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A role for procarboxypepidase U (TAFI) in thrombosis

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1. ABSTRACT

The maintenance of the equilibrium between coagulation and fibrinolysis is crucial for normal haemostasis. In contrast, pathologic consequences of imbalance manifest tendencies of bleeding or thrombosis. Procarboxypeptidase U (proCPU, TAFI) is recognized as an important link between the coagulation system and fibrinolysis. Following activation by thrombin (IIa), carboxypeptidase U (CPU) exerts an antifibrinolytic effect by abolishing the cofactor function of partially degraded fibrin in plasminogen (Pg) activation. This review article focuses on the role of the proCPU/CPU system in the balance between fibrin deposition and removal. How a disturbed system can lead to a higher thrombotic tendency is discussed, while CPU inhibition as a new drug target for fibrinolytic therapy is extensively reviewed.

2. INTRODUCTION

Humans have evolved an intricate hemostatic system that is designed to maintain blood in a fluid state

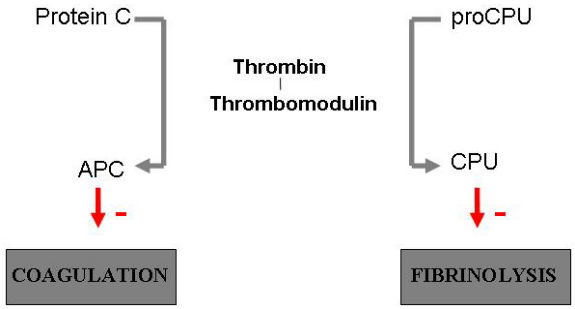

Figure 1. The thrombin-thromodulin complex (IIa-Tm) activates protein C (PC) and procarboxypeptidase U (proCPU) to the enzymes activated protein C (APC) and CPU, which respectively downregulate the coagulation and fibrinolytic cascades. The proCPU pathway provides a regulatory link between the two cascades such that activation of coagulation suppresses fibrinolysis.

under physiologic conditions, while it is primed to react to vascular injury in an explosive manner to stop blood loss by sealing the defect in the vessel wall.

In vasculature system, there are two well coordinated coagulation and fibrinolytic cascades that operate to stop blood flow at the site of an injury and to maintain blood fluidity elsewhere. These cascades involve plasma proteins, formed elements of blood, particularly platelets, and cells lining the blood vessel wall. In response to vascular injury, the coagulation cascade is upregulated to convert prothrombin (II) to IIa that converts fibrinogen to fibrin, thereby producing a blood clot. In response to fibrin, the fibrinolytic cascade is turned on converting plasminogen (Pg) to plasmin (PL), which digests fibrin into soluble fibrin degradation products (1-2). Imbalance of coagulation and fibrinolysis could present bleeding or thrombosis episode. Figure 1 depicts such delicate balance between fibrin deposition and removal. In the presence of thrombomodulin (TM), IIa acts as not only a potent anticoagulant, but also an antifibrinolytic enzyme. The thrombin-thrombomodulin (IIa-TM) complex converts the zymogen protein C to anticoagulant activated protein C (APC) that downregulates IIa formation through a negative feedback loop known as APC anticoagulant pathway. The IIa-TM complex also activates the zymogen procarboxypeptidase U (proCPU) to the active enzyme CPU, which suppresses the fibrinolytic rate. Thus, proCPU activation by the IIa-TM complex provides an explicit molecular link between the coagulation and the fibrinolytic cascades (2-3).

Thrombosis may occur if the haemostatic stimulus is unregulated, either because the capacity of inhibitory pathways is impaired or, more commonly, because the capacity of the natural anticoagulant mechanism is overwhelmed by the intensity of the stimulus (1). The relation of the protein C pathway with thrombosis has been demonstrated by the existence of severe thrombosis in the congenital absence of protein C and the elevated risk of thrombosis in the condition known as activated protein C resistance, associated with factor V_{Leiden} (1-3).

This review focuses on the role of the proCPU/CPU system in the balance between fibrin deposition and removal to elucidate how a disturbed system can lead to an enhanced thrombotic tendency. The biochemical and functional aspects necessary to understand the role of the CPU system in thrombosis will be discussed in full.

3. BIOCHEMICAL CHARACTERISTICS OF PROCPU

3.1. General considerations

Carboxypeptidases are a group of enzymes capable of cleaving a single amino acid from the C-terminus of peptide or protein substrates (4). Procarboxypeptidase U (proCPU, EC 3.4.17.20) is a carboxypeptidase B-like proenzyme which circulates in plasma at a concentration of 4-15 µg/ml (5-7). Since its first discovery by Hendriks in 1988 (8), the active enzyme carboxypeptidase U (CPU) has been independently described by three other groups named as carboxypeptidase R, plasma carboxypeptidase B, and activated thrombin activable fibrinolysis inhibitor (TAFIa) (9-11). CPU is a basic metallocarboxypeptidase meaning that Zn^{2+} is essential for catalytic action and that only basic amino acids (lysine and arginine) can be cleaved off from peptides and proteins (4, 12-13).

proCPU is synthesized in the liver as a prepropeptide consisting of 423 amino acids, composed of a 22 aa signal peptide, a 92 aa activation peptide and a 309 aa catalytic domain (Figure 2). The N-terminal signal peptide is efficiently cleaved off during secretion, resulting in a 401 aa glycoprotein (proCPU) with an apparent molecular mass of 60 kDa on SDS-page (10). A single cleavage at Arg92 removes the glycosylated activation peptide and liberates the 36-kDa non glycosylated catalytic unit, CPU (10, 11, 14). proCPU is also synthesized by the megakaryocyt during the process of megakaryocytopoiesis. This platelet proCPU is present intracellularly in α–granule–like structures and is secreted when platelets are activated by IIa. Such platelet proCPU may boost the local proCPU concentrations at the site of blood clotting (15).

It is suggested that proCPU circulates in a complex with Pg in plasma, although direct evidence is still lacking (13-14). proCPU binds with a 10-fold higer affinity to Lys-plasminogen compared with Glu-plasminogen (Kd 0.035 and 0.3 µmol/L, respectively). The binding of Pg is likely to be mediated by the glycosylated activation peptide of proCPU in view of the reduction in the affinity for Glu- and Lys-plasminogen by 7-and 10-fold, respectively, upon proCPU activation (14).

