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Thrombolysis by chemically modified coagulation factor Xa

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Summary

Background—Enzymatic thrombolysis carries the risk of hemorrhage and re-occlusion must be evaded by co-administration with an anticoagulant. Toward further improving these shortcomings, we report a novel dual-functioning molecule, Xai-K, which is both a non-enzymatic thrombolytic agent and an anticoagulant. Xai-K is based on clotting factor Xa, whose sequential plasmin-mediated fragments, FXaβ and Xa33/13, accelerate the principal thrombolytic agent, tissue plasminogen activator (tPA), but only when localized to anionic phospholipid.

Methods—The effect of Xai-K on fibrinolysis was measured in vitro by turbidity, thromboelastography and chromogenic assays, and measured in a murine model of occlusive carotid thrombosis by Doppler ultrasound. The anticoagulant properties of Xai-K were evaluated by normal plasma clotting assays, and in murine liver laceration and tail amputation hemostatic models.

Results—Xa33/13, which participates in fibrinolysis of purified fibrin, was rapidly inhibited in plasma. Cleavage was blocked at FXaβ by modifying residues at the active site. The resultant Xai-K (1 nm) enhanced plasma clot dissolution by ~7-fold in vitro and was dependent on tPA. Xai-K alone (2.0 μg g−1 body weight) achieved therapeutic patency in mice. The minimum primary dose of the tPA variant, Tenecteplase (TNK; 17 μg g−1), could be reduced by > 30-fold to restore blood flow with adjunctive Xai-K (0.5 μg g−1). TNK-induced systemic markers of fibrinolysis were not detected with Xai-K (2.0 μg g−1). Xai-K had anticoagulant activity that was somewhat attenuated compared with a previously reported analogue.

Conclusion—These results suggest that Xai-K may ameliorate the safety profile of therapeutic thrombolysis, either as a primary or tPA/TNK-adjunctive agent.

Addendum

E. L. G. Pryzdial designed and analyzed experiments and wrote the manuscript. S. C. Meixner, K. Talbot, L. J. Eltringham-Smith, J. R. Baylis and F. M. H. Lee designed and performed experiments. C. J. Kastrup and W. P. Sheffield designed and analyzed experiments.

Disclosure of Conflict of Interests

E. L. G. Pryzdial is the inventor on ‘C-Terminally tethered amino acids and their fibrinolytic therapeutic uses’ (patent pending) with relevance to the current work.

Introduction

The initiating branches of blood coagulation have a common biochemical role: to activate factor (F) X to FXa. This multifunctional protease is localized to the site of clot formation by procoagulant phospholipid (proPL)-binding [1]. Not until sufficient FXa production overcomes the physiological anticoagulant threshold can enough downstream thrombin accumulate to generate a clot. FXa activity is consequently a prominent drug target with broad-ranging application, including anticoagulation [2, 3], anticoagulation reversal [4], procoagulation [5, 6] and cancer suppression [7]. Recently, we reported that after proteolytic modulation, proPL-bound FXa enhances *in vitro* fibrinolysis [8, 9], suggesting an additional therapeutic application. In the current study we hypothesized that this novel FXa activity could be tailored to facilitate localized thrombolysis.

The prevailing thrombolytic therapeutic, Alteplase (rtPA) [10], is the recombinant form of tissue plasminogen activator (tPA), the physiological initiating protease of fibrinolysis. The currently favored model of fibrinolysis is largely based on therapeutic levels of tPA and emphasizes fibrin as the sole tPA cofactor necessary to surpass the inherent antifibrinolytic activity of plasma. Fibrin accelerates tPA-mediated activation of plasminogen (Pg) to plasmin, the direct clot-dissolving enzyme [11]. There are intrinsic binding sites for tPA and Pg on fibrin [12, 13]. However, additional sites are generated primarily for Pg [14] via limited plasmin-mediated proteolysis that exposes important C-terminal lysines (CTLs) [15, 16]. These latter sites prime the fibrin for enhanced cofactor function through positive feedback, inducing a burst of plasmin activity and the lytic phase of fibrinolysis. Thus, fibrin itself is pivotal within the fibrinolysis mechanism, as both a tPA cofactor and as a sentry to localize plasmin production.

There is consensus that fibrin is the ultimate fibrinolysis cofactor [17]. However, at circulating plasma levels of tPA and its predominant inhibitor, PAI-1, we have shown that the intact form of FXa, FXaα, acquires auxiliary cofactor activity by proteolysis, even at concentrations overwhelmed by fibrin [8]. Fibrinolysis experiments are usually conducted at much higher therapeutic-level tPA concentrations. Because tPA has intrinsic enzymatic activity, at these concentrations it can bypass the requirement of a cofactor to prime the fibrin for rapid plasmin production. Thus a role for auxiliary cofactors has been previously overlooked.

Purified FXa is typically a mixture of FXaα and the proteolyzed β-form (FXaα/β). The latter is produced by excision of a short C-terminal peptide by either autoproteolysis [18–20] or more rapidly by plasmin [21]. When generated in the presence of proPL and Ca2+, FXaβ or the subsequent plasmin-mediated non-covalent heterodimer [22], Xa33/13, have been shown to bind Pg (Fig. 1). FXaα/β and Xa33/13 both enhance tPA-dependent Pg activation and fibrin solubilization [21]. These earlier studies were conducted using purified proteins, and later extended to plasma, where the production of Xa33/13 antigen was detected during fibrinolysis [9]. The addition of purified FXaα/β significantly shortened the time of plasma fibrinolysis, as did the respective FXa-serpin adduct [9]. Combined, these data indicate a non-enzymatic role for FXa in the early stages of fibrinolysis that may provide novel thrombolytic potential and avoid certain shortfalls inherent to rtPA.

Despite its benefits, rtPA can lead to hemorrhage in up to 3% of patients with myocardial infarction or 7% with stroke [23]. Because the prescribed dose exceeds the physiological concentration of endogenous tPA by many orders of magnitude, its normal cofactor dependence may be circumvented and contribute to undesired systemic [24] rather than localized generation of plasmin. The ability to reduce the dose yet maintain efficacy would conceivably help to resolve this problem [25].

Re-occlusion has also been a significant challenge in thrombolytic therapy [26]. Once the clot is cleared, exposure of an underlying procoagulant surface may lead to platelet activation and thrombin generation. The standard practice is to co-administer the lytic agent with drugs that inhibit platelet activation, such as clopidogrel and aspirin. However, the outcome of this treatment is ineffective in as many as 40% of patients [27] and alternative anticoagulants are being pursued [28].

