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The Ligand Occupancy of Endothelial Protein C Receptor Switches the Signaling Specificity of Thrombin from a Disruptive to a Protective Response in Endothelial Cells

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1. 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. In addition to its anticoagulant role, APC also possesses cytoprotective and antiinflammatory properties, which have lead to FDA approval of recombinant APC as a therapeutic drug for treating severe sepsis. The mechanism of the antiinflammatory function of APC is not understood. 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 the same receptor with an 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 cytoprotective activities through the cleavage of PAR-1 in the presence of thrombin. The EPCR and PAR-1-dependent mechanism of the cytoprotective and anti-inflammatory activities of APC have been extensively studied in lung endothelial cells, primary human umbilical vein endothelial cells (HUVECs). These and other such studies have established that thrombin signaling through PAR-1 has a disruptive permeability barrier effect and also 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 the endothelial cell death. Intriguingly, it has been noted that picomolar concentrations of thrombin, similar to APC, exhibit a barrier protective effect in endothelial cells and high concentrations of APC, similar to thrombin, exerta disruptive effect, leading to the hypothesis that the level of PAR-1 activation may dictate the type of the response. 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 g-carboxyglutamic acid (Gla) domain of the thrombin intermediate was substituted with the corresponding domain of protein C (PC-Gla/meizothrombin). 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 signaling 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 a physiologically relevant concentration of a protein C mutant in which the catalytic residue Ser-195 was substituted with Ala (PC-S195A). The results revealed that when endothelial EPCR is occupied by its ligand protein C, the cleavage of PAR-1 by thrombin also elicits protective signaling responses in endothelial cells. Hence, the beneficial effect of therapeutic APC in severe sepsis may not be mediated via a PAR-1 cleavage dependent mechanismin endothelial cells since thrombin is a far more efficient activator of PAR-1. Furthermore, the typeof proinflammatory properties that have been attributed to thrombin in vitro, based on cultured vein endothelial cells, need to be reevaluated.

2. Materials and Methods

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 HUVEC cells.

Apoptosis assay; The protective effect of different proteases (APC, 0-100 nM; thrombin and meizothrombin, 0-20 nM) in HUVEC cells in the absence or presence of protein C S195A (0-80 nM) was evaluated in a staurosporine-induced apoptosis assay. The number of apoptotic cells was expressed as the percentage of TUNEL-positive cells of the total number of nuclei determined by Hoechst staining.

Permeability assay; HUVEC cell permeability in response to thrombin (5 nM for 10 min) following treatment with increasing concentrations of APC (0-100 nM 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 HUVEC cell monolayers using a modified 2-compartment chamber model. For the function-blocking antibody treatments of the monolayers, medium was removed and antibodies (25 mg/mL) were added for 30 min in serum-free medium followed by analysis of the permeability. 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 the permeability of HUVEC cells pretreated with PC-S195A (0-80 nM) for 15 min. Cell permeability assays were also car-

ried out in the presence of the pertussis toxin (PTX). In this case, the endothelial cells were pretreated with the toxin (100 ng/mL) for 16 h prior to their incubation with the PAR-1 agonists.

3. Results and Discussion

It is known that thrombin disrupts the permeability barrier of EA.hy926 cells, whereas APC exerts a potent protective effect. Paradoxically, both the disruptive effect of thrombin and the protective effect of APC require the cleavage of PAR-1 by these proteases. This assay was utilized to evaluate the nature of the signaling effects of both meizothrombin and PC-Gla/meizothrombin. In agreement with previous results, treatment of EA.hy926 cells with thrombin resulted in a partial protective effect at ~ 50 pM thrombin, but an enhanced permeability effect above 50pM which was effectively reversed by APC in a concentration dependent manner (Fig. 1). Meizothrombin exhibited an effect that was essentially identical to that observed with thrombin (Fig. 1). On the other hand, PC-Gla/meizothrombin functioned like APC with the exception that the barrier protective effect ofthe mutant was observed at far lower concentrations of the protease (0.5-2 nM). As with the disruptive effect of the higher concentrations of APC, PC-Gla/meizothrombin also exhibited a disruptive effect when its concentrations exceeded 2 nM (Fig. 1).

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 further test this hypothesis, we evaluated the effect of thrombin in the presence of PC-S195A that can interact with normal affinity with EPCR, but cannot cleave PAR-1 to elicit cellular responses. Interestingly, thrombin in the presence of the mutant zymogen elicited a protective response identical to that observed with PC-Gla/meizothrombin (Fig. 1).

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. 2, 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 protective for up to 2 nM protease (Fig. 2). These results support the hypothesis that the ligand occupancy of EPCR recruits PAR-1 to a protective pathway in endothelial cells.

The activation of PAR-1 by the thrombin receptor agonist peptide (TRAP) is known to mimic the proinflammatory effect of thrombin in HUVECs. TRAP invoked a dis-

ruptive response, thus enhancing the permeability barrier in endothelial cells. 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 disruptive to a protective response, confirming the conclusion that when EPCR is occupied by its natural ligand, PAR-1 activation elicits a protective response in endothelial cells. The barrier protective effect of low concentrations of thrombin was abolished in the presence of either thrombomodulin (TM) or the function-blocking anti-PAR-1 antibody, suggesting that the protective effect of very low concentrations of thrombin is mediated through the activation of PAR-1.

