Activated protein C protects against ventilator-induced pulmonary capillary leak


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Acute lung injury (ALI) is a devastating illness with an annual incidence of ~200,000 and a mortality of 40% (51). Most commonly seen in the setting of sepsis (9, 26, 50), ALI is a complex inflammatory syndrome marked by increased vascular permeability resulting in tissue edema and organ dysfunction (58). The vascular endothelium is a key target and critical participant in the pathogenesis of sepsis-induced organ dysfunction and is central to the pathophysiology of both sepsis and ALI. Unfortunately, recent insights into the mechanisms of ALI have not translated into improved therapy, and treatment remains largely supportive and dependent on mechanical ventilation (MV). However, MV can contribute to increases in vascular permeability, particularly at high tidal volumes, a phenomenon known as ventilator-induced lung injury (VILI), and a lung-protective strategy of ventilation aimed at minimizing alveolar overdistention attenuates the inflammatory response (47) and results in decreased mortality (3). Although the current understanding of the pathophysiology of VILI remains incomplete, a critical deleterious component of MV appears to be mechanical stress that, in experimental models, activates the GTPase Rho pathway and contributes to increased vascular permeability (7, 45). Rho-associated kinase (ROCK-1), the downstream effector of Rho in pulmonary endothelium, enhances myosin light chain phosphorylation and endothelial stress fiber formation, which leads to endothelial gap formation (60).

It has become increasingly appreciated that a deficiency of regulatory proteins involved in coagulation, such as activated protein C (APC), participates in the widespread inflammation of sepsis and ALI (38, 59) and that treatment of patients with sepsis with the endogenous anticoagulant APC reduces mortality (6). Although APC is known to have anticoagulant and anti-inflammatory properties, the exact mechanisms through which it benefits patients with severe sepsis (and potentially ALI) remain undefined. We (16) have previously demonstrated that APC enhances pulmonary endothelial barrier function in vitro via alterations in the endothelial cytoskeleton and that APC-mediated intracellular signaling and endothelial barrier protection is dependent on the APC receptor, the endothelial protein C receptor (EPCR).

Although APC is known to promote vascular integrity and improve survival in sepsis (6, 16), a beneficial effect in ALI is unproven. Moreover, whether APC can attenuate the deleterious effects of MV remains unknown. We hypothesized that...
APC could be protective in VILI. Using a well-characterized murine model of ventilator-induced vascular leak (1, 35, 44), we demonstrate that MV-mediated increases in pulmonary permeability are associated with increased expression of ROCK-1 and loss of pulmonary, membrane-bound EPCR. Furthermore, our results indicate that loss of pulmonary EPCR is critical to the generation of stress-mediated pulmonary vascular permeability; APC treatment attenuates both stretch-mediated vascular leak and ROCK-1 expression and stabilizes pulmonary EPCR expression. Finally, microarray analysis of lung tissue identified several gene ontologies as well as specific genes that might be participating in APC-mediated vascular barrier protection.

MATERIALS AND METHODS

Johns Hopkins University Institutional Animal Care and Use Committee approved all animal protocols.

Experimental protocol and animal exposure to MV. Male C57BL/6j mice age 8–10 wk (The Jackson Laboratory, Bar Harbor, ME) were studied in a pathogen-free facility. Animals were first anesthetized, underwent intubation, and were subjected to MV (Harvard Apparatus, Boston, MA) with room air for 0 h (sham) or 0.5–4 h with low (7 mL/kg; \( \text{i.v.} \)) and high (20 mL/kg; \( \text{i.v.} \)) tidal volumes. Animals were pretreated 1 h before MV with either human APC (100 \( \mu \)g/kg iv; Eli Lilly, Indianapolis, IN) in a single bolus dose (APC-treated) or the equivalent volume phosphate-buffered saline (untreated). In separate experiments, animals were given APC at 30 and 150 min after initiation of MV and were ventilated for a total of 4 h. All animals were given a 500-\( \mu \)L bolus of lactated Ringer solution intravenously at the initiation of MV to maintain adequate mean arterial blood pressure (~80 Torr). The respiratory rate was set at 160 breaths/min for all tidal volumes, and the dead space was adjusted by increasing the length of ventilator tubing in the HV group to maintain an arterial pH between 7.35 and 7.45. The adequacy of MV settings on gas exchange was confirmed in preliminary experiments in which arterial blood gases obtained via catheterization of a femoral artery and analyzed by automated blood gas analyzer (Instrumentation Laboratories, Lexington, MA) revealed stable levels of arterial oxygen and carbon dioxide and an arterial pH ranging between 7.35 and 7.45. No positive end-expiratory pressure (PEEP) was applied, and airway pressures, continuously measured during MV, revealed that end-expiratory pressures remained around 0–2 cmH\text{2}O throughout the 4-h period for both LV and HV. At the end of MV, the animals were administered an intraperitoneal lethal dose of the anesthetic agent before the lungs were harvested.

