Full Length Article

C1-esterase inhibitor enhances thrombin generation and spatial fibrin clot propagation in the presence of thrombomodulin

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ABSTRACT

Package inserts for C1-esterase inhibitor (C1INH) products include warnings for an elevated risk of possible thrombosis in certain individuals, referring to thromboembolic events (TEEs) that were reported to occur after C1INH infusions. However, the mechanism(s) that could explain possible development of TEEs due to C1INH remains unknown. In this work, we evaluated plausible impact of C1INH on the protein C (PC) anticoagulant system. We performed thrombin generation (TG) assays (TGA) and analyzed spatial fibrin clot propagation using thrombodynamics in plasma of individual donors after the addition of thrombomodulin (TM) and C1INH. The addition of C1INH was consistent with the plasma concentrations resulting from doses currently approved for the HAE treatment up to ones consistent with off-label use in patients with risk of inflammation. 16 IU/ml of C1INH significantly enhanced thrombin peak (TP) generation in the presence of 12 and 15 nM TM. TG enhancement was observed by the addition of C1INH to make concentrations equal to 2 and 4 IU/ml in some donor plasmas. C1INH addition in the presence of TM enhanced the stop time of spatial clot growth in Thrombodynamics assay. A chromogenic activity assay demonstrated that C1INH inhibited PC activation by thrombin in the presence of TM. Substitution of TM with APC in TGA attenuated the TP enhancing effect of C1INH. The collective results of the present study suggest a concentration dependent C1INH interaction with the PC system. This study introduces a plausible TM-dependent mechanism, that may explain reported TEEs via suppressed production of APC in the presence of C1INH.

1. Introduction

C1-esterase inhibitor (C1INH) is a multifunctional anti-inflammatory plasma protein, a vital down-regulator of complement, contact activation and fibrinolytic amplification cascades belonging to serpin superfamily [1–3]. While C1INH is best recognized as the only inhibitor of C1s and C1r of the complement system, thus preventing complement activation, it possesses a wide range of inhibitory activities toward several other proteases including factors XIa, XIIa and kallikrein in contact activation of the coagulation system, and plasmin and tissue plasminogen activator of fibrinolytic system [1,2]. C1INH also inhibits thrombin, although exhibits only a mild inhibitory effect on this coagulation enzyme [4,5]. The normal plasma concentration of C1INH in healthy individuals varies in the range of 0.15–0.30 mg/ml [2] which [corresponds approximately to 0.5–1.5 IU/ml [6,7]. C1INH quantitative or qualitative deficiency was identified as the primary cause of

hereditary angioedema (HAE), a rare potentially fatal autosomal disease, clinically manifested by recurrent acute attacks as swelling in the face, throat, hands, feet or abdomen [1,8]. Since 2008, several C1INH-based products have been licensed in the US for the amelioration of acute attacks or for prophylaxis of HAE [9–11] in doses ranging from 20 to 100 IU/kg and dosing results in restoration and maintenance of plasma concentrations. C1INH preparations have been proposed for and used off-label for other clinical conditions including sepsis, myocardial infarction and ischemic stroke at significantly higher doses than those indicated for C1INH replacement therapy in patients with HAE [2,12,13]. In 2000, formation of severe thrombus in the upper venous system was reported when patients were dosed with up to 500 IU/kg of C1INH [13]. As of 2011, according to the US FDA adverse event reporting system database, several types of thromboembolic events (TEEs); including deep vein thrombosis, pulmonary embolism and ischemic stroke were associated with C1INH over a range of indicated

