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REGULAR ARTICLE

Protein disulfide isomerase has no stimulatory chaperone effect on factor X activation by factor VIIa-soluble tissue factor

Egon Persson *

Haemostasis Biochemistry, Novo Nordisk A/S, Novo Nordisk Park (G8.2.76), DK-2760 Måløv, Denmark

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Abstract

Introduction: It was recently reported that protein disulfide isomerase (PDI) stimulates factor X (FX) activation by factor VIIa (FVIIa) bound to soluble tissue factor (sTF) in a purified system and that PDI may be responsible for activating cellular tissue factor (TF) and switching it between its roles in blood coagulation and cellular signalling. This study further investigates the former effect of PDI.
Method: FX activations by FVIIa-sTF_{1-219} were carried out in the presence of different forms of PDI, with annexin V or detergent present in the system and using various forms of FVIIa and FX. In addition, FVIIa-lipidated TF was used as the FX activator.
Results: Recombinant human PDI did not influence FX activation by FVIIa-sTF_{1-219}, whereas PDI purified from bovine liver enhanced the activation rate in a dose-dependent manner. The inclusion of annexin V or detergent abolished the stimulatory effect. Removal of the phospholipd-interactive γ-carboxyglutamic acid (Gla)-containing domain from either FVIIa or FX obliterated the bovine PDI-induced enhancement of FX activation, as did the introduction of F4A or L8A mutation in FVIIa. The presence of 25 nM bovine PDI lowered the apparent K_m for FX from far above 10 μM to 1–2 μM. No PDI effect was seen when FVIIa-lipidated TF was the FX activator.
Conclusions: FX activation is insensitive to PDI *per se* and a phospholipid contaminant in the bovine PDI preparation acts stimulatory when sTF, but not lipidated TF, is the cofactor. Strong support is provided by the lacking effect of bovine PDI after removal or modification of the Gla domain in either FVIIa or FX as well as by the effects of annexin V and detergents and the decreased K_m value.
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Abbreviations: FVIIa, activated factor VII; FX, factor X; FXa, activated factor X; Gla, γ-carboxyglutamic acid; PDI, protein disulfide isomerase; sTF, soluble tissue factor; TF, tissue factor.

* Tel.: +45 4443 4351; fax: +45 4466 3450.
E-mail address: egpe@novonordisk.com.

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Introduction

Tissue factor (TF) is the integral membrane cofactor and receptor for the enzyme factor VIIa (FVIIa). The binary complex initiates blood coagulation via activation of factors IX and X and mediates cell signalling events through cleavage of protease-activated receptor 2 [1]. Protein disulfide isomerase (PDI) is a protein normally present in the endoplasmic reticulum where it catalyses protein disulfide rearrangements and assists protein folding [2,3]. It was reported that cryptic, i.e. signalling but non-coagulant, TF contains unpaired Cys^{186} and Cys^{209} residues, whereas these cysteines are engaged in a disulfide bond in procoagulant TF and that extracellular PDI, by employing its oxidoreductase activity, regulates the thiol status of TF and the distribution between signalling (and coagulantly dormant) and procoagulant forms [4–6]. However, conflicting data exist which question the involvement of PDI and favour the phospholipid composition as the principal TF switch [7]. Independent of this, PDI has been shown to enhance factor X (FX) activation by FVIIa bound to soluble TF (sTF) by a chaperone mechanism not related to its oxidoreductase activity [8].

The studies that suggest a regulatory [5,6] and stimulatory [8] role of PDI employed endogenous cellular PDI and PDI purified from bovine liver, respectively. Two studies [5,8] presented circumstantial evidence of a direct association between PDI and TF using immunoprecipitation visualised by Western blotting. In addition, data from the purified system indicated that PDI facilitated formation of the ternary FVIIa-sTF-FX complex and the subsequent release of the product factor Xa (FXa) [8], but the molecular details pertinent to the PDI-induced stimulation of FX activation remain unknown. The ostensible biological importance of PDI in the context of TF certainly warrants efforts aiming at elucidating the underlying mechanism. I have obtained data suggesting that PDI does in fact not influence FX activation by means of a chaperone mechanism. Recombinant human PDI is unable to enhance FX activation by FVIIa-sTF, whereas liver-derived bovine PDI increases the rate as previously observed, but not when lipidated TF is the cofactor [8]. The enhancement in the presence of the bovine PDI preparation is abolished by annexin V and detergents, by removal of the γ-carboxyglutamic acid (Gla) domain of either FVIIa or FX or by mutations of the hydrophobic side chains in positions 4 and 8 of FVIIa critical for phospholipid binding. Moreover, the inclusion of bovine PDI reduces the K_m of FVIIa-sTF for FX. Thus, PDI itself appears to be without any effect on FX activation. Instead, a contaminant in the bovine PDI preparation with the properties of phospholipid appears to cause the increase in the rate of FX activation.

