Inhibition of staurosporine-induced apoptosis of endothelial cells by activated protein C requires protease-activated receptor-1 and endothelial cell protein C receptor

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In a model of staurosporine-induced apoptosis using EAhy926 endothelial cells, inhibition of apoptosis by activated protein C was dose-dependent and required the enzyme's active site, implicating activated protein C-mediated proteolysis. Consistent with this implication, both protease-activated receptor-1 (PAR-1) and endothelial cell protein C receptor (EPCR) were required for the anti-apoptotic effects of activated protein C.

Key words: activated protein C, apoptosis, endothelial cell protein C receptor (EPCR), protease-activated receptor (PAR).

INTRODUCTION

The protein C pathway provides a natural anti-coagulant feedback mechanism [1]. Activation of protein C is mediated by thrombin which binds to thrombomodulin on the endothelial surface such that the procoagulant activity of thrombin involving its exosite I is blocked while its anti-coagulant properties, i.e. activation of protein C, are enhanced [2]. Activation of protein C by the thrombin–thrombomodulin complex is facilitated by localization of protein C on the endothelial surface by binding to the endothelial cell protein C receptor (EPCR). Activated protein C (APC), aided by its cofactor protein S, degrades the activated cofactors factor VIIIa and factor Va that are required to sustain thrombin formation through the intrinsic coagulation pathway [1].

Components of the protein C pathway contribute not only anti-coagulant activity but also anti-inflammatory functions, as shown in various animal models or cell-culture studies [3]. The anti-inflammatory effects of thrombomodulin, recently attributed to its lectin-like domain, can protect mice against neutrophil-mediated tissue damage [4]. The murine centrosomal protein CCD41, or centrocyclin, involved in cell-cycle regulation is identical to murine EPCR lacking the N-terminal 31 amino acids [5,6]. Structural similarity of EPCR to the MHC class 1/CD1 family of proteins, most of which are involved in inflammatory processes, suggests that the function of EPCR may not be limited to its ability to localize APC or protein C on the endothelial membrane [7]. APC can down-regulate pro-inflammatory cytokine production and favourably alter tissue factor expression or blood pressure in various models [8–10]. In baboons, APC can provide EPCR-dependent protection against the lethal effects of *Escherichia coli* infusion [11]. On a cellular level, APC is capable of inhibiting staurosporine-induced apoptosis [12]. This recently described anti-apoptotic activity of APC, as well as its anti-inflammatory activity, may help to explain the remarkable ability of APC to reduce all-cause 28-day mortality by 19 % in patients with severe sepsis [13], because potent anti-coagulant agents such as antithrombin III and recombinant tissue-factor-pathway inhibitor have failed in similar phase III clinical trails [14,15]. Thus the success of clinical trials of recombinant APC in humans suggests that the direct cellular effects of APC are physiological.

In spite of the numerous *in vivo* studies documenting the beneficial effects of APC, there is limited information about the molecular mechanisms responsible for APC's direct anti-inflammatory and anti-apoptotic effects on cells. APC can directly modulate gene expression in human umbilical vein endothelial cells (HUVECs) with notable effects on anti-inflammatory and cell-survival genes [12,16]. This direct effect of APC on certain cells requires protease-activated receptor-1 (PAR-1) and EPCR [16], although no data for functional correlation of PAR-1-dependent signalling by APC were provided. It is not known whether the APC-mediated inhibition of staurosporine-induced apoptosis [12] is dependent on PAR-1 and EPCR. Here we describe a modified model of staurosporine-induced apoptosis with EAhy926 endothelial cells that is used to demonstrate that inhibition of staurosporine-induced apoptosis by APC is dependent on PAR-1 and EPCR. These findings are consistent with recent work showing that inhibition of hypoxia-induced apoptosis of human brain endothelial cells requires PAR-1 [17].