Interestingly, (pro)CPU is as a substrate for transglutaminases, such as factor XIIIa. This interaction might have importance in proCPU activation and CPU stability. Since CPU can retard fibrinolysis, the crosslinking to fibrin by FXIIIa during coagulation is of particular interest (16). Immunohistochemical examination of either *in vitro* or *ex vivo* made thrombi demonstrates that proCPU colocalizes with fibrin, which is consistent with

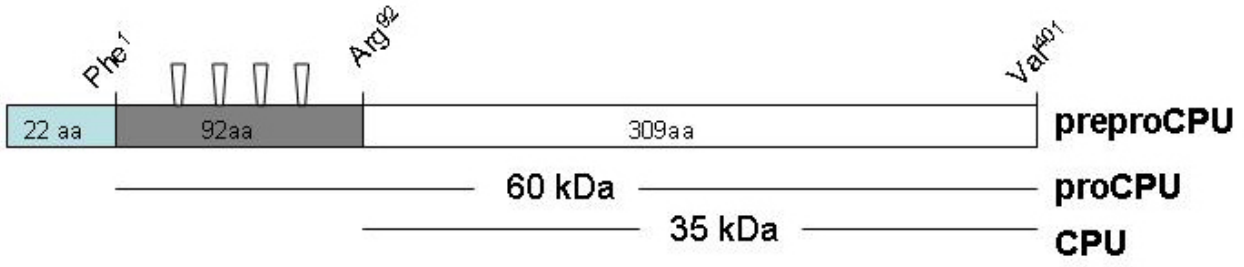

Figure 2. Presentation of preproCPU. ProCPU is synthesised as a prepropeptide: a 22 aa prepeptide (blue), a 92 aa activation peptide (grey) containing four Asn-linked glycosylation sites (white arrows) and the 309 aa enzyme domain (white). The proenzyme has an apparent Mr of 60,000, the active CPU enzyme 35,000.

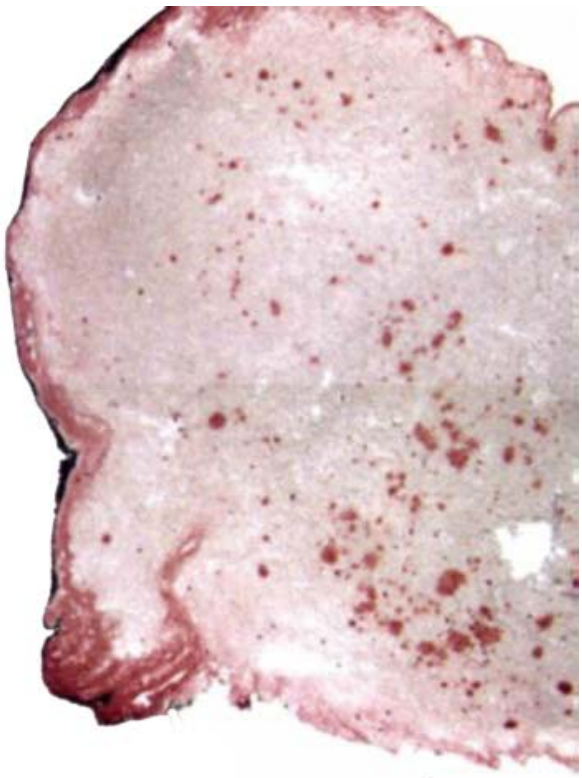

Figure 3. Staining for (pro)CPU in an *in vitro* formed whole blood clot. Thrombi were made in an artificial circulation (Chandler loop) using whole blood. Immunohistochemical staining was performed using antibodies that recognise both proCPU and CPU. The main immuno-reactive material (dark brown-red regions) was found on the surface of the clot along the fibrin strands which is consistent with cross-linking and binding of (pro)CPU to fibrin. In addition, (pro)CPU was detected in platelet rich areas present in the core of the thrombus, which is in agreement with the presence of (pro)CPU in platelets as discussed under 2.

such cross-linking and binding to fibrin (Figure 3) (17 and unpublished data from Hendriks and his associates).

3.2. proCPU gene polymorphisms

proCPU gene maps to chromosome 13q14.11., containing eleven exons and spanning approximately 48 kb of genomic DNA (18-19). Thus far, 16 single-nucleotide polymorphisms (SNPs) have been identified in the proCPU gene: 10 in the 5' flanking region (-2599G/C, -2345 1G/2G, -1925T/C, -1690G/A, -1102T/G, -1053T/C, -530C/T, -438A/G, -298G/A, -152A/G), three in the 3' flanking region (+1364G/A, +1542C/G, +1583A/T) and three in the coding region (+505A/G, +678C/T, +1040C/T). The 505A/G SNP results in T147A, and the 1040C/T SNP leads to T325I. 678C/T SNP is a silent mutation (20-23).

A wide range of variation with up to a ten-fold difference in proCPU plasma concentration between individuals has been reported (24) and it was suggested that proCPU levels are likely under genetic control supported by the fact that environmental factors poorly explain proCPU level variability. Several studies showed that proCPU gene polymorphisms were highly associated with the levels of circulating proCPU. Using a commercially available antigen kit it was stated that proCPU gene polymorphisms explained up to 60% of the variability of

proCPU levels indicating that proCPU levels are under strong genetic control (20-22, 24-25).

The polymorphisms in the 5'flanking region and the 3' UTR associated with proCPU plasma concentration are in strong linkage disequilibrium with each other and with the 505A/G SNP polymorphism in the coding region (20-23). The T147A mutation has the strongest association with antigen levels but is in strong linkage disequilibrium with the T325I (21, 23). In contrast, Gils *et al* demonstrated that T325I substitution can have a large impact on the immunoreactivity of the isoform so that the association reported in studies between the polymorphism and proCPU antigen levels may reflect differences in assay sensitivity rather than proCPU levels (26).

