Role of the endothelial cell protein C receptor in thrombosis

P. Medina1, S. Navarro1, E. Bonet2, L. Martos1, E. Zorio1, A. Estellés1, F. España3
1 Hemostasis and Thrombosis Unit of the Research Centre; 2 Clinical Pathology Service; and 3 Cardiology Service. La Fe University Hospital. Valencia, Spain

ABSTRACT

The protein C anticoagulant pathway plays a crucial role as a regulator of the blood coagulation cascade. The endothelial protein C receptor (EPCR) was first revealed as the key molecule for efficient protein C activation, displaying its essential role in haemostasis. However, the discovery of the cytoprotective functions of activated protein C and the requirement of EPCR in the endothelial cell membrane scenario to activate the signaling cascades, highlighted EPCR as a molecule of paramount importance in the crosstalk between coagulation and inflammation.

The recent discovery of novel EPCR ligands has widened the perspective in the search of new EPCR functions. The interaction of EPCR with FVII/FVIIa and FX/FXa might result in physiologically relevant consequences. Additionally, many efforts are being made to elucidate the association of PROCR polymorphisms with the risk of thrombosis, alone or in combination with other risk factors. This review highlights the role of the endothelial protein C receptor in disease and discusses the association of its mutations with the risk of thrombosis.

Keywords: activated protein C, endothelial protein C receptor, factor VII, venous thrombosis, myocardial infarction

Introduction

The protein C (PC) anticoagulant pathway plays a crucial role in the regulation of fibrin formation via proteolytic inactivation of the procoagulant cofactors Factor (F) Va and FVIIIa. PC is a vitamin K-dependent glycoprotein that circulates in plasma as an inactive zymogen, which is activated to activated PC (APC) on the surface of endothelial cells by the thrombin-thrombomodulin (TM) complex. Another endothelial cell-specific protein, the endothelial cell PC receptor (EPCR) binds PC on the endothelial cell surface, which enhances the rate of PC activation 10- to 20-fold by decreasing the $K_m$ of PC for its activation by the thrombin-TM complex.

EPCR is mainly expressed on the endothelium of large vessels, which counterbalances the relative low presence of TM and ensures an efficacious PC activation in these vessels. This occurs because EPCR is located in the lipid rafts of the membrane where it co-localizes with TM, and PC binding to EPCR through its Gla domain makes it much more accessible to the thrombin-TM complex. Moreover, EPCR is also present, albeit at lower levels, on the surface of other cell types such as trophoblasts, monocytes, neutrophils, eosinophils, brain endothelial cells, lymphocytes, osteoblasts, gastric epithelial cells, chondrocytes, tenocytes, epidermal keratinocytes, human vascular smooth muscle cells, murine CD8+ dendritic cells, hematopoietic stem cells, cardiomyocytes, and in a variety of cancer cells.

Given the fact that EPCR is essential for APC to exert its properties, EPCR expression in these cell types may have a relevant role yet fully unexplored.

Once activated, APC may dissociate from EPCR, bind to its cofactor protein S and exhibit its anticoagulant functions, or it may remain bound to EPCR and display cell-signaling cytoprotective activities.

Recent studies point at EPCR as a cornerstone molecule in APC’s anticoagulant and cytoprotective activities, in addition inflammatory stimuli like tumor necrosis factor-α (TNF-α) or an atherosclerotic setting have been shown to reduce its expression.

The PROCR gene spans approximately 6 kb, is located on chromosome 20q11.2, and consists of 4 exons.

EPCR is a 46-kD type I transmembrane protein comprised of 238 aa. The crystal structure of EPCR showed that a tightly bound phospholipid resides in the groove typically involved in antigen presentation, which is necessary to preserve the ligand binding properties of EPCR. Very recently, it has been demonstrated that the major phospholipid bound to human sEPCR is phosphatidylcholine, which could also be exchanged for lysophosphatidylcholine and platelet activating factor, therefore impairing the protein C binding ability of sEPCR.

A soluble form of EPCR (sEPCR), which lacks the transmembrane and cytoplasmic tail domain, is present in normal human plasma and is shed by the metalloproteinase TNF-α converting enzyme or ADAM17. A variety of mediators increase EPCR shedding from the endothelium. Moreover, ADAM17 promotes the release of pro-inflammatory and adhesion molecules, and TNF-α significantly decreases the expression of EPCR and TM in several human endothelial cells, sEPCR binds PC and APC with the same affinity as the membrane-bound form, however its binding to PC impairs its activation and its binding to APC inhibits its anticoagulant and anti-inflammatory properties.

sEPCR levels show a bimodal distribution in a healthy population. Around 50-80% of plasma sEPCR variations are under genetic control and most subjects with elevated sEPCR levels carry the H3 haplotype, one of the PROCR haplotypes described, but this will be discussed in detail later. H3 can also increase sEPCR levels by al-
ternative splicing of its mRNA rendering a truncated EPCR lacking the sequence encoding the transmembrane and intra-cytoplasmic domains.

