

Endogenous protein C is essential for the functional integrity of human endothelial cells

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Abstract Circulating protein C (PC) plays a vital role as an anti-coagulant and anti-inflammatory mediator. We show here that human endothelial cells produce PC that acts through novel mediators to enhance their own functional integrity. When endogenous PC or its receptor, endothelial protein C receptor (EPCR), was suppressed by small interfering (si) RNA, human umbilical cord endothelial cell (HUVEC) proliferation was decreased and apoptosis elevated. Interestingly, PC or EPCR siRNA significantly increased HUVEC permeability, which is likely via reduction of the angiopoietin (Ang)1/Ang2 ratio and inhibition of the peripheral localization of the tight junction protein, zona occludens-1. In addition, PC or EPCR siRNA inhibited type IV collagen and matrix metalloproteinase-2, providing the first evidence that PC contributes to vascular basement membrane formation. These newly described actions of endogenous PC act to stabilize endothelial cells and enhance barrier function, to potentially promote the functional integrity of blood vessels.

Keywords Activated protein C · Endothelial protein C receptor · Permeability · Angiopoietin

Abbreviations

| | |
|-------|----------------------------------------|
| APC | Activated protein C |
| EPCR | Endothelial protein C receptor |
| HUVEC | Human umbilical cord endothelial cells |
| MMP | Matrix metalloproteinase |
| siRNA | Small interfering RNA |
| ZO-1 | Zonula occludens-1 |

Introduction

Protein C (PC), a vitamin K-dependent zymogen, is converted to activated PC (APC) on the endothelial surface when thrombin binds to thrombomodulin [1, 2]. The endothelial PC receptor (EPCR), a 46-kDa type I transmembrane glycoprotein homologous to major histocompatibility complex class I/CD1 family proteins [3, 4], is the specific receptor for, and binds equally to, PC and APC [1, 2, 5]. EPCR accelerates PC activation, and blocking EPCR prevents the presentation of PC to the thrombin–thrombomodulin complex and reduces PC activation rates by 80% [2]. The PC pathway plays a key role in the regulation of blood coagulation. In the presence of its cofactor, protein S, APC degrades the coagulation factors, Va and VIIIa, and inhibits thrombin generation. The importance of APC as an anticoagulant is reflected by the findings that deficiencies in PC result in severe familial disorders of thrombosis [6]. Replenishment of PC in patients with systemic or local hypercoagulation can reverse the abnormality.

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APC also acts via EPCR to exert a broad range of cytoprotective actions on endothelium, including suppression of inflammation and stabilisation of endothelial barrier function [7, 8]. Endothelial cells normally form a dynamically regulated stable barrier at the blood–tissue interface, and breakdown of this barrier is a key pathogenic factor in inflammatory disorders, such as sepsis. APC can enhance endothelial barrier integrity by converting sphingosine to sphingosine-1-phosphate (S1P), which signals intracellularly or through its G-protein-coupled receptor, S1P1, to stabilize the cytoskeleton and reduce endothelial permeability [9, 10]. Alternatively, by binding to EPCR and cleaving protease-activated receptor (PAR)-1, APC can transactivate S1P1 in endothelial cells leading to barrier protection [9]. Interestingly, barrier stabilization is more effective when APC is derived endogenously and functions in an autocrine manner than when the source of APC is exogenous [11].

In the current study, we show that PC derived from HUVEC acts via novel pathways involving angiopoietin(Ang)s, zona occludens (ZO)-1, and the basement membrane protein, type IV collagen, to directly or indirectly enhance barrier function via EPCR.

Materials and methods

Cells isolation, culture and treatment

Human umbilical vein endothelial cells (HUVEC) were isolated and maintained as described previously [12] in accordance with local ethical regulations. HUVEC were grown in Biorich medium containing 20% fetal calf serum (FCS) plus 50 µg/ml endothelial cell growth supplement (Sigma, St. Louis, MO) and 50 µg/ml heparin (Sigma), and cultured at 37°C with 5% CO₂ in a humidified incubator. Confluent cells were trypsinized and seeded onto 6- and 24-well culture plates. Once confluent, cells were switched to Biorich medium containing 5% FCS without growth supplement or heparin (Basal medium), and treated with siRNA, recombinant APC (Xigris; Eli Lilly, Indianapolis, Indiana), and/or EPCR blocking antibody RCR252, EPCR non-blocking antibody RCR92 (gift from Professor Fukudome, Department of Immunology, Saga Medical School, Nabeshima, Saga, Japan), thrombin, PC, and hirudin (Sigma). Cells were used at passage 1–3.

Small interfering (si) RNA preparation and nucleofection

siRNA duplex oligonucleotides were purchased from ProLigo (Sigma). The designed siRNAs for PC were: sense 5′GAGGUGAGCUUCCUCAAUUGC, antisense 5′AAUU

GAGGAAGCUCACCUCGC; and for EPCR were: sense 5′GUGGACGGCGAUGUUAUUUAC, antisense 5′AAUUAACAUCGCCGUCCACCU′. The scrambled forms of PC and EPCR siRNAs were used as negative controls. HUVEC were adjusted to 1 × 10⁵ cells/ml in growth medium and subjected to nucleofection using the HUVEC nucleofector™ kit and Amaxa nucleofector™ II machine according to the manufacturer's instructions (Amaxa Biosystems, Cologne, Germany). Transfected cells were allowed to attach overnight, and then trypsinized and seeded into either 24-well plates, 8-well permanox™ slides (Nalge Nunc International, Rochester, NY) or 96-well plates (2 × 10³ cells/well). When attached, cells were refed with Basal medium and incubated for 48 or 72 h. The specificity of PC and EPCR siRNA was confirmed by validated siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA). The specificity of EPCR siRNA was also confirmed by the EPCR blocking antibody, RCR252.

