The ligand occupancy of endothelial protein C receptor switches the PAR-1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells

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Running Title: EPCR recruits PAR-1

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Abstract

Recent studies have indicated that activated protein C (APC) may exert its cytoprotective and antiinflammatory activities through the endothelial protein C receptor (EPCR)-dependent cleavage of protease activated receptor 1 (PAR-1) on vascular endothelial cells. Noting that i) the activation of protein C on endothelial cells requires thrombin, ii) relative to APC, thrombin cleaves PAR-1 with ~3-4-orders of magnitude higher catalytic efficiency, and iii) PAR-1 is a target for the proinflammatory activity of thrombin, it is not understood how APC can elicit a protective signaling response through the cleavage of PAR-1 when thrombin is present. In this study, we demonstrate that EPCR is associated with caveolin-1 in lipid rafts of endothelial cells and that its occupancy by the Gla-domain of protein C/APC leads to its dissociation from caveolin-1 and recruitment of PAR-1 to a protective signaling pathway through coupling of PAR-1 to the pertussis toxin sensitive G_{i}-protein. Thus, when EPCR is bound by protein C, the PAR-1 cleavage-dependent protective signaling responses in endothelial cells can be mediated by either thrombin or APC. These results provide a new paradigm for understanding how PAR-1 and EPCR participate in protective signaling events in endothelial cells.
Introduction

Activated protein C (APC) is a plasma serine protease that down-regulates thrombin generation by degrading the procoagulant cofactors Va and VIIIa by limited proteolysis. APC is generated when thrombin forms a complex with thrombomodulin (TM) on endothelial cell surface to activate the zymogen protein C. The anticoagulant function of APC in degradation of both cofactors is stimulated by protein S. The importance of APC in regulation of blood coagulation can be illustrated by the observation that a heterozygous protein C deficiency is associated with high risk of venous thrombosis and its homozygous deficiency causes purpura fulminans, which is fatal unless treated by protein C replacement therapy. In addition to its anticoagulant role, APC also possesses antiinflammatory properties, which have lead to the FDA approval of recombinant APC as a therapeutic drug for treating severe sepsis. The mechanism of the antiinflammatory function of APC is not well understood, however, it has been hypothesized that complex formation of APC with endothelial protein C receptor (EPCR) renders the protease capable of cleaving protease activated receptor 1 (PAR-1), thereby eliciting protective signaling responses in endothelial cells. However, it is known that thrombin can cleave PAR-1 with at least three orders of magnitude higher catalytic efficiency than APC to initiate proinflammatory events in endothelial cells. Since thrombin is the only known physiological activator of protein C, there is controversy as to whether APC can exert a protective activity through the cleavage of PAR-1 when thrombin is also present in the same environment.

The EPCR and PAR-1 dependent antiinflammatory activities of APC have been extensively studied in lung endothelial cells, primary human umbilical vein endothelial cells (HUVECs) and transformed HUVECs (EA.hy926 cells). These and other previous studies have established that thrombin signaling through PAR-1 enhances permeability and initiates a pro-apoptotic cycle in cultured endothelial cells. On the other hand, the cleavage of PAR-1 by the APC-EPCR complex exerts an opposite effect, thus restoring the permeability to a baseline
state and also inhibiting endothelial cell death. Interestingly, it has been noted that picomolar concentrations of thrombin, similar to APC, exhibits a protective effect in endothelial cells and high concentrations of APC, similar to thrombin, enhances permeability, leading to the hypothesis that the level of PAR-1 activation may dictate the type of the response, with a low dose receptor activation by APC invoking protective and a high dose receptor activation by thrombin invoking barrier-disruptive responses.

To investigate whether the level of receptor activation by thrombin and APC determines the type of response in endothelial cells, we engineered a chimeric meizothrombin in which the γ-carboxyglutamic acid (Gla) domain of the thrombin intermediate was substituted with the corresponding domain of APC. This meizothrombin derivative retained its high specific activity toward PAR-1 and interacted with EPCR with normal affinity. We discovered that PAR-1 cleavage by this meizothrombin derivative elicits a protective response in endothelial cells, suggesting that the binding of Gla-domain of APC to EPCR determines the type of response. To investigate the mechanism of this effect, we studied the effect of PAR-1 cleavage by thrombin in endothelial cells which were treated with the catalytically inactive Ser-195 to Ala substitution mutant of protein C. The results revealed that when endothelial EPCR is occupied by its ligand protein C, the cleavage of PAR-1 by thrombin elicits only protective signaling responses in endothelial cells.
Materials and Methods