EXPERIMENTAL

APC, S360A-APC and the anti-APC monoclonal antibody C3 were prepared as described in [18,19]. Antibodies against PAR-1 (WEDE-15 and ATAP-2) were obtained from Dr L. Brass, antibodies against PAR-2 (SAM-11) were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.) and an anti-peptide antibody against EPCR was from Zymed (South San Francisco, CA, U.S.A.), who also provided the EPCR-based peptide antigen which neutralizes the anti-EPCR antibody. PAR-1 and PAR-2 agonist peptides, TFLLRNPNDK and SLIGRL respectively, were obtained from Ana Spec (San Jose, CA, U.S.A.) and thrombin (3281 units/mg) was from Enzyme Research Labs (South Bend, IN, U.S.A.). Heat-denatured APC was prepared by placing it in a boiling-water bath for 10 min. The heat denaturation abolished all detectable chromogenic activity of APC. EAhy926 endothelial cells were from Dr C. J. S. Edgell and were maintained in Dulbecco's modified Eagle's medium high glucose (Gibco) with 10 % fetal bovine serum (Omega Scientific, Tarzana, CA, U.S.A.) and 2 mM

Abbreviations used: Ac-DEVD-amc, acetyl-DEVD-7-amino-4-methylcoumarin; PAR-1, protease-activated receptor-1; EPCR, endothelial cell protein C receptor; APC, activated protein C; HUVEC, human umbilical vein endothelial cell.

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glutamine (Gibco) in a humid atmosphere containing 5 % CO_2 in air as described in [20].

Staurosporine-induced apoptosis of endothelial cells was initiated using modifications of the assay described previously [12]. Briefly, 12 mm circular coverslips (Fisherbrand, Pittsburgh, PA, U.S.A.) were acid-washed, rinsed with distilled water and 95 % ethanol, dipped approx. 10 times in gelatin (0.5 % gelatin provided with the Apopercentage dye) until a homogeneous drop was formed and air-dried. EAhy926 cells were grown to confluency on gelatin-coated coverslips in 24-well plates and incubated with APC for various times as indicated prior to apoptosis induction. After the preincubation with the various proteins and antibodies, apoptosis was induced by 10 μM staurosporine (Calbiochem, San Diego, CA, U.S.A.) for 1 h in the presence of the Apopercentage dye (Biocolor, Belfast, Northern Ireland, U.K.) as per the manufacturer's instructions, after which the cells were washed and photographed. An average of six fields at 100× magnification were photographed per coverslip and apoptotic cells were counted using the image analysis software Cell Counter (Dr L.O. Mosnier). For each experiment representative fields of the cells were photographed using phase contrast and the total number of cells present was counted. The percentage of apoptosis is expressed as the number of apoptotic cells relative to the total number of cells. Repeated control experiments were performed (MTT-based assay, where MTT is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H* tetrazolium bromide; Celltiter 96 Aqueous non-radioactive cell proliferation assay; Promega, Madison, WI, U.S.A.) to ascertain that the cells did not become detached. In addition, when disruption of the confluent cell layer was occasionally observed, the data point was excluded from further analysis and repeated. In some experiments, before being washed and photographed, cells were incubated with the apoptosis-specific dye, YO-PRO-1 (at 10 μM for 5 min; Molecular Probes, Eugene, OR, U.S.A.) as described in [21], or for 20 min with the synthetic substrate for caspase 3-like enzymes, acetyl-DEVD-7-amino-4-methylcoumarin (Ac-DEVD-amc) (Calbiochem).

RESULTS AND DISCUSSION

Staurosporine, an ATP analogue and inhibitor of protein kinase C, is a well-known and potent inducer of apoptosis. Apopercentage dye allows measurement of the expression of phosphatidylserine on the outside surface of the cell membrane, which is therefore similar to what is measured by traditional annexin-V labelling. The transfer of phosphatidylserine to the outside surface of the cell membrane permits the unidirectional transport of the Apopercentage dye inside the cell, where it is retained and accumulates. The accumulated dye has a red/purple colour and is visible under a conventional microscope (illustrated in Figure 1, left panels) [12]. We used this dye to monitor apoptosis. Using a staurosporine-induced apoptosis model, we were able to reproduce the anti-apoptotic effects of APC after significant modifications of the published assay, which was not reproducible in our hands as described in [12]. The modifications involved culturing the cells on gelatin-coated coverslips, changing the staurosporine concentration and optimizing the APC preincubation times before addition of staurosporine. Staurosporine induced time- and concentration-dependent apoptosis in EAhy926 endothelial cells, as determined by Apopercentage staining (results not shown).

