexin 2 (PN2) is secreted from α -granules into plasma and inhibits FXIa (32–34). PN2, a kunitz-type inhibitor, is ~120 kDa isoform of the Alzheimer's β -amyloid protein precursor, which is a potent and physiologically relevant inhibitor of FXIa (32, 33, 35–38), with minimal or absent detectable inhibitory activity against other coagulation enzymes, including thrombin, FIXa, FXa, FXIIa, plasmin and kallikrein (39). The KPI domain of PN2 (PN2KPI) is 57 residues in length, comprising Glu²⁸⁹-Ile³⁴⁵ in the 751 amino acid isoform of PN2, and contains the totality of the FXIa inhibitory function of PN2 (36–38, 40). Although the release of PN2 from activated platelets inhibits plasma FXIa activity in the environment of the haemostatic thrombus, platelet-bound FXIa is protected from inactivation by PN2 (37, 41, 42).

We have examined the effect of rKPI on thrombus development in a murine carotid artery injury thrombosis model (43). Intravenous administration of rPN2KPI into WT mice dramatically decreased the progress of FeCl₃-induced thrombus formation in the carotid artery as well as middle cerebral artery thrombosis. These studies strongly support the conclusion that inhibition of FXIa by KPI is a promising approach to antithrombotic therapy.

We have also carried out studies to compare FXIa inhibition by PN2KPI with FXIa inhibition by basic pancreatic trypsin inhibitor (BPTI) (39). PN2KPI was ~775-fold more potent than BPTI in FXIa inhibition. Studies of FXIa inhibition by PN2KPI and BPTI and P1 site swap mutants (PN2KPI-Arg¹⁵Lys, BPTI-Lys¹⁵Arg) demonstrated that mutation of P1 impaired FXIa inhibition by PN2KPI-Arg¹⁵Lys ~14-fold, and enhanced FXIa inhibition by BPTI-Lys¹⁵Arg ~150-fold. Arginine at the P1 site of either PN2KPI or BPTI was shown to confer high affinity and specificity for FXIa. Moreover, recent studies have demonstrated that the presence of hydrophobic residues at the P2' position in their substrates or kunitz inhibitors

confers enhanced anticoagulant activity, whereas the presence of basic residues at this position confers enhanced antifibrinolytic activity (44). Therefore, in the present study, we have examined the effects of mutations at various residues, focusing specifically on the P1 and P2' residues within KPI, on the specificity of the inhibitor for various coagulation and fibrinolytic enzymes, including FXIa and plasmin, as well as BPTI and on thrombus formation in a murine model of FeCl₃-induced carotid artery thrombosis. The major purpose of the present work was to examine the mechanism by which PN2KPI inhibits thrombosis and the relationship between its antithrombotic properties and its FXIa-inhibitory activity.

Materials and Methods

Reagents

The site-directed mutagenesis kit (Quikchange™) was purchased from Stratagene (La Jolla, CA, USA). Lipofectamine 2000 Reagent was from Invitrogen (Carlsbad, CA, USA). The chromogenic substrates (all obtained from Chromogenix, DiaPharma Group, Inc., OH, USA) and their final concentrations for the reactions were: 200 mM of S-2366 (L-pyroGlu-Pro-Arg-pNA-HCl) for FXIa and thrombin; 160 or 200 mM of S2765 (N-a-Z-D-Arg-Gly-Arg-pNA.2HCl) for trypsin or FXa, respectively; 300 mM of S2302 (Pro-Phe-Arg-pNA.2HCl) for plasma kallikrein; and 400 mM of S2403 (pyroGlu-Phe-Lys-pNA.HCl) for plasmin. Bovine serum albumin, penicillin/streptomycin, glutamine, *O*-phenylenediamine (OPD), aprotinin and cyanogen bromide-activated sepharose 4B were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's Medium was obtained from Mediatech (Herndon, VA, USA). Geneticin G418 was purchased from Gibco (Grand Island, NY, USA). All other reagents were of analytical grade and were the best quality commercially available.