**Role of EPCR as multi-ligand receptor**

Recent investigations have pointed out a new role of EPCR as multi-ligand receptor, extending the perspective of EPCR functions. Light has been shed about PC/APC not being the only molecules able to interact with EPCR. In fact, FVIIa/FVIIa and FXa are also able to bind to EPCR. Both are serine proteases that play a central role in haemostasis and are also involved in signaling processes of wound healing, tissue remodelling, inflammation or metastasis.

The EPCR-dependent cytoprotective properties of APC have been well established. APC, when bound to EPCR in the lipid rafts, can cleave thus activating the protease-activated receptor-1 (PAR-1), which triggers signaling pathways ultimately responsible for the anti-apoptotic, neuroprotective and endothelial barrier-protective effects of APC. In fact, the APC-EPCR-PAR-1 axis has been proven to reduce organ damage in models of stroke, ischaemic injury, sepsis and autoimmune diseases. Moreover, a clinical trial is under study of APC as a promising pharmacological molecule for stroke, although in contrast its use for septic shock treatment in patients has been recently cancelled.

Recently and almost simultaneously, three groups found evidence that FVII/FVIIa were able to bind to EPCR with high affinity, similar to that of PC/APC, encouraged by the high degree of homology of the FVIIa Glα domain with that of PC, and the conservation of all the residues directly involved in the binding to EPCR. FVIIa is a serine protease that binds to tissue factor (TF) and initiates the coagulation cascade, and its interaction with EPCR on endothelial cells dose-dependently reduce the activation of PC, specially in situations such as haemophiliac or severe trauma patients under anti-haemorrhagic treatment with FVIIa. In addition, EPCR mediates the internalization of FVIIa bound to it on the cell surface, indicating that it may play a role in FVIIa clearance from the circulation to the extravascular space. Even though the physiologically circulating levels of FVII are very low compared to those of PC, 10 vs 60 nM respectively, when they were studied in a large healthy population, individuals carrying the Gly allele of the p.Ser219Gly polymorphism which also showed higher sEPCR levels and probably less membrane-bound EPCR, showed significantly higher levels of circulating FVII and of the marker prothrombin F1+2.

More recently, it has been shown that endothelial cells may down-regulate the slight FXa-dependent generation of FVIIa through EPCR binding. This regulation occurs by moving FVII from phosphatidyserine-rich regions, suggesting a new anticoagulant role for EPCR in normal haemostasis. Therefore, all these findings suggest that the interaction between FVII and EPCR may influence the basal activation of coagulation.

FVIIa, upon binding to the EPCR on the lipid rafts of the endothelial cell surface, activates the endogenous PAR-1 with similar efficiency to APC, and induces PAR-1-mediated p44/42 mitogen-activated protein kinase (MAPK) activation, thus providing a barrier-protective effect. However, research on this field is scarce, and further experimental evidence might be needed to confirm this barrier-protective effect of the FVIIa-EPCR complex.

It must be noted that, very recently, FXa has been shown to bind to EPCR, but whether it represents a high-affinity ligand of EPCR and, thus, shows any clinical relevance remains unsolved. Opposite results have been obtained for the signaling mechanisms triggered after FXa-EPCR binding on endothelial cells, suggesting that the relevance of the interaction between FX and EPCR is questionable.

**Anti-EPCR autoantibodies**

The presence of high titers of anti-EPCR autoantibodies has been described in patients with antiphospholipid syndrome, fetal death, deep vein thrombosis in the general population, and women with acute myocardial infarction. Also, a case report described a patient with stroke and massive cutaneous necrosis who had high titers of anti-EPCR autoantibodies. Two of these autoantibodies blocked the binding of PC to EPCR, and thus inhibited the generation of APC on the endothelium. For this reason, anti-EPCR autoantibodies may play a causative role in thrombosis, since low APC levels have been associated with an increased risk of venous and arterial thrombosis.

There is an association between elevated levels of the anti-EPCR autoantibodies, high levels of coagulation activity estimated by D-dimer levels, and levels of sEPCR, which could be related with endothelial injury induced by these autoantibodies. Anyhow, the mechanisms by which anti-EPCR autoantibodies confer a risk for thrombotic events are not fully understood.

