Microparticle-associated endothelial protein C receptor and the induction of cytoprotective and anti-inflammatory effects

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The online version of this article contains a supplementary appendix.

ABSTRACT

Background

The endothelial protein C receptor plays an important role within the protein C pathway in regulating coagulation and inflammation. Recently, we described that endothelial protein C receptor can be released *in vitro* in microparticulate form from primary endothelial cells by exogenous activated protein C. Activated protein C bound to this endothelial protein C receptor retains anticoagulant activity and we hypothesize that this microparticulate endothelial protein C receptor-activated protein C complex can also cleave endothelial protease-activated receptor 1 to modulate inflammation and increase cell survival. Our main objective was, therefore, to study the effect that microparticle-associated endothelial protein C receptor-activated protein C has on endothelial function.

Design and Methods

Mini-arrays were used and probed with cDNA obtained from endothelial cells after treatment with microparticle-associated endothelial protein C receptor-activated protein C and results were confirmed by real time polymerase chain reaction. The functional relevance of changes at gene level were further analyzed by endothelial apoptosis and permeability assays, in the presence and absence of specific blockade of endothelial protein C receptor, protein C and protease-activated receptor 1.

Results

Gene profiling of endothelial cells stimulated by 40 nmol/L activated protein C on microparticles showed significant changes in anti-apoptotic and inflammatory pathways. This was accompanied by protease-activated receptor 1-dependent anti-apoptotic and barrier protective effects, the latter of which also involved sphingosine 1-phosphate receptor and vascular endothelial growth factor receptor-2/ kinase insert domain receptor. Protein C blockade reversed these effects showing specificity for activated protein C on microparticles. Furthermore, confocal microscopy and enzyme-linked immunosorbent assay of plasma obtained from septic patients during recombinant activated protein C treatment showed evidence of their presence *in vivo*.

Conclusions

Activated protein C on microparticle-associated endothelial protein C receptor release can induce protease-activated receptor 1-dependent endothelial effects. The mechanisms underlying barrier protection involve sphingosine 1-phosphate receptor and kinase insert domain receptor.

Key words: activated protein C, endothelial protein C receptor, microparticles, protease activated receptor, vascular endothelial growth factor receptor-2.

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Introduction

The role of microparticles in health and disease has been increasingly recognized in recent years. Microparticles are plasma membrane fragments that are shed from cells in response to stimulation and retain many of the ligands and receptor-proteins expressed by the original cells.[1] As such, microparticles can interact with other cells and, through the expressed proteins or other factors, deliver signals that induce distinct patterns of gene expression in these cells.[2] There is also a general ability of microparticles to escape phagocytosis by nature of their size and extent of phosphatidylserine exposure.[1] The size of microparticles (0.2-1 μm) differs from that of exosomes (30-100 nm) and this difference enables their differential separation by centrifugation.[3,4]

We recently showed that activated protein C (APC), but not protein C, can induce microparticle release from human umbilical vein endothelial cells (HUVEC) and monocytes in a time- and concentration-dependent manner.[5] In addition to acting as an anticoagulant,[6] APC also has anti-inflammatory and anti-apoptotic properties.[7] Although the relative contribution of each of these properties to the beneficial effects of APC in human sepsis is presently unclear, recombinant human APC (rhAPC) is now approved for the treatment of sepsis.[8]

The mechanism of microparticle generation by APC requires the presence of the endothelial protein C receptor (EPCR) at the cell surface and the released microparticles retain the EPCR-APC complex in an anticoagulant-active conformation. This contrasts with the truncated, soluble EPCR form whose bound APC can no longer inactivate coagulant factor Va.[9] APC induction of microparticle-associated EPCR (MP-EPCR) also depends on protease activated receptor-1 (PAR1) at the cell surface. Riewald *et al.* reported that APC utilizes EPCR as a co-factor in activating PAR1 to induce anti-inflammatory and cytoprotective genes.[10] In the absence of EPCR, APC is unable to activate PAR1 and the physiological relevance of this EPCR-APC-PAR1 pathway has been demonstrated in experimental models.[11,12]

We hypothesized that the EPCR-APC complex on microparticles can induce PAR1-dependent cytoprotective and anti-inflammatory effects on endothelial cells. In order to test this hypothesis, we first screened for an effect of *in vitro*-generated APC-derived microparticles on gene arrays related to inflammation and cytoprotection and then focused more specifically on selected genes. As we previously showed that APC at physiologically achievable concentrations induces MP-EPCR release *in vitro*, we speculated that such microparticles could circulate in septic patients undergoing treatment with rhAPC. If this were to be the case, it might also clarify the current uncertainty over whether endothelial PAR1 activation by APC is important in patients with sepsis, given the PAR1-dependence of MP-EPCR release.