and off-label doses [14]. TEEs in HAE patients following C1INH therapy are rare and have been associated with underlying risk factors and with very high doses of C1INH [12]. Nonetheless, prescribing information for currently approved plasma-derived C1INH products contains warning for a risk of TEEs that may accompany C1INH replacement therapies within the recommended dose ranges [12,14], as well as C1INH administration at higher doses for unapproved indications [12,13,15]. Since the first report of TEEs associated with C1INH, the biochemical mechanisms of the thrombosis development in certain patients remain unclear. The literature on C1INH and its influence on hemostasis suggest controversial results based of clinical and biochemical studies [12,16–20]. In vitro coagulation assays [17–19] suggest that C1INH may inhibit the coagulation activity of plasma, however, most of these studies did not evaluate the C1INH impact on the process of fibrinolysis or used the methods which were not precise enough. The exception is the study [20], that reports fibrinolytic activity in plasmas from HAE patients collected during acute attack with decreased C1INH levels is significantly higher than in plasmas from healthy controls with normal levels of C1INH. This observation was associated with elevation of soluble TM in plasma. However, this study did not specifically investigate thrombin generation and fibrinolytic processes in the presence of C1INH exceeding normal physiologic levels. Carefully considering all these studies and potential sites of C1INH interactions with coagulation we realized that the impact of C1INH on anticoagulation pathways of the Protein C (PC) system [21,22] has not been evaluated. Critical to this pathway is activation by the cofactor thrombomodulin (TM) which is normally located on the vessel wall and is not present in plasma. In this pathway thrombin cleaves PC after complex formation with TM, converting it to the activated form (APC). Cleavage of activated factors V and VIII by APC inactivates the positive feedback loops in blood coagulation [23]. TM stops spatial fibrin clot propagation in blood plasma [24–26], thereby TM is an important modulator of the clot size. It was previously shown that in the presence of TM, artificial thrombin inhibitors caused a paradoxical enhancement of thrombin generation (TG) [27–29]. It was suggested that these inhibitors also inhibit thrombin in the thrombin-TM complex, as well as free thrombin. In the present work, we hypothesized that C1INH may exhibit similar effect at certain concentration levels, which can vary between different patients. If correct, this hypothesis would suggest that C1INH may lead to a slower activation of PC and, as a result, lead to the formation of a greater amount of generated thrombin and enhanced fibrin clot growth. To investigate this plausible scenario, we evaluated individual donor plasma using a thrombin generation assay (TGA) and a novel technique called Thrombodynamics (TD) which allows the spatial propagation of fibrin clot investigation (Fig. 1).

2. Materials and methods

2.1. Plasma samples

Fresh acid-citrate-dextrose drawn whole blood from 48 healthy donors was obtained from the Department of Transfusion Medicine, Clinical Center, National Institutes of Health (NIH), Bethesda, MD. According to NIH Research Donor Program, the blood donors were at least 18 years old, weighted more than 110 pounds, were in excellent health without known heart, lung, kidney, or bleeding disorders, do not have a history of hepatitis since age 11, do not have a history of illicit drug use within the past 5 years, have not received clotting factor concentrates in the past 5 years, have not accepted money or drugs in exchange for sex in the past 5 years, and never have engaged in high risk activities for exposure to the AIDS virus. A health history questionnaire, examination of blood pressure and heart rate, evaluation of arm veins, and blood tests to determine blood cell counts and detect exposure to HIV and hepatitis viruses was done at the donor's assessment visit. Each donor signed the informed consent to participate in the study. Platelet poor plasma (PPP) was prepared using 15 min

centrifugation at 1500g. For TD assays thawed 20 donors-pooled plasma (Affinity Biologicals, Ancaster, ON, Canada) was used. All samples were obtained with NIH and FDA IRB approval under informed consent.

2.2. TGA

TG was measured using a specific thrombin fluorogenic substrate Z-Gly-Gly-Arg-AMC (Bachem, Bubendorf, Switzerland) initially developed by H.C. Hemker [30–32]. TGA was performed using the previously described original method that does not require thrombin-α_2-macroglobulin calibrator but does all the corrections according to the inner filter effect and substrate consumption [33–35]. Briefly, 68 μl of PPP was mixed with 20 μl of fluorogenic substrate (3.08 mM), 12 μl of C1INH (Berinert, CSL Behring, King of Prussia, PA, USA) in different concentrations or buffer A (20 mM HEPES and 150 mM NaCl, Sigma-Aldrich, St. Louis, MO, USA) in the wells of 96-well Costar plate (Fisher Scientific, Hampton, NH, USA). TM (Haematologic Technologies, Essex Junction, VT USA) and APC (Sigma-Aldrich, St. Louis, MO, USA) were added in different concentrations. The final concentration of the substrate was 513 μM. TG was triggered by PPP-reagent (Diagnostica Stago, Asnières sur Seine, France) containing tissue factor and phospholipids with added CaCl$_2$ (100 mM final concentration, Sigma-Aldrich, St. Louis, MO, USA). Fluorescence was measured using Synergy HTX plate reader by BioTek, Winooski, VT, USA. Obtained TG curves after background subtraction were calibrated on the special well containing PPP, substrate and 10 μM 3-methyl-4-aminocoumarin (AMC). Thrombin concentration was calculated using the Michaelis-Menten equation using the obtained values of AMC production rate. The data was processed by Origin software (OriginLab, Northampton, MA, USA). Undetectable thrombin generation was considered as a sample with thrombin peak (TP) equal to zero. In our work, we designed a modelling approach to a situation where different amounts of TM could contact plasma (either vessel wall bound or soluble) as is the case in different vessels that contact blood in circulation. Thus, we are showing data in the case of high vessel wall TM content that C1INH elevates TG. In this case, the comparison between the samples with both TM and C1INH cannot be directly compared to either C1INH or TM alone. Therefore, samples without TM or C1INH are not the control for the sample with both of agents.

