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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.

BSA at a final concentration of 1 mg/mL was analyzed in the assay, as a protein control sample. Heat denatured BSA (dBSA) formed by heating BSA (60 mg/mL) for 35 min at 65 °C was also analyzed at a final concentration of 50 μg/mL.

The experiments were followed for 140 min at 37 °C with agitation and OD recordings (405 nm) every minute on a Tecan Sunrise microtiter reader (Tecan Group Ltd., Männedorf, Switzerland).

2.3.3. FXII activation

An SDS-PAGE WB based bandshift assay, using purified FXII, PK and C1PIV, was used to determine the FXII-activating properties of C1PIV. Sixty nanomolar FXII, 0.9 μM PK and 2.9 μM C1-inh polymers were incubated for 15 min in PBS at 37 °C, and 5 μL of this mixture was at time points 0, 5, 10 and 15 min transferred to SDS-PAGE WB reducing sample buffer (Bio-Rad). A parallel control experiment was included, wherein C1PIV was replaced with 2.9 μM monoclonal nonsense antibody. Samples were boiled for 8 min at 96 °C, and 15 μL was loaded onto 4–15% Mini-PROTEAN® TGX™ precast gels (Bio-Rad) using an SDS-PAGE Tris–glycine buffer (Bio-Rad). A control experiment was performed, wherein C1PIVs were replaced with APTT reagent diluted 1:10 (Actin FS, Siemens, Erlangen, Germany). Another control experiment was included wherein only zymogen FXII and FXIIa were analyzed (15 ng/lane). Gels were run, blotted and blocked as described above. The membrane was incubated for 1 h at room temperature with 1.1 μg/mL of a biotinylated pool of monoclonal antibodies against the heavy chain of FXII [16]. The membrane was washed thrice in PBS-TW, and incubated for 1 h with HRP-conjugated streptavidin (Invitrogen) diluted 1:4000 (v/v) in PBS-TW. The membrane was developed as described above.

2.3.4. (Pre)kallikrien ELISA

An in house ELISA method that recognizes PK, kallikrein, C1-inh:kallikrein complexes and (pre)kallikrein:HMWK complexes to the same extent, was used to measure total (pre)kallikrein levels in HAE patients. Briefly 96-well polystyrene flat bottom MicroWell™ MaxiSorp™ plates (Thermo Fisher Scientific) were coated with 0.2 μg MAb PK 10-8-11 per mL. EDTA plasma samples were analyzed at a 1:3000 dilution, and PK (Enzyme Research Laboratories) was used as calibrator. Biotinylated MAb PK 10-8-32 (0.2 μg/mL) was used as detection antibody, and plates were visualized using HRP-conjugated streptavidin and ortho phelylenediamine (OPD)/H_2O_2. The plates were read at 492 nm with 650 nm as reference using a Tecan Sunrise ELISA reader. EDTA plasma samples from 69 polymer negative, 6 polymer positive HAE patients and 238 healthy individuals were analyzed.

2.3.5. Ethics

All subjects gave informed written consent to blood sampling and mutational analysis, and the study was conducted in accordance with

