



# Activated protein C binds directly to Tie2: possible beneficial effects on endothelial barrier function

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**Abstract** Activated protein C (APC) is a natural anticoagulant with strong anti-inflammatory, anti-apoptotic, and barrier stabilizing properties. These cytoprotective properties of APC are thought to be exerted through its pathway involving the binding of APC to endothelial protein C receptor and cleavage of protease-activated receptors. In this study, we found that APC enhanced endothelial barrier integrity via a novel pathway, by binding directly to and activating Tie2, a transmembrane endothelial tyrosine kinase receptor. Binding assays demonstrated that APC competed with the only known ligands of Tie2, the angiopoietins (Angs). APC bound directly to Tie2 (K<sub>d</sub> ~ 3 nM), with markedly stronger binding affinity than Ang2. After binding, APC rapidly activated Tie2 to enhance endothelial barrier function as shown by Evan's blue dye transfer across confluent cell monolayers and *in vivo* studies. Blocking Tie2 restricted endothelial barrier integrity. This study highlights a novel mechanism by which APC binds directly to Tie2 to enhance endothelial barrier integrity, which helps to explain APC's protective effects in vascular leakage-related pathologies.

**Keywords** Endothelial barrier integrity · Protein C · Sepsis · RCR252 · Protease-activated receptor ·

SDS-PAGE · Biacore · Immunoprecipitation · Solid-phase binding assay

## Introduction

Activated protein C (APC) is a vitamin K-dependent serine protease that plays a key role in the regulation of blood coagulation but also exerts a broad range of cytoprotective actions on endothelium including suppression of inflammation and stabilization of endothelial barrier function [1–3]. APC-mediated cytoprotective signaling requires endothelial protein C receptor (EPCR) and cleavage of protease-activated receptor (PAR)1 at Arg46 [4], which may occur in caveolin-1-enriched lipid rafts [5]. In addition, other receptors, such as sphingosine-1-phosphate receptor 1 (S1P1), epidermal growth factor receptor (EGFR), PAR3, and Tie2, may independently or co-operatively contribute to APC-mediated protective effects on endothelium [2, 6–8]. For example, APC protects against endothelial barrier disruption by cross-activating S1P1 [2] or by stimulating Ang1 whilst inhibiting Ang2 to activate Tie2 [6]—both these mechanisms require signaling through EPCR and PAR1.

Tie2 is a transmembrane endothelial tyrosine kinase receptor that not only regulates vessel maturation and remodeling angiogenesis, but also controls endothelial inflammation and permeability [9, 10]. Tie2-deficient mice are not viable and have severe vascular abnormalities [11, 12]. The angiopoietin (Ang)s are the only known ligands for Tie2 with Ang1 and Ang2 being the most abundant and best studied. Ang1 acts as an agonist and Ang2 as an antagonist by blocking the stabilizing action of Ang1. Ang1 ligation to Tie2 results in tyrosine phosphorylation, which subsequently promotes vascular endothelial

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cell survival and migration, maintains vessel integrity, inhibits vascular leakage, suppresses inflammatory gene expression, and prevents recruitment and transmigration of leukocytes [13–15]. Ang1/Tie2 signaling benefits pathologies of vascular activation, such as sepsis, stroke, diabetic retinopathy, rheumatoid arthritis, and asthma [16]. Ang2 competes with the binding of Ang1–Tie2 and sensitizes the endothelium to inflammatory cytokines, resulting in injury and vascular leakage which directly contributes to the adverse outcomes in sepsis [17, 18]. The balance between Ang1 and Ang2 regulates baseline endothelial barrier function and its response to injury and is, therefore, considered a gatekeeper of endothelial activation [19].

In this study, we revealed that APC can act as novel ligand for Tie2 without requiring EPCR or PAR-1, to initiate downstream signaling pathways and maintain vascular integrity.

## Experimental procedures

### In vivo permeability assay

C57BL/6 male mice were obtained from the Kearns facility at Kolling Institute, The University of Sydney. Six-to-eight-week-old C57BL/6 male mice were used for this study. To block Tie2, Tie2 kinase inhibitor (Tie2-I, 25 mg/kg) (Calbiochem) was injected intraperitoneally (i.p.). After 30 min, mice were treated with APC (2 mg/kg) [20] (Eli Lilly) by tail intravenous (i.v) injection. Hyper-permeability was induced by intraperitoneal injection of 1 ml lipopolysaccharide (LPS, derived 1 from *Escherichia coli* serotype 055:B5, 5 or 10 mg/kg), 5 min after APC administration [21–24]. Evan's blue (EB) dye (Sigma-Aldrich) was dissolved at a concentration of 30 mg/ml in phosphate-buffered saline (PBS). To this solution, bovine serum albumin (BSA) was added to a final concentration of 40 mg/ml (Sigma-Aldrich), mixed well with a magnetic stirrer, and then sterile filtered through a 0.22  $\mu$ m syringe filter to prepare evans blue albumin (EBA). One hundred  $\mu$ l EBA was injected i.v. into mice and allowed to circulate for 30 min. The dye was collected, measured, and normalized as previously described [25, 26]. Briefly, while under anesthesia, the mice were perfused with PBS through the portal vein. Immediately after perfusion, mice were terminated and organs (lungs and kidneys) were removed. The collected organs were dried before homogenising in formamide at 60 °C for 16 h to extract the dye and centrifuged at 12,000 $\times$ g with 3 ml of PBS. Absorbance of supernatant was measured at 620 and 740 nm using a spectrophotometer. The organs of mice that were not injected with EBA were also harvested and similarly

treated to calculate the tissue-specific turbidity correction factors.

Use of animals was approved by the Royal North Shore Hospital Animal Care and Ethics Committee in accordance with the National Health and Medical Research Council of Australia.

### Cell isolation and culture

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously described [6]. In some experiments, cells were blocked for 1 h with 5  $\mu$ g/ml Tie2 blocking antibody (aTie2) or 2  $\mu$ g/ml soluble (s)Tie2 (R&D Systems) that blocked the activation of Tie2, RCR252 to block EPCR receptor, or PAR1 inhibitor (PAR-1, SCH79797, Abcam) blocking PAR1 receptor. Cells were then treated with tumor necrosis factor (TNF)- $\alpha$  (100 ng/ml), LPS (10  $\mu$ g/ml), vascular endothelial growth factor 165 (VEGF) (200 ng/ml) (R&D Systems), and/or APC. Passage 1–4 cells were used in experiments.