**PROCR polymorphisms and thrombosis**

As mentioned before, normal APC generation depends on the precise assembly of thrombin and PC to their respective receptors, TM and EPCR, on the surface of endothelial cells. Any change in the efficiency of this coupling may cause altered APC generation and a modification in the risk of thrombosis. In fact, several mutations and polymorphisms have been reported in the PROCR gene, some of them associated with the risk of venous or arterial thrombosis.

**PROCR and venous thromboembolism**

Up to 4 haplotypes of PROCR have been reported: H1, H2, H3, and H4; 3 of which contain 1 or more single-nucleotide polymorphisms (SNPs) that are haplotype-specific. Two of these autoantibodies blocked the binding of PC to EPCR, and thus inhibited the generation of APC on the endothelium. For this reason, anti-EPCR autoantibodies may play a causative role in thrombosis, since low APC levels have been associated with an increased risk of venous and arterial thrombosis.

The H1 haplotype, tagged by the rare allele of g.4678G>C (rs9574), has been associated with increased circulating APC levels and a reduced risk of VTE in 2 independent studies. H1 also reduced the risk of thrombosis in carriers of FV Leiden. In patients with the FV Leiden mutation, the mean age at the first thrombosis was significantly higher in H1H1 propositi than in non-carriers of the H1 haplotype. Recently, a study evaluating the association of the PROCR haplotypes with the risk of VTE in Behçet disease, a rare autoimmune
It is known that oral anticoagulants reduce plasma APC levels in a haplotype. Bold numbers indicate supposed numbering for these single-nucleotide polymorphisms according to this numbering since the sequence described by Simmonds RE and Lane DA does not reach these positions.

EPCR is essential for normal embryonic development and plays a key role in preventing thrombosis at the maternal-embryonic interface. Moreover, it has been shown that the PROCR H1 haplotype protects Behçet’s patients from VTE. Furthermore, increased APC levels apparently protect patients from developing posterior uveitis. In contrast, 2 other groups found no association of the H1 haplotype with the risk of thrombosis, and this effect was also confirmed for VTE patients which was found not to be significant between PROCR H1 and the risk of thrombosis. A recent study reveals that carriers of PROCR H3 are protected against the clinical manifestations associated with Behçet’s disease, and that this protection is likely due to the increased levels of sEPCR that are associated with this haplotype. The presence of PROCR H3 and concomitantly elevated sEPCR plasma levels in carriers of the 2 dysfunctional PC variants, p.Arg-1Cys and p.Arg-1Leu, is associated with severe thrombotic manifestations. In addition, it has been observed that PROCR H3 increases the risk of VTE in carriers of the prothrombin g.20210A mutation, probably due to its association with increased sEPCR levels. Furthermore, H3 carriers experienced the first VTE episode at a young age. Additionally, the maternal PROCR H3 allele has been found to be a mild risk factor for iliac VTE during pregnancy and puerperium. A recent meta-analysis in which 12 candidate genes and 13 genome-wide association studies were analyzed, shows that the risk of VTE significantly increases by a factor of 1.22 (95% confidence interval, 1.11–1.33, P<0.001) for every additional copy of the G allele. Overall, the thrombogenicity of the PROCR H3, even if weak, does not seem anecdotal. First, the high incidence of this polymorphism in the Caucasian Mediterranean population (21.4%) and the fact that it may potentiate the prothrombotic effect of other thrombophilias, like the prothrombin g.20210A allele, suggests that its contribution towards a VTE event may not be negligible. Second, the H3 haplotype may be a risk factor not only for VTE but also for pregnancy loss. Further studies are required to identify which polymorphism/s is responsible for the observed associations, although some efforts are being made.

The H3 haplotype, tagged by the rare allele of g.4600A>G (rs867186), is associated with increased plasma levels of sEPCR, but its association with the risk of VTE is controversial. One study reported that carriers of the H3 haplotype have an increased risk of VTE in men but not in women, whereas others did not find a significant association between PROCR H3 and the risk of thrombosis. A recent study reveals that carriers of PROCR H3 are protected against the clinical manifestations associated with Behçet’s disease, and that this protection is likely due to the increased levels of sEPCR that are associated with this haplotype. The presence of PROCR H3 and concomitantly elevated sEPCR plasma levels in carriers of the 2 dysfunctional PC variants, p.Arg-1Cys and p.Arg-1Leu, is associated with severe thrombotic manifestations. In addition, it has been observed that PROCR H3 increases the risk of VTE in carriers of the prothrombin g.20210A mutation, probably due to its association with increased sEPCR levels. Furthermore, H3 carriers experienced the first VTE episode at a young age. Additionally, the maternal PROCR H3 allele has been found to be a mild risk factor for iliac VTE during pregnancy and puerperium. A recent meta-analysis in which 12 candidate genes and 13 genome-wide association studies were analyzed, shows that the risk of VTE significantly increases by a factor of 1.22 (95% confidence interval, 1.11–1.33, P<0.001) for every additional copy of the G allele. Overall, the thrombogenicity of the PROCR H3, even if weak, does not seem anecdotal. First, the high incidence of this polymorphism in the Caucasian Mediterranean population (21.4%) and the fact that it may potentiate the prothrombotic effect of other thrombophilias, like the prothrombin g.20210A allele, suggests that its contribution towards a VTE event may not be negligible. Second, the H3 haplotype may be a risk factor not only for VTE but also for pregnancy loss.