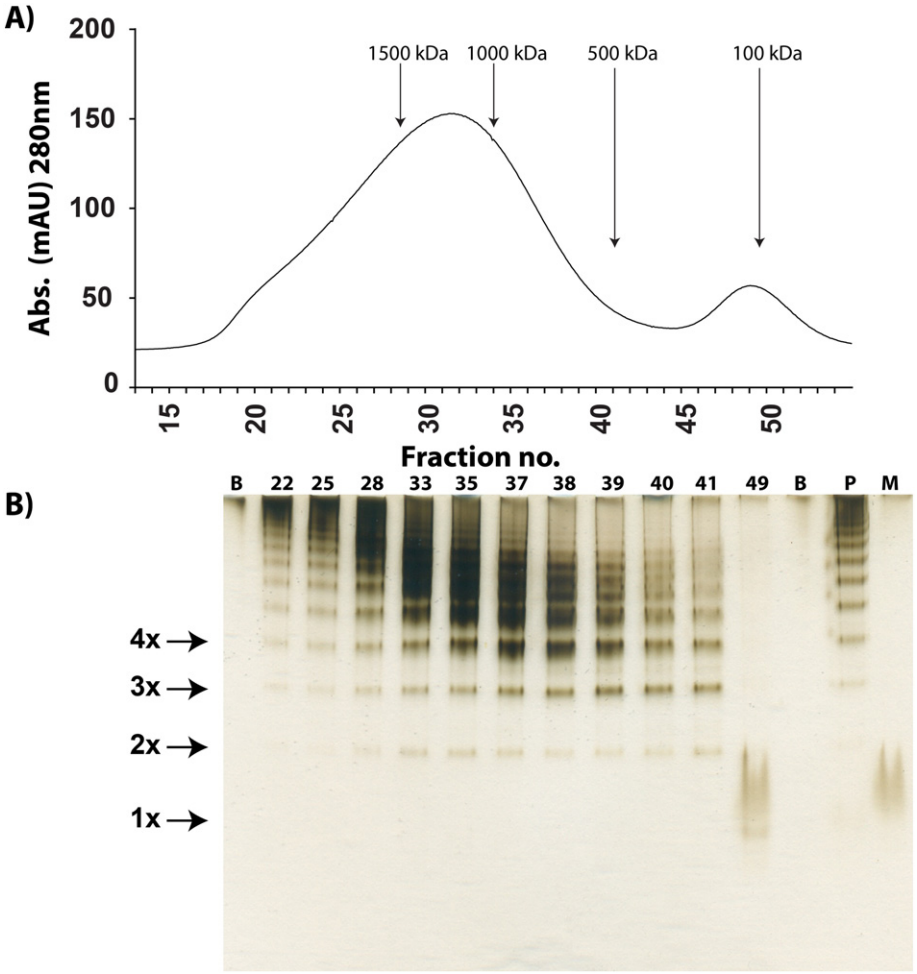

Fig. 1. Purification C1-inh polymers. A) Chromatogram obtained from gel filtration of C1-inh polymers formed using heat denaturation. The y-axis represents absorbance (mAU) measured at 280 nm and the x-axis represents fraction numbers. B) Native PAGE analysis of selected fractions from gel filtration of C1-inh polymers formed using heat denaturation. "B" represents a blank well, "P" represents C1-inh polymers formed using heat denaturation, and "M" represents monomeric C1-inh. Numbers correspond to fraction numbers and proteins were visualized by silver staining. Samples were loaded at the top and "1×" to "4×" represent number of C1-inh molecules in the complex.

the Helsinki II Declaration and approved by the Regional Scientific Ethics Committee for Southern Denmark (S-20110047) and the Danish Data Protection Agency (2008-58-0035). Animal care was in agreement with institutional guidelines.

3. Results

3.1. Preparation of in vivo sized C1-inh polymers

In the chromatogram from the gel filtration of heat-induced C1-inh polymers we observed two peaks (Fig. 1). Fractions 18 to 43 contained polymers of decreasing sizes, and the second peak spanning fractions 45 to 55 contained monomeric C1-inh. From fraction 40 polymers approximated the sizes observed *in vivo*, and fractions 40 to 43 were pooled and further purified by ion exchange chromatography. Native PAGE analysis of fraction A8–A11 from the ion exchange confirmed the localization of the polymers primarily in fraction A9. The native PAGE analysis also revealed that fraction A9 primarily contained di- to hexameric polymers of C1-inh (Fig. 2).