Construction, Expression and Purification of Recombinant Proteins

Construction and expression of protein C and its Ser-195 → Ala (PC-S195A) (chymotrypsinogen numbering)\textsuperscript{20} variant in human embryonic kidney (HEK-293) cells has been described.\textsuperscript{21} The prothrombin mutant in which the protease cleavage sites Arg-155, Arg-271 and Arg-286 were eliminated by their substitutions with Ala has been described.\textsuperscript{22} Upon activation by the Taipan snake venom, this mutant yields an active product named meizothrombin.\textsuperscript{23} A prothrombin chimera in the background of this mutant was constructed in which its Gla-domain was substituted with the corresponding domain of human protein C (PC-Gla/Proth/155,271,286A). The prothrombin mutant was activated by the Taipan snake venom and the active product (PC-Gla/meizothrombin) was separated from the activator using an FPLC Mono Q ion exchange column as described.\textsuperscript{24} Protein C was activated by thrombin and APC was purified as described.\textsuperscript{21,24} The concentration of APC was determined by its absorbance at 280 nm and stoichiometric titration of the enzyme with known concentrations of recombinant protein C inhibitor as described.\textsuperscript{25} The concentration of PC-Gla/meizothrombin chimera was determined by an amidolytic activity assay and by stoichiometric titration with known concentrations of antithrombin as described.\textsuperscript{26}

Antibodies blocking (H-111) and non-blocking (S-19) the activation of PAR-1 on HUVECs,\textsuperscript{21} the anti-PAR-2 (SAM-11) and anti-PAR-4 (H-120) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and function-blocking EPCR antibody (clone RCR-252) was purchased from Cell Sciences (Canton, MA). The antibody concentrations in the blocking assays were 25 µg/mL in all experiments. Anti-caveolin-1 antibody was purchased from BD Biosciences (San Jose, CA). Pertussis toxin (PTX), staurosporine, bacterial lipopolysaccharide (LPS) and hirudin were purchased from Sigma (St. Louis, MO). Thrombin
receptor agonist peptide (TRAP) for PAR-1 (H-Ser-Phe-Leu-Leu-Arg-Asn-NH2) was purchased from Bachem Bioscience (Torrance, CA).

**PAR-1 Cleavage Assay**

The cleavage rate of PAR-1 at the endothelial cell surface by different proteases was evaluated using an alkaline phosphatase-PAR-1 (ALP-PAR-1) reporter plasmid transiently transfected to EA.hy926 cells (kindly provided by Dr. C. Edgell from University of North Carolina at Chapel Hill, NC) as described. The results are expressed as mean ±SEM and all experiments were repeated three times.

**Apoptosis Assay**

The protective effect of different proteases (APC, 0-100 nM; thrombin and meizothrombin, 0-20 nM) in EA.hy926 cells in the absence or presence of PC-S195A (0-80 nM) was evaluated in a staurosporine-induced apoptosis assay as described. The number of apoptotic cells was expressed as the percentage of TUNEL-positive cells of the total number of nuclei determined by Hoechst staining as described. Results are expressed as mean ±SEM and all experiments were repeated three times. All APC assays were carried out in the presence of 4 units/mL hirudin.

**Permeability Assay**

EA.hy926 cell permeability in response to thrombin (5 nM for 10 min) following treatment with increasing concentrations of APC (0-100 nM plus 4 units/mL hirudin for 3 h) or meizothrombin derivatives (0-10 nM for 3 h) was quantitated by spectrophotometric measurement of the flux of Evans blue-bound albumin across functional EA.hy926 cell monolayers using a modified 2-compartment chamber model as described. For the function-blocking antibody treatments of the monolayers, medium was removed and antibodies were added for 30 min in
serum-free medium followed by analysis of the permeability as described. Results are expressed as mean ±SEM and all experiments were repeated at least three times.

The same assay was employed to evaluate the effect of thrombin (0-10 nM for 3 h) or TRAP (0-2 mM for 3 h) on permeability of EA.hy926 cells pretreated with PC-S195A (0-80 nM) for 15 min. The cell permeability assays were also carried out in presence of pertussis toxin (PTX). In this case, endothelial cells were pretreated with the toxin (100 ng/mL) for 16 h prior to their incubation with PAR-1 agonists. EA.hy926 cell permeability in response to lipopolysaccharide (LPS) was carried out using the same methods except that instead of 5 nM thrombin for 10 min, LPS (10 ng/mL) for 4 h was used to induce permeability.

**RNA Interference**

EA.hy926 cell permeability in response to thrombin (0-10 nM) or APC (0-100 nM) was evaluated following the knockdown of PAR-4 expression by a pool of three target-specific 20-25 nucleotide siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer’s instruction. A non-targeting 20-25 nucleotide siRNA obtained from the same company was used as a negative control.

**Immunoprecipitation, SDS-PAGE and Western blotting**

Total cellular proteins were extracted by sonication with PBS containing 25 µM proteosome inhibitor MG132 (Sigma-Aldrich) and complete protease inhibitor cocktail (Roche Molecular Systems, Summerville, NJ) as described. Lysates were combined with 3 µg of each specific antibody (H-111 for PAR-1, SAM-11 for PAR-2 and H-120 for PAR-4), and incubated for 2 h at 4°C. Immunoprecipitates were collected with protein A/G Agarose (Santa Cruz, CA), fractionated on a 10% SDS-PAGE, transferred to membranes, and subjected to Western-blotting with appropriate primary and horseradish peroxidase-conjugated secondary antibodies as
described. The immunoreactive protein bands were visualized by SuperSignal West Pico (Pierce, Rockford, IL).