APC inhibited staurosporine-induced apoptosis in EAhy926 endothelial cells as determined by Apopercentage staining, uptake of the fluorescent nuclear YO-PRO-1 dye (which identifies cells undergoing apoptosis) and appearance of caspase 3-like activity (Figure 1). In the absence of apoptotic stimuli, APC inhibited baseline apoptosis by ≈50 %, demonstrating its cell-survival activity. Inhibition of apoptosis by APC was dose- and time-dependent (Figure 2). Half-maximum inhibition of staurosporine-induced apoptosis was achieved at 1.7 nM APC using a 24 h preincubation. When 50 nM APC was preincubated with EAhy926 cells for various times before adding staurosporine, 50 % inhibition of apoptosis occurred when the preincubation with APC was for 46 min and maximal inhibition was obtained with a 4 h preincubation time (Figure 2, inset).

To determine what structural properties of APC were required for its anti-apoptotic activity, different forms of APC or protein C were assayed for their anti-apoptotic activity. The specificity for APC of the observed inhibition of staurosporine-induced apoptosis was established, as the inhibition of apoptosis was blocked by pretreatment of APC with an anti-APC monoclonal antibody or by heat denaturation of APC (Figure 3A). APC-mediated inhibition of staurosporine-induced apoptosis required APC's active site, since the inactive protein C zymogen as well as an inactive APC mutant, in which the active-site Ser was replaced by Ala, S360A-APC [19], were devoid of anti-apoptotic activity (Figure 3A). This implied that the anti-apoptotic activity of APC was mediated by proteolysis.

Consistent with the implication that APC's proteolytic active site was required for inhibition of apoptosis, preincubation of cells with blocking antibodies against PAR-1, but not against PAR-2, abolished the APC-mediated inhibition of staurosporine-induced apoptosis (Figure 3B). Furthermore, APC's anti-apoptotic activity was abolished by an anti-EPCR antibody that blocks binding of APC to EPCR (Figure 3B), and controls showed that this effect of the anti-EPCR antibody was neutralized by preincubation of the antibody with its peptide immunogen (Figure 3B). Therefore, based on antibody blocking studies, PAR-1 and EPCR are required for APC to inhibit the staurosporine-induced apoptosis of endothelial cells.

This requirement for PAR-1 and EPCR for inhibition of staurosporine-induced apoptosis of EAhy926 endothelial cells is similar to the finding that these receptors are also important for APC's anti-apoptotic activity in the setting of hypoxic brain microvascular endothelial cells [17]. It also is consistent with the report by Riewald et al. [16], who demonstrated that PAR-1-dependent signalling by APC was dependent on EPCR, although they provided no data that related any APC functional activity with PAR-1-dependent signalling.

What is the role of PAR-1 in the inhibition of apoptosis by APC? APC can cleave a synthetic extracellular N-terminal PAR-1 polypeptide at Arg-41, the thrombin cleavage site [22]. Cleavage of this synthetic PAR-1 polypeptide by APC is 5000 times slower than by thrombin [22]. When thrombin cleaves PAR-1 at Arg-41, potent cell-signalling pathways are initiated [23]. It is likely that APC cleavage of PAR-1 at Arg-41 initiates cell signals, including phosphorylation of mitogen-activated protein kinase [16]. In brain endothelial cells subjected to hypoxia, an early result of APC signalling is the inhibition of increases in the levels of p53 [17]. Based on previous studies, APC directly alters the gene-expression profiles of HUVECs, such that several anti-apoptotic genes are up-regulated [12,16] and APC specifically down-regulates levels of the pro-apoptotic factor Bax, while it up-regulates levels of the anti-apoptotic factor Bcl-2, in brain endothelial cells [17]. The specific alteration of the critical ratio Bax/Bcl-2 is likely to be of key importance for apoptosis. Other than these events, little can be stated about the mechanisms of PAR-1-dependent APC signalling. It is interesting to note that the PAR-1 agonist peptide, TFLLRNPNDK,

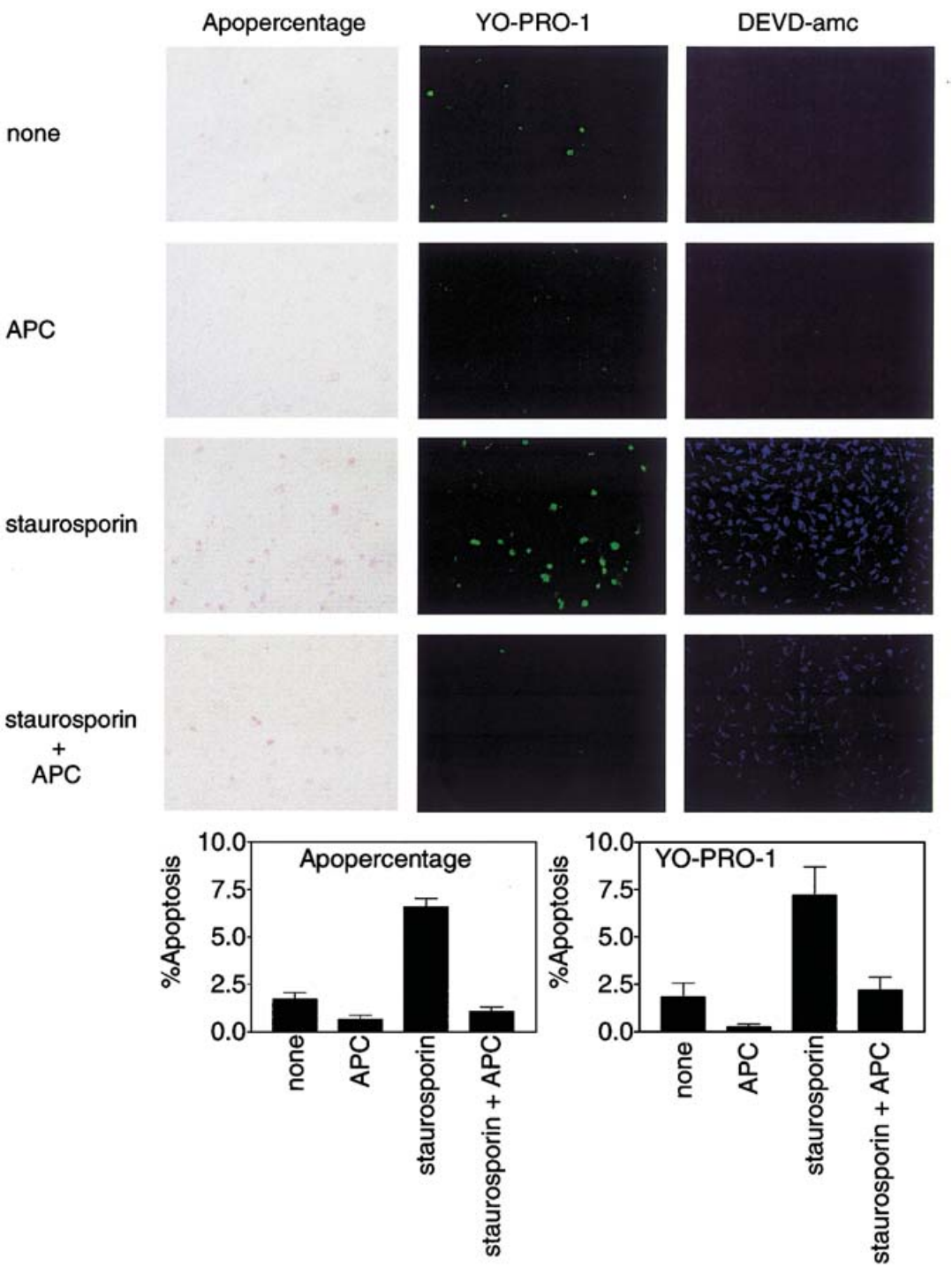

Figure 1 Inhibition of staurosporine-induced apoptosis by APC

EAhy926 endothelial cells were grown to confluency on gelatin-coated coverslips in 24-well plates and incubated with APC (5 nM) as indicated for 24 h prior to induction of apoptosis by addition of 10 μM staurosporine for 1 h. Apoptosis was analysed by light or fluorescence microscopy at 200$\times$ magnification using either the Apopercentage dye (left panels and left-hand graph), YO-PRO-1 (middle panels and right-hand graph) or the synthetic substrate for caspase 3-like enzymes, Ac-DEVD-amc (right panels). These results are representative of at least three independent experiments (top panels) and each point represents the mean $\pm$ S.E.M. from at least three independent experiments (left- and right-hand graphs).

exhibited no protection against staurosporine-induced apoptosis of EAhy926 cells, whereas this agonist provided partial rescue of brain endothelial cells from hypoxia-induced apoptosis, suggesting there are subtle but significant differences between APC's PAR-1-dependent anti-apoptotic activities in these two models.

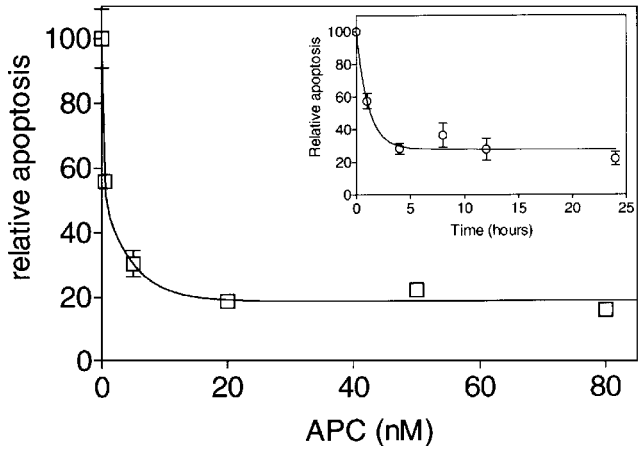

Figure 2 Dose- and time-dependent inhibition of apoptosis by APC

EAhy926 endothelial cells were grown to confluency on gelatin-coated coverslips in 24-well plates and incubated with APC at the doses indicated for 24 h prior to apoptosis induction. Apoptosis was induced by addition of 10 μM staurosporine for 1 h and analysed by measuring uptake of Apopercentage dye. Inset: time-dependence of the effect of preincubation time with APC (50 nM). Each point represents the mean $\pm$ S.E.M. from at least three independent experiments.