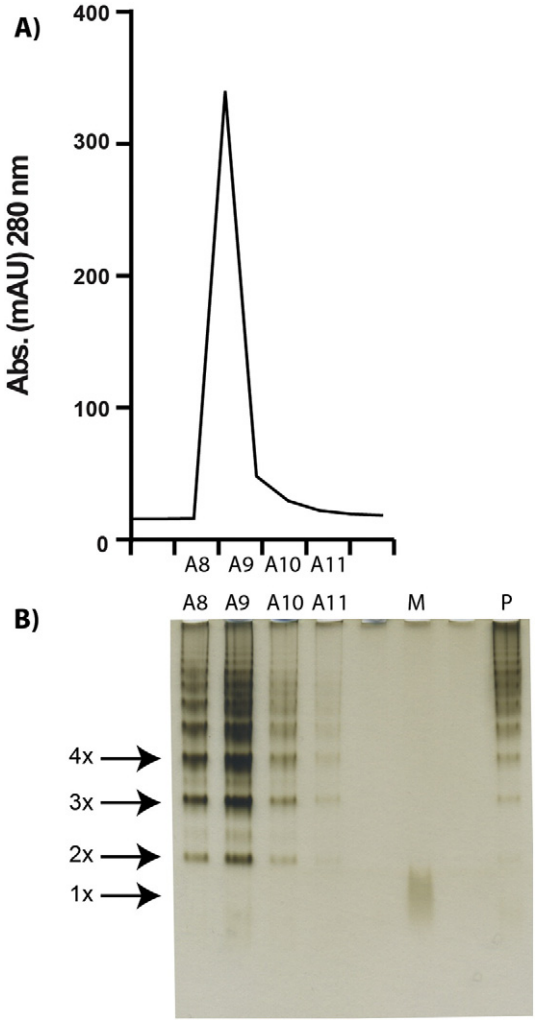

Fig. 2. Native PAGE analysis of selected fractions from ion exchange chromatography of pooled gel filtration fractions. A) Chromatogram obtained from anion exchange of C1-inh polymers subjected to gel filtration. The y-axis represents absorbance (mAU) measured at 280 nm, and the x-axis represents fraction numbers. Bound proteins were eluted with 1 M NaCl, pH 7.7. B) Native PAGE analysis. A8–A11 represent fractions obtained from ion exchange, "M" represents monomeric C1-inh and "P" represents C1-inh polymers formed using heat denaturation. Samples were loaded at the top and proteins were visualized by silver staining. "1×" to "4×" represent number of C1-inh molecules in the complex.

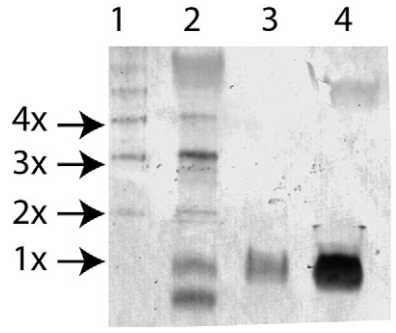

Fig. 3. Native PAGE WB of *in vivo* sized C1-inh polymers (lane 1), a polymer positive HAE patient plasma samples (lane 2), monomeric C1-inh (lane 3) and a plasma pool from 30 healthy individuals (lane 4). Samples were loaded at the top. "1×" to "4×" represent number of C1-inh molecules in the complex. Membranes were visualized using anti C1-inh MAb (12-27-15).

3.2. Comparison of in vivo sized C1-inh polymers with HAE patient plasma C1-inh polymers

3.2.1. Native PAGE Western blot analysis

The polymers in the C1PIV preparation migrated with the same electrophoretic mobility as the polymers in a plasma sample from an HAE patient (Fig. 3). A few polymer bands in the C1PIV preparation exceed the polymer size observed in the patient plasma samples. The electrophoretic mobility of monomeric C1-inh from HAE plasma sample, purified monomeric C1-inh preparation and the healthy plasma sample were identical.

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

3.3.1. Kallikrein and FXIIa inhibition

C1-inh inhibits proteases by forming complexes that are stable, even in the presence of SDS. Unlike monomeric C1-inh, C1PIV failed to form such complexes with kallikrein, and complex formation with FXIIa was minimally compared with monomeric C1-inh (Fig. 4).

