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REVIEW

The endothelial cell protein C receptor: cell surface conductor of cytoprotective coagulation factor signaling

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Abstract Increasing evidence links blood coagulation proteins with the regulation of acute and chronic inflammatory disease. Of particular interest are vitamin K-dependent proteases, which are generated as a hemostatic response to vascular injury, but can also initiate signal transduction via interactions with vascular receptors. The endothelial cell protein C receptor (EPCR) is a multi-ligand vitamin K-dependent protein receptor for zymogen and activated forms of plasma protein C and factor VII. Although the physiological role of the EPCR-FVII(a) interaction is not well-understood, protein C binding to EPCR facilitates rapid generation of APC in response to excessive thrombin generation, and is a central requirement for the multiple signal-transduction cascades initiated by APC on both vascular endothelial and innate immune cells. Exciting recent studies have highlighted the emerging role of EPCR in modulating the cytoprotective properties of APC in a number of diverse inflammatory disorders. In this review, we describe the structure–function relationships, signal transduction pathways, and cellular interactions that enable EPCR to modulate the anticoagulant and anti-inflammatory properties of its vitamin K-dependent protein ligands, and examine the relevance of EPCR to both thrombotic and inflammation-associated disease.

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Background

In addition to their well-established hemostatic functions, vitamin K-dependent (VKD) coagulation proteins can also modulate immune response upon infection or injury [1]. These immuno-regulatory effects require initiation of intracellular signaling networks in vascular and non-vascular cells. To trigger signaling, VKD coagulation proteases must first interact with extracellular receptors. In some instances, this interaction alone is sufficient to trigger signal transduction [2]. More commonly, however, extracellular receptor interaction positions VKD proteases such that protease-sensitive receptor activation and intracellular signal transduction can occur. These 'effector' receptors constitute a family of cleavage-sensitive G-protein coupled receptors (GPCRs) called protease-activated receptors (PARs) [3, 4]. PAR activation commonly occurs upon presentation of the VKD protease by a proximal coagulation protein co-receptor [5–8]. The endothelial cell protein C receptor (EPCR) fulfils this role for several VKD coagulation factors. VKD protein interaction with EPCR does not, however, serve solely to localize VKD proteases for PAR activation. Rather, EPCR binding can directly influence the subsequent nature of downstream PAR-activated signaling outcomes [9]. EPCR-dependent signaling by VKD proteins is implicated in a myriad of 'cytoprotective' responses to injury or infection. This review highlights recent advances in our understanding of EPCR physiological function, biochemistry and expression, and further explores the central position of EPCR in the regulation of coagulation and immune cell function.

EPCR expression, structure, and post-translational modification

EPCR was initially characterized by Esmon and colleagues as a high-affinity endothelial cell surface receptor for zymogen and activated protein C [10]. Although originally identified as an endothelial cell receptor, EPCR has since been detected in a number of cell types, including vascular smooth muscle cells [11], eosinophils [12], neutrophils [13], monocytes [14], keratinocytes [15], hippocampal neurons [16], cardiomyocytes [17], and placental trophoblasts [18]. EPCR was recently shown to be expressed on the surface of bone marrow [19] and fetal liver embryonic hematopoietic stem cells (HSCs) [20]. EPCR$^+$ HSCs exhibit potent hematopoietic reconstitution activity and demonstrate durable and equivalent differentiation for all blood lineages [19]. Consequently, EPCR expression is now an established HSC phenotypic marker.

The human EPCR gene (*PROCR*) is located on chromosome 20q11.2, spanning approximately 8 kilobases (kb) [21]. *PROCR*, consisting of four exons and three introns, encodes a protein of 238 amino acids [10, 21] to yield a mature protein of approximately 46 kDa [22]. Exon 1 encodes amino acid residues 1–24, which comprise the 5′-untranslated region and signal peptide. Exons 2 and 3 encode amino acids 24–108 and 108–201 respectively, which constitute the majority of the extracellular region. Exon 4 encodes amino acids 201–238, which includes the remaining extracellular domain, the transmembrane region (comprising 21 amino acid residues; 211–231), a short cytoplasmic tail (amino acid residues 232–238), and 3′-untranslated region [21]. Two major transcription initiation sites have been identified and are located 79 and 82 bp upstream of the transcription start site [23]. *PROCR* expression is tightly regulated by multiple *Sp1* transcription sites contained within the 5′ flanking region [24].