Design and Methods

Materials

Human protein C, APC and protein C blocking antibody (AHPC-5071) were acquired from Haematological Technologies (Vermont, USA), and S2366 from Chromogenix Laboratory (Milan, Italy). Anti-rat immunoglobulin G (IgG, whole molecule) labeled with fluorescein isothiocyanate, isotype control rat IgG (whole molecule), bovine serum albumin (BSA) and Evans blue were from Sigma (Poole, UK). CD13-phycoerythrin, corresponding IgG class control and goat-anti-mouse-phycoerythrin were from BD Biosciences (San Jose, CA, USA). PAR1 antagonist peptide H-Met-Ser-Arg-Pro-Ala-Cys-Pro-Asn-Asp-Lys-Tyr-Glu-OH (T1) was from Bachem (St. Helens, UK), and cleavage-blocking antibodies ATAP2 and WEDE15 were from Santa Cruz (Santa Cruz, CA, USA) and Beckman Coulter (High Wycombe, UK), respectively. The EPCR antibodies RCR-2, RCR-49 and RCR-252 used were as previously described.[5,13] Soluble EPCR was kindly provided by Dr H Nakatake (Chemo-Sero-Therapeutic Research Institute, Saga, Japan). The inhibitors SU5416 and LY294002 were from Calbiochem (Nottingham, UK).

Microparticulate endothelial protein C receptor

MP-EPCR were generated and isolated from HUVEC as previously described[5] with some modifications. In brief, endothelial cells were stimulated with 100 nmol/L APC overnight and microparticles were isolated from the conditioned media by sequential centrifugation. Cellular debris was eliminated by centrifugation of the media at 5,000 x g for 5 min, followed by further centrifugation at 18,000 x g for 30 min to pellet the microparticles. The concentration of APC on the induced microparticles was quantified by enzyme-linked immunosorbent assay, (ELISA) as described elsewhere, using the specific chromogenic substrate S2366 and an APC standard curve.[5] The concentration of APC on microparticles used for gene expression analysis was 40 nmol/L, as this was the mean value obtained from *in vitro*-generated microparticles. *In vivo* microparticles were obtained from patients diagnosed as having severe sepsis (American College of Chest Physicians criteria),[14] who also fulfilled the National Institute of Clinical Excellence (England and Wales) criteria[15] for treatment with rhAPC [Drotrecogin alfa (activated)] (Xigris®, Eli Lilly, The Netherlands). Samples were obtained from four patients receiving a 96-hour rhAPC (24 μg/kg/h) infusion. With Local Research Ethics Committee approval, blood samples were collected into 0.105 mol/L trisodium citrate with 0.1 mol/L benzamidine. Microparticles were isolated by centrifugation at 5,000 g for 10 min followed by 18,000 g for 30 min twice at 4°C. The concentration of APC on MP-EPCR was estimated by ELISA by capture with RCR-2 EPCR antibody and detection using S2366, as previously described.[5]

Determination of endothelial gene expression by cDNA array

HUVEC were serum-starved and incubated in serum-free Iscove's modified Dulbecco medium with 40 nmol/L APC in free or microparticulate-bound form for 4 h at 37°C and in 5% CO_2. RNA extraction, gene expression analysis and quantitative real time polymerase chain reaction (qRT-PCR) are described in the *Online Supplemental Data* to this paper.

Functional assays

Apoptosis was induced in confluent HUVEC using staurosporine (10 μmol/L) for 1h. The effect of pre-incubation with free or microparticulate-bound APC (*in vitro* or patient-derived) for 3h prior to staurosporine was also examined. Apoptotic cells were detected with an APOPercentage apoptosis assay (Biocolor, Newtonabbey, Northern Ireland). Cells were incubated with APOPercentage dye for 30 min and excess dye was washed off with phosphate-buffered saline (PBS) before photography or treatment with a dye release reagent. The level of released dye was measured in a Spectramax plate reader. APC specificity was examined by pre-treatment with anti-protein C (10 μg/mL) or isotype control. For PAR1, T1 (50 μmol/L) or ATAP2 (20 μg/mL) was added before APC inclusion in free or microparticulate form. Images of the stained cells were taken using a Olympus CK2 microscope with 10X objective lenses at room temperature; the microscope was attached to a Nikkon Digital Camera DXM1200 and images were taken with the software provided (Nikkon ACT-1).

The permeability of a monolayer of endothelial cells was analyzed in a dual chamber system using Evans blue-labeled BSA, as described elsewhere.[16] In brief, EAhy926 cells (a kind gift from Dr Cora Edgell) were seeded on 6.5-mm diameter Transwell polycarbonate membranes of 3 μm pore size (Costar Corning, NY, USA). The upper and lower chambers were filled with 100 μL and 500 μL of growth medium, respectively. Cells were grown for 2 days to obtain monolayers that were *subconfluent*. This level of permeability was defined as 100% to enable comparison with cells after treatment with APC in free or microparticulate form for 3h. Permeability was assessed by adding Evans Blue-BSA (0.67 mg/mL Evans blue in medium containing 4% BSA) to the top chamber. After 10 min, an aliquot was taken from the lower chamber and diluted 1:2 in medium for measurement of absorbance at 650 nm. To clarify their respective roles, blocking antibodies to EPCR (RCR-252 at 20 μg/mL), APC (protein C antibody at 10 μg/mL) and PAR1 (ATAP2 and WEDE15 at 20 μg/mL) were added to the monolayers 30 min before the addition of APC in free or microparticulate form. Pharmacological inhibitors to phosphoinositide 3-kinase (PI3K) [LY294002 10 μmol/L] and kinase insert domain receptor (KDR) [SU5416 10 μmol/L] were added to the cells for 2h prior to the addition of APC. In all cases, cells were exposed to APC in free or microparticulate form.