Using antigen assays insensitive to the isoform dependent artefact, a recent study reported proCPU polymorphisms contributing to 20% instead of the earlier 60 % (20-22, 24-25) of proCPU variability. In addition, the variability is only a 2 to 3-fold difference between the lower and higher antigen value as was reported previously using an HPLC-assisted activity assay (27-28). Interestingly, single-locus and haplotype analysis revealed that two polymorphisms, C-2599G and Ala147Thr (or T + 1583A that is in nearly complete association with it) had additive effects on proCPU levels and explained > 18% of proCPU variability. Moreover the authors stated that the haplotype carrying the Thr147 allele was associated with increased risk of coronary heart disease (CHD) in France where the reverse tended to hold in the Norhern Ireland population (29). The relation between proCPU gene polymorphisms and the relation to diseases remains under current investigation.

3.3. Measurement of the plasma proCPU concentration

Either immunologic assays (5-7) (sandwich ELISA) or activity based assays (27, 30-33) can measure proCPU concentration in plasma. Both methodologies show difficulties and pitfalls compromising straightforward measurement of proCPU in a routine laboratory setting. Unfortunately, many clinical studies in the past were performed with inadequately validated assays, forcing reconsideration of conclusions made in those reports. This complicated matter is reviewed in dept in (30).

To measure the extent of proCPU activation, Neill *et al.* recently developed a very sensitive and selective method to detect CPU concentrations in the picomolar range based on the attenuation by CPU of the Pg activator cofactor activity of high-molecular soluble fibrin degradation products (34). Ceresa & coworkers developed ELISA's that selectively react with the released activation peptide or with CPU reporting that subjects with hyperlipidemia had significantly higher plasma levels of both the activation peptide and CPU, whereas the difference for intact plasma proCPU did not reach the conventional level of statistical significance (35). Both recently developed assays could add benefit to evaluating the relationship between the proCPU/CPU system with cardiovascular disease.

4. ACTIVATION OF PROCARBOXYPEPTIDASE U

Thombin, plasmin, trypsin and neutrophil elastase are known to catalyze a single proteolytic cleavage at the Arg92-Ala93 bond, resulting in proCPU activation. (10-11, 14, 36-39)

4.1. Activation by thrombin: a role for the intrinsic coagulation pathway

IIa alone is a relatively weak activator of proCPU with a K_m of 2.14 μM and a k_{cat} of 0.0021 s^{-1}(38). proCPU is activated by IIa alone at a rate and extent to influence fibrinolysis, provided the IIa level is very high. The small amount of IIa generated by the extrinsic pathway is sufficient to convert fibrinogen to fibrin, while much higher IIa levels are required to activate proCPU. These concentrations are reached in the propagation phase of IIa formation in which the intrinsic pathway is triggered through the IIa-catalyzed activation of factor XI, and a relatively enormous burst of IIa occurs within the clot (40-42). In normal plasma following clotting, the burst of IIa is sufficient to activate proCPU to suppress subsequent Pg activation and fibrinolysis (41-42).

4.2. Activation by the thrombin-thrombomodulin (IIa-TM) complex

Either soluble or cellular form of the endothelial cell receptor TM drastically upregulates thrombin-induced proCPU activation up to a k_{cat} of 0.4-1.2 s^{-1} (38). The IIa-TM complex is thought to be the physiologic activator for proCPU activation. Similarly, the IIa-TM complex catalyzes the conversion of the zymogen protein C (PC) to the anticoagulant enzyme, activated-protein C (APC) that in turn inactivates factor Va and VIIIa and thereby downregulates IIa formation (43).

Distinct domains in TM have been identified to mediate either proCPU or PC activation. IIa binds to the epidermal growth factor (EGF)-like domains 5 and 6 of TM (44). The additional presence of EGF domain 4 is essential for PC activation (45), while proCPU activation requires the c-loop of EGF domain 3 with 13 a.a. connected to the EGF domain 4 to 6 of TM (45-46). Interestingly, the oxidation of Met-388 in TM, which can occur in the presence of neutrophils, reduces the rate of PC activation without effect on proCPU activation (46-47). This suggests that in an inflammatory milieu, a strong shift in the balance between fibrin deposition and removal could occur in favour of thrombosis, because the anticoagulant pathway through PC would be severely attenuated, but the antifibrinolytic pathway through proCPU would not (46-47)

TM potentiates fibrinolysis by down-regulating the generation of thrombin via PC activation while inhibiting fibrinolysis by making IIa more effective in proCPU activation. The net effect seems to depend on the TM concentration. proCPU activation is stimulated at low TM concentrations (<5 nM). In contrast, PC is activated at higher TM concentrations (~ 10 nM). As a result, TM downregulates IIa generation as well as proCPU activation (48).

In vivo, TM concentrations vary throughout the body in different compartments of the vasculature. Larger arteries contain lower concentrations, whereas the concentration increases when the blood moves into smaller capillaries. These variations in TM concentrations are likely to alter the outcome of the balance between the procoagulant and antifibrinolytic activities of TM (48).

4.3. Activation by plasmin

PL, the key protease of fibrinolysis, is also able to cleave proCPU at Arg92. The catalytic efficiency of PL is about eight-fold higher than the activation by IIa alone. Such PL-mediated activation is stimulated substantially by glycosaminoglycans resulting in a catalytic efficiency that is one tenth that of the IIa-TM complex (39). Similar to TM, glycosaminoglycans such as heparine could prevent fibrin deposition by stimulating the inhibition of coagulation while at the same time they could prevent fibrin dissolution by stimulating proCPU activation. The physiological role of PL in the regulation of CPU activity is unclear. Since IIa generation will usually precede PL formation, the importance of PL-mediated proCPU activation may be limited (49).

4.4. *In vivo* relevance of proCPU activation pathways

It is easy to demonstrate *in vitro* that a variety of enzymes can activate proCPU. However the ability of an enzyme to carry out this function *in vivo* will be largely dependend on its localisation and the presence at that site of cofactors, other substrates and inhibitors.

The IIa-TM complex mediated activation may be of particular importance in intact microvessels where the local concentration of endothelium-associated TM is very high (48, 50). Moreover, the concentration of soluble TM is elevated under certain pathological conditions such as disseminated intravascular coagulation (DIC), diabetes mellitus with angiopathy, and systemic lupus erythematosus (51).

In circulation, inhibitors such as antithrombin (AT) rapidly inactivate IIa. However inside a thrombus, clot bound IIa is protected from inhibition by AT and might contribute to activation of proCPU (52-53). PL-mediated proCPU activation could also be relevant upon exposure to extracellular matrix glycosaminoglycans after vessel injury (39).