EPCR shares both sequence and three-dimensional structural homology with the major histocompatibility class 1 (MHC)/CD1 family of proteins, in particular murine CD1d [10]. The EPCR α_1 and α_2 domains consist of two antiparallel α-helices that sit upon an eight-stranded β-sheet platform. An MHC-like hydrophobic lipid-filled groove is formed between the two helices [25] (Fig. 1). Unlike MHC class I/CD1 receptors, however, EPCR lacks the α-3 domain and is therefore unlikely to associate with β-2 microglobulin.

EPCR undergoes significant post-translational modification prior to extracellular expression. EPCR contains four putative *N*-linked glycan attachment sites (Asn-30, Asn-47, Asn-119, and Asn-155), although the precise function of each individual glycan chain is unknown. Recombinant soluble EPCR variants in which each individual *N*-linked glycan attachment site was deleted by site-directed mutagenesis did not exhibit reduced ligand binding [26]. EPCR contains an intracellular C-terminal unpaired cysteine (Cys-221), which constitutes a putative palmitolyation site [10]. EPCR palmitolyation may contribute to the membrane localization and intracellular trafficking of EPCR, although a precise definition of its contribution to EPCR function is unresolved.

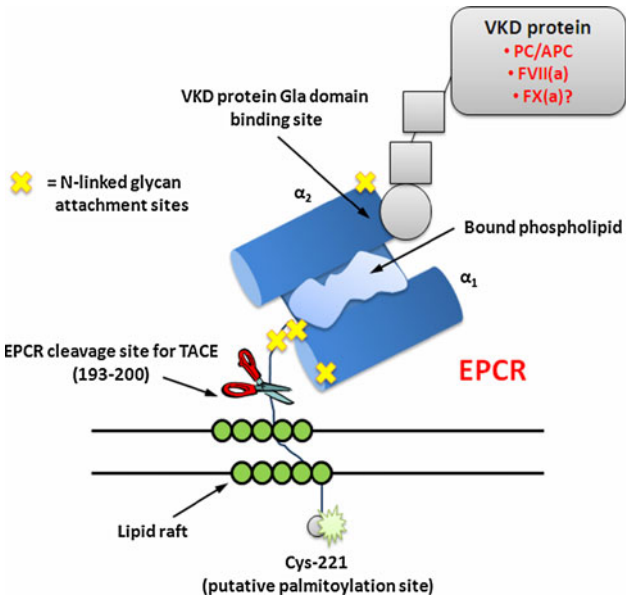

Fig. 1 EPCR structure–function relationships: EPCR is structurally homologous to CD1d (approximately 28% amino acid sequence similarity) and consists of a characteristic eight-strand β-sheet platform for two anti-parallel α-helical domains (α_1 and α_2). The α_1 and α_2 segments form a binding surface for VKD ligand binding, which is largely conserved across bovine and murine species. A crystal structure of recombinant soluble EPCR revealed the presence of a phospholipid moiety, tightly bound in the central groove, removal of which prevented protein C interaction. EPCR shedding is mediated by ADAM17/TACE upon cleavage of a region between EPCR amino acids 193–200. The cellular microenvironment of EPCR is crucial to its functional role in PAR activation and EPCR localization in lipid rafts has been shown to be necessary for APC-EPCR-PAR1 signaling on endothelial cells. EPCR has four solvent-exposed *N*-linked glycosylation consensus sequences at Asn-30, Asn-47, Asn-117, and Asn-155, and a putative palmitoylation site at the intracellular C terminus (Cys-221)