Assessment of pulmonary capillary permeability. Evans blue dye (EBD; 20 mL/kg) was injected into the external jugular vein 30 min before termination of the experiment to assess vascular leak as previously described (1, 35, 45).

Bronchoalveolar lavage protein concentration. Bilateral bronchoalveolar lavage (BAL) was performed with 1 mL of saline for determination of BAL fluid protein concentration as previously described (45).

SDS-PAGE and immunoblotting. Western blotting using EPCR antibody (R&D Systems, Minneapolis, MN) was performed as previously described after tissue was homogenized and quantified using a Bradford protein assay (18). ROCK expression in lung tissue was assessed via Western blotting using a specific ROCK-1 or -2 antibody (BD Transduction Laboratories, Lexington, KY) as previously described (20).

Membrane-fraction enrichment. To determine the effect of MV on surface EPCR expression, membrane enrichment was obtained as described previously (24, 32) and subjected to Western blotting as above.

Measurement of thrombin-antithrombin complexes. Mice [wild-type (WT) and Tie2-EPCR] were exposed to 4-h MV with and without APC as above. Blood was drawn into syringes containing 3.2% citrate and 100 mM benzamidine and centrifuged at 3,000 rpm for 15 min. Thrombin-antithrombin complexes (TATc) were determined via commercially available ELISA (Enzygnost; Dade Behring).

Transcript profiling with Illumina oligonucleotide array. Illumina profiling was performed at the Lowe Family Genomics Core. The total lung mRNA was isolated from parenchymal tissues. The processed RNA (quality monitored on an Agilent 2100 Bioanalyzer) was hybridized to Mouse_Ref-8_V2 microarray (25,697 transcripts), and hybridization signals were analyzed using BeadStudio version 1.5.0.34 software (Illumina). Resulting digitized matrix was processed by modified Illumina platform approach as described previously (23).

Briefly, the significance of hybridization signals was tested, and “Present” [BeadStudio detection (BSD) < 0.05] and “Absent” (BSD ≥ 0.05) transcripts was identified. The chip background and brightness were computed using high quartile and whole set of Absent hybridization signals, respectively. The expression data were stratified by experimental conditions (n = 3), and hybridization of each transcript was evaluated for each cluster. The transcripts that were Present and produced a signal at least twice as high as that of background in at least 2 of 3 hybridizations in any given group of mice were considered expressed. To normalize expression values between different BeadChips, each background adjusted value was divided by a corresponding chip brightness coefficient.

Computational identification of ventilation-affected candidate genes. Significance Analysis of Microarrays (SAM 2.20; Ref. 57) was conducted using full permutation of 3 control and 3 HV or HV + APC samples (720 permutations) without application of arbitrary restrictions (34). Genes with 50% changes in expression and 5% false discovery rate (FDR) were considered significantly affected by HV or HV + APC.

Gene ontology analysis. The Affymetrix identities for the matching Illumina probe were retrieved from NetAffx (http://www.affymetrix.com). Affymetrix and Illumina probe identities were matched by gene symbols using Microsoft Access and corresponding Affymetrix identities assigned to probes that comprise the Mouse_Ref-8_V2 microarray. The MAPPFinder-compatible files were prepared using GeneMAPP converting tool as we (21) described previously. This approach identified 9,188 genes linking 5,314 genes to known gene ontology term, of which 53 genes were affected by APC.

