Thrombin activatable fibrinolysis inhibitor

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Keywords
Thrombin activatable fibrinolysis inhibitor, TAFI, proCPU, fibrinolysis

Summary
Thrombin activatable fibrinolysis inhibitor (TAFI) was discovered two decades ago as a consequence of the identification of an unstable carboxypeptidase (CPU), which was formed upon thrombin activation of the respective pro-enzyme (proCPU). The antifibrinolytic function of the activated form (TAFIa, CPU) is directly linked to its capacity to remove C-terminal lysines from the surface of the fibrin clot. No endogenous inhibitors have been identified, but TAFIa activity is regulated by its intrinsic temperature-dependent instability with a half-life of 8 to 15 min at 37 °C. A variety of studies have demonstrated a role for TAFI/TAFIa in venous and arterial diseases. In addition, a role in inflammation and cell migration has been shown. Since an elevated level of TAFIa it is a potential risk factor for thrombotic disorders, many inhibitors, both at the level of activation or at the level of activity, have been developed and were proven to exhibit a profibrinolytic effect in animal models. Pharmacologically active inhibitors of the TAFI/TAFIa system may open new ways for the prevention of thrombotic diseases or for the establishment of adjunctive treatments during thrombolytic therapy.

Zusammenfassung

Discovery and nomenclature of TAFI

In 1989, an unstable basic carboxypeptidase activity, unrelated to the constitutively active plasma carboxypeptidase N (CPN) was detected in freshly prepared serum. Because of its instability, it was named “unstable” carboxypeptidase (CPU) (1). In an independent study, Campbell and Okado reported the identification of a protein with an arginine carboxypeptidase activity which was named arginine carboxypeptidase (CRP) (2). A third independent study revealed the presence of a plasminogen-binding protein in plasma with an amino acid sequence similar to pancreatic carboxypeptidase B. Therefore, it was named plasma procarboxypeptidase B (plasma proCPB) (3). In 1995, Bajzar et al. (4) reported the discovery of a carboxypeptidase zymogen that, upon activation by thrombin, attenuates clot lysis and therefore was named thrombin activatable fibrinolysis inhibitor (TAFI). These results provided an explanation for the previously observed antifibrinolytic effect upon activation of prothrombin during t-PA-induced fibrinolysis (5). Ultimately, amino-terminal sequencing revealed that proCPU, proCRP, plasma proCPB and TAFI were identical (6).

TAFIa is a metallo-carboxypeptidase

Activated TAFI (TAFIa) is a member of the family of metallo-carboxypeptidases (E.C. 3.4.17.20). These exopeptidases are zinc-dependent and cleave carboxy-terminal peptide bonds. The metallo-carboxypeptidases are subdivided in subfamily A and B according to the MEROP database (7).

* All members of subfamily A are zymogens and contain an N-terminal acti-
viation peptide of ~90 amino acids and a catalytic domain of ~300 amino acids (7). TAFI and the intensively studied pancreatic carboxypeptidase B are both members of this subfamily and have a mutual identity of 42 percent at amino acid level (8).

- Members of subfamily B (e.g. carboxypeptidase N, CPN) lack an activation peptide and therefore are constitutively active. As CPN circulates in plasma, it compromises measurements of TAFIa activity in plasma (9).

### Physiological role of TAFI

#### Attenuation of fibrinolysis

Fibrinolysis is initiated by the activation of plasminogen to plasmin by t-PA at a fibrin clot surface. Plasmin then degrades the fibrin clot into partially degraded fibrin resulting in the exposure C-terminal lysines residues at the fibrin surface. These C-terminal lysines are important in the regulation of fibrinolysis, since they act as a cofactor for plasmin formation, resulting in a burst of plasmin generation due to the following events:

- Binding of plasminogen to partially degraded fibrin through the C-terminal lysines not only leads to an accumulation of plasminogen leading to plasmin generation at the clot, but also induces conformational changes in plasminogen, making it a better substrate for t-PA (10).
- Not only plasminogen, but also plasmin binds to the C-terminal lysines on partially degraded fibrin. Fibrin-bound plasmin is protected from inactivation by α2-antiplasmin (11).
- Partially degraded fibrin also acts as a cofactor in the plasmin-mediated conversion of Glu-plasminogen to Lys-plasminogen, which is a better substrate for t-PA (11).