EPCR is a multi-ligand coagulation protein receptor

Zymogen protein C and activated protein C (APC) bind EPCR with comparable affinity [10, 22]. Both share an identical EPCR-binding motif, which is located within the N-terminal γ-carboxyglutamic acid rich (Gla) domain [27]. Resolution of the crystal structure of extracellular soluble EPCR (sEPCR) bound to a protein C Gla domain fragment identified a number of protein C amino acid residues that are crucial for EPCR interaction [25]. This interactive surface is

surprisingly small: Phe-4 and Leu-8 located in the conserved 'ω-loop' of the protein C/APC Gla domain mediate hydrophobic interactions with Tyr-154 and Thr-157 at the distal end of EPCR α_1 and α_2 chains. Additional hydrogen bonding between protein C Gla residues Gla-7, Gla-27, and Gla-29 and EPCR residues Glu-86, Arg-87, and Gln-150 were also identified. Site-directed mutagenesis studies have also confirmed that additional EPCR amino acids proximal to the site of ligand binding may also contribute to protein C-EPCR interaction [26]. The amino acid residues of the protein C/APC Gla domain involved in modulating EPCR binding are highly conserved across other VKD proteins, and are completely identical in factor FVII and its activated form, activated factor VII (FVIIa) [28]. Recent studies have confirmed that FVII(a) constitutes an additional ligand for both recombinant soluble [28] and cell-bound EPCR [29, 30]. Activated factor X (FXa) has also been reported to require EPCR to initiate PAR-dependent signaling [7], although the affinity of human FXa for EPCR in comparison to APC and FVIIa is unknown.

In addition to differences between the ability of individual VKD proteins to bind to EPCR, sequence and functional binding analyses suggest species-specific EPCR binding. Rat APC, for example, possesses a valine at position 8, which has been shown to be incompatible with EPCR binding in the human APC Gla domain [28]. Interestingly, the murine APC-EPCR binding motif differs slightly to that of human PC/APC/FVII(a) in that Leu-8 is replaced by methionine at this position. This EPCR binding motif is also present in human FX, which interacts with murine [31], but not human EPCR [32]. On this basis, the presence of Met at position 8 in the murine protein C ω-loop would preclude binding to human EPCR. The specific requirements for EPCR ligand interaction are, therefore, incompletely understood. Due to the almost complete conservation of the VKD protein binding region in EPCR from species characterized thus far [33], amino acid residues proximal to the VKD protein binding site must ultimately contribute to the affinity of EPCR-ligand interactions [26]. For example, in the crystal structure of sEPCR bound to the protein C ω-loop, direct interactions between Leu-8 of protein C with Leu-86 of sEPCR were observed [25]. Leu-86 is conserved in murine EPCR, but is surrounded by multiple amino acid residues which are not shared between human and murine EPCR, including a 2-amino acid insertion in the murine EPCR sequence at positions 88 and 89 [26, 33]. Consequently, this subtle alteration may provide an optimal binding surface for Met-8 (rather than Leu-8)-containing VKD protein ω-loops and thereby enable interaction with murine protein C/APC and non-murine VKD proteins that possess a Met-8 ω-loop motif, such as human FX. The specificity of the VKD protein ω-loop binding pocket is further highlighted by the failure of murine FVII to bind either human or murine sEPCR [32]. Murine FVII possesses a Leu at position 4 of its ω-loop, whereas Phe is present in all other VKD protein EPCR ligands. This indicates that substitution of Phe-4 is not compatible with EPCR binding, irrespective of species origin.

EPCR has been reported to also bind non-VKD protein ligands, although the functional significance of these interactions is not fully understood. A phosphatidylethanolamine moiety bound with high affinity to the central groove of the EPCR molecule was identified upon sEPCR crystallization [25]. Interestingly, the EPCR homolog CD1d utilizes the same groove to bind lipid antigens and present them to natural killer T (NKT) cells, resulting in their activation [34]. However, no role for EPCR in lipid presentation to NKT cells has been ascribed to date. The bound lipid may contribute to EPCR structural integrity, as detergent removal of lipid from EPCR resulted in significant loss of protein C/APC binding affinity [25]. Cell surface and recombinant sEPCR also bind to the neutrophil serine protease proteinase-3 (PR3)[35], which is secreted from neutrophils upon activation or expressed on the activated neutrophil cell surface. Optimal sEPCR binding to PR3 on PMA-activated neutrophils requires Mac-1, and may presage cell-bound EPCR cleavage by PR3 [36].