Laser scanning confocal microscopy

Microparticles were stained with anti-protein C for 1h at room temperature in PBS/0.2% Triton, washed at 18,000g for 20 min and then incubated with goat-anti-mouse-phycoerythrin and RCR-49-fluorescein isothiocyanate in PBS/0.2% Triton for 30 min in the dark before further washing. Corresponding isotypic and second layer controls were done simultaneously. Stained microparticles were spotted onto poly-L-lysine (Sigma)-coated glass slides and mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) for microscopy on an Olympus BH-2 using a Bio-Rad MicroRadiance confocal scanning system and Laser Sharp 2000 software.

Activated protein C replacement assay

APC-induced microparticles from HUVEC were isolated and quantified for APC content as previously described. Equal concentrations of microparticles, standardized by APC content, were incubated in HBS (20 mmol/L HEPES, 0.15 mol/L NaCl, pH 7.4)/2.5 mmol/L $CaCl_2$/ 1 μmol/L hirudin at varying concentrations of protein C (0, 60, 100 nmol/L) at room temperature. After 30 min, the sample was centrifuged at 18,000g for 20 min. Following separation from the supernatant, the microparticles were washed in HBS/ 2.5 mmol/L $CaCl_2$. The supernatant and microparticles were then respectively incubated with S2366 and the absorbance read in a Spectramax plate reader (Molecular Devices). Results were estimated from an APC standard curve.

To test whether APC on microparticles is displaced by soluble EPCR, equal concentrations of APC-MP were incubated with increasing concentrations of soluble EPCR (0, 250, 500, 1000 nmol/L) in HBS/2.5 mmol/L $CaCl_2$. S2366 was used to measure APC levels on microparticles and the corresponding supernatants after incubation for 30 min and centrifugation. To examine the possibility of APC becoming unbound to act as free APC, buffer was removed at the end of the incubation period and centrifuged at 18,000 x g for 30 min to pellet any residual microparticles. The supernatant was checked with S2366 and no specific APC signal was obtained.

Statistical analysis

SPSS v15.0 software was utilized with data expressed as mean and standard deviation (SD) of at least four independent experiments. Student's paired *t* test was used to compare groups and $p<0.05$ indicated statistical significance.

Results

Effect of in vitro microparticles/endothelial protein C receptor on endothelial cell gene expression.

APC (40 nmol/L) bound to HUVEC-derived microparticles induced a change of 2-fold or more in the signal ratio of genes involved in cell activation, apoptosis, angiogenesis and cytokine production pathways (*Online Supplementary Table S1*). The up- or down-regulatory effect of microparticle-bound APC was similar to that of an equivalent concentration of free APC, although the overall gene expression effect seemed to be higher with microparticle-bound APC. Protein C blockade demonstrated that, with the exception of angiopoietin 1 and ICAM2, induction of all other genes by MP-EPCR required bound APC. Pre-incubation with T1 antagonist peptide or ATAP2 antibody demonstrated PAR1 involvement in this effect with the main exception of caspase 1, CRADD and KDR (*Online Supplementary Table S1*). As the induction of these PAR1-independent genes was APC-specific, the results implied activation of some PAR1-independent pathways by APC. qRT-PCR was utilized to confirm array results and this demonstrated that APC in both forms significantly upregulated gene expression of A20, MCP-1, IL-8 and GM-CSF, an effect which was abrogated by protein C and PAR1 inhibition (Figure

1). However, qRT-PCR showed that KDR gene expression was also APC- and PAR1-dependent, differing from the initial array result but corroborating the screening nature of arrays in comparison to the more sensitive qRT-PCR. After monolayer incubation with microparticles, Bcl-xL expression was significantly increased but pro-apoptotic Bax was significantly reduced by APC in either form.

The effect of activated protein C on in vitro *microparticles/endothelial protein C receptor and apoptosis*

To examine whether APC on microparticles could induce cytoprotective effects, HUVEC were incubated with an equal amount of APC (40 nmol/L) in free or microparticulate form prior to the addition of staurosporine (n=3), as done in a previously described model.[11] While untreated HUVEC did not undergo apoptosis, as demonstrated by the absence of dye uptake, staurosporine induced apoptosis, with the apoptotic cells showing high dye uptake (Figure 2). Cells pre-treated with either form of APC (40 nmol/L) showed very low dye uptake. This indicates that APC on microparticles is equally capable as free APC of inhibiting staurosporine-induced apoptosis. Inhibition by protein C blocking antibody reversed this anti-apoptotic action, while an isotypic control did not. The cytoprotection was, therefore, specific to APC on MP-EPCR. Similarly, PAR1 dependence was demonstrated by loss of APC–induced cytoprotection against staurosporine in the presence of T1 or ATAP2 (*data not shown*).