Proteins

FXa, FXIa and plasmin were purchased from Haematologic Technologies Inc., VT, USA, whereas thrombin and plasma kallikrein were obtained from Enzyme Research Laboratories, IN, USA and bovine trypsin was purchased from Sigma Chemical Co., St. Louis, MO, USA. Kunitz-type protease inhibitor was purified from transfected *Pichia pastoris* as previously described (45).

Native and mutant inhibitor gene constructs for *P. pastoris* expression

From the full-length human PN2 gene the PN2KPI domain was amplified by polymerase chain reaction (PCR) as described earlier (39, 45). PCR-based site-directed mutagenesis (QuikChange, Stratagene, USA) was utilized to introduce an Ala, Arg or Lys mutation at the P1, P2' and other sites, inserting the codon preferentially used in yeast, in the PN2KPI domain, such as PN2KPI-R¹⁵K, M¹⁷K, M¹⁷R and F³⁴A, and double mutants R¹⁵K-M¹⁷K and R¹⁵K-M¹⁷R. As a PCR amplification product from bovine liver cDNA (Biochain Institute, Inc., USA), BPTI was obtained as a 206-bp length region and ligated into the yeast expression vector, pPICZαA (Invitrogen, USA). The pPICZαA plasmids containing native and mutant inserts were sequenced from 5' to the α -mating factor secretion signal using an AOX5 primer to confirm the mutations at the desired sites as well as the reading frame integrity.

PN2KPI domain and mutant protein expression in yeast cells

By recombinational cloning, the pPICZαA plasmid containing PN2KPI, BPTI and mutant gene constructs were incorporated into the methylotrophic yeast, *P. pastoris* X33, competent cell genome (Invitrogen, USA) and spread on to Zeocin-YPD plates. Yeast clones were zeocin selected and were lysed either by lyticase (Sigma, USA) or by repeated freeze-thaw cycles. Using gene-specific forward and reverse primers, the lysates were checked for correct insert size by PCR. Successful clones containing the correct insert were propagated at 30°C for 96 h in buffered medium containing

glycerol and yeast nitrogen base and expressed in 0.1 or 1 l of buffered medium containing methanol and yeast nitrogen base under vigorous agitation. The culture was supplemented with 0.5% methanol (final concentration) every 24 h.

Purification of recombinant Kunitz inhibitory domain proteins

Purification of recombinant PN2KPI and its mutant protein followed the protocol explained earlier (39, 45). Purification of BPTI was carried out using a modification of the PN2KPI protocol. Purified proteins were estimated for their concentration either by the bicinchoninic acid assay (Pierce Biotechnology, Inc., USA) or using their extinction coefficients, which were derived from the protein sequence (ProtParam, ExPASy, Swiss Institute of Bioinformatics). On silver-stained SDS-PAGE gels under reducing conditions, the purified proteins were single, sharp bands at 6.3–6.5 kDa.

Determination of equilibrium inhibition constant

To establish the equilibrium inhibition constant (K_i) values for inhibition by the kunitz inhibitors, FXa, FXIa, plasmin, thrombin, plasma kallikrein or trypsin, assays were carried out in 50 mM Tris, 150 mM NaCl, 0.1% BSA, pH 7.5 buffer using appropriate chromogenic substrates to measure residual activity after incubation with the kunitz inhibitors. The final enzyme concentrations used were FXIa (1 nM), thrombin (1 nM), FXa (1 nM), plasmin (10 nM), plasma kallikrein (1 nM) or trypsin (100 pM), which were incubated in a 135 μ l volume for 45 min at 37°C in a microtitre plate to establish equilibrium between the inhibitor and the enzyme. The appropriate substrate (15 μ l) was added to this pre-incubation mixture to a final reaction volume of 150 μ l. The substrate concentrations used were at or above their determined K_m values for their respective enzymes. The initial reaction velocities of chromogenic substrate cleavage were determined for 10 min at 37°C in a microplate reader at 37°C (Thermomax, Molecular Devices, USA) and were converted to fraction of amidolytic activity remaining. The IC₅₀ values were determined by plotting residual enzyme activity against inhibitor concentration using KaleidaGraph v3.5 software and equilibrium K_i values were calculated using the equation explained in the 'Data analyses' section.

