Treatment of Sepsis-Induced Acquired Protein C Deficiency Reverses
ACE-2 Inhibition and Decreases Pulmonary Inflammatory Response

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ABBREVIATIONS: ACE, angiotensin converting enzyme; AngII, angiotensin II; APC, activated protein C; iNOS, inducible nitro oxide synthase; MIP, macrophage inflammatory protein; PAI-1, plasminogen activator inhibitor-1; PC, protein C; TARC, Thymus and activation-regulated chemokine

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ABSTRACT

The protein C (PC) pathway plays an important role in vascular and immune function, and acquired deficiency during sepsis is associated with increased mortality in both animal models and in clinical studies. However, the association of acquired PC deficiency with the pathophysiology of lung injury is unclear. We hypothesized that low PC induced by sepsis would associate with increased pulmonary injury and that replacement with activated protein C (APC) would reverse the activation of pathways associated with injury. Using a cecal ligation and puncture (CLP) model of polymicrobial sepsis, we examined the role of acquired PC deficiency on acute lung injury assessed by analyzing changes in pulmonary pathology, chemokine response, iNOS and the angiotensin pathway. Acquired PC deficiency was strongly associated with an increase in lung inflammation and drivers of pulmonary injury including AngII, TARC, PAI-1 and iNOS. In contrast, the protective factor ACE-2 was significantly suppressed in animals with acquired PC deficiency. The endothelial protein PC receptor, required for the cytoprotective signaling of APC, was significantly increased post-CLP, suggesting a compensatory up-regulation of the signaling receptor. Treatment of septic animals with activated PC (APC) reduced pulmonary pathology, suppressed the MIP family chemokine response, iNOS expression and PAI-1 activity, and up-regulated ACE-2 expression with concomitant reduction in AngII peptide. These data demonstrate a clear link between acquired PC deficiency and pulmonary inflammatory response in the rat sepsis model, and provide support for the concept of APC as a replacement therapy in acute lung injury associated with acquired PC deficiency.
Introduction

Acute lung injury (ALI) leading to acute respiratory distress syndrome (ARDS) is a significant issue in the ICU, with an incidence of 10-15% (Vincent and Zambon, 2006). Despite advances in the understanding of the pathophysiology of ALI/ARDS, and improved supportive care with optimal ventilation and fluid balance, mortality rates range from 30 to 50% (Bastarache et al., 2006). ARDS is a systemic disease with the majority of patients dying from multiorgan dysfunction (Vincent and Zambon, 2006), and there remains a significant need for new therapeutic interventions.

Infection is a common cause of pulmonary injury, and in sepsis, the lung is the most often affected organ (Vincent et al., 2003; Bastarache et al., 2006). The lung is a major source of various inflammatory mediators that affect both local and systemic immune response (Ritter et al., 2005), and recent studies have identified specific markers associated with pulmonary dysfunction. For example, chemokines associated with leukocyte migration and activation such as MIP-1α and MIP-2 have been found in close association in pulmonary lesions (Sun et al., 2006) and associated with respiratory dysfunction (Bonville et al., 2006). In addition, the T-cell chemotactic factor TARC has been found to be elevated both in humans (Manabe et al., 2005) and models of lung inflammation (Ritter et al., 2005). The induction of nitric oxide (NO) through inducible NO synthase (iNOS) also plays a significant role in the inflammatory response, and its inhibition attenuates endotoxin-induced lung injury in rats (Xia et al., 2007; Su et al., 2007). Excessive production of angiotensin II (AngII) peptide, which is generated by elevated angiotensin converting enzyme-1 (ACE-1) expression, has also been implicated in models of lung injury (reviewed in Kuba et al., 2006). Recent studies also have begun to highlight the involvement of coagulation and fibrinolysis in the pathogenesis ALI/ARDS (Schultz et al., 2006) and the potential for therapies modulating microvascular coagulation.
Therefore, multiple factors play a role in the balance of endothelial and leukocyte activation and dysfunction contributing to the pathogenesis of sepsis-induced ALI.