Barrier-protective effect of activated protein C and microparticles on a subconfluent endothelial model

To further investigate a role of APC bound to MP-EPCR in inflammation, we examined the effect of APC in either form on endothelial permeability. In dose-response experiments, APC showed a barrier-protective effect

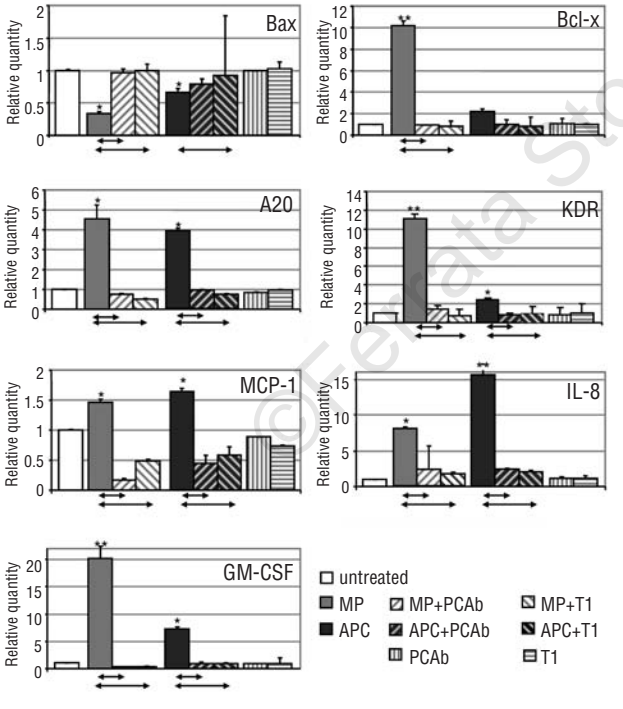

Figure 1. Effect on the differential expression of selected genes by free or microparticle (MP)-bound activated protein C. HUVEC were stimulated in serum-free media with 40 nmol/L APC (free or MP-bound) for 4 h at 37°C. Anti-protein C antibody (PCAb) (10 μg/mL) and PAR1 antagonist peptide T1 (50 μmol/L) were included where indicated. Gene expression, as evaluated by qRT-PCR, of Bax, Bcl-x, A20, KDR, MCP-1, IL-8 and GM-CSF, is shown. The data are expressed as relative quantity to the untreated control set at 1. **p*<0.05 and ***p*<0.005 denote statistical significance between treatments *vs.* untreated. Significance changes from antibody blockade/antagonism are indicated as arrow bars at the base of the bar charts to signify activated protein C or PAR1 specificity.

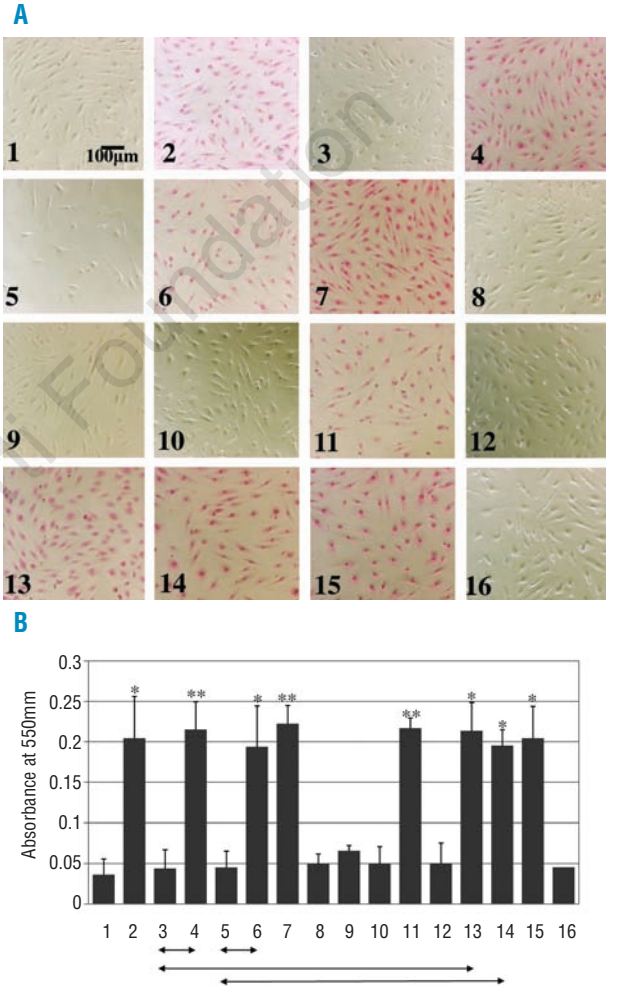

Figure 2. Cytoprotective effect of APC on MP-EPCR in an endothelial cell model of apoptosis. The effect of 40 nmol/L APC (free or microparticle-bound) for 3 h on staurosporine (10 μM)-induced apoptosis of HUVEC was assessed by an ApoPercentage assay. (A) Light microscopy (X100 magnification) shows HUVEC: [1] untreated, and after treatment with [2] staurosporine, [3] APC + staurosporine, [4] APC + anti-protein C antibody (PCAb) + staurosporine, [5] APC-MP + staurosporine, [6] APC-MP + PCAb + staurosporine, [7] PCAb + staurosporine, [8] PCAb, [9] APC + IgG + staurosporine, [10] APC-MP + IgG + staurosporine, [11] IgG + staurosporine, [12] IgG, [13] T1 + APC + staurosporine, [14] T1 + APC-MP + staurosporine, [15] T1 + staurosporine, and [16] T1. (B) The bar chart shows corresponding release of uptaken dye; data are expressed as mean±SD from four separate experiments (**p*<0.05 and ***p*<0.005 significance between treatment effects vs. untreated). Significant changes from results with PCAb or T1 are indicated by error bars at the base of the bar chart.