Statistics. Values are shown as means ± SE, with \( n \) = 4 for each experimental condition. Data were analyzed by a one-way ANOVA with Bonferroni correction. Significance in all cases was defined as \( P < 0.05 \). For gene expression analysis, the SAM-generated \( q \) value was used, which represents the lowest FDR for a gene to be called significant (54). The \( q \) value is an approximated equivalent of \( P \) value, adapted to the analysis of a large number of genes, and significance in transcriptional changes was considered as \( q < 5 \).

Gene ontologies that were represented by at least one APC-affected gene in the “child” node of a biological processes tree, three APC-affected genes (>5% of detected by MAPPFinder genes) in the “parental” node of the same tree, and \( z \)-score >1.96 were considered as significantly affected by APC.

RESULTS

Increased pulmonary vascular permeability in response to HV. We have previously demonstrated that 2-h HV MV causes significant pulmonary vascular leakage compared with control, spontaneously breathing mice and mice exposed to LV. However, the minimum duration of MV required to induce injury is unknown. To determine the time of onset of capillary vascular leak in response to MV, C57BL/6j mice were exposed to either HV (20 mL/kg) or LV (7 mL/kg) for 30...
min to 2 h and compared with controls. Figure 1 demonstrates that 2 h of HV₇, but not shorter times, caused significant pulmonary leak, as measured by increases in BAL total protein and lung tissue EBD extravasation. LV₇ failed to elicit increases in permeability over 2 h (data not shown).

**Effect of APC on MV-mediated increases in lung vascular permeability.** We (16) have previously reported that APC confers endothelial barrier protection in human pulmonary endothelial cells exposed to thrombin. Therefore, we tested whether APC could protect against mechanical stress-induced capillary leakage in vivo. Mice were ventilated for 2 h with tidal volumes of 20 ml/kg (HV₇), with or without pretreatment with APC (1 h before MV, 100 μg/kg iv as described in MATERIALS AND METHODS), before measurement of BAL total protein and lung EBD extravasation. As shown in Fig. 2A, the increase in BAL total protein produced by HV₇ MV was significantly reduced by pretreatment with APC. Similarly, lung extravasation of EBD was significantly attenuated by pretreatment with APC (Fig. 2B). APC had no effect on BAL protein or EBD leak in unventilated mice (data not shown), and LV₇ MV showed no differences in measured indices of capillary permeability compared with control (Fig. 2). These data indicate that APC is protective against the development of increased pulmonary capillary permeability in an in vivo model of mechanical injury.

We have previously shown that mechanical stress imparted by HV₇ MV [e.g., activation of signaling pathways such as p38 MAPK, inducible nitric oxide synthase, and xanthine oxidoreductase (1, 44)] is an early phenomenon occurring within minutes of MV. Therefore, we sought to determine whether APC could be protective against vascular permeability after initiation of MV. Mice were ventilated for 4 h as described above, and APC treatment was given at 30 and 150 min after initiation of MV. As shown in Fig. 3, treatment with APC resulted in significant attenuation of HV₇-mediated vascular leak as measured by changes in BAL protein and EBD extravasation, consistent with a protective effect of APC on pulmonary vascular permeability after the onset of mechanical stress. At 4 h, LV₇ resulted in a small but significant increase in BAL protein but no increase in EBD extravasation. Delaying APC treatment beyond 30 min (e.g., 1 h or beyond) was not effective (results not shown).

**Effect of MV and APC on lung EPCR.** EPCR is the receptor for both protein C and APC and is an essential component in APC-mediated vascular protection (16). Nonmechanical inflammatory stimuli have been shown to result in EPCR shedding from the endothelial cell surface (3, 10, 33, 52). Whether a mechanical stimulus alters EPCR expression in vivo, mice were exposed to 2-h HV₇ MV, following which, lung EPCR levels were assessed using SDS-PAGE and Western blotting with an EPCR-specific antibody. As shown in Fig. 4, both 2 and 4 h of HV₇ MV resulted in decreased lung tissue EPCR expression, which was significantly attenuated by pretreatment with APC, whereas shorter exposure to HV₇ MV, and LV₇ of any duration had no effect on tissue EPCR. Taken together, these studies reveal that HV₇ MV results in decre-
ments in lung tissue EPCR and increased pulmonary vascular permeability, whereas the barrier-protective administration of APC results in restoration of tissue EPCR. Effect of MV and APC on lung tissue membrane EPCR. Although EPCR is expressed on the surface of endothelial cells, decreases in EPCR expression (as shown in Fig. 4) may result from changes in intracellular stores of EPCR rather than specific membrane expression. Moreover, we hypothesized that EPCR must be on the endothelial surface, available for APC ligation, to effect intracellular signaling. To assess endothelial surface EPCR expression, lung tissue collected from mice exposed to HVT MV with and without APC treatment, LVT, and spontaneously breathing controls was fractionated into membrane and cytosolic components. SDS-PAGE was done on the membrane fraction. As can be seen in Fig. 5, HVT results in significant decreases in membrane EPCR compared with LVT and controls, which are restored to normal levels by pretreatment with APC. These findings demonstrate that mechanical stretch-induced pulmonary vascular injury is accompanied by specific loss of membrane EPCR, which can be prevented by APC. Effect of MV on pulmonary vascular function and tissue EPCR levels in EPCR-overexpressing mice. We (16) have previously demonstrated that EPCR is a necessary participant in APC-mediated barrier protection in cultured endothelial cells. The association of increased murine pulmonary vascular permeability with loss of membrane EPCR further supports that intact membrane EPCR expression participates in APC-mediated pulmonary vascular barrier protection. Mice that overexpress EPCR (Tie2-EPCR mice) on the endothelial surface, and obtained from a C57BL/6J background strain, have been shown to have higher circulating levels of APC and decreased mortality when exposed to lethal doses of LPS (36). To define further the role of EPCR in VILI, we exposed Tie2-EPCR mice to HVT MV and measured indices of pulmonary vascular permeability. In contrast to WT mice, Tie2-EPCR mice exposed to HVT were significantly protected from MV-induced vascular permeability as measured by changes in
BAL protein and EBD extravasation (Fig. 6). The finding that EPCR overexpression confers enhanced barrier protection from MV compared with WT mice provides further evidence that EPCR is a critical participant in vascular barrier functional integrity.

ROCK expression in MV. Although cyclic stretch has been shown to activate Rho pathway signaling, the effect of HV on Rho signaling and its downstream effector, ROCK, in vivo is unknown. Therefore, lung tissue from mice exposed to HV with and without APC treatment was assessed via Western blotting. ROCK-1 expression was significantly increased in mice exposed to 4-h HV compared with controls. APC treatment (30 and 150 min after initiation MV) significantly attenuated ROCK-1 expression in WT mice (Fig. 7A). In contrast, ROCK-1 expression was only mildly and not significantly increased by HV in Tie2-EPCR mice (Fig. 7B). We saw no changes in ROCK-2 expression (data not shown).

Effect of MV on TATc. Given that ALI is characterized by enhanced coagulation, we tested whether there was increased thrombin production in our model and whether the APC-mediated protection observed was a function of attenuating thrombin production. Thrombin generation in response to MV was assessed via plasma measurements of TATc as described in MATERIALS AND METHODS. Although intratracheal LPS (used as a positive control) caused a significant elevation in TATc (91.93 vs. 30.54 μg/l control), HV failed to increase TATc at 4 h in WT mice compared with controls (Fig. 8A). APC treatment caused a reduction in TATc, however, this was not significant. Tie2-EPCR mice had significantly less TATc compared with WT (Fig. 8, B–D) and similarly had no increase in TATc in response to MV.

Differential pulmonary gene expression by MV and APC. Previous studies have reported that APC can influence expression of genes that might be relevant to lung injury (29), however, how APC might affect changes in expression in the lung in response to mechanical stress is unknown. We have previously described broad changes in gene expression in mouse (43) and rat (41) models of VILI. Several VILI-related candidate genes common to the two animal models were significantly upregulated. These include particularly genes related to tissue remodeling, coagulation, regulation of inflammation, chemotaxis and cell motility, cell proliferation and adhesion, and redox signaling. To assess the specific effects of APC treatment on changes in gene expression in response to HV MV exposure, we utilized Affymetrix gene expression profiling on whole lung RNA from mice exposed to 4-h HV with and without APC treatment (provided at 30 and 150 min).
compared with controls. All genes were filtered to include those with a difference in gene expression between HV$_T$ alone vs. HV$_T$ + APC of 1.5-fold or greater.

Individual genes were categorized into distinct biological pathways using MAPPFinder, a tool that combines annotations of the Gene Ontology (GO) Project with the free software package GenMAPP. As shown in Table 1, GO analysis identified specific ontologies relevant to vascular permeability such as blood vessel development, blood vessel morphogenesis, cell adhesion, hemopoiesis, and inflammation, which were significantly affected by APC treatment. Moreover, expression analysis identified specific genes pertinent to endothelial biology and lung injury, such as MAPK-activated protein kinase-2 (MK2), integrin-β1, bone morphogenetic protein 1, and Bcl-2-like-1, for which expression was significantly decreased by APC treatment compared with HV$_T$ alone (Table 2).

**DISCUSSION**

ALI is characterized by pulmonary vascular permeability resulting in severe tissue inflammation, profound hypoxia, and respiratory failure requiring support with MV. However, it has become increasingly apparent, both in animal models and in humans, that MV, although necessary for life support, can directly injure the pulmonary vascular endothelium (14) resulting in loss of integrity of barrier function and formation of a protein-rich edema in the alveolar space.

We (16) have previously demonstrated that APC confers protection against the endothelial barrier-disrupting agonist thrombin in cultured pulmonary endothelial cells, an effect that is dependent on EPCR. Here, we build on those findings and report that APC protects against a murine model of pulmonary vascular permeability and that EPCR plays a critical role in vascular barrier protection in the intact animal. To test the effects of APC and EPCR expression on pulmonary endothelial integrity, we used a well-characterized model of mechanical stress-induced pulmonary vascular injury that does not rely on a second injurious stimulus, such as endotoxin or hyperoxia, and is characterized predominantly by pulmonary vascular leakage (1, 35, 44). One notable aspect of our model is that it lacks a significant inflammatory component (as measured by increased histological and lavage neutrophil infiltration, data not shown). Differences from other models characterized by increased neutrophils (13, 25) can be explained by variations in

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**Table 1. Differential gene expression changes by HV$_T$ and APC**

<table>
<thead>
<tr>
<th>GO Name</th>
<th>Number changed, %</th>
<th>Z-Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood vessel morphogenesis</td>
<td>4 (6.45)</td>
<td>4.3</td>
</tr>
<tr>
<td>Blood vessel development</td>
<td>4 (5.56)</td>
<td>3.9</td>
</tr>
<tr>
<td>Organ morphogenesis</td>
<td>7 (3.66)</td>
<td>3.8</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>4 (4.94)</td>
<td>3.6</td>
</tr>
<tr>
<td>Cell adhesion</td>
<td>6 (3.05)</td>
<td>2.9</td>
</tr>
<tr>
<td>Hemopoiesis</td>
<td>3 (3.41)</td>
<td>2.3</td>
</tr>
<tr>
<td>Regulation of transcription from RNA polymerase II promoter</td>
<td>4 (2.68)</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Values are means (SD). HV$_T$, high tidal volume; APC, activated protein C; GO, Gene Ontology.
tidal volume or MV duration. However, the consistent endothelial barrier dysfunction and capillary leak observed in response to mechanical stress (1, 35, 44) makes our model suitable for in vivo validation of our previous work in vitro on endothelial permeability (16). There was a small but significant increase in BAL protein at 4-h LVT, however, without any increase in EBD extravasation. Although it is possible that EBD might result in some subtle increase in vascular permeability, it is likely that these changes reflect damage to the alveolar epithelium, whereas EBD leakage reflects endothelial barrier dysfunction.

The present study extends known, in vitro APC-mediated vascular barrier protection to an in vivo model of VILI and potentially provides a mechanistic basis for the protective effect of APC. These findings, taken together with our previous data demonstrating a protective effect of APC in response to thrombin in vitro (16), suggest that APC-mediated protection is not specific to inflammatory (or coagulation-related) stimuli but also extends to vascular protection against mechanical stress. It is notable that our model results in increased vascular permeability without activating coagulation, as measured by levels of Tatc. Not surprisingly, APC decreases Tatc, although this was not significant. Furthermore, EPCR-overexpressing mice, which have enhanced protein C conversion to APC and increased endothelial localization of APC (36), have lower Tatc levels compared with WT mice. Although it is possible that enhanced coagulation and thrombin production may be operative in other models of ALI, we found no evidence for APC-mediated protection through decreased thrombin production in our model.

Increasing evidence suggests that ROCK, the downstream effector of the GTPase Rho, plays a role in vascular barrier disruption by agents such as thrombin and VEGF (11, 39, 55). The Rho pathway promotes actin stress fiber formation resulting in endothelial cell contraction, paracellular gap formation, and increased permeability. Cyclic stretch has been shown to induce the Rho pathway in vitro (53); however, the role of ROCK in stretch-induced permeability in vivo has so far been untested. Our data demonstrate that injurious MV is associated with increased pulmonary ROCK-1 expression. Furthermore, we provide novel data that APC attenuates stretch-induced ROCK expression and that ROCK upregulation in response to MV was absent in EPCR-overexpressing mice. These results suggest that APC-mediated vascular barrier protection involves inhibition of ROCK-1, a mechanism that should be further explored.

These experiments suggest a critical role of EPCR in APC biology and vascular permeability. Structurally similar to the major histocompatibility class I/CD1 family of molecules, EPCR binds protein C, presenting it to the thrombin-thrombomodulin complex, thereby increasing the activation of protein C by ~20-fold (17, 42, 56). Importantly, APC can also bind EPCR, and although the bound form of APC loses its extracellular anticoagulant activity (37), increasing evidence indicates that much, if not all, of APC intracellular signaling is EPCR-dependent. APC inhibits apoptosis and alters endothelial gene expression in an EPCR-dependent manner (12, 48), and APC-mediated changes in phospho-myosin light chain and barrier protection require EPCR ligation (16). Inflammatory stimuli result in EPCR shedding from the endothelial surface, and a soluble form of EPCR (sEPCR) is elevated in plasma from patients with sepsis and lupus (33). Moreover, elevated sEPCR is associated with increased inflammation and tissue injury suggesting that membrane-associated EPCR has some protective effect, perhaps through endogenous APC signaling (52).

In the present study, we define a temporal relationship between MV-induced pulmonary vascular injury and loss of membrane EPCR, suggesting that loss of tissue EPCR is a critical component of VILI. Moreover, APC therapy is associated with prevention of EPCR downregulation and endothelial barrier protection, even after the onset of mechanical stress. Although significant changes [such as activation of p38 MAPK signaling (1, 35)] and activation of other signaling processes [e.g., activation of prooxidant enzymes such as inducible nitric oxide synthase (44) and xanthine oxidoreductase (1)] occur within a few minutes of mechanical stress, the effect of APC treatment in protecting against capillary leakage can be delayed after initiation of mechanical stress (as shown in Fig. 3) but has to precede EPCR membrane loss (2 h) since APC given at later time points after initiation of MV failed to protect against capillary leakage at 4 h (results not shown). Given that EPCR facilitates protein C activation to APC and serves as the required receptor for APC-mediated endothelial signaling, alterations in tissue EPCR expression have two potentially significant consequences, changes in APC formation (via EPCR-facilitated protein C conversion to APC) and changes in EPCR-dependent APC signaling.

Table 2. Selected genes regulated by APC

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>HIVr APC vs. HVr Ratio</th>
<th>q Value, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear receptor subfamily 1, group D, member 1 (Nrd1d)</td>
<td>0.24</td>
<td>0.00</td>
</tr>
<tr>
<td>Amiloride binding protein 1 (amine oxidase, copper-containing) (Abp1)</td>
<td>0.57</td>
<td>0.00</td>
</tr>
<tr>
<td>Chemokine (C-X-C motif) ligand 15 (Cxcl15)</td>
<td>0.42</td>
<td>0.00</td>
</tr>
<tr>
<td>Acyl-coenzyme A oxidase-like (Acox1)</td>
<td>0.43</td>
<td>0.00</td>
</tr>
<tr>
<td>Gene model 129, (NCBI) (Gm129) XM_907670</td>
<td>0.46</td>
<td>0.00</td>
</tr>
<tr>
<td>Tubulin folding cofactor E-like (Tbc1)</td>
<td>0.64</td>
<td>1.0</td>
</tr>
<tr>
<td>Acyl-CoA thioesterase 7 (Aco7t)</td>
<td>0.62</td>
<td>1.0</td>
</tr>
<tr>
<td>Integrin beta 6 (Itgb6)</td>
<td>0.62</td>
<td>2.0</td>
</tr>
<tr>
<td>RIKEN cDNA E130012A19 gene (E130012A19Rik)</td>
<td>0.64</td>
<td>2.0</td>
</tr>
<tr>
<td>ATPase family, AAA domain containing 4 (Atad4)</td>
<td>0.68</td>
<td>2.0</td>
</tr>
<tr>
<td>Protein arginine N-methyltransferase 8 (Prmt8)</td>
<td>0.73</td>
<td>2.7</td>
</tr>
<tr>
<td>Arginine vasopressin-induced 1 (Avpi1)</td>
<td>0.66</td>
<td>2.7</td>
</tr>
<tr>
<td>Transmembrane protein 82 (Tmem82)</td>
<td>0.69</td>
<td>3.4</td>
</tr>
<tr>
<td>Ras-related C3 botulinum substrate 3 (Rac3)</td>
<td>0.68</td>
<td>3.4</td>
</tr>
<tr>
<td>Phosphatidic acid phosphatase 2a (Ppap2a)</td>
<td>0.61</td>
<td>3.5</td>
</tr>
<tr>
<td>Transcript variant 2</td>
<td>0.61</td>
<td>3.5</td>
</tr>
<tr>
<td>Tumor-associated calcium signal transducer 2 (Tact2d)</td>
<td>0.66</td>
<td>3.5</td>
</tr>
<tr>
<td>Interleukin-1 receptor-associated kinase 2 (Irk2)</td>
<td>0.71</td>
<td>3.5</td>
</tr>
<tr>
<td>Transmembrane protein 192 (Tmem192)</td>
<td>0.73</td>
<td>3.5</td>
</tr>
<tr>
<td>RIKEN cDNA 4732473B16 gene (4732473B16Rik)</td>
<td>0.72</td>
<td>4.4</td>
</tr>
<tr>
<td>Brk-II-like 1 (Bcl21l), nuclear gene encoding mitochondrial protein</td>
<td>0.74</td>
<td>4.4</td>
</tr>
<tr>
<td>CTTNB2P N-terminal like (Ctnb2pnl)</td>
<td>0.72</td>
<td>4.4</td>
</tr>
<tr>
<td>Bone morphogenetic protein 1 (Bmp1)</td>
<td>0.66</td>
<td>5.0</td>
</tr>
<tr>
<td>S100 calcium binding protein G (S100 g)</td>
<td>0.67</td>
<td>5.0</td>
</tr>
<tr>
<td>MAP kinase-activated protein kinase 2 (MK2)</td>
<td>0.71</td>
<td>5.0</td>
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</table>
The notion that EPCR is central to vascular barrier protection is supported by the finding that EPCR-overexpressing mice (obtained from a C57BL/6J background mouse strain) are protected against VILI in our model. Generated with an endothelial-specific Tie2 promoter to target endothelial overexpression of EPCR, these mice have >100-fold increased pulmonary EPCR expression compared with WT. Furthermore, they have increased EPCR on both pulmonary macro- and microvascularity, a critical feature given that vascular permeability is mediated at the capillary level where EPCR expression is normally relatively low. Despite unchanged protein C mRNA levels, these mice have specific redistribution of protein C to the endothelium and resultant increased protein C activation. Phenotypically normal under basal conditions, Tie2-EPCR mice have improved survival after LPS infusion compared with WT mice (36). Further evidence of the importance of EPCR in vascular function, EPCR-deficient mice exhibit increased hemodynamic and cardiac derangements as well as increased mortality in response to endotoxin challenge (27). Our findings are the first to demonstrate that mechanical stress can alter EPCR expression and suggest a dynamic interaction between EPCR and APC: injury is a result of loss of tissue EPCR, whereas APC protects via EPCR stabilization and resultant EPCR-dependent signaling (16).

Although the effect of MV and VILI on pulmonary gene expression has been recently reported by us and other investigators (22, 41, 43), the effect of APC treatment on pulmonary gene expression in response to MV has been unexplored. Supportive of our hypothesis and evidence that APC is protective against vascular barrier dysfunction, we demonstrate that APC treatment is associated with downregulation of specific genes that participate in endothelial barrier regulation. For example, MK2 is known to mediate endothelial actin redistribution (30) and increases in vascular permeability (8) induced by hypoxia. This is of particular interest since we (16) have previously demonstrated that APC prevents thrombin-induced actin cytoskeletal changes and is barrier-protective in cultured endothelial cells. Given that endothelial cyclic stretch results in actin redistribution in vitro (7), it is possible that the increased pulmonary vascular leak observed in HV$_T$ is via MK2, a mechanism that is under investigation in our laboratory but beyond the scope of the present study.

Similarly, integrin-β$_6$, which is regulated by APC in our model, is particularly relevant to lung injury. This member of the α$_v$-integrin subfamily is highly expressed in the lungs and has been incriminated in the pathogenesis of ALI induced by lipopolysaccharide and bleomycin (46). Of note, Jenkins et al. (28) have demonstrated in a mouse model of VILI that ligation of the thrombin receptor protease-activated receptor (PAR)-1 induces integrin-β$_6$-dependent activation of transforming growth factor-β (TGF-β) and that deletion of integrin-β$_6$ protects against pulmonary edema observed in their VILI model. Integrin-β$_6$-mediated activation of TGF-β has been directly linked to and is dependent on the actin cytoskeleton (40). Furthermore, integrin-β$_6$ blockade attenuates the barrier disruptive effect and actin stress fiber formation induced by thrombin in endothelial cells. Given the coordinated signaling of APC and thrombin (4, 5, 49) and the known barrier-protective effects of APC on the endothelial cytoskeleton, it is possible that part of the protective effect of APC in VILI seen in our model is via downregulation of integrin-β$_6$ and downstream decreases in activation of TGF-β. Interestingly, another gene that was downregulated in response to APC treatment was bone morphogenetic protein 1, which is part of the TGF superfamily signaling system and can activate TGF-β (17). In addition, our data indicating that APC downregulates interleukin-1 receptor-associated kinase-2 (IRAK-2), a serine/threonine kinase that associates with the interleukin-1 receptor and participates in Toll-like receptor signaling to activate NF-κB (31), supports previous work that APC can alter NF-κB expression (29). Our finding that APC decreases expression of Bcl-2-like-1, a known regulator of apoptosis, is also notable in light of our recent report that VILI is associated with increased alveolar cell apoptosis (35). Furthermore, our data are supportive of other work that identified increased Bcl-2-like-1 expression in pulmonary endothelium in response to hyperoxia (61). Finally, we have identified novel potential VILI targets, such as Nr1d1 (Rev-erbalpha), a nuclear receptor involved in maintaining circadian rhythms (15), which is significantly elevated in mouse lung in response to HV$_T$ but returns to normal levels after APC treatment.

In summary, 2 h of HV$_T$ MV is required to induce pulmonary vascular permeability and is temporally related to decreased surface EPCR protein expression and increased ROCK-1 expression, whereas treatment with APC significantly attenuates MV-mediated leak and is associated with restoration of tissue EPCR protein and normalization of ROCK-1. These findings, taken together with our previous report that APC enhances pulmonary endothelial barrier function in an EPCR-dependent manner, strongly suggest that the protein C system is a crucial participant in vascular barrier homeostasis. Understanding the importance of EPCR in injury development and treatment might lead to novel therapies for ALI.

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DISCLOSURES

C. T. Esmon has a license with Baxter for a method to produce Ceprotin (human protein C).

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