Protection by Recombinant $\alpha_1$-Antitrypsin Ala$^{357}$ Arg$^{358}$ against Arterial Hypotension Induced by Factor XII Fragment

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Abstract

The specificity of serpin superfamily protease inhibitors such as $\alpha_1$-antitrypsin or C1 inhibitor is determined by the amino acid residues of the inhibitor reactive center. To obtain an inhibitor that would be specific for the plasma kallikrein–kinin system enzymes, we have constructed an antitrypsin mutant having Arg at the reactive center P1 residue (position 358) and Ala at residue P2 (position 357). These modifications were made because C1 inhibitor, the major natural inhibitor of kallikrein and Factor XIIa, contains Arg at P1 and Ala at P2. In vitro, the novel inhibitor, $\alpha_1$-antitrypsin Ala$^{357}$ Arg$^{358}$, was more efficient than C1 inhibitor for inhibiting kallikrein. Furthermore, Wistar rats pretreated with $\alpha_1$-antitrypsin Ala$^{357}$ Arg$^{358}$ were partially protected from the circulatory collapse caused by the administration of $\beta$-Factor XIIa.

Introduction

The plasma kinin–forming system comprises the serine protease zymogens Factor XII and prekallikrein and the nonenzymatic cofactor high–molecular-weight kininogen (1). Upon activation, Factor XII is proteolytically converted into $\alpha$- and $\beta$-Factor XIIa (2), and prekallikrein into $\alpha$- and $\beta$-kallikrein (3). When zymogen activation takes place on a negatively charged surface, high–molecular-weight kininogen functions as a cofactor because it increases the rate of reciprocal activation of Factor XII by kallikrein and of prekallikrein by Factor XIIa (4). High–molecular-weight kininogen also serves as a substrate for $\alpha$- and $\beta$-kallikrein and for Factor XIIa (5–7). At an early stage of high–molecular-weight kininogen proteolytic cleavage, kinins such as the nonapeptide bradykinin are released (5–7). These peptides are potent inflammatory mediators that enhance vascular permeability and cause arterial vasodilation and venous constriction (8).

Septic shock and angioedema attacks are associated with activation of the plasma kinin–forming system (1). However, it is not known whether this mechanism induces the symptoms observed in these disease states or whether activation of this pathway merely represents an accompanying phenomenon. This question could be addressed by examining the influence of a specific and efficient inhibitor of Factor XIIa and kallikrein on the clinical course of these pathological conditions. Furthermore, if a favorable effect is detectable, the availability of such an agent would represent a significant step toward the development of new therapeutic strategies.

In normal plasma, $\alpha$- and $\beta$-Factor XIIa as well as kallikrein are predominantly controlled by the serine protease inhibitor C1 inhibitor (9), a molecule whose existence was first recognized by Ratnoff and Lepow (10). This inhibitor is an $\alpha_2$-glycoprotein that belongs to the serpin superfamily (11, 12), a recently identified class of related proteins that also includes $\alpha_1$-antitrypsin and antithrombin III. The nature of the amino acid residue located at position P1 of the reactive center peptide bond plays an important part in dictating the specificity of serpins. Studies with natural or recombinant $\alpha_1$-antitrypsin mutants have shown that a Met $\rightarrow$ Arg mutation at P1 (position 358) changes the specificity of $\alpha_1$-antitrypsin from neutrophil elastase to the Arg-specific proteases thrombin, kallikrein, $\beta$-Factor XIIa, Factor XIa, Factor Xa, and plasmin (13–18). To obtain an inhibitor with narrower specificity, i.e., a molecule that would be more specific for kallikrein and Factor XIIa, we have now produced by oligonucleotide-directed mutagenesis of cloned $\alpha_1$-antitrypsin complementary DNA, an antitrypsin mutant with Met $\rightarrow$ Arg at P1 and Pro $\rightarrow$ Ala at P2. The modification at P2 was made because C1 inhibitor, which inhibits Factor XIIa and kallikrein, also has Ala at P2 (11, 12). The novel inhibitor, $\alpha_1$-antitrypsin Ala$^{357}$ Arg$^{358}$, was then studied for in vitro reactivity with $\beta$-kallikrein, $\beta$-Factor XIIa, and thrombin. Thereafter, we have examined whether the in vivo administration of this mutated $\alpha_1$-antitrypsin molecule had an effect on the consumptive time of Wistar rats or influenced the kinin-dependent circulatory collapse induced in these animals by the administration of $\beta$-Factor XIIa (or Factor XIIa active fragment), a $\beta$-Factor XIIa active fragment, a $\beta$-Factor XIIa, and thrombin.