Regulation of coagulation by EPCR–ligand interactions

Protein C-EPCR complex formation on endothelial cells accelerates APC generation by the thrombin–thrombomodulin complex, and thus enhances the anticoagulant response upon thrombin generation [37]. EPCR binding results in approximately a fourfold reduction in K_m for protein C activation by thrombin–thrombomodulin complex on endothelial cells [37, 38]. The molecular basis of EPCR-dependent increased protein C activation is unknown, but current evidence suggests that EPCR positions protein C for optimal thrombin cleavage of the protein C activation peptide. Surprisingly, no direct interaction between thrombomodulin and EPCR has been described, and their requisite proximity on the surface of endothelial cells may be a consequence of co-localization in caveolin-rich lipid microdomains [39]. In baboons, co-infusion of thrombin with an anti-EPCR monoclonal antibody reduced APC generation 20-fold [40], suggesting the role of EPCR in protein C activation in vivo may be more prominent than is observed using cultured endothelial cells. Normal protein C activation upon thrombin challenge was observed upon EPCR$^{-/-}$ mouse bone marrow transplantation into EPCR$^{+/-}$ mice, demonstrating that non-hematopoietic cell (presumably endothelial) EPCR is the main site of protein C activation in vivo [41].

In contrast to protein C-EPCR binding, the physiological significance of FVII(a) and/or FX(a) interactions with EPCR are less well understood. EPCR has been shown to attenuate FXa generation by the FVIIa-TF complex [30, 31] via EPCR interactions with FVIIa or FX. FVII activation by FXa is also reduced by the presence of EPCR, which is presumably achieved by reduction in FVII/FXa interaction with cell-surface phospholipids upon EPCR complex formation [42]. Additionally, FVII binding to EPCR may have an indirect procoagulant role [29, 30]. The apparent affinity of FVII for EPCR suggests it is likely to remain bound to EPCR in vivo [28]. However, given the normal FVII plasma concentration (10nM) compared to protein C (70nM) it is unlikely to represent an effective inhibitor of protein C activation. Nonetheless, administration of pharmacological does of recombinant FVIIa may serve to inhibit APC generation on endothelial cells [29, 30].

The importance of EPCR in the regulation of coagulation in vivo has been characterized using transgenic mice. Mice completely deficient in EPCR ($EPCR^{-/-}$) die in utero by embryonic day 10. In contrast, mice with heterozygous EPCR deficiency ($EPCR^{+/-}$) survive to birth and are phenotypically similar to $EPCR^{+/+}$ mice [43]. Absence of EPCR expression on giant trophoblast cells, rather than embryonic cells, is responsible for the early embryonic loss associated with $EPCR^{-/-}$ mice [44]. However, the role played by EPCR in regulating hemostasis at the feto-maternal interface is not fully understood. Transgenic mice expressing approximately 1% of normal EPCR expression ($EPCR_{LOW}$) are viable and exhibit no thrombotic tendency, indicative of a requirement for only limited EPCR expression for embryonic viability [45, 46]. Conversely, a transgenic mouse line ($EPCR_{HIGH}$), with at least eightfold increased EPCR expression, exhibits higher circulating APC levels and as a result, rapidly reduced thrombin generation and fibrin deposition upon procoagulant challenge with FXa and phospholipid vesicle infusion [47].

Soluble EPCR

sEPCR, comprising the EPCR extracellular domain, is detectable in plasma [48]. sEPCR retains the ability to bind protein C/APC with similar affinity to membrane-bound EPCR [49], and is released from the endothelial surface by metalloproteinase (tumor necrosis factor-α converting enzyme (TACE)/ADAM17)-dependent shedding [50, 51]. sEPCR plasma concentration is bimodally distributed in the general population, with significantly elevated levels in approximately 10% of adults and 20% of children [52]. Unlike the membrane bound receptor, sEPCR acts to inhibit APC anticoagulant function. First, sEPCR competes with membrane-bound EPCR to bind zymogen protein C and thereby inhibits APC generation. Second, sEPCR also blocks the interaction of APC with cell-surface phospholipids [49, 53], preventing membrane complex formation with cofactor protein S and substrate activated factor V. Pro-inflammatory cytokines IL-1β and TNF-α, phorbol esters and thrombin enhance EPCR shedding from endothelial cell surfaces via activation of MAP kinase signaling pathways [54, 55]. Accordingly, higher levels of sEPCR have been reported in patients with systemic inflammatory diseases and conditions associated with increased thrombin production [56].

