Increased endothelial cell (EC) permeability is central to the pathophysiology of inflammatory syndromes such as sepsis and acute lung injury (ALI). Activated protein C (APC), a serine protease critically involved in the regulation of coagulation and inflammatory processes, improves sepsis survival through an unknown mechanism. We hypothesized a direct effect of APC to both prevent increased EC permeability and to restore vascular integrity after edemagenic agonists. We measured changes in transendothelial electrical resistance (TER) and observed that APC produced concentration-dependent attenuation of TER reductions evoked by thrombin. We next explored known EC barrier-protective signaling pathways and observed dose-dependent APC-mediated increases in cortical myosin light chain (MLC) phosphorylation in concert with cortically distributed actin polymerization, findings highly suggestive of Rac GTPase involvement. We next determined that APC directly increases Rac1 activity, with inhibition of Rac1 activity significantly attenuating APC-mediated barrier protection to thrombin challenge. Finally, as these signaling events were similar to those evoked by the potent EC barrier-enhancing agonist, sphingosine 1-phosphate (S1P), we explored potential cross-talk between endothelial protein C receptor (EPCR) and S1P1, the receptors for APC and S1P, respectively. EPCR-blocking antibody (RCR-252) significantly attenuated both APC-mediated barrier protection and increased MLC phosphorylation. We next observed rapid, EPCR and PI 3-kinase-dependent, APC-mediated phosphorylation of S1P1 on threonine residues consistent with S1P1 receptor activation. Co-immunoprecipitation studies demonstrate an interaction between EPCR and S1P1 upon APC treatment. Targeted silencing of S1P1 expression using siRNA significantly reduced APC-mediated barrier protection against thrombin. These data suggest that novel EPCR ligation and S1P1 transactivation results in EC cytoskeletal rearrangement and barrier protection, components potentially critical to the improved survival of APC-treated patients with severe sepsis.

Sepsis is a devastating inflammatory syndrome producing life-threatening end organ damage including acute lung injury (ALI) (1). Essential and defining pathophysiological features of sepsis include the activation of the coagulation cascade and marked increases in vascular permeability, processes that result in tissue and organ dysfunction (2, 3). The coagulation system, long recognized as an integral aspect of inflammation, contributes to enhanced vascular permeability via generation of procoagulant proteins, which directly perturb the integrity of the endothelial cell (EC) monolayer. For example, both thrombin, the central bioregulatory molecule of hemostasis, and fibrin, which is rapidly generated by thrombin-mediated fibrinogen cleavage, directly increase EC permeability in vivo and in vitro (4, 5). Further, intravascular fibrin microthrombi are uniformly present at autopsy in fatal sepsis and ARDS (6). Once generated from its circulating zymogen, thrombin exerts potent edemagenic responses catalyzed by cleavage of the thrombin receptor known as PAR1 (7, 8).

The prognosis of severe sepsis remains dismal and whereas therapies aimed at reducing coagulation in sepsis and ALI, such as anti-thrombin III and tissue factor pathway inhibitor, were successful in animal models, human trials failed to demonstrate improved survival (9–12). In contrast, in the landmark, multicenter Prowess trial, the anticoagulant serine protease, activated protein C (APC) reduced levels of the inflammatory marker interleukin-6 and improved survival in severely septic patients (13). Protein C circulates as an inactive zymogen and is activated by the newly formed thrombomodulin/thrombin complex allowing down-regulation of the thrombin-generating cascade. Both APC and non-activated protein C bind to the endothelial protein C receptor (EPCR), a receptor structurally similar to the major histocompatibility class I/CD1 family of molecules. EPCR is a crucial participant in the protein C pathway (14) as EPCR ligation increases the activation of protein C by ~20-fold (15). The mechanism underlying the prosurvival effects of APC in sepsis is currently unknown but presumed to be related to beneficial APC effects on coagulation and inflammation. Whereas the anticoagulant properties of protein C and APC are