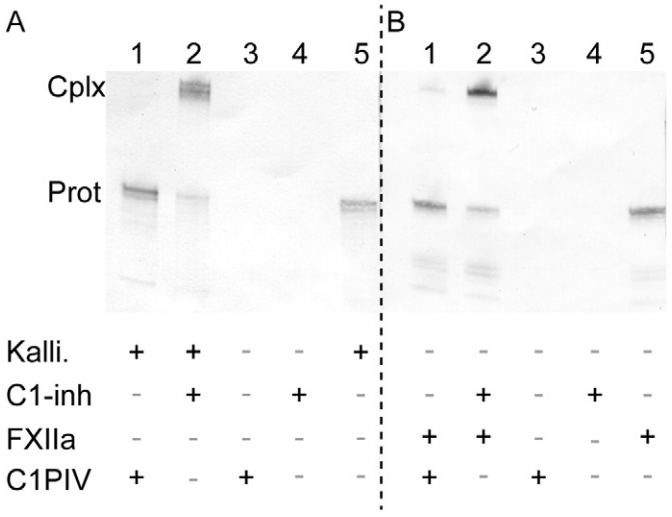

Fig. 4. Detection of SDS-stable C1-inh complexes with kallikrein and FXIIa by SDS-PAGE WB. In panel A, "kallikrein", complex formation of kallikrein was analyzed using anti-PK MAb (PK 10-8-11). In panel B "FXIIa", complex formation of FXIIa was analyzed using anti-FXII MAb (FXII 10-11-37). "Cplx" represents localization of complex bands and "Prot" represents localization of the proteases (kallikrein or FXIIa). Contents of the lanes are as follows 1: protease and C1PIV; 2: protease and C1-inh monomer; 3: C1PIV; 4: C1-inh monomer; 5: protease.