The high sEPCR levels associated with the H3 haplotype might be responsible for the increase in the thrombotic risk. Among the SNPs comprised in the PROCR H3, the g.4600G (p.219Gly) allele

Figure 1. The 4 haplotypes of the PROCR gene. Numbering according to Simmonds RE and Lane DA. Circled letters correspond to specific alleles for each haplotype.
arises as the more obvious candidate responsible for the association of the H3 haplotype with increased sEPCR levels, in view of the fact that the cleavage of the membrane-bound EPCR to generate sEPCR occurs around its position in exon 4. The Ser 219 to Gly substitution predicts a conformational change in the protein rendering an EPCR more susceptible to cleavage by ADAM17, and leads to a truncated mRNA through alternative splicing.

Recently, a study that looked for genetic determinants for PC levels has shown that the PROCR H3 is associated with higher levels of plasma PC (39,40). Additionally, the H3 haplotype has also been associated with higher levels of FVII (11,12), which could hypothetically confer its risk of thrombosis. An alternative explanation for the thrombogenicity that the H3 haplotype may induce is that the increased shedding of EPCR could reduce the amount of EPCR at the endothelial surface. In favor of this argument is the fact that inducing EPCR shedding in cells bearing the H3 haplotype notably reduced their ability to sustain PC activation as compared with non-H3 cells (41).

Finally, the PROCR H4 was reported to be associated with a slight increase in the risk of VTE (32), although no further studies have confirmed these results.

Additionally, two other mutations in PROCR have been reported, although its association with thrombosis could not be demonstrated. A 23-pb insertion in exon 3 generates a STOP codon downstream of the insertion point, which impairs the localization of the protein on the cell surface and, subsequently, APC generation. However, given its low population frequency (0.48% for VTE and 0.38% for arterial thrombosis) (11,45), its correlation with the risk of thrombosis could not be assessed. Finally, the relevance of the mutation c.2769C>T (p.Arg96Cys) could not be demonstrated in vitro (44) and its relevance could not be evaluated by any other case-control study due to its anecdotal prevalence.

**Final considerations**

Ever since the identification of EPCR as the essential receptor for efficient protein C activation, new discoveries highlighting additional roles of EPCR converts it in the central player in the cross-talk between coagulation and inflammation.

The recent discovery of novel EPCR ligands has opened new venues in the role of EPCR in serine-protease signaling pathways. FVII/FVIIa binds to EPCR with similar affinity than PC/APC. Although FVII’s concentration in circulation is six times lower than that of PC, increasing evidence demonstrates that such an interaction results in physiologically relevant consequences. However, further work is needed to address whether or not the interaction between EPCR and FX/FXa is biologically significant. Overall, these results do not clarify whether EPCR is part of the inflammatory response or if, conversely, it orchestrates signaling pathways towards protective actions. Furthermore, whether or not functional polymorphisms in the PROCR gene increase or decrease the risk of thrombosis, alone or in combination with other risk factors, has not been undoubtedly demonstrated and requires further investigation.

In conclusion, EPCR initially considered as the mere receptor for protein C activation, seems to have arisen as a key regulatory protein in diverse pathological scenarios. Therefore, a great outburst regarding new EPCR functions seems just around the corner.

**Conflict of interest statement**

The authors state that they have no conflict of interest.

**Acknowledgment**

This work was supported by the PN de I+D+I 2008-20011 of Instituto de Salud Carlos III-Fondo de Investigación Sanitaria and FEDER (PS09/00610 and Red Temática de Investigación RECAVA RD06/0014/0004), Conselleria de Educación-Generalitat Valenciana (Prometeo2011/027), and Fundación para la Investigación del Hospital Universitario La Fe (2007-0185), Spain. Pilar Medina is a Miguel Servet Researcher (ISCIII CP09/00065).

**References**