Real time RT-PCR

Total RNA was extracted from HUVEC using Tri Reagent (Sigma) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from total RNA using AMV reverse transcriptase and Oligo (dT)₁₅ as a primer (Promega, Madison, WI.). The levels of mRNA were semi-quantified using real-time PCR on a Rotor-gene 3000A (Corbett Research, Sydney, Australia). Samples were normalized to the housekeeping gene RPL13A and results were reported for each sample relative to the control. Primers used were as follows: PC (213 bp): sense 5′TCTTCGTCCACCCCAACTAC and anti-sense 5′GGT TTCTCTTGGCCTCCTTC; EPCR (91 bp): sense 5′TCC TACCTGCTCCAGTTCCA and antisense 5′AAGATGC CTACAGCCACACC; RPL13A (152 bp): sense 5′AAGCCTACAAGAAAGTTTGCCTATC and anti-sense 5′TGT TTTCCGTAGCCTCATGAGC.

Zymography

Cells were incubated with media containing 5% gelatinase-free FCS. The culture medium was collected for MMP-2 detection using gelatin zymography under non-reducing conditions, as described previously [13].

Permeability assay

Membranes (0.45-µm pore and 12 mm diameter; Millipore, Billerica, MA) were coated with 0.2% gelatin for 10 min before seeding with HUVEC. Cells were incubated with growth medium for 3–4 days until confluent. To prevent cell proliferation, cells were pre-treated with mitomycin C (10 µg/ml; Sigma), which was applied to the cells 2 h

before treatment and removed with three phosphate buffered saline (PBS) washes. Medium was changed to growth medium containing 5% FCS, and cells were treated with recombinant APC and/or blocking and non-blocking antibodies. After 24 h of treatment, permeability was detected using Evan's blue-albumin method as described previously [14]. After washing with PBS, 1.5 ml of Biorich medium was added to the lower chamber and 0.5 ml of Evans blue binding to albumin (200 µg/ml) was added to the upper chamber. Medium (60 µl) was collected at intervals of 30 min for 2 h, transferred to a 96-well plate and dye concentration analyzed spectrophotometrically at 630 nm.

Enzyme-linked immunosorbent assay (ELISA)

Protein levels in cell culture supernatants or whole cell lysates were measured by ELISA kits for Ang1, Ang2, EPCR (R&D systems, MN) and PC (Technoclone, Vienna, Austria) according to the manufacturers' instructions.

MTT assay

The colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to quantitate the effect of different test agents on cell growth and viability. Briefly, 2×10^3 cells/well were seeded into a 96-well microplate to a final volume of 200 µl, and incubated for 4 h to allow cells to attach. Cells were then treated with different test agents and, 3 h prior to the completion of the treatment, 10 µl of 5 mg/ml MTT (Sigma) was added per well. After a further 3 h incubation, the MTT solution was removed and replaced by 100 µl dimethyl sulfoxide. The optical density of each well was determined at a wavelength of 570 nm with a reference wavelength of 630 nm.

Apoptosis detection

Apoptotic cells were detected by measuring active caspase-3 (see "[Immunofluorescent staining](#)") and using an in situ cell death detection kit according to the manufacturer's instructions (Roche Diagnostics Australia, NSW, Australia). Briefly, cells were permeabilised with 0.1% Triton X-100 in freshly prepared 0.1% sodium citrate, and incubated with terminal deoxynucleotidyl transferase in the presence of fluorescein-labeled dUTP (60 min at 37°C). TUNEL positive cells were visualized using an anti-fluorescein peroxidase (POD) conjugated antibody and POD substrate reaction. Sections were counterstained with DAPI. The frequency of apoptotic cells was determined by a blinded investigator by counting TUNEL positive cells and total cell number under a high magnification view ($\times 40$) and calculating the percentage of TUNEL positive cells.

Western blot

HUVEC were washed three times with PBS and lysis buffer (0.15 M NaCl, 0.01 mM PMSF, 1% NP-40, 0.02 M Tris, 6 M urea/H₂O) supplemented with protease inhibitor and phosphate inhibitor (Roche, Indianapolis, IN, USA) was added. Cell lysates were centrifuged at 10,000g for 15 min and supernatants were separated by 10% sodium-dodecyl-sulphate polyacrylamide-gel electrophoresis (SDS-PAGE) and transferred to a PDVF membrane. The primary antibodies used were: rabbit anti-human EPCR (Invitrogen Australia, VIC, Australia); rabbit anti-human PC (Sigma); mouse anti-human ZO-1 and rabbit anti-human Tie2 (Invitrogen); and mouse anti-human type IV collagen (MP Biomedicals, Aurora, OH). Immunoreactivity was detected using the ECL detection system (Amersham, Piscataway, NJ). Anti-human β -actin antibody was included to normalize against unequal loading.