2.3. PC activation chromogenic assay

The assay was designed close to one described earlier [36]. 3.75 nM thrombin, 25 nM TM and C1INH in concentrations equal to 2 and 8 IU/ml were mixed in buffer A containing 2 mM of CaCl$_2$ in the wells of 96-well plate and incubated for 10 min under 37 °C. After that 50 nM of PC was added to the wells and the plate was incubated for 10 min. To stop the reaction 12.5 U/ml of hirudin (Sigma-Aldrich, St. Louis, MO, USA) was added to each well. The chromoganic substrate S-2366 (Chromogenics, Uppsala, Sweden) was added such that a final concentration of 500 μM per well was achieved prior to measurement of APC activity at 405 nm. The absorption data was processed using Origin software. The same procedure was carried out with 2 nM APC (Sigma-Aldrich, St. Louis, MO, USA) and increasing concentrations of C1INH to demonstrate that APC was not inhibited by C1INH.

2.4. TD measurements

Spatial propagation of the fibrin clot with and without thrombin was measured by continuous registration of side scattering of the clot and fluorescence using a Thrombodynamics Analyzer System Model T2-T with all reagents purchased from the manufacturer (HemaCore LLC, Moscow, Russian Federation) as described previously [25,26,35,37–41]. Briefly, 120 μl of pooled PPP containing 16 IU/ml C1INH and various concentrations of TM or buffer at the same volume
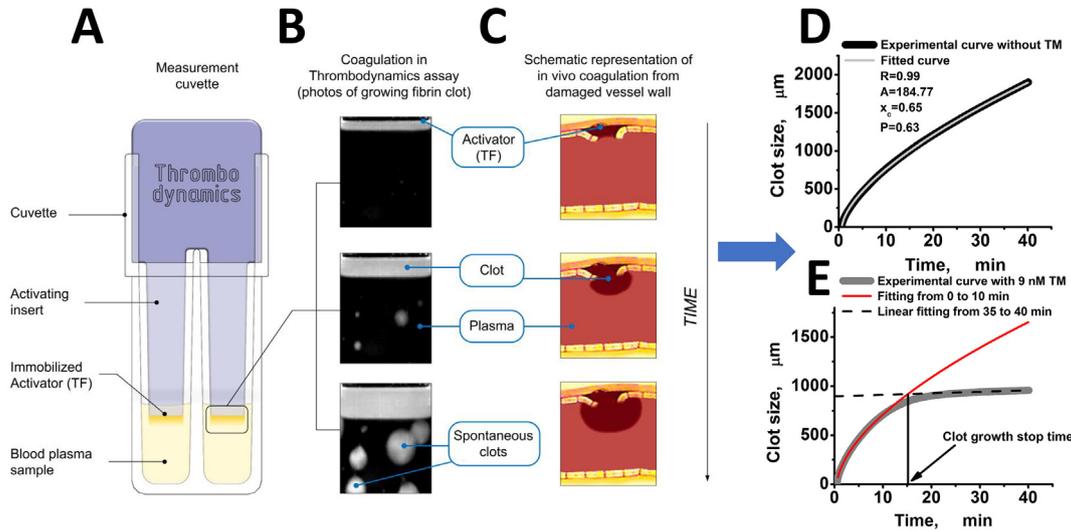
Fig. 1. Basic principles of TD assay and results proceeding. A. Schematic image of experimental cuvette with activator placed inside for TD measurement. B. Sequential side scattering photographs of the clot growth from the activator, and spontaneous clots in the bulk of the sample. C. Schematic picture of clot growth from a damaged vessel wall in vivo. D. Representative dependence of clot size versus time without TM addition. Curve fitting was determined with the following equation: $y(x) = A \cdot (x - x_C)^P$. E. Determination of the time dependence associated with stopping clot growth in the plasma with TM addition. The experimental curve in fitted by $y(x) = A \cdot (x - x_C)^P$ from 0 to 10 min and with line from 35 to 40 min. The time of the crossing of these two curves is defined as the clot growth stopping time.