In the current study, the FXaα/β active site was chemically modified, tethering an additional CTL to combine the novel fibrinolytic [8, 9] and known anticoagulant properties [29] in a single compound (Xai-K). Unlike FXaα/β [21] or Xa33/13, Xai-K facilitated fibrinolysis of plasma at low concentrations (nM). Furthermore, Xai-K induced reperfusion as either an adjunct to otherwise sub-therapeutic levels of the tPA variant, Tenecteplase (TNK), or as a primary treatment in a murine carotid model of occlusive thrombolysis. Systemic markers of plasmin activation were consequently avoided. Cumulatively, these results suggest a novel auxiliary cofactor approach to vascular recanalization.

Materials and methods

Reagents and proteins

Tetrasodium ethylenediaminetetraacetic acid (EDTA) and sodium chloride (Fisher Scientific, Nepean, ON, Canada), calcium chloride (EMD Chemicals, Inc., Gibbstown, NJ, USA), chromogenic substrate N-a-Benzyloxycarbonyl-D-Arg-Gly-Arg-pnitroaniline-dihydrochloride (S2765, DiaPharma, West Chester, OH, USA), HEPES and PEG 8000 were obtained from Sigma-Aldrich (Oakville, ON, Canada). Small unilamelar vesicles consisting of 75% phosphatidylcholine and 25% phosphatidylserine were used as a source of proPL [30]. Na-[(acetylthio)acetyl]-D-Phe-Pro-Arg-$CHCl_2$ (ATA-FPRck) was purchased from Molecular Innovations (Novi, MI, USA). α-lysyl-([N-maleimidopropionamido]-tetraethylene glycol) ester (Lys-mTEG) was synthesized through an academic affiliation with the Centre for Drug Research and Development (Vancouver, BC, Canada). Purified Human FXaα/β, thrombin, Lys-plasminogen and plasmin were obtained from Haematologic Technologies (Essex Junction, VT, USA). rtPA and the variant Tenecteplase (TNK) were from Genentech (San Francisco, CA, USA). VisuCon-F Calibrator plasma was from Affinity Biologicals (Ancaster, ON, Canada).

Lys-mTEG preparation

To succinimidyl-([N-maleimidopropionamido]-tetra-ethyleneglycol) ester (0.058 mm, Thermo Scientific #22104) in dimethylformamide (0.2 mL) was added N-ε-tertiary-butoxycarbonyl-L-lysine (0.065 mM, Novabiochem #8.54105.0005) followed by diisopropyl

ethylamine (0.17 mM). The reaction mixture was stirred at room temperature for 2 h. Diethyl ether (3 mL) was added and the resultant suspension was centrifuged (3000 × g, 5 min) to isolate the white precipitate. To facilitate de-protection, the solid was resuspended in dichloromethane (1 mL) and trifluoroacetic acid (0.5 mL) was added. The reaction mixture was stirred at room temperature for 1 h and then concentrated. The crude product was purified by C18 preparative HPLC chromatography (Waters XBridge, Mississauga, ON, Canada) and subjected to MALDI-TOF mass spectrometry for identification and quality assurance.

Xai-K preparation

FXaα/β (46 μM) was combined with ATA-FPRck (350 μM) in 5 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer containing 0.3 M NaCl and 1 mM EDTA, pH 6 [31]. The resulting FXaα/β-FPR-ATA was assayed with substrate S2765 [9] to ensure > 99.8% inhibition. Unreacted ATA-FPRck was removed by buffer exchange into 10 mM HEPES, 300 mM NaCl, EDTA (1 mM), pH 7.0 (HEPES-EDTA), by Sephadex G25 chromatography. FXaα/β-FPR-ATA (14 μM), Lys-mTEG (70 μM) and NH_2OH (0.1 M) were then combined in HEPES-EDTA for 1 h with mixing, then dialyzed against phosphate buffered saline (PBS; 10.1 mM, Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.2).

Plasma fibrinolysis

Fibrinolysis was followed by turbidity at 37 °C, as described previously [8], where plasma clot formation was initiated by thrombin (3 nM) and $CaCl_2$ (20 mM) in the presence of PenStrep (1%) and tPA (25 pM; diluted in 0.1% Tween 20). To derive the time required to achieve 50% lysis, the kinetic data were fit to an inverse sigmoidal curve using GRAPHPAD PRISM 4 software.

Thromboelastography was conducted using a TEG® 5000 Hemostasis Analyzer at 37 °C. Briefly, 330 μL of citrated normal pooled plasma was added to TEG cups containing a 30-μL mixture, giving final concentrations of $CaCl_2$ (10 mM), tPA (0.7 nM) and Xai-K (titrated). Clotting was initiated with 3 nM thrombin and clot integrity was followed for the machine maximum of 180 min. TEG traces were differentiated to determine the maximal slope during the lytic phase at each Xai-K concentration using GRAPHPAD PRISM 4 software.

Chromogenic assay

At room temperature, the conversion of purified Lys-plasminogen (0.5 μM) to plasmin by tPA or urokinase was followed using the chromogenic substrate S2251 (Chromogenix-Diapharma, West Chester, OH, USA), as previously described [8].

Immunoblots and ^{125}I-plasminogen ligand blots

Following sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed with anti-FX monoclonal antibody (Green Mountain, Burlington, VT, USA) [8, 32] or ^{125}I-labelled Lys-Pg (^{125}I-Pg), as previously described.

Plasma clotting assays

Clot formation in normal human pooled plasma in the presence or absence of Xai-K or FXa treated with dansyl-Glu-Gly-Arg-chloromethylketone (Xai-DEGR; prepared as previously described [8]) was measured by manual tilt assays at 37 °C. Activated partial thomboplastin time (APTT) reagent was incubated with normal human pooled plasma for 5 min and clotting was initiated by the addition of $CaCl_2$ (8 mM). Prothrombin time (PT) assays were conducted by initiating clotting with a source of recombinant tissue factor (Innovin; Siemens, Oakville, Ontario, Canada). Reagent dilutions and concentrations were followed according to those of an automated Diagnostica Stago STart analyzer protocol, as previously described [33].

