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Endothelial cell protein C receptor-dependent signaling

Usha R. Pendurthi and L. Vijaya Mohan Rao

Department of Cellular and Molecular Biology, The University of Texas Health Science Center at Tyler, Tyler, Texas, USA

Abstract

Purpose of review—Endothelial cell protein C receptor (EPCR), a transmembrane glycoprotein present on the surface of endothelial cells and other cell types, is an essential component of the protein C (PC) anticoagulant system. EPCR is also shown to play a critical role in mediating activated protein C (APC)-induced cytoprotective signaling. The purpose of this review is to outline the mechanisms of EPCR-dependent cell signaling and discuss recent findings made in this area.

Recent findings—Recent studies showed that the cleavage of protease-activated receptor (PAR)1 at a noncanonical site by APC–EPCR or the canonical site by thrombin when PC occupies EPCR induces β -arrestin-2-mediated biased cytoprotective signaling. Factor VIIa binding to EPCR is also shown to induce the cytoprotective signaling. EPCR is found to be a reliable surface marker for identifying human hematopoietic stem cells in culture. EPCR, binding to diverse ligands, is thought to play a role in the pathogenesis of severe malaria, immune functions, and cancer by either blocking the APC-mediated signaling or by mechanisms that are yet to be elucidated.

Summary—Recent studies provide a mechanistic basis to how EPCR contributes to PAR1-mediated biased signaling. EPCR may play a role in influencing a wide array of biological functions by binding to diverse ligands.

Keywords

activated protein C; cytoprotective signaling; endothelial cell protein C receptor; factor VIIa; protease-activated receptors

INTRODUCTION

Endothelial cell protein C receptor (EPCR), originally identified as an endothelial cell-specific transmembrane glycoprotein capable of binding to protein C (PC) and activated protein C (APC) [1], is expressed in many cell types, including hematopoietic stem cells (HSCs) and tumor cells. EPCR plays a key role in the PC anticoagulant pathway by promoting the activation of PC by thrombin–thrombomodulin complex [2]. EPCR plays an

Correspondence to Usha R. Pendurthi, Department of Cellular and Molecular Biology, The University of Texas Health Science Center at Tyler, 11937 US Highway 271, Tyler, TX 75708, USA. Tel: +1 903 877 7342; Usha.Pendurthi@uthct.edu.

Conflicts of interest

There are no conflicts of interest.

important role in facilitating APC-mediated cytoprotective effects through protease-activated receptor (PAR)-mediated cell signaling. Recent studies demonstrated that EPCR also binds other ligands, including coagulation protein factor VIIa (FVIIa), *Plasmodium falciparum* erythrocyte membrane protein 1 (pfEMP1), and T-cell receptor (TCR) present on a subset of V δ 2 $^{-}$ γ δ T cells (Fig. 1). These observations raise a possibility that EPCR could mediate novel signaling pathways. In this review, we focus primarily on reviewing selected literature published in last 2 years on EPCR-dependent signaling. We recommend recently published extensive reviews for a comprehensive view on EPCR-mediated cell signaling [3–7].

ENDOTHELIAL CELL PROTEIN C RECEPTOR-DEPENDENT SIGNALING

EPCR is a type 1 transmembrane protein that exhibits sequence and three-dimensional structural homology with the major histocompatibility class 1/CD1 family of proteins, particularly CD1d [1,8]. As EPCR has only a short cytoplasmic tail (Arg-Arg-Cys-COOH), the induction of direct cell signaling by EPCR via its cytoplasmic tail is unlikely. However, EPCR-cytoplasmic domain may play an indirect role in the modulation of EPCR-dependent signaling as the palmitoylation of COOH-terminal Cys residue [9] may selectively localize EPCR into lipid rafts or caveolae [10] that are enriched with a plethora of signal mediators. The primary role of EPCR in cell signaling is to facilitate its protease ligands to activate PARs, particularly PAR1 by anchoring the protease proximity to PARs. The primary signaling ligand of EPCR is APC, and thus most of the studies in literature were on the delineation of APC–EPCR-mediated cell signaling pathways and their relevance in health, disease, and therapy. APC–EPCR activation of PAR1 initiates signaling via β -arrestin-2 and results in the activation of phosphatidylinositol 3-kinase/Akt survival pathway, transactivation of Gi protein-coupled sphingosine-1-phosphate receptor 1 (S1P1), and activation of Rac1 GTPase. These signaling events lead to cell survival, protection from barrier disruption, and suppression of the inflammatory nuclear factor kappa light chain-enhancer of activated B cells (NF- κ B) signaling pathway [7,11]. A recent study showed that EPCR also plays a role in APC inhibition of neutrophil extracellular trap formation [12■]. Although the mechanism by which EPCR–APC inhibits neutrophil extracellular traps (NET)osis is unclear, it was shown to require macrophage-1 antigen (Mac-1) and PAR3 to induce intracellular cytoprotective signaling that results in the downstream inhibition of NET formation [12■]. Many preclinical studies established that APC reduces the damage caused by diverse injuries or diseases, from sepsis to stroke, and most of the cytoprotective activities of APC were dependent on EPCR [7].