5. INACTIVATION OF CPU

5.1. General considerations

Unlike most coagulation and fibrinolysis enzymes being downregulated by protease inhibitors including antithrombin (AT), antiplasmin, and Pg activator inhibitor-1 (PAI-1) there is no physiologic inactivator for CPU. CPU spontaneously loses its activity over time, which presumably represents physiological down-regulation (12-13, 54-56). The CPU decay is highly temperature-dependent; the half-life is approximately 10 minutes and 2 hours at body and room temperature, respectively. At 0°C, CPU is highly stable (12-13, 54-56). Its instability is reflected in the name that we gave in 1988 to this enzyme, i.e. carboxypeptidase U, the "U" standing for "unstable" (8, 12-13, 57).

It is generally accepted that CPU inactivation is due to a conformational instability rather than a proteolytic cleavage. In fact, proteolysis of CPU by IIa at Arg 302 occurs after the inactivation of CPU by a conformational change (55-56). An Arg302Gln mutant was not proteolytically cleaved as seen on SDS PAGE but still inactivated on incubation at 37°C (55). Moreover, CPU inactivation is accompanied by a substantial decrease in the intrinsic fluorescence of CPU, implying that CPU is indeed converted to an inactive form by a conformational change (55, 58).

5.2. Stabilising effect of inhibitors

CPU can be stabilized not only by decreasing the temperature but also in the presence of competitive inhibitors like guanidinoethylmercaptosuccinic acid (GEMSA) and potato tuber carboxypeptidase inhibitor (PTCI) where the free energy of the interaction with the inhibitor is used to stabilize the active conformation (59). Most likely for the same reason, the enzymatic activity is preserved in the presence of an excess of substrate, a feature that has been proven very useful in the development of CPU activity assays (27, 30-33).

5.3. Mechanism of CPUs instability

Little is known about the mechanism of CPUs instability. A naturally occurring variation in human proCPU was detected at position 325, being either a Thr or an Ile residue (21). The substitution has a significant effect on the stability of the active enzyme, CPU 325 Ile being twice as stable as CPU 325 Thr (15 min versus 8 min at 37°C) (60). To date, this Thr325Ile polymorphism is the only known amino acid variation that increases the CPU half-life. Several other proCPU mutants, created by site directed mutagenesis, with mutations in the proximity of residue 325, i.e. Arg302, Arg320 and Arg 330 all showed decreased stability of their respective active forms (55).

Interestingly, CPU is about 40% identical to pancreas CPB. Despite all similarities between proCPU and proCPB, pancreas CPB is in contrast to CPU a stable protease. Marx *et al* created a highly stable proCPU-CPB chimera by replacing amino acids 293 to 401 of proCPU to the corresponding ones in proCPB. The activated mutant displayed a markedly prolonged half-life of 1.5 h, indicating that the determinants of CPU stability are in the C-terminal part of the protein. However this chimera did not bind to Pg or fibrinogen and could not prolong clot lysis time in proCPU-deficient plasma as efficiently as wild-type proCPU (61). Recently Knecht *et al*. reported that single as well as a few (2-4) mutations in human CPU can prolong the half-life at 37°C from 0.2 h to 0.5-5.5 h (62). One mutant with H333Y and H335Q shows a half-life of 1.5 h. This CPU mutant YQ is the first reported stable CPU form with conserved wild-type characteristics and antifibrinolytic potential, making it an adequate surrogate of native proCPU for studying the physiological role of CPU and for structural characterization in the future (62).

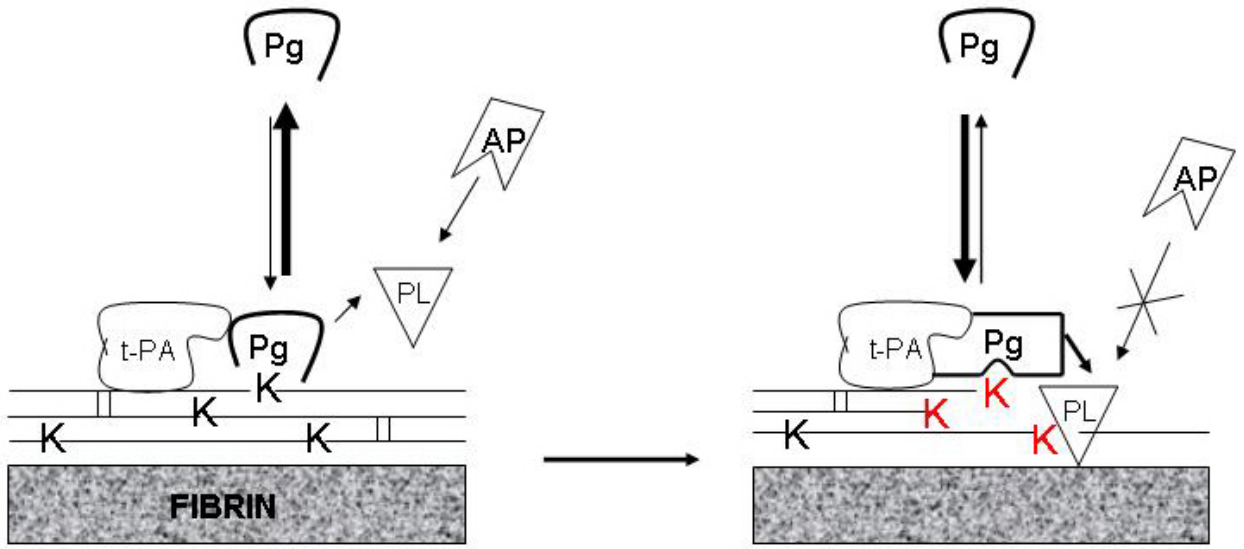

Figure 4. The importance of C-terminal lysine residues on partially degraded fibrin. In the initial phase of fibrinolysis, both Pg and t-PA bind to fibrin resulting in limited PL formation. PL cleaves fibrin after lysine residues (K) thereby creating partially degraded fibrin which contains free C-terminal lysine residues (K). These C-terminal lysine residues are high affinity binding sites for Pg leading to an accumulation of Pg on the fibrin surface. Moreover they evoke a conformational change in the Pg molecule making it a better substrate for t-PA. PL, which is normally cleared very fast by the action of α_2-antiplasmin (AP), can also bind to these C-terminal lysine residues and this binding protects PL from inhibition by AP. As a result the fibrinolytic rate accelerates dramatically.

