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C1-inhibitor polymers activate the FXII-dependent kallikrein–kinin system: Implication for a role in hereditary angioedema

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ABSTRACT

Background: The FXII-dependent kallikrein–kinin system (KKS) is tightly regulated by the serine protease inhibitor (serpin) C1-inhibitor (C1-inh). When regulation of the FXII-dependent KKS fails, which is the case in hereditary angioedema (HAE), patients consequently experience invalidating edema attacks. HAE is caused by mutations in the C1-inh encoding gene, and we recently demonstrated that some mutations give rise to the presence of polymerized C1-inh in the plasma of HAE patients.
Methods: C1-inh polymers corresponding to the size of polymers observed *in vivo* were produced using heat denaturation and gel filtration. The ability of these polymers to facilitate FXII activation was assessed *in vitro* in an FXII activation bandshift assay. After spiking of plasma with C1-inh polymers, kallikrein generation was analyzed in a global kallikrein generation method. Prekallikrein consumption in the entire Danish HAE cohort was analyzed using an ELISA method.
Results: C1-inh polymers mediated FXII activation, and a dose dependent kallikrein generation in plasma spiked with C1-inh polymers. An increased (pre)kallikrein consumption was observed in plasma samples from HAE patients presenting with C1-inh polymers *in vivo*.
Conclusion: Polymerization of the C1-inh transforms the major inhibitor of the FXII-dependent KKS, into a potent activator of the very same system.
General significance: The C1-inh polymers might play a role in the pathophysiology of HAE, but several diseases are characterized by the presence of serpin polymers. The role of serpin polymers has so far remained elusive, but our results indicate that such polymers can play a role as inflammatory mediators through the FXII-dependent KKS.

1. Introduction

Activation of coagulation factor XII (FXII) initiates the kallikrein–kinin system (KKS), which propagates through activated FXII (FXIIa)-induced activation of prekallikrein (PK) and kallikrein-induced formation of bradykinin (BK), mediating vasodilatation through binding to the BK B2 receptor on vascular endothelial cells [1]. FXII independent PK activation may, however, also occur as demonstrated in studies of the chaperone protein heat shock protein 90 [2].

Recent studies have demonstrated that a number of aggregated and misfolded proteins are potent activators of the FXII-dependent KKS [3,4]. In particular the role of aggregated β amyloid protein as an activator of the FXII-dependent KKS is well established [5]. The activity of both FXIIa and kallikrein, however, is tightly regulated by complement C1 esterase inhibitor (C1-inh) belonging to the serine protease inhibitor (serpin) superfamily. In general serpins fulfill their inhibitory role, when an active protease cleaves their reactive center loop (RCL), and a stable inactive serpin:protease complex is formed. The RCL cleavage induces a thermodynamic transition of the serpin, from an unstable conformation characterized by a relatively high free energy, to a low energy highly stable conformation [6]. The multimeric polymerized form of the serpin represents another stable conformation and polymerization is potentially associated with gene mutations in the serpin encoding gene [7,8]. The conformational instability of mutated serpin molecule leads to

Abbreviations: BSA, bovine serum albumin; C1-inh, complement C1 esterase inhibitor; C1PIV, *in vivo* sized C1-inh polymers; CPDA, citrate phosphate dextrose adenine; CPK, chromozyme PK; FXII, coagulation factor XII; FXIIa, activated FXII; dBSA, heat denatured BSA; HAE, hereditary angioedema; HMWK, high molecular weight kininogen; HRP, horseradish peroxidase; KKS, kallikrein–kinin system; MAb, monoclonal antibody; O/N, overnight; OD, optical density; OPD, ortho phenylenediamine; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PBS-TW, phosphate buffered saline with 0.05% Tween-20; PBS-TW skim, PBS-TW with 2.5% skimmed milk powder; PK, prekallikrein; PVDF, polyvinylidene fluoride; RCL, reactive center loop; RT, room temperature; SDS, sodium dodecyl sulfate; Serpin, serine protease inhibitor; WB, Western blot; FXIIa, activated FXII

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serpinopathies, characterized by formation of ordered serpin polymers and uncontrolled proteolytic activity [9]. The serpinopathies are expressed in clinical conditions such as emphysema, hereditary familial dementia and hereditary angioedema (HAE) [10–12].