Liver laceration hemostasis model

All procedures involving animals were approved by the University of British Columbia or McMaster University Animal Care Committee and performed in accordance with the guidelines established by the Canadian Council on Animal Care. As previously described [34], 6-week-old female CD-1 mice (Charles River, Montreal, Canada) were anesthetized by inhalation of vaporized isofluorane and the liver was accessed through a 3-cm transverse incision. Xai-K or TNK were injected via the tail vein. A 2 mm × 2 mm incision was made in the liver and blood loss after 10 min was quantified by weight absorbed onto filter paper.

Tail clip hemostasis model

CD1 mice were anesthetized with vaporized isofluorane and weighed to calculate dose of experimental drug. The mouse was then placed supine on a heating pad and isofluorane was maintained at 1.5% through a nose cone at a rate of 1 L O2 min^{-1}. Xai-K or TNK was injected into the tail vein and allowed to circulate for 5 min. A 4-mm section of tail was cut from the tip using a scalpel blade. The tail was immediately immersed in 10 mL 0.9% saline at 37 °C in a 15-mL centrifuge tube and blood was collected for 15 min. Blood loss was quantified by lysing the cells with 150 mM NH_4Cl, 1 mM $KHCO_3$ and 0.1 mM EDTA and measuring OD_{405}.

Carotid thrombosis ultrasound

As previously described [35], the jugular vein of a CD1 mouse was surgically exposed and cannulated, while a 0.5-mm Transonic Doppler ultrasound flow probe was fixed to the exposed carotid artery (ADInstruments, Colorado Springs, CO, USA). Data were acquired with a Powerlab system and LABCHART SOFTWARE (ADInstruments). TNK, Xai-K or a combination of both was injected via the jugular vein after 5 min of complete occlusion. The time to restoration of flow was defined when re-occlusion was absent for at least 60 s. The experimental endpoint was 1 h. As some carotids underwent unstable reperfusion during this period, the data were also reported as relative Doppler blood flow to obtain statistics. The area of the Doppler ultrasound curve was determined (volts/s). The total blood flow varied between mice prior to occlusion and was relativized by using the difference between pre- and post-occlusion blood flow voltages. In less than 10% of mice, the blood flow upon reperfusion shifted slightly compared with the pre-occlusion value, which was not accounted for in the analyses.

Results

Xa33/13 is inactivated in plasma

Our previous studies showed that Xa33/13 enhanced dissolution of purified fibrin [8]. Here we evaluated its fibrinolytic effect on clots generated in plasma. Relative ^{125}I-Pg-binding to Xa33/13 is many fold greater than to FXaα/β [21]. Because the latter is known to accelerate fibrinolysis of plasma-derived clots [9], it was unexpected that purified Xa33/13 did not significantly enhance fibrinolysis in plasma, even at greatly supra-physiological concentrations (Fig. 2A). To explain the different effect of Xa33/13 on fibrinolysis of plasma-derived vs. purified clots, Fig. 2(B) shows that both Xa33 antigen and ^{125}I-Pg-binding are substantially reduced from the time of clot initiation (T_o) to ~50% solubilized ($T_{½}$) in plasma. In contrast, Xa33 antigen and ^{125}I-Pg-binding remained nearly constant by $T_{½}$ in a purified fibrin dissolution experiment (Fig. 2B). The appearance of a trace antigenic band at ~28KDa during the lytic phase was comparable to a previously shown plasmin-mediated fragment [8, 36], that correlated with lost Xa33/13 fibrinolytic activity in a purified system [8]. All ^{125}I-Pg binding was inhibited in the presence of ε-aminocaproic acid (10 mM) as before [9, 21], indicative of CTL-specific binding (not shown).

Chemical modification of FXaα/β blocks Xa33/13 production

To stabilize the fibrinolytic activity of FXaα/β in plasma, a modification was introduced that enabled conversion to FXaβ but prevented formation of Xa33/13, which is rapidly inhibited. Although previous amino acid sequencing correlated the conversion of FXaβ to Xa33/13 with the exposure of a single new N-terminus at Lys330 [21], successive proteolysis may also take place at nearby basic residues, Lys317 and Arg326. To avoid the probable need for multi-site mutagenesis, we speculated that cleavage by plasmin could be controlled chemically. Covalent occupancy of the FXaα/β active site by a fluorescent tripeptidyl chloromethyl ketone has been shown to allow cleavage of FXaα to FXaβ by plasmin, while minimizing the generation of Xa33/13 [8]. In the current study, the fluorophore was substituted with a more biocompatible alternative, tetraethylene glycol, measuring ~25 Å. This homogeneous polymer was selected for its orbital radius to provide appropriate steric protection from proteolysis without impeding the proPL binding site within the γ-carboxyglutamic acid rich (Gla)-domain, which is ~70 Å from the active site [37]. The interaction of FXaα/β with proPL has been shown to alter the cleavage sites to produce a form of FXaβ that binds Pg [21] and is considered important for localizing the potential fibrinolytic effect of the stabilized FXaβ *in vivo*. A CTL was tethered to the tetraethylene glycol, to augment the anticipated intrinsic fibrinolytic activity. This active site-inhibited version of FXaα/β is referred to as Xai-K (after the single letter abbreviation for lys) and is depicted in Fig. 3 as also having an endogenous CTL that would originate from FXaβ.

Over a 15-min digestion in the presence of $CaCl_2$ (Fig. 4), the Coomassie Blue protein stain of PVDF showed complete conversion of Xai-Kα to Xai-Kβ. No further proteolysis was detectable. This differed from unmodified FXaα/β, which was rapidly converted by plasmin from FXaα to FXaβ and then to Xa33/13 [21]. These PVDF protein profiles were first subjected to ^{125}I-Pg ligand blotting. Although < 0.2% of the original FXaα/β chromogenic activity (S2765, not shown) remained after modification to Xai-K, demonstrating

stoichiometric active site modification with the addition of a respective CTL, ^{125}I-Pg binding to Xai-Kα was not detected by this technique for unknown reasons. Unlike Xai-K, after treatment of unmodified FXaα/β with plasmin, the predominant ^{125}I-Pg-binding species was Xa33/13, as previously described [21]. Because of the chemical modification, the bands corresponding to the α/β-forms of Xai-K migrated with higher apparent molecular weight than unlabeled FXaα/β (not shown). Thus, Xai-K is converted by plasmin to a stable Xai-Kβ form.