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1 The abbreviations used are: ALI, acute lung injury; APC, activated protein C; ARDS, acute respiratory distress syndrome; co-IP, co-immunoprecipitation; DN, dominant-negative; EC, endothelial cell(s); EPCR, endothelial protein C receptor; HGF, hepatocyte growth factor; HPAECs, human pulmonary artery endothelial cells; MLC, myosin light chain; MLCK, myosin light chain kinase; PAR1, protease-activating receptor 1; S1P, sphingosine 1-phosphate; TER, transendothelial electrical resistance; TNF, tumor necrosis factor; PI, phosphatidylinositol; siRNA, short-interfering RNA; PDGF, platelet-derived growth factor; VEGF, vascular endothelial growth factor.
APC-mediated S1P<sub>1</sub> Transactivation

**FIG. 1.** APC attenuates thrombin-induced human lung EC barrier disruption. A depicts the concentration-dependent (0.1–50 μg/ml) effect of APC on thrombin-induced EC barrier disruption as assessed by changes in TER across human pulmonary artery EC monolayers. Human pulmonary EC were pretreated with APC 0.1, 1.0, 10, and 50 μg/ml for 1 h and then challenged with thrombin 0.2 units/ml. Results are expressed as a percentage of barrier protection versus thrombin, i.e., percent difference in nadir resistance between thrombin alone and APC + thrombin. Comparisons of TER between thrombin and APC with thrombin were made at maximal barrier disruption (i.e., consistently 30 min (± 5 min) after thrombin). n = 3–6; *, p ≤ 0.05 (via Neuman-Keul). Inset, real time evolution of thrombin-induced declines in normalized electrical resistance in the presence or absence of EC pretreatment with APC (1 μg/ml, 1 h). B demonstrates the effect of APC (1.0 μg/ml) on TER as a function of time. EC are pretreated with APC for 1 or 4 h prior to thrombin (0.2 units/ml) exposure. In addition, the effect of APC treatment 5 min after thrombin (0.2 units/ml) exposure is demonstrated. n = 3–6; *, p ≤ 0.05.

evident in patients with inherited protein C deficiency (16–18), APC binding to EPCR results in the loss of anticoagulant activity by the bond APC (19). Similarly, the anti-inflammatory properties of the protein C pathway have been implicated in APC survival benefit, as APC inhibits NF-kB activation in monocytes exposed to lipopolysaccharide (LPS), as well as decreases LPS-induced TNF-α production in mice (20, 21). APC infusion in humans given endotoxin-improved hemodynamics, but failed to alter inflammatory, thrombotic, or fibrinolytic indices, suggesting an alternate mechanism of APC-mediated protection (22). The exact mechanism of APC survival benefit, therefore, remains enigmatic.

In this report, we have hypothesized that one mechanism of APC benefit may be related to inhibition of the widespread increases in vascular permeability, characteristic of severe sepsis, with accelerated recovery from the untoward effects of edemagenic agonists. We recently reported that sphingosine 1-phosphate (S1P), the major barrier-protective agent released from platelets (23) via its specific receptor S1P<sub>1</sub> (also known as Edg1), represents a novel anti-edemagenic strategy both in vitro (24) as well as in animal models of sepsis (25, 26). S1P<sub>1</sub> signaling induces barrier-protective cell-cell and cell-matrix tethering to the EC cytoskeleton (24, 27), compared with the barrier-disruptive forces produced by agents such as TNF-α and thrombin. These edemagenic agonists induce EC barrier disruption via alterations in cytoskeletal proteins actin and myosin that result in transcellular actin stress fibers causing cellular contraction and paracellular gap formation (4, 28–30).

Given the central role of increased vascular permeability in inflammatory syndromes such as sepsis, therapies that reverse vascular permeability via targeting of the EC cytoskeleton would have obvious clinical utility. We explored whether APC-mediated survival benefit in sepsis may involve APC-induced EC barrier regulation. Our studies indicate that APC evokes human lung EC signaling paradigms, which target the Rho family GT-Pase, Rac1, with subsequent Rac1-dependent cytoskeletal rearrangement and profound EC barrier protection. Finally, we demonstrate that APC-mediated protection from the edemagenic effects of thrombin occurs via novel transactivation of S1P<sub>1</sub>, the potent barrier-enhancing receptor for S1P.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Unless otherwise specified, reagents were obtained from Sigma. LY294002 was purchased from Calbiochem (La Jolla, CA). Rabbit anti-diphosphorylated and pan-MLC antibodies were purchased from Cell Signaling Technology (Beverly, MA). PAR1 rabbit polyclonal IgG and normal rat monoclonal IgG antibodies were obtained from Zymed Laboratories (San Francisco, CA) were commercially obtained. APC was generously supplied by Eli Lilly (Indianapolis, IN).