from 12.5 nmol/L with a significant difference from the untreated control at 20 nmol/L; similar levels of protection were obtained with 50 and 100 nmol/L (*data not shown*). We, therefore, used APC at 20 nmol/L for all permeability experiments. Figure 3A shows that free APC reduced the permeability of a subconfluent monolayer to a level of approximately 70% and that blocking with antibodies to EPCR, APC or PAR1-cleavage site reversed this protective effect. Microparticles with an equal APC concentration of 20 nmol/L reduced the permeability to approximately 60%. This protective effect was APC- and PAR1-dependent. However, blocking EPCR on the cell surface did not abrogate the barrier protection by APC-MP.

The involvement of phosphoinositide 3-kinase and vascular endothelial growth factor receptor-2 in the endothelial barrier-protective effect of activated protein C in its free or microparticulate form

It has been reported that the barrier-protective role of APC occurs through transactivation of sphingosine 1-phosphate receptor ($S1P_1$) involving PI3K-dependent phosphorylation[17,18] although neither of these studies provided direct evidence that inhibiting $S1P_1$ phosphorylation through inhibition of PI3K would result in a leaky monolayer. We tested this by incubating subconfluent endothelial monolayers with the PI3K inhibitor LY294004 (10 μmol/L) for 2 h followed by treatment with APC in free or microparticulate form. The presence of this inhibitor abolished the barrier-protection by both forms of APC (Figure 3B). This finding fully supports the proposed $S1P_1$ phosphorylation through the PI3K pathway as the mechanism of barrier protection by APC.

To further clarify the role of APC in barrier protection, we investigated the possible involvement of the vascular endothelial growth factor (VEGF) pathway. Since VEGF receptor 2 (VEGFR2)/ KDR is phosphorylated and activated by signals from S1P,[19] we treated the monolayer with the specific KDR inhibitor SU5416 prior to APC-MP incubation. Figure 3B shows that KDR inhibition completely abolished the beneficial effect of APC and microparticles on endothelial permeability.

Microparticles/endothelial protein C receptor and activated protein C treatment in patients with sepsis

Since rhAPC is now used in the treatment of severe sepsis, we considered that this might release MP-EPCR into the circulation. Samples were taken from four patients during rhAPC infusion and their own pre-treatment samples served as individual controls. The isolated microparticles were stained for EPCR and protein C along with corresponding isotypic controls for analysis by laser-scanning confocal microscopy (Figure 4). The analyzed microparticles showed that in samples prior to

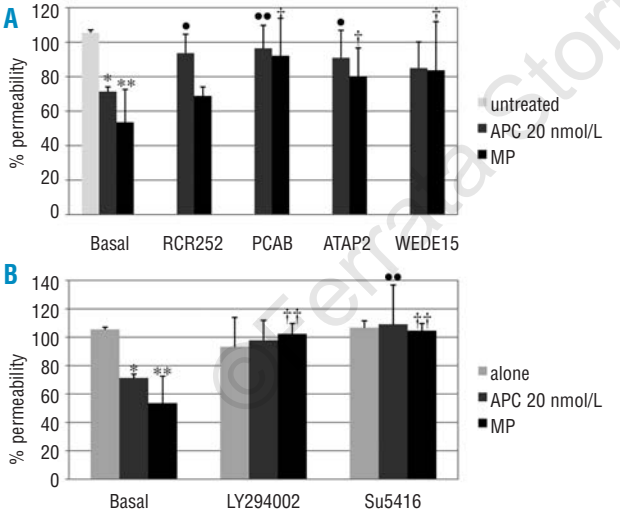

Figure 3. Effect of APC in free or microparticulate form on endothelial permeability. Endothelial cells seeded onto Transwell plates were incubated with 20 nmol/L APC [free or bound to microparticles (MP)] for 3 h and permeability was assessed using Evans-blue-BSA. (A) Dependence on EPCR binding was assessed by inclusion of the blocking antibody RCR252 (20 μg/mL); specificity of APC and PAR1 was investigated by inclusion of protein C antibody (PCAB) (10 μg/mL) and PAR1-specific antibodies (ATAP2 and WEDE15 at 20 μg/mL). Antibody blockade was performed for 30 min before incubation with free or MP-bound APC. (B) To establish the pathways involved in barrier protection, cells were pre-incubated with specific inhibitors to PI3K (LY294002 10 μmol/L) and KDR (SU5416 10 μmol/L) for 2 h prior to treatment with APC or MP. *p<0.05 and **p<0.005 denote statistical significance in relation to untreated baseline; •p<0.05 and ••p<0.005 denote statistical significance between free APC and blockade; †p<0.05 and ††p<0.005 denote statistical significance between MP and blockade.