Animal model of carotid artery thrombosis

A model of FeCl₃-induced carotid artery thrombosis was established in 7- to 11-week-old female C57BL/6 mice following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the protocol approved by the Temple University Institutional Animal Care and Use Committee exactly as previously described (43). All experimental manipulations and observations were carried out by an individual (H.L.) blinded to the treatment assignment, i.e. WT mice given rPN2KPI, normal saline or the double mutant molecule (PN2KPI-R¹⁵K,M¹⁷K).

Inhibition of fibrinolysis by PN2KPI and PN2KPI-R¹⁵K,M¹⁷K

The inhibition of fibrinolysis in whole plasma was measured by monitoring changes of the absorbance at 405 nm with plasma coagulation initiated by thrombin and fibrinolysis facilitated by plasmin. Pooled human plasma (200 μ l, George King Bio-Medical Inc, KS, USA) was mixed with 50 μ l of TBS buffer containing PN2KPI or PN2KPI-R¹⁵K,M¹⁷K and 200 μ l of this mixture was added to 50 μ l of TBS buffer containing final concentrations of 10 nM thrombin, 20 U/ml streptokinase and 5 mM CaCl₂. The absorbance of the mixture was monitored in a Spectromax M2 plate reader (Molecular Devices, CA, USA) at 37°C for 30 min.

Molecular modelling

In the modelling procedure, the crystal structure of the FXIa/PN2KPI complex (45) (PDB: 1ZJD) was compared with the crystal structure of plasmin (46) (PDB: 1BUI). To identify interactions of native PN2KPI with FXIa and possible interactions with plasmin, 1ZJD and 1BUI were superimposed using the Align software (Pymol, Schrödinger, Portland, USA). The plasmin structure (1BUI) and the KPI structure (1ZJD) were used to construct a model of the plasmin/PN2KPI complex. The PN2KPI mutations at P1 and P2' sites were introduced using Pymol molecular modelling

software and were used to construct the molecular models of the complexes of the mutants with plasmin. Energy minimization was carried out in AMBER7 F99 force field utilizing SYBYL software (version 7.2, from Tripos Inc., St. Louis, MO, USA). The cutoff point for the termination of the energy minimization calculation was either the completion of 10,000 iterations or a gradient value of 0.5 kcal mol⁻¹ Å⁻¹. The energy-minimized structures of the complex of plasmin with the two mutants, PN2KPI-R¹⁵K,M¹⁷K and PN2KPI-R¹⁵K,M¹⁷R were superimposed with the modelled wtPN2KPI/plasmin complex structure to monitor possible changes in interactions between mutant PN2KPI molecules and plasmin.

Data analyses

Values of K_i eq were derived from their IC₅₀ values using the following equation:

$$K_{i\text{eq}} = \text{IC}_{50} / (1 + ([S]/K_m)) \quad (1)$$

where [S] is substrate concentration and K_m is the Michaelis constant.

All animal model data are shown as mean values $\pm$ standard error of the mean (SEM) and differences between the PN2KPI groups and the control group were further evaluated using an unpaired two-tailed *t*-test with statistical significance determined as $P < 0.05$.