Materials and methods

Proteins and reagents

Recombinant human PDI was purchased from ProSpec Tany TechnoGene (Rehovot, Israel). Bovine PDI (purified from liver) and annexin V were from Sigma (St Louis, MO, USA). Triton X-100, CHAPS and Tween 80 were from Sigma or Merck. FX and FXa were from Enzyme Research Laboratories (South Bend, IN, USA), whereas relipidated TF (Innovin) was from Dade Behring (Marburg, Germany). FVIIa was obtained as described [9] and Gla-domainless FVIIa (des(1–38) and des(1–44)) was isolated after autodigestion and cathepsin G-mediated cleavage, respectively [10,11]. sTF_{1-209} and sTF_{1-219} were prepared from *E. coli* inclusion bodies, reduced and refolded as described [12]. Gla-domainless FX (des(1–44)) was isolated after limited proteolysis with chymotrypsin [13]. The F4A, L8A and F4A/L8A mutations were introduced into FVII using the QuikChange kit (Stratagene, La Jolla, CA, USA) and the human FVII expression plasmid pLN174 [14]. The following sense primers (and complementary reverse primers) were used, with base substitutions in italic and changed codons underlined: F4A, 5'-CGCGCCAACGCG*GC*CCTGGAGGAGCTGC-3'; L8A, 5'-CCTGGAGGAG*GC*GCGGCCGGGCTCCC-3'; F4A/L8A, 5'-GCCAACGCG*GC*CCTGGAGGAG*GC*GCGGCCGGGC-3'. Plasmid preparations, baby hamster kidney cell transfections and selection and protein expression were performed as described [15,16]. The mutants were purified by chromatography on Q Sepharose Fast Flow (Ca^{2+} elution) and sTF-Sepharose (elution by Ca^{2+} chelation). All three FVII mutants autoactivated to completion in 5–6 days at room temperature without signs of degradation and displayed the same amidolytic activity as the wild-type enzyme.

FX activation assays

All proteins were normally diluted in and the assays run in 20 mM HEPES, pH 7.4, containing 130 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.1% (w/v) PEG 8000 and 0.01% (v/v) Tween 80. To assess the effect of PDI on FX activation by FVIIa bound to sTF, 5 nM FVIIa, 25 nM sTF_{1-219} and 150 nM FX were incubated for 10 minutes at room temperature in the absence or presence (25 nM) of PDI (total volume 100 μl), optionally in the presence of 500 nM annexin V, 0.1 or 0.5% (v/v) Triton X-100, 1% (w/v) CHAPS or 1% (v/v) Tween 80. F4A-, L8A- and F4A/L8A-FVIIa were tested under the same conditions but without optional agent. In some experiments, the concentration of sTF_{1-219} (25-400 nM) or PDI (0.8–100 nM (and only 2 nM FVIIa)) was varied, sTF_{1-209} was used instead of sTF_{1-219}, or Mg^{2+} was omitted from the buffer. FX activation by FVIIa bound to relipidated TF was measured by incubating 150 nM FX with 1 nM FVIIa and 1 pM TF for 10 min with or without 25 nM PDI, optionally with annexin V or detergent present. A decreased function of F4A-, L8A- and F4A/L8A-FVIIa (at 1 and/or 0.25 nM) due to an expected diminished phospholipid affinity was verified using lipidated TF. To determine the K_m of FVIIa-sTF_{1-219} for FX, 50 pM FVIIa and 25 nM sTF_{1-219} were incubated with 150 nM-10 μM FX for 30 minutes. The incubation time was reduced to 10 minutes when the experiment was performed in the presence of 25 nM bovine PDI. All activation reactions were terminated by adding 50 μl buffer containing 20 mM EDTA, followed by the addition of 50 μl of a 2-mM solution of S-2765 (Chromogenix, Instrumentation Laboratory, Milan, Italy) to quantify the formed FXa by continuous absorbance measurement in a SpectraMax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

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