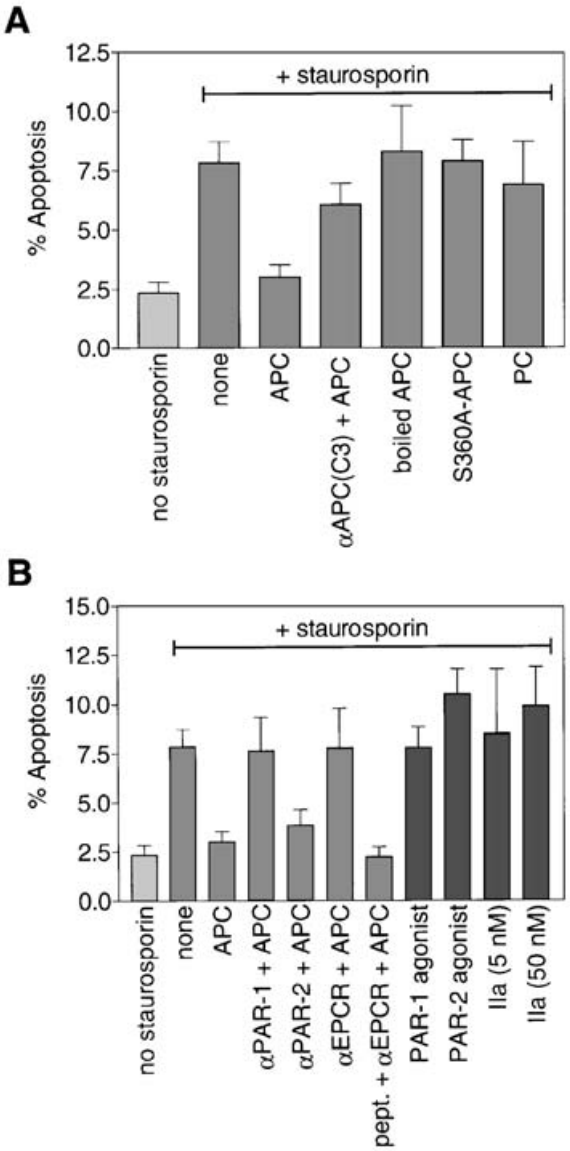

Figure 3 Inhibition of apoptosis by APC requires APC's active site, PAR-1 and EPCR

(**A**) The effects of different forms of APC (50 nM, 4 h pretreatment) on inhibition of apoptosis were analysed in EAhy926 endothelial cells grown to confluency on gelatin-coated coverslips in 24-well plates and incubated with either buffer or 50 nM of the following prior to apoptosis induction: APC, APC pretreated with a 10-fold molar excess of anti-APC antibody (monoclonal antibody C3), heat-denatured APC (boiled APC), the mutant S360A-APC or protein C zymogen (PC). (**B**) APC (50 nM) was preincubated with cells for 4 h and APC-mediated inhibition of apoptosis was determined in the presence of blocking antibodies against PAR-1 (a combination of WEDE-15 at 20 μg/ml and ATAP-2 at 15 μg/ml), PAR-2 (SAM-11 at 20 μg/ml), EPCR (rabbit anti-EPCR at 20 μg/ml) or anti-EPCR in the presence of a 20-fold molar excess of its peptide immunogen (pept.). The effects on apoptosis of PAR-1 agonist peptide (10 μM), PAR-2 agonist peptide (100 μM) or thrombin (IIa; 5 or 50 nM) in the absence of APC were determined. Except as noted, antibodies were incubated with their immunogen for 30 min prior to the start of the assay. Apoptosis was induced by addition of 10 μM staurosporine for 1 h and analysed by the uptake of Apopercentage dye. Each point represents the mean $\pm$ S.E.M. from at least three independent experiments.

What is the role of EPCR in the inhibition of apoptosis by APC? EPCR was discovered originally as a receptor capable of binding protein C and APC with equal affinity [6], and EPCR was shown to enhance the activation of protein C by the thrombin–thrombomodulin complex [24], apparently by optimizing the spatial localization of protein C for efficient activation by thrombomodulin-bound thrombin. Presumably EPCR binds APC to the endothelial surface and positions APC's active site in proximity to the PAR-1 cleavage site at Arg-41. Paradoxically, although EPCR function might be anti-coagulant by stimulating protein C activation [24], EPCR actually inhibits APC anti-coagulant activity when APC is bound to EPCR [25]. Because binding of APC to EPCR is essential for APC's anti-apoptotic activity, we conclude that the anti-apoptotic activity of APC is independent of its anti-coagulant activity. This suggests that certain APC mutants may lack anti-coagulant activity but retain anti-apoptotic activity; such mutants could be therapeutically useful if they provided patients with direct cell-survival activity without increased risks of bleeding. A scheme summarizing the interactions that generate APC on the endothelial surface where it interacts with EPCR and PAR-1 to effect anti-apoptotic activity is depicted in Figure 4.

In vivo data are consistent with an important distinction between the anti-coagulant and cell-protective activities of APC, as depicted in Figure 4. APC-induced neuroprotective effects in a murine ischaemia/reperfusion injury model were observed at low APC doses that had no effect on fibrin deposition or on the restoration of blood flow, indicating that APC's neuroprotective effects, at least in part, were independent of APC's anti-coagulant activity [17].