Results

Equilibrium inhibition assays

Initially equilibrium assays were carried out to determine values of K_i for the inhibition of various serine proteases (FXIa, plasmin, kallikrein, FXa, trypsin and thrombin) by the various kunitz inhibitors and their mutants (wild-type PN2KPI, i.e. wtPN2KPI, PN2KPI-R¹⁵K, PN2KPI-M¹⁷A, PN2KPI-M¹⁷K, PN2KPI-M¹⁷R, PN2KPI-R¹⁵K,M¹⁷R, PN2KPI-R¹⁵K,M¹⁷K, PN2KPI-F³⁴A, PN2KPI-M¹⁷A,F³⁴A and BPTI). The results of selected experiments focusing on the inhibition of these proteases by wtPN2KPI and BPTI, as well as the double mutants, PN2KPI-R¹⁵K,M¹⁷R and PN2KPI-R¹⁵K,M¹⁷K, are shown in Fig. 1. The mean K_i values (nM $\pm$ SD) derived for inhibition of all the proteases (except FXa and thrombin) by all the inhibitors are detailed in Table I. These studies show that wtPN2KPI has much greater inhibitory activity against FXIa (K_i 2.2 nM) than against plasmin (K_i 196 nM), kallikrein (K_i 240 nM), FXa (K_i 861 nM) or thrombin (no inhibition), whereas BPTI is a more potent inhibitor of plasmin (K_i 4.1 nM) and kallikrein (K_i 40 nM), than against FXIa (K_i 270 nM), FXa or thrombin, neither of which was inhibited by BPTI. Trypsin inhibitory activity was remarkably similar for all kunitz inhibitors and mutants (K_i 0.44–1.67 nM). Mutation of arginine to lysine at the P1 site (Arg¹⁵) resulted in decrease of inhibitory activity against FXIa (K_i 34 nM) and kallikrein (K_i 8059 nM) and gain of inhibitory activity against plasmin (K_i 107 nM), consistent with our previous demonstration of a preference for arginine at the P1 residue for FXIa inhibition and a preference for lysine for plasmin inhibition (39). Mutation of the hydrophobic residue methionine at the P2' site (Met¹⁷) to alanine had very little effect on FXIa inhibition (K_i 2.32 nM), and resulted in a modest enhancement in plasmin inhibition (K_i 145 nM) and a marked enhancement in kallikrein inhibition (K_i 8 nM). In contrast, when the P2' site was changed from a hydrophobic to a basic

Fig. 1 Inhibition of selected serine proteases by native PN2KPI, PN2KPI-R¹⁵K,M¹⁷K, PN2KPI-R¹⁵K,M¹⁷R and BPTI. (A) Inhibition of plasmin (Panel I), FXIa (II) and plasma kallikrein (III) by wtPN2KPI, by PN2KPI-R¹⁵K-M¹⁷R (IV–VI), by PN2KPI-R¹⁵K,M¹⁷K (VII–IX) and by BPTI (X–XII). (B) Inhibition of FXa (Panel I), thrombin (II) and trypsin (III) by wtPN2KPI, by PN2KPI-R¹⁵K-M¹⁷R (IV–VI), by PN2KPI-R¹⁵K,M¹⁷K (VII–IX) and by BPTI (X–XII). Equilibrium inhibition constants (K_i values) are shown in respective graphs, and are mean values derived from two to four assays, carried out in duplicate. Mean K_i values (nM $\pm$ SD) are detailed in Table I.

residue (either arginine or lysine), FXIa (K_i 53–94 nM) and kallikrein (K_i 2760–3820 nM) inhibition were drastically impaired, whereas plasmin (K_i 7.4–26 nM) inhibition was enhanced. The two double mutant molecules, KPI-R¹⁵K,M¹⁷R and KPI-R¹⁵K,M¹⁷K,

were characterized by remarkable loss of activity against FXIa (K_i 706.8 and 3081.4, respectively) and gain of activity against plasmin (K_i 7.89 and 7.91, respectively), with complete loss of inhibitory activity against kallikrein and FXa.

Fig. 1 Continued.

The effects of P1 and P2' mutations on the inhibition by PN2KPI of carotid artery thrombosis in a mouse model

We have previously shown that PN2KPI is a potent inhibitor of FXIa and an effective inhibitor of both FeCl₃-induced carotid artery thrombosis and middle cerebral artery thrombosis in mice (43). These observations suggest but do not prove that the inhibition of thrombosis by PN2KPI is a direct consequence of

FXIa inhibition. To further investigate this possibility, we examined the effects of PN2KPI and the double mutant PN2KPI-R¹⁵K,M¹⁷K on FeCl₃-induced carotid artery thrombosis in the mouse. Since simultaneous mutations at the P1 and P2' sites shift its specificity from FXIa towards plasmin by virtually eliminating FXIa inhibition and dramatically enhancing plasmin inhibitory activity, the double mutant

Table I. Inhibitory activities of PN2KPI, its mutants and BPTI against FXIa, plasmin and other serine proteases.