6. CARBOXYPEPTIDASE U: AN ATTENUATOR OF THE FIBRINOLYTIC RATE

Fibrin deposition results from the activation of the coagulation cascade, while fibrin removal begins with the activation of the fibrinolytic cascade where Pg is converted to the enzyme PL digesting the fibrin clot to soluble fibrin degradation products. The imbalance between fibrin deposition and removal leads to the pathologic consequences, manifesting as bleeding or thrombosis (2, 63).

6.1. The fibrinolytic system

Intravascular fibrinolysis is initiated when Pg is converted to PL by tissue-type Pg activator (t-PA). PL then degrades fibrin, yielding soluble fibrin degradation products. Although PL displays a broad trypsin-like substrate specificity, fibrin-specific proteolysis is accomplished by formation of PL within a fibrin clot. This localization of PL is mediated by one or more of the five kringle domains that constitute the amino-terminal heavy chain through the expression of lysine binding sites which recognize lysine residues in fibrin. Activation of Pg also is rendered clot-specific by virtue of the affinity of the activator, t-PA for fibrin and the resulting fibrin-dependent stimulation of t-PA catalyzed Pg activation (63-65).

Fibrin thus binds both t-PA and Pg and thereby functions not only as the ultimate fibrinolytic substrate but also as a cofactor for Pg activation. Indeed, as a functional consequence of t-PA and Pg interaction with fibrin, the catalytic efficiency of t-PA mediated Pg activation is 2-3 orders of magnitude greater in the presence of fibrin than in its absence (66). PL cleaves fibrin after arginine or lysine residues; the resulting partially degraded fibrin contains C-terminal arginine and lysine residues (initial phase of fibrinolysis) The C-terminal lysine residues in partially degraded fibrin present high affinity binding sites for new plasmin(ogen) molecules in propagating fibrinolysis (Figure 4) (67-70). The action of PL on fibrin increases not only the accumulation of Pg on the fibrin surface, but the interaction of Pg with the newly exposed C-terminal lysine residues also evokes a conformational change in the Pg molecule thereby making it a better substrate for the activation by t-PA and u-PA (Figure 4) (71). Partially degraded fibrin also serves as a cofactor in the PL-mediated conversion of glu-plasminogen to its lysine counterpart, lys-plasminogen that is a better substrate for t-PA (66). In addition, the C-terminal lysine residues provide additional binding sites for PL and increase the protection of PL from inhibition by α_2-antiplasmin (Figure 4) (72-73). As a result, the fibrinolytic efficiency increases dramatically known as the acceleration phase of fibrinolysis.

6.2. CPU action: Threshold mechanism

Fibrinolysis is controlled by serine protease inhibitors of both the Pg activators (Pg activator inhibitors, PAI's) and plasmin (α_2-antiplasmin), and also by CPU. The basic carboxypeptidase, CPU, is able to cleave off the C-terminal lysine residues generated by the action of PL on fibrin, abrogating the enhanced cofactor activity of partially degraded fibrin and promoting the inhibition of PL by α_2-antiplasmin (11, 67-68, 72-73)

CPU does not inhibit fibrinolysis per se, but eliminates the positive feedback; it thereby substantially

attenuates the rate of Pg activation and fibrinolysis (74-75). CPU attenuates the fibrinolytic rate through a threshold dependent mechanism (74-75). As long as CPU is present at or above a key threshold value, fibrin degradation essentially ceases; the acceleration gains once CPU decays to the level below the threshold value (62, 74-75).

The time interval over which the CPU level stays above the threshold is determined by both its initial input concentration and half-life (first order decay). It becomes clear when the decay of CPU is expressed following a simplified equation: $N = N_0 \times e^{-k/t}$ where $k = \ln(2)/T$, T= half-life of CPU. Rearrangment of this formula gives the equation: $t = (T \log(2)^{-1}) \times (\log (N_0/N))$, where t is the time above the threshold, N_0 is the initial CPU activity, and N denotes the threshold activity value. This equation indicates that the time above the threshold, and hence the clot lysis time (CLT), is linearly related to the CPU half-life and only logarithmically with the initial CPU activity (generated from proCPU by first order kinetics) (62, 74). The generation of a sustained low level of CPU activity in excess of the threshold value is therefore far more efficient in retarding clot lysis than an efficient proCPU conversion during a short time interval. IIa formation and inactivation, TM concentration, proCPU concentration, and the thermal stability of CPU will determine the period of time that CPU activity remains above this threshold (62, 74).

It is important to note that the threshold CPU concentration is dependent on the concentrations of Pg activator, Pg and antiplasmin (74-75). Leurs *et al.* showed that the CPU threshold increases by increasing the t-PA concentration. Low t-PA concentration produces a much more pronounced effect of the CPU pathway on prolonging the CLT (74).

7. THE PROCPU/CPU SYSTEM AND THROMBOSIS

Figure 5 highlights the 3 distinct components (proCPU concentration, CPU stability and proCPU activation) in the proCPU/CPU system playing roles in thrombosis

7.1. Plasma proCPU concentration

7.1.1. Plasma proCPU concentration and the risk for thrombosis

proCPU circulates in plasma at a concentration of 70-250 nmol/L (4.4-15.0 µg/mL) (5-7). Given that the proCPU concentration in plasma is far below its K_m for the IIa or IIa-TM mediated activation (around 2 µM), the formation of CPU will be directly proportional to the proCPU concentration (30, 38). Consequently, a moderate change in the average plasma proCPU concentration will result in a similar change in the amount of CPU formed and thereby will have a modest effect on the CLT, since the relation between CLT and CPU concentration is logarithmic (62, 74). A low or a high proCPU concentration might therefore tip the balance between profibrinolytic and antifibrinolytic pathways and thereby cause a predisposition to bleeding or thrombosis. Plasma proCPU concentrations therefore have been analyzed in different clinical settings.

7.1.2. Clinical studies

Several epidemiological studies demonstrated a relationship of plasma proCPU concentration with thrombotic tendency. An overview of all clinical studies investigating the role of proCPU as a risk factor for cardiovascular diseases is summarized in reference 76.