A key factor regulating the balance of endothelial and leukocyte function is activated protein C (APC) (O’Brien et al., 2006). Various studies have demonstrated cytoprotective and anti-inflammatory actions of APC that are mediated by its interaction with the endothelial protein C receptor (EPCR) (reviewed in Mosnier et al., 2007). In addition, low PC levels are predictive of early death during sepsis in both human (Macias and Nelson, 2004) and rat model of polymicrobial sepsis (Heuer et al., 2004). Previous studies have demonstrated that patients with ALI/ARDS exhibit low PC levels relative to normal subjects regardless of the underlying etiology of lung injury (Ware et al., 2006), and this reduction in PC levels has been associated with adverse clinical outcomes. These studies signify the role of PC pathway in response to infection, along with the demonstrated efficacy of recombinant human APC (rhAPC) in treatment of severe sepsis (Bernard et al., 2001).

Previous studies have demonstrated that APC can attenuate ALI in lung injury models (reviewed in Robriquet et al., 2006), and rhAPC decreased neutrophil migration into the airspaces and reduced local coagulation in volunteers given pulmonary endotoxin (Abraham, 2005; van der Poll et al., 2005). In a ewe model of sepsis, APC-treated animals had lower pulmonary arterial pressure and lung wet/dry ratio (Wang et al., 2007b). In the present study, we have explored the relationship between acquired PC deficiency and markers of lung injury in a rat cecal ligation and puncture (CLP) model of polymicrobial sepsis, a model known to be accompanied by acute lung failure (Martin et al., 2003). We demonstrate that acquired PC deficiency following sepsis is associated with increased tissue pathology and markers of lung inflammation, but reduced pulmonary ACE-2 expression, a factor that functions as a negative regulator of AngII production (Donoghue et al., 2000). Moreover, treatment with APC results in
suppression of lung pathology and the markers of injury and inflammation, and restoration of ACE-2 levels and reducing AngII. The data presented here suggest that low endogenous PC levels during systemic inflammatory response may be pathophysiologically related to lung injury by reducing the ability to control the cascading inflammatory responses in the lung following infection.
Methods

Anesthesia and CLP Surgery. The rat cecal ligation and puncture model of polymicrobial sepsis has been described in detail previously (Heuer et al., 2004). Briefly, Sprague-Dawley rats (245-265 g) were purchased from Harlan (Indianapolis, IN) and allowed to acclimate a minimum of 6 days prior to surgery. Animals were anesthetized with 3% isoflurane (1:1.5 with O₂) and polyethylene catheters (Strategic Applications, Inc. Libertyville, IL) were implanted surgically into the femoral vein. Immediately following femoral catheterization, CLP was performed with a single puncture with a 16G needle and care was taken to ligate the same length of cecum (1 cm measured by a ruler on the scalpel). Sham rats received identical surgery (except for CLP). Following surgery, the rats were given ketoprofen (2 mg/kg) intramuscularly for pain relief, injected subcutaneously with 5 mls of prewarmed saline and then continuously infused with 5 % dextrose in 0.9 % saline (Abbott Laboratories, North Chicago, IL) at a rate of 2 mls/hr via the femoral catheter until endpoint of the study at 22 hrs. Treatment with rat APC, prepared as previously described (Gerlitz and Grinnell, 1996), was infused at 200 ug/kg/hr starting 10 hrs post-CLP. This recombinant rat APC has been shown to be effective in preventing mortality in the rat CLP model (personal communication, Dr. Toan Huynh). Each animal was sacrificed at the 22 hr time point for collection of lung tissue for pathology, protein and mRNA analysis. The 22 hr time point was selected base on the Kaplan-Meyer survival curve for the rat CLP model as described previously (Heuer et al., 2004). At this time point, the level of PC was highly predictive of mortality outcome in this model, where early death was associated with low protein C levels. Saline vehicle was used, as previous studies have demonstrated that active site inhibited APC has no in vivo activity (Kerschen et al., 2007). All
experimental methods were approved by the Institutional Animal Care and Use Committee and were in accordance with the institutional guidelines for the care and use of laboratory animals.