Inhibitor	Protease					
	FXIa K_i nM $\pm$ SD	Plasmin K_i nM $\pm$ SD	Kallikrein K_i nM $\pm$ SD	FXa K_i nM $\pm$ SD	Trypsin K_i nM $\pm$ SD	Thrombin
KPIwt	2.22 $\pm$ 0.59	196.2 $\pm$ 14.3	239.7 $\pm$ 18.6	861.1 $\pm$ 40.9	0.89 $\pm$ 0.06	NI ^a
KPI-R ¹⁵ K	33.8 $\pm$ 0.83	107.5 $\pm$ 6.1	8059 $\pm$ 1568		0.81 $\pm$ 0.02	
KPI-M ¹⁷ A	2.32 $\pm$ 0.52	145.1 $\pm$ 16.1	7.9 $\pm$ 0.73		1.12 $\pm$ 0.00	
KPI-M ¹⁷ K	94.3 $\pm$ 6.37	7.41 $\pm$ 1.3	3820 $\pm$ 2.9		0.44 $\pm$ 0.00	
KPI-M ¹⁷ R	52.5 $\pm$ 0.95	25.8 $\pm$ 2.3	2760 $\pm$ 0.12		1.31 $\pm$ 0.02	
KPI-R ¹⁵ K,M ¹⁷ R	706.8 $\pm$ 72.2	7.89 $\pm$ 0.3	NI ^a	NI ^a	0.79 $\pm$ 0.01	NI ^a
KPI-R ¹⁵ K,M ¹⁷ K	3081.4 $\pm$ 117.9	7.91 $\pm$ 0.4	NI ^a	NI ^a	0.77 $\pm$ 0.10	NI ^a
KPI-F ³⁴ A	8.6 $\pm$ 0.17	722.9 $\pm$ 21.2	392.5 $\pm$ 3.16		1.67 $\pm$ 0.01	
KPI-M ¹⁷ A,F ³⁴ A	3.18 $\pm$ 0.01	118.2 $\pm$ 2.9	24.5 $\pm$ 0.35		0.86 $\pm$ 0.04	
BPTI	270.04 $\pm$ 2.26	4.07 $\pm$ 0.1	39.7 $\pm$ 1.39	NI ^a	0.66 $\pm$ 0.04	NI ^a

^aNo inhibition observed at the highest inhibitor concentration (8 μ M).

PN2KPI-R¹⁵K,M¹⁷K provides an ideal opportunity to determine whether the antithrombotic activity of PN2KPI is dependent upon its inhibitory effect against FXIa. As shown in Fig. 2, compared with a control mouse treated with placebo (Fig. 2A), a representative mouse treated with the double mutant PN2KPI-R¹⁵K,M¹⁷K (Fig. 2B) developed FeCl₃-induced carotid artery thrombosis that was indistinguishable in extent from the control animal. As demonstrated previously, wtPN2KPI was a potent inhibitor of FeCl₃-induced carotid artery thrombosis (data not shown). Identical studies carried out in five mice in each group (Fig. 2C) demonstrated that the extent of thrombosis developing in PN2KPI-R¹⁵K,M¹⁷K-treated animals was not significantly different ($P > 0.1$) from that observed in control animals. The major conclusion to be drawn from these experiments is that the antithrombotic activity of PN2KPI is a direct consequence of its FXIa-inhibitory activity.

