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Full Length Article

Stimulation of thrombin- and plasmin-mediated activation of thrombin-activatable fibrinolysis inhibitor by anionic molecules

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ABSTRACT

Background: Thrombin-activatable fibrinolysis inhibitor (TAFI) is a proenzyme that, once activated, attenuates fibrinolysis by removing C-terminal lysine residues from partially degraded fibrin. TAFI can be activated by thrombin or plasmin via a cleavage at Arg92 that removes the activation peptide from the enzyme, TAFIa. Thrombomodulin enhances thrombin-mediated TAFI activation and glycosaminoglycans enhance plasmin-mediated TAFI activation. The aim of this study was to investigate whether there are other anionic molecules that function as a cofactor for thrombin- or plasmin-mediated TAFI activation.
Methods: TAFI activation by thrombin or plasmin was studied in the presence of physiological anionic molecules (polyphosphate, heparin, hyaluronan, DNA and dermatan sulfate) and the non-physiological sodium dodecyl sulfate (SDS). Additionally, the effect of these molecules on TAFIa stability and on thrombin-mediated protein C activation was determined.
Results: Unfractioned heparin, calcium-saturated polyphosphate with an average chain length of 100 monomers (Ca-PolyP100) and SDS significantly enhanced TAFI activation by thrombin and plasmin. Dermatan sulfate and polyphosphates with sodium as counter ion (Na-PolyP700, Na-PolyP100 and Na-PolyP70) enhanced plasmin-mediated but not thrombin-mediated TAFI activation. Additionally, unfractioned heparin, Ca-PolyP100 and SDS enhanced thrombin-mediated protein C activation. The different nature of anionic molecules capable of enhancing TAFI and protein C activation suggests a general mechanism.
Conclusions: Several anionic molecules function as (potent) cofactors for thrombin- and plasmin-mediated TAFI activation and thrombin-mediated protein C activation. This may imply that thrombin and plasmin activity is regulated in the vasculature by more cofactors than currently appreciated.
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1. Introduction

The product of the CPB2 gene known as thrombin-activatable fibrinolysis inhibitor (TAFI) is a zinc-dependent metallocarboxypeptidase (EC 3.4.17.20) that is synthesized in the liver and released into the bloodstream. TAFI circulates in plasma at a concentration of 70–275 nM [1]. TAFI is also known as procarboxypeptidase B2 (proCPB2), procarboxypeptidase U (proCPU), procarboxypeptidase R (proCPR) and plasma procarboxypeptidase B [2–5]. Once active, TAFIa attenuates fibrinolysis by cleaving C-terminal lysine residues from partially degraded fibrin that function as cofactor for plasminogen activation.

TAFI is activated by thrombin or plasmin via a proteolytic cleavage at Arg92 that separates the activation peptide (Phe1-Arg92) from the catalytic domain (Ala93-Val401). Thrombin-mediated TAFI activation is inefficient but can be enhanced three orders of magnitude in the presence of thrombomodulin [6]. Plasmin is an ~8-fold more efficient TAFI activator than thrombin, and glycosaminoglycans enhance plasmin-mediated TAFI activation ~16-fold [7].

Thrombin is the central enzyme in coagulation. Its main function is to assist in the formation of a blood clot by activating platelets and by converting soluble fibrinogen into insoluble fibrin fibers. Thrombin contains a Na^{+} binding loop and two anion binding sites (exosites 1 and 2) that can change thrombin's substrate specificity when occupied by cofactors [8,9]. In the absence of cofactors, thrombin can cleave fibrinogen, FV, FVIII and FXIII. In a blood clot, thrombin can bind to fibrin via exosite 1 to enhance FXIII activation [10]. Furthermore, thrombomodulin, a membrane protein present on the endothelium, can also bind to exosite 1 and promotes TAFI and protein C activation [11]. GpIbα, present on platelets, can bind to exosite 2 to promote PAR1 activation and GpV cleavage and binding of thrombin to GPIb leads to FXI activation [10].

