Thrombosis Research 134 (2014) 132–137

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Regular Article

# Inhibitory effects of LDL-associated tissue factor pathway inhibitor ☆

Cecilia Augustsson, Ida Hilden, Lars C. Petersen *

*Haemophilia, in vitro Biology, Biopharmaceutical Research Unit, Novo Nordisk A/S, Måløv, Denmark*

ARTICLE INFO

*Article history:*
Received 4 February 2014
Received in revised form 13 March 2014
Accepted 26 March 2014
Available online 1 April 2014

*Keywords:*
Anticoagulant
Haemostasis
lipoprotein
tissue factor

ABSTRACT

*Introduction:* Tissue factor pathway inhibitor (TFPI) is present in plasma as full-length free TFPI (TFPIα) and as C-terminally degraded forms mainly associated with low-density lipoprotein particles (LDL-TFPI). Addition of TFPIα to plasma induces a prolongation of the clotting time when tested in a diluted prothrombin time (dPT) assay, whereas no prolongation is observed with LDL-TFPI or truncated recombinant TFPI ($TFPI_{1-161}$). The aim was to further characterize kinetic properties of purified LDL-TFPI in thrombin generation and chromogenic activity assays.

*Materials and Methods:* LDL-TFPI was purified from human plasma by sequential flotation ultracentrifugation. Thrombin generation was measured in human plasma or in FVIII-immunodepleted plasma using either 1 pM tissue factor and 4 μM phospholipids or 0.5 nM factor Xa (FXa) and 4 μM phospholipids, respectively.

*Results:* TFPIα prolonged the lag-phase and decreased the thrombin peak in tissue factor-induced thrombin generation, whereas LDL-TFPI exclusively decreased the peak height of thrombin without effecting the lag phase. Steady-state and transient kinetics showed that LDL-TFPI was a more potent inhibitor of FXa than TFPIα and $TFPI_{1-161}$, indicating that FXa inhibition was not rate determining for the lag phase, whereas it appeared to affect thrombin generation during the propagation phase. This was supported by FXa-induced thrombin generation showing that LDL-TFPI, compared with TFPIα, more actively decreased the peak height.

*Conclusions:* Our results suggest that LDL-TFPI affects thrombin generation during the propagation phase, and is kinetically different from $TFPI_{1-161}$. It may therefore play a more prominent physiological role *in vivo* than hereto anticipated from dPT measurements.



## Introduction

Tissue factor pathway inhibitor (TFPI) is a regulator of the initiation phase in the extrinsic pathway of coagulation [1,2]. TFPIα is the originally isolated form of TFPI consisting of 276 amino acids with a predicted molecular mass of 32 kDa and an observed mass of 43 kDa [3,4]. TFPIα contains three tandemly arranged Kunitz-type protease inhibitor (KPI) domains, KPI-1, KPI-2 and KPI-3, framed by a negatively charged amino terminus and a positively charged carboxy terminus. An isoform of TFPI, TFPIβ, lacks the KPI-3 and instead has an alternative carboxy terminus with a glycosyl phosphatidyl inositol (GPI) anchor binding motive [5,6]. The vascular endothelium is the major *in vivo* source of TFPIα and TFPIβ. In addition, platelets express TFPIα that is released upon their activation [7–9]. The total plasma concentration of TFPI in normal individuals is approximately 1–2.5 nM [10,11]. A major proportion of plasma TFPI is bound to lipoproteins (80%), and mostly to low-density lipoprotein (LDL) particles [9]. The exact association of TFPI with LDL has not been determined, but LDL-associated TFPI (LDL-TFPI) appears to consist of a C-terminally truncated form of TFPIα which lacks a part of, or the entire KPI-3 domain [12–14]. The predominant form of LDL-associated TFPI has a molecular mass of ~34 kDa [13]. The remaining 20% of the plasma TFPI circulates as free TFPIα, with both full-length TFPIα and C-terminally truncated variants [15].

TFPI regulates coagulation by inhibiting both factor Xa (FXa) and the tissue factor (TF)/factor VIIa (FVIIa) complex. Direct FXa inhibition occurs in a biphasic reaction with the generation of a loose TFPI-FXa complex, which slowly rearranges into a tight complex [16]. The KPI-2 domain binds and blocks the active site of FXa [17,18]. The FXa-dependent inhibition of FVIIa/TF involves the formation of the quaternary TF/FVIIa/FXa/TFPI complex [16]. Binding of FVIIa involves KPI-1, and all KPI domains of TFPI are important for complete inhibition of the TF/FVIIa complex whereas the KPI-3 and C-terminus of TFPI have been shown not to be a prerequisite for the quaternary complex formation [17–19].

