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Regular Article

Heparin affinity of factor VIIa: Implications on the physiological inhibition by antithrombin and clearance of recombinant factor VIIa

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ABSTRACT

Factor VIIa (FVIIa), a trypsin-like serine protease, plays an essential role in haemostasis by initiating the coagulation in complex with its cofactor, tissue factor (TF). The TF pathway inhibitor is the main physiological inhibitor of FVIIa-TF complex, but FVIIa can also be inhibited by antithrombin, although little is known about this process.

Functional analyses by second order kinetic determination and identification of FVIIa-antithrombin complex by electrophoresis, evaluating the effect of different cofactors: pentasaccharide, low molecular weight heparin (LMWH) and unfractionated heparin (UFH), confirmed that any activation of antithrombin significantly enhanced the inhibition of FVIIa. The analysis of the binding of FVIIa to heparin by surface plasmon resonance identified a high affinity interaction under physiologic conditions ($K_D = 3.38$ μM, with 0.15 M of ionic strength) strongly dependent on Ca^{2+} and ionic strength. This interaction was verified in cell models, indicating that FVIIa also binds to the surface of endothelial cells with similar requirements. Structural modeling suggests the presence of a potential exosite II in FVIIa. However, the binding of heparin did not display significant changes on both the intrinsic fluorescence and the associated functional consequences of FVIIa.

These results indicate that FVIIa binds to exposed glycosaminglycans of the endothelium through an exosite II, structurally similar to that reported for thrombin and suggested for FIXa. This binding may favor its inhibition by antithrombin in the absence of TF, contributing to the physiological control of this protease. This process may also play an important role in the clearance of recombinant FVIIa administered to patients.

Introduction

Most procoagulant reactions of the clotting cascade are carried out by enzymes that belong to the family of trypsin-like serine proteases. The first actor of this cascade is factor VII (FVII), a serine protease that circulates in blood in the zymogen form. The active form of FVII (FVIIa), comprising a light and heavy chain linked by a disulfide bond, is generated by the cleavage of the Arg-152–Ile-153 peptide bond by different proteases, including FXa [1–4], FIXa [4–6], thrombin [7] or, in an autocatalytic manner of FVIIa after binding to its main cofactor tissue factor (TF) [8–14], which is a transmembrane glycoprotein that is exposed upon vascular injury [15,16]. TF-bound FVIIa is an efficient activator of FIX and FX which, on the surface of activated platelets, ultimately results in a burst of thrombin, fibrin deposition and the formation of a haemostatic plug [17]. Moreover, recombinant FVIIa (rFVIIa) is an increasingly used agent that efficiently reduces hemorrhage in different settings [18]. The crystal structure of FVIIa in complex with the extracellular part of TF has been solved [19] and the dependency of calcium for its catalytic activity has also been described [20]. The enzymatic activity of FVIIa is regulated by two physiological inhibitors. The FVIIa-TF complex is efficiently inhibited by the Kunitz inhibitor TF Pathway Inhibitor (TFPI) in the presence of FXa by forming an inhibitory TFPI-FXa-FVIIa-TF quaternary complex [21,22]. FVIIa can also be inhibited by the serpin antithrombin [20], which is generally considered to be the primary plasma inhibitor of FVIIa *in vivo* [23–25].

Antithrombin is the main haemostatic anticoagulant, exhibiting a suicide inhibition mechanism over its target proteases [26]. In an

Abbreviations: FVIIa, Factor VIIa; TF, Tissue Factor; rFVIIa, recombinant FVIIa; LMWH, Low molecular weight heparin; UHF, Unfractionated heparin; SPR, Surface Plasmon resonance; TFPI, Tissue factor pathway inhibitor; RU, Response units.

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allosteric process, antithrombin becomes a more effective inhibitor after binding of heparin, which acts as a cofactor [27]. Nevertheless, all heparins do not display the same activation effect. Thus, effective pentasaccharide and low molecular weight heparins (LMWH) are sufficient for a fast inhibition of FXa, while heparin chains with more than 20 saccharides, present in unfractionated heparin (UFH) and to a minor degree, in LMWH, is required for effectively enhancing inhibition of thrombin [28,29]. In the latter case, the crystal resolution of this complex revealed a ternary interaction between antithrombin-thrombin-heparin with the UFH acting as a bridge that propagates the protease to the reactive centre loop of the inhibitor, restricting it to a two-dimensional reaction [28]. Additionally, it has been recently described that among those glycosaminoglycans located in the endothelium, only few of them are able to completely activate antithrombin, due to the absence of the required activating pentasaccharide [30]. In the case of the antithrombin-FVIIa complex, little is known on the requirements for an optimal interaction. Moreover, it is not clear how fast and to what extent FVIIa reacts with antithrombin *in vivo* and what implications this might have on the elimination of FVIIa from the circulation.

In this study, we have determined the heparin affinity of rFVIIa by surface plasmon resonance (SPR) and the binding of rFVIIa to long chain heparins as those exposed on the surface of the endothelial cells. Thus, our results may contribute to a better understanding of the mechanistic and physiological relevance of FVIIa/rFVIIa, its inhibition by antithrombin and its binding to the exposed glycosaminoglycans in the endothelium.

Materials and methods

Materials

Human thrombin was purchased from Calbiochem. rFVIIa was obtained from Novoseven (Novo Nordisk, Denmark). Purified antithrombin and plasma were used to perform our studies.

N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide (EDC) and biotin-XX-hydrazide were from Sigma (Madrid, Spain). Pentasaccharide (Fondaparinux; Arixtra®) was from Sanofi-Synthelabo, and LMWH (Bemiparin) or UFH were from Rovi (Madrid, Spain). The BIAcore X biosensor, HBS-EP buffer and streptavidine sensor chips (SA chip) were from BIAcore (GE Healthcare, Barcelona, Spain). The biotin quantitation kit was obtained from Pierce (Rockford, IL, USA).