### Permeability assay

Endothelial permeability was assessed using transwell Evan's blue albumin (EBA) as previously described [27] with modifications [6]. HUVEC were cultured on gelatin-coated membrane supports (0.45  $\mu$ m pore, 12 mm diameter) until confluent. Medium was changed to Biorich plus 2% fetal calf serum (FCS) before treatment. Cells were treated with or without with a Tie2 or non-blocking isotype for 1 h and then treated with or without TNF- $\alpha$  (100 ng/ml), LPS (10  $\mu$ g/ml), VEGF (200 ng/ml), or Dimethyl sulfoxide (DMSO) for 30 min before the addition or not (no APC control) of APC (1  $\mu$ g/ml) for 24 h. After treatment, medium in upper luminal chamber was replaced with 0.5 ml of 2% Evans blue in Biorich medium with 4% BSA and lower abluminal chamber with 1.5 ml of Biorich medium. The volume of media added to both chambers allowed for the levels to be equal, thus avoiding formation of hydrostatic pressure which could result in movement of medium in one direction. Medium (75  $\mu$ l) was then collected from the lower chamber at selected timepoints (up to 80 min) and dye concentration analyzed spectrophotometrically at 630 nm.

### Immunoprecipitation and western blot

Immunoprecipitation was performed on HUVEC lysates using Protein A/G PLUS-Agarose (Santa Cruz), as described previously [28] with minor changes. Proteins were analyzed by immunoblotting [29] with rabbit anti-human Tie2 (aTie2, 1:1000, Santa Cruz), rabbit anti-human

Phosphorylated (P)-Tie2 (1:500, R&D Systems), and mouse anti-human PC antibody (1:1000, Abcam).

### Enzyme-linked immunosorbent assay (ELISA)

Commercial Duo Set ELISA kits for total (T)-Tie2 and P-Tie2 (R&D Systems) were used according to manufacturer's instructions.

### Biacore binding assay

The binding interaction between APC (1  $\mu\text{g/ml}$ ) and sTie2-Fc (25  $\mu\text{g/ml}$ ) was observed by surface plasmon resonance using a Biacore 3000 sensor (GE Healthcare). The surface of carboxymethylated dextran matrix of a CM5 sensor chip (Biacore AB) was prepared using Amine Coupling Kit (Biacore AB). sTie2-Fc (25  $\mu\text{g/ml}$ ) was immobilized on prepared sensor chip. APC was then injected at a flow rate of 10  $\mu\text{l/min}$  over the sTie2-Fc for 6 min, and binding signals were recorded in response unit (RU) [30, 31].

### SDS-PAGE-based binding assay

The binding of APC to the soluble extracellular domain of Tie2-Fc (R&D Systems) was analyzed using SDS-PAGE-based binding assay. APC (20 ng) was mixed with sTie2-Fc (100 ng) and incubated in 500  $\mu\text{l}$  NaCl-Tris buffer (50 mM Tris, 100 mM NaCl, pH 7.4) containing 0.02% TritonX-100 at 4  $^{\circ}\text{C}$ . After 2 h, 20  $\mu\text{l}$  of protein A/G Plus-Agarose was added and incubated for 1 h at 4  $^{\circ}\text{C}$ . The protein-A conjugated samples were washed with NaCl-Tris buffer containing 0.02% TritonX-100. Samples were eluted with non-reducing sample buffer, separated by 6% PAGE with 0.5% SDS, and analyzed with mouse anti-human protein C antibody.

### Solid-phase binding assay

sTie2-Fc (2  $\mu\text{g/ml}$  in PBS) was coated onto a 96-well plate overnight at 4  $^{\circ}\text{C}$  followed by six washings with PBST (PBS + 0.05% Tween 20). The coated wells were blocked with blocking buffer (Thermo Scientific) for 4 h at room temperature, and then washed with PBST. Ang1, Ang2 (R&D Systems), or APC in 100  $\mu\text{l}$  of PBS was added to coated wells and incubated overnight at 4  $^{\circ}\text{C}$  [32, 33]. Ang1, Ang2, or APC bound to sTie2 was detected by incubating with their respective primary antibodies. To determine significance of calcium ion ( $\text{Ca}^{2+}$ ) in APC-Tie2 binding, we performed binding assay experiments with HEPES buffer (20 mM, pH 7.5, 140 mM NaCl) with 1 mM  $\text{Ca}^{2+}$ , 1 mM  $\text{Mg}^{2+}$ , and 1 mM  $\text{Mn}^{2+}$ . All washing steps included 1 mM  $\text{Mg}^{2+}$  and 1 mM  $\text{Mn}^{2+}$  in HEPES buffer. For time-dependent binding interaction analysis,

APC was incubated with sTie2 for 2 min, 1 h, and O/N in the presence and absence of  $\text{Ca}^{2+}$ . The absorbance was measured at 450 nm, and the absorbance at 570 nm was used as correction factor. For the  $\text{Ca}^{2+}$  experiments, the substrate enzyme was incubated for 10 min rather than the 5 min used for other experiments. For the competition binding assays, the binding of APC to sTie2 immobilized on the 96-well plate was performed in the presence or absence of Ang1 or Ang2 [34, 35].

### Statistics

Statistical analysis was performed with two-tailed Student's *t* test between two groups. ANOVA was used to compare means among three or more independent groups followed by Newman-Keuls post hoc test. All results are presented as mean  $\pm$  SEM. A *P* value of less than 0.05 was considered statistically significant.