**Human Lung Endothelial Cell Cultures—**Human pulmonary artery EC (HPAEC) were obtained from Cambrex (Walkersville, MD) used at passages 3–8 and maintained in the manufacturer’s recommended EGM-2 medium with 10% fetal bovine serum as we have previously described (24, 27). Cells were incubated at 37 °C in 5% CO<sub>2</sub> and 95% air. Two hours prior to all experimentation, cells were incubated in serum-free, endothelial basal medium.

**Measurement of Transendothelial Electrical Resistance (TER)—**Human EC were grown to confluence over evaporated gold microelectrodes connected to a phase-sensitive lock-in amplifier as we previously described (24, 31). TER was measured using an electrical cell-substrate impedance sensing system (ECIS; Applied BioPhysics Inc., Troy, NY). As cells adhere and spread out on the microelectrode, TER increases, whereas cell retraction, rounding, or loss of junctional adhesion is reflected by a decrease in TER. These measurements provide a sensitive biophysical assay that indicates the state of cell shape and focal adhesion reflective of changes in paracellular permeability. All comparisons of TER were made using normalized resistances, with actual starting resistances ranging between 1200 and 1800 ohms. Comparisons of TER between thrombin and APC with thrombin were made at maximal barrier disruption and expressed as a percentage of barrier protection versus thrombin, i.e., percent difference in nadir resistance between thrombin alone and APC + thrombin.

**MLC Phosphorylation in Intact Endothelium—**Human EC were analyzed for MLC phosphorylation by sodium dodecyl sulfate-polyacryl-
APC-mediated S1P, Transactivation

Fig. 2. APC directly and rapidly increases EC MLC phosphorylation. A depicts the concentration-dependent effect of APC on phosphorylation of MLC, an event that results in increased actin-myosin cross-bridge formation. MLC phosphorylation was detected by Western blotting of APC-challenged EC lysates using antisera specific for MLC phosphorylated on serine 19, threonine 18 residues (Phospho-MLC) in confluent EC and expressed as relative densitometry units (RU). EC were treated with APC 0.1 μg/ml, 1.0 μg/ml, or thrombin 0.2 units/ml for 5 min, and levels of phospho-MLC were measured and compared with unstimulated control. n = 3–6; *; p ≤ 0.05 (Student’s t test). B demonstrates the time-dependent effect of APC on MLC phosphorylation compared with controls. EC are treated with APC 1.0 μg/ml at 1, 5, 10, 15, and 60 min. Thrombin control (0.2 unit/ml, 5 min) is again shown for comparison. Representative Western blots are shown as insets in both A and B. n = 3–6; *; p ≤ 0.05 (Student’s t test).

Determination of Serine/Threonine Phosphorylation of the S1P, Receptor—EC monolayers were serum-starved for 1 h followed by either vehicle (methanol, control) or 1 μM S1P challenge for 5 min or pretreated with control IgG, anti-EPCR or anti-PARI blocking antibody (20 μg/ml) for 1 h followed by APC (1 μg/ml) or thrombin (0.2 units/ml) exposure (5 min) or pretreated with vehicle (MeSO, control) or 10 μM LY294002 for 1 h followed by APC treatment (1 μg/ml, 5 min). Cells were subsequently solubilized in extraction buffer (1% Triton X-100, 0.1% SDS, 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl2, 0.4 mM Na3VO4, 40 mM NaF, 50 mM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture 3). The samples were then immunoprecipitated with rabbit anti-S1P1, antibody followed by SDS-PAGE in 4–15% polyacrylamide gels and transferred onto ImmobilonTM membranes (Millipore Corp., Bedford, MA). After blocking nonspecific sites with 5% bovine serum albumin, the blots were incubated with either rabbit anti-S1P1 antibody, rabbit anti-phosphoserine antibody, or rabbit anti-phosphothreonine antibody followed by incubation with horseradish peroxidase-labeled goat anti-rabbit IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence (Amersham Biosciences).

Determination of Complex Formation between EPCR and S1P, Receptor—EC monolayers were serum-starved for 1 h followed by treatment with control IgG or anti-EPCR blocking antibody (20 μg/ml) for 1 h followed by APC (1 μg/ml) exposure (5 min). Cells were subsequently solubilized in complex buffer (1% Nonidet P-40, 50 mM HEPES (pH 7.5), 150 mM NaCl, 20 mM MgCl2, 0.4 mM Na3VO4, 40 mM NaF, 50 mM okadaic acid, 0.2 mM phenylmethylsulfonyl fluoride, 1:250 dilution of Calbiochem protease inhibitor mixture). The samples were then immunoprecipitated with rabbit anti-EPCR antibody followed by SDS-PAGE in 4–15% polyacrylamide gels and transferred onto ImmobilonTM membranes (Millipore Corp.). After blocking nonspecific sites with 5% bovine serum albumin, the blots were incubated with either rabbit anti-S1P1 antibody or rabbit anti-phosphothreonine antibody followed by incubation with horseradish peroxidase-labeled goat anti-rabbit IgG. Visualization of immunoreactive bands was achieved using enhanced chemiluminescence.

Statistics—Either Student’s t test or analysis of variance with a Neuman-Keul posthoc test was used to compare the means of data from two or more different experimental groups as indicated in the individual figure legends. Results are expressed as means ± S.D.

RESULTS

Effect of APC on Thrombin-mediated Reductions in TER—Our initial experiments characterized the time-dependent effect of APC on human lung EC barrier function utilizing measurements of TER, a sensitive measure of barrier integrity (24, 34). Fig. 1A demonstrates that EC pretreatment with APC...
(0.1–50 μg/ml, 1 h) attenuates thrombin-mediated (0.2 units/ml) TER decreases in a concentration-dependent manner. All measurements of electrical resistance were made at maximal barrier disruption, i.e. nadir resistance, regardless of time. Of note, nadir resistance occurs consistently at 30 min (± 5 min) after thrombin challenge in all experiments. APC has minimal effect on the timing of thrombin-induced barrier disruption. Pretreatment of human lung EC with APC (1 μg/ml) for durations of 1–4 h significantly reduced the thrombin-induced EC barrier-disrupting response (Fig. 1B), although this protective effect was not observed after 20 h of APC exposure (data not shown). Importantly, EC with established thrombin-mediated barrier dysfunction and subsequently challenged with APC (1 μg/ml, 5 min), demonstrated significant reversal of the thrombin-induced decline in TER (Fig. 1B).

**Effect of APC on EC Cytoskeletal Rearrangement and MLC Phosphorylation**—Phosphorylation of MLC results in increased actin-myosin cross-bridge formation and plays a central role in

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**Fig. 3.** APC rapidly increases EC cytoskeletal rearrangement and MLC phosphorylation in a spatially restricted manner. Immunofluorescence staining of HPAECs demonstrates alterations in the cytoskeletal proteins actin (red) and phosphoMLC (green) under agonist challenges. The Actin and phosphoMLC images show matched cell fields for each condition. A, untreated. B, thrombin (0.2 unit/ml, 10 min). C, APC (1 μg/ml, 5 min). D, APC (1 μg/ml, 5 min) 5 min after thrombin (0.2 units/ml). Scale bar represents 10 μm.