7.1.2.1. Venous thromboembolic disease

Verdu *et al.* reported that in patients, the proCPU levels above the 90th percentile of the controls increased the risk for venous thromboembolic disease by 4-fold compared with proCPU levels below the 90th percentile (OR= 4.0; 95% confidence interval: 1.4-10.9) (77). This was in agreement with Eichinger *et al.* who demonstrated that high proCPU levels were associated with a 2-fold higher risk for recurrence of DVT (78) and Van Tilburg *et al.* who showed that proCPU levels above the 90th percentile increased the risk for thrombosis nearly 2-fold compared with proCPU levels below the 90th percentile (79).

7.1.2.2. Arterial thrombosis and coronary artery disease

Several studies have suggested that proCPU levels are associated with the risk of arterial thrombosis but results have been contradictory. Three retrospective studies using an activity based proCPU assay have reported that proCPU plasma levels are significantly increased in individuals with myocardial infarction at a young age (80), acute CHD (81), or stable angina pectoris (82). Leebeek *et al.* showed that functional proCPU levels were higher in ischaemic stroke patients than in controls (19.5 ± 4.2 vs 17.7 ± 3.7 min, $p<0.05$). Individuals with the highest quartile functional proCPU showed an increased risk of ischemic stroke compared with the lowest quartile (odds ratio (OR) 4.0, 95% confidence interval: 1.6-9.8). The persistently high proCPU levels after 3 months indicates that proCPU levels after stroke are not caused by an acute phase reaction. (83). Similar positive correlations were reported by Montaner *et al* (84) and Santamaria *et al* (85).

In sharp contrast, high proCPU levels were found to be protective against myocardial infarction reported by Prospective Epidemiological Study of Myocardial Infarction (PRIME) study (86) and to be negatively correlated with refractiveness in individuals with unstable angina pectoris (87). Reanalysis of the samples from the PRIME study with an ELISA insensitive to proCPU isoforms, however, revealed no significant association of proCPU levels with the risk of angina pectoris. Nor was there a significant association with heart coronary events (29).

7.2. The coagulation system and the generation of CPU

It was hypothesized that the thrombotic tendency in subjects carrying FV^{Leiden} relates to the proCPU/CPU pathway (88). proCPU and protein C are both activated by the IIa-TM complex. Consequently, the generation of IIa can in principle lead to downregulation of both the coagulation and fibrinolytic cascades. Indeed, *in vitro* studies with FV^{Leiden} disclosed not only reduced factor Va inactivation, but also attenuated fibrinolysis. As a result,