**Isolation of Lipid Rafts/Caveolae**

Lipid rafts/caveolae proteins were isolated by slight modification of procedures as described. Briefly, confluent cells in 150-mm Petri dishes were washed with ice-cold PBS and scraped into Triton X-100 containing Tris lysis buffer (25 mM Tris-HCl, 250 mM sucrose, 2 mM EDTA and 1% Triton X-100). Cell pellets were then homogenized with a tight-fitting Dounce homogenizer followed by 3 x 20 sec bursts of ultrasonic sonicator on ice. The lysate was adjusted to 45% sucrose by the addition of an equal volume of 90% sucrose in 25 mM MES-buffered saline (pH 6.5) and placed at the bottom of an ultracentrifuge tube. Two solutions (1.7 mL each) of 35% and 5% sucrose were laid sequentially on the top of the 45% sucrose solution. After ultracentrifugation at 35,000 rpm with Beckman SW Ti55 rotor for 16–20 h, 10 x 0.5-mL fractions were collected from the top of tubes, and a portion of each fraction was analyzed by SDS-PAGE, transferred to nitrocellulose membranes and subjected to Western blotting with appropriate primary and secondary antibodies.

**Interaction with EPCR**

The affinity of PC-Gla/meizothrombin for interaction with EPCR was evaluated by an ELISA-based binding assay using HPC4-tagged recombinant soluble EPCR (sEPCR) (kindly provided by Dr. Esmon from Oklahoma Medical Research Foundation) as described.
Results

PC-Gla/meizothrombin Interaction with EPCR and Cleavage of PAR-1

PC-Gla/meizothrombin interacted with soluble EPCR with a normal affinity (Fig. 1A), exhibiting a \( K_{d(app)} \) of \( \sim 35 \pm 1 \) nM that is similar to the value obtained for the interaction of APC with the receptor \( K_{d(app)} = 34 \pm 1 \) nM. APC cleaved the Arg-41 scissile bond of PAR-1 with \( \sim 3 \) orders of magnitude lower catalytic efficiency than thrombin as determined by an alkaline phosphatase activity assay using a PAR-1 cleavage reporter plasmid transfected to EA.hy926 cells (Fig. 1B). Both meizothrombin and PC-Gla/meizothrombin cleaved PAR-1 with an efficiency that was only \( \sim 20\% \) lower than the cleavage rate by thrombin, suggesting that the exosite-1 of meizothrombin derivatives can interact with the hirudin-like sequence of PAR-1 to effectively catalyze the cleavage of the Arg-41 peptide bond on the PAR-1 exodomain (Fig. 1B).

Barrier Protective Effect of PC-Gla/meizothrombin in Endothelial Cells

Previous studies have indicated that both thrombin \(^{17}\) and TNF-\( \alpha \) \(^{19}\) disrupt the permeability barrier of EA.hy926 cells and that APC exerts a potent protective effect. Paradoxically, both the permeability-enhancing effect of thrombin and the barrier-protective effect of APC require the cleavage of PAR-1 by these proteases.\(^{17,19}\) However, the protective effect of APC requires its interaction with EPCR.\(^{9,17}\) This assay was utilized to evaluate the nature of the signaling effect of both meizothrombin, which cannot interact with EPCR, and PC-Gla/meizothrombin which exhibits a normal affinity for the receptor. In agreement with previous results, treatment of EA.hy926 cells with thrombin resulted in a partial protective effect at \( \sim 50 \) pM thrombin,\(^{17}\) but an enhanced permeability effect above 50 pM which was effectively reversed by APC in a concentration dependent manner reaching saturation at 10 nM APC (Fig. 2A). Meizothrombin exhibited an effect that was essentially identical to that observed with thrombin (Fig. 2A). On the other hand, PC-Gla/meizothrombin functioned like APC with the exception that...
its barrier protective effect was observed at much lower concentrations of the protease (0.5-2 nM). Like an enhanced permeability induced by the higher concentrations of APC, PC-Gla/meizothrombin also exhibited a similar effect when its concentrations exceeded 2 nM (Fig. 2A). Function-blocking antibodies to either EPCR or PAR-1 eliminated the barrier protective effects of both APC and PC-Gla/meizothrombin, confirming that the EPCR-dependent cleavage of PAR-1 mediates the cellular effects of both proteases (data not shown).