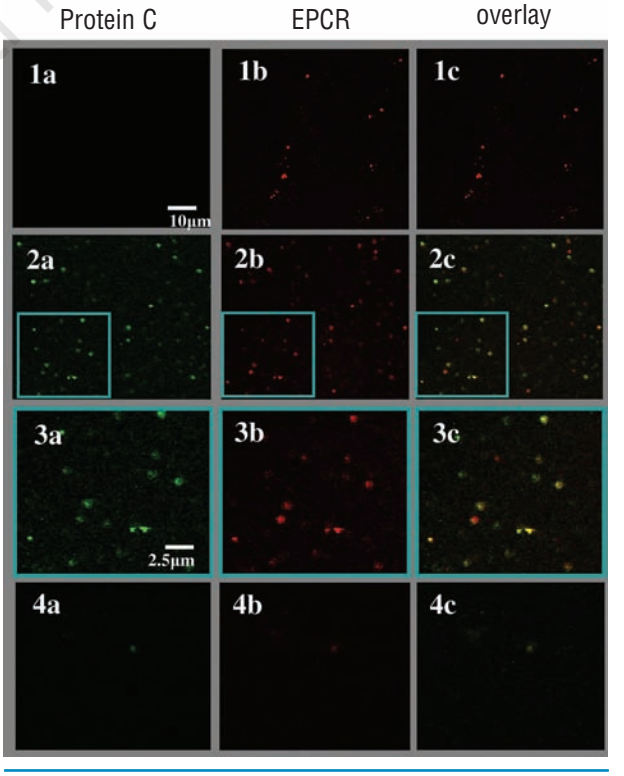

Figure 4. Activated protein C on MP-EPCR is found in human plasma. Microparticles from septic patients were isolated by sequential centrifugation and stained with anti-PC-FITC [column a] and anti-EPCR (RCR49)-phycoerythrin [column b] for laser-scanning confocal microscopy (X630 magnification). Overlaid images [column c] indicate co-localization. Rows 1 and 2 show microparticles from before and during rhAPC treatment, respectively. Row 3 shows further magnification of the boxed fields from row 2. Row 4 shows isotypic controls.

rhAPC-treatment there was staining for EPCR only. It was during rhAPC treatment (Row 2) that dual staining for EPCR and protein/APC occurred, demonstrated by the yellow color resulting from co-localization of EPCR and protein/APC. Although the anti-protein C antibody does not discriminate between zymogen protein C and APC, we assume that microparticle staining during rhAPC treatment indicates APC rather than protein C given that protein C does not induce MP-EPCR release.[5] Furthermore, the absence of protein C staining on patients' microparticles prior to rhAPC treatment suggests that MP-EPCR might not readily bind and retain protein C unlike cell-surface EPCR. According to S2366 amidolytic assays, it was only during rhAPC treatment (days 1 and 4) that APC levels on EPCR-captured microparticles were significantly above the levels of individual pre-treatment controls and unrelated, non-rhAPC-treated patient controls (Figure 5A).

These findings raise questions over whether zymogen protein C can influence the EPCR-APC complex on microparticles given that soluble EPCR and EPCR on endothelial cells can bind both protein C and APC with similar affinity.[20,21] To resolve this question, we measured the displacement of APC by zymogen protein C from *in vitro* APC-induced microparticles, using a binding assay based on the method of Regan *et al.*[21] We found that levels of APC on microparticles in the absence or presence of protein C were similar, indicating that no significant amount of APC was replaced by the zymogen (Figure 5B). Concordantly, levels of APC in the separated supernatant remained unchanged even at the highest protein C concentration. To further verify that APC on MP-EPCR was not easily displaceable, we investigated the effect of increasing concentrations of soluble EPCR since the binding affinity of APC for soluble EPCR is reportedly similar to that for cell surface EPCR.[19] Levels of APC retained on microparticles after incubation remained unchanged, further emphasizing, the strong binding of APC to MP-EPCR (Figure 5C).