Abbreviations: TAFI, thrombin-activatable fibrinolysis inhibitor; TAFIa, activated thrombin-activatable fibrinolysis inhibitor; SDS, sodium dodecyl sulfate; PolyP, polyphosphate; PPACK, H-D-Phe-Pro-Arg-chloromethylketone; CMC, critical micelle concentration.

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Additionally, polyphosphates have been shown to interact with thrombin exosite 2 to promote FV activation [12]. Furthermore, heparin can bind to exosite 2 which will promote thrombin inactivation by antithrombin [13].

Plasmin is responsible for dissolving the fibrin network and is therefore the central enzyme in fibrinolysis. Plasmin activity is enhanced in the presence of glycosaminoglycans. However, plasmin activity can be inhibited via an allosteric site [14]. It has been a matter of debate whether thrombin or plasmin is the physiological activator of TAFI. In a baboon sepsis model, the importance of thrombin–thrombomodulin mediated TAFI activation was revealed [15]. However, the importance of plasmin-mediated TAFI activation has been demonstrated in vitro using a monoclonal antibody that specifically inhibits plasmin-mediated TAFI activation [16] suggesting that both enzymes contribute to TAFI activation [17]. Despite the low catalytic efficiency of thrombin for TAFI activation, in vitro assays in the absence of thrombomodulin show that thrombin is capable of activating enough TAFI to attenuate fibrinolysis [18]. It has been proposed that the rapid increase of thrombin generation during the propagation phase in coagulation results in sufficient TAFI activation to attenuate fibrinolysis [19].

Many of the molecules known to affect thrombin and plasmin activity are (poly)anionic in nature. Therefore, the aim of our study was to perform a direct comparison of the effects of polyanionic molecules on thrombin- and plasmin-mediated TAFI activation. We selected physiological anionic molecules (different forms of polyphosphates, heparin, hyaluronan, DNA and dermatan sulfate). During this study we coincidentally discovered that sodium dodecyl sulfate (SDS) was a very potent stimulator of thrombin- and plasmin-mediated TAFI activation. SDS is not a physiologically relevant molecule but after this finding we included it in the study as it may provide valuable information on the mechanism of TAFI activation enhancement by cofactors.

2. Materials and methods

Unfractioned heparin was from LEO Pharma bv. (Breda, Netherlands, 1 U/ml is estimated to correspond to ~2 μg/ml or ~0.15 μM). Sodium dodecyl sulfate (SDS) and Tween-20 were from Merck (Darmstadt, Germany). H-D-Phe-Pro-Arg-chloromethylketone (PPACK) was purchased from Bachem (Bubendorf, Switzerland). Plasmin (the concentration of the stock was 10 U/ml, corresponding with ~1.25 mg/ml or ~14.7 μM) and aprotinin were from Roche Nederland (Woerden, Netherlands). Hyaluronan (MW: 60 K, 500 K and 15 M) was from Sigma Aldrich (St. Louis, MO, USA). Thrombin was a generous gift of Dr. W. Kisiel (University of New Mexico, Albuquerque, NM, the concentration of the stock was 167 NIH units/ml, corresponding to ~50 μg/ml or ~1.3 μM). Calcium-saturated polyphosphate with an average chain length of 100 monomers (Ca-PolyP100) was a generous gift of Dr. Thomas Renné (Karolinska Institutet, Stockholm, Sweden), PolyP700 and PolyP100 with sodium as counter-ion (Na-PolyP700 and Na-PolyP100) were from Kerafast (Boston, MA, USA) and PolyP70 with sodium as counter-ion (Na-PolyP70) was from BK Giuliani GmbH (Ladenburg, Germany). Batroxobin and rabbit lung thrombomodulin were from American Diagnostica (Greenwich, CT). Thrombomodulin was used directly from the commercial stock. Chromogenic substrate S-2366 was purchased from Chromogenix (Mölndal, Sweden). Human DNA was purified from whole blood using Gentra Puregene bloodkit (Qiagen, Valencia, CA, USA). Plasma TAFI was purified as previously described [20].

