Procoagulant Activity of Reversibly Acylated Human Factor Xa

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The plasma clotting factors used to treat hemophiliacs who have developed inhibitory antibodies have a shared history of limited clinical safety and utility. To improve on existing bypass factors, we have developed a reversibly acylated form of human plasma factor Xa capable of providing a time-dependent release of procoagulant activity. Factor Xa was treated with p-amidinophenyl p'-anisate to generate anisoyl Xa. The chemical modification of the protein involves acylation of the active site serine residue of factor Xa. Anisoyl Xa deacylated in a time, pH, and temperature-dependent manner. Active factor Xa generated on deacylation of anisoyl Xa exhibited amidolytic and prothrombinase complex activities in vitro assays, the level being comparable to those of untreated factor Xa. When Anisoyl Xa was infused into rabbits, active factor Xa was generated on deacylation of the acylated enzyme, which shortened the activated partial thromboplastin time (APTT) in a dose-dependent manner. The duration of effect on rabbit APTT could be directly correlated to the level of human plasma factor Xa. Because anisoyl Xa bypasses the “tenase” complex that is compromised in hemophilia A and B and is unaffected by inhibitory antibodies, it has the potential to be used as an effective bypass therapy.

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Hemophilia A and B are two common disorders of the blood coagulation pathway. The deficiencies are linked to the absence of functional cofactor VIII (hemophilia A) and factor IX (hemophilia B), both proteins being important in the intrinsic pathway of coagulation. Factors VIIIa and IXa form a calcium-dependent complex on phospholipid surfaces to catalyze the conversion of factor X to factor Xa: the deficiency of either factor VIII or factor IX results in defective clot formation. Current treatment of hemophilia A consists of supplementation of plasma-derived or recombinant factor VIII to alleviate the severity of bleeding episodes. Factor VIII inhibitors develop in approximately 10% to 15% of people with severe hemophilia A, while the percentage of patients with inhibitors to factor IX is 1% to 2% in hemophilia B. Recent studies raise the concern that the use of recombinant factor VIII may be associated with rapid development and higher levels of inhibitors in previously untreated patients. Although much is known about the nature of the inhibitory antibodies, treatment options are restricted to the infusion of bypass clotting factors, which have proven to be of limited efficacy and safety. Activated concentrates such as Autoplex (Hyland) or Feiba (Immuno) are designed to be used specifically for patients with factor VIII inhibitors. However, no reliable in vitro method exists for predicting in vivo efficacy of these agents. Porcine factor VIII concentrate is considered a last-resort therapy due to cost and the possibility of anamnestic response during subsequent treatment. Thus, the management of inhibitor patients continues to pose difficult therapeutic dilemmas. Because of the limitations of existing therapies, alternate strategies have been sought to restore normal coagulation either in hemophiliacs deficient in factors VIII or IX or in hemophiliacs resistant to the factors due to development of neutralizing antibodies. The idea of bypassing intrinsic and extrinsic tenase complexes by using factor Xa has already been developed by Giles et al. Infusion of factor Xa in combination with phospholipid vesicles has been shown to correct the bleeding diathesis of hemophilic dogs deficient in factor VIII. The procoagulant mixture of factor Xa and phospholipid vesicles bypasses the normal role of factor VIII in the generation of stimuli that promote the formation of a sustainable hemostatic plug. The use of this approach for the management of human hemophilia has not yet been reported.

The catalytic mechanism of serine proteases has provided strategies for the temporal release of active protease from chemically modified protein. The active site serine residue can be acylated by specific groups with slow deacylation rates. Stable acyl-derivatives of human tissue plasminogen activator have been reported and the concept of reversible deacylation is currently used in thrombolytic therapy. Acylated plasminogen-streptokinase activator complex (APSAC, anistreplase) undergoes a controlled deacylation of the catalytic center on administration resulting in sustained fibrinolytic activity. We have extended this concept to another serine protease of clinical applicability, namely factor Xa. This article reports the development of a unique form of reversibly acylated plasma factor Xa. The novel proenzyme form of factor Xa is produced on inactivation of the catalytic site serine residue of the proteolytic enzyme. Under physiological conditions, the enzyme undergoes activation to the functional form responsible for generation of thrombin, thereby bypassing the tenase complexes (IXa/VIIIa) affected by inhibitory antibodies.