The sites on FXaα/β cleaved by plasmin are known to be altered by the Ca^{2+}-dependent interaction with proPL [21]. Rather than Xa33/13, a 40 kDa species is rapidly generated by omitting either $CaCl_2$ or proPL from the proteolysis mixture. Interestingly, Xai-K is similarly cleaved, and neither Xai-K40 nor Xa40 interact with ^{125}I-Pg (Fig. 4, no $CaCl_2$). Thus Xai-K requires Ca^{2+} and proPL for persistent ^{125}I-Pg-binding in the presence of plasmin.

Xai-K accelerates fibrinolysis in plasma and is tPA dependent

Blocking the proteolytic step leading to Xa33/13 production enabled the fibrinolytic function of FXaα/β in plasma. Figure 5(A) shows that 10 nM Xai-K shortened the $T_{½}$ of plasma clot dissolution by ~8.5-fold and was dose dependent. At a concentration of Xai-K as low as 0.1 nM, plasma fibrinolysis was still enhanced by ~5-fold. The inset shows the difference between plasma clot dissolution profiles in the presence and absence of Xai-K. Confirming that chemical modification was required to enhance lysis by low concentrations of FXaα/β, at 10 nM the unmodified protein had no effect on the rate of fibrinolysis. Fibrinolysis was also not affected by identically modified factor VIIa or trypsin at 10 nM, demonstrating protein specificity. A similar fibrinolysis profile was observed as a function of Xai-K concentration when monitored by thromboelastography (Fig. 5B). When supplemental tPA was omitted from either experiment, Xai-K had no effect (not shown). Confirming the specificity of Xai-K for tPA, conversion of purified Pg to plasmin was followed in a chromogenic assay (Fig. 5C). Here, Xai-K enhanced the rate of plasmin formation by tPA, but not urokinase-type plasminogen activator (uPA).

Xai-K has anticoagulant properties

Xai-DEGR and other active-site blocked versions of FXa are known to have anticoagulant activity through competition against FXa/FVa/proPL complex assembly (i.e. prothrombinase). Shown in Fig. 6(A) is the effect of Xai-K on PT (panel A) and APTT (panel B) compared with Xai-DEGR. As predicted, Xai-K prolonged both PT and APTT. However, at the same level of Xai-DEGR (concentration confirmed in Fig. 6C), the anticoagulant effect of Xai-K was less, suggesting that the differing chemical modification affected interactions with other coagulation factors. The anticoagulant properties of Xai-K were furthermore evaluated in animal models of hemostasis. As anticipated, Xai-K clearly enhanced blood loss in tail vein transection (Fig. 6D) and exhibited a trend towards enhanced blood loss in liver laceration (Fig. 6E) models. Although TNK is known to cause bleeding as a result of systemic effects on fibrinolysis, in these models it had an insignificant effect.

Xai-K enhances thrombolysis as an adjunct or primary treatment *in vivo*

To assess the efficacy of Xai-K as a thrombolytic agent, a $FeCl_3$-induced murine model of occlusive carotid thrombosis was used [35]. The tPA variant TNK was injected through the cannulated jugular vein and the time to stable reperfusion was followed by Doppler ultrasound. Figure 7(A) shows that at the minimal dose of TNK found to consistently achieve complete reperfusion in ~30 min post thrombus induction (17 μg g^{-1}), adjunctive treatment with 0.5 μg g^{-1} Xai-K significantly reduced the reperfusion time by ~50% (28 ± 13 vs. 14 ± 7 min). This implied that the TNK concentration could be decreased considerably assuming a 1 : 1 stoichiometry, because a molar ratio was selected to favor Xai-K saturation at ~24-fold excess of TNK. The TNK dose was consequently decreased to a sub-therapeutic level (9 μg g^{-1}), which alone did not restore patency by the 60-min experimental endpoint. Supplementing 9 μg g^{-1} TNK with 0.5 μg g^{-1} adjunctive Xai-K (molar ratio ~13 : 1, TNK : Xai-K) restored stable blood flow without significantly changing the reperfusion time (17 ± 8 min) compared with the higher TNK dose. The molar ratio was then dropped to ~1:0.7 (0.5 μg g^{-1} TNK). Although several results were considered unstable with re-occlusion by 60 min, most thrombi were completely cleared at this very low TNK dose.

The question of whether Xai-K may facilitate reperfusion independent of added TNK was next addressed. Figure 7(A) shows that at the level of Xai-K (0.5 μg g^{-1}) used for adjunctive treatment, blood flow was stably restored in 2/8 carotids and an additional 4/8 showed evidence of thrombolysis with unstable reperfusion. Doubling the Xai-K (1.0 μg g^{-1}) furthered this trend; of 10 examples, no thrombi were completely resistant to treatment and six achieved stable reperfusion. A further doubling of the Xai-K (2.0 μg g^{-1}) achieved patency in all mice tested at ~50% of the time that was required for therapeutic TNK (17 μg g^{-1}).

To perform statistical analysis inclusive of data that resulted in unstable reperfusion or no reperfusion by the 60-min endpoint (Fig. 7A), total blood flow was quantified (Fig. 7B). Using this method, it is interesting to note that differences were insignificant between TNK used as a primary therapeutic (17 μg g^{-1})and either low-dose TNK + Xai-K adjunctive treatment or Xai-K as a primary therapeutic at either of the two lower doses used (0.5 or 1.0 μg g^{-1}). This method of analysis clearly supports the higher level of Xai-K alone (2.0 μg g^{-1}) as superior to the therapeutic dose of TNK alone (17 μg g^{-1}).

Xai-K inhibits systemic fibrinogen degradation

A potential side-effect of current thrombolytic agents is hemorrhage involving systemic fibrinogen degradation and plasmin activation. Immediately after thrombolysis, plasma was obtained by cardiac puncture and the fibrinogen (Fig. 8A) and Pg (Fig. 8B) fragmentation profiles of five mice for each treatment were compared. When saline was used instead of thrombolysis treatment, several fibrinogen fragments (~17–35 KDa) were noted and no detectable plasmin. In the presence of the high-dose TNK (17 μg g^{-1}), additional fibrinogen fragments were observed and plasmin generation was found, consistent with systemic fibrinolysis activation. When Xai-K was added as an adjunct to this level of TNK, even more degradation of fibrinogen and plasmin formation was noted. These were decreased as the

dose of TNK was reduced. In the absence of TNK, the highest level of Xai-K (2.0 μg g^{-1}), which had the fastest reperfusion rate of all treatments that were evaluated, showed no markers of systemic fibrinolysis activation.