Methods

**Proteins.** Plasma kallikrein in the $\beta$ form (19) and $\beta$-Factor XIIa (20) were purified as previously described. Human thrombin (T 6759; 3,000 NIH [National Institutes of Health] U/mg) was purchased from Sigma Chemical Co., St. Louis, MO. $\alpha_1$-Antitrypsin Arg$^{358}$ and $\alpha_1$-antitrypsin Val$^{358}$ were prepared from *E. coli* strains previously described (16). For
the production of α₁-antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>, the α₁-antitrypsin coding sequence was modified by cassette mutagenesis (17) to generate Ala and Arg codons at positions 357 and 358. These modifications were verified by DNA sequencing. The double mutant was then synthesized and purified from <i>E. coli</i> cultures using the methods described for the other α₁-antitrypsin variants (16, 17).

**Kinetic studies.** Kallikrein, β-Factor XIIa, or thrombin were preincubated at room temperature with α₁-antitrypsin Arg<sup>358</sup> or α₁-antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>. At various times, aliquots were assayed for residual kallikrein or β-Factor XIIa amylolytic activity using the chromogenic substrate H-D-Pro-Phe-Arg-p-nitroanilide (S-2302), whereas residual thrombin activity was assessed on H-D-Phe-Pip-Arg-p-nitroanilide (S-2238). S-2302 and S-2238 were obtained from Kabi Diagnostica, Stockholm, Sweden. With S-2302, a 0.6 mM solution was prepared in 85 mM sodium phosphate buffer, pH 7.6, containing 127 mM NaCl. With S-2238, a 0.12 mM solution was prepared in 97 mM sodium phosphate buffer, pH 7.6, containing 145 mM NaCl. 10 μl of the solution to be tested was added to 330 μl of substrate at 37°C, and the absorbance change at 405 nm was continuously recorded with a Cary 11 spectrophotometer (Varian Associates, Inc., Palo Alto, CA). Under these conditions, the hydrolysis rate of S-2302 was 82 amol/min with 1 mg of kallikrein and 18 amol/min with 1 mg of β-Factor XIIa, whereas 1 mg of thrombin hydrolyzed S-2238 at a rate of 201 amol/min. Second-order reaction rate constants were derived from pseudo-first-order plots (21) or directly calculated using an integrated form of the second-order equation (22).

**Animal studies.** Male Wistar rats (Madarin, Fullinsdorf, Switzerland), weighing 232–271 g, were used for this study. For the experiments designed to evaluate the anticoagulant action of α₁-antitrypsin mutants, the animals were anesthetized with ether during the entire experiment and they were not exposed to exogenous heparin. After cannulation of the right femoral vein with a PE-10 catheter, the animals received a bolus injection of the agent under investigation. 5 min later, 2 ml of blood were obtained by cannulation of the right external iliac artery with a PE-50 catheter. Blood samples were immediately transferred into plastic tubes containing 0.1 vol of 0.13 M sodium citrate, and plasma samples were prepared by centrifugation at 3,000 g for 15 min. The thrombin time was the coagulation time obtained on addition of 1 vol of thrombin at 12 U/ml (Topostasin, Hoffman La Roche, Basel, Switzerland) to 1 vol of plasma. Under these conditions, the thrombin time of normal rat plasma was 26–29 s, whereas values ranging from 12.5 to 13.0 s were observed with normal human plasma. For the experiments designed to evaluate influence of α₁-antitrypsin mutants on β-Factor XIIa-induced arterial hypotension, the following protocol was employed. Under light ether anesthesia, the right external iliac artery of the animals was cannulated with a PE-50 catheter and the right femoral vein with a PE-10 catheter. All catheters contained a heparinized 5% dextrose solution. The rats were then placed in a plastic tube for the restriction of their movements, where they were left to recover from anesthesia. Arterial pressure and heart rate were monitored using a pressure transducer (Statham, Hato Rey, PR) connected to an electrogalvanometer (Philips 2000, Eindhoven, Netherlands), and recorded on a light-sensitive oscillograph (Mannarp 150, Electronic Institute, London, UK). The study was initiated 90–120 min after the end of anesthesia, when blood pressure and heart rate were stable and normal. Over a period of 5 min, the animals were pretreated by a continuous intravenous perfusion of the agent under investigation. 5 min after the end of the pretreatment period, a bolus intravenous injection of β-Factor XIIa (1.0 μg) was administered. Blood pressure and heart rate were continuously recorded during 15 min, from the start of the pretreatment injection (Fig. 1, time = 0) until 5 min after the bolus administration of β-Factor XIIa (Fig. 1, time = 15 min). Blood pressure changes were expressed as mean±SD, and groups were compared using one-way analysis of variance and Student's t tests.