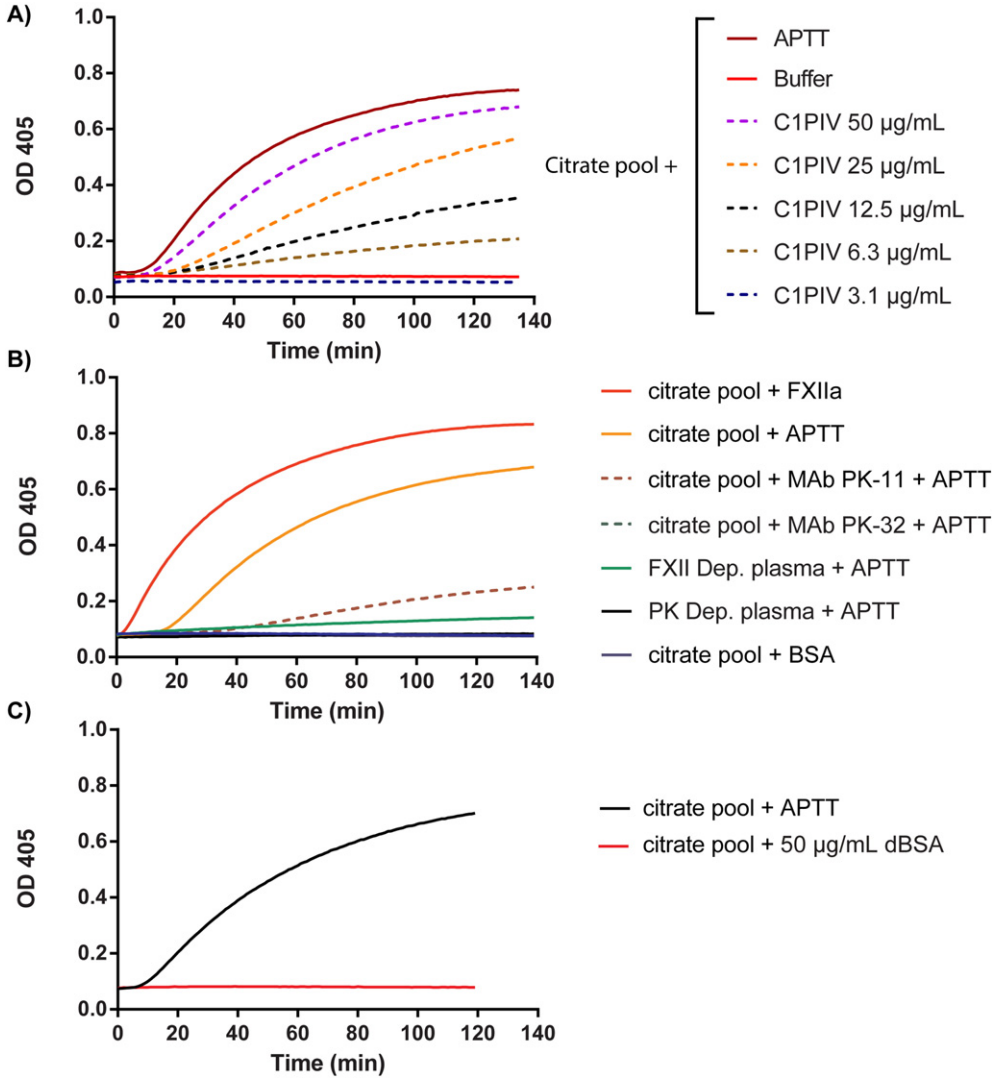

Fig. 5. C1PIV mediated PK activation. A) Kallikrein generation in a diluted citrate plasma pool in the presence of APTT reagent. A dilution series of C1PIV and buffer was measured using the chromogenic kallikrein sensitive substrate CPK. *In vivo* sized C1-inh polymers were analyzed at five different final concentrations. APTT reagent was used as positive control, and buffer was tested as a negative control. Y-axis represents OD measured at 405 nm, and x-axis represents time in minutes. B) Specificity test and verification of the kallikrein activity assay used above. The citrated diluted plasma pool was tested in the presence of: FXIIa, APTT reagent, APTT reagent plus anti PK MAb (PK 10-8-11), APTT reagent plus anti PK MAb (PK 10-8-32) and BSA. The citrated plasma pool was replaced with FXII-depleted plasma and PK depleted plasma both in the presence of APTT reagent. C) Assessment of the ability of heat denatured BSA to facilitate kallikrein generation. dBSA was tested at 50 µg/mL, and APTT-reagent was included as a positive control.

3.3.2. Prekallikrein activation

C1PIVs were potent stimulators of kallikrein generation, and a dose dependency between C1PIV concentrations and kallikrein activity was observed (Fig. 5). At a C1PIV concentration of 3.1 µg/mL no kallikrein activity was observed, which was also the case for the buffer sample. APTT activator was used as a positive control, and mediated a kallikrein activity, which exceeded that observed for the highest C1-inh polymer concentration (Fig. 5A).

A line of control experiments was carried out to characterize the performance and specificity of the kallikrein activity assay (Fig. 5B). Excess amounts of FXIIa mediated an immediate burst in kallikrein activity, which exceed that observed when APTT activator was added. The two anti PK antibodies partially (PK 10-8-11) or completely (PK 10-8-32) inhibited the kallikrein generation. Minimal levels of kallikrein activity were still observed when a FXII-depleted plasma pool was activated with APTT reagents, but no kallikrein activity was measured when a PK depleted plasma pool was activated with APTT reagent. Negative controls containing BSA did not mediate kallikrein generation. dBSA caused no kallikrein generation, indicating that kallikrein generation is not related to heat denatured proteins in general.

3.3.3. FXII activation

C1-inh polymers mediated FXII activation in a system containing purified FXII and PK, and it was confirmed that the activity was not mediated by the protein content in the samples, as a nonsense monoclonal antibody did not cause activation (Fig. 6A). Omission of the nonsense antibody caused no activation either (data not shown). In Fig. 6B, a well-known activator of the FXII-dependent KKS (APTT reagent) was used as a positive control instead of C1-inh polymers. This reagent caused also activation of FXII. Fig. 6C confirms that our antibodies recognize both inactive single chain FXII and the heavy chain of FXIIa.

3.3.4. Levels of (pre)kallikrein in healthy individuals and HAE patients

A statistically significant difference in the (pre)kallikrein levels was observed between the C1-inh polymer positive patients and the reference population (medians = 24.8 µg/ml and 31.0 µg/ml, respectively). No significant difference in total (pre)kallikrein levels was observed between C1-inh polymer negative HAE patients and the reference population (medians = 28.9 µg/mL and 31.0 µg/mL, respectively) or between C1-inh polymer positive and negative patients (Fig. 7).