was mixed with Reagent I which contained inhibitor of contact pathway activation and fluorogenic substrate and with 5 μl of standard lipid suspension. After 15 min incubation, PPP was mixed with Reagent II containing CaCl₂ and transferred to a channel of 2-channel experimental cuvette (Fig. 1A). The experiment was started after the plastic activator with tissue factor immobilized on its surface was inserted into the cuvette (Fig. 1B). At the end of each experiment clot size as a function of time was obtained (Fig. 1D). In the case of added TM, the clot growth stop time was calculated as shown in Fig. 1E. The data was processed using software provided by the manufacturer and by Origin (OriginLab Corp.) (Fig. 1, D, E).

3. Results

3.1. Supra-physiological concentrations of C1INH enhance TG in donor plasma

Fig. 2 shows that addition of 16 IU/ml C1INH (approximately 500 IU/kg) without TM significantly decreases TG. All the donors demonstrated reduced TP without TM (Fig. S1A, Table S1). As the concentration of TM increased, the inhibitory effect was decreased. When TM concentrations reached 9 nM the TP distributions were not different. Finally, at concentrations of 12 and 15 nM TM, the addition of increasing C1INH lead to greater TP values (up to 3 times) than without the addition of C1INH. Most donors in this study demonstrated greater TP values after addition of C1INH (Fig. S1B, Table S2).

3.2. C1INH in different concentrations increases TG in the presence of TM

Fig. 3 demonstrates the dependence of TP on C1INH concentration in the presence of 12 and 15 nM TM. As observed for PPP samples from 14 healthy donors, addition of C1INH to the level of 2 to 8 IU/ml (final concentration in PPP samples) does not lead to a significant increase of the TP (Fig.3 A, B). 2 and 4 IU/ml C1INH content significantly decreased TP, but in several donors, an increase was observed (Tables S3-S4). C1INH concentrations equal to 12 and 16 IU/ml resulted in TP values that were significantly higher than those without C1INH.
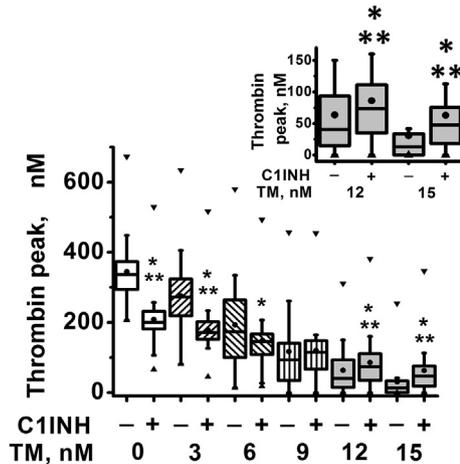
Fig. 2. TM changes the effect of C1INH on thrombin generation. Box plot of TP distribution in the presence of different concentrations of TM and 16 IU/ml C1INH added to individual donor PPPs. Upper horizontal line of box indicates the 75th percentile, and the lower horizontal line of box – indicates the 25th percentile, horizontal line inside box – median, circle inside box – mean value, horizontal lower and upper bars outside box – respectively 10th and 90th percentiles, ▼ – maximal value, ▲ – minimal value, * - significant difference between the TP values from PPPs with and without C1INH by Wilcoxon test, ** - significant difference between the TP values from PPPs with and without C1INH by sign test, N = 20, p = 0.05.