**Results**

Interaction of blood proteolytic enzymes with α₁-antitrypsin mutants: *in vitro* studies. The inactivation of kallikrein, β-Factor XIIa, and thrombin by α₁-antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>, and α₁-antitrypsin Arg<sup>358</sup> was studied using enzyme/inhibitor molar ratios ranging from 1:1 to 1:20. α₁-antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> inactivated kallikrein with a second-order rate constant of 3.6 × 10<sup>5</sup> M<sup>−1</sup> s<sup>−1</sup>, a value that was 5.2 times greater than the value observed with α₁-antitrypsin Arg<sup>358</sup> and 21.2 times greater than the value determined with Cl inhibitor (Table I). However, α₁-antitrypsin Ala<sup>357</sup> Arg<sup>358</sup> was less efficient than α₁-antitrypsin Arg<sup>358</sup> for the inactivation of β-Factor XIIa (3.8 times) as well as of thrombin (4.9 times) (Table I).

**Anticoagulant effect of α₁-antitrypsin mutants.** Anesthetized male Wistar rats weighing 232–271 g received an intravenous bolus of α₁-antitrypsin Arg<sup>358</sup> or α₁-antitrypsin Ala<sup>357</sup> Arg<sup>358</sup>. 5 min later, the anticoagulant effect of these treatments was assessed using the thrombin time. Control values were obtained by studying animals treated with α₁-antitrypsin Val<sup>358</sup>, an oxidation-resistant variant that possesses the same inhibitory spectrum as α₁-antitrypsin Met<sup>358</sup> and therefore does not exhibit anticoagulant activity (16). Two animals treated with 0.7 mg of α₁-antitrypsin Arg<sup>358</sup> had a prolonged thrombin time, respectively by a factor of 2.3 and 1.4, whereas a 1.1-fold prolongation was detectable in the animal that received half this dose (Table II). In contrast, rats treated with 0.7 mg of α₁-antitrypsin Ala<sup>357</sup>.
Arg358 had thrombin time values similar to those observed in controls (Table II).

Effect of α1-antitrypsin mutants on β-Factor XIIa–induced arterial hypotension. Conscious normotensive male Wistar rats weighing 241–268 g were pretreated intravenously during 5 min with either saline (Fig. 1 A), 0.7 mg of α1-antitrypsin Val358 (Fig. 1 B), or 0.7 mg of α1-antitrypsin Ala357 Arg358 (Fig. 1 C). 5 min after the end of pretreatment, the animals received an intravenous bolus of 1.0 μg of β-Factor XIIa (Fig. 1, vertical arrows). Nearly identical peak arterial blood pressure reductions were recorded in rats pretreated with saline (−26±1 mmHg, n = 4; Fig. 1 A) or α1-antitrypsin Val358 (−27±2 mmHg, n = 4; Fig. 1 B), whereas significantly smaller blood pressure decreases were observed in animals pretreated with α1-antitrypsin Ala357 Arg358 (−14±3 mmHg, n = 4, P < 0.01; Fig. 1 C).