2.1. TAFI activation

TAFI (100 nM, all concentrations are final concentrations), derived from human plasma, was added to a premix of thrombin (200 nM) or plasmin (200 nM) with or without $CaCl_2$ (5 mM) in 50 mM Hepes, 50 mM NaCl, pH 7.4 in the absence or presence of unfractioned heparin, dermatan sulfate, Ca-PolyP100, Na-PolyP100, Na-PolyP700, Na-PolyP70, SDS with 0.01% Tween-20, hyaluronan or DNA for 15 min at RT. After incubation, PPACK or aprotinin (both 1 μM) was added to inhibit thrombin or plasmin activity respectively. TAFIa activity was measured using the method developed by Willemse et al. [21] as previously described [20].

2.2. TAFI activation time course

TAFI (100 nM) was added to a premix of thrombin (8 nM or 200 nM) and $CaCl_2$ (5 mM) or plasmin (8 nM or 200 nM) in 50 mM Hepes, 50 mM NaCl, pH 7.4 in the absence or presence of 5 U/ml unfractioned heparin, 50 μg/ml Ca-PolyP100 or 70 μg/ml SDS with 0.01% Tween-20 at 22 °C. In time, aliquots of the mix were withdrawn and added to PPACK (1 μM, for thrombin) or aprotinin (1 μM, for plasmin) and placed on ice. For $t = 0$, PPACK (1 μM, to inhibit thrombin activity) or aprotinin (1 μM, to inhibit plasmin activity) was added to the activation mix and incubated for 2 min before adding TAFI. The $T = 0$ mix was then incubated for 30 min at 22 °C. TAFIa activity was measured as described above.

2.3. TAFIa thermal stability

The half-life of TAFIa in the absence or presence of anionic molecules was determined by incubating 125 nM of TAFI with a premix of thrombin (8 nM), thrombomodulin (16 nM) and $CaCl_2$ (5 mM) in 50 mM Hepes, 50 mM NaCl, pH 7.4 for 15 min at 22 °C. After activation, PPACK (1 μM) was added to stop thrombin activity. After 1 min, TAFIa (100 nM) was added to 5 U/ml unfractioned heparin, 50 μg/ml Ca-PolyP100 or 70 μg/ml SDS with 0.01% Tween-20 and TAFIa was preheated to 37 °C for 2 min. In time, aliquots of TAFIa were placed on ice and activity was measured as described above. The first aliquot was assigned as $t = 0$ and set at 100% activity. The half-lives were determined with nonlinear regression with Graphpad Prism version 5.01 (Graphpad, San Diego, CA, USA). Statistical analysis was performed in Graphpad Prism using an unpaired *t*-test with Welch's correction.

2.4. Protein C activation

Protein C (20 μM) was added to a premix of thrombin (40 nM) and $CaCl_2$ (5 mM) in 50 mM Hepes, 50 mM NaCl, pH 7.4 in the absence or presence of unfractioned heparin, Ca-PolyP100 or SDS with 0.01% Tween-20 for 45 min at 37 °C. After incubation, 5 μl of the mix was added to a mix of chromogenic substrate S-2366 (1 mM) and hirudin (20 U/ml) in 50 mM Hepes, 50 mM NaCl, pH 7.4 (final volume 50 μl). Protein C activity was measured kinetically for 10 min at 405 nm at 37 °C in a Versamax 96-well plate reader. APC activity in the absence of a cofactor was set to 100%.

2.5. Thrombin activity

Thrombin (10 nM), was added to unfractioned heparin, Ca-PolyP100 or SDS with 0.01% Tween-20 for 1 min at 37 °C. Thrombin activity was measured kinetically after the addition of 0.5 mM chromogenic substrate S-2366 for 10 min at 405 nm at 37 °C in a Versamax 96-well plate reader. Thrombin activity in the absence of an anionic molecule was set to 100%.

2.6. Fibrin generation

Thrombin (5 nM) or batroxobin (4 BU/ml or 40 μg/ml) was added to fibrinogen (5 mg/ml) in the absence or presence of unfractioned heparin, Ca-PolyP100 or SDS with 0.01% Tween-20 at 37 °C. Turbidity was measured kinetically at 405 nm at 37 °C in a Versamax 96-well plate reader and the “clot time” was defined as the time to half-maximal turbidity. The time to half-maximal coagulation in the absence of an anionic molecule was set to 100%.

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