Based on these results, we hypothesized that the occupancy of EPCR by its ligand alters the specificity of the PAR-1 signaling pathway in endothelial cells. To test this hypothesis, we evaluated the effect of thrombin in the presence of PC-S195A, which can interact with normal affinity with EPCR, but cannot cleave PAR-1 to elicit a cellular response. Interestingly, thrombin in the presence of the mutant zymogen elicited a protective response identical to that observed with PC-Gla/meizothrombin (Fig. 2A). Interestingly, a similar level of protective effect for thrombin in the presence of PC-S195A was observed if LPS was used to induce hyperpermeability, suggesting that when EPCR is ligated, thrombin plays a protective role in response to other proinflammatory stimuli (Fig. 2B).

Activity of APC and Thrombin in the Apoptosis Assay

It is known that thrombin, proinflammatory cytokines and the kinase inhibitor staurosporine can induce apoptosis in endothelial cells that can be reversed by APC by an EPCR and PAR-1-dependent mechanism. As presented in Fig. 2C, the treatment of EA.hy926 cells with staurosporine induced apoptosis and APC inhibited cell death by a concentration dependent manner. As with the permeability assay, thrombin exhibited a partial cytoprotective effect at low pM concentrations, but in the presence of PC-S195A, the signaling effect by thrombin was cytoprotective for up to 2 nM protease (Fig. 2C). These results support the hypothesis that the ligand occupancy of EPCR recruits PAR-1 to a cytoprotective pathway in endothelial cells.
The concentration dependence of the protective effect of PC-S195A was studied by a permeability assay. As presented in Fig. 2D, the protective effect of thrombin reached saturation at 10 nM mutant zymogen. These results clearly suggest that it is the occupancy of EPCR by its ligand that determines the specificity of PAR-1 signaling not the type of the protease that is cleaving PAR-1.

**Protective Effect of Thrombin in the Presence of PC-S195A can be Mimicked by TRAP**

The activation of PAR-1 by the TRAP peptide is known to mimic the proinflammatory effect of thrombin in HUVECs.\textsuperscript{17,28} As presented in Fig. 3A, TRAP enhanced the permeability barrier in endothelial cells. As with the protective effect of thrombin at pM concentrations, TRAP also elicited a similar response at relatively low concentrations (Fig. 3A). As observed with thrombin, the treatment of endothelial cells with either protein C or PC-S195A switched the specificity of signaling by TRAP from a permeability-enhancing to a barrier-protective response, confirming the conclusion that when EPCR is occupied, PAR-1 activation elicits a protective response in endothelial cells (Fig. 3A). To ensure that the protective effects of low pM concentrations of thrombin is mediated through activation of PAR-1, the permeability assay was also carried out following treatment of endothelial cells with thrombin in complex with a saturating concentration of TM456 (100 nM) which blocks the exosite-1-dependent interaction of thrombin with PAR-1. The barrier protective effect of low pM concentrations of thrombin was abolished in the presence of TM456 (Fig. 3A). The same results were obtained if endothelial cells were incubated with the function-blocking anti-PAR-1 antibody, indicating that the protective effect of very low concentrations of thrombin is mediated through the activation of PAR-1 (Fig. 3A).

In all permeability assays described above, endothelial cells were initially treated with different PAR-1 agonists for 3 h before inducing permeability with 5 nM thrombin. In a variation
of this assay, we directly measured the effect of thrombin and TRAP on cell permeability in the absence and presence of PC-S195A following 3 h incubation of cells with different concentrations of each agonist, omitting the 5 nM thrombin treatment step. As presented in Fig. 3B, both thrombin and TRAP dramatically enhanced the permeability barrier in a concentration dependent manner with the maximal effects reaching saturation at 2 nM thrombin and ~20 μM TRAP. Similar to results presented above, PC-S195A switched the signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response for up to 2 nM thrombin, whereas the zymogen mutant switched the effect of TRAP at both low and high concentrations so that it could no longer elicit a permeability-enhancing response (Fig. 3B). These results strongly suggested that 2 nM thrombin is sufficient to cleave all available cell surface PAR-1 in endothelial cells and that PAR-1 signaling is protective in HUVECs if EPCR is occupied by its ligand. This hypothesis is derived from the observation that, unlike the high concentration of thrombin, a high concentration of TRAP in the presence the zymogen mutant did not exhibit a hyperpermeability effect (Fig. 3B). These results raised the possibility that a high concentration of thrombin in the presence of the mutant zymogen elicits a barrier disruptive response through the cleavage of other candidate PARs present at the surface of endothelial cells.