EPCR's role in cell signaling is not limited to promoting APC-induced signaling as EPCR can also bind other protease ligands, notably FVIIa [13–15]. FVIIa binds EPCR with the same affinity as APC as all the residues in PC that were shown to interact with EPCR are fully conserved in human FVIIa [14]. Although not all studies were successful in demonstrating that FVIIa bound to EPCR can activate PAR1 and prevent thrombin-induced endothelial cell barrier permeability [14,16], our studies showed that EPCR-bound FVIIa could activate endogenous PAR1 in primary endothelial cells and induce downstream protective signaling similar to APC [17]. A subsequent study showed that FVIIa reduces lipopolysaccharide (LPS)-induced and vascular endothelial growth factor-induced vascular

permeability *in vivo* in an EPCR-dependent fashion and involves the activation of PAR1 [18].

In addition to supporting FVIIa-induced cell signaling, EPCR may also modulate the signaling initiated by tissue factor (TF)–FVIIa–factor Xa (FXa) ternary complex. It had been shown that EPCR promoted more efficient cleavage of PAR1 and PAR2 by TF–FVIIa–FXa but had no effect on TF–FVIIa cleavage of PAR2 [19]. Liang *et al.* [20] showed that EPCR-dependent PAR2 activation by the ternary TF–FVIIa–FXa complex was required for LPS induction of toll-like receptor 3/4 signaling adaptor protein Pellino-1 and the transcription factor interferon regulatory transcription factor 8. Mice lacking EPCR failed to fully initiate an interferon-regulated gene expression program in response to LPS challenge [20]. These data suggest that EPCR acts as an essential coreceptor for the ternary TF coagulation complex-initiated cell signaling. More recently, it had been suggested that EPCR selectively modulates TF–FVIIa–FXa-induced signaling pathways [21]. In this study, Yuan *et al.* [21] showed that the activation of p44/42 mitogen-activated protein kinase signaling initiated by TF–FVIIa–FXa on activated endothelial cells was EPCR-dependent, whereas TF–FVIIa–FXa inhibition of p65 NF- κ B signaling was independent of EPCR.

ENDOTHELIAL CELL PROTEIN C RECEPTOR OCCUPANCY: BIASED SIGNALING

For many years, it was perplexing to understand how EPCR-dependent APC activation of PAR1 elicits cytoprotective signaling when the activation of the same receptor by thrombin induces a proinflammatory response and disrupts the endothelial barrier. The first clue to answering this conundrum came from an observation that EPCR and a subpopulation of PAR1 were localized in lipid rafts/caveolae microdomains in endothelial cells [22,23]. Next, Soh and Trejo [24] showed that APC activation of PAR1 initiates signaling via β -arrestin-2 and not through the G proteins as it happens with thrombin activation of PAR1. Mosnier *et al.* [25] found that APC cleaves PAR1 at both canonical Arg41 thrombin cleavage site as well as a novel noncanonical Arg46 cleavage site; and a peptide mimicking the N-terminus of PAR1 cleaved at the Arg46 site (PAR1 residues 47 and 66, TR47) initiates APC-like signaling response. The authors hypothesized that APC cleavage of PAR1 at a different site from that of thrombin allosterically modulates PAR1 and thereby APC-activated PAR1 specifically recruits β -arrestin-2 rather than interacting with G proteins and thus induce biased protective signaling. The occupancy of EPCR by its ligand, PC, and/or one or more proteins located in lipid rafts/caveolae microdomains in which EPCR and PAR1 reside may also play a role in allosteric modulation of PAR1 [22,23]. It had been shown that EPCR interacts with caveolin-1 within lipid rafts in endothelial cells and the occupancy of EPCR by PC leads to dissociation of EPCR from caveolin-1 and the initiation of PAR1-mediated cytoprotective signaling irrespective of whether PAR1 was cleaved by thrombin or APC [11,16]. A recent study showed that administration of PC to septic mice reduced not only levels of proinflammatory cytokine IL-6 and the extent of apoptosis in the septic lung but also decreased caveolin-1 expression in the lung [26]. The authors suggest that PC suppression of caveolin-1 facilitates the interaction between EPCR and PC that drives PAR1 signaling toward cytoprotection [26].