Discussion

α1-Antitrypsin Ala357 Arg358 differs from α1-antitrypsin Arg358 in possessing Ala instead of Pro at position P2 of the reactive center. This additional modification modifies the relative inhibition of kallikrein and thrombin (Table I). α1-Antitrypsin Ala357 Arg358 is a better inhibitor of kallikrein than of thrombin (4.9-fold), in contrast to α1-antitrypsin Arg358, which is more efficient against thrombin than against kallikrein (5.2-fold). Thus, it can be calculated that at inhibitor concentrations causing identical rate of kallikrein inhibition, α1-antitrypsin Ala357 Arg358 will induce 25 times less inhibition of thrombin than α1-antitrypsin Arg358. Another characteristic of α1-antitrypsin Ala357 Arg358 is that the mutated recombinant protein is more than one order of magnitude more efficient in inhibiting kallikrein and β-Factor XIIa than C1 inhibitor, an unexpected finding since C1 inhibitor is the most potent natural inhibitor of these enzymes (9). Furthermore, because C1 inhibitor and α1-antitrypsin Ala357 Arg358 have the same P1 and P2 residues, our results demonstrate that additional factors other than the residues located in the immediate vicinity of the reactive site contribute to the reactivity of protease inhibitors of the serpin superfamily.

Infusion of certain plasma protein fraction lots has induced circulatory collapse in surgical patients. Because the preparations involved contained β-Factor XIIa (24), it was proposed that the mechanism responsible for the hypotensive reaction included prekallikrein activation by β-Factor XIIa, followed by high–molecular-weight kininogen cleavage by kallikrein leading to the release of bradykinin. Subsequent studies showed that arterial hypotension is also observed when purified and catalytically active β-Factor XIIa is employed instead of plasma protein fraction (25, 26). In these experiments, the hypotensive response is mediated by bradykinin/kinin molecules. Indeed, more severe hypotension is observed in rats treated with captopril (25, 26), a kininase II inhibitor that causes decreased bradykinin catabolism. Moreover, animals treated with the bradykinin competitive inhibitor B4162 are partly protected from the hypotensive reaction to β-Factor XIIa (27). A similar observation is now made in animals pretreated with α1-antitrypsin Ala357 Arg358 (Fig. 1 C), thereby demonstrating that the double mutant can prevent a well documented kinin-mediated reaction. The effect of α1-antitrypsin Ala357 Arg358 is related to its specific and efficient inactivation of the proteolytic enzymes of the kallikrein-kinin system. Indeed, α1-antitrypsin Val358, which is active against neutrophil elastase but not against β-Factor XIIa or kallikrein (18), did not prevent the hypotensive response to β-Factor XIIa (Fig. 1 B). Furthermore, because α1-antitrypsin Ala357 Arg358 prevents the kinin-dependent consequences of prekallikrein and Factor XII activation, it is likely that this inhibitor will also inhibit the kinin-independent manifestations that are observed when these enzymes are activated. These reactions include activation of the blood coagulation and fibrinolytic pathways and of blood neutrophils (1).

In vivo manifestation of the specificity of α1-antitrypsin Ala357 Arg358 and further indication of its clinical potential was obtained by studying the anticoagulant properties of the double mutant. With a dose of 0.7 mg of α1-antitrypsin Ala357 Arg358, which reduced the hypotensive reaction to β-Factor XIIa (Fig. 1 C), no prolongation of the thrombin time was detectable, whereas with the same dose of α1-antitrypsin Arg358, the thrombin time was prolonged significantly (Table II). Therefore, in vivo, α1-antitrypsin Ala357 Arg358 can efficiently inhibit the enzymes of the plasma kinin–forming system without causing increased inhibition of thrombin, an observation that confirms and amplifies the results obtained by our kinetic studies in purified systems.

Further studies are now required to establish the inhibitory activity of α1-antitrypsin Ala357 Arg358 toward other Arg-specific proteases of the coagulation, complement, and fibrinolytic systems. These studies will determine whether α1-antitrypsin Ala357 Arg358 is sufficiently specific for the enzymes of the plasma kinin–forming system or whether additional mutants should be constructed. If new molecules must be created, the detailed sequence information now available for most plasma protease inhibitors of the serpin superfamily should permit the generation of a new set of molecules with the desired specificity.

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References