The Permeability-enhancing Effect of Higher Concentrations of both Thrombin and APC are Mediated via the Cleavage of PAR-4

In addition to PAR-1, HUVECs are also known to express PAR-2 and PAR-4. To determine whether the permeability-enhancing effect of a higher concentration of thrombin is mediated through activation of PAR-2, we conducted the permeability assay for thrombin and TRAP (Fig. 3B) in the presence of a function-blocking anti-PAR-2 (SAM-11) antibody. The ability of SAM-11 antibody to block PAR-2 signaling was first confirmed by its ability to block the PAR-2-dependent barrier protection in endothelial cells mediated by factor Xa as previously
reported (data not presented). The results (not shown) was essentially identical to those presented in Fig. 3B, suggesting that the permeability-enhancing effect of higher than 2 nM thrombin in the presence of PC-S195A is not mediated through the activation of PAR-2. Since we have no knowledge as to whether or not the commercial anti-PAR-4 antibody is a function-blocking antibody, we decided to use the siRNA approach to investigate the contribution of the PAR-4 cleavage to the hyperpermeability effect of high concentrations of either thrombin in the presence of PC-S195A or APC in endothelial cells. The knockdown of PAR-4 in endothelial cells by siRNA was very efficient with no detectable expression of the receptor (Fig. 4A). Interestingly, while the control siRNA had no effect on the cellular responses elicited by thrombin in the absence and presence of PC-S195A, the specific PAR-4 siRNA eliminated the hyperpermeability effect induced by a high concentration of thrombin in the presence of the mutant zymogen, suggesting that the permeability-enhancing effect of higher than 2 nM thrombin is mediated through the cleavage of PAR-4 (Fig. 4B). The observation that thrombin in the presence of PAR-4 siRNA (in the absence of PC-S195A) enhanced the permeability of endothelial cells implies that the thrombin cleavage of PAR-1 on these cells is proinflammatory unless EPCR has been occupied by its ligand. The hyperpermeability effect of a high and non-physiological concentration of APC was also found to be mediated through the cleavage of PAR-4, since siRNA also inhibited this response (Fig. 4C). Similar studies indicated that the loss of anti-apoptotic activity observed at thrombin concentrations of above 2 nM (Fig. 2B) is also due to the thrombin activation of PAR-4 (data not shown). The PAR-4-dependent thrombin enhancement of cell permeability does not require protein C since thrombin can induce similar hyperpermeability in the presence of function-blocking anti-PAR-1 antibody in the absence of protein C (Fig. 2A, filled circles).

The Ligand Occupancy of EPCR Couples PAR-1 to G Protein
PAR-1 can signal via coupling to different G proteins.\textsuperscript{18,31,32} It is known that the signaling function of G\textsubscript{i} is sensitive to pertussis toxin (PTX).\textsuperscript{31} To understand the mechanism by which EPCR switches the signaling specificity of thrombin, we investigated the cellular effect of thrombin in the absence and presence of PC-S195A in endothelial cells pretreated with PTX. We found that the permeability-enhancing effect of thrombin is mediated via coupling of PAR-1 to a non-G\textsubscript{i} protein as PTX did not influence the response of endothelial cells to thrombin (Fig. 4D). On the other hand, the protective effect of APC was totally abolished by PTX, suggesting that the EPCR-bound APC signals via PAR-1 coupled to G\textsubscript{i} protein (Fig. 4D). Interestingly, similar to APC, the protective signaling by thrombin was PTX-sensitive in the presence of PC-S195A (Fig. 4D), clearly suggesting that the occupancy of EPCR by its ligand couples PAR-1 to G\textsubscript{i} protein, thereby altering the signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response.

**EPCR is Associated with Caveolin-1 in Lipid Rafts**

To understand the mechanism by which the occupancy of EPCR by its ligand recruits PAR-1 to a protective pathway, we isolated lipid rafts of non-treated and PC-S195A treated endothelial cells and analyzed the protein contents of different low and high buoyancy fractions by immunoblotting using antibodies against EPCR, PAR-1 and caveolin-1. As presented in Fig. 5, all three proteins were found to be colocalized within the cholesterol rich lipid rafts/caveolae membrane microdomains of endothelial cells in both the absence (Fig. 5A) and presence of PC-S195A (Fig. 5B). To explore a possible association between these molecules, we next immunoprecipitated the total protein extract of endothelial cells with anti-caveolin-1, anti-PAR-1 or anti-EPCR antibodies and subjected them to a 10\% reducing SDS-PAGE. Western blot analyses of immunoprecipitates indicated that the antibody specific for either protein co-immunoprecipitates the other two proteins from the extract derived from non-treated endothelial
cells (Fig. 5C), suggesting that they are located in the same membrane microdomains. Immunoblotting of membrane lipid rafts derived from primary HUVECs using the same anti-PAR-1 and anti-EPCR antibodies yielded identical results (not shown). Interestingly, when endothelial cells were treated with PC-S195A, while both EPCR and PAR-1 pairs and caveolin-1 and PAR-1 pairs remained localized to the same microdomains, EPCR and caveolin-1 were not co-immunoprecipitated by the antibody pairs (Fig. 5C), suggesting that the occupancy of EPCR with its ligand leads to its dissociation and/or its migration out of the caveolar compartment, a process which appears to couple PAR-1 to G_i protein, thereby changing the specificity of the PAR-1 signaling from a permeability-enhancing to a barrier-protective response independent of the protease cleaving the receptor. Thus, thrombin cleavage of PAR-1 in endothelial cells would be protective as long as they express EPCR and there is sufficient ligand (protein C) to occupy the receptor.