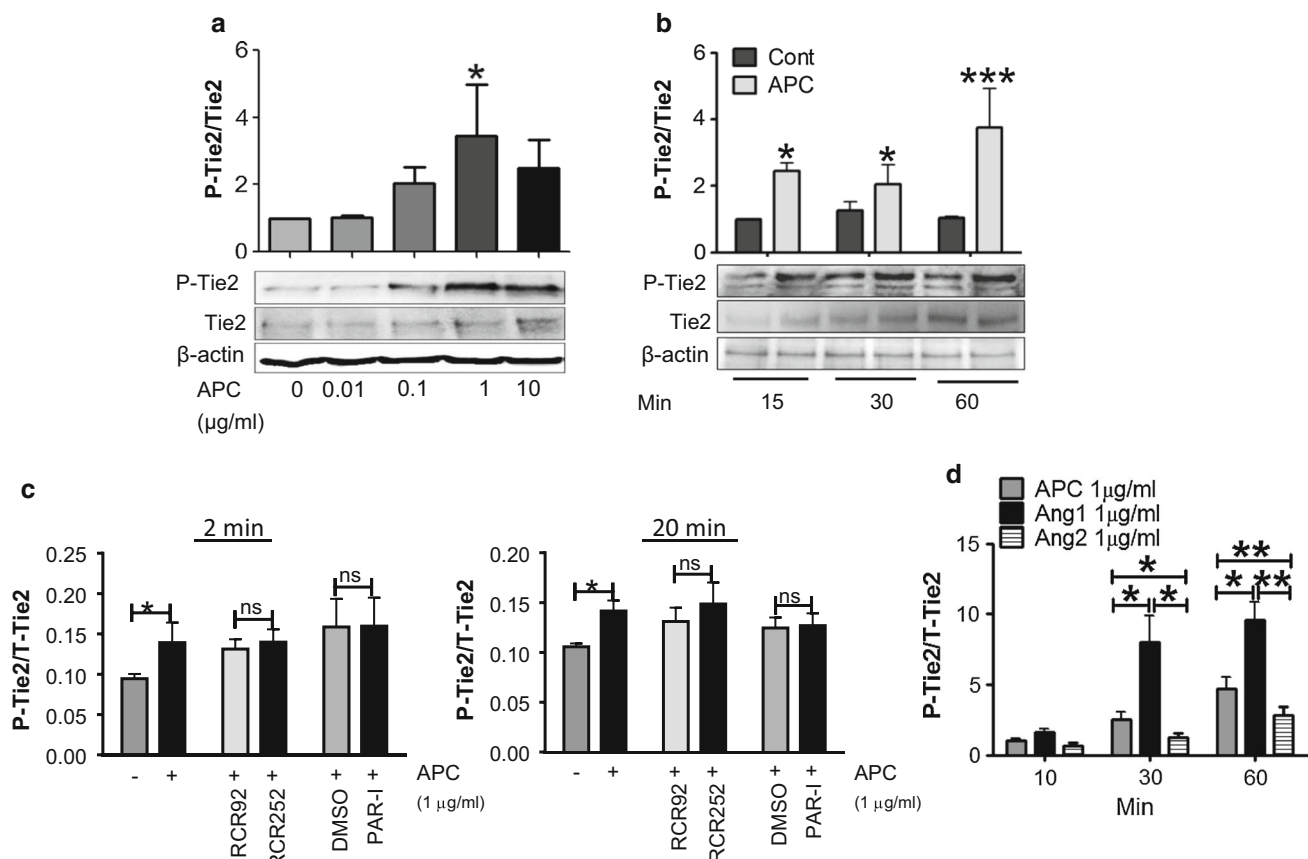
## Results

### APC rapidly activates the Tie2 receptor

When HUVEC were treated with APC (0.01–10  $\mu\text{g/ml}$ ) for 1 h, P-Tie2 was dose-dependently increased, peaking at 1  $\mu\text{g/ml}$  APC (Fig. 1a). APC also increased activation of Tie2 in a time-dependent manner, from  $\sim$ twofold at 15 min to  $\sim$ 4.5-fold at 60 min (Fig. 1b). To determine if this early activation of Tie2 by APC requires EPCR or PAR1, cells were treated with their respective blocking antibodies, RCR252 or PAR1-I for 1 h prior to APC treatment and Tie2 activation was assessed at 2 and 20 min. Confirmation that RCR252 works under these conditions is shown in Supplementary Fig. 1. ELISA results showed that blocking EPCR or PAR1 receptors had no significant effect on the activation of Tie2 by APC either at 2 or 20 min (Fig. 1c), indicating that neither of these receptors were involved in activation of Tie2. We then compared APC-induced Tie2 phosphorylation to that induced by the native ligands of Tie2, Ang1, or Ang2. When used at 1  $\mu\text{g/ml}$ , Ang1 was the most potent activator with APC and Ang2 showing comparable levels of Tie2 activation (Fig. 1d).

### Does APC bind to Tie2 receptor?

The above results raised the possibility of direct interaction between APC and Tie2. To investigate, we used several strategies. First, Biacore assay was used to detect the specificity of binding between APC and Tie2 in real time. APC showed specific binding to sTie2 compared to the control (blank chip surface). APC associated rapidly with



**Fig. 1** APC rapidly activates Tie2 in HUVEC. HUVEC were incubated with Biorich medium +2% FCS for 24 h before treatment. **a** Western blot for phosphorylated (P)-Tie2 and total(T)-Tie2 treated for 1 h with APC (1  $\mu$ g/ml). *Graph* shows mean  $\pm$  SD from  $n = 3$  independent experiments. **b** HUVEC were treated with APC (1  $\mu$ g/ml) for 15, 30, or 60 min, and cell lysates were assessed by western blotting. *Graph* shows mean  $\pm$  SD from  $n = 4$  independent experiments. **c** HUVEC were pre-incubated in Biorich medium +2% FCS for 1 h with blocking antibodies RCR252 (10  $\mu$ g/ml) and PAR-1-

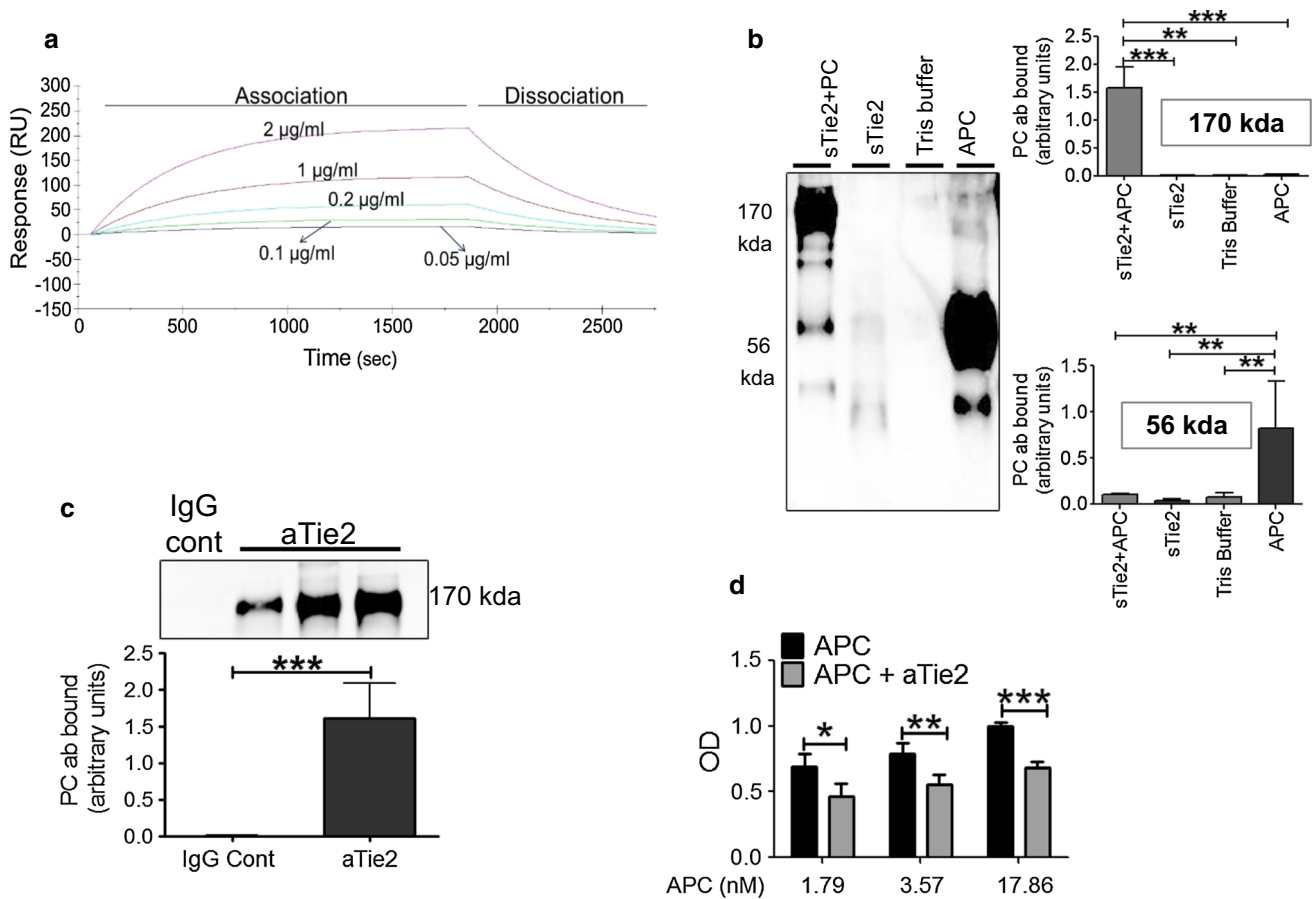
(SCH79797) (1 $\mu$ M) and their respective controls (non-blocking antibody RCR92 and DMSO) then treated with (+) or without (-) APC for 2 (*left panel*) or 20 (*right panel*) min. Cell lysates were analyzed for P-Tie2 and T-Tie2 by ELISA. *Graphs* show mean  $\pm$  SD from three separate experiments. **d** HUVEC were treated with APC, Ang1, or Ang2 for different times, and cell lysates were assessed for P/T-Tie2 by ELISA. *Graph* shows mean  $\pm$  SD from  $n = 6$  independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . *ns* not significant