LDL-TFPI, like TFPIα, is capable of inhibiting FXa when tested in various chromogenic assays [13,20,21]. A detailed study of LDL-TFPI

*Abbreviations:* BSA, bovine serum albumin; CAT, calibrated automated thrombogram; dPT, diluted prothrombin time; FVIIa, activated factor VII; FX, factor X; FXa, activated factor X; GPI, glycosyl phosphatidyl inositol; HBS, hepes buffered saline; IP, immuno pull down; $K_I$, inhibitor constant; $k_{obs}$, apparent rate constant; KPI, Kunitz-type protease inhibitor; PL, phospholipids; TBS, tris buffered saline; TFPI, tissue factor pathway inhibitor; LDL, low-density lipoprotein.

☆ Presented in abstract form at the ISTH, The Netherlands, 2013.

* Corresponding author at: Novo Nordisk A/S, Novo Nordisk Park, 2760 Måløv, Denmark. Tel.: +45 30754345.
*E-mail address:* lcp@novonordisk.com (LC. Petersen).

http://dx.doi.org/10.1016/j.thromres.2014.03.043
0049-3848/

inhibition kinetics is, however, still lacking. Addition of TFPIα to plasma induces a prominent dose-dependent prolongation of the clotting time when tested in a diluted prothrombin time (dPT) assay [21]. In contrast, addition of LDL-TFPI was shown not to prolong the clotting time when tested in a dPT assay [21]. The anticoagulant activity of TFPI in human plasma was consequently assumed to be almost entirely due to the action of TFPIα with no or only minor contributions from LDL-TFPI; and this notion was further supported by the observation that, like in the case of LDL-TFPI, addition of a C-terminally truncated variant, $TFPI_{1-161}$, did not prolong dPT [21]. The variant ($TFPI_{1-161}$) lacks the KPI-3 and C-terminal regions, but in contrast to LDL-TFPI, it is not lipid associated. $TFPI_{1-161}$ binds with a lower affinity than TFPIα to FXa. The affinity of the separate KPI-2 domain is even further decreased indicating that exosites outside the KPI-2 domain in both ends contribute to fine-tune TFPIα's interaction with FXa [18,22].

The aim of this study was to further characterize the kinetic properties of purified LDL-TFPI, using thrombin generation and chromogenic assays. Our work suggests that plasma LDL-TFPI is kinetically distinct from truncated $TFPI_{1-161}$ and provides new information on the anticoagulant properties of LDL-TFPI.

## Materials and Methods

### Materials

Human citrate plasma was purchased from Innovative Research (MI, USA) and Precision BioLogic (Dartmouth, Canada) for use in ultracentrifugation and thrombin generation respectively. FXa and FX were from Enzyme Research Laboratories (IN, USA). FVIIa (NovoSeven®) was produced in-house. Synthetic chromogenic substrate for FXa, S2765, was from Chromogenix (Milan, Italy). Tris–HCl, Hepes, NaCl, bovine serum albumin (BSA) and $CaCl_2$ were purchased from Sigma-Aldrich (MO, USA). Tris buffered saline (TBS) consisted of 70 mM Tris–HCl and 150 mM NaCl, pH 7.5. Hepes buffered saline (HBS) consisted of 20 mM Hepes, 140 mM NaCl pH 7.4. Procoagulant phospholipids (PLs) were purchased from Rossix (Mölndal, Sweden). EDTA stop buffer, Titriplex III, was from Merck (Darmstadt, Germany). Relipidated tissue factor (TF, Dade-Innovin®) was from Siemens Healthcare (Germany). Reagents for thrombin generation assay were purchased from Thrombinoscope (Maastricht, Netherlands) if nothing else stated. Polyclonal sheep anti human factor VIII antibody was from Haematologic Technologies Inc. (VT, USA). Monoclonal antibody against KPI-2 of TFPI [23,24], was produced in-house using standard procedures. Goat polyclonal anti-human TFPI antibody was produced in-house using standard procedures [25]. TFPIα (full-length $TFPI_{1-276}$) and $TFPI_{1-161}$ were produced in-house as previously described [26,27].