In a more recent study, Roy *et al.* [27] demonstrated that EPCR occupancy by PC initiated β -arrestin-2-mediated biased PAR1 signaling independent of whether PAR1 was cleaved at the canonical site or noncanonical site. This study provided further mechanistic insights into the biased PAR1 signaling (Fig. 2). The study demonstrated that occupancy of EPCR by either APC or catalytically inactive PC zymogen (PC_{S195A}) results in the recruitment of G-protein-coupled receptor kinase (GRK)5 to the plasma membrane. When GRK5 was recruited to the plasma membrane, the cleavage of PAR1 either by thrombin at the Arg41 site or APC at Arg46 site results in the GRK-dependent phosphorylation of the N-terminal cytoplasmic domain of the cleaved PAR1. The phosphorylation of PAR1 cytoplasmic domain by GRK5 inhibits the interaction between the PAR1 cytoplasmic domain and G proteins. EPCR occupancy by its ligand also results in recruitment of β -arrestin-2 and disheveled 2 (Dvl2) scaffolding proteins to the PAR1 cytoplasmic domain and thereby transmits the PAR1 signal through the β -arrestin-2-dependent pathway. PAR1-mediated signaling via β -arrestin-2 leads to activation of Rac1 GTPase, inhibition of NF- κ B pathway, and barrier protection in endothelial cells. Although EPCR- and PAR1-dependent APC cytoprotective responses require cross-talk with S1P1 signaling, it is not entirely clear currently the mechanism responsible for this cross-talk. Although the above study illustrates the importance of EPCR in regulating the PAR1-dependent signaling specificity, it does not answer how the dissociation of caveolin-1 from EPCR following its occupancy is linked to the recruitment of GRK5, β -arrestin-2, and Dvl2 to PAR1. APC binding to EPCR was also shown to induce the biased cytoprotective signaling through the cleavage of PAR3 at a noncanonical site, but the mechanism involved in the PAR3-mediated biased signaling is not entirely known [28]. Currently, it is unknown whether FVIIa occupancy of EPCR also results in similar responses as EPCR occupancy by PC. A recent study showed that EPCR occupancy by PC–FVII chimera containing the N-terminal domain of FVII with the conserved EPCR-binding site failed to mimic barrier stabilization induced by EPCR occupancy by PC upon PAR1 proteolysis by thrombin [29]. These data suggest that residues in PC outside of the EPCR binding site may play a role in enabling EPCR-dependent PAR1-mediated cytoprotective signaling.

ENDOTHELIAL CELL PROTEIN C RECEPTOR: IMMUNE FUNCTIONS

The apparent structural similarity between EPCR and CD1/major histocompatibility complex (MHC) class 1 superfamily [30] and the presence of a tightly bound phospholipid in the hydrophobic groove typically involved in antigen presentation raised a possibility that EPCR may present lipid antigens to T cells and play a role in host defense from infection. However, no evidence was found so far that EPCR presents antigens to T cells. But, Willcox *et al.* [31] found that EPCR binds to a TCR of a subpopulation of $\gamma\delta$ T cells (V δ 2 $\gamma\delta$ T cells). Although structural similarity of EPCR with MHC molecules and its lipid-binding analogous to CD1d suggest that the lipid-binding surface of EPCR could be responsible for binding to the TCR, mutagenesis studies showed that structural moieties of β -sheet of EPCR, and not the lipid-binding surface, are crucial for the EPCR–TCR interaction [31]. The binding of EPCR to TCR alone is not sufficient to activate T cells bearing $\gamma\delta$ TCRs; other costimulatory molecules that are expressed in response to cytomegalovirus infection or malignancy are required for $\gamma\delta$ T-cell activation. The above data indicate EPCR as a