The inhibition of fibrinolysis by PN2KPI and PN2KPI-R¹⁵K,M¹⁷K

To examine the effects of PN2KPI and the double mutant PN2KPI-R¹⁵K,M¹⁷K on fibrinolysis in whole plasma, an assay was developed in which thrombin was added to pooled normal human plasma to induce clot formation and streptokinase was added to activate endogenous plasminogen. The progress of clot formation and dissolution was monitored as changes of absorbance at 405 nm. As shown in Fig. 3A and B, in the absence of PN2KPI, the absorbance increased rapidly after adding thrombin due to fibrin formation reaching a peak at ~ 2 –3 min, whereas fibrinolysis of the plasma clot by streptokinase-activated plasmin resulted in gradual subsequent decreases in absorbance at 405 nm. Adding wild-type PN2KPI (Fig. 3A) and the double mutant PN2KPI-R¹⁵K,M¹⁷K (Fig. 3B) inhibited clot lysis and stabilized the fibrin clot in a concentration-dependent manner. Shown in Fig. 3C is the percentage inhibition of fibrinolysis calculated from the absorbance changes 30 min after adding either wtPN2KPI or PN2KPI-R¹⁵K,M¹⁷K. The percent inhibition of

fibrinolysis by wtPN2KPI and PN2KPI-R¹⁵K,M¹⁷K at concentrations $> 2.5 \mu$ M was similar ($P > 0.1$), whereas at concentrations $< 1 \mu$ M, the percent inhibition by PN2KPI-R¹⁵K,M¹⁷K was significantly greater ($P < 0.05$) than that obtained with wtPN2KPI. This result is consistent with the conclusion that PN2KPI-R¹⁵K,M¹⁷K is a much more potent inhibitor of plasmin (K_i 196 nM) than is wtPN2KPI ($K_i \sim 8$ nM, Table I).

Discussion

The present studies were initiated for the primary purpose of determining the structural requirements for FXIa-inhibition by PN2KPI (39, 45), the mechanism by which PN2KPI inhibits cerebral thrombosis (43) and the relationship between the two. The results of our experiments demonstrate the critical role of the P1 and P2' residues in the inhibition of FXIa and other serine proteases, including plasmin. It can be concluded that mutation of the P1 residue from arginine to lysine or mutation of the hydrophobic P2' residue to lysine or arginine results in loss of inhibitory activity against FXIa and gain of activity against plasmin, so that the double mutant PN2KPI-R¹⁵K,M¹⁷K no longer inhibits FXIa but is a potent inhibitor of plasmin. The fact that PN2KPI-R¹⁵K,M¹⁷K loses both its FXIa-inhibitory activity and its antithrombotic activity in murine FeCl₃-induced carotid artery thrombosis strongly supports the conclusion that the antithrombotic activity of wtPN2KPI is a direct consequence of its FXIa-inhibitory activity.

The fact that PN2KPI-R¹⁵K,M¹⁷K, compared with wtPN2KPI, also gains antifibrinolytic activity as a potent plasmin inhibitor has interesting therapeutic implications because the inhibition of fibrinolysis has become a very effective strategy in the management of bleeding in various clinical scenarios, such as post-surgical or post-traumatic bleeding and spontaneous bleeding in haemophilia. The BPTI (aprotinin; Trasylol®), is an inhibitor of several human serine proteases, including trypsin, plasmin, kallikrein, activated protein C and FXIa (47, 48) and has been commonly

Fig. 2 The inhibition of thrombus formation in the mouse common carotid artery induced by FeCl₃. Thrombus formation, in pairs of mice selected for study on a single day was visualized by an increase of rhodamine fluorescence recorded by fluorescence microscopy. The image of thrombus growth was captured from video clips recorded at different time points. Panel A: thrombus growth in a representative control mouse treated with vehicle only (i.e. without PN2KPI-R¹⁵K,M¹⁷K treatment). Panel B: thrombus growth observed in a representative mouse, treated with PN2KPI-R¹⁵K,M¹⁷K. The time points are 0, 1, 5, 7, 10, 15, 20, 30 and 40 min. The increase in fluorescence intensity, which represents the size of the thrombus, was plotted against time and is presented in Panel C. No statistically significant differences ($P > 0.1$) in fluorescence intensity at any time point were observed between mice in the control group (squares) and mice treated with PN2KPI-R¹⁵K,M¹⁷K (circles). The results shown are mean values ($\pm$ SEM) of five animals in each group.

utilized clinically in the past to control plasma proteolysis during and after cardiopulmonary bypass surgery. Although BPTI is an effective fibrinolytic inhibitor, complications (e.g. acute myocardial infarction, stroke, renal insufficiency and immunological or hypersensitivity reactions) prevent its clinical use. As there is a need to replace BPTI with an effective and safe human protein, it seems feasible to examine the therapeutic effects of variants derived from the KPI domain of PN2, a human protein with high structural homology to BPTI.