TAFIa removes the C-terminal lysines from partially degraded fibrin, thereby abrogating the fibrin cofactor function of plasminogen activation. The prevention of the progression of fibrinolysis into the propagation phase results in a dramatic decrease of the fibrinolytic efficiency of t-PA (12, 13).

#### Regulation of the antifibrinolytic effect – The threshold mechanism

So far, there is no evidence for an endogenous physiological TAFIa inhibitor in plasma. The observation that the antifibrinolytic effect is in part dependent on the stability of TAFIa led to the hypothesis that the intrinsic instability of TAFIa is one of the regulatory mechanisms of its antifibrinolytic activity. Two research groups independently discovered that TAFIa attenuates fibrinolysis through a threshold-dependent mechanism (14, 15). As long as TAFIa is present above a certain threshold value, fibrinolysis stays in the initial phase. Only when the TAFIa level drops below the threshold, the rate of fibrinolysis accelerates through an exponential increase in C-terminal lysine residues. The threshold value is influenced by the plasmin concentration, which in turn is dependent on the t-PA concentration and the α2-antiplasmin concentration. The time during which the TAFIa level remains above the threshold value depends on several factors:

- TAFI concentration in plasma and the extent of TAFI activation, since the TAFI concentration in plasma is far below the Km values for TAFI activation
- most importantly by the stability of TAFIa, since only low TAFIa concentrations (1% of total TAFI protein) are required to produce an antifibrinolytic effect.

Therefore, the generation of a sustained low level of TAFIa activity above the threshold is more efficient than a high but short TAFI activation (14, 15).

#### Other TAFIa functions

Apart from partially degraded fibrin several other substrates for TAFIa have been described. Bradykinin, thrombin-cleaved osteopontin and activated complement factors C3a and C5a are inactivated by TAFIa, at least as good as by the other carboxypeptidase CPN which is the major anaphylatoxin inhibitor (16–18). The physiological relevance of TAFIa activity in processes such as blood pressure regulation and inflammation revealed conflicting data [reviewed in (19)]. For example, in wild-type mice bradykinin-induced hypotension can be reduced by pre-infusion with a thrombin variant exhibiting minimal pro-coagulant properties but normal antifibrinolytic function through activation of TAFI. In contrast, in TAFI-deficient mice this thrombin variant had no effect on hypotension indicating that the effect of the thrombin variant is mediated through the activation of TAFI (19). On the other hand, in a bleomycin-induced lung fibrosis model no significant differences in body temperature and blood pressure were observed between wild-type and TAFI-deficient mice, rather suggesting that TAFIa does not contribute to bradykinin clearance under these conditions (20). Results from studies with TAFI-deficient mice to unravel the (patho)physiological function of TAFI in inflammatory disease processes have recently been reviewed in (21).

On the other hand, it should be noted that the plasminogen/plasmin system plays also an important role in wound healing and cellular events. It has been reported that TAFIa also cleaves C-terminal lysines and arginines from cellular receptors of plasminogen and thereby inhibits cellular processes such as cell migration in vivo (22) and angiogenesis in vitro (23). TAFI-deficient mice were shown to have delayed and altered wound healing with disturbed keratinocyte migration probably due to excessive plasmin formation and subsequent weakening of the extracellular matrix (24).

### TAFI synthesis and distribution

Eaton et al. (3) were the first to isolate and characterize the cDNA of TAFI. The TAFI encoding gene, CPB2, was mapped to chromosome 13 (13q14.11), contains 11 exons and spans approximately 48 kb of genomic DNA (25). In total, 19 single nucleotide polymorphisms (SNP) have been reported in CPB2. Ten in the 5’-flanking region, six in the coding region and three in the 3’ un-
translated region (3’ UTR) (26). Two of the six SNP in the coding region result in an amino acid substitution: +505G/A and +1040C/T corresponding to a 147Ala/Thr and 325Thr/Ile polymorphism, respectively. These polymorphisms result in the existence of four isoforms (27), i.e.  
- TAFI-A\textsuperscript{147T}I\textsuperscript{325}\textsuperscript{V}  
- TAFI-A\textsuperscript{147I}T\textsuperscript{325}\textsuperscript{V}  
- TAFI-T\textsuperscript{147T}I\textsuperscript{325}\textsuperscript{V}  
- TAFI-T\textsuperscript{147I}T\textsuperscript{325}\textsuperscript{V}  

While the polymorphism at position 147 has no major effect on the functional properties, the polymorphism at position 325 has an important impact on the stability of TAFI as well as on the reactivity of TAFI in immunological assays.