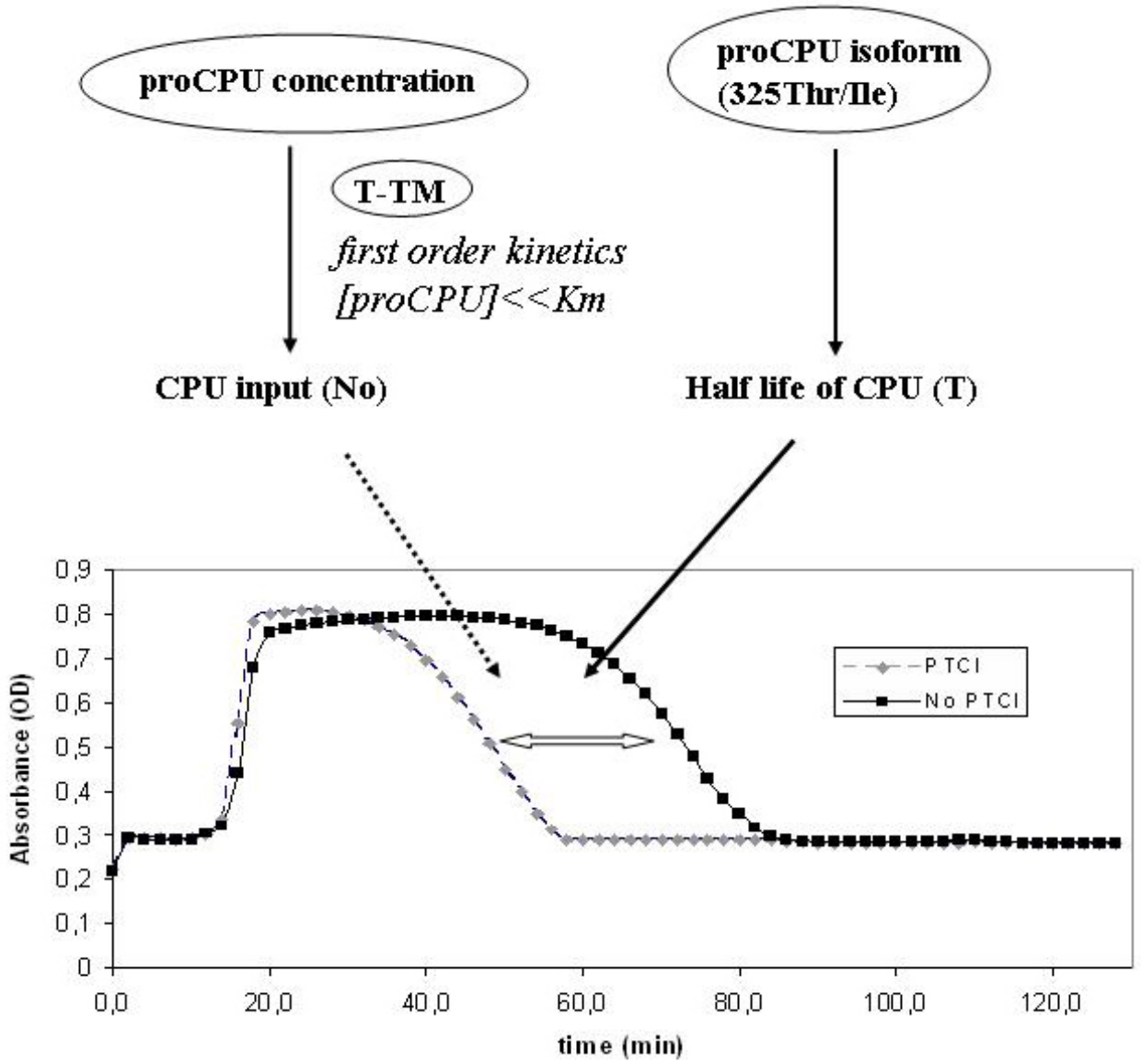

Figure 5. The proCPU/CPU system and the risk for thrombosis. The impact of the CPU system on fibrinolysis can be visualised using clot lysis experiments in the presence and absence of PTCI (a selective CPU inhibitor). CPU attenuates fibrinolysis through a threshold dependent mechanism. The time that CPU stays above the threshold and hence the clot lysis time is logarithmically (dashed line) related with the initial CPU input and linearly with the half-life of CPU. The proCPU concentration in plasma is far below its K_m for the IIa or IIa-TM mediated activation (around 2 μM), the formation of CPU will therefore be directly proportional to the proCPU concentration. The stability of CPU is influenced by a naturally occurring polymorphism at position 325. Thus proCPU plasma concentration, CPU generation and CPU stability are three distinct risk factors for thrombosis.

proCPU activation could adversally contribute to the thrombotic tendency in patients with FV^{Leiden} (2, 88). Similarly, the higher prothrombin levels associated with mutation G20210A in the untranslated region of the prothrombin gene, leads to more IIa generation, resulting in more proCPU activation and subsequent inhibition of fibrinolysis (89).

The increased thrombotic risk associated with protein S deficiency could also be due to an increased antifibrinolytic activity due to ineffective downregulation of IIa formation and subsequent proCPU activation regardless of APC-dependency or independency. Besides an APC cofactor activity, protein S also exhibits an APC-independent anticoagulant function. APC -dependent and -independent pathways were demonstrated to be involved in the downregulation of prothrombin and subsequent proCPU activation (90).

Meijers *et al.* described that an elevated factor XI level is a risk factor for venous thrombosis, with a doubling of the risk at levels that are present in 10 percent of the population. The involvement of the proCPU/CPU system

could partially account for the thrombotic risk. The high levels of factor XI result in the enhanced or sustained secondary generation of IIa, leading to a prolonged down-regulation of fibrinolysis and therefore a risk to thrombose (91).

7.3. Stability of CPU

7.3.1. The 325ThrIle polymorphism

The naturally occurring variation in human proCPU was detected at position 325 being either a Thr or an Ile residue, which has a significant effect on the CPU half life. The Ile- variant of CPU has a half-life that is double that of the Thr-variant; in addition, it is approximately 60% more potent as an antifibrinolytic agent (60). Therefore, the measurement of proCPU concentrations combined with the determination of differences in genotype distribution for this polymorphism between controls and patients were performed in many clinical studies after the discovery of this functional polymorphism. It has been reported that 10% of the population is homozygous for the more stable variant and 40% for the less stable one, although some populations (e.g. Chilean) show a strikingly low percentage of the Ile/Ile form (3%) (21, 92).

No significant differences in distribution could be seen in young patients with myocardial infarction (82), in an elderly Japanese population with cerebral infarction (93), in patients with CHD (29) and in patients with mucocutaneous bleeding (92) compared to controls. There is one report that describes a relation between this polymorphism and clinical outcome (survival or death) in meningococcal disease. The genotype of survivors and many of their relatives were determined, as were the genotypes of relatives of the nonsurvivors. The analysis indicated that patients whose parents were carriers of the CPU Ile 325 genotype had a 1.6-fold (CI, 0.7-3.7) higher risk of contracting meningococcal disease and a 3.1 fold (CI, 1.0-9.5) increased risk of dying from the disease compared with all other genotypes (94).

7.3.2. CPU inhibitors

Potato tuber carboxypeptidase inhibitor (PTCI also referred to as PCI or CPI) is the most widely used CPU inhibitor both in *in vitro* and in *in vivo* studies to specifically inhibit CPU but not the other carboxypeptidase present in plasma, CPN (95). PTCI not only inhibits CPU, but also stabilizes the catalytic domain (59). It was recently shown that PTCI could either enhance or inhibit CPU activity as well as the antifibrinolytic potential of CPU in a plasma system depending on the concentration of inhibitor, t-PA concentration, and CPU concentration. This can be explained by the notation that free and bound CPU stays in equilibrium. While the free CPU inactivates irreversibly, the bound form protected against spontaneous inactivation is released to replenish the free pool as to keep the equilibrium. As long as the free CPU concentration stays above the t-PA dependent threshold value, fibrinolysis will stay in its initial phase. Similarly, the arginine analog GEMSA, a reversible inhibitor of CPU, shows such a paradoxical effect (59). It remains unclear whether PTCI and other similar inhibitors can trigger an antifibrinolytic effect *in vivo*; however, no such effects have so far been reported using PTCI or other CPU inhibitors in animal models.

8. CPU: A NEW DRUG TARGET FOR FIBRINOLYTIC THERAPY

proCPU knockout mice have been obtained recently (96). These mice are viable and the absence of proCPU does not create a phenotype, which suggests that CPU inhibition would not be unacceptably dangerous. A role for proCPU was demonstrated in models of pulmonary embolism and peritoneal inflammation by backcrossing proCPU deficient mice to a heterozygous Pg background, indicating that proCPU can modulate the *in vivo* functions of plasmin(ogen) in fibrinolysis and cell migration (97).

8.1. Improvement of endogenous fibrinolysis

In a batroxobin-induced pulmonary embolism model, proCPU knock out mice (with normal Pg status) displayed a lower retention of fibrin in the lungs than did WT littermates, thus suggesting that proCPU deficiency enhances endogenous fibrinolysis (98). Furthermore, i.v. administration of *ex vivo* activated human proCPU dose-dependently impaires endogenous clot lysis in a rat model of batroxobin-induced fibrin deposition in the lungs, which is consistent with the concept that increased circulating CPU reduces the fibrinolytic rate (99).