We recently demonstrated certain mutations in the *SERPING1* gene cause polymerization of C1-inh *in vivo* [13], and we speculate whether such polymers in addition to loss of inhibitory properties have the capability to activate the FXII-dependent KKS. In the present study we tested this hypothesis with the use of protein chemistry and enzymatic methods. Both lines of experiments suggest that C1-inh polymers cause activation of the FXII-dependent KKS.

2. Materials and methods

Unless otherwise stated, reagents were obtained from Merck, Darmstadt, Germany. Affinity purified human FXII, FXIIa, PK and kallikrein were purchased from Enzyme Research Laboratories, Swansea, UK.

2.1. Preparation of in vivo sized C1-inh polymers (C1PIV)

C1-inh (Berinert P 500) was obtained from CSL Behring, Lyngby, Denmark. Berinert P 500 was dissolved in 10 mL H_2O and dialyzed against 5 L phosphate buffered saline (PBS) pH 7.4 at 4 °C for 72 h in a Spectra/Por® Dialysis Membrane MWCO 6–8000 (Spectrumlabs®, Rancho Dominquez, CA, US). C1-inh polymers were formed by heating 50 μL of C1-inh (6.5 mg/mL) in PBS at 55 °C for 35 min on a Peltier Thermal Cycler PTC-200 (MJ Research Inc., Waltham, MA, US). C1-inh polymers were frozen in 50 μL aliquots at −80 °C immediately after formation.

Two milliliters of 6.4 mg/mL heat denatured C1-inh as described above were separated according to polymer size using a 110 mL Superose 6 preparation grade 17-0489-01 gel filtration column (GE Healthcare, Piscataway, NJ, US). The column was equilibrated with PBS at 1.5 mL/min, and the polymers were passed over the column at a flow rate of 0.5 mL/min. One milliliter fractions were collected and frozen at −80 °C immediately after the gel filtration. Fractions from the gel filtration were diluted 1:20 in native PAGE sample buffer (Bio-Rad, Hercules, CA, US) and separated by native-PAGE in 4–15% Mini-PROTEAN® TGX™ precast gels (Bio-Rad) using a native PAGE Tris–glycine buffer system according to manufacturer's description. The gel was run for 120 min at 150 V, and proteins were visualized using silver staining according to Nesterenko et al. with the modifications described previously [14,15].

Fractions containing C1-inh polymers corresponding to sizes observed *in vivo* were pooled and concentrated using anion exchange chromatography on a 1 mL Resource Q column (GE Healthcare). The pooled fractions were passed over the column at a flow rate of 1 mL/min using 50 mM Tris HCl pH 7.7. The polymers were eluted using 50 mM Tris HCl pH 7.7 containing 1 M NaCl, and collected in 250 μL fraction. The fractions were subsequently dialyzed against PBS. The concentration of the polymers was calculated using the molar extinction coefficient for monomeric C1-inh (0.382 mL mg^{-1} cm^{-1}). The sizes of the polymers were confirmed using native PAGE. This polymer preparation is denoted “in vivo sized C1-inh polymers (C1PIV)”.

2.2. Comparison of HAE patient plasma C1-inh polymers with C1PIV

2.2.1. Native PAGE Western blot analysis

A native PAGE Western blotting (WB) based method was used to compare the sizes of C1-inh polymers in a plasma sample from an HAE patient with the C1PIV. The HAE patient is classified as HAE type I, and bears a mutation affecting helix C of the C1-inh protein (DNA:c.566C>A, protein: p.Thr167Asn) [13]. Analyses were done using a protein content of 60 ng C1PIV per lane, a citrate phosphate dextrose adenine (CPDA) plasma sample from the HAE patient diluted 1:10, monomeric C1-inh with a protein content of 50 ng/lane and a CPDA plasma from a healthy individual diluted 1:10. CPDA plasma samples were collected using Vacuette® CPDA tubes (Greiner Bio-One, Wemmel, Belgium). The native PAGE gel was run as described above and proteins were transferred to a polyvinylidene fluoride (PVDF) membrane using the Trans-Blot® Turbo™ Blotting System (Bio-Rad) with Trans-Blot® Transfer Pack consumables (Bio-Rad). The membrane was blocked in phosphate buffered saline (PBS) pH 7.4 with 0.05% Tween 20 and 2.5% skimmed milk powder (PBS-TW skim) for 1 h with agitation at room temperature (RT), and incubated overnight (O/N) at 4 °C with 2 μg/mL of a monoclonal antibody raised against C1-inh polymers (MAb 12-27-15) in PBS-TW skim. The membrane was washed thrice in PBS with 0.05% Tween 20 (PBS-TW) and incubated for 1 h at RT with horseradish peroxidase (HRP)-conjugated rabbit anti mouse IgG antibody (ZyMAX™, Invitrogen, Tåstrup, Denmark) diluted 1:4000 (v/v) in PBS-TW. The membrane was visualized using 0.4 mg/mL 3-amino-9-ethylcarbazole in 50 mM acetate buffer pH 5.0 with 0.015% H_2O_2, and the coloring reaction was stopped by dilution with H_2SO_4.