Discussion

Earlier *in vitro* experiments showed that FXaβ and its proteolyzed derivative, Xa33/13, are auxiliary tPA cofactors, but not the intact precursor FXaα [8, 20]. Interestingly, the current study demonstrates an as yet unidentified mechanism in plasma that rapidly degrades Xa33/13 antigen and its related Pg-binding capacity. Thus, Xai-K was developed as a novel version of FXaα/β that is completely converted to FXaβ, but does not subsequently transition from FXaβ to Xa33/13. This chemically stabilized agent for the first time demonstrates that an auxiliary tPA cofactor in the nm range can markedly reduce the plasma clot dissolution time by nearly an order of magnitude *in vitro*. This is counterintuitive because the exceptionally high concentration of fibrin, also a tPA cofactor, should preclude the relevance of other potential cofactor proteins in the clot vicinity. A probable explanation is that plasmin cleaves and feedback-modulates FXaα much faster than fibrin [8], enabling comparatively rapid acquisition of enhanced tPA cofactor function. These data suggest a model where prior to fibrin acquiring CTLs, transient plasma cofactors auxiliary to fibrin may contribute to early plasmin generation. Other auxiliary cofactors have been reported [36, 38–41], but are difficult to individually verify because of redundancy in the complex plasma milieu.

Here we have utilized the low intrinsic tPA concentration and assessed the fibrinolytic cofactor function of Xai-K as a primary therapeutic *in vivo*. Xai-K was observed to restore blood flow in a murine carotid thrombosis occlusion model. The plasmin-mediated exposure of Pg binding sites on Xai-K *in vitro* was only found under conditions that facilitated Ca^{2+}-dependent association with proPL. Therefore, localized binding of Xai-K to proPL and presumably localized secretion of tPA by endothelial cells [42] probably contribute to the negligible induction of systemic plasmin generation markers during the *in vivo* experiments. This was in sharp contrast to the administration of TNK as a primary thrombolytic agent, which resulted in systemic fibrin(ogen) fragment and plasmin antigen appearance. The highest dose of Xai-K evaluated (2.0 μg g^{-1}) was not only superior to therapeutic TNK alone (17 μg g^{-1}) in terms of minimizing systemic fibrinolytic markers, but reperfusion was achieved in ~12 vs. ~26 min. These results suggest that Xai-K is an alternate thrombolytic agent that may have a lower hemorrhagic bleeding frequency than current treatments.

In addition to application as a primary thrombolytic agent, Xai-K was evaluated as an adjunct to TNK. When administered with a therapeutic dose of TNK, reperfusion was greatly enhanced. To improve the safety profile of current thrombolysis, TNK could be decreased to a sub-therapeutic dose in the presence of Xai-K without affecting the reperfusion rate. Although optimization of the relative TNK:Xai-K dose has not yet been conducted, these studies show that TNK concentration could be reduced by ~97% with all occluded carotids responding to treatment, although several displayed unstable reperfusion. This adjunctive strategy may have an advantage in treating resistant clots while reducing the hemorrhagic risk of tPA/TNK in some patients.

Although a high healthcare priority, only modest advances have been made over the past several decades toward improving the safety and efficacy of clinical thrombolysis [43, 44]. Of note, a cofactor approach has recently been reported that is based on the S100A10-annexin A2 heterodimeric protein complex, which accelerates tPA [45, 46]. In a rat stroke model, annexin A2 reduced the tPA required for thrombolysis by 4-fold [47]. In the current work we introduce Xai-K as a unique cofactor-based thrombolytic agent, which also has an anticoagulant function. Although the latter effect was designed to avoid re-occlusion, Xai-K was shown here to not only decrease the required dose of TNK by ~34-fold with diminished systemic effects, but also exhibited promise as a primary agent for therapeutic thrombolysis.

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Essentials

- Factor Xa (FXa) acquires cleavage-mediated tissue plasminogen activator (tPA) cofactor activity.
- Recombinant (r) tPA is the predominant thrombolytic drug, but it may cause systemic side effects.
- Chemically modified, non-enzymatic FXa was produced (Xai-K), which rapidly lysed thrombi in mice.
- Unlike rtPA, Xai-K had no systemic fibrinolysis activation markers, indicating improved safety.

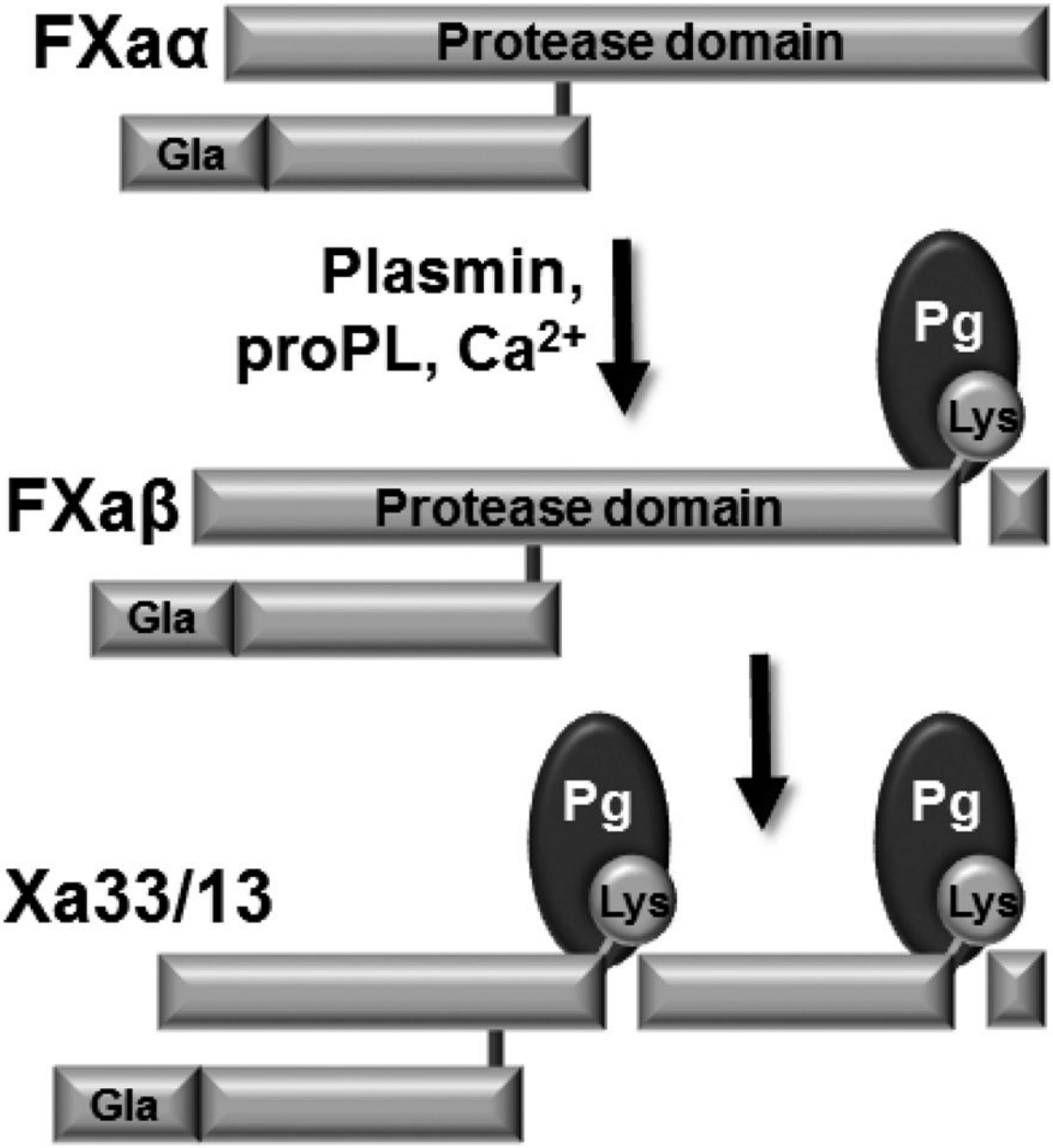

Figure 1.

Fragmentation of FXa. When bound to a proPL-containing membrane in the presence of Ca^{2+}, intact FXaα is consecutively converted to FXaβ and Xa33/13 by plasmin cleavage or more slowly by autoproteolysis. Proteolytic exposure of C-terminal lysine (indicated; Lys) facilitates plasminogen-binding and is known to correlate to enhanced tPA-mediated fibrinolytic activity. The protease-domain-containing heavy chain and γ-carboxyglutamic acid (Gla) domain-containing light chain are shown connected by a single disulfide bond.

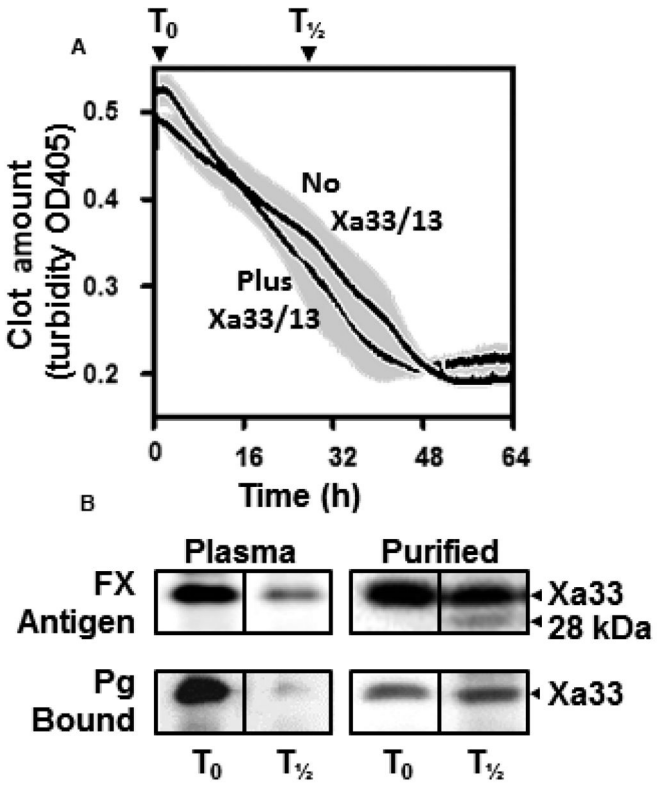

Figure 2.

Xa33/13 is stable during dissolution of a purified clot, but not a plasma clot. Purified Xa33/13 (0.1 μM) was added to fibrinolysis assays using light scattering at 405 nm to follow dissolution of clots formed from either purified proteins [8] or plasma [9] (purified, 1 ρM tPA; plasma, 35 ρM tPA). (A) Turbidity profiles of plasma lysis are shown. Solid line, averaged data ± SD indicated by grey shade ($n = 3$). (B) Identical samples were prepared in parallel and stopped with denaturing Laemmle sample buffer under non-reducing conditions just after clot formation (T_o) and at ~50% lysis ($T_{½}$). The samples were analyzed by immunoblotting using a FX mAb that recognizes FXa33 antigen [9] and by ligand blot using ^{125}I-Pg. The Xa33 band is shown. Consistent loading in each lane was ensured (not shown): for plasma, Coomassie blue R250 staining of the PVDF revealed the same amount of total protein in each lane; for purified, the total antigen recognized by the FX antibody did not vary over the experimental duration.

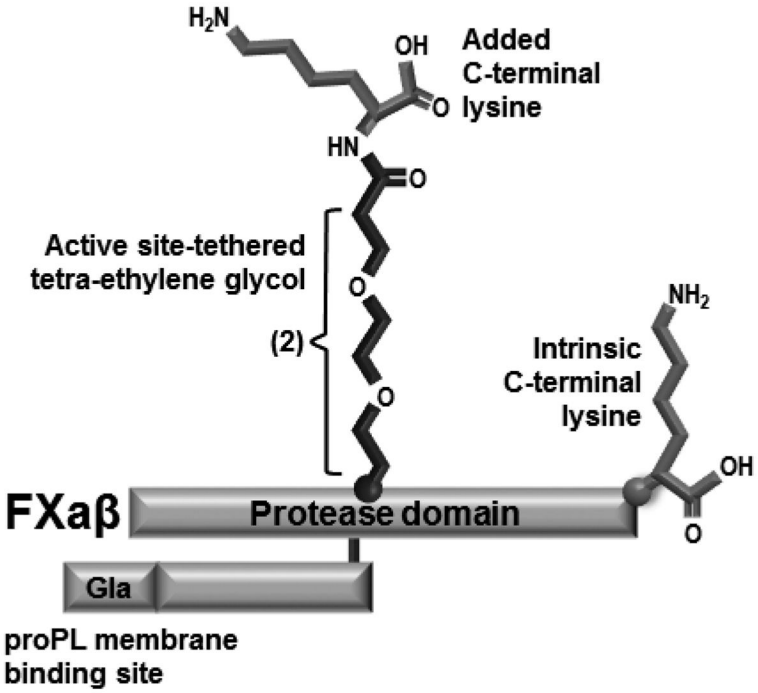

Figure 3.

Xai-K: chemically stabilized FXaβ. To form Xai-K, a C-terminal lysine linked to a tetraethylene glycol spacer is tethered to FXaα/β. Attachment is facilitated by stoichiometric modification of the active site His using chloromethyl ketone chemistry. Consequently, the coagulation enzyme properties are inhibited. Xai-K is resistant to conversion to Xa33/13 by plasmin and forms a stable FXaβ-like species including exposure of an intrinsic peptidyl C-terminal lysine.

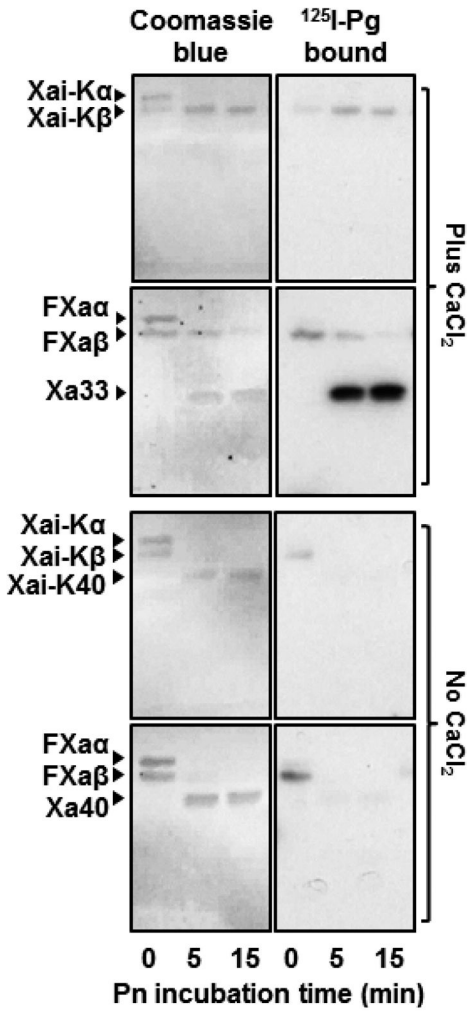

Figure 4.

Xai-K is protected from conversion to Xai-K33/13. Purified FXaα/β or Xai-K (5 μM) was treated with plasmin (0.2 μM) for the indicated times, in the presence of 5 mM $CaCl_2$ and 50 μM proPL for 20 min at 22 °C. Samples were subject to SDS-PAGE under non-reducing conditions and transferred to PVDF to follow ^{125}I-Pg-binding by ligand blot. The PVDF was subsequently stained for protein with Coomassie Blue R250.

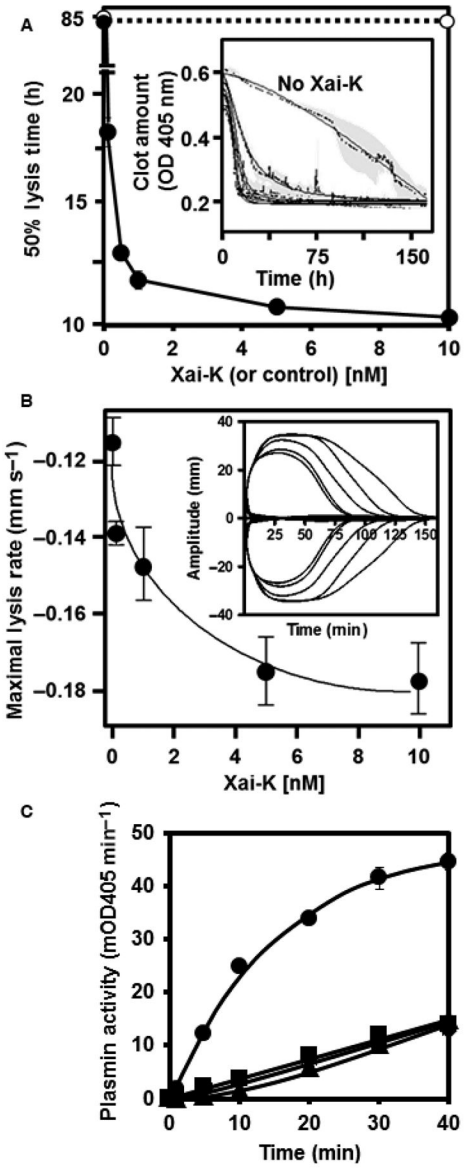

Figure 5.

Xai-K enhances fibrinolysis dependent on tPA. (A) Normal citrated plasma (VisuCon-F calibrator plasma) was supplemented with 35 pM tPA and clotting was induced with 20 mM Ca^{2+} and 3 nM thrombin. Clot amount was followed by turbidity at 405 nm and the time to 50% lysis was determined. Inset: turbidity profiles of the Xai-K titration. Dotted line shows averaged data (±SD indicated by grey shade) ($n = 3$); the solid line is an inverse sigmoidal fit. (B) Clotting of normal plasma was initiated as in (A) and at 0.7 nM tPA. Clot amount was followed by thromboelastography and the maximal slope during the fibrinolysis phase was plotted. Inset: average TEG traces at concentrations of Xai-K from right to left: 0, 0.5, 1.0, 5.0 and 10.0 nM. $n = 3 \pm$ SD. (c) Plasmin generation by tPA (1 nM; ●, ▲) or uPA (0.2 nM; ■) in the presence (●, ■) or absence of 10 nM Xai-K (▲, ◆). tPA or uPA was required for S2251 color formation (not shown). $n = 3 \pm$ SD.

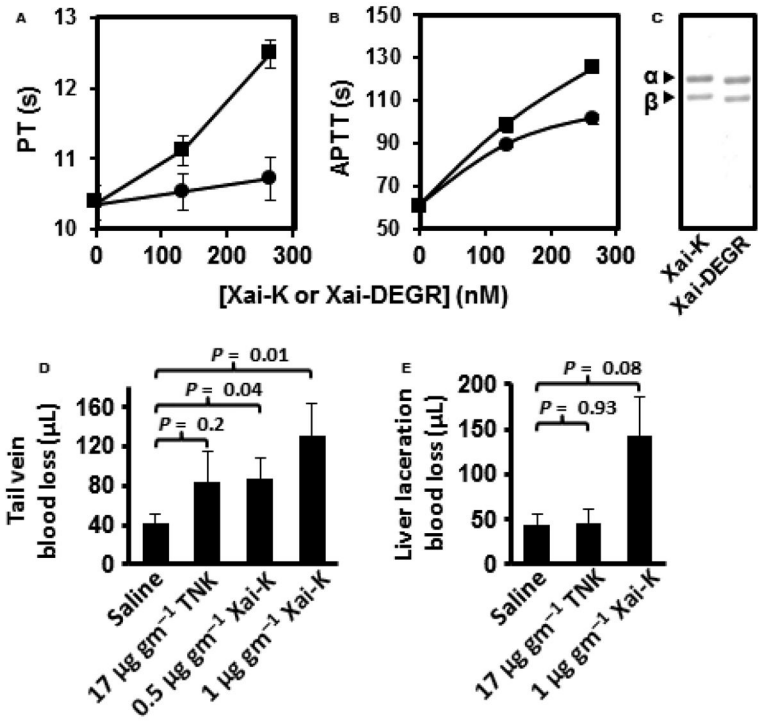

Figure 6.

Xai-K has anticoagulant activity. (A) PT was measured after combining an equal volume of Innovin (recombinant tissue factor) and pooled plasma in the presence or absence of Xai-K (■) or Xai-DEGR (●). ($n = 3$; ± SD). (B) APTT was determined after an equal volume of APTT reagent was preincubated for 5 min with normal pooled plasma prior to the addition of Xai-K (■) or Xai-DEGR (●) and Ca^{2+} (15 mM final). ($n = 3$; ± SD). (C) Xai-K and Xai-DEGR (1 µg) were run side-by-side on non-reducing SDS-PAGE (10% acrylamide) to confirm the protein concentration determined by BCA assay; 'α' and 'β' correspond to the respective forms of FXa. (D) Tail vein amputation ($n = 6$–10) and (E) liver laceration ($n = 6$) models were used to evaluate the effects of TNK and Xai-K on hemostasis recovery. Note: 1.0 µg g^{-1} corresponds to ~266 nM Xai-K.

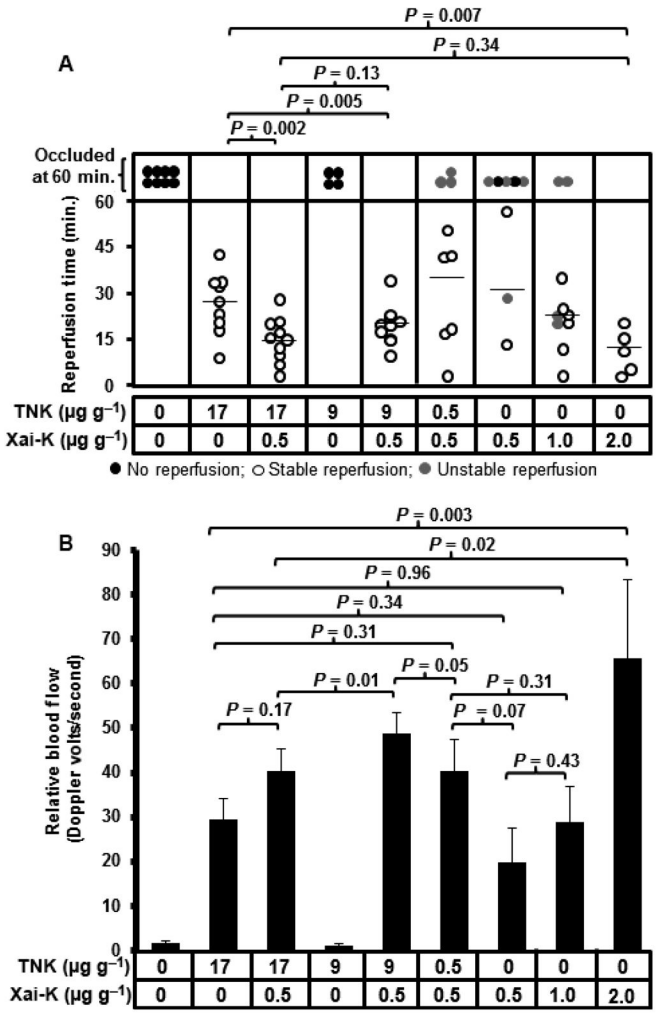

Figure 7.

Xai-K enhances reperfusion in a carotid occlusion model. (A) Thrombi were induced in exposed carotid arteries of CD1 mice by FeCl3 and blood flow was measured by Doppler ultrasound. TNK and Xai-K were injected adjunctively or alone through the jugular vein. For each mouse, the time to reach stable (open) or no reperfusion (solid) by the 60-min experimental end-point is indicated. In some cases reperfusion was ‘unstable’ (grey). Bars indicate average for times when stable reperfusion occurred. Student’s *t*-test was performed when all experiments achieved stable reperfusion. (B) The data in panel A were analyzed to obtain total blood flow over the duration of the experiment to include the examples of unstable reperfusion and lack of reperfusion in the statistical analyses.

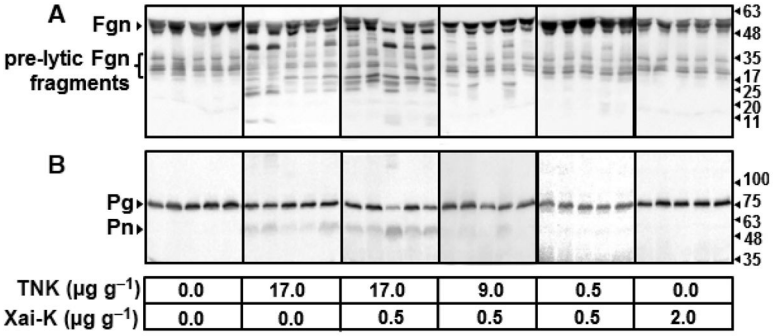

Figure 8.

Xai-K does not induce systemic effects like TNK. At the close of carotid occlusion experiments (Fig. 7) citrated plasma was prepared from blood obtained by cardiac puncture. After plasma was subjected to SDS-PAGE under reducing conditions and transferred to PVDF, immunoblotting was conducted for (A) fibrinogen or (B) plasminogen using polyclonal Abs on samples from five different mice treated as indicated. Pg, plasminogen; Pn, plasmin; Fgn, fibrinogen