prototypic stress-regulated molecule that elicits the increase in the number of $\gamma\delta$ T cells in response to infection or malignancy. It had been proposed that the constitutive expression of EPCR on endothelial cells and tumor cells could direct $\gamma\delta$ T cells toward cell types Vascular biology that are most relevant for the control of infection or malignancy and could establish thresholds for the activation of $\gamma\delta$ T cells [31]. Although the above findings raised a novel signaling function to EPCR in immunological functions, there were no follow-up studies yet to delineate molecular mechanisms by which EPCR recognition contributes to activation and expansion of $\gamma\delta$ T-cell subset and its role in subsequent viral clearance or clearance of tumor cells. A recent review provides a comprehensive discussion on how endothelial cells could contribute to the immune response by expressing EPCR and other MHC class I-related molecules [32■].

ENDOTHELIAL CELL PROTEIN C RECEPTOR AND SEVERE MALARIA

Endothelial cell dysfunction that results from sequestration of infected erythrocytes in the microvasculature is a central pathogenic mechanism in severe malaria [33]. The recent discovery that in severe malaria infected erythrocytes expressing a specific set of PfEMP1 bind EPCR on the endothelium gave new insights into the pathogenesis of severe malaria [34]. PfEMP1 proteins are highly polymorphic and contain a series of adhesion domains, Duffy-binding like and cysteine-rich interdomain region (CIDR) [35]. Turner *et al.* [34] showed that PfEMP1s containing CIDR α 1 domain that associated with severe malaria bind EPCR. They also showed that PfEMP1 binds EPCR near or at the same region as APC. The interaction between PfEMP1 and EPCR is thought to contribute to the pathogenesis of severe malaria as the PfEMP1 binding to EPCR blocks the binding of PC and APC to the EPCR, thus reducing PC activation and dampening APC-induced anti-inflammatory and barrier-protective effects [34,36,37]. Reduced PC activation results in an increased thrombin generation, which promotes proapoptotic, proinflammatory, and barrier-disruptive signaling pathways in endothelial cells [11]. The subsequent generation of fibrin also activates proinflammatory pathways and promotes monocyte recruitment to the microvasculature [38]. This vicious cycle of inflammation, loss of barrier integrity, and thrombosis has been thought to be responsible for the pathogenesis of severe malaria associated with the parasite binding to EPCR [36,37]. In-vitro studies of Petersen *et al.* [39] provided robust evidence to the above concept by showing PfEMP1 (CIDR α 1) severely impaired the activation of PC, blocked APC-mediated activation of PAR1, and the associated barrier protective effects of APC on endothelial cells.

EPCR-binding CIDR α 1 domains are extremely diverse, including in the residues that directly contact EPCR [40]. But, the conserved structural features in the diverse CIDR α 1 domains allow the CIDR α 1 domain containing PfEMP1 to retain the EPCR-binding phenotype [40]. However, subtle differences exist among various CIDR α 1 domains in their binding footprints as they differ in their APC-blockade activity, indicating distinct PfEMP1 variants in severe malaria may bind with different affinities to EPCR [41]. Although the binding sites of PfEMP1 and APC overlap, there are significant differences between their binding as the CIDR α 1-binding site is much larger than that of PC [40]. Such differences between PfEMP1 and APC binding to EPCR might allow development of decoy molecules that could bind PfEMP1 but not to APC, such as soluble EPCR variant (sEPCR_{E86A}) [39].

When sEPCR_{E86A} variant was used as a decoy to capture PfEMP1, it reduced cytoadhesion of infected erythrocytes to endothelial cells and allowed normal activation of PC and APC-mediated barrier-protective effect [39].

Recent studies showed that intracellular adhesion molecule-1 (ICAM-1) is a coreceptor for a subset of EPCR-binding parasites and the complementary receptor interactions of EPCR-binding PfEMP-1 with ICAM-1 amplifies development of severe malaria [42,43]. Analysis of expression of different subsets of PfEMP1 transcripts from Ugandan children with cerebral malaria showed that increasing severity of illness was associated with increasing levels of EPCR-binding PfEMP1 [44]. Levels of EPCR binding-PfEMP1 transcripts were also associated with plasma levels of angiopoietin 2 (Ang2) in severe malaria [45]. Ang2 is a growth factor released from Weibel–Palade bodies and elevated plasma concentrations of Ang2 reflect endothelial activation [46]. These data suggest that PfEMP1 binding to EPCR results in endothelial activation in severe malaria. At present, it is unknown whether increased thrombin generation or EPCR-PfEMP1-mediated direct effect is responsible for endothelial activation in severe malaria patients.