TAFI is predominantly synthesised in the liver and circulates in the blood as a zymogen (28). The measured molecular mass of 56 kDa is in contrast with the predicted 46 kDa, based on the 401 amino acids. However, TAFI harbours five N-glycosylation sites, four in the activation peptide (Asn\textsuperscript{22}, Asn\textsuperscript{51}, Asn\textsuperscript{63}, Asn\textsuperscript{86}) which are always glycosylated and one potential glycosylation site in the TAFI moiety (Asn\textsuperscript{219}) that is not glycosylated.

Different studies indicated a concentration of TAFI between 75 and 275 nmol/l (corresponding to 4 - 15 μg/ml) in plasma (29, 30). The apparently large variation in plasma concentration can be explained to a large extent by the 325Thr/Ile polymorphism influencing the reactivity of TAFI in some commercially available ELISAs. These findings imply that several TAFI measurements have been biased (31). Eventually, only 25% of the variation in plasma concentration is due to a difference in gene expression or mRNA stability in association with the SNP (26, 32).

TAFI was also identified in platelets and is secreted upon platelet activation. Even though platelet TAFI represents only 0.1% of total blood TAFI, release from platelets at the site of thrombus formation may result in a local boost of TAFI. This might have an impact on fibrinolysis, as relatively small amounts of TAFI have been found to influence clot lysis (29, 30). A specific role for TAFI generated from platelets has also been suggested to be linked to platelet-mediated resistance towards fibrinolysis (33). The enzymatic characteristics of platelet-derived TAFI (e.g. activation by thrombin/thrombomodulin, TAFI stability) are comparable to that of plasma-derived TAFI. Only the glycosylation pattern differs, indicating that the synthesis occurs in megakaryocytes (34).

Activation of TAFI

The zymogen TAFI can be activated to TAFI upon proteolytic cleavage of the Arg\textsuperscript{92}–Ala\textsuperscript{93} bond by thrombin or plasmin, resulting in the generation of the active protease moiety TAFIa (Ala\textsuperscript{93}–Val\textsuperscript{301}; 36 kDa) and the activation peptide (Phe\textsuperscript{1}–Arg\textsuperscript{92}; 20 kDa) (Fig. 1).

Thrombin is a weak activator of TAFI. However, in complex with thrombomodulin, the efficiency of TAFI activation is increased 1250-fold (35). Thrombomodulin is a transmembrane protein expressed on the surface of endothelial cells. Upon binding to thrombomodulin, the substrate specificity of thrombin is changed. Fibrinogen and other procoagulant substrates are no longer recognized, but instead, thrombin/thrombomodulin (T/TM) generates activated protein C (APC) as an effective natural anticoagulant and TAFIa with antifibrinolytic effect is formed. Through this dual activity massive coagulation is prevented on the one hand and protecting the generated clot is secured on the other hand.

TAFI can also be activated by plasmin, which is a stronger activator than thrombin. The efficiency of plasmin-mediated activation is enhanced by glycosaminoglycans such as heparin (36). It has been postulated that T/TM is the major physiological activator of TAFI, since the catalytic efficiency of T/TM is 10-fold higher than that of plasmin/heparin. Also in an in vivo study with an antibody that selectively inhibits T/TM-mediated TAFI activation it appeared that T/TM is the predominant activator of TAFI (37). However, recent studies using different monoclonal antibodies that mainly inhibit plasmin-mediated TAFI activation, revealed that also plasmin plays an important role in the activation of TAFI in vitro in clot lysis experiments (38, 39) as well as in vivo in a thromboembolism model (38).