**Fig. 4.** APC-mediated EC barrier function is Rac1-dependent. A, levels of activated Rac1 are measured in EC as described under “Experimental Procedures” after treatment with APC 1.0 μg/ml (5min) and compared with control. Levels of Rac1 are expressed as RDU. n = 3; *, p ≤ 0.05 (Student’s t test). B, the effect of APC (1-h pretreatment, 50 μg/ml) on thrombin- (0.2 unit/ml) induced barrier disruption is assessed in EC infected with a Rac dominant negative adenovirus. EC infected with an empty vector and untransfected EC treated with APC are used as controls. Results are expressed as percent barrier protection versus thrombin treatment alone. n = 3–6; *, p ≤ 0.05 (Student’s t test).
the barrier-regulatory properties of agents such as S1P, hepatocyte growth factor (HGF) as well as edemagenic agents such as TNF and thrombin (24, 32, 35, 37, 39). Given the barrier protection observed with APC treatment, we next investigated the effect of APC on MLC phosphorylation via Western blotting using an antibody specific for MLC phosphorylated on serine 19, threonine 18 residues (phospho-MLC) in confluent EC. Fig. 2 demonstrates the concentration- and time-dependent effect of APC on MLC phosphorylation. Peak MLC phosphorylation occurred with brief (5 min, 1 µg/ml) durations of APC exposure. Thrombin (0.2 units/ml), an agonist well known to increase levels of phospho-MLC via coordinate Rho kinase and myosin light chain kinase (MLCK) activation (37, 38), served as a positive control. We next explored the spatially defined effects of APC on the actin cytoskeleton, a key participant in maintaining intercellular adhesion- and barrier-regulatory properties of the confluent EC monolayer. EC barrier-disruptive agents such as thrombin cause the formation of transcellular actin stress fibers resulting in increased tension, cellular contraction, and paracellular gap formation (Fig. 3), whereas in contrast, APC (1.0 µg/ml, 5 min) stimulates strong actin-phospho-MLC rearrangement at the cell periphery and reduced central stress fiber formation (Fig. 3). Finally, APC (1.0 µg/ml) treatment 5 min after thrombin (0.2 µ/ml) exposure results in marked attenuation of stress fiber formation and peripheral cortical actin-phospho-MLC arrangement (Fig. 3).

Role of Rac GTPase in APC-induced EC Cytoskeletal Rearrangement and Barrier Protection—We have previously demonstrated that the barrier-protective cortical actin distribution induced by S1P, simvastatin, and HGF, is mediated by activation of the small Rho family GTPase, Rac1 (24, 33, 39). We next investigated the role of Rac1 in APC-mediated EC barrier protection. Fig. 4A demonstrates that Rac activation occurs in APC-challenged EC monolayers within 1 min (50% increase). Activated Rac levels returned to baseline over the following 30 min (data not shown). To assess the role of Rac1 in APC-mediated barrier function, we infected EC with a Rac1 dominant-negative (DN) adenovirus and determined that reductions in Rac activity attenuate the APC-mediated barrier-protective response (~50% reduction) (Fig. 4B).

Role of EPCR on APC-mediated Barrier Protection and MLC Phosphorylation—As EPCR is the recognized receptor for both protein C and APC, we next sought to determine the role of EPCR in APC-mediated cytoskeletal activation and barrier protection. Pretreatment with RCR-252, an effective EPCR blocking antibody thereby inhibiting APC binding (40, 41), nearly abolished APC-mediated EC barrier protection, as measured by TER (~70% reduction in protective effect compared with control antibody incubation) (Fig. 5A). Pretreatment with a nonspecific rat IgG had no effect on APC-mediated barrier protection, whereas rat IgG and RCR-252 pretreatment had no effect on basal TER levels (data not shown). Furthermore, RCR-252 pretreatment completely abolished APC-induced EC MLC phosphorylation (Fig. 5B), but did not alter basal TER values, basal levels of phospho-MLC, or thrombin-induced increases in phospho-MLC. These results clearly indicate that APC-mediated EC barrier protection evolves via EPCR ligation eliciting downstream signals, which target the EC cytoskeleton.