3.3. C1INH extends fibrin clot growth time when added to donor plasma

As it is shown at Fig. 4A, in the absence of TM the addition of C1INH reduces the clot growth velocity. Video 1 shows the experiments in absence of TM with and without C1INH respectively. In the presence of 9 nM TM, C1INH enhances the time of stopping of clot growth (Fig. 4 A, B). Video 2 shows the experiments in presence of TM with and without C1INH respectively. The videos are accelerated, and 1 s in the videos represents 2 min of experiments. Thus, TD confirms the results that were obtained by TGA.
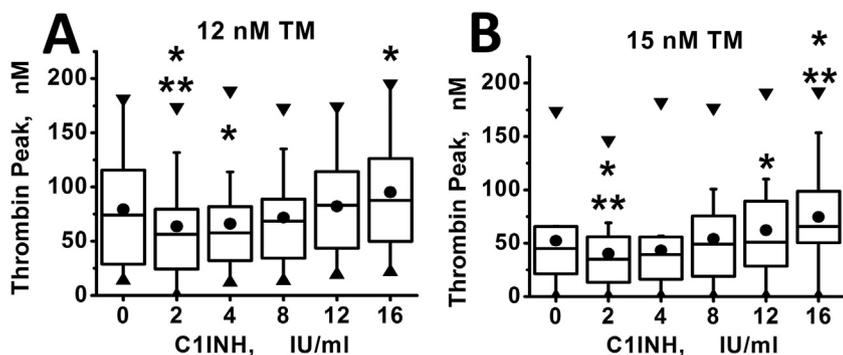
Fig. 3. Thrombin generation with TM addition is affected by C1INH in differently and is dependent on C1INH concentration added to individual donor PPP. A, B. Box plots of TP distributions in the presence of 12 nM (A) and 15 nM (B) TM and different concentrations of C1INH. Upper horizontal line of box indicates the 75th percentile, lower horizontal line of box – 25th percentile, horizontal line inside box – median, circle inside box – mean value, horizontal lower and upper bars outside box – respectively 10th and 90th percentiles, ▼ – maximal value, ▲ – minimal value, * - significant difference by Wilcoxon test, ** - significant difference by sign test, $N = 14$, $p = 0.05$ between the TP values from PPPs with different concentrations of C1INH comparing to the left box of TP values from PPPs without C1INH. C. Representative dependence of TP on C1INH concentration with added 12 and 15 nM TM obtained in PPP of donor 13 in Tables S3 and S4.
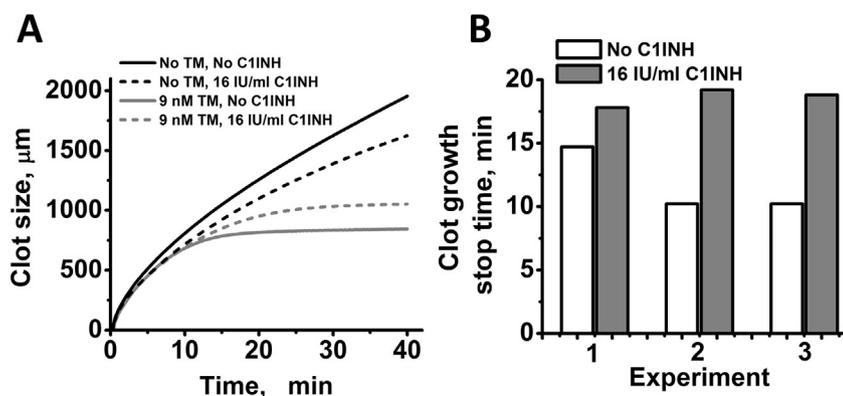
Fig. 4. Influence of C1INH on spatial clot growth in TD assay. A. Representative curves of clot size versus time with the presence or absence of C1INH and TM added to individual donor PPP. B. Clot growth stop times values obtained in 3 similar experiments in 2-channel cuvettes in pooled PPP with 9 nM TM in the presence and absence of added C1INH. White column represents the channel with PPP without C1INH, the grey one represents the channel with PPP in the presence of C1INH.

3.4. C1INH inhibits APC production by thrombin-TM complex

Fig. 5 demonstrates that C1INH reduces chromogenic activity of APC generated from PC by thrombin in the TM presence. That suggests that C1INH addition to plasma in TG and TM experiments leads to a reduction in APC production and the addition of C1INH did not inhibit APC itself as it was shown previously [42]. Moreover, our experiment suggests that APC activity was slightly enhanced in the presence of 16 IU/ml C1INH (Fig. S2).
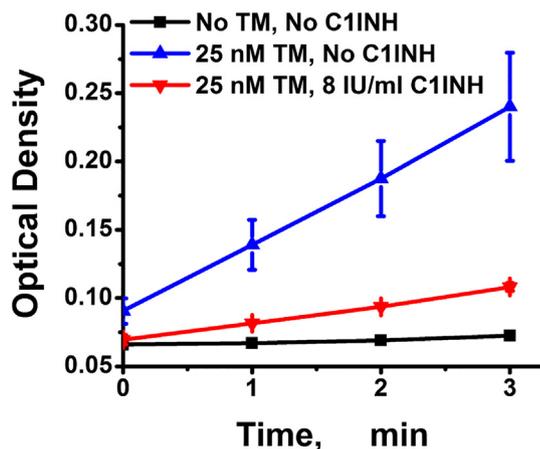
3.5. Substitution of TM with APC attenuates the effect of C1INH on TP in donor plasma

If the complex thrombin-TM is the only mechanism of enhancing TP by C1INH, substituting TM by APC, which is not inhibited by C1INH [42] may revert TP enhancement to TP reduction. As a result, TP values in the presence of C1INH should be lower than without C1INH in the presence of APC. In half of the donors C1INH indeed showed the shift from TG enhancement to reduction (Fig, S3A, Table S5). But the results from 6 donors out of 14 demonstrated the same effect of increasing of TP by C1INH (Fig. S3B. Table S5). Taken together C1INH did not change TP significantly in the presence of APC (Fig. 5A). This result suggests that C1INH suppresses the PC system by additional mechanisms. For example, these mechanisms may involve interactions with protein S and complement system.

4. Discussion

In this study, we evaluated whether C1INH inhibits the thrombin-TM complex and the plasma concentrations at which this inhibition may be relevant. The results reported here support a C1INH concentration dependent inhibition of thrombin-TM complex and suggests that the PC system is the likely site where C1INH contributes to thrombosis. Further, we demonstrate that addition of TM to PPP changes thrombin generation upon C1INH addition. In experiments presented here, C1INH acted as a dose-dependent promoter of coagulation in the presence of TM. The amount of TM that comes into contact with blood in vivo differs across blood vessel types and sizes, therefore, we evaluated a wide range of TM concentrations of added to donor plasma samples. The concentrations used in the present study exceed the reported concentrations of soluble TM in human plasma [20,43], nonetheless circulatory TM (plasma and luminal vascular wall bound) that comes in contacts with blood is in excess of plasma concentrations alone, since TM is primarily located on the vessel wall. It should be also

Fig. 5. C1INH inhibits APC production. The representative result of influence of C1INH on the optical density rate of p-nitroaniline from chromogenic substrate S-2366 cleaved by APC generated from PC in the presence of 3.75 nM thrombin, 25 nM TM and 8 IU/ml of C1INH added to buffer A. Each curve is the mean of two wells of the 96-well plate with the same content.

mentioned that soluble TM is released into plasma by enzymatic cleavage of the non-soluble form attached to the vascular endothelium [44,45]. Paired statistical assays demonstrated that only additions of 12 and 15 nM TM to donor plasmas enhanced TP significantly. However, in 12 donors, this enhancement was observed in the presence of lower concentrations (Tables S1, S2, donors 2, 3, 6, 7, 8, 9, 11, 13, 14, 16, 19, 20). Therefore, the effect we observed could also be donor specific. This observation is consistent with the infrequency of thrombotic events in HAE patients treated with C1INH, but also suggests that coagulation assays sensitive to the PC system could be useful to help to define safe dosing levels for novel C1INH indications where higher plasma concentrations are required.

To expand on this, we also investigated PPP by adding increasing concentrations of C1INH to induce TG in the presence of 12 and 15 nM TM. We evaluated these concentrations because they showed statistically significant enhancement of TP upon C1INH addition in PPP. Here, we were interested in defining the highest concentration of C1INH that did not induce TG following addition to PPP. Upon completion of experiments, we found that the switch from TG inhibition to TG promotion depends not only on the donor and TM concentration, but also on C1INH concentration added to PPP (Tables S3 and S4). 2 and 4 IU/ml of added C1INH resulted in a reduction of TP that was observed for most donor samples. Significant TP enhancement was observed when greater than 8 IU/ml of C1INH were added to PPP. The fact that in the presence of 12 nM of TM in Fig. 3A there was no significant C1INH enhancing effect opposite to Fig. 2. That also indicates that the effect of C1INH on TG is very donor specific. In our studies plasma concentration corresponding to 8 IU/ml of C1INH would be achieved by a dose of approximately 250 IU/kg, which is in a range of doses reported to be associated with TEEs [13,15]. It should be mentioned that we observed the effect of TG enhancement by C1INH only at concentrations of C1INH that are much greater than physiological. Nonetheless, these higher concentrations were suggested as a possible cause for the reported cases of TEEs that occurred after C1INH administration [13,15]. Also, these elevated levels of C1INH can be reached locally at venous access sites. Thus, our in vitro results are consistent with the clinical data describing thrombogenic events. Therefore, the plausible mechanism that we consider here by demonstrating TMs key role in turning C1INH inhibitory potency from anticoagulant effect to a potentially procoagulant one, provides a viable explanation for the reported TEEs associated with C1INH in some patients. Interestingly, it is important that in some donors' plasmas we observe addition of C1INH to achieve concentrations of 2 to 4 IU/ml did enhance TP. Our in vitro addition of 2 IU/ml C1INH to PPP would be an expected plasma concentration after a C1INH dose of approximately to 60 IU/kg and is consistent with approved doses [10]. A 2014 review by Crowther et al. [12] suggests that TEEs associated with C1INH therapy are rare and mostly occur in the patients with already existing underlying thromboembolic risk factors. Indeed, as an anti-inflammatory and anti-coagulation agent, C1INH should not contribute to thrombosis in patients or toward indicators of thrombosis in donor plasma. However, the present study evaluates an unexplored mechanism where C1INH interacts with the PC system in our in vitro model. This newly defined point of interaction between C1INH and the coagulation system may be relevant in some patients with thrombogenic risk factors. It should be also mentioned that even the modern methods of thrombosis diagnostics still leave some cases undiagnosed, especially in the patients with small thrombosis or peripheral (military) pulmonary embolization [46].

To verify the results, we obtained by TGA, we tested PC system function of modulating the spatial propagation of fibrin clot. TM addition to plasma stops the clot growth, but does not influence on its initial phase [24–26]. In our experiments, TM changed the behavior of spatial clot growth in the same way. This is consistent with our initial experimental results. In the absence of TM, C1INH acts as an inhibitor of the intrinsic coagulation pathway, at the level of factor XIa. This

pathway was shown to play a crucial role in promoting spatial propagation of fibrin clot formation and growth in plasma when the coagulation activator is far away from the clot border [24,25,40]. In the absence of TM, C1INH inhibited the rate of this propagation (Fig. 4A). After the addition of TM, this propagation phase disappeared, and the effect of C1INH inhibition on intrinsic pathway was no longer of relevance. However, we observed that addition of TM and C1INH delayed the stopping time of clot growth and enhanced final fibrin clot size in PPP (Fig. 4B).

To confirm that C1INH reduced PC activation by the complex of thrombin-TM we performed an APC generation chromogenic activity assay. As far as C1INH is known to interact with thrombin [4], we supposed that it can also interact with thrombin-TM complex. We have shown that C1INH reduces the activity of generated APC (Fig. 5). Interestingly, C1INH is not considered to be a strong thrombin inhibitor [4]. Therefore, further investigation of the mechanism of C1INH interaction with thrombin-TM complexes will provide more information on C1INH role in hemostasis.

C1INH inhibits thrombin [4] in the presence and absence of TM, but does not inhibit APC [42] (Fig. S2). That's why the substitution of TM with APC in TG assay could switch the C1INH effect on TG from enhancement to reduction. Our results are consistent with previous data that investigated the impact of APC on TG [29,30,47,48]. The TG inhibition effect of APC is reached by the same concentrations that were used in this study (Fig. 6 and Table S5). For some donor plasma samples we observed a TG reduction after the addition of C1INH and APC (Fig. S3A, Fig. 6), while increased TG was observed in other donor plasma samples (Fig. S3B, Fig. 6). This observation suggests that there is at least one additional mechanism that can influence TG induction after addition of TM and C1INH to donor plasmas. One possibility is that an interaction could also occur between C1INH and the APC cofactor protein S [49,50] with complement.

It should be mentioned that we used only PPP from healthy donors not patients with HAE or other diseases where C1INH products are used. However, prior to using the actual patients' blood it was necessary to define the plausible mechanism(s) of C1INH thrombogenicity and to explain why C1INH was never identified as a possible pro-coagulant in the earlier reported in vitro experiments [18,19]. The protocol we suggest in this study will be next used to investigate plasmas of patients with various clinical conditions for which treatment C1INH products
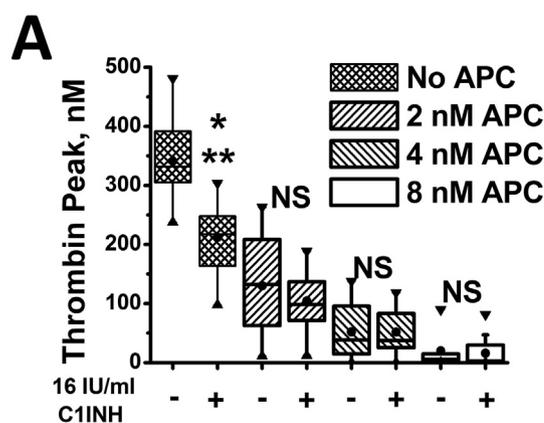
Fig. 6. Substitution of TM by APC partially removes the effect of C1INH enhancing of TG. Box plot of TP distributions in the presence of different concentrations of APC with and without C1INH after addition of each to donor PPP. Upper horizontal line of box means 75th percentile, lower horizontal line of box – 25th percentile, horizontal line inside box – median, circle inside box – mean value, horizontal lower and upper bars outside box – respectively 10th and 90th percentiles, ▼ – maximal value, ▲ – minimal value, * - significant difference by Wilcoxon test between the TP values from PPPs with and without C1INH, ** - significant difference by sign test between the TP values from PPPs with and without C1INH, $N = 14$, $p = 0.05$.

are currently used. Further studies of TG in the presence of TM on plasmas from the patients with HAE, during attacks of edema and on C1INH therapy will help develop a better understanding of the effects of C1INH on TG, the PC system and hemostasis in general.

The other limitation of this study is that it was not focused on the other potential reason of TEEs development caused by C1INH. This other potential mechanism can be linked with fibrinolytic system inhibition. The further studies using plasmin generation assay will be conducted in the future to study this possibility.

In conclusion, the present study performed in our in vitro model systems provides data to support that thrombin inhibition can enhance coagulation. It is known that synthetic thrombin inhibitors can increase thrombin generation [27–29]. We demonstrate that C1INH, a natural thrombin inhibitor, behaves similarly, and show that C1INH may enhance thrombin production in the presence of TM. This effect is paradoxical to C1INHs expected role in coagulation, but may help reconcile the observation of thrombosis in some patients receiving therapy as well as some reports of thrombotic risk associated with high dose therapy. Taken together with previous studies, the mechanism of thrombin-TM complex inhibition might be universal for all the drugs that have antithrombin activity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.thromres.2019.02.013.

Disclosure of conflict of interests

The authors state that they no conflict of interest. The findings and conclusions in this article have not been formally disseminated by the Food and Drug Administration and should not be construed to represent any Agency determination or policy.

Addendum

P.W. Buehler and E. Karnaukhova developed the research concept, designed and supervised the project, and were involved in the data analysis and interpretation, critical writing and revisions of the manuscript. I. D. Tarandovskiy designed and conducted experiments, analyzed the data, drafted the manuscript and was involved in the manuscript processing and revisions. F. I. Ataullakhanov evaluated the research concept and was involved in the data analysis and interpretation and reviewed the manuscript for scientific content. The manuscript has been approved by all authors.

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