Instability of TAFIa

Activated TAFI is characterized by a pronounced, temperature-dependent instability, showing a spontaneous decay with a half-life of eight min at 37°C or several hours at 22°C (12). This instability of TAFIa is also dependent on the polymorphism at position 325 with a two-fold prolonged half-life for the T\textsuperscript{325}variant (40). The spontaneous inactivation of TAFIa plays a role in the regulation of its antifibrinolysis.
nolytic activity in vivo, as no physiological inhibitors have been described. This is in line with the observation that more stable TAFIa variants also exert an increased antifibrinolytic activity (41–43). The inactivation of TAFIa is associated with conformational changes (44), which result in the exposure of a cryptic cleavage site at Arg302 (45) (Fig. 1). The subsequent proteolytic cleavage by thrombin or plasmin results in the degradation of inactive TAFIa (46). Mutation studies also revealed that one particular region (residues 300–330) is responsible for the destabilization of TAFIa (44, 47). The subsequent elucidation of the three-dimensional structure of TAFI provided a molecular explanation for the instability of TAFIa (45).

**Inhibition of TAFIa or prevention of TAFI activation**

To date no physiological inhibitors of TAFIa have been found. Several low-molecular weight, relatively non-specific inhibitors have been characterized [reviewed in (48)]. Like all other metallo-carboxypeptidases, TAFIa is inhibited by chelating agents such as EDTA and o-phenanthroline. Reducing agents (e.g. dithiothreitol or mercaptoethanol) and small synthetic substrate analogs, such as MERGEPTA (2-mercaptopentyl-3-guanidinopentyl-thiopropanoic acid) and GEMSA (guanidinomethylmercaptopuscinic acid) have also been reported to inhibit TAFIa (1, 3, 49). A major drawback of these inhibitors is their inhibitory capacity towards other plasma-circulating carboxypeptidases, such as CPN. During the last decade, many other potent synthetic TAFIa inhibitors that are less reactive towards CPN have been generated. Although little side effects are expected, it should be noted that these inhibitors also inhibit the pancreatic carboxypeptidase CPB (50–57).

Apart from synthetic inhibitors, some naturally occurring inhibitors have been reported. Potato tuber carboxypeptidase inhibitor (PTCI) competitively inhibits TAFIa with a Ki in the nanomolar range. Since PTCI does not inhibit CPN, it is a widely used inhibitor in clot lysis assays. Carboxypeptidase inhibitors have also been isolated from leech and ticks (LCI and TCI, respectively) (58, 59). Remarkably, GEMSA and PTCI show a biphasic effect, attenuating clot lysis at low concentrations and enhancing lysis at high concentrations. The former effect can be explained by the fact that binding to an inhibitor prevents conformational changes that cause the inactivation of TAFIa. The TAFIa-inhibitor complex slowly dissociates, permitting TAFIa to exert its antifibrinolytic function during a longer time interval (60).

Besides TAFIa inhibitors, the formation of TAFIa can also be inhibited by impairing the activation of TAFI. Several monoclonal antibodies and nanobodies with such properties have been generated against TAFI (29, 38, 39, 61–63).

**Protease activity associated with the TAFI zymogen**

In 2006, it was reported for the first time that the TAFI zymogen shows enzymatic activity, depending on the substrate used (64). Characterization of this activity revealed that TAFI zymogen not only hydrolyses small synthetic substrates such as hirpuryl-arginine, but also larger synthetic fibrinogen-derived peptides, thereby impairing in vitro plasminogen binding (65). The TAFI zymogen has a 41-fold decreased catalytic rate in comparison with TAFIa. This is partially compensated by a 2-fold increased affinity for the substrate, resulting in an 18-fold lower catalytic efficiency (65). There is still some controversy whether or not the zymogen activity of TAFI has a physiological role in the regulation of fibrinolysis. One study reported that the observed down regulation of fibrinolysis by TAFI in a clot lysis assay without addition of exogenous thrombomodulin must be due to the zymogen activity of TAFI since nearly no TAFIa is generated under these conditions (65). However, results of another study suggested that there was sufficient TAFIa generated under these conditions (66). In an independent study, it was proposed that the zymogen activity cannot attenuate fibrinolysis, since, according to their experiments, TAFI zymogen is unable to cleave plasmin-modified fibrin degradation products (67).