Two recent studies have demonstrated that EPCR also acts as a cellular receptor for factor VIIa on endothelium, exhibiting an affinity comparable to that of APC/protein C. However, factor VIIa was unable to recruit PAR-1 to a protective pathway since, unlike protein C, the treatment of endothelial cells with factor VIIa failed to prevent the enhanced permeability induced by thrombin (Fig. 6). These results suggest that the protective effect of the EPCR occupancy is specific for APC/protein C in endothelial cells. The data in Fig. 6 also show that the prior incubation of endothelial cells with blocking anti-EPCR and anti-PAR-1 antibodies, but not with the non-blocking anti-PAR-1 antibody, eliminate the protective activity of APC.
Discussion

We have demonstrated here that the occupancy of EPCR by protein C switches the specificity of PAR-1-dependent signaling by thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells. Further support for this proposal was provided by the observation that the thrombin agonist peptide TRAP, also increased the permeability barrier in endothelial cells, but PC-S195A reversed the hyperpermeability effect of PAR-1 signaling by TRAP, clearly indicating that the occupancy of EPCR recruits PAR-1 to a protective pathway. The observation that PC-Gla/meizothrombin and thrombin plus PC-S195A both elicited a protective response in both apoptosis and permeability assays is also in line with the overall conclusion of this study.

The protective signaling activity of thrombin in the presence of PC-S195A was dependent on the concentration of the protease, exhibiting an optimal effect at ~2 nM thrombin and thereafter decreasing and reversing to a barrier disruptive effect at 10 nM thrombin. To determine whether this biphasic effect of thrombin is mediated through the cleavage of PAR-1, we incubated endothelial cells with 2 nM thrombin in the presence of PC-S195A for 10 min and then treated cells with the function-blocking anti-PAR-1 antibody followed by incubation of cells with 10 nM thrombin. However, blocking PAR-1 did not block the enhanced permeability in endothelial cells, suggesting that the hyperpermeability effect of the high concentration of thrombin may not be mediated via the cleavage of PAR-1 (data were not presented). Further support for this hypothesis was provided by the observation that the hyperpermeability effect of TRAP in the absence of PC-S195A and its protective effect in the presence of the mutant zymogen were both mono-phasic reaching saturation at ~10 µM TRAP. Thus, we hypothesize that a higher concentration of thrombin may elicit a barrier-disruptive response in endothelial cells via the activation of other PARs. In addition to PAR-1, HUVECs are known to express PAR-2 and PAR-4. Thrombin is not known to cleave PAR-2 and the function-blocking anti-PAR-2 antibody
further ruled out a role for the thrombin cleavage of this receptor in endothelial cells. However, the knockdown of PAR-4 expression by siRNA eliminated the hyperpermeability effect of the high concentration of thrombin in the presence of PC-S195A, suggesting that when EPCR is occupied by its ligand, the thrombin cleavage of PAR-1 elicits only a protective response. A similar enhanced permeability in HUVECs has been observed in response to supraphysiological concentrations of APC (above 100 nM). The siRNA knockdown of PAR-4 also eliminated the hyperpermeability effect of a high concentration of APC, suggesting that this effect is also mediated though the cleavage of PAR-4 in cultured endothelial cells.

PAR-1, as a seven transmembrane G protein coupled receptor, can signal through interaction with different members of the G protein subfamilies including G, G, and G. It has been hypothesized that thrombin disrupts endothelial barrier function through PAR-1 coupled to G and/or G, but APC may signal through the cleavage of PAR-1 coupled to G. Among these G proteins, only the signaling function of G is sensitive to pertussis toxin (PTX). The observation that the PAR-1-dependent permeability-enhancing effect of thrombin was insensitive to PTX, but the protective response of APC and thrombin plus PC-S195A were both sensitive to PTX suggested that the ligand occupancy of EPCR recruits PAR-1 to a protective pathway by coupling the receptor to G protein in the lipid rafts of endothelial cells. Thus, thrombin cleavage of PAR-1 on vascular endothelial cells would be protective as long as they express EPCR and there is sufficient amount of ligand to occupy the receptor. The mechanism by which protein C binding to EPCR changes the G protein coupling specificity of PAR-1 is not known. However, it appears that EPCR is associated with caveolin-1 and that the occupancy of EPCR by its ligand results in dissociation and/or migration of EPCR out of the caveolar compartment. The EPCR dissociation from caveolae somehow couples PAR-1 to G protein, thereby switching the specificity of the receptor signaling by thrombin from a barrier-disruptive to a barrier-protective pathway. The EPCR-dependent switch in the specificity of PAR-1 signaling was unique for
protein C and not recapitulated by factor VIIa, though factor VIIa can bind to EPCR with a high affinity. The basis for this observation is not clear, but it is known that the ω-loop residues of the Gla-domain of protein C, which are critical for interaction with EPCR, have been entirely conserved in factor VIIa, thereby accounting for the similar affinity of both proteases for the receptor. It appears that interactions outside of the ω-loop, which must be specific for protein C, are required for the EPCR-dependent protective signaling. The findings of the current study are consistent with the report that factor VIIa-bound EPCR does not elicit cell signaling by activation of PARs.