As EPCR is crucial for effective APC generation, genetic aberrations leading to diminished membrane EPCR expression or function may be expected to contribute to an increased risk of thrombosis. A 23-bp insertion in *PROCR* was identified in individuals with early myocardial infarction and deep vein thrombosis. In vitro expression studies showed that a severely truncated EPCR molecule with diminished function [57] is generated as a consequence of this insertion, although its broader contribution to thrombotic risk is difficult to establish due to its rarity.

Of the four defined *PROCR* haplotypes, the A3 haplotype is most associated with an elevated risk of thrombosis [58, 59]. This haplotype results in elevated sEPCR levels compared to other haplotypes, and is characterized by a glycine substitution at Ser-219 in the EPCR transmembrane region [58]. This substitution has been shown to result in increased sensitivity to EPCR cell surface shedding by ADAM17 [60]. Recent evidence indicates that an additional means by which the A3 haplotype contributes to elevated sEPCR levels is via expression of an alternatively spliced truncated *PROCR* mRNA [61, 62]. This alternatively spliced mRNA isoform is truncated at the 3′ end of exon 3, and therefore does not encode exon 4. The expressed EPCR therefore does not contain the EPCR transmembrane and intracellular domains, but instead possesses a C-terminal 56-amino acid tail. Transfection of a vector encoding alternatively-spliced EPCR cDNA into HEK 293 cells resulted in direct EPCR secretion and no membrane retention, highlighting the mechanism by which this EPCR mRNA transcript could lead to elevated sEPCR levels in individuals carrying the A3 haplotype [61]. Interestingly, the presence of the Ser219Gly substitution has been shown to result in significantly increased plasma levels of both protein C and factor VII in healthy middle-aged men, which is likely a consequence of reduced vascular membrane localization of these EPCR ligands [63].

Molecular basis of EPCR-dependent signaling by coagulation proteases

Important recent studies have suggested that APC administration may have therapeutic applications in a range of

diverse settings, including sepsis, post-ischemic stroke, diabetic nephropathy, multiple sclerosis, cancer, inflammatory bowel disease, and neurodegenerative disease [64–70]. EPCR binding is required for the beneficial effects of APC in each of these disease models. APC binding enables PAR1 activation and APC-specific PAR1/G-protein coupling [5]. EPCR blockade prevents APC activation of PAR1 therefore EPCR is an obligate receptor for APC signaling via PAR1 on endothelial cells [5]. APC can cleave and activate PAR1, but not PAR2 on endothelial cells. However, APC has been shown to activate PAR2 on EPCR/PAR2 transfected cells, and PAR1 cleavage by APC may initiate PAR2 activation by an unknown *trans*-activation mechanism [5, 71, 72]. APC induces multiple cytoprotective benefits, including down-regulation of injury-induced vascular barrier permeability both in vitro [6] and in vivo [73], down-regulation of inflammatory cytokines (IL-6, TNF-α) [74, 75] and up-regulation of IL-10 [76]. Furthermore, APC-EPCR-dependent PAR1 activation attenuates apoptosis in both endothelial cells [77] and neurons [78, 79].

The cellular microenvironment is crucial for EPCR-dependent PAR1 signaling by APC. Chemical disruption of lipid rafts disables EPCR-dependent protective PAR1 signaling by APC against thrombin-induced endothelial cell barrier permeability and TNF-α-induced apoptosis in endothelial cells [39]. PAR1 recruitment with EPCR to caveolin-rich microdomains alters G-protein coupling specificity, and thus the downstream signaling response upon PAR1 activation [9]. Specifically, APC-EPCR binding preceding PAR1 proteolysis alters PAR1 activation-dependent G protein coupling from $G_{q/12/13}$ to G_i [9]. As such, caveolae formation is crucial for EPCR-dependent APC signaling via PAR1 [80]. The physical means by which EPCR associates with lipid rafts in endothelial cells is unknown, but may be linked to interaction with caveolin-1 [39]. C-terminal EPCR palmitolyation is also expected to contribute to EPCR raft localization.