**Plasma and Tissue Measurements.** Whole blood (90 ul) was collected from the retro-orbital sinus into a tube containing 10 ul of 3.8% sodium citrate and 500 mM benzamidine-HCl, and plasma collected and stored frozen until analysis. The ELISA for measurement of PC levels was performed as described previously (Heuer et al., 2004). Measurements of MIP-2, MIP1α and MIP1β were by immunoassay using the Rodent Multi-Analyte Profile (Rules Based Medicine; Austin, TX). PAI-1 activity was assayed using and a functional PAI-1 activity assay HPAIKT following the manufacturer's recommendations (Molecular Innovations Inc. Southfield, MI). Total RNA was purified from lung tissue samples that had been preserved in RNA-later (Ambion Austin, TX) using the RNeasy kit (Qiagen Valencia CA) with RNA integrity determined by agarose gel electrophoresis. DNase treated total RNA was used for first-strand cDNA synthesis primed with random hexamers using the Superscript II cDNA synthesis system (Invitrogen, Carlsbad, CA) and parallel control reactions (-RT) were performed in the absence of reverse transcriptase. TaqMan® Gene Expression Assays for TARC, iNOS, ACE-1 and ACE-2 were purchased from Applied Biosystems, (ABI, Foster City, CA).

**Determination of concentration of AngII in rat lung tissue.** Ang II levels were measured using the kit supplied by Phoenix pharmaceuticals (Belmont, CA, USA) following the manufacturer’s instructions. To determine lung AngII levels, tissue samples were homogenized in lysis buffer (10mM tris, pH 7.5). The homogenate was centrifuged at 1600xg, for 15min at 4°C. Tissue AngII concentration was measured after extraction through the Sep-Pak C-18 column supplied by the manufacturer (Phoenix peptide, CA). The peptide was eluted very slowly with 3 ml of 60% acetonitrile in 1% trifluoroacetic acid (TFA) and the fluent was collected in a polypropylene tube. The eluent was lyophilized and the residue was then dissolved in 250 µl of
RIA buffer and assayed for AngII according to manufacturer specifications. Results were normalized to the amount of protein per sample, as determined by BCA assay (Pierce, Rockford, IL). The rat AngII assay has no cross-reactivity with endothelin-1, substance P, [Arg8]-Vasopressin, and Ang1 peptides.

**EPCR Western Analysis.** Protein lysates were prepared for Western analysis using the T-PER reagent (Pierce, Rockford, IL) containing complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) from lung tissues that had been preserved in RNA-later (Ambion Austin, TX). The protein lysates were quantified by BCA assay (Pierce, Rockford, IL) and equal concentrations of each lysate were loaded for SDS PAGE and electroblotting. EPCR was detected using anti-EPCR antibody (1:1000; Zymed laboratories, San Francisco, CA). The blots were stripped and re-probed using a monoclonal antibody to β-actin (Sigma St Louis, MO) for normalization. Levels of EPCR and β-actin were quantified by analyzing the pixel density of each band from scanned autoradiograms using UnScanIt software (Silk Scientific Corporation, Orem, UT).

**Tissue Pathology and Immunohistochemistry.** For pathology and immunohistochemistry, tissues were fixed, sectioned and stained as described previously (Gupta et al., 2007). For EPCR staining, 10µg/ml of the anti-EPCR antibody (or irrelevant control antibody) followed by a biotinylated secondary antibody plus streptavidin-horseradish peroxidase kit (Dako LSAB2) was utilized along with a DAB chromagen and peroxide substrate to detect the bound antibody complexes. For iNOS, lungs were fixed in 4% paraformaldehyde and embedded in paraffin. 5µm sections were immunostained for iNOS using the automated Ventana Discovery XT staining module (Ventana Medical Systems, Tuscon, AZ). The sections were incubated with rabbit anti-mouse iNOS (5µg/ml, BD Transduction Labs) for 60 minutes followed by biotinylated goat anti-rabbit IgG (1:200, DAKO) for 20 minutes. Detection was performed
using Ventana’s DAPMap kit and sections were taken offline for routine counterstaining with hematoxylin. MIP1α staining was performed similarly on the Ventana using a rabbit anti-rat MIP1α (Peprotech Catalog#500-P77) for 1 hour followed by the secondary Dako anti-rabbit. The slides were reviewed using light microscopy to evaluate the intensity and localization of the staining.