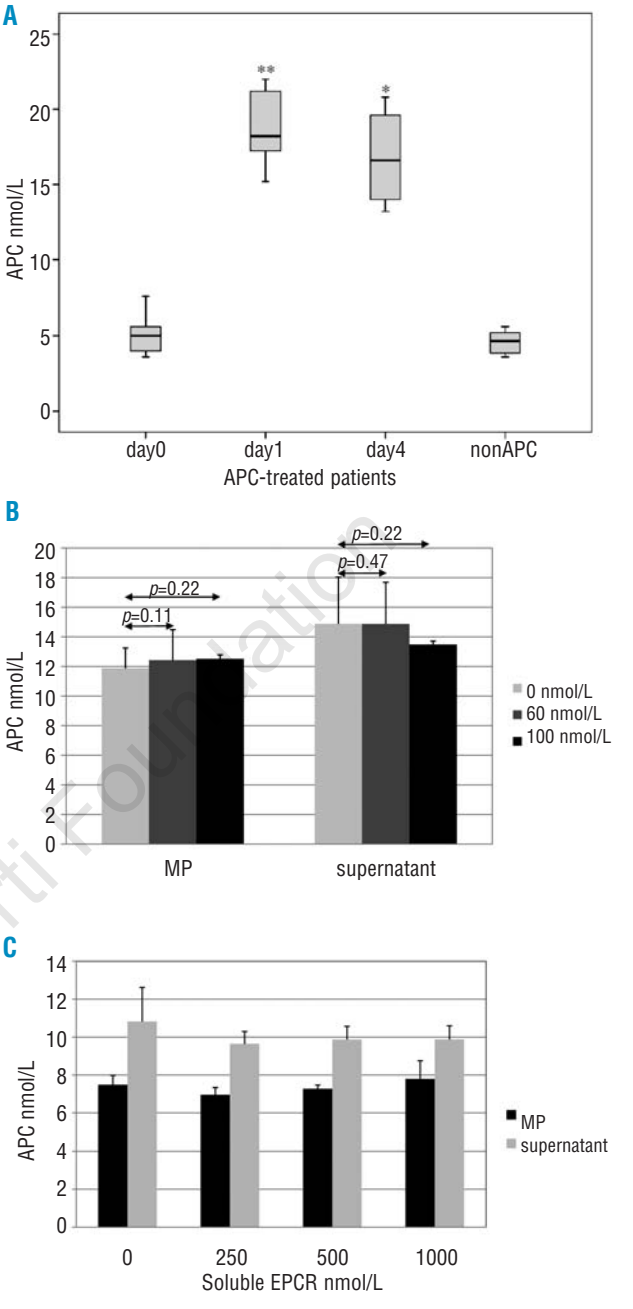

Figure 5. Circulating microparticles (MP) express activated protein C (APC). (A) MP from four septic patients, taken at days 0, 1 and 4 of rhAPC infusion, were compared with MP from four non-APC-treated septic patients. MP were captured with anti-EPCR antibody RCR-2 and APC quantified with chromogenic S2366. Data are expressed as mean APC concentration (nmol/L) on MP. *$p<0.05$ and **$p<0.005$ (paired *t* test) indicate statistical significance between treated to untreated and time-point comparisons. (B) To test whether APC on MP is displaced by zymogen protein C, equal concentrations of APC-MP were incubated with protein C (0, 60, 100 nmol/L) in the presence of hirudin. Chromogenic S2366 was used to determine APC levels on MP from *in vitro* APC stimulation as well as in the respective supernatant separated after 30 min of incubation with protein C (0, 60 and 100 nmol/L). (C) To test whether APC on MP is displaced by soluble EPCR, equal concentrations of APC-MP were incubated with increasing concentrations of soluble EPCR (0, 250, 500, 1000 nmol/L). S2366 was used to measure APC levels on MP and in corresponding supernatants after incubation for 30 min and centrifugation. All data are mean values from four independent experiments. **$p<0.005$ denotes statistical significance between MP and the corresponding supernatant.

Discussion

We recently reported the novel finding that APC induces the release of MP-EPCR from HUVEC and primary monocytes. That these were indeed microparticles was confirmed by electron microscopy and expression of cell membrane markers (*Online Supplementary Figure S1*). We have now found that APC on MP-EPCR influences the expression of endothelial genes involved in regulating apoptosis and inflammation through the activation of PAR1, which is in agreement with the results of others who described an anti-inflammatory role of free APC.[7,10,22] The anti-inflammatory effects of APC have been linked to suppression of the nuclear factor κB (NFκB) pathway. Joyce *et al.* found that treatment of monocytes with lipopolysaccharide and rhAPC down-regulated levels of the p50 subunit of NFκB[7] and Cheng *et al.* also found that APC inhibited NFκB activation in an ischemic mouse endothelium model.[12] In our model of unperturbed growing cells, treatment with APC in free or microparticulate form did not induce NFκB activation as measured by

nuclear levels of RelA (NκB p65) (*Online Supplementary Figure S2*). This suggests that in the absence of inflammatory stimuli, APC may not have a significant influence on NFκB activation and that the main effect of this protein seems to be the preservation of cell viability and endothelial barrier function. Anti-apoptotic effects and permeability barrier protection by APC require EPCR binding and PAR1 activation[17,23,24] and these can be induced through both gene expression and post-translational modification of cellular proteins. The finding that genes related to anti-apoptosis are highly up-regulated in our system could explain the inhibition of staurosporine-induced apoptosis by APC on microparticles observed in the present study. Clearly, more work needs to be carried out to fully understand the complexity of signaling and cell stimulation by APC under different experimental conditions.

Endothelial barrier protection by APC has been reported previously and was found to depend on $S1P_1$ in addition to PAR1. In separate reports, Finigan *et al.*[17] and Feistritzer *et al.*[18] showed that silencing of $S1P_1$ expression abrogates this barrier protection. By using western blotting, Finigan showed that the PI3K inhibitor LY294002 blocked APC-induced $S1P_1$ phosphorylation and that the kinase responsible was Akt. Our study corroborates this involvement of PI3K and shows that the same protection can be obtained by incubation of the endothelial monolayer with either APC in free or MP-EPCR form. Moreover, activation of $S1P_1$ leads to activation of endothelial nitric oxide synthase, which also depends on the activation of KDR.[19] Since KDR is one of the genes that was significantly up-regulated by both free APC and APC on microparticles in our system, it was important to determine whether this up-regulation played any role in our permeability assay. When monolayers were incubated with the specific KDR inhibitor SU5416, we observed an increase rather than a decrease in monolayer permeability. Moreover, pre-incubation with SU5416 abrogated the barrier-protective effect of APC and in the case of cell stimulation with APC on microparticles, permeability increased above pre-treatment levels. These results imply that barrier permeability protection requires KDR.