Whether APC-mediated PAR-1 activation alone is sufficient to account for the anti-apoptotic effects of APC or whether it is merely a first step in more complex mechanisms is currently unknown. No inhibition of staurosporine-induced apoptosis of EAhy926 cells was observed with either PAR-1 or PAR-2 agonist peptides in the absence of APC; moreover, thrombin, the archetypal activator of PAR-1, did not inhibit staurosporine-induced apoptosis (Figure 3B). This indicates that the PAR-1-dependent anti-apoptotic effects of APC for staurosporine-induced apoptosis are specific for APC, because other activators of PAR-1, fail to provide cell-survival activity. We can speculate that when EPCR-bound APC cleaves and activates PAR-1, a significant modulation of PAR-1's intracellular signalling occurs

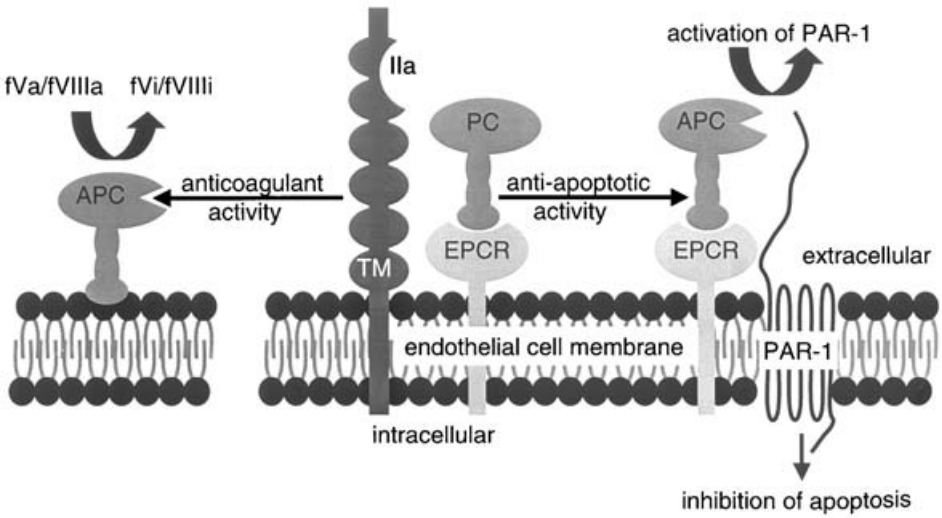

Figure 4 Schematic model of the generation of APC and of APC's anti-apoptotic and anti-coagulant activities

Protein C (PC) is activated by thrombin (IIa) bound to thrombomodulin (TM) located on the endothelial cell surface. EPCR localizes protein C on the endothelial cell membrane and enhances the activation of protein C. Whereas the anti-coagulant activity of APC is inhibited when APC is in complex with EPCR, EPCR is a required cofactor for the anti-apoptotic activity of APC. Activation of PAR-1 by APC is EPCR-dependent. When APC dissociates from EPCR, it can express its anti-coagulant activity, especially when bound to negatively charged phospholipids (e.g. phosphatidylserine) on activated platelet or endothelial cell membranes. As an anti-coagulant, APC cleaves the activated cofactors factor Va (fVa) and factor VIIIa (fVIIIa) to yield the inactivated cofactors fVi and fVIIIi.

compared with signals triggered by thrombin or the PAR-1 agonist peptide. Another potential source of complexity may arise from the reported ability of EPCR to mediate nuclear translocation of APC [26]. Studies are clearly needed to elucidate the intracellular signals and pathways that cause inhibition of apoptosis by APC in various cell model systems.

Finally, one must note that EPCR-dependent signalling via PAR-1 by APC is certainly physiologically relevant because the APC-induced neuroprotective effects in a murine ischaemia/reperfusion injury model required PAR-1 and EPCR [17]. It is not clear whether the anti-apoptotic activity of APC plays a major role in APC's ability to reduce mortality in patients with severe sepsis [13], although it is tempting to speculate that such is the case. However, it is clear that the staurosporine-induced apoptosis model described for EAhy926 cells will be useful for studies dissecting mechanisms for inhibition of apoptosis by APC and that such mechanisms will probably have clinical implications.

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