In order to engineer the variants of PN2KPI functioning similar to BPTI, two critical issues have to be addressed, i.e. the loss of the inhibitory function of PN2KPI against FXIa and the gain of the inhibitory activity against plasmin. The P1 site of kunitz-type inhibitors has been demonstrated to be essential to their serine protease inhibitory function; e.g. mutations at

the P1 site of BPTI, i.e. Lys15 to alanine largely, lost its trypsin inhibitory activity (49). Previous studies in our laboratory on the crystal structure and functional activities of the FXIa/PN2KPI complex showed that the P1 residue of PN2KPI, i.e. Arg15, was critical to its binding and inhibition of FXIa. The mutant PN2KPI-R¹⁵A lost its activity against FXIa completely (45).

Although the P1 site residues of PN2KPI and BPTI are both positively charged residues with similar backbone structures and orientations, the differences in side chain bulkiness, length and consequent charge distribution may cause significant differences in their interactions with the S1 pocket of serine proteases. The replacement of P1 sites of PN2KPI, i.e. R¹⁵, by its counterpart from BPTI, lysine, leads to partial loss of its inhibitory function against FXIa, which is consistent with previous reports that wtPN2KPI is

Fig. 3 The inhibition of fibrinolysis by PN2KPI and PN2KPI-R¹⁵K,M¹⁷K. Whole plasma coagulation and fibrinolysis was observed by the changes in plasma absorbance at 405 nm. Fibrin formation caused by thrombin increased the absorbance, whereas fibrinolysis catalysed by streptokinase-activated plasmin decreased the rate of decay of absorbance. With different concentrations of wtPN2KPI (Panel A) and PN2KPI-R¹⁵K,M¹⁷K (Panel B) added to plasma, fibrinolysis was inhibited as shown by decreased rates of the absorbance decay of the plasma. However, the inhibition of fibrinolysis is more prominent with PN2KPI-R¹⁵K,M¹⁷K at lower concentrations (<1 μM) compared with wtPN2KPI, as shown in Panel C, in which the percent of fibrinolysis inhibition was measured 30 min after the reaction started. The inhibition of fibrinolysis by PN2KPI-R¹⁵K,M¹⁷K (open bars) is significantly greater ($P < 0.05$) from that effected by wtPN2KPI (black bars) at concentrations <1 μM.

~14-fold more potent than the mutant PN2KPI-R¹⁵K in FXIa inhibition (39, 50). However, the mutant PN2KPI-R¹⁵K is ~10-fold more active in plasmin inhibition (50). In the current study, we found that this mutant also partially gains plasmin inhibitory function, which is ~2-fold more potent than its parent molecule. Although the P1 site is important in the specificity of PN2KPI in FXIa inhibition, the mutation at the P1 site alone is not sufficient to switch PN2KPI to a potent plasmin inhibitor.