APC has been approved by the FDA as a drug for treating severe sepsis. The question as to whether or not the beneficial effect of APC is mediated through the activation of PAR-1 is not settled. An EPCR and PAR-1 dependent protective activity for APC has been observed in mouse models of ischemic stroke, supporting the hypothesis that the beneficial effect of APC in severe sepsis may also be mediated through the same mechanism. However, other investigators disagree with this hypothesis, arguing that PAR-1 activation by APC cannot contribute to its beneficial effect in severe sepsis. The controversy primarily stems from the observation that thrombin exhibits a proinflammatory effect in HUVECs, and has an ~3-4 orders of magnitude higher catalytic efficiency than APC toward PAR-1. Thus, it has been justifiably contended that thrombin would cleave all available cell surface PAR-1, leaving no opportunity for APC to function through the cleavage of the same receptor. Our observation that EPCR recruits PAR-1 to a protective signaling pathway in endothelial cells supports this contention and explains the basis for the paradoxical effects of PAR-1 cleavage by either thrombin or APC as observed in the cultured endothelial cells. Our data suggests that, in endothelial cells expressing EPCR, thrombin would also invoke an antiinflammatory response when protein C is present. Thus, the EPCR occupancy by protein C is the missing link in the cross-talk between the two receptors that has hindered a better understanding of the PAR-1 cleavage-dependent signaling by
thrombin and APC in cultured endothelial cells. Assuming that the results obtained in HUVECs are relevant to endothelial cells in vivo for which there is some evidence,\textsuperscript{39} thrombin would primarily be responsible for the cleavage of the EPCR-dependent G\textsubscript{i}-protein-coupled PAR-1 in intact endothelial cells. This is in contrast to the traditional view that thrombin elicits inflammatory responses in endothelial cells such as in pulmonary edema. Noting that the protein C concentration in plasma is high enough to yield at least a 50\% occupancy of EPCR on the endothelial cells, the findings of this study predict that thrombin would play a protective role through the EPCR-dependent activation of PAR-1. These findings further suggest that a complete inhibition of thrombin activity by anticoagulants during an inflammatory disorder such as sepsis may adversely affect the course of the disease. The down-regulation of EPCR expression of endothelial cells by endotoxin and proinflammatory cytokines in sepsis may also abolish the protective role of thrombin. Hence, the type of proinflammatory properties that have been attributed to thrombin in cultured vein endothelial cells may not reflect the true function of the protease under physiological conditions.
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AUTHOR CONTRIBUTIONS

J.-S. Bae performed research, L. Yang constructed the mutant constructs, C. Manithody purified recombinant proteins and A.R. Rezaie designed the experiments, analyzed the data and wrote the paper.

The authors declare that they have no competing financial interests.
References


Figure Legends

Figure 1. Binding of protein C and PC-Gla/meizothrombin to soluble EPCR and the cleavage of PAR-1 exodomain by the proteases. (A) An ELISA-based assay using the monoclonal antibody, HPC4 (1 µg/mL), was used to measure the affinity of either protein C (○) or PC-Gla/meizothrombin (■) for interaction with soluble EPCR (0.5 µM). (B) EA.hy926 cells were transiently transfected with ALP-PAR-1 reporter plasmid and incubated with increasing concentrations of thrombin (○), meizothrombin (□), PC-Gla/meizothrombin (■) and APC (▲) for one h. The activity of soluble alkaline phosphatase (ALP) was measured as described under “Methods”. The activity of 10 nM thrombin is presented as 100%.

Figure 2. The permeability barrier enhancing and pro-apoptotic effects of thrombin are reversed by the ligand occupancy of EPCR. (A) EA.hy926 cells were incubated with increasing concentrations of thrombin (○), thrombin + anti-PAR-1 antibody (●), meizothrombin (□), PC-Gla/meizothrombin (■), thrombin + PC-S195A (50 nM) (▲) and APC (▲) for 3 h before inducing permeability with 5 nM thrombin for 10 min. (B) The same as (A) except that following incubation of endothelial cells with increasing concentrations of thrombin (○), thrombin + PC-S195A (▲) and APC (▲), the permeability was induced with LPS (10 ng/mL) for 4 h. (C) EA.hy926 cells were treated with increasing concentrations of thrombin (○), thrombin + PC-S195A (▲) and APC (▲) for 24 h followed by induction of apoptosis with staurosporine (5 µM) for 4 h. The number of apoptotic cells is expressed as the percentage of TUNEL-positive cells of the total number of nuclei. The number of TUNEL-positive cells in the absence of staurosporine was 10-15%. (D) The same as (A) with the exception that the concentration dependence of the protective effect of PC-S195A in the presence of 2 nM thrombin (○) is measured in the permeability assay. The extent of the protective effect of 10 nM APC (▲) is also presented.