Immunofluorescent staining

Cells were grown onto 8-well chamber slides coated with 0.2% gelatin and fixed in ice-cold acetone for 90 s. After washing with PBS, cells were blocked by 5% horse serum in PBS and incubated with mouse anti-human ZO-1, type IV collagen, Ang1 and 2, and rabbit anti-human Tie2, PC, EPCR, active caspase-3 (R&D Systems) antibodies for 2 days at 4°C. After three washes with PBS, cells were incubated with anti-rabbit IgG conjugated with FITC (green) and anti-mouse IgG conjugated with Cy3 (red) (1:400; Invitrogen). After washing, slides were mounted with Gold anti-fade reagent with DAPI (Invitrogen) and observed using a fluorescence microscope (Nikon ECLIPSE 80i; Nikon Australia, Lidcombe NSW). Images were acquired and processed using a Nikon digital camera and software (Diagnostic Instruments, Australia) and Image J (<http://rsb.info.nih.gov/ij>).

Results

Inhibition of endogenous PC or EPCR with siRNAs suppresses HUVEC proliferation and promotes apoptosis

HUVEC expressed substantial amounts of PC and EPCR as detected by RT-PCR, ELISA, immunofluorescent staining, and western blotting (Fig. 1). The efficacy of the siRNAs was examined at 48 h by real-time PCR and at 72 h by ELISA and western blot (Fig. 1). PC and EPCR siRNAs dose-dependently reduced their corresponding mRNA levels by $\sim 75\%$ when used at 500 nM (Fig. 1a, b). Accordingly, secreted protein levels of PC/APC and cell-

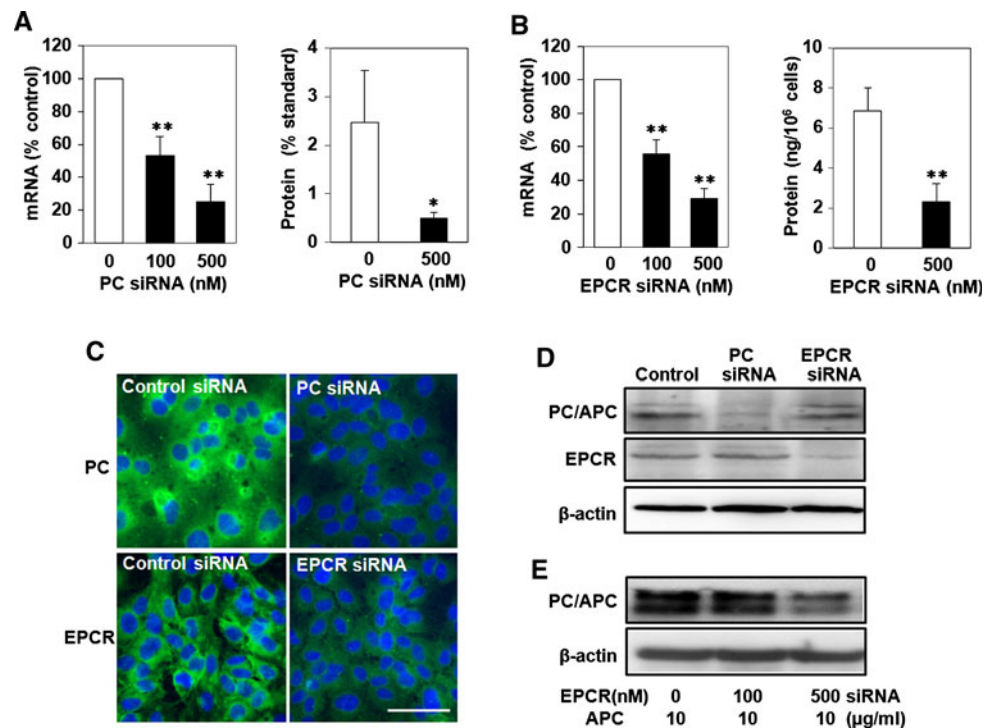


Fig. 1 The efficacy of PC or EPCR siRNA on HUVEC. HUVEC were treated with PC or EPCR siRNA at 100 and 500 nM. Cells and culture supernatants were collected at 48 or 72 h after siRNA treatment. **a,b** PC and EPCR mRNA and protein levels detected by real-time PCR and ELISA at 48 h following siRNA treatment. Data are expressed as mean \pm SEM ($n = 4$). **c** PC and EPCR protein levels in HUVEC in control and siRNA treated cells, detected by

immunofluorescent staining. *Scale bar* 20 μ m. **d** PC and EPCR protein levels in whole cell lysates detected by western blot at 72 h following siRNA treatment. **e** Cell-associated PC/APC after addition of 10 μ g/ml APC for 24 h at 72 h following EPCR siRNA treatment, detected by western blot in whole cell lysates. β -actin was included as an internal standard

associated EPCR were substantially decreased by 80 and 66%, respectively (Fig. 1a, b). Immunofluorescent staining and western blotting also confirmed that cell-associated PC and EPCR were reduced following 500 nM siRNA treatment (Fig. 1c, d). When recombinant APC (10 μ g/ml) was added to EPCR siRNA-treated cells for 24 h, there was considerably less cell-associated PC/APC compared to control siRNA-treated cells. This confirmed the expected reduction in the PC/APC binding ability to endothelial cells when EPCR is reduced (Fig. 1e).

The addition of recombinant APC to cultured HUVEC stimulates their proliferation [15]. Here, we examined the role of endogenous PC and its receptor, EPCR, on HUVEC proliferation and apoptosis. PC siRNA (500 nM) reduced HUVEC proliferation by approximately 35% at 72 h compared to the control (Fig. 2a). This inhibition was partially reversed ($P < 0.01$) by adding 1 μ g/ml recombinant APC. Adding exogenous PC (2 μ g/ml) also partially reversed cell proliferation (73.9% of control, $P = 0.045$) when compared to PC siRNA-treated cells alone (Fig. 2a). Higher concentrations of exogenous PC or APC (up to 20 μ g/ml) could not completely rescue the effect of PC siRNA (data not shown). EPCR siRNA also reduced

HUVEC proliferation at a similar rate to PC siRNA; however, adding recombinant APC (Fig. 2a) or exogenous PC had no rescue effect (data not shown), providing evidence that APC acts through EPCR to stimulate proliferation. To substantiate the effect of EPCR siRNA, cells were treated with RCR252, an antibody which prevents APC binding to EPCR. RCR252 not only inhibited APC-stimulated cell growth, but also dose-dependently inhibited unstimulated HUVEC proliferation (Fig. 2b). The non-blocking antibody to EPCR, RCR92, when used at the same concentration has no or minimal effect (data not shown). This confirms the stimulatory effect of endogenous PC on cell growth via EPCR. Overall, these results indicate that endogenous PC/APC acts through EPCR to, at least partially, contribute to the normal proliferation of HUVEC in basal conditions.

The effect of endogenous PC on HUVEC apoptosis was detected by immunofluorescent evaluation of active caspase-3, a marker for apoptosis. Considerably stronger staining for active caspase-3 was observed in PC siRNA- or EPCR siRNA-treated cells, when compared to scrambled control siRNA-treated cells (Fig. 2c). Active caspase-3 was not only localized in the cytoplasm but was also present in

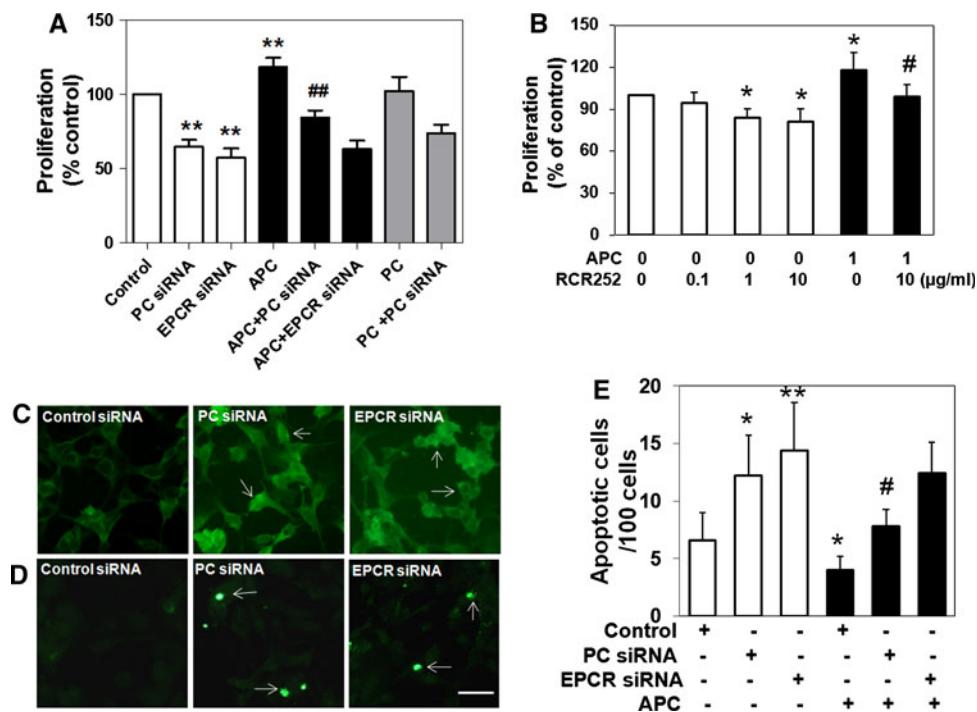


Fig. 2 PC or EPCR siRNA treatment inhibits growth and promotes apoptosis of HUVEC. **a** Proliferation rate of HUVEC in response to control, PC siRNA or EPCR siRNA (500 nM) treatment in the presence or absence of exogenous APC or PC, as detected by MTT assay. Cell proliferation is expressed as a percentage of control (mean \pm SD) over 72 h. ** $P < 0.01$ compared to control (1st bar), ## $P < 0.01$ compared to PCsiRNA (2nd Bar). **b** Proliferation of HUVEC in response to a blocking antibody to EPCR (RCR252) in the presence or absence of recombinant APC. Cell proliferation is expressed as a percentage of control (mean \pm SD) over 72 h. * $P < 0.05$ compared to control (1st bar), # $P < 0.05$ compared to APC alone (5th bar). **c–e** HUVEC were treated with control, PC

siRNA or EPCR siRNA (both at 500 nM). After 36 h, transfected cells treated with recombinant APC (1 μ g/ml) for 12 h, and cells were harvested at 48 h. Active caspase-3 was detected by immunofluorescent staining (white arrows indicate active caspase-3 positive cells) (c) and apoptotic cells were detected by in situ cell death detection kit (white arrows indicate apoptotic cells) (d). Images represent one of three independent experiments. Scale bar 40 μ m. **e** Apoptotic cells (from d) were quantitated by counting cells co-stained with DAPI under the microscopy. Data are expressed as the average number of apoptotic cells under a high magnification (40 \times) (mean \pm SEM $n = 3$). * $P < 0.05$, ** $P < 0.01$ compared to control (1st bar), # $P < 0.05$ compared to PCsiRNA treatment (2nd bar)

the nucleus. Additionally, a commercial in situ cell apoptosis detection kit was used to confirm these results. PC or EPCR siRNA treatment resulted in an approximate doubling of the number of apoptotic cells compared to scrambled siRNA treatment at 48 h (Fig. 2d, e). Adding recombinant APC decreased apoptosis in control and PC siRNA-treated but not EPCR siRNA-treated cells (Fig. 2e).

Inhibition of endogenous PC or EPCR decreases barrier function of HUVEC

Barrier protection is an essential task of the endothelium and is enhanced by APC [9]. We found that recombinant APC (1 μ g/ml) significantly reduced HUVEC permeability (Fig. 3a), whereas treatment of unstimulated HUVEC with PC siRNA or EPCR siRNA significantly induced cell permeability (Fig. 3a). The effect of EPCR siRNA was mimicked by the EPCR-blocking antibody, RCR252 (5 μ g/

ml). Addition of recombinant APC (1 μ g/ml) almost totally recovered the reduced permeability of PC siRNA-treated cells, but not the cells treated with EPCR siRNA (Fig. 3a), indicating that APC's effect on permeability requires EPCR.

The barrier function of endothelium is dependent on the actions of many cytoskeletal and membrane proteins that are involved in endothelial junctional complexes including the intracellular protein, ZO-1, and the angiotensin receptor, Tie2 [16, 17]. Confluent untreated HUVEC expressed ZO-1 and Tie2 (Fig. 3b, c), with prominent staining around the cell periphery (Fig. 3b). PC siRNA treatment for 48 h marginally reduced the expression of ZO-1 and Tie2 (Fig. 3c), but markedly shifted the localization of both proteins from the periphery to a more diffuse pattern throughout the cell (Fig. 3b). Adding APC (1 μ g/ml) for 24 h stimulated expression of ZO-1 and Tie2 (Fig. 3c) as well as re-localizing these proteins to the cell periphery in both control and PC siRNA-treated cells (Fig. 3b). This

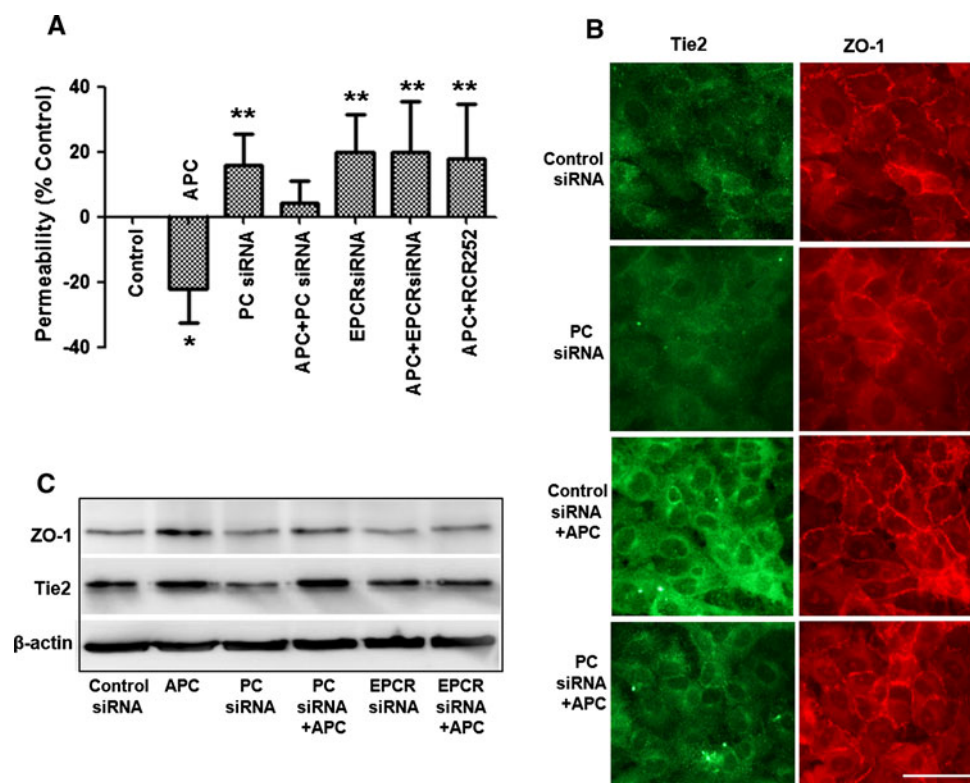


Fig. 3 PC/EPCR siRNA treatment decreases barrier function of HUVEC. HUVEC were treated with control, PC siRNA or EPCR siRNA (both at 500 nM) and, after 24 h, cells were trypsinized and seeded into a modified Boyden chamber and allowed to attach for 4 h. Confluent cells were then incubated for further 24 h in the presence or absence (control) of 1 μ g/ml APC. Cells were pre-incubated for 1 h with RCR252 (5 μ g/ml) before APC treatment. Cell permeability was detected by measuring transfer of Evan's blue-albumin dye across chamber with absorbance at 630 nm and is expressed as a percentage of control of average of four measurements within 2 h (mean \pm SD).

Graph represents one of three independent experiments. * $P < 0.05$, ** $P < 0.01$. **b** Cells following siRNA treatment for 36 h were incubated for further 12 h at the presence or absence of APC (1 μ g/ml) and were harvested at 48 h following siRNA treatment. The expression of ZO-1 and Tie2 was detected by immunofluorescent staining. Scale bar 40 μ m. **c** The expression of ZO-1 and Tie2 in whole cell lysates of HUVEC was detected by western blot at 48 h post transfection with siRNA and 12 h APC treatment. β -actin was included as an internal standard

recovery effect of APC was absent in EPCR siRNA-treated cells (Fig. 3c). These results show that exogenous or endogenous PC/APC increase peripheral localization of Tie2 and ZO-1 in HUVEC which may contribute to APC's ability to reduce endothelial permeability. Furthermore, these effects of APC are mediated, at least partly, via EPCR.

Inhibition of PC pathway differentially regulates Ang1 and 2 expression

Ang1 and Ang2 play a pivotal role in angiogenesis and endothelial permeability [18–20], HUVEC culture supernatant contained both Ang1 and 2 under basal conditions (Fig. 4a). In response to PC siRNA treatment, the levels of Ang1, a positive regulator of cell barrier function, was decreased by $\sim 30\%$ ($P < 0.05$) in cell culture supernatants, whereas the negative regulator, Ang2, increased by

$\sim 27\%$ ($P < 0.05$) (Fig. 4a). These changes were rescued by replenishing with recombinant APC (1 μ g/ml). In addition to secreted Angs, immunofluorescent staining showed that inhibition of PC expression by PC siRNA suppressed cell-associated Ang1 and stimulated cell-associated Ang2 (Fig. 4b).

APC upregulates type IV collagen production by HUVEC

Type IV collagen is the major component of vascular basement membranes and does not only provide a mechanical support for blood vessel formation and its stabilization, but also influences cellular behavior such as differentiation and proliferation [21]. Following APC treatment (1 μ g/ml) for 48 h, protein expression of type IV collagen in the extracellular space of HUVEC was enhanced as detected by immunofluorescent staining

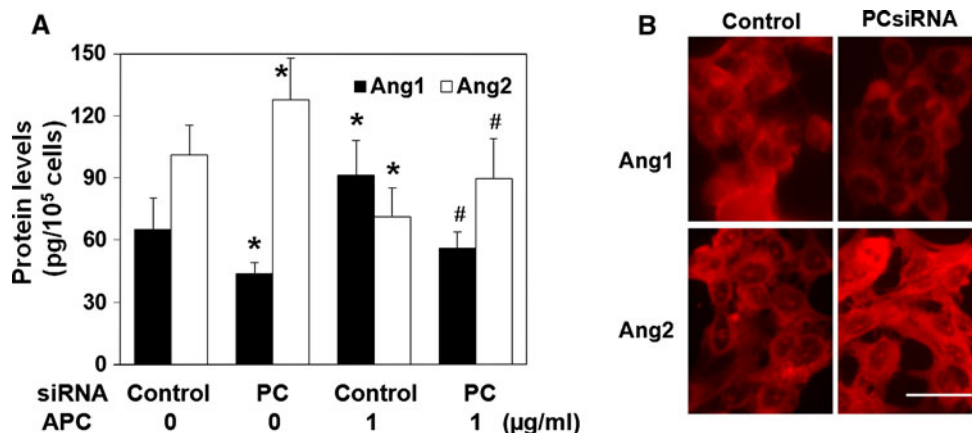


Fig. 4 PC siRNA treatment differentially regulates Ang1 and 2. HUVEC were treated with control or PC siRNA. After transfection (48 h), cells were treated with recombinant APC (1 μ g/ml) for 24 h. Cell culture supernatants were collected for detection of Ang1 and

Ang2 levels by ELISA (a) and cells were processed for immunofluorescent staining of Ang1 and Ang2 (b). Graph is displayed as mean \pm SEM ($n = 3$). * $P < 0.05$ when compared to control. # $P < 0.05$ when compared to PC siRNA treatment. Scale bar 20 μ m

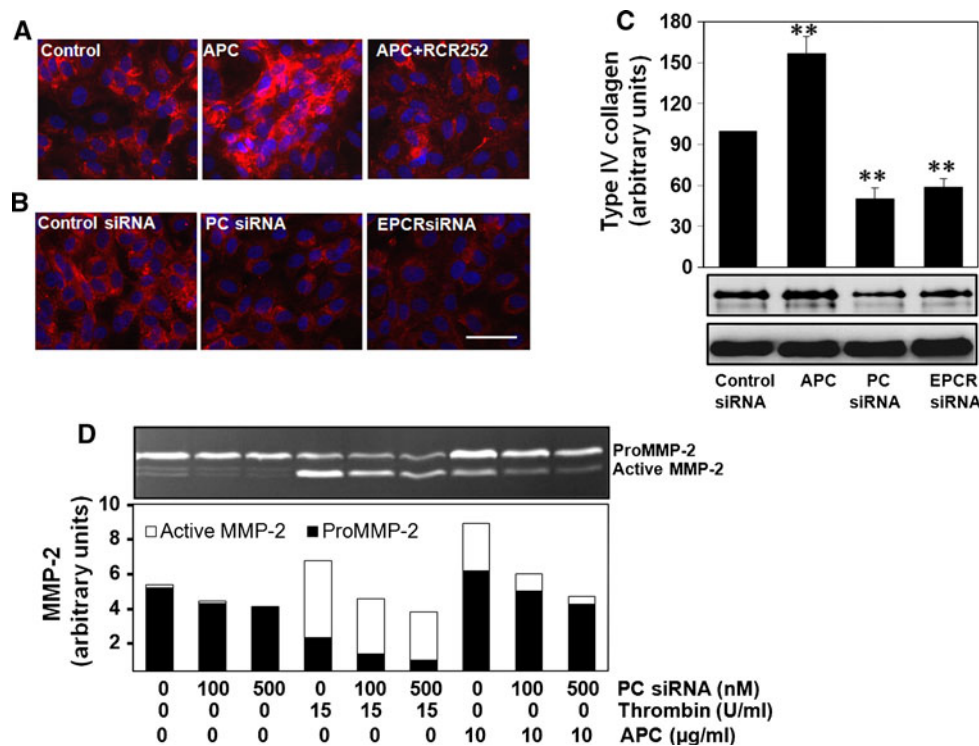


Fig. 5 PC or EPCR regulates type IV collagen production and MMP-2 activation by HUVEC. **a** HUVEC were treated with recombinant APC (1 μ g/ml) in the presence or absence of RCR252 (5 μ g/ml) for 48 h and processed for immunofluorescent staining using a type IV collagen primary antibody. **b** HUVEC were treated with control, PC siRNA or EPCR siRNA (both at 500 nM). After 72 h, type IV collagen expression was detected by immunofluorescent staining. Scale bar 30 μ m. **c** Type IV collagen in the whole cell lysates was

detected by western blotting after treatment for 48 h and semi-quantitated by image analysis software. ** $P < 0.01$ when compared to control. **d** HUVEC were treated with PC siRNA and, after 48 h, treated with recombinant APC (10 μ g/ml) or thrombin (15 U/ml) for a further 24 h. Cell culture supernatants were collected for MMP-2 detection by zymography and semi-quantified by image analysis. The gel images represent one of three experiments. Data on graph is shown as mean \pm SEM ($n = 3$)

(Fig. 5a) and western blotting (Fig. 5c). This increase was abolished by pre-treatment with RCR252 (5 μ g/ml), indicating that APC stimulates type IV collagen via EPCR.

Treatment of cells with PC siRNA or EPCR siRNA further decreased the basal type IV collagen protein levels collagen by more than 40% (Fig. 5b, c).

Inhibition of endogenous PC/APC decreased MMP-2 expression and activation

MMP-2 is constitutively produced by HUVEC and is essential for endothelial cell migration, vascular matrix remodeling, and the regulation of growth factors [22, 23]. Recombinant APC stimulated and activated MMP-2 in HUVEC (Fig. 5d), as previously described [12, 24]. Interestingly, the addition of thrombin (15 U/ml) caused a reduction in pro-MMP-2, which was due to a sharp increase in active enzyme. Treatment of unstimulated HUVEC with PC siRNA (500 nM) reduced the expression of proMMP-2 by ~25% (Fig. 5d), suggesting that endogenous PC contributes to basal MMP-2 production. PC siRNA also inhibited total MMP-2 in a dose-dependent manner in the presence of exogenous APC or thrombin. The partial inhibition of thrombin-induced MMP-2 by PC siRNA suggests that thrombin acts at least partly through endogenous PC.

Discussion

Tanabe et al. [25] have shown that human endothelial cells synthesize functional PC which is activated on HUVEC to exert anticoagulant function. They found a time-dependent and saturable accumulation of APC on the surface of HUVEC in the absence of exogenous stimuli [25], indicating that endothelium contains all the components to activate PC [25, 26]. Thus, it is likely that, in our system, endogenous PC is secreted, activated via EPCR, thrombomodulin, and thrombin, and then acts through EPCR to exert its protective effects, although the exact mechanism is yet to be elucidated. A similar autocrine cytoprotective action of PC has recently been described for human skin keratinocytes [27].

Inhibition of PC or EPCR by siRNA caused a significant suppression in cell growth. Surprisingly, the addition of relatively high concentrations (up to 20 $\mu\text{g/ml}$) of recombinant APC could not completely rescue this inhibitory effect. Since HUVEC accumulate considerably less than 20 $\mu\text{g/ml}$ PC/APC in culture supernatant (data not shown), this connotes that endogenous PC plays an essential role in basal HUVEC growth and may be more effective than exogenous APC. In a similar manner, Feistritzer et al. [11] have shown that barrier stabilization is more effective when endogenous PC is activated on the endothelial surface rather than when the source of APC is exogenous.

In concert with the effect on endothelial growth, inhibition of PC or EPCR increased endothelial apoptosis. Caspase-3 is one of the most prevalent caspases in the cell and is ultimately responsible for many of the apoptotic effects [28, 29]. Following PC or EPCR siRNA treatment,

activated caspase-3 was not only elevated in the cytoplasm of HUVEC but also present in the nuclei, which indicates a later stage of cell death [30].