Bajaj *et al.* (44) recently showed that although the coagulation proteases prefer hydrophobic residues at the P2' position in their substrates/inhibitors, plasmin prefers a basic residue at this position. We have, therefore, carried out modelling studies of the interaction between plasmin and PN2KPI P1 and P2' mutants (Fig. 4A). The plasmin/PN2KPI complex model showed that R¹⁵ of PN2KPI was inserted deeply into a pocket formed by the loop structure 189–196 (DSCQGDSG) and 211–219 (VTWGLGC); however, there was no significant interaction of plasmin with the P2' Met¹⁷ of PN2KPI (Fig. 4A). As BPTI is a potent inhibitor of plasmin ($K_i = 4.07 \pm 0.1$), we replaced the P1 residue of PN2KPI with that of BPTI (i.e. by mutating Arg¹⁵ to Lys) in the plasmin/PN2KPI model and did not find a significant structural difference (Fig. 4C). Accordingly, the PN2KPI-R¹⁵K-mutant expressed in *P. pastoris* exhibited an ~10-fold decrease in affinity for FXIa and ~2-fold increase of inhibition of plasmin (Table I). When the P2' site Met¹⁷ is replaced by the basic residue Arg or Lys (Fig. 4B), the sidechains of residue Arg¹⁷/Lys¹⁷ of the mutants were inserted into a cavity in the plasmin structure interacting closely with the backbone Phe⁴¹ of plasmin and its side chain also interacted with the backbone of Trp¹⁴¹ and Gln¹⁹² (Fig. 4D). In the FXIa inhibition assay, both mutants showed a significant increase in plasmin inhibition ($K_i = 25.8 \pm 2.3$ nM and 7.41 ± 1.3 nM, respectively), while still maintaining relatively potent inhibitory activity against FXIa ($K_i = 52.5 \pm 0.95$ nM and 94.3 ± 6.37 nM, respectively). The double mutants PN2KPI-R¹⁵K,M¹⁷R and PN2KPI-R¹⁵K,M¹⁷K have the combined changes of the interactions of P1 and P2' sites with plasmin as shown in single mutants of each site, respectively.

The secondary mutation at the P2' site in addition to the P1 site mutations also further decreases the inhibitory activity of PN2KPI against FXIa. When administered *in vivo*, wtPN2KPI effectively diminished thrombus formation in murine FeCl₃-induced carotid artery thrombosis. Due to the dramatically decreased inhibitory effect against FXIa, ~1,700-fold less than wtPN2KPI, the mutants with P1 and P2' mutations have an insignificant effect in thrombosis development in the mouse model. Owing to its human origin, the double mutant PN2KPI-R¹⁵K,M¹⁷K, which acquires antifibrinolytic activity and loses FXIa inhibitory and antithrombotic function, is potentially an effective antifibrinolytic agent when used in humans.

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Fig. 4 Molecular modelling of the interaction between plasmin and PN2KPI P1 and P2' mutants. The interaction with plasmin of the P1 and P2' residues of wtPN2KPI and mutants involving the P1 and P2' sites are shown in Panels A–D. The surfaces in Panels A and B are coloured red for negative charge and blue for positive charge. Panel A: the positively charged side chain R¹⁵ of wtPN2KPI is inserted deeply into a negatively charged pocket of plasmin and there is no interaction of M17 of wtPN2KPI with plasmin. Panel B: in the double mutant KPI-R¹⁵K, M¹⁷K, the mutation of R¹⁵K, does not have a major effect on the interaction between the P1 site with plasmin. However, the mutation of M¹⁷ to a positively charged residue K¹⁷ results in an intimate interaction of the positively charged lysine side chains with a negatively charged pocket in plasmin. These interactions between K¹⁵ or K¹⁷ of PN2KPI with plasmin in the double mutant PN2KPI-R¹⁵K, M¹⁷K are magnified in Panels C and D. Panel C shows that K¹⁵ of PN2KPI-R¹⁵K, M¹⁷K is inserted into the cavity formed by 189–196 (DSCQGDSG) and 211–219 (VTSWGLGC) of plasmin with the positively charged side chain of K¹⁵ interacting closely with D¹⁸⁹ and S¹⁹⁰ of plasmin. Panel D: the side chain of K¹⁷ of the double mutant PN2KPI-R¹⁵K, M¹⁷K interacts intimately with the side chain of the F⁴¹ residue of plasmin and also with the backbone of W¹⁴¹ and Q¹⁹² of plasmin. Similar interactions were demonstrated for the combined interactions of the P1 and P2' residues of the double mutant PN2KPI-R¹⁵K, M¹⁷R with plasmin (data not shown).

Conflict of interest

None declared.

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