MATERIALS AND METHODS

Human factors Xa, Va, and prothrombin were obtained from Haematologic Technologies (Essex Junction, VT) and Enzyme Research Laboratories (South Bend, IN). Amidolytic activity of factor Xa and thrombin were quantitated by measuring the extent of cleavage of Chromozym X (N-methoxycarbonyl-D-norleucylglycylargyl-4-nitranilide acetate, Boehringer Mannheim, Indianapolis, IN) and Chromozym TH (tosylglycylprolylarginyl-4-nitranilide acetate), respectively. Preparation of phospholipid vesicles and prothrombinase assays were carried out as described. Specific details are described in the figure legends.

Acylation of factor Xa. The reagent, p-amidinophenyl p'-anisate, was prepared as a stock solution (0.1 mol/L) in dimethyl sulfoxide. The standard protocol of modification involved treatment of human factor Xa with a 3- to 10-fold molar excess of anisate reagent at room temperature. The reaction was allowed to proceed for 10 to 30 minutes and monitored for loss of amidolytic activity over the time course. Acylation was terminated by gel filtration chromatography on Sephadex G-25 (PD 10; Pharmacia, Uppsala, Sweden) in a
pH 5.0 buffer (25 mmol/L MES NaOH, 0.15 mol/L NaCl). The protein was stored at −70°C before use. For comparison purposes, acylation of human factor Xa was also performed by p-nitrophenyl p'-guanidinobenzoate HCl (NPGB).12

Acylation of human factor Xa. The catalytic site serine residue of human factor Xa can be reversibly acylated by reacting the protein with an excess of p-amidinophenyl p'-anisate (Fig 1). Acylation results in the loss of catalytic activity of human factor Xa and can be monitored by the loss of amidolytic activity. As shown in Fig 2, when factor Xa is treated with a threefold excess of p-amidinophenyl p'-anisate, only 0.2% residual activity remains after a 3-minute incubation. NPGB has been used for acylation of active site serine residues in several serine proteases.12 The kinetics of acylation of human factor Xa using NPGB is comparable to that of p-amidinophenyl p'-anisate. However, the percent residual activity remaining is 2.5 to 6-fold higher even after a 20-minute incubation using tenfold molar excess of reagent. For labeling purposes, acylation of factor Xa was performed by a modified protocol involving an initial labelling using p-amidinophenyl p'-anisate followed by the addition of unlabelled reagent to complete the inactivation. Deacylation was monitored both by the loss of amidolytic activity and by the recovery of amidolytic activity as a measure of active enzyme (Fig 3). The rates of deacylation as determined by the two methods were in agreement (46% active by amidolytic activity and 49% active by 3H counts at the end of 4 hours of incubation).

We have observed comparable amidolytic, prothrombinase complex and plasma clotting activity on assaying a particular batch of deacylated anisoyl Xa. For a given batch of acyl Xa, the recovered factor Xa activity on deacylation at pH 7.5 at room temperature for 1 hour was 5.5%, 5.5%, and 4.2% respectively. A variation in activity of unmodified human factor Xa contributed to variation in acyl Xa activity.
However, the possibility that certain batches of anisoyl Xa may be more readily deacylated than others cannot be ruled out.