Endothelial cells adhere to each other through junctional transmembrane proteins that are linked to specific intracellular structural and signaling complexes. In endothelial tight junctions, the extracellular domains of occludin or claudin maintain cell–cell contact, while intracellular domains, such as ZO-1, provide junctional stability through their linkages with the cytoskeleton [31]. ZO-1 also interacts with several cytoplasmic and signaling molecules that regulate cell growth and survival [32]. In confluent cells that are bound by tight junctions, ZO-1 localizes around the cell circumference [33]. In the current study, when cells were treated with either PC or EPCR siRNA, the basal expression of ZO-1 was decreased and peripheral location was less pronounced. The addition of recombinant APC reversed these effects caused by PC siRNA but not EPCR siRNA, which emphasizes the crucial role of EPCR in enhancing barrier function. EPCR is not a signaling receptor, but by utilizing EPCR as a co-receptor, APC can cleave protease-activated receptor (PAR)-1 on the endothelial surface and exert cytoprotective effects [34]. Both thrombin and APC can cleave PAR-1 at identical locations. Whereas thrombin cleavage promotes inflammation and increases vascular permeability, APC cleavage of PAR-1 strongly inhibits vascular permeability and prevents inflammation [34]. These opposing effects of APC and thrombin can be explained by the presence of lipid rafts in cell membranes [35, 36]. When APC binds to EPCR in the lipid raft, caveolin-1 is replaced with PAR-1 which couples with the pertussis toxin-sensitive Gi-protein to initiate a protective signaling pathway. In contrast, cleavage of PAR-1 by thrombin outside the lipid raft appears to cause signaling via Gq and/or G12/13 which exerts inflammatory effects [35]. Recombinant APC requires PAR-1 to exert its barrier stabilization functions [37]. Whether these mechanisms are involved in the beneficial effects of endogenous PC/EPCR on barrier integrity needs to be determined.

Mice with PC or EPCR deletion do not survive the neonatal period [38, 39]. The survival rates are strongly dependent on PC/EPCR levels. With as low as 18% PC or 10% EPCR expression, mice are able to support male and female virility, as well as embryonic development, birth, and survival to adulthood [40, 41]. Clearly, the siRNA-treated HUVEC in our *in vitro* studies express at least 18% PC and 10% EPCR. So why are the changes observed in the current study, using HUVEC, seemingly not apparent *in vivo*? One likely explanation is that other protective mechanisms may compensate *in vivo*, such as production of Ang1 by pericytes which acts to improve endothelial barrier integrity in a paracrine manner [42].

Tie2 belongs to the receptor tyrosine kinase family and functions as a receptor for Ang1/2. Gene-targeting analyses of either Ang1 or Tie2 in mice reveal a critical role of Ang1–Tie2 signaling in developmental vascular formation [18–20]. Tie2 maintains the vascular integrity of mature vessels by enhancing endothelial barrier function and inhibiting apoptosis of endothelial cells [18, 20, 43]. While Ang1 is a Tie2 agonist, Ang2, which binds Tie2 with similar affinity to Ang1, acts as an antagonist on endothelium. In our hands, suppression of endogenous PC reduced the levels of Ang1 and Tie2 and elevated Ang2, resulting in an increase in endothelial permeability. These data, together with the positive effects on ZO-1, clearly implicate endogenous PC as a requirement for maintaining endothelial barrier function. The involvement of the Ang–Tie2 signaling pathway in this process was confirmed by addition of exogenous APC, which stimulated Tie2 and Ang1 expression [37] (Figs. 3, 4). Our findings that PC siRNA caused diffuse distribution of Tie2, and that APC relocated Tie2 to the peripheral cell–cell contact borders of HUVEC, concurs with the recent report showing that trans-association of Tie2 contributes to maintenance of vascular quiescence by enhancing endothelial survival and integrity [16].

The inner lining of quiescent capillary blood vessels consists of a continuous barrier of vascular endothelium surrounded by a basement membrane, which forms a sleeve around the endothelial tubes and provides a scaffold essential for blood vessel organization. Type IV collagen is the major component of the vascular basement membrane structure and provides stability to the blood vessel [44, 45]. The current study is the first to show that APC stimulates type IV collagen production by HUVEC. Using immunofluorescence, the collagen appeared to be localized pericellularly, which concords with its role as a basement membrane protein. One of the major proteases responsible for basement membrane turnover is matrix metalloproteinase (MMP)-2, which belongs to the gelatinase subfamily of MMPs. In endothelial cells, recombinant APC upregulates the activity of MMP-2 [12]. In this study, endogenous PC upregulated MMP-2 expression and activation by HUVEC. MMP-2 not only degrades matrix components such as type IV collagens but also cleaves growth factors, cytokines/chemokines, by proteolysis [46, 47], and is directly involved in the regulation of cell migration, proliferation, inflammation, and death. The protective effect of endogenous PC/APC on endothelial cells may be partially mediated via regulation of MMP-2.

In conclusion, our data are the first to show that endogenous PC exerts cytoprotective effects by acting via Angs, ZO-1, and collagen type IV and reduces endothelial permeability. These novel actions of PC/APC are likely to enhance the stability of blood vessels.

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