![Fig. 2 Structure of TAFI. Ribbon drawing of TAFI based on the structure of Marx et al. (45) with the activation peptide shown in grey, the catalytic domain in green and the dynamic flap region (residues 296–350) in cyan. Residues involved in hydrolysis, substrate binding and zinc binding are shown in magenta, yellow and blue spheres, respectively. The four glycosylation sites are represented as orange sticks. The two thrombin cleavage sites are shown as red spheres.](https://www.haemostaseologie-online.com)
Structure of TAFI

The metallo-carboxypeptidases of subfamily A show high structural similarity, consisting of a globular shape with two separate moieties: the activation peptide and the catalytic domain. Pancreatic carboxypeptidase B (pCPB) is the prototype for this subfamily and shares 42% of sequence identity with TAFI. Therefore, a three-dimensional model of human TAFI was initially derived based on the structure of the stable enzyme pCPB (8). In 2008, Marx et al. succeeded in the elucidation of the structure of human TAFI by crystallographic analysis, using recombinant TAFI with homogeneous N-linked glycans (► Fig. 2). The catalytic domain is folded into an 8-stranded mixed β-sheet, flanked by 9 α-helices in a typical αβα-hydrolase fold. The amino-terminal activation peptide can be subdivided into two parts. The first part (Phe1-Val76) forms an open sandwich antiparallel αβα-fold with four α-strands and two α-helices. This part is connected to the catalytic domain by a linker region (Glu72-Arg82) which is partially α-helical (45).

Within the catalytic domain, a dynamic flap region could be distinguished (Phe296-Trp350) (► Fig. 2), characterized by a higher mobility. In non-activated TAFI, the activation peptide stabilizes this dynamic flap region due to interactions of Val35 and Leu39 with Tyr341. Since the dynamic flap is part of the catalytic cleft wall, it is proposed to be involved in the (in)stability of TAFIa. In non-activated TAFI this region (Phe296-Trp350) is stabilized by interactions with the activation peptide. Activation of TAFI results in a dislocation of the activation peptide, a subsequent disruption of the stabilizing interactions resulting in an increased mobility in the dynamic flap, eventually leading to conformational changes that disrupt the catalytic site (45).

Elucidation of the structure of TAFI in complex with GEMSA revealed that the dynamic flap is stabilized upon binding of GEMSA within the active site, which is in line with the observation that GEMSA stabilizes TAFIa (60, 68). Almost simultaneously, Anand et al. (69) reported the structure of bovine TAFI. In line with the findings of Marx et al., a region in the TAFIa moiety (Ser305-His335) with increased flexibility was identified. This region also contains sulphate ions which are putative binding sites for heparin, previously being reported to exert a stabilizing effect on TAFIa (70), and further supporting the hypothesis that this region is responsible for the intrinsic instability of TAFIa (69). Recently, Sanglas et al. reported the structure of activated (bovine) TAFI, which was stabilized through interactions with the inhibitor TCI (71). The structure of the active site of TAFIa was found to be identical to the active site in non-activated TAFI, which is in line with the proposal of Marx et al. that the active site in non-activated TAFI is already in the active conformation. The three-dimensional structure of human TAFIa in complex with TCI adds to previous structural work and confirms that TAFIa is structurally unstable, poorly soluble and prone to aggregation (72).

Pathophysiology of TAFI

TAFI/TAFIa as thrombotic risk factor

Thrombotic disorders are caused not only by increased thrombin formation, but also by impaired fibrinolysis, possibly due to an increased TAFIa activity. As expected, epidemiological studies revealed that elevated TAFI levels in plasma are correlated with a slightly elevated risk for venous thrombosis (73, 74). Surprisingly, several studies claim that there is a relation between TAFI levels and arterial thrombosis, whereas others don’t (75–77). One study even reported a protective effect of extremely high TAFI levels against myocardial infarction (78). There are several possible explanations for the discrepancies between the conclusions drawn from these different studies. • Different methods were used to determine the TAFIa activity and/or TAFI antigen levels, including different calibrators and reference samples (29, 30, 79–82). • Various isoforms of TAFI display different reactivities in some commercially available ELISAs leading to an underestimation of the isoform (TAFI-I325) that exhibits a more pronounced antifibrinolytic effect (31). It has been suggested that the measurement of the extent of activation (either through measurement of the released activation peptide, the formation of TAFIa or the consumption of TAFI) might be a more relevant diagnostic marker (83–86). Taking into account the threshold mechanism (14, 15) and the fact that the TAFI concentration in plasma is far below the K_M values for TAFI activation, total TAFI concentration, extent of TAFI activation as well as TAFIa stability must be considered as risk factors for thrombotic disorders. It should be remembered that the function of TAFI/TAFIa is not limited to its role in fibrinolysis. Since TAFI has also been associated with inflammation, cell migration and wound healing, it might provide a direct link between thrombosis and atherosclerosis (87).