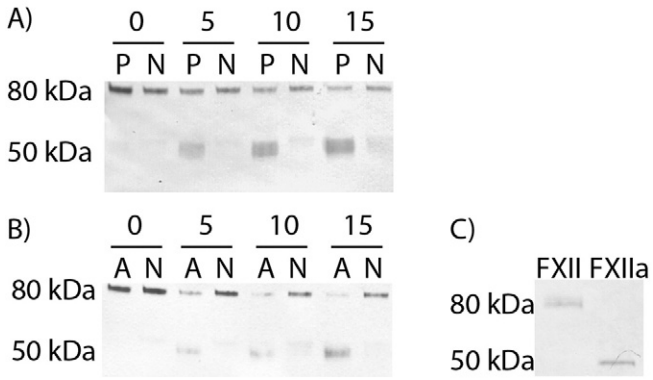

Fig. 6. C1PIV-mediated activation of FXII. A) SDS-PAGE WB based FXII activation bandshift assay. Kallikrein mediated FXII cleavage in the presence of C1PIV and nonsense MAb was monitored for 15 min. Numbers above the lines represent time points in minutes, "P" represents C1PIV and "N" represents nonsense MAb as probe. The 80 kDa bands represent intact single chain non cleaved FXII, and the 50 kDa bands represent cleaved and activated FXIIa heavy chain. All samples were analyzed under reducing conditions and membranes were visualized using a pool of anti FXII heavy chain antibodies. B) APTT reagent mediated FXII activation is visualized over 15 min. "A" represents that FXII and PK were incubated with APTT reagent, and "N" represents that FXII and PK were incubated with a nonsense MAb. C) The reactivity of the anti FXII antibodies is demonstrated. "FXII" represents that inactive single chains FXII were analyzed, and "FXIIa" represents the FXIIa was analyzed.

4. Discussion

The *SERPING1* gene encodes C1-inh, which is the major inhibitor of the KKS that controls formation of bradykinin. Loss of function mutations in the *SERPING1* gene cause the serpinopathy HAE [1], which is characterized by recurrent bradykinin mediated- and potentially life-threatening edema attacks induced by activation of the KKS. We presently confirm, as shown in a limited number of studies [17–19], that polymers of mutated C1-inh exist in plasma samples from a subgroup of HAE patients, and we further found that polymerized C1-inh fails to inhibit the KKS [20].

The current line of experiments were aimed to test the hypothesis that polymerization of C1-inh transforms this major inhibitor of the KKS into an activator of the very same system. Our results point to the conclusion that C1-inh polymers have an effect on the FXII-dependent KKS, similar to that reported for other aggregated proteins [3,5].

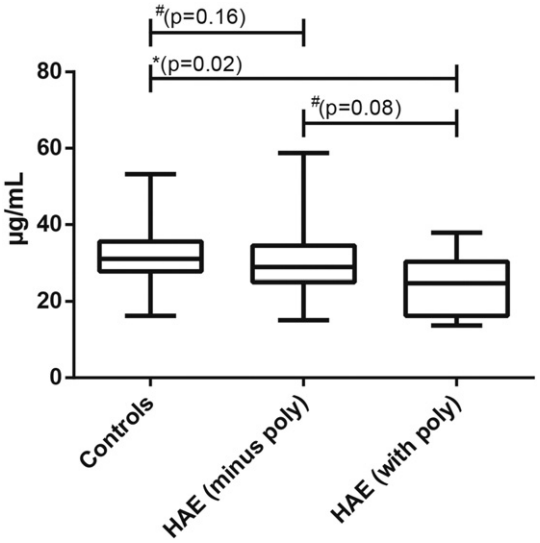

Fig. 7. Levels of (pre)kallikrien levels in healthy individuals and HAE patients. Y-axis depicts the (pre)kallikrein concentration. Boxes represent 25th to 75th percentiles, and medians are represented by horizontal lines. Whiskers represent minimum and maximum measurements. "Controls" represents healthy individuals ($n = 278$), "HAE (minus poly)" represents HAE patients ($n = 69$) that do not present with C1-inh polymers in plasma and "HAE (with poly)" represents HAE patients ($n = 6$) with C1-inh polymers in plasma. *Significantly different (Mann–Whitney *U* test). #Not significantly different (Mann–Whitney *U* test).