**pH dependence of deacylation.** Rate constants for deacylation of acyl-chymotrypsin derivatives have been studied extensively. The studies show that rates of deacylation are affected by a variety of factors including the nature of acyl group, pH, and composition of incubation medium. In the absence of reported studies on acyl derivatives of factor Xa or related vitamin K-dependent proteins, the existing information on serine protease derivatives was not sufficient to predict the nature of deacylation of anisoyl Xa. Furthermore, to investigate the in vivo properties of anisoyl Xa, we needed to stabilize the acyl derivatized version of the enzyme. The deacylation reaction was largely dependent on the pH of the incubation medium (Fig 4). The recovery of catalytic activity was slow at pH between 4 and 6 and progressively faster at higher pH up to 8 (the highest pH studied). At the physiologic pH of 7.4, deacylation proceeded faster at 37°C than at room temperature (data not shown). Preparations of anisoyl Xa are stored frozen at pH 5 before use. The relative stability of anisoyl Xa at pH 5 at room temperature has allowed us to perform in vivo experiments without the threat of thrombotic complications due to a prior deacylated factor Xa.

**Deacylation in the presence and absence of cofactor Va.** The catalytic moiety responsible for thrombin generation is the prothrombinase complex and not factor Xa alone. Chloromethyl ketone inactivated factor Xa is capable of assembly into the prothrombinase complex so we investigated if anisoyl Xa before deacylation is capable of associating with factor Va. The inhibition of activation of prothrombinase in a plasma free system was performed by an adaptation of a previously described procedure. Different concentrations of glutamylglycylarginyl chloromethyl ketone inactivated human factor Xa (EGR Xa) or anisoyl Xa were preincubated with factor Xa (0.5 nmol/L) and factor Va (2 nmol/L) in the presence of phospholipids and calcium. Withdrawn aliquots were assayed for cleavage of prothrombin. The standard curve of thrombin generation by an equivalent amount of untreated factor Xa after similar incubations was used for calculation of percent activity. Under these assay conditions, the concentration of anisoyl Xa (3 nmol/L) required for half maximal inhibition of prothrombinase activity is comparable to that of EGR Xa (5 nmol/L) (data not shown).

Because anisoyl Xa is capable of associating with factor Va in a manner analogous to EGR Xa, we wanted to determine if the rate of deacylation was affected by the presence of other components of the prothrombinase complex, namely phospholipid vesicles, calcium ions, and cofactor Va. As shown in Fig 5, the recovery of catalytic activity was not dependent on whether anisoyl Xa was preincubated with the other components of the membrane bound complex or free in solution.

**Anisoyl Xa as a procoagulant agent in rabbits.** Anisoyl Xa reactivates to the procoagulative form in a time-dependent manner, thus at any given time during our in vivo experimentation, a proportion of the infused protein will remain catalytically inactive. Rabbits were chosen as the species to investigate physiologic activity because of our previous experience with EGR Xa, a catalytically inactive protein. Anesthetized rabbits exhibit no ill effects to high doses (250 μg/kg bolus followed by an infusion of 4.8 μg/kg/min for 2 hours) of EGR Xa and hematologic parameters are unaffected. In the current study, anisoyl Xa was admin-

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**Fig 3.** Deacylation of anisoyl Xa on incubation in pH 7.5 buffer at 37°C. Activity recovered in amidolytic assay. Anisoyl Xa (7 μg) was incubated in buffer A (1 mL) and during the course of reactivation withdrawn aliquots (70 ng) were assayed as described in Fig 2. **(O)** 

**Fig 4.** Effect of variation of pH on deacylation of anisoyl Xa. The incubations were performed at room temperature as discussed in Materials and Methods. Anisoyl Xa (10 μg) was incubated in 1 mL of appropriate buffer, at various time points aliquots (100 ng) were assayed at pH 7.5 as described in Fig 2. pH values: (□), 6.0; (○), 6.5; (■), 7.2; (●), 7.0; (□□), 6.0; (◇), 5.0; (△), 4.0.