Data and Statistical analyses. One way ANOVA or multivariate analyses were used to determine statistical significance with JMP5.1 software (SAS Institute). Data are presented as the mean +/- the SEM, unless indicated otherwise. A p value of < 0.05 was considered significant. Receiver operator characteristic (ROC) curves generated from logistic regression models were performed using JMP5.1 software.
Results

**Endogenous protein C and lung inflammation.** Previous studies in the rat CLP model have demonstrated that a subset of animals exhibit a rapid drop in plasma PC level, and this acquired deficiency in PC is predictive of poor outcome (Heuer et al., 2004). The rapid drop in PC to a level of <60% baseline demonstrated a 100% sensitivity and specificity for early death by ROC analysis (Berg et al., 2006). We were interested in determining a possible relationship between pulmonary dysfunction and low PC level. Shown in Fig. 1A is the distribution of plasma PC levels at 22 hrs post CLP compared to surgical sham animals as a control population. Approximately half (14/25) of the animals had a significant reduction in PC levels, using the previously defined cut-off of < 60% of baseline (Berg et al., 2006). As indicated in the introduction, the activation of the MIP chemokines and TARC have been characterized as markers of pulmonary dysfunction, so we analyzed levels of these markers with respect to the PC deficiency. As shown in Fig. 1B, animals with low PC had a significant elevation in pulmonary TARC expression (3.5 +/- 0.3 fold), compared to no change in TARC in those animals that maintained PC levels within the normal range. Similarly, the MIP chemokines were significantly elevated in the low PC animals (Fig.1C) but not in the normal PC animals. Moreover, there were significant negative correlations between plasma PC and TARC ($r^2 = -0.62$, $p<0.001$), MIP2, ($r^2 = -0.61$, $p < 0.005$), MIP1α ($r^2 = -0.63$, $p < 0.002$), and MIP1β ($r^2 = -0.70$, $p < 0.0005$). Unlike these chemokines, the cytokines IL1, TNFα and IL6 were not associated with PC deficiency at 22 hrs, likely because they peak earlier that the chemokines, which continue to increase from 10hrs to the 22hr time point (Supplemental Table S1).

Acute lung injury results in increased coagulation, and locally suppressed fibrinolysis as a result of strongly elevated levels of PAI-1 (Schultz et al., 2006). As shown in Fig. 1D, animals
with PC deficiency had significantly elevated levels of pulmonary PAI-1 activity compared both to sham animals and animals whose PC level had not dropped. Although IL6 levels in the plasma were not significantly different in low PC animals, we assessed levels directly in the lung, as IL6 is strongly induced by local thrombin generation (Shin et al., 1999). As shown in Fig. 2A, there was a significantly higher level of pulmonary IL6 in the low PC animals, with normal PC animals no different than shams. We also examined the level of MCP1 as another inflammatory activator induced by thrombin (Wang et al., 2007a) and observed significant elevation in the low PC animals. Moreover, the correlation between the level of pulmonary IL6 and MCP1 was $r=0.93 \ p<0.00001$, $n=25$.

**Endogenous protein C, lung inflammation and pulmonary iNOS.** To further explore the relationship of low PC and tissue injury, we examined the degree of pulmonary inflammation and pathology at 22 hrs post-CLP. As shown in Fig. 3A, animals with low PC showed increased pulmonary congestion and edema, alveolar fluid accumulation and had a significantly greater degree of leucocyte infiltration and margination to the vascular endothelium vs. animals with normal PC levels. This increased margination was clearly observed using immunohistochemistry with ED1 (Fig. 3B) and MPO (data not shown). We also stained for MIP1α chemokine expression and as shown in Fig. 3C observed significant margination of MIP1α–positive cells in the low PC animals but not in those with normal PC. As would be suggested by the ED1 staining, the majority of the MIP1α–positive cells were mononuclear (Supplemental Fig. S1).

We also assessed the level of iNOS expression in the lung and found a large increase (mean of 25 fold) in the low PC population relative to the normal PC group (Fig. 4A), which was highly negatively correlated with the change in plasma PC levels in the study (Fig. 4B). Using immunohistochemistry for iNOS expression, we observed a significant increase in the number of
iNOS positive cells in the tissue, and in the degree of margination of iNOS positive leukocytes to the vasculature (Fig. 4C). As shown in Supplementary Fig. S2, these iNOS-stained cells were predominately mononuclear as was observed with the ED1 and MIP1α staining.