Prior studies utilizing PAR1 and PAR2 reporter constructs in heterologous cell expression systems indicated that FVII(a) binding to EPCR does not facilitate PAR1 cleavage and fails to prevent thrombin-induced endothelial cell barrier permeability [29]. Furthermore, FVIIa activation of PAR1 did not take place on EPCR-expressing immortalized endothelial cells [9]. However, recent studies indicate that EPCR-bound FVIIa can activate PAR1 on primary endothelial cells (HUVECs) and results in similar downstream protective signaling to that of APC [8]. EPCR-dependent FVIIa-PAR1 signaling on endothelial cells results in endothelial barrier stabilization, MAPK phosphorylation and Rac1 activation. Furthermore, EPCR has recently been described to interact with the extrinsic tenase complex (FX-TF-FVIIa) to modulate PAR activation in this setting [31].

FXa can also elicit cytoprotective signaling via PAR1/2 cleavage that is dependent upon EPCR [7, 81]. FXa binds endothelial cells and EPCR-transfected Chinese Hamster Ovary (CHO) cells. The presence of a monoclonal anti-EPCR antibody inhibits FXa activation of PAR1 and consequently ERK1/2 phosphorylation and endothelial cell barrier protection. These data suggest that many of the PAR-dependent signaling properties of FXa on endothelial cells require interaction with EPCR as a co-receptor.

EPCR-bound APC initiates alternative signal transduction pathways independent of PAR1 activation. Apolipoprotein E receptor 2 (ApoER2)-bound APC induces disabled 1 and glycogen synthase 3β phosphorylation via PI3 K/Akt signaling pathway on monocytic U937 cells. An antibody that inhibited APC-EPCR interaction attenuated APC inhibition of TF-mediated procoagulant activity via this pathway on LPS-treated monocytes. PAR1 blockade, however, had no effect [2]. This indicates that the APC-EPCR complex can stimulate anti-inflammatory signal transduction in a PAR1-independent manner on myeloid cells.

Ligand-bound EPCR determines PAR1 signaling outcome

In addition to EPCR-dependent PAR1 signaling by APC, recent research by the Rezaie laboratory has shown that zymogen protein C-bound EPCR is also important in the control of PAR1 proteolysis-dependent signaling [9]. EPCR bound to an enzymatically inactive APC variant containing an active site mutation that disrupts the APC active site, exhibits protection from thrombin-induced endothelial cell barrier permeability and staurosporine-induced apoptosis [9], amongst other cytoprotective benefits [82–85]. Similarly, PAR1 activation by PAR1 peptide agonists and thrombin in the presence of protein C-bound EPCR inhibits P-selectin secretion from Weibel-Palade bodies in endothelial cells, limiting leukocyte adhesion upon cytokine stimulation [86]. The molecular basis for thrombin-PAR1 signaling in the presence of protein C-bound EPCR on endothelial cells has been further defined. EPCR occupancy by protein C induces EPCR dissociation from caveolin-1-rich lipid rafts on the endothelial cell surface, altering the G-protein coupling specificity of PAR1 from $G_q/G_{12}/_{13}$ to $G_{i/o}$ [9]. This in turn leads to sphingosine-1 phosphate receptor 1 ($S1P_1$) phosphorylation via the PI3 K/Akt pathway [87]. Rac1 becomes activated and NF-κB down-regulated. Recently, a positive feedback loop has been identified whereby Rac1 activation up-regulates Ang1/Tie2 expression, amplifying PI3 K/Akt signaling and $S1P_1$ phosphorylation. The importance of the PI3 K/Akt signaling pathway was evidenced using a

specific PI3 K/Akt inhibitor LY294002, which completely ablated protective thrombin-PAR1 signaling on endothelial cells in the presence of protein C-bound EPCR [84, 87].

Despite convincing evidence for the protective benefits of PAR1 activation in the presence of protein C-bound EPCR on endothelial cells, a key role for EPCR occupancy in determination of PAR1 signaling outcome remains to be characterized in vivo, and is complicated by the complex role played by PAR1 activation during sepsis [88]. Additionally, administration of active-site inhibited APC is unable to confer protection from LPS-induced mortality in murine models of endotoxemia [89], suggesting EPCR occupancy alone by protein C/APC is not sufficient to enable sepsis survival benefit.

It is not known what role EPCR occupancy by alternative EPCR ligands FVII and FX may have on the unperturbed endothelium. FX can maintain endothelial cell barrier integrity in a similar manner to protein C (i.e., via alteration of thrombin-PAR1 signaling), enabling Rac1 activation and blocking NF-κB activation in the presence of thrombin and PAR1 agonists [90]. However, unlike protein C, EPCR was not found to be important in mediating this phenomenon [90]. Despite binding to EPCR with equal affinity as zymogen protein C, EPCR occupancy by FVII failed to yield any cytoprotective benefit upon PAR1 activation by thrombin or PAR1 agonists [8, 9]. Given the limited EPCR binding motif on PC/FVII required for FVII(a)-EPCR interaction, the inability of FVII(a)-bound EPCR to modulate PAR1 signaling specificity when activated by thrombin is incongruous with our current understanding of ligand-bound EPCR dependent reversal of PAR1 signaling. Furthermore, this discrepancy may indicate the involvement of hitherto unidentified mechanisms by which protein C-bound EPCR can modulate PAR1 signaling that are inaccessible to FVII.

In addition to direct regulation of cell surface signal transduction via PARs, exciting recent data suggest that EPCR can be internalized in the presence or absence of its ligands [91]. APC or FVIIa-bound EPCR undergo dynamin and caveolin-1-dependent endocytosis in both endothelial and EPCR-transfected cells. Upon internalization, the majority of EPCR-ligand complexes are trafficked to recycling endosomes before transportation back to the cell surface, making it unlikely that EPCR exerts a major role in the plasma clearance of its ligands [91], but may provide a means by which EPCR ligands can be delivered to the extravascular environment.

The role of EPCR in APC therapy of severe sepsis

The prototypal EPCR ligand APC exhibits pleiotropic signaling functions in vivo, regulating inflammatory, barrier-function and apoptosis pathways. Baboons challenged with a sublethal dose of LPS in conjunction with an anti-EPCR antibody that blocks EPCR ligand interaction died more rapidly than those treated with a non-blocking anti-EPCR antibody [92]. As such, ligand binding to EPCR was established as a crucial step in host response to sepsis. Later studies in which $EPCR_{HIGH}$ transgenic mice were challenged with LPS showed reduced mortality compared to wild-type mice [47]. As EPCR is expressed on both vascular endothelial cells and leukocytes, mice solely expressing hematopoietic or non-hematopoietic EPCR were utilized to define the cellular source of EPCR required for protective benefit from sepsis [41]. Interestingly, mice deficient in hematopoietic cell EPCR exhibited a similar response to LPS challenge as wild-type mice, indicating non-hematopoietic-derived EPCR is crucial for maintaining endogenous immune response to LPS challenge [41]. Further information on the role of EPCR in host response to sepsis is provided by transgenic $EPCR_{LOW}$ mice using the same endotoxemia model. As expected, $EPCR_{LOW}$ mice exhibited reduced survival compared to wild-type mice, and administration of APC had limited protective effect in these mice [89].

Efforts to characterize the cellular source of EPCR required to mediate the protective effects of exogenously administered APC in murine models of sepsis identified $EPCR^{+}CD8^{+}CD11c^{hi}$ dendritic cells and $EPCR^{+}$ hematopoietic cellular precursors as important for APC—reduced mortality [93]. This suggests that the cellular target for pharmacological APC is distinct from that utilized by the endogenous protein C-EPCR pathway to dampen the inflammatory response to LPS. Further clinical studies are necessary to elucidate the role of $EPCR^{+}$ dendritic cells in recombinant APC protection from sepsis-induced mortality.

Crucial role of EPCR interaction with APC beyond sepsis

There is increasing evidence that diminished EPCR expression and/or function can be a contributory factor in conditions characterized by chronic inflammation and autoimmune disease. For example, individuals with active inflammatory bowel disease (IBD) exhibit depleted EPCR expression and increased EPCR shedding on their colonic mucosal microvasculature, caused by local generation of TNF-α and IL-1β in the inflamed local environment [69]. The deleterious effect of vascular EPCR depletion in vivo was highlighted by mice with dextran-sodium sulphate-induced colitis, which lost the ability to effectively generate APC. 'Replacement' of missing APC by recombinant APC administration reduced disease activity, weight loss

and mucosal inflammation by inhibition of chemokine production and leukocyte adhesion to the colonic microvascular endothelium [69].

The presence of anti-EPCR auto-antibodies has been associated with an increased risk of deep vein thrombosis [94], myocardial infarction [95], and fetal death [96]. Anti-EPCR antibodies could feasibly inhibit APC anticoagulant activity by limiting protein C-EPCR binding and slowing protein C activation. Indeed, an anti-EPCR IgM antibody isolated from an individual with anti-phospholipid syndrome effectively inhibited protein C activation on endothelial cells [96]. Furthermore, given the established link between chronic inflammation and vascular disease, impairment of EPCR-dependent APC cytoprotective function may also be expected to contribute to the deleterious effect of anti-EPCR auto-antibodies in vascular disease.

APC can also mediate multiple neuroprotective benefits, which are dependent upon its interaction with EPCR on central nervous system (CNS) cells and the blood–brain barrier (BBB) [97]. EPCR mediated APC-PAR1 signaling enhances brain endothelial barrier integrity [6, 98] and stimulates angiogenesis [99–101]. APC–EPCR interaction promotes BBB integrity, and in doing so, reduces the passage of neurotoxic circulatory proteins into the CNS [70] and leukocyte migration across the BBB [102]. APC also directly inhibits microglial inflammation via activation of PAR1 [70] and neuronal cell apoptosis via PAR1 and PAR3 activation [78, 79, 103]. This neuroprotective effect of APC on CNS cells is indirectly facilitated by EPCR, as APC–EPCR complex formation is required for APC transcytosis across the BBB and delivery into the cerebrospinal fluid [104]. Recombinant APC administration is anti-inflammatory, neuroprotective, and increases neurological function and survival in murine models of ischemic stroke [64, 102]. Interestingly, this therapeutic benefit was not observed in transgenic $EPCR_{LOW}$ mice, highlighting the importance of EPCR in the neuroprotective effect of APC in vivo [64]. APC administration is similarly neuroprotective in murine models of embolic stroke [105], hemorrhagic brain injury [65], and amyotrophic lateral sclerosis (ALS) [70]. Recombinant non-anticoagulant APC administration to mice with a superoxide dismutase (SOD) mutation causing ALS-like symptoms enhanced BBB integrity and enabled cytoprotective neuronal signaling. This was evidenced by diminished SOD mutant expression and proinflammatory cytokine markers, reduced disease severity and increased lifespan. APC was unable to modulate BBB integrity or neuroprotective signaling in $EPCR_{LOW}$ mice, demonstrating the crucial role of EPCR interaction in the protective effects of APC in this mouse model. These studies highlight a potential role for non-anticoagulant APC as a drug target for multiple neurological diseases.

EPCR expression has been detected in a number of cancer cell lines, including monoblastic leukemia, glioblastoma, osteosarcoma, erythroleukemia, and prostate cancer cells [106]. Interestingly, EPCR can dictate divergent cancer cell behaviors: EPCR-dependent APC cleavage of PAR1 increases invasion and chemotaxis of breast cancer cells, without alteration of cell proliferation [107]. In contrast, $EPCR_{HIGH}$ mice with melanoma metastasis are less prone to metastatic infiltration into both the lungs and liver compared to wild-type mice. In vitro analysis of APC-treated cultured B16-F10 melanoma cells indicate that this function is a consequence of APC-EPCR complex-dependent down-regulation of tumor adhesion and transendothelial migratory functions [108].

Summary

Since its identification as a co-receptor for protein C activation by the thrombin–thrombomodulin complex on endothelial cells, discovery of additional roles in regulating VKD protease signal transduction has established EPCR as a central player in the convergent pathways of hemostasis and inflammation. EPCR binding unlocks the signaling potential of APC and potentially other ligands on numerous cell types, initiating down-regulation of multiple inflammatory processes. EPCR interaction is central to many of the potential therapeutic applications proposed for recombinant APC, and its prominent role in regulating endogenous response to infection has been unveiled in numerous animal models of inflammatory disease. Significant advances have been made in identifying novel EPCR ligands, but further efforts are required to establish what role, if any, these interactions may have in modulating hemostasis and/or immunity. Future studies will aid our understanding of the part played by EPCR in recently identified PAR-dependent and -independent signaling pathways, and further cement EPCR's position as the conductor of the complex symphony of VKD protease cytoprotective signaling.

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