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istered as a bolus (200 μg/kg), and ex vivo clotting parameters were monitored in six rabbits. A quantitative ELISA detecting the human protein in rabbit plasma was used to measure circulating plasma concentrations. Plasma half-life was calculated using first order rate kinetics and determined to be approximately 40 minutes (data not shown). As deacylation of anisoyl Xa is slow relative to its plasma clearance in rabbits, we administered the protein by constant infusion. During evaluation of procoagulant effect of the agent, a dose-dependent reduction was observed in APTT measurements. All plasma clotting assay values are expressed as a ratio of parameters obtained before the administration of drug (Fig 6A). The plasma concentration (Fig 6B) showed good correlation to the clotting activity. At the doses studied, there was no statistically significant changes in hematologic parameters. These data indicate that infusion of anisoyl Xa demonstrated a procoagulative effect in rabbits and further studies in hemophilic dogs are needed to evaluate its potential in correcting deficiencies in clinically relevant animal models.

DISCUSSION

Factor VIII inhibitors are circulating antibodies that specifically neutralize factor VIII procoagulant activity. The patients include those with classic hemophilia, postpartum women, people with autoimmune disorders, and older adults. Despite the low prevalence of such disorders, clinical management is made difficult by the limited clinical safety and high cost of currently available bypass clotting factors. Proposed alternative therapeutics include recombinant factor VIIa and phospholipid encapsulated human factor Xa. Preliminary evidence of efficacy of recombinant factor VIIa...
was observed in a phase I study of 15 hemophilic patients. Optimized of the potential therapeutic benefits in a larger study is yet to be reported. As in the case of factor Xa with phospholipids, our strategy has focused on the prothrombinase complex as the likely target for bypass activity. The prothrombinase complex is the only known source of thrombin formation in the vasculature; a controlled increase in its activity should harbor a potential prohemostatic effect during a hemorrhagic episode.

We have pursued a chemically modified human plasma factor Xa to improve on conventional therapies. An easily synthesized amidinophenyl derivative was used for reversible acylation of human factor Xa. On deacylation, anisoyl Xa exhibited the expected catalytic activity in cleavage of tripeptide substrates, prothrombin, and in plasma clotting. Our studies show that the loss of p-anisoyl moiety and recovery of catalytic activity follow a parallel course. The specific activity of p-amidinophenyl-p'-(ring 3H) anisate provided as an ethanol stock precluded complete modification using radioactive reagent for stoichiometric titration. Acyl group radiolabelling has demonstrated that active site acylation of plasmin with p-amidinophenyl-p'-anisate was quantitative for one serine residue per molecule. The possibility of modification of more than one serine residue per molecule of factor Xa cannot be excluded. The capacity of anisoyl Xa to bind cofactor Va on a phospholipid membrane and the time-dependent deacylation that ensues on injection would ensure that the contribution of administered therapy can be optimized.

The procoagulant activity of anisoyl Xa in normal rabbits indicates that the desired controlled deacylation can be achieved under physiologic conditions. However, the procoagulant activity, which is deduced from an ex vivo clotting assay, is a secondary parameter dependent on synthetic reagents and phospholipid membranes as required by the in vitro test. The true test of acyl factor Xa to normalize hemostatic parameters would be dependent on both the rate of deacylation and its half-life of plasma clearance. Manipulating the rate of deacylation to produce more active factor Xa before clearance from plasma might significantly increase the therapeutic benefit. Structure-activity relationships for acylated fibrinolytic enzymes have used Hammett constants for predicting the rate of deacylation of several serine proteases. Taft constants for various acyl groups have also been used for similar predictions for aprotinin. Both strategies can be used for deducing the optimal acyl Xa with desired in vivo deacylation profile. Further studies using other acylating agents and in vivo experiments in hemophic dogs are currently under way. These should be helpful in elucidating the exact therapeutic potential of anisoyl Xa.

ACKNOWLEDGMENT

We thank Robert Scarborough, Alan Laibelman, Jerry Nzerem, Alice Gunn, and Kathy Needham for their contributions to this project.

REFERENCES


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