An interesting question rises whether the inhibition of CPU or proCPU activation promotes endogenous fibrinolysis, thereby offering long-term protection from undesirable thrombotic events (100). Several studies have evaluated whether a CPU inhibitor alone improves endogenous thrombolysis. Minnema *et al.* described that incorporation of anti-factor XI antibodies in jugular vein thrombi resulted in an almost twofold increase in endogenous thrombolysis compared with a control antibody. CPU inhibition also results in a twofold increase in clot lysis, while inhibition of both factor XI and CPU activity shows no additional effect. It leads to the postulation that inhibition of factor XI activity enhances thrombolysis resulting from diminished proCPU activation (101). Wang *et al.* demonstrated that PTCI significantly inhibited murine thrombosis without administration of t-PA in a ferric chloride (3.5 %)-induced vena cava thrombosis model. In contrast, PTCI had no effect on 3.5% $FeCl_3$ –induced carotid artery thrombosis (102). Similarly, Suzuki *et al.* and Klement & coworkers did not observe any effect of a CPU inhibitor on endogenous fibrinolysis in an arterio-venous shunt model in rat and an arterial thrombosis model using rabbit aorta, respectively (103-104). Hashimoto and his associates, however, demonstrated the enhancement of endogenous fibrinolysis upon addition of PTCI or argatroban (a direct IIa inhibitor) in an arterial thrombolysis model using rat mesenteric arterioles (105). Some studies only report a significant effect of a CPU inhibitor when administered prior to but not after thrombus formation (101) while in other studies this effect is seen regardless of whether administration occurred before or after thrombus formation (105-106).

The efficiency of the administration of a CPU inhibitor alone in venous and arterial thrombosis models remains unclear and warrants further investigation

8.2. Adjuvants for thrombolytic therapy

Thrombolysis consists of the pharmacological dissolution of a blood clot by intravenous infusion of plasminogen activators that activate the fibrinolytic system. The clinical benefits of thrombolytic therapy in patients with acute myocardial infarction are well documented; however, all available thrombolytic agents still have significant shortcomings, including the need for large therapeutic doses, limited fibrin specificity and significant associated bleeding tendency and reocclusion (107). It is well known that thrombolytic therapy by t-PA or other agents induces a local procoagulant state. The observation of increased plasma fibrinopeptide A following such therapy is consistent with release of IIa in the vasculature, and could help explain the observations of rethrombosis following thrombolysis (53, 108). It appears that IIa inhibitors accelerate thrombolysis and improve clinical outcome (108-109).

Upon activation of platelets, proCPU can be secreted from α-granules boosting local proCPU concentrations at the site of the clot (15). The finding that proCPU can be crosslinked to fibrin by factor XIIIa (16) can have an important clinical relevance in that proCPU and IIa come in close proximity to each other at the surface of the thrombus leading to efficient CPU generation and that the interaction of CPU with fibrin can possibly lead to a stabilisation of this carboxypeptidase increasing its antifibriolytic potential dramatically (16, 62, 74).

Adjunctive therapy with a CPU inhibitor may therefore present a powerful tool to potentiate the thrombolytic effect and could simultaneously reduce the dose of Pg activators required, potentially reducing unfavourable side effects. Moreover, by its mode of action – on and within the thrombus only – a CPU inhibitor administered along with a Pg activator could be a major improvement in target specific thrombolysis.

Klement *et al.* investigated the impact of a CPU inhibitor (PTCI) on t-PA induced clot lysis in a rabbit model of arterial thrombolysis (104). They found that when the inhibitor administered along with t-PA enhanced the efficiency of thrombolysis. The time to reperfusion was reduced by an approximate factor of three. Vessel patency was similarly improved. Similar studies were carried out by Nagashima *et al.* in a rabbit model of venous thrombolysis, where the impact of PTCI on t-PA-mediated clot lysis was determined (110). When PTCI was administered along with t-PA, the clot weight was reduced to approximately half of the control weight. t-PA alone at the same dose reduced clot weight only 26%. This combined effect of t-PA and PTCI could be obtained with three times the dose of t-PA in the absence of PTCI. Thus the CPU inhibitor effectively tripled the effect of a given dose of t-PA. Mattsson *et al.* showed that local proCPU activation during thrombolytic treatment in a dog model of coronary artery thrombosis can be inhibited with a direct, small molecule IIa inhibitor (melagatran). This suggests that the profibrinolytic effects of direct IIa inhibitors may, at least partly, be due to an inhibition of IIa mediated activation of proCPU (112). Indeed, the shortening in lysis time when rt-PA was combined with melagatran is comparable to the reduced lysis time seen on addition of MERGETPA (a carboxypeptidase inhibitor) to rt-PA in the same model (111). As inhibition of CPU leaves the coagulation system intact, it can be hypothesized that CPU inhibitors can, with respect to bleeding complications, be a safer alternative for adjunctive therapy with t-PA than direct IIa inhibitors. However, this hypothesis warrants further investigations (111).

9. CONCLUSIONS AND PERSPECTIVES

Both *in vitro* and *in vivo* studies show that the proCPU/CPU pathway is an important link between coagulation and fibrinolysis. Upon activation by IIa, CPU prevents lysis from proceeding into the acceleration phase; therefore, this system is considered to be a potential target for the treatment of thrombotic disorders. The understanding of the proCPU/CPU system has prompted the development of several new selective CPU inhibitors, which will have to prove their merit for treating thrombotic disorders in the near future. Studies of proCPU knockout mice without an overt phenotype suggest that CPU inhibition may not be unacceptably dangerous. A possible role for CPU in the regulation of other physiological and pathophysiological processes such as inflammation and wound healing (reviewed in ref 76 & 113) warrants thorough investigation.

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Abbreviations: IIa: thrombin, APC: activated protein C, AT: antithrombin, CHD: coronary heart disease, CLT: clot lysis time, CPB: carboxypeptidase B, CPU: carboxypeptidase U, DIC: disseminated intravascular coagulation, EGF: epidermal growth factor, ELISA: enzyme-linked immunosorbent assay, GEMSA: guanidinoethylmercaptosuccinic acid, PAI-1: plasminogen activator inhibitor I, Pg: plasminogen, PL: plasmin, proCPU: procarboxypeptidase U, PTCI: potato tuber carboxypeptidase inhibitor, SDS-PAGE: sodium dodecyl

sulphate-polyacrylamide gel electrophoresis, SNP: single nucleotide polymorphism, TAFI: thrombin activable fibrinolysis inhibitor, TAFIa: activated thrombin activable fibinolysis inhibitor, TM thrombomodulin, t-PA: tissue type plasminogen activator, u-PA: urokinase-related plasminogen activator, WT: wild type

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