ENDOTHELIAL CELL PROTEIN C RECEPTOR: A NOVEL STEM CELL MARKER WITH STEM CELL FUNCTIONS

A small fraction of HSCs in the murine fetal liver and adult bone marrow were shown to express EPCR on their surface, and these cells were found to be endowed with the highest bone marrow long-term repopulation compared with HSCs lacking EPCR [47,48]. Recent studies by Gur-Cohen *et al.* [49] showed that EPCR-mediated signaling plays a key role in the retention and recruitment of long-term-HSCs in the bone marrow. All the components required for EPCR-mediated signaling, that is, thrombin : thrombomodulin complex that converts PC to activated protect C and PAR1, were expressed in the bone marrow, endothelial cells in bone marrow, and HSCs themselves [49]. APC–EPCR–PAR1 signaling in bone marrow was shown to limit NO generation by increasing endothelial nitric oxide synthase phosphorylation at the negative regulatory site Thr495 and reduced phosphorylation at the positive regulatory site Ser1177. Low NO levels, in turn, limit Cdc42 activity and enhance integrin very late activation antigen 4 affinity, the processes that facilitate long-term-HSCs adhesion and bone marrow retention. The observation that mice expressing very low levels of EPCR showed defects in the homing of HSCs in the bone marrow and increased levels of HSCs in the circulation further supported the importance of EPCR-mediated signaling in retention of HSCs in the bone marrow. The initial studies of EPCR expression in HSCs were limited primarily to murine cells, but recently Fares *et al.* [50] showed that EPCR is a highly reliable, robust surface marker for expanded human long-term-HSCs. In contrast to other stem-cell markers, such as CD38, EPCR expression in long-term-HSCs was maintained irrespective of culture conditions and duration. Transcription profiling of EPCR– and EPCR+ HSCs sorted from expanded cord blood showed that genes encoding stem-cell surface markers are preferentially expressed in EPCR + cells. The role of EPCR in human long-term-HSCs appears to differ from that of murine long-term-HSCs as the EPCR silencing disrupted HSC function by affecting self-renewal than the homing ability [50]. The demonstration of EPCR as a novel stem-cell marker may

allow the identification and separation of human long-term-HSCs from both unmanipulated and ex-vivo expanded cord blood HSCs. The analysis of transcription factors upregulated in self-renewing EPCR+ HSCs may identify genetic pathways governing HSC 'stemness'. A recent study showed that EPCR is also a potential human epidermal stem-cell marker [51]. This study showed that EPCR+ human epidermal keratinocytes were associated with the highest levels of stem-cell markers p63 and integrin β 1 and EPCR play a role in keratinocyte survival and proliferation.

ENDOTHELIAL CELL PROTEIN C RECEPTOR AND CANCER

Aberrant expression of EPCR was detected in tumors of different origin, and the EPCR expression in tumors was found to correlate with clinical outcome [52,53,54–59]. As EPCR/APC-mediated cell signaling typically activates cell survival and antiapoptotic pathways [6,60], it is believed that EPCR expression in tumor cells promotes tumor growth and metastasis [61,62]. Consistent with this notion, the EPCR was shown to promote tumor growth and metastasis in lung tumorigenesis as the EPCR/APC-mediated signaling increased the survivability of tumor cells by downregulating apoptosis [55]. In a recent study [53], Perurena *et al.* examined the role of EPCR in primary and metastatic tumor growth by analyzing the correlation between EPCR expression and clinical outcome in a cohort of 286 breast cancer patients and using murine xenograft models. High EPCR expression levels associated with a poor clinical outcome in this cohort. Studies in murine models showed that EPCR silencing reduced primary tumor growth as well as metastasis in the skeleton and the lungs. Transcriptomic analysis of EPCR-silenced tumors revealed that EPCR silencing downregulated several genes related to tumor progression, including SPOCK1/testican 1, a member of the SPARC family of matricellular proteins. Additional studies showed that SPOCK1 silencing impaired breast tumorigenesis and metastasis, suggesting that EPCR promotes breast cancer progression through upregulation of SPOCK1/testican 1. The above study also showed that EPCR-mediated effects on tumor growth and metastasis were independent of APC.