Over the last decade the potential role of TAFI/TAFIa has been investigated in many disease states, including venous and arterial disease, ischemic stroke, sepsis, disseminated intravascular coagulation, obesity, diabetes, hepatic disease, renal disease, cancer. A review of these epidemiological studies demonstrates that TAFI/TAFIa plays an important role in the fibrinolytic process in vivo and constitutes a potential risk factor in thrombotic diseases as well as in many other pathologies (13, 88).

TAFI inhibitors have a profibrinolytic potential

Currently used thrombolytics have been developed to reduce side effects of therapy by increasing fibrin specificity and serum half-life (89). However, clinical trials suggested also an increased incidence of severe bleedings using these third generation thrombolytics. Taking into account that TAFIa does not inhibit but attenuate fibrinolysis, TAFIa might be in interesting drug target (48). Adjunctive therapy with TAFI inhibitors may potentiate the thrombolytic efficacy of current thrombolytics and subsequently allow a reduction of the required doses needed for efficient thrombolysis, thereby reducing the risk for side effects as well.

In a rabbit arterial thrombosis model, perfusion times were significantly im-
proved when PTCI was incorporated before thrombolysis (90). Similar findings were observed by Nagashima et al. (91) in a rabbit jugular vein thrombolysis model. Moreover, required t-PA concentrations for efficient thrombolysis were decreased 3-fold. However, infusion of solely PTCI without t-PA did not enhance endogenous thrombolysis (90, 91). In contrast, in two other studies promising results were obtained: Without addition of t-PA, microthrombi could be efficiently lysed after PTCI injection, even after the formation of the thrombi (82, 92). Alternatively, inhibition of activation of TAFI in a mouse thromboembolism model, using a monoclonal antibody, resulted in a significantly increased survival rate (38).

TAFI-deficient mice

Three groups independently generated TAFI-deficient mice (22, 24, 93). TAFI-deficient mice are indistinguishable from wild-type littersmates concerning survival, development and fertility and did not show any major physical or haematological abnormalities. Also, upon haemostatic challenges, no significant differences could be observed between wild-type and TAFI-deficient mice in various thrombosis models (22, 24, 93). In one venous thrombosis model, TAFI-deficient mice revealed reduced thrombus formation (94) and in a batroxobin-induced pulmonary embolism model a lower degree of fibrin deposition in the lung was observed in TAFI-deficient mice compared to that in wild-type mice (51, 95). On the other hand, full TAFI-deficiency combined with heterozygous deficiency in plasminogen provided additional evidence for the role of TAFI in thrombosis as well as in inflammation (22).

Treatment of bleeding disorders by TAFI

Since TAFI activation is mediated by thrombin, deficiencies in the coagulation cascade may result in the formation of clots that are relatively unprotected against lysis. Considering the fact that factor XIa significantly contributes to generation of thrombin, the bleeding tendency of patients with factor XI deficiency might be explained by a diminished TAFI activation which results in a lack of protection of the clot against fibrinolysis. This hypothesis is strengthened by the observation that these patients mostly bleed in to tissues with locally increased fibrinolytic activity. Indeed, in vivo studies in animal models confirmed that factor XI-dependent inhibition of fibrinolysis is exerted by thrombin-generated TAFIa (96).

Also, deficiencies in factor VIII and IX led to premature lysis of in vitro formed clots, suggesting that the increased bleeding tendency in patients with haemophilia A and B is not only due to a failure of clot formation, but may also be due to premature clot lysis in consequence to inadequate TAFIa generation (97–99).

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