Stimulation of KDR is known to promote endothelial cell growth and survival, which could also play a role in barrier protection. It is, however, important to note that internal KDR stimulation by autocrine VEGF has very different effects on endothelial cytoprotection as compared to stimulation of KDR on the cell surface by exogenous VEGF. In our previous study using SU5416 and anti-VEGF antibodies to block either KDR or exogenous VEGF, we demonstrated that only internal KDR stimulation prolonged cell viability.[25] Indeed, a recent study demonstrated that abolition of internal KDR stimulation resulted in widespread vascular disruption with intravascular thrombosis,[26] again indicating that endothelial integrity depends on this internal KDR stimulation. The results we present here indicate a cross-talk between PAR1 and KDR via $S1P_1$ for APC signaling to induce barrier protection and cell survival, as illustrated in Figure 6.

The role of *in vitro*-generated microparticles by APC has relevance if translated to a clinical setting, such as

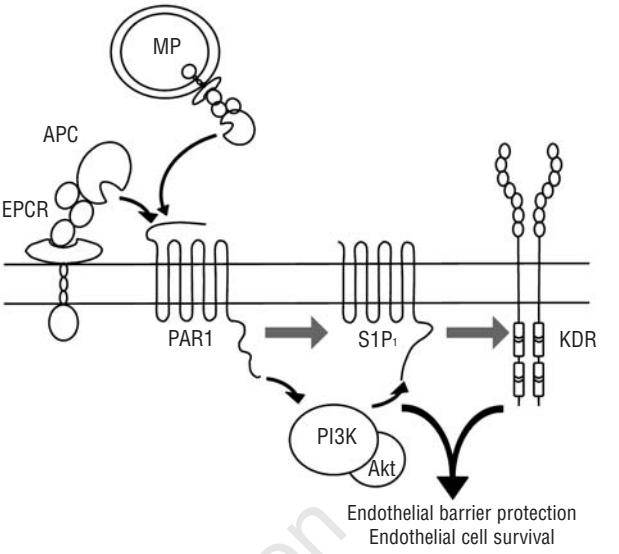

Figure 6. Proposed mechanism of endothelial barrier protection and cell survival by activated protein C (APC) and APC on microparticles (MP). We propose that APC binding to EPCR at the endothelial cell surface and APC on MP-EPCR can cleave and activate PAR1, leading to the transactivation of $S1P_1$ via PI3K and Akt with the subsequent transactivation of KDR. This would mediate cellular interactions and promote cell proliferation with overall barrier protective and cell survival effects.

sepsis, in which endothelial dysfunction is common and protein C levels can be considerably depleted. We now show that microparticles with bound APC are generated upon treatment of septic patients with rhAPC. As the literature suggests that both protein C and APC have similar affinity for EPCR *in vitro*,[20,21] we examined whether APC on MP-EPCR could be displaced by protein C at normal physiological concentrations or by increasing concentrations of soluble EPCR. Our findings indicate that the binding of APC to EPCR embedded within microparticles is stable: in relation to a mechanism underlying this lack of dissociation, Regan *et al.* found that binding of APC to EPCR facilitates further protein-protein and protein-phospholipid interactions.[21] Dai *et al.* found that when the Ca^{2+}-dependent conformation of the protein C-Gla domain was altered (by disrupting the hexapeptide disulfide loop between Cys residues 17 and 22), the change in secondary structure increased, rather than decreased, EPCR binding.[27] Crystal structure studies have also suggested that bound phospholipid is important for maintaining EPCR interactions with protein C-APC.[28] This may be particularly relevant to microparticles in retaining membrane-bound conformations and interactions, especially as the aromatic stack of protein C-APC is also essential for phospholipid binding.[29]

Further understanding of structure-function relationships with emphasis on how APC is retained by EPCR and then held for PAR1 activation will be important in defining the mechanism involved. The PAR1-dependent mechanism of MP-EPCR release suggests that the EPCR-APC-PAR1 signaling pathway may be functional *in vivo*, at least in this specific clinical and pharmacological setting. Whether these rhAPC-induced microparticles have

physiological relevance remains uncertain. One possibility is that these microparticles could circumvent the need for cell-type specific co-localization of EPCR and PAR1 to facilitate APC signaling.[11] Our *in vitro* data of endothelial barrier protection by APC on MP-EPCR, despite blocking of cell surface EPCR, would support this. In particular, cell surfaces that are EPCR-deficient or depleted due to cleavage by pro-inflammatory cytokines could still produce APC-PAR1-dependent cytoprotection.[30,31] These postulated mechanisms are currently the subject of further investigation.

Authorship and Disclosures

MPC designed experiments, performed research, analyzed data and drafted the manuscript; CD and BCM performed research and analyzed data; KF contributed essential reagents; MZ designed experiments and wrote the manuscript; CHT designed research and wrote the manuscript.

The authors reported no potential conflicts of interest.

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