Figure 3. The barrier enhancing effect of TRAP and its reversal by the ligand occupancy of EPCR. (A) EA.hy926 cells were incubated with increasing concentrations of thrombin (○),
thrombin + thrombomodulin fragments 456 (TM456) (●), TRAP (□), TRAP + wild-type protein C (■), TRAP + PC-S195A (△), TRAP + wild-type protein C + blocking anti-PAR-1 (H-111) (▲) and TRAP + PC-S195A + anti-PAR-1 (H-111) (▽) for 3 h before inducing permeability with 5 nM thrombin for 10 min. (B) The same as (A) with the exception that the permeability was directly monitored as a function increasing concentrations of thrombin (○), thrombin + PC-S195A (▲), TRAP (□) and TRAP + PC-S195A (■) by incubation of EA.hy926 cells with different agonists for 3 h (the 5 nM thrombin step for inducing permeability has been omitted in this assay). The first data point on the y-axis represents the baseline permeability level.

**Figure 4. The effect of thrombin on the permeability of endothelial cells in the presence of protein C S195A and siRNA for PAR-4.** (A) EA.hy926 cells were transiently transfected with siRNA for PAR-4 or control siRNA (1 µg each). Following three days of incubation at 37 °C, total cellular proteins were extracted, separated on 10% SDS-PAGE and Western-blotted with antibodies directed to either PAR-4 or PAR-1. An antibody against actin was used as internal control. (B) EA.hy926 cells were incubated with increasing concentrations of thrombin (○), thrombin with prior treatment of cells for three days with control siRNA (●), thrombin with prior treatment of cells with siRNA for PAR-4 (□), thrombin + PC-S195A (■), thrombin + PC-S195A with prior treatment of cells with control siRNA (△) and thrombin + PC-S195A with prior treatment of cells with siRNA for PAR-4 (▲) for 3 h before inducing permeability with 5 nM thrombin for 10 min. (C) The same as (B) with the exception that the barrier protective effect of APC was monitored in the absence (△) and presence of control siRNA (●) or siRNA for PAR-4 (□). (D) The barrier protective effect of thrombin + PC-S195A in endothelial cells is sensitive to PTX. EA.hy926 cells were incubated with increasing concentrations of thrombin (○), thrombin with prior treatment of cells with 100 ng/mL PTX for 16 h (●), APC (□), APC with prior treatment of cells with 100 ng/mL PTX for 16 h (■), thrombin + PC-S195A (△), thrombin
+ PC-S195A with prior treatment of cells with PTX (▲) for 3 h before inducing permeability with 5 nM thrombin.

**Figure 5. SDS-PAGE, immunoblotting and co-immunoprecipitation of lipid raft/caveolae preparations derived from EA.hy926 cells with and without treatment with PC-S195A.** (A) Following SDS-PAGE of membrane fractions prepared by discontinuous sucrose gradient ultracentrifugation, they were immunoblotted with antibodies directed to PAR-1, EPCR and caveolin-1. (B) The same as (A) except that the fractions were derived from EA.hy926 cells treated with 50 nM PC-S195A. (C) Total cellular proteins from non-treated and PC-S195A treated EA.hy926 cells were immunoprecipitated with anti-caveolin-1, anti-PAR-1 and anti-EPCR antibodies, separated on SDS-PAGE and immunoblotted with different pairs of the same antibodies.

**Figure 6. The permeability barrier enhancing effect of thrombin is reversed by the ligand occupancy of EPCR with PC-S195A, but not with factor VIIa.** EA.hy926 cells were incubated with APC (10 nM) and thrombin (2 nM) in the absence and presence of either PC-S195A or factor VIIa (50 nM) for 3 h before inducing permeability with 5 nM thrombin for 10 min. B-αEPCR is blocking anti-EPCR (clone RCR-252), B-αPAR-1 (H-111) is blocking anti-PAR-1 and NB-αPAR-1 (S-19) is non-blocking anti-PAR-1 which have been incubated with endothelial cells for 30 min prior to treatment with APC. Baseline represents the permeability of untreated control cells not stimulated with thrombin.
Figure 1

A. Graph showing the relationship between OD_{405} (nm) and [Ligand], μM for PC and PC-Gla/MeizoTh.

B. Graph showing the ALP Activity (% Th Max) against [Protease], nM for Th, APC, MeizoTh, and PC-Gla/MeizoTh.
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
The ligand occupancy of endothelial protein C receptor switches the PAR-1-dependent signaling specificity of thrombin from a permeability-enhancing to a barrier-protective response in endothelial cells

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