EPCR could play different roles in different cancers. Our studies on the progression of malignant pleural mesothelioma (MPM) revealed that EPCR functions as a negative regulator of cancer progression in MPM [63]. Analysis of in-vivo tumorigenicity of multiple MPM cell lines showed that MPM cells that lack EPCR expression grew rapidly and formed large-sized tumors in the pleural space, whereas MPM cells that express EPCR had the least tumorigenicity. Transduction of *EPCR* gene expression to aggressive MPM cells that express oncogenic TF but lack EPCR markedly attenuated the tumorigenicity of MPM cells, whereas EPCR silencing in TF expressing nonaggressive MPM cells that constitutively express EPCR increased the tumorigenicity of the nonaggressive MPM cells [63]. A follow-up recent study showed that EPCR overexpression makes MPM cells highly susceptible to $\text{TNF}\alpha$ + $\text{IFN}\gamma$ -induced cell death, whereas EPCR silencing increased the resistance to $\text{TNF}\alpha$ + $\text{IFN}\gamma$ -induced apoptosis [64]. The proapoptotic function of EPCR appears to be independent of EPCR–APC or EPCR–FVIIa-mediated cell signaling as APC, FVIIa, or EPCR blocking antibody that prevents APC and FVIIa binding to EPCR did not affect the EPCR-mediated apoptosis. Consistent with the observation that EPCR acts as a tumor suppressor, *EPCR* gene delivery to an established MPM xenograft in a mouse significantly

reduced the progression of tumor growth when compared with tumor growth observed in Vascular biology mice treated with a control adenovirus. EPCR expression in MPM cells was found to alter the transcription profile in MPM cells, favoring suppression of genes that encode antiapoptotic proteins. The signaling mechanisms by which EPCR affects the transcription profile in MPM cells and make them susceptible to cytokine-mediated cell death are unknown. The two recent studies discussed above indicate that EPCR functions may vary based on the tumor type or tumor microenvironment and ligands other than APC and FVIIa can also drive the EPCR-mediated signaling.

CONCLUSION

Most of our current understanding of the role of EPCR in cell signaling comes from studies of APC-induced PAR-mediated signaling. Recent studies provided mechanistic insights into how EPCR contributes to the biased PAR1 signaling. It will be interesting to investigate in the future whether FVIIa, a protease ligand that binds EPCR with a similar affinity as of APC, mimics APC in inducing EPCR-dependent cytoprotective signaling. The observation that EPCR binds diverse ligands suggests that EPCR may play a role in many functions beyond its well known barrier protective and anti-inflammatory functions. EPCR-dependent signaling observed in specific cell types, independent of APC, indicates that there may be other ligands to EPCR that are yet to be identified. Discovery of these ligands will expand the importance of EPCR in regulating many pathophysiological processes and provide clues for the development of new therapeutic options to treat diseases from severe malaria to cancer.

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KEY POINTS

- EPCR occupancy by its ligand PC or APC induces β -arrestin-2-dependent biased PAR1 cytoprotective signaling upon PAR1 activation by either APC or thrombin by recruiting G-protein-coupled receptor kinase 5, β -arrestin-2, and disheveled 2.
- Factor VIIa, which binds EPCR in a similar fashion as of APC, induces EPCR-dependent cytoprotective signaling.
- EPCR is a robust and reliable cell-surface marker in detecting human hematopoietic stem cells in culture.
- EPCR binds divergent ligands, including *Plasmodium falciparum* erythrocyte membrane protein 1 and a T-cell receptor, suggesting that EPCR could play a role in the pathogenesis of severe malaria and immune functions.
- EPCR influences cancer pathogenesis, but the effect may differ from one cancer to the other.

FIGURE 1.

Various ligands of endothelial cell protein C receptor and endothelial cell protein C receptor-dependent signaling responses.

FIGURE 2. Schematic representation of endothelial cell protein C receptor-dependent biased protease-activated receptor 1 signaling. Modified from [27■].