Fluorescence measurements

The effect of heparin binding and calcium on rFVIIa was analyzed by fluorescence emission spectra on a Cary Eclipse spectrofluorometer. Intrinsic tryptophan emission spectra of purified rFVIIa incubated with UFH were monitored by an excitation at 295 nm and emission at 305 to 405 nm. The slit-widths were set at 5 nm for both excitation and emission and a constant scan rate of 400 nm/min was used. The final concentration of rFVIIa and UFH were 600 nM and 10 U, respectively.

Formation of antithrombin-FVIIa complexes and rates of inhibition

The formation of covalent complexes between antithrombin and rFVIIa were evaluated by mixing 0.5 μM antithrombin with 0.25 or 0.5 μM rFVIIa at 37 °C in a buffer containing 0.1 M Hepes, 150 mM NaCl, 10 mM $CaCl_2$, 2.5 mM $MgCl_2$, pH 7.5, in the presence or absence of 10 μM heparin (Pentasaccharide, LMWH or UFH). The complexes formed were detected by 8% SDS-PAGE under reducing conditions and western blot, as previously reported [31].

Second order association rate constants ($M^{-1}\cdot s^{-1}$) for inhibition of FVIIa by antithrombin and its complexes with different heparins were evaluated essentially as previously described [32]. Briefly, the rates of inactivation of rFVIIa by plasma antithrombin in the absence of heparin were measured under pseudo first-order conditions using a discontinuous assay. One hundred-microliter samples containing 10 μM inhibitor and 1 μM protease were incubated in the presence of 0.05 mg/mL polybrene in I = 0.15 buffer at room temperature (23 ± 2 °C). Reactions were quenched at various times by the addition of 1 mL of 10 mM S-2288 (Chromogenix, Izasa, Spain). The residual protease activity was measured from the initial rates of substrate hydrolysis monitored at 405 nm. For the heparin-catalyzed rates, 20-μL samples containing 0.5 μM antithrombin, 50 nM protease, and 0.1-1 μM heparin saccharide were incubated in the same buffer at room temperature, and the rates were measured at 405 nm over 60 seconds on a microplate reader (Synergy HT, Bioteck, UK). The pseudo first-order rate constants (k_{obs}) were obtained from the slopes of the plots of the natural logarithm of residual protease activity versus time of incubation. Second-order rate constants for the uncatalyzed reactions were obtained by dividing the observed pseudo first-order rate constant (k_{obs}) by the inhibitor concentration. Second-order rate constants, $k_{cat}/K_{m,}$ for the heparin-catalyzed reactions were taken as the slope of k_{obs} versus the total heparin concentration, since K_d under these conditions was always significantly less than the initial antithrombin concentrations.

FVIIa and antithrombin binding to endothelial cells

Py-4-1 is a hemangioma derived endothelial cell line isolated from a transgenic mouse carrying the polyoma virus early region. The cell line was kindly supplied by Dr. A. Rouzaut (CIMA, Navarra, Spain). Cells were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2. Cells were continuously passaged at confluence after treatment with trypsin-ethylenediaminetetraacetic acid buffered solution for over 100 passages. The human EA.hy 926 endothelial cell line was a kind gift from Dr C.-J. S. Edgell (University of North Carolina, USA). The cells were cultured in 75 cm^2 flasks in DMEM-GlutaMAX containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 mg/ml). The cells were sub-cultured twice a week at a ratio of 1:4.

Cells were plated at a density of $2.86\cdot10^5$ cells/cm^2 in 96-well dishes in DMEM containing serum.

The binding of rFVIIa and antithrombin to human endothelial cells was evaluated by adding 30 μL of 4.5 μM rFVIIa or 0.26 μM antithrombin to confluent cells extensively washed with saline solution, and incubated during 10 minutes. rFVIIa or antithrombin were pre-incubated in the presence and absence of 50 μM UFH for 3 min to distinguish the specificity of the interaction with glycosaminoglycans. Binding of rFVIIa was also evaluated at different ionic strengths from 0.05 to 0.3 M. Afterwards, supernatants were removed and transferred to a new 96-well plate, where 15 μL of 10 mM S-2288 substrate was added for evaluating the residual rFVIIa activity, or 33 μL of 8.57 nM thrombin and 20.9 μL of 1 mM S-2238 substrate (Chromogenix, Izasa, Spain) was supplemented to measure the residual inhibitory activity of antithrombin. The absorbance of the reaction was measured at a wavelength of 405 nm using a plate reader (Synergy HT, Bioteck, UK).

Biotinylation of heparin and immobilization onto SA chip

UFH (50 mg) was dissolved in 5 mL 0.1 M Mes, pH 4.5 and mixed with 0.6 mL of 1 mg/mL Biotin-XX-hydrazide previously prepared in dimethyl formamide and diluted 1:4 in 0.1 M Mes, pH 4.5. The mixture was initially incubated for 30 min at 25 °C with 7 mg of EDC. An additional 7 mg of EDC was added and incubated for further 30 min. Unincorporated biotin was removed with a PD-10 column (GE Healthcare, Barcelona, Spain) connected to a LC-Biologic system (Biorad, Madrid, Spain), using 10 mM NaH_2PO_4, pH 7, as the exchange buffer. The rate of biotinylation of heparin was then estimated using Pierce biotin quantitation kit following the manufacturer's instructions.

The immobilization of biotinylated heparin onto SA chip was carried out as previously described [33].