In a variation of the permeability assay described above, we directly measured the effect of thrombin and TRAP on endothelial cells in the absence and presence of PC-S195A. 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 ~10 mM TRAP. PC-S195A switched the signaling specificity of thrombin from a disruptive effect to a protective effect 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 disruptive signaling response. These results strongly suggest that 2 nM thrombin is sufficient to activate all available PAR-1 in endothelial cells and that PAR-1 signaling is protective if EPCR is occupied by its ligand. Thus, the disruptive effect of a high concentration of thrombin in the presence of the mutant zymogen is likely due to the cleavage of other candidate PARs present at the surface of endothelial cells.

To understand the mechanism by which the occupancy of EPCR by its ligand recruits PAR-1 to a protective signaling pathway, we isolated the lipid rafts of non-treated and PC-S195A treated endothelial cellsand 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. 3, all three proteinswere found to be colocalized within lipid rafts/caveolae membrane microdomains in both the absence and presence of PC-S195A. To explore whether there is a physical association between these molecules, we 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% SDS-PAGE. Western blot analyses of the 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. 3), possibly suggesting that they are physically associated at the membrane surface. Interestingly, when endothelial cells were treated with PC-S195A, while both EPCR and PAR-1 pairs and caveolin-1 and PAR-1 pairs remained associated with each other, EPCR and caveolin-1 were not

co-immunoprecipitated by the antibody pairs (Fig. 3), suggesting that the occupancy of EPCR with its ligand leads to its dissociation and/or migration out of the caveolar compartment.

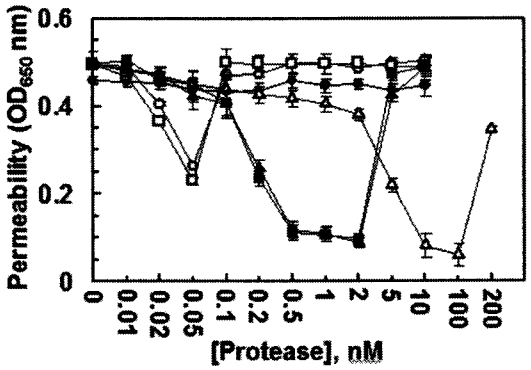

Fig. 1. HUVEC cells were incubated with increasing concentrations of thrombin (○), thrombin + anti-PAR-1 antibody (●), meizothrombin (□), PC-Gla/meizo-thrombin (■), thrombin + PC-S195A (50 nM) (▲) and APC (△) for 3 h before inducing permeability with 5 nM thrombin for 10 min.

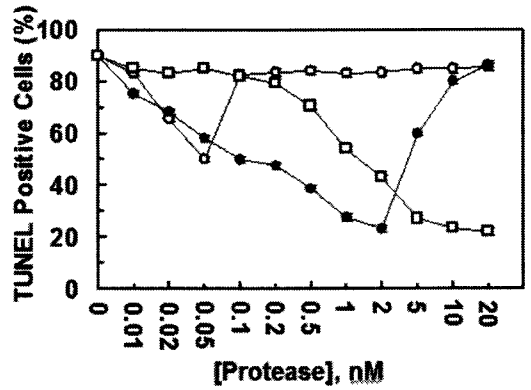

Fig. 2. HUVEC cells were treated with increasing concentrations of thrombin (○), thrombin + PC S195A (●) and APC (□) for 24 h followed by induction of apoptosis with staurosporine (5 uM) for 4 h. The number of apoptotic cells is expressed as the percentage of TUNEL-positive cells of the total number of nuclei. The number of TUNEL-positive cells in the absence of staurosporine was 10-15%.

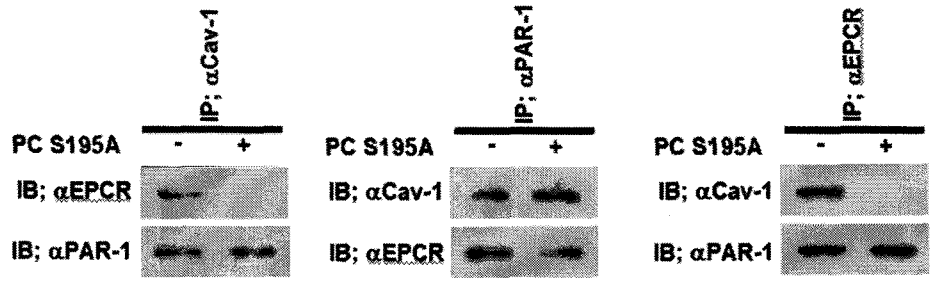

Fig. 3. Total cellular proteins from non-treated and PC-S195A treated EA.hy926 cells were immunoprecipitated with anti-caveolin-1, anti-PAR-1 and anti-EPCR antibodies, separated on SDS-PAGE and immunoblotted with different pairs of the same antibodies.

4. Abstract

Activated protein C (APC) is thought to exert antiinflammatory activitiesthrough the endothelial protein C receptor (EPCR)-dependent cleavage of protease activated receptor 1 (PAR-1) in endothelial cells. Since thrombin cleaves PAR-1 with ~3-4-orders of magnitude higher efficiency, and PAR-1 is a target for proinflammatory activities of thrombin, it is not understood how APC can elicit protective responses through the cleavage of PAR-1. In this study, we demonstrate that EPCR is associated with caveolin-1 in endothelial lipid rafts, but its occupancy by protein C leads to its dissociation from caveolin-1 and subsequent recruitment of PAR-1 to protective signaling pathways through the coupling of PAR-1 to Gi-protein. When EPCR is bound by protein C, the PAR-1-dependent protective response in endothelial cells can be mediated by either thrombin or APC. These results provide a new paradigm for understanding the mechanism through which PAR-1 and EPCR participate in cellular signaling events in endothelial cells.

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