sTie2 captured on sensor chip and dissociated slowly (Fig. 2a), confirming the specificity of binding between APC and Tie2. Second, co-immunoprecipitation was employed to examine whether APC can bind or associate with Tie2. Recombinant APC (~56 kDa) was co-incubated with sTie2 (~130 kDa) for 1 h and subjected to immunoblotting with mouse anti-human protein C antibody. Results showed a band at ~170 kDa (Fig. 2b), indicating that the molecules were bound together. To investigate whether APC binds to Tie2 in endothelial cells, APC was incubated with HUVEC and whole lysates were subjected to co-immunoprecipitation with rabbit anti-human Tie2 antibody, and then subjected to western blot detection with mouse anti-human protein C antibody. Again, a strong band at ~170 kDa was distinct, providing evidence that APC is associated with Tie2 in cells (Fig. 2c). Third, a solid-phase binding assay was

performed to measure APC binding to Tie2 in the presence or absence of aTie2. On blocking the Tie2 receptor, binding between APC and Tie2 was inhibited significantly irrespective of APC concentration (0.1, 0.2, and 1  $\mu$ g/ml) (Fig. 2d).

### Binding affinity between APC and Tie2

A solid-phase saturation binding assay was performed between APC and Tie2 to determine half maximal binding concentration, referred to as BC50, and compared to Ang1 and Ang2 under similar binding conditions. The BC50 s for APC, Ang1, and Ang2 binding were ~3.14, 2.16, and 36.52 nM, respectively (Fig. 3a–c). Thus, compared to Ang1, ~1.5-fold higher concentration of APC was necessary to induce half maximal response in Tie2; however,



**Fig. 2** APC–Tie2 interaction. **a** Various doses of APC were passed over a Biacore sensor chip covalently coupled with Tie2-Fc (25 µg/ml). Binding is shown as Resonance Units (RU). **b** sTie2 (100 ng) and APC (20 ng) were co-incubated in Tris buffer and analyzed by immunoblotting (APC + sTie2) for protein C with mouse anti-human protein C antibody. sTie2 (100 ng) alone (in Tris buffer), Tris buffer alone, and APC (20 ng) alone (in Tris buffer) were treated similarly and used as controls. Graphs show PC antibody (ab) binding (y-axis) to APC + sTie2, sTie2, control Tris buffer, and APC for two molecular weights, 170 and 56 kDa. Y-axis is in arbitrary units

(mean ± SD), for three separate experiments. **c** APC was incubated with HUVEC for 1 h, and then, whole lysates were subjected to co-immunoprecipitation with rabbit anti-human Tie2 antibody (anti-tie2) or IgG control, followed by western blot detection with mouse anti-human protein C antibody (PC). Graph shows arbitrary units (mean ± SD), for three separate experiments. **d** Tie2 receptor was blocked with a blocking antibody (aTie2, 8 µg/ml) for 1.5 h before incubating with different concentrations of APC. y-axis shows binding affinity as OD (450–570) nm. \**P* < 0.05, \*\**P* < 0.01, \*\*\**P* < 0.001, *n* = 3

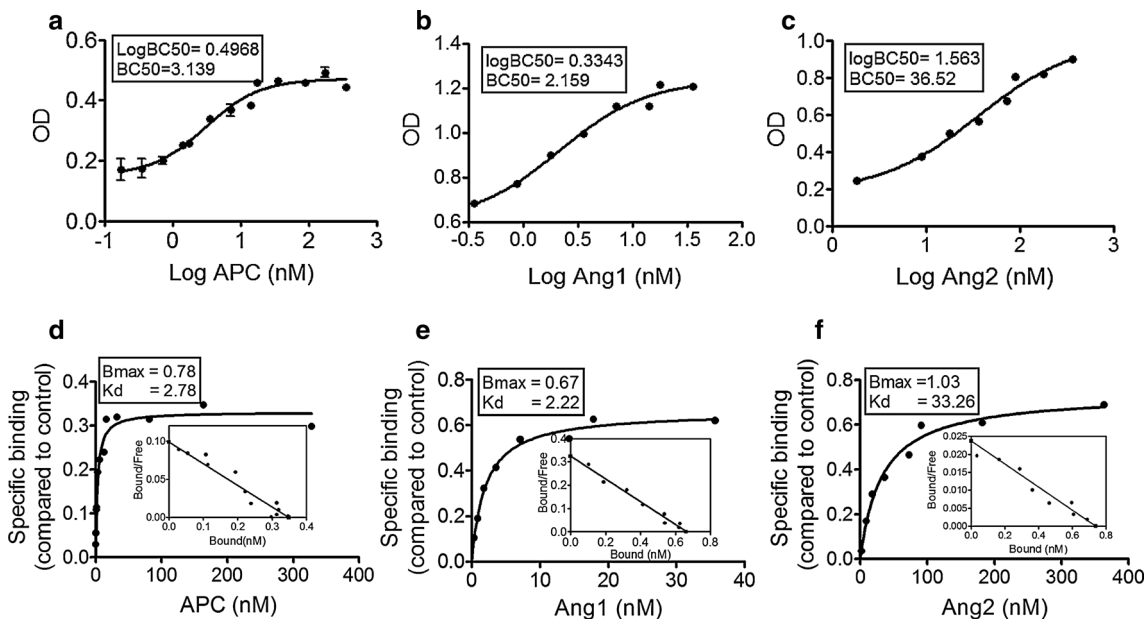
compared to Ang2, ~11.6-fold lower level of APC was required.