### Isolation of LDL-Associated TFPI

Low-density lipoprotein, LDL, ($1.0068 < d < 1.068$ g $mL^{-1}$) was isolated from human citrate plasma by sequential flotation ultracentrifugation as described earlier [28], and dialyzed against HBS prior to storage at −80 °C. The isolated LDL fraction was characterized by separation on gel filtration column, Superose 6 10/300 GL (GE Healthcare, Stockholm, Sweden), and HBS was used as running buffer. Eluted fractions were analysed for total protein content (absorbance at 280 nM), TFPI protein by total TFPI Asserachrom ELISA, (Diagnostica Stago Inc., NJ, USA), and total cholesterol (Wako Diagnostics, VA, USA). The TFPI protein content in the isolated LDL fraction was determined with the total TFPI Asserachrom ELISA. For molar concentration calculations of LDL-TFPI a molecular weight of 35 kDa was used (assuming the observed molecular weight reflects the apparent molecular weight), and typically a concentration of around 10 mg/mL total protein, with 200 ng/mL (6 nM) of total TFPI was obtained in the purified LDL fraction. For use of LDL-TFPI in thrombin generation assay, samples were concentrated using a spin column with 10 000 molecular weight cut-off (Merck Millipore, MA, USA).

### Immuno Pull Down Assay

Immuno pull down of TFPI was done using dynabeads M280 (Invitrogen, CA, USA) coupled with TFPI KPI-2 antibody using recommended protocol. Samples (Plasma, LDL or TFPIα) were incubated with antibody coupled beads for 1 h at room temperature. Beads were washed three times in HBS and SDS/PAGE sample buffer was added to beads in order to elute the proteins from pull down. Samples were run on 4-12% SDS/PAGE non-reduced gel and immunoblot was visualized with a polyclonal TFPI antibody.

### FXa Activity Measurements

Assay buffer for FXa inhibition assays was TBS supplemented with 0.1% BSA and 5 mM $CaCl_2$. Steady-state inhibition of FXa was performed by mixing 0.4 nM FXa with various (0–0.8 nM) concentrations of TFPI derivatives (TFPIα, $TFPI_{1-161}$ or LDL-TFPI) in assay buffer in absence or presence of 20 μM PLs and/or 80 nM TFPI KPI-2 antibody. Samples were incubated at room temperature for 30 min to allow inhibition of FXa by TFPI. The reaction was stopped with 100 mM stop buffer (Titriplex III) and residual FXa activity was measured with 0.4 mM chromogenic substrate S2765. FXa activity (maximum velocity, $V_{max}$) was measured at room temperature for 15 min at 405 nm and plotted against the TFPI concentration. Inhibitor constant, $K_I$, was calculated using the Morrison tight binding non-linear curve fitting.

Progression of TFPI inhibition was determined from progress curves of FXa-catalyzed p-Nitro aniline generation produced when 0.05 nM FXa was added to a reaction mixture containing 0.25 mM S2765 and various (0–1 nM) concentrations of TFPI derivatives (TFPIα, $TFPI_{1-161}$ or LDL-TFPI). The reaction was followed at 405 nm for 3 h at 37 °C in assay buffer in absence or presence of 20 μM PLs and/or 100 nM TFPI KPI-2 antibody. Data from the progress curves (Absorbance, Y, versus time, t) were fitted by nonlinear regression to the equation for slow binding inhibition: $Y = A_0 + (v_S{*}t) + (v_0\text{-}v_S){*}(1\text{-}\exp(-k_{obs}{*}t))/k_{obs}$ [29]. Where, $A_0$ represent the initial absorbance at 405 nm, $v_S$ is the final steady-state velocity, $v_0$ is the initial velocity and $k_{obs}$ is the apparent rate constant.

### FVIIa/TF/FX Activity Measurements

TFPI inhibition of FVIIa/TF-mediated FXa generation was measured by mixing 1 nM FVIIa, 20 pM TF (Innovin®) and 320 nM FX with various concentrations (0–1 nM) of TFPI derivatives (TFPIα, $TFPI_{1-161}$ or LDL-TFPI) in absence or presence of 10 nM TFPI KPI-2 antibody in TBS with 0.1% BSA and 5 mM $CaCl_2$. Samples were incubated at room temperature for 15 min. TF/FVIIa activation of FX was then stopped with 100 mM stop buffer (Titriplex III) and the amount of FXa generated was assessed at room temperature by measuring the FXa activity at 405 nm with 0.4 mM chromogenic substrate S2765. Data were fitted by nonlinear regression to the equation for Inhibition $Y = A/(1 + x/K_I)$. A represents the maximal FXa generation without inhibitor (x) present and $K_I$ is the inhibitor constant.

### Thrombin Generation Measurements

Assay buffer for thrombin generation assay was HBS supplemented with 2% BSA. Thrombin generation assay was performed according to the calibrated automated thrombogram (CAT) method [30]. Human citrate platelet poor plasma (80 μL) was supplemented with 10 μL TFPI derivatives (TFPIα or LDL-TFPI, various final concentration of 0–1 nM) in 96-well plate (Nunc, Denmark). Samples (90 μL) were placed at 37 ° C for 5 min and followed by the addition of 10 μL PPP-reagent low (final concentrations of 1 pM tissue factor and 4 μM phospholipids). Clotting