We confirmed the results reported by others that C1-inh polymerization causes loss of function of the C1-inh (Fig. 4) [21]. More importantly, we tested whether C1-inh polymers have the capacity to activate the FXII-dependent KKS by two methodologically different approaches. In the first line of experiments we used enzymatic methods where the activation of the FXII-dependent KKS in plasma spiked with C1-inh polymers was analyzed. A dose dependent relationship between C1-inh polymer concentration and kallikrein generation was demonstrated (Fig. 5A). An explanation for this finding could be that the KKS activating capability of C1-inh polymers was mediated by unspecific heat denaturation of the protein. We observed, however, that heat denatured BSA did not give rise to kallikrein generation (Fig. 5C).

In the enzymatic experiments FXII-dependent KKS activation required the presence of both FXII and PK, as use of FXII or PK depleted plasmas quenched the kallikrein generation (Fig. 5B). However, small amounts of kallikrein generation were observed when FXII depleted plasma was utilized. This activity may be caused by an FXII independent PK activation or by the presence of trace amounts of FXII in the FXII depleted plasma pool.

In the second line of experiments we employed an SDS-PAGE WB based FXII activation bandshift assay, where a distinct C1-inh polymer specific FXII activation was demonstrated (Fig. 6). In both lines of experiments we observed, that activation was not dependent upon unspecific protein interactions, as replacement of C1-inh polymers with irrelevant proteins did not affect activation of the FXII-dependent KKS. Omission of PK from FXII activation bandshift also annihilated the FXII activation (data not shown). In the enzymatic experiments plasma samples spiked with C1-inh polymers supported that C1-inh polymers activate the FXII-dependent KKS in a plasma milieu.

In vivo sized polymers of C1-inh were formed using heat denaturation of monomeric C1-inh and gel filtration. The size of the polymers formed *in vitro* resembled the size of polymers in HAE patient plasma, but obviously further studies are needed to analyze their structural resemblance. However a previous report suggests that heat denaturation represents an approach that produces polymers concordant to polymers observed *in vivo* [22]. It is an intriguing observation, that C1-inh polymers circulate the plasma of an HAE type I patient, as type I patients according to Rosen et al. [23] do not secrete the mutated form of the C1-inh protein. However, usually HAE patients are classified by utilizing C1-inh antigen ELISA methods and such methods do not necessarily recognize the misfolded C1-inh polymers. These methodological issues may explain why patients presenting with C1-inh polymers are classified as type I patients. Furthermore, we have demonstrated the presence of C1-inh polymers in three Danish HAE type I families [13], and Eldering et al. [18] have done similar observations.

The potential clinical relevance of C1-inh polymers is supported by preliminary data demonstrating significantly lower plasma PK concentrations in HAE patients presented with polymers when compared to a healthy reference population (Fig. 7). These results indicate that an ongoing activation of the KKS takes place in HAE patients with polymerogenic *SERPING1* mutations, but obviously it is speculative to conclude that such activation is caused solely by the presence of C1-inh polymers.

From this line of experiments we cannot conclude if the polymerization of mutated C1-inh takes place intracellularly, or if the polymers form in the blood stream. Furthermore it can be speculated that polymers of mutated C1-inh behave in an infectious manner and incorporate native C1-inh in the polymers. This phenomenon has been shown for other mutated polymerogenic SERPINS, i.e. antithrombin [24].

Our experiments find evidence, that polymerization of C1-inh transforms the major inhibitor of the FXII-dependent KKS to an activator of the very same system. In the subgroup of HAE patients presenting with C1-inh polymers, the polymers may play a central role as a trigger

of HAE attacks. So far *SERPING1* mutations associated with HAE have been regarded as loss of function mutations, but our results demonstrate, that polymerogenic mutations may also be classified as gain of function mutations. Other serpinopathies with extracellular serpin polymer depositions (e.g. α_1-antitrypsin deficiency) are also characterized by an increased inflammatory response [25], and we speculate that activation of the FXII-dependent KKS system might contribute to the inflammatory state observed in these diseases [10].

Conflict of interest

None of the authors have any conflict of interest.

Acknowledgments

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