APC Transactivates S1P1, the Barrier Protective Receptor for Sphingosine 1-Phosphate—Careful analysis of the integrated biochemical and physiologic EC responses to APC revealed remarkable and extensive similarities between APC-mediated barrier protection and the signaling pathways and cytoskeletal rearrangement evoked by the potent anti-edemagenic agonist, S1P (24, 26, 27). As the barrier-enhancing S1P receptor, S1P1 (24), is known to exhibit transactivation following ligation of growth factor receptors such as PDGF and VEGF (42, 43), we next examined whether APC-induced EC barrier protection involves receptor cross-talk between EPCR and S1P1. Immunoprecipitation of S1P1 from APC-challenged EC followed by antithreonine immunoblotting revealed strong evidence of APC-mediated, EPCR-dependent threonine (but not serine) phosphorylation (Fig. 6A). These results are consistent with the recognized role of threonine (Thr236) phosphorylation in S1P1 activation observed after S1P challenge (44) and are highly consistent with EPCR-S1P1 cross-talk. To further define EPCR-mediated S1P1 transactivation, co-immunoprecipitation studies were performed that demonstrate APC-induced interaction between EPCR and S1P1, which was inhibited by EPCR blocking antibody (Fig. 6B). Given that S1P induces phosphorylation of the S1P1 receptor via the PI 3-kinase-Akt pathway, we sought to determine the role of this pathway in APC-mediated S1P1 phosphorylation. Use of the PI 3-kinase inhibitor LY294002 blocked APC-induced phosphorylation of S1P1, Thr236, indicating involvement in APC signaling of PI 3-kinase and possibly Akt, a known downstream effector of PI 3-kinase (Fig. 6C). Consistent with a primary role of S1P1 transactivation by EPCR, silencing RNA specific for S1P1, attenuated (as did EPCR blocking antibody) APC- and S1P-mediated EC barrier protection (Fig. 6D). Thus, APC is a potent barrier-protec-
tive agonist that signals through S1P1 to produce EC barrier protection and restoration.

DISCUSSION

In this study, we demonstrate a direct effect of APC on human pulmonary EC barrier integrity resulting in protection from thrombin-induced EC vascular permeability. Mechanistically, in a spatially defined and cortically distributed manner, APC stimulates increased levels of phosphorylated MLCs and actin polymerization thereby reversing established barrier disruption produced by thrombin. APC-mediated barrier protection was rapid and sustained, exerting a protective effect for up to 4 h. Given that estimates of APC half-life are generally in the 110–25 min range, these results indicate a real potential for cytoskeletal remodeling as a key component in the sustained barrier protection, an effect we have previously noted with S1P, another potent barrier enhancing agonist (24, 27).

We (37, 45) as well as others (46) have reported thrombin to increase, in a central locale, the levels of phosphorylated MLC, actin polymerization, and stress fiber formation via the coordinated action of MLCK and Rho kinase. In contrast, APC (Fig. 3) induces cortical actin and peripheral MLC phosphorylation, which persist despite concomitant treatment with thrombin. The effects of APC on Rac1 and cortical actin are similar to those seen with S1P (24, 27), which is known to increase phosphorylation of MLC and induce Rac1-dependent cortical cytoskeletal rearrangement conferring EC barrier protection. Rac1 is a member of the Rho family of GTPases linked to actin cytoskeletal organization and lamellipodia formation (47). We previously reported that S1P causes prominent actomyosin rearrangement, which is dependent on S1P1 ligation, and activation of p21-associated kinase (known as PAK), a downstream effector of Rac1 (24). The exact mechanism through which Rac1 activation results in cortical actin formation and decreased EC permeability is not completely understood, however, our data clearly confirm the important role of Rac1 in maintaining EC barrier function. Our findings of S1P1 transactivation and Rac1 activation by APC are consistent with previous data concerning S1P1 signaling. S1P causes Akt-mediated phosphorylation of S1P1 at Thr236 and this transactivation of S1P1 is required for Rac1 activation and cortical actin redistribution (44). The elevated levels of phosphorylated MLCs are spatially-localized within the enhanced cortical ring (Fig. 3), a locale where increased tensile strength occurs with enhanced affinity between adherens junctions and focal adhesion components and the cortical cytoskeleton (48). This peripheral distribution of actin and phosphorylated MLC evoked by APC is a pattern seen with other barrier-protective agents such as S1P, HGF, shear stress, and simvastatin as well as oxidized phospholipids (24, 33, 39, 49, 50). It should be noted that while S1P directly increases baseline electrical resistance, APC does not. Both agonists however, exhibit a protective effect in the presence of the barrier-disruptive agent, thrombin. This “rescue effect” is similar.
to the barrier protective agent simvastatin (33) and is particularly relevant given our data that APC is barrier protective after thrombin exposure, a scenario similar to treatment of septic patients with APC.