2.3. FXII-dependent KKS activity and C1-inh polymers

2.3.1. Kallikrein and FXIIa inhibition

The ability of C1PIV to inhibit kallikrein and FXIIa was investigated by assessment of SDS-stable complex formation of kallikrein and FXIIa with *in vivo* sized polymers using SDS-PAGE WB. C1PIV (125 μg/mL) was incubated with kallikrein (25 μg/mL) or FXIIa (25 μg/mL) for 1 h at 37 °C. C1-inh monomer (125 μg/mL) was incubated with kallikrein (25 μg/mL) or FXIIa (25 μg/mL) for 1 h at 37 °C. Kallikrein (25 μg/mL), FXIIa (25 μg/mL), C1PIV (125 μg/mL) and C1-inh monomer (125 μg/mL) were analyzed as controls. All samples were diluted 1 + 1 in SDS sample buffer and boiled for 8 min at 96 °C, and subsequently 20 μL of each sample was separated on 4–15% Mini-PROTEAN® TGX™ precast gels (Bio-Rad) as described above. Membranes were blocked in PBS-TW for 30 min with agitation at RT, and incubated (O/N) at 4 °C with either mouse anti-human-PK MAb (0.6 μg/mL, PK 10-8-11) or mouse anti-human-FXII MAb (0.8 μg/mL, FXII 10-11-37) in PBS-TW [16]. PK 10-8-11 is an in house prepared antibody that recognizes apple domain 4 of PK. Membranes were visualized as described above.

2.3.2. Prekallikrein activation

A PK activation assay was established using the kallikrein sensitive chromogenic substrate Chromozym PK (CPK) (Roche Diagnostics GmbH, Mannheim, Germany) and a citrate stabilized plasma pool. A buffer made of 0.05 M Tris–HCl pH 7.4 with 6 μM of $ZnCl_2$ (THZ-buffer), prewarmed to 37 °C was used throughout the experiments. NUNC non-binding polystyrene plates (cat. 269620 Thermo Fisher Scientific, Roskilde, Denmark) were blocked for 2 h in bovine serum albumin (BSA, 0.5 mg/mL) (Sigma-Aldrich, Vallensbaek, Denmark) diluted in THZ-buffer. CPK was dissolved to 1 mM in THZ-buffer and 50 μL of this solution was added to each well. A citrate stabilized plasma pool collected from 30 healthy individuals was diluted 1:36 in THZ-buffer, and 40 μL of this solution was added to each well. Ten-microliter samples containing the compounds of interest were added to the appropriate wells. C1PIV was analyzed at final concentrations of 50, 25, 12.5, 6.3 and 3.1 μg/mL. APTT reagent (STA-PPT A 5, Stago, Parsippany, NJ, US) at a final dilution of 1:200 was used as a positive control. The FXII-dependency of the assay was assessed by using FXIIa as a sample at a final assay concentration of 0.5 μg/mL. The PK dependency of the assay was analyzed by spiking the citrate plasma pool with two inhibitory monoclonal anti-PK antibodies (PK 10-8-11 and PK 10-8-32) (final assay concentration 0.7 μg/mL) before addition of APTT reagent. PK 10-8-32 is an in house prepared antibody, which recognizes the region between apple domain 3 and 4 of PK. FXII-depleted plasma (in house preparation, previously described in [16]) and PK depleted plasma (Affinity Biologicals Inc., Ancaster, ON, Canada) replaced the citrate plasma pool and were also analyzed with APTT reagent as activator.