Next, we determined binding affinity between Ang1, Ang2 or APC, and Tie2 by scatchard and saturation analysis, which produces values for maximum binding capacity (Bmax) and binding affinity (Kd) between interacting proteins, where lower Kd values signify stronger interaction. Figure 3d shows a specific binding analysis between APC and Tie2, where Bmax was 0.78 and Kd was 2.78 nM (Fig. 3d). Ang1–Tie2 interaction showed Bmax of 0.67 and Kd of 2.22 nM (Fig. 3e) and for Ang2–Tie2 Bmax was 1.03 and Kd was 33.26 nM (Fig. 3f). These results confirm the strong binding affinity between APC and Tie2, which is ~12 times higher than Ang2–Tie2. These results also confirm the previous findings that Ang1 binding to Tie2 is stronger than Ang2 [34].

To determine if additional Ca<sup>2+</sup> is required for the interaction between APC and Tie2, binding assay was performed in the presence or absence of Ca<sup>2+</sup>. BC50 was approximately equal, i.e., ~3.54 and ~3.53 nM in the presence and absence of Ca<sup>2+</sup> (Fig. 4a). The Kd value between APC and Tie2 in the presence of Ca<sup>2+</sup> was 2.47 nM (Fig. 4b) and in its absence 2.78 nM (Fig. 3a). The protein–protein interaction remained unaffected in the presence of Ca<sup>2+</sup> up to the 1 h timepoint (Fig. 4c). These results demonstrate that APC–Tie2 binding does not require additional Ca<sup>2+</sup>.

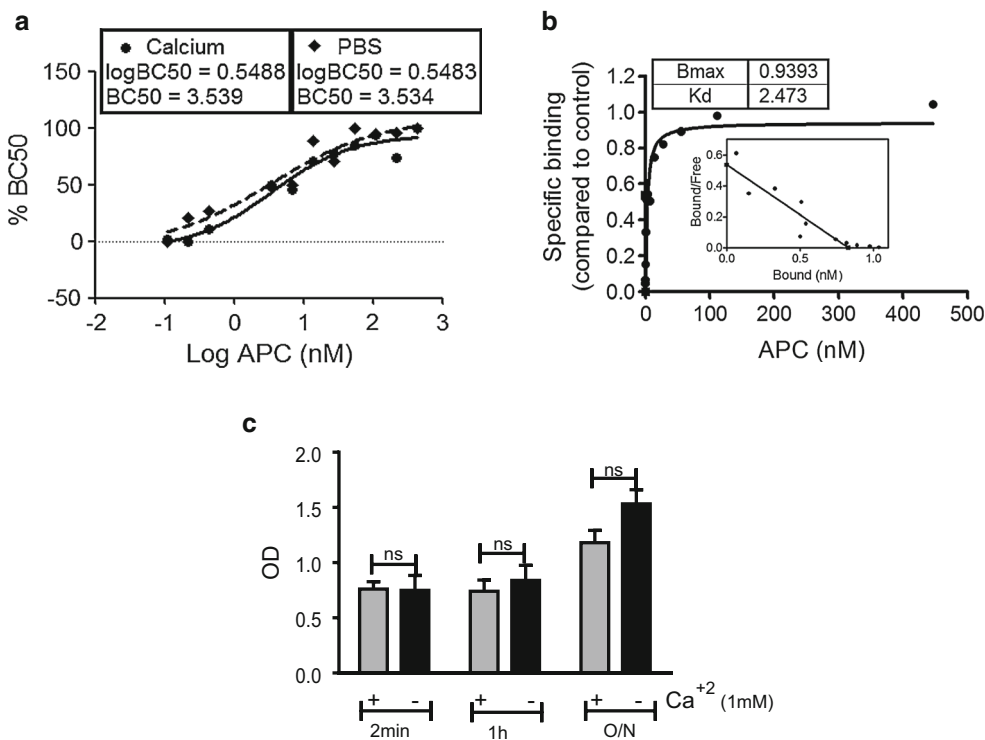
**Competitive binding between Tie2 ligands**

To determine how the presence of either Ang1 or Ang2 affects the binding interaction between APC and Tie2, we



**Fig. 3** Binding affinity between APC and Tie2. The graphs show BC50 for binding. **a** APC ( $n = 5$ ); **b** Ang1 ( $n = 3$ ); **c** Ang2 ( $n = 3$ ), binding to Tie2, using different concentrations of ligands under similar conditions, as described in “Experimental procedures”. OD

was measured by spectrophotometry at 450 and 570 nm. Graphs show mean  $\pm$  SE. The graphs show saturation binding curve and scatchard analysis (*inset*) for: **d** APC and Tie2 ( $n = 5$ ); **e** Ang1 and Tie2 ( $n = 3$ ); and **f** Ang2 and Tie2 ( $n = 3$ )



**Fig. 4** Binding affinity between APC and Tie2 in the presence of calcium ion ( $Ca^{2+}$ ). **a** Graph shows BC50 for APC binding to Tie2, using different concentrations of ligands in the absence (*dashed line*) or presence (*full line*) of  $Ca^{2+}$ , as described in “Experimental procedures”. OD was measured by spectrophotometry at 450 and 570 nm and converted to % BC50. Graph represents mean  $\pm$  SE from three separate experiments. **b** Graphs shows saturation binding curve and scatchard analysis.  $n = 3$ . **c** Graph represents binding between

APC and soluble Tie2 in a time-dependent manner in the presence or absence of  $Ca^{2+}$ . sTie2 and APC were incubated for different time periods [2 min (min), 1 h, and overnight (O/N)] and analyzed by an ELISA-based binding assay, as described in “Experimental procedures”. Graph shows OD (mean  $\pm$  SD), for three separate experiments, with the no APC controls subtracted from all values. ns non-significant

performed competitive binding experiments. Approximate BC50 concentrations of each protein were used as determined previously in Fig. 3a–c, i.e., 1.75 nM Ang1, 35.09 nM Ang2, and 3.57 nM APC. The binding between APC and Tie2 was inhibited by ~66% in the presence of Ang1 and ~29% in the presence of Ang2 (Fig. 5a, b). When the concentration of APC was increased fivefold to 17.86 nM, inhibition of APC–Tie2 binding by Ang1 and Ang2 was reduced to ~58 and ~31%, respectively (Fig. 5c, d).

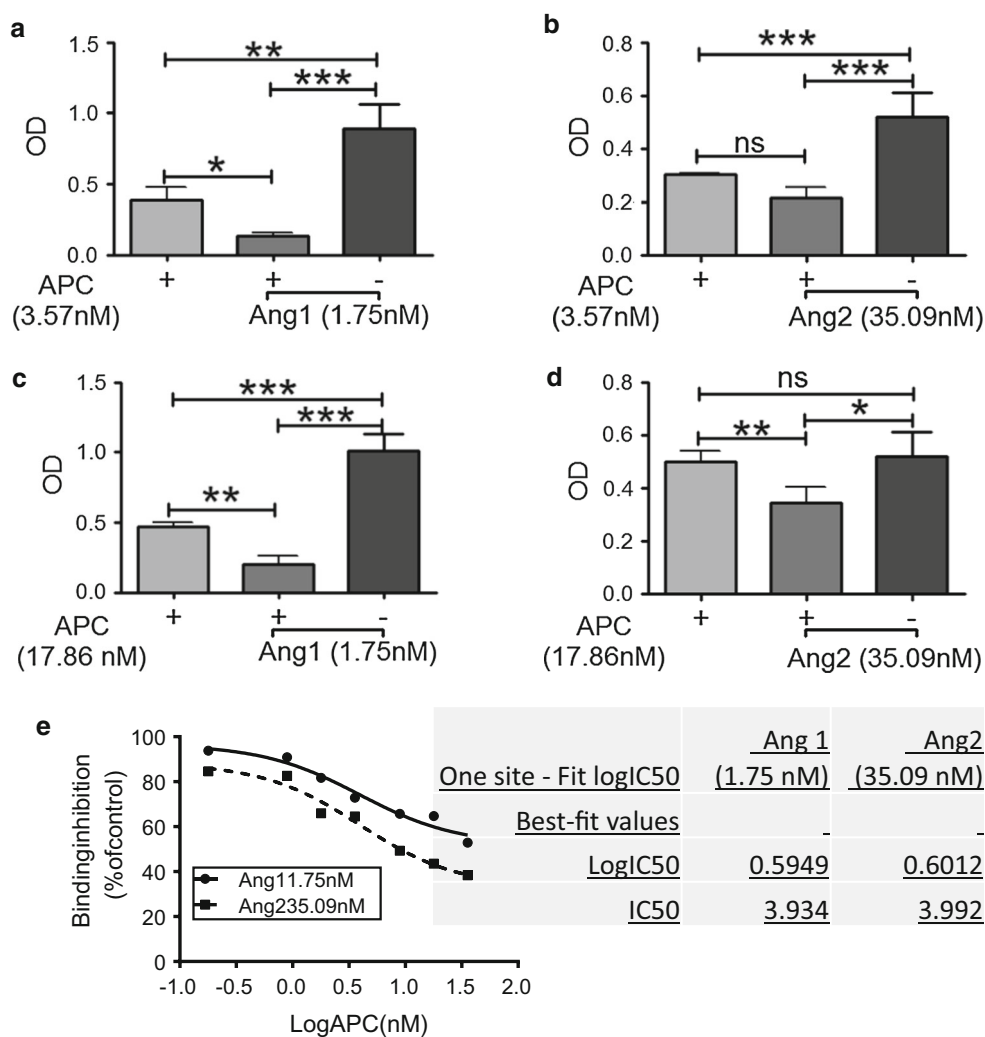
Competitive binding was performed using different concentrations of APC (0.179–35.71 nM) and either 1.75 nM Ang1 or 35.09 nM Ang2. The binding of either Ang1 or Ang2–Tie2 was inhibited when co-incubated with APC (Fig. 5e). Half maximal inhibitory concentration (IC50) of APC was calculated by one site-fit logIC50 analysis (GraphPad Prism) to be 3.9 nM for inhibition of Ang1, or 4.0 nM for Ang2, binding to Tie2 (Fig. 5e). At a ten-fold higher concentration, APC inhibited Ang2

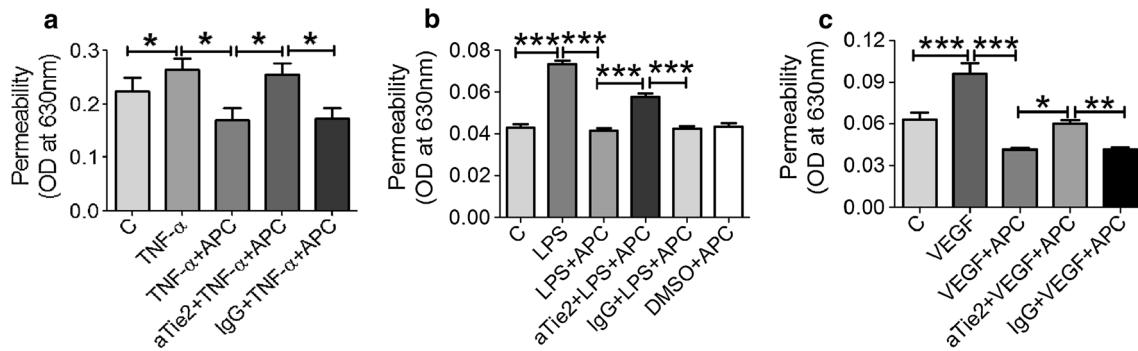
by ~90% and Ang1 by ~75%. Tie2 binding to Ang2 was inhibited comparatively more than Ang1, at any given concentration of APC.

### APC rapidly suppresses endothelial permeability via Tie2

To determine whether APC induces permeability changes in endothelium directly via binding Tie2, hyper-permeability was induced in HUVEC with TNF- $\alpha$  (100 ng/ml) (Fig. 6a), LPS (10  $\mu$ g/ml) (Fig. 6b), or vascular endothelial growth factor (VEGF) (200 ng/ml) (Fig. 6c) in the presence or absence of APC, with or without Tie2 blocking antibody and permeability was measured after 1 h using EBA dye transfer. As expected, APC significantly reduced permeability compared to TNF- $\alpha$  (1.6-fold), LPS (1.8-fold), or VEGF (2.3-fold) treatment alone. Pre-treatment of aTie2 resulted in a significant reversal of APC-induced endothelial integrity (Fig. 6a–c).

**Fig. 5** Competitive binding. **a, b** Competitive binding to Tie2 was assessed, as described in “Experimental procedures”, using BC50 concentrations of APC (3.57 nM), Ang1 (1.75 nM), and/or Ang2 (35.09 nM). **c, d** Similar to a and b, except APC was used in excess at a dose of 17.86 nM. **a–d** Graphs show binding affinity as OD (mean  $\pm$  SD) from three separate experiments. The no APC control was subtracted from each data point. **e** Binding assays were conducted with increasing concentrations of APC and BC50 concentrations for Ang1 (full line) and Ang 2 (dashed line). Results using the stated concentration of competitor are expressed as a percentage inhibition of binding to Tie2 in the absence of competitor. Each curve represents results from three separate experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ,  $n = 3$ . IC50 data on right was obtained from competitive binding assays and derived from three independent experiments, conducted in duplicate





**Fig. 6** APC signals via Tie2 to reduce endothelial permeability. **a–c** HUVEC were incubated in the presence or absence (control) of 1  $\mu\text{g/ml}$  APC for 1 h. Cells were pre-incubated for 1 h with aTie2 (5  $\mu\text{g/ml}$ ) and/or 30 min pre-treatment with **a** TNF- $\alpha$  (100 ng/ml), **b** LPS (10  $\mu\text{g/ml}$ ), **c** VEGF (200 ng/ml), or control non-blocking isotypes

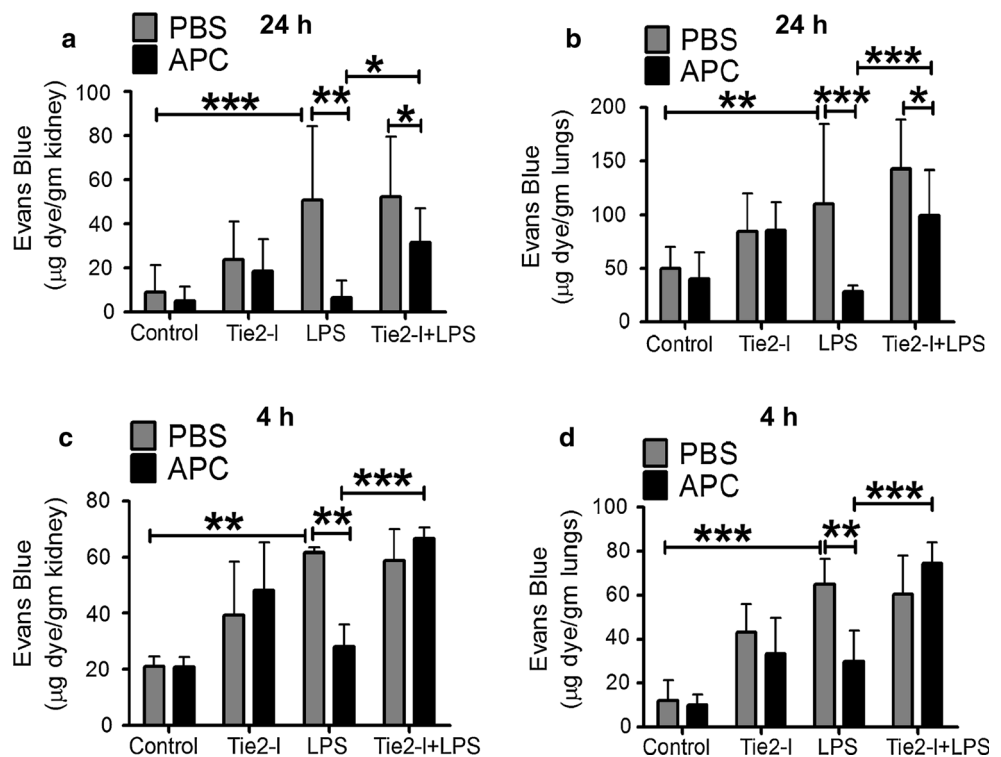
(IgG) or DMSO before APC treatment for 1 h. Permeability was measured using a modified Boyden chamber and results displayed as absorbance at 630 nm. *Graphs* show mean  $\pm$  SD from three replicates for **a** and eight replicates for **b** and **c**. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$

### APC signals through Tie2 to reverse vascular leakage in vivo

To determine whether Tie2 is required for APC's inhibition of vascular leakage, mice were treated with Tie2 inhibitor(I) for 30 min prior to APC treatment in the presence or absence of LPS [36] and assessed at two timepoints. After 24 h, LPS (5 mg/kg) significantly increased Evan's blue albumin (EBA) dye leakage by  $\sim 5.6$ -fold in the kidneys and  $\sim$ twofold in the lungs, compared to control (PBS-treated). Pre-treatment with APC almost completely prevented this effect

(Fig. 7a, b). Tie2-I alone did not affect EBA leakage into the kidneys or lungs; however, the protective effect of APC was significantly reversed by Tie2-I in all experiments (Fig. 7a, b), indicating APC signals through Tie2. To rule out possible effects of APC on promoting Ang1, which requires at least 16 h [6], we performed experiments over the shorter duration of 4 h. Inhibiting Tie2 with or without LPS (10 mg/kg) treatment resulted in increased vascular permeability and also prevented APC from exerting its protective effect (Fig. 7c, d), confirming the direct effects of Tie2 on APC's actions.

**Fig. 7** APC prevents LPS-induced renal and pulmonary vascular injury in mice. Mice were injected with APC (2 mg/kg) before administering LPS (5 mg/kg for 24 h or 10 mg/kg for 4 h) or the same volume of PBS. The *graphs* show results for EBA amount extracted from kidneys and lungs of mice, treated with APC or PBS for: **a**, **b** 24 h and **c**, **d** 4 h. Mice were injected with Tie2 inhibitor (Tie2-I) (25 mg/kg) before treatment with APC (2 mg/kg) or equal volume of PBS for 24 h, plus or minus LPS (5 mg/kg for 24 h or 10 mg/kg for 4 h). Each group consisted of six mice. Statistical analysis using ANOVA. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$





## Discussion

This report shows that APC is a direct agonist for Tie2; it binds to and initiates rapid activation of the receptor which in turn manifests in barrier protection of endothelial cells. These actions of APC mimic those of Ang1, albeit less efficiently, and are supported by the functional similarities between Ang1 and APC. Mice that are completely deficient in protein C exhibit neonatal lethality in a similar fashion to Ang1 or Tie2 knockout mice [11, 37, 38]. Mice genetically predisposed to severe protein C deficiency exhibit exacerbated acute inflammation and reconstitution of low-protein C mice with recombinant APC reduces inflammation and extends survival after LPS challenge [38]. Recombinant Ang1 or genetically overexpressing Ang1 also protects against inflammation and/or vascular leakage and improves survival in endotoxin-induced shock or hyperoxia-induced lung injury [12, 39–41]. The anti-inflammatory properties of both APC and Ang1 signaling in endothelium are mediated, at least partly, via inhibition of NF- $\kappa$ B-mediated expression of leucocyte adhesion molecules, such as intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 [19, 42].

APC is well known for its cytoprotective actions on endothelial cells, most of which are mediated through EPCR-dependent cleavage and activation of PARs, particularly PAR1. For example, after binding EPCR and cleaving PAR-1, APC stimulates endothelial sphingosine kinase-1 which enhances S1P production, subsequently selectively activating Rac1 to exert the barrier-protective function by stabilizing the endothelial cytoskeleton [2, 3, 43]. Results from the current study suggest that the rapid activation of Tie2 by APC does not require EPCR or PAR1, rather that APC acts as a direct ligand for Tie2. Such a mechanism may provide an alternative protective means for APC's actions when EPCR or PAR receptors are not available. For example, group V secretory phospholipase A2 (sPLA<sub>2</sub>V) inhibits EPCR anticoagulant and antiapoptotic properties by accommodating lysophosphatidylcholine or platelet activating factor (PAF) in the hydrophobic groove and prevents APC binding EPCR on the endothelial surface [44]. The same phenomenon occurs in rheumatoid synovial fibroblasts [45] due to the inflammatory effects of sPLA<sub>2</sub>V [46, 47]. In such situations, APC would be free to bind Tie2 and exert cytoprotective signaling. However, our results should be interpreted with caution as the blocking antibody and antagonist only show that the effects of APC are independent of the actual binding sites on EPCR and PAR1, respectively, that the inhibitor blocks, and do not eliminate the possibility that these receptors are functionally involved in APC's actions on Tie2, possibly via lateral associations.

In other cell types, APC has been found to directly bind and function through receptors other than EPCR and PAR1 to exert cytoprotective effects. In human monocytic-like U937 cells, binding to ApoER2 by APC signals via Dab1 phosphorylation and subsequent activation of PI3 K and Akt and inactivation of GSK3beta contributes to APC's beneficial effects [48]. Cao et al. [49] have shown that the anti-inflammatory activity of APC on macrophages is dependent on it binding integrin CD11b/CD18, but not EPCR, within lipid rafts and facilitates APC cleavage and activation of PAR1, leading to enhanced production of S1P and suppression of the inflammation. APC directly binds to  $\beta_1$  and  $\beta_3$  integrins and inhibits neutrophil migration via its Arg-Gly-Asp (RGD) sequence, both in vitro and in vivo [50].

In vivo, the protective effect of APC in a mouse model of vascular leakage was reversed by Tie2-kinase inhibitor at 4 h. Considering that APC cannot induce Ang1 or suppress Ang2 synthesis over this short duration [6], these results suggest direct participation of Tie2 in APC's actions. Taken together, our in vitro and animal studies show that APC signals directly and rapidly through Tie2 to induce vascular barrier integrity. However, there are at least three other mechanisms via which APC can activate Tie2. First, APC stimulates Ang1 and inhibits Ang2 to activate Tie2 and reduce endothelial permeability, with the first sign of protection occurring after 16 h [6]. Second, APC can enhance barrier integrity in human skin keratinocytes by a complex mechanism which requires binding to EPCR, cleaving, and activating PAR1 which causes intracellular transactivation of epidermal growth factor receptor (EGFR) followed by transactivation of Tie2 tyrosine kinase [51]. Whether this mechanism is functional in endothelium is unknown. Third, Stavenuiter and Mosnier [52] have recently shown that noncanonical cleavage of PAR3 by APC promotes a novel pathway for Tie2 activation and stabilization of vascular integrity. In addition, protein C inhibits rapid mobilization of Ang2 from Weibel–Palade bodies in endothelial cells which would effectively promote the actions of Ang1 and APC on Tie2 [53]. Considering Tie2's vital role in barrier function, this apparent mechanistic redundancy of Tie2 activation by APC may serve to ensure continuous protection of endothelium from barrier disruption.

Solid-phase binding assays as well as scatchard and saturation analysis indicated strong binding affinity of APC–Tie2, which was less than that of Ang1 and Tie2, but remarkably  $\sim 12$  times higher than that between Ang2 and Tie2. The higher Bmax value of APC–Tie2 binding analysis shows that larger numbers of binding sites are involved between APC and Tie2 compared to Ang1–Tie2. Although, Bmax for APC–Tie2 is less than Ang2–Tie2, the

lower K<sub>d</sub> for the former signifies that interaction between amino acids at these binding sites is more favorable. Competitive inhibition studies indicated that APC competes with both Ang1 and Ang2 for binding to Tie2, suggesting that APC and Angs share some similar binding sites to Tie2. It has been shown previously that APC and Angs require Ca<sup>2+</sup> for structural stability and function [54, 55]; however, additional Ca<sup>2+</sup> was not required for either Angs or APC to bind to Tie2 receptor. The exact nature of molecular interaction between APC and Tie2 awaits further structural analysis and in vitro molecular interaction analysis.

Ang2 can activate Tie2 in HUVEC and promote endothelial cell survival through the ERK1/2 and PI3K pathways [34, 56]. In our hands, Ang2 also partially induced Tie2 activation in HUVECs (Fig. 1d). However, Ang2 is generally considered as a non-signal-transducing ligand that inhibits Tie2 activation [57] and provokes inflammation and vascular hyper-permeability [58, 59]. Ang2 is elevated in sepsis, a very serious condition characterized physiologically by an aberrant systemic inflammatory response and microvascular dysfunction. Plasma levels of Ang2 correlate with the severity of illness and directly contribute to morbidity and mortality [59, 60]. Ang2 administration in healthy adult mice triggers sepsis-like changes, such as vascular leakage and lung injury [58, 59]. In sepsis models, Ang2 (+/−) mice exhibit reduced tissue inflammation, lower levels of both renal failure and lung injury, and show improved survival over wild-type litters [17]. Using mice engineered to inducibly overexpress Ang2, Ziegler et al. [61] showed that Ang2-mediated microvascular disintegration not only contributes to septic shock but that inhibition of the Ang2/Tie2 interaction during sepsis is a potential therapeutic target. Results here showing the competitive inhibition between APC and Ang2 together with the favorable binding affinity of APC to Tie2 suggest that APC could protect against Ang2-mediated disease by restoring Tie2 activation.

When endogenous protein C levels are lower than normal, endotoxic [62] and septic responses occur [63] and circulating protein C levels exhibit a strong inverse correlation with sepsis prognosis [64]. The PROWESS clinical trial demonstrated efficacy of APC in severe sepsis [65] and APC was subsequently FDA-approved (marketed as Xigris, Eli Lilly) to treat patients with severe sepsis. However, in 2011, APC was voluntarily withdrawn from the market by the supplier, due to questions on efficacy (PROWESS-SHOCK ClinicalTrials.gov, NCT00604214). A recent Cochrane review suggested that APC is not effective for treating patients with severe sepsis or septic shock and that it is associated with a risk of bleeding [66]. Nonetheless, reports continue to show that patients with septic shock who were treated with APC had a reduced in-

hospital mortality compared with those not treated with APC [67]. Furthermore, in animal models, APC prevents endotoxin-induced sepsis by inhibiting activated leukocytes, cytokines, and vascular permeability [3, 68–70] (Fig. 7). The current study adds a new mechanism of action to the growing body of evidence that APC has potential to benefit sepsis and other diseases associated with vascular pathologies, such as rheumatoid arthritis and cancer.

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#### Compliance with ethical standards

**Conflict of interest** CJ and MX have patents and commercial interests in molecular variants of APC and skin conditions.

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