Our findings are also consistent with other reports that have addressed the importance of the APC receptor, EPCR, in APC signaling. APC inhibits apoptosis and alters gene expression in EC in an EPCR-dependent manner (51, 52). Our studies indicate that the novel APC-mediated EC MLC phosphorylation and barrier-protective responses we have described appear to evolve in an EPCR-dependent manner. The most potentially critical and highly novel finding of our studies, however, is the observation that APC mediates EC barrier protection via transactivation of S1P1 (aka Edg1), the receptor for the barrier-enhancing, lipid growth factor, S1P. Both APC (Fig. 4) and S1P (24, 27) exhibit Rac1-dependent barrier protection and induce cortical actin polymerization as well as retard the edemagenic effects of thrombin. Utilizing S1P1 phosphorylation on threonine (Thr236) residues as the accepted readout of S1P1 activation (44), we convincingly demonstrated S1P1 transactivation by APC-ligated EPCR as APC-induced robust S1P1 phosphorylation, which was eliminated by EPCR blocking antisera. Co-immunoprecipitation studies strongly suggest an interaction between EPCR and S1P1 (Fig. 6B). Furthermore, use of the PI 3-kinase inhibitor LY294002, blocked APC-induced S1P1 Thr236 phosphorylation, demonstrating that APC phosphorylates S1P1 via the PI 3-kinase-Akt pathway (Fig. 6C). Finally, and of critical importance, S1P1 silencing abrogates the APC-mediated EC barrier protection indicating that S1P1 expression is essential for APC-mediated barrier protection. S1P1 transactivation is not a novel observation and has been previously described in the context of ligation of growth factor receptors such as PDGF and VEGF. S1P increases tyrosine phosphorylation of the VEGF receptor, Flk-1/KDR, and S1P-mediated phosphorylation of Akt and EC nitric-oxide synthase is VEGF-dependent (53). Moreover, the defective migration of fibroblasts to PDGF stimulation in S1P1-null mice is corrected only after S1P1 transfection, indicating PDGF and S1P1 transactivation (54). Similarly, S1P treatment of rat vascular smooth muscle cells results in tyrosine phosphorylation of PDGF β-receptor and epidermal growth factor receptor, which is S1P1 receptor-dependent (55). The exact mechanism by which growth factor receptor-S1P1 transactivation occurs is unknown but has been suggested to involve increases in sphingosine kinase activity and newly generated S1P secretion allowing S1P1 ligation to occur (56). Nevertheless, together our data indicate that S1P1 is a key target for APC/EPCR-mediated barrier protection and suggests that S1P1 may, in fact be central to plasma membrane signaling sequences which evolve to enhance vascular integrity (Fig. 7).

In summary, increased endothelial permeability is central to the pathophysiology of inflammatory syndromes such as sepsis and ALI. Our data strongly indicate that APC prevents increases in endothelial permeability, and restores vascular integrity after edemagenic agonists such as thrombin. Intracellular signaling pathways linked to known EC barrier-protective alterations in the EC cytoskeleton resulted in cortically distributed MLC phosphorylation, increased calcium (data not shown) and polymerized actin. These APC-mediated biochemical and physiologic effects were attributed to increased Rac1 activity. A highly novel finding from our work was the observation that the sequence of APC-mediated Rac1 activation, actomyosin rearrangement, and barrier protection were dependent upon signaling events evoked by the potent EC agonist, S1P, with strong evidence of cross-talk between EPCR and S1P1. Together, these data suggest that EPCR ligation, EPCR/S1P1 cross-talk to the endothelial cytoskeleton, and modulation of EC barrier function may be critical components in the improved survival observed in patients with sepsis treated with APC.

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