**Low PC and ACE-2.** ACE-2 is a negative regulator of AngII production and its deficiency has been shown to be associated with worsening of lung function and leukocyte accumulation in acute lung injury (Imai et al., 2005). Therefore, we sought to determine if there was any relationship between the PC pathway and this protective factor. As shown on Fig. 5A, animals with low PC had significantly lower level of ACE-2 expression in the lung compared to animals whose PC level had not dropped. In contrast, the level of ACE-1 expression in the lung was increased by ~50% (p <0.001) in the CLP animals in both the low PC and normal PC animals (data not shown). As an increase in ACE-1 should drive higher levels of AngII, whereas ACE-2 should counter-regulate the induction, we measured the level of AngII peptide in the lung. As shown in Fig. 5B, there was an 8-fold increase in the AngII level in the low PC animals but significantly lower levels in the normal PC animals, consistent with the reduced ACE-2 in the low PC group. Taken together, the above data suggest a strong association between acquired PC deficiency following polymicrobial sepsis and the degree of pulmonary injury and inflammation.

**The receptor for APC is up-regulated in rat and human lung injury.** The anti-inflammatory and cytoprotective effects of APC have been shown to require EPCR (Mosnier et al., 2007); however, little is know regarding its regulation during pulmonary injury. We examined the levels of EPCR in the lungs following CLP and found a 2.5 fold increase in mRNA, which was significantly higher in animals with low PC (Fig. 6A), and a corresponding increase in the level of protein expression by Western blot analysis (Fig. 6B). We also assessed EPCR by immunohistochemistry and as shown in Fig. 6C, we observed staining for EPCR in the
small vessels, consistent with previous reports (Gupta et al., 2007). As was observed with the mRNA and protein analyses, the level of EPCR by immunohistochemistry was elevated in the low PC animals as shown by increased staining in the vasculature as well as in the infiltrating leukocytes. Thus, injury to the lung appears to result in an increase in the key vascular factor mediating the anti-inflammatory signaling of APC.

**Treatment with APC reduces pulmonary pathology, iNOS and inflammation.** The low level of circulating PC likely compromises the ability to naturally generate APC, thereby resulting in a reduction in the endogenous mechanism to protect from pulmonary injury. To test this hypothesis, we examined the effect of infusing rat APC on pulmonary pathology, level of iNOS expression, leukocyte margination and chemokine inflammation markers. Animals were subjected to CLP, and 10 hrs later infused with vehicle or APC for 12 hrs prior to sacrifice. As shown in Fig. 7A, treatment of animals with APC starting at 10 hrs post CLP resulted in a significant decrease in the level of iNOS mRNA expression in the lung. As the level of iNOS mRNA was highly related both to the degree of leukocyte margination and tissue pathology in the untreated animals above, we assessed the effect of APC treatment on pulmonary iNOS expression by immunohistochemistry. As shown in Fig. 7B, following APC treatment we did not observe the high degree of iNOS positive cells marginating to the vasculature that was seen in untreated animals, as shown in Fig. 4C.

As the MIP chemokines were significantly associated with lung injury and low PC, and were highly correlated with the level of iNOS ($r^2 = 0.84$, $p < 0.001$), we assessed the effect of APC treatment on level of MIP chemokine family members. As shown in Fig. 8, MIP2, MIP1α and MIP1β were significantly reduced by the APC treatment. These data suggest that APC down-regulates chemokine-mediated responses during CLP that may account for the observed improvement in lung pathology. As was observed with iNOS, we found very few
MIP1α-positive cells by immunohistochemistry following APC treatment (data not shown). Although we observed a significant suppression of leukocyte margination in the lung by APC treatment, we observed no significant effect on peripheral leukocyte populations (data not shown).

We examined the effect of APC treatment on both IL6 and MCP1 in the lung and as shown in Fig 9, both of these thrombin-activated cytokines were significantly suppressed by APC treatment. The reduction in local IL6 in the lung, but not in the plasma, by APC treatment suggests the effect of APC may be directly on lung. However, we also examined the effect of APC on the systemic inflammatory response, using multiplex profiling as previously described (Heuer et al., 2004). As shown in Supplemental Table S2, which lists the significant fold changes in plasma level following treatment, APC broadly reduced inflammatory markers associated with leukocyte activation and infiltration, suggesting a significant effect on the systemic inflammatory component in this model. However, as also shown, APC reduced tissue factor, increased fibrinogen levels and decreased d-dimer. Thus, APC treatment does result in significant changes consistent with an anti-inflammatory role, but also reduces systemic coagulopathy as would be expected. Further studies will be required to better define if the effect of APC on the lung is local, or the result of suppression of systemic inflammatory and coagulopathic responses.

As previous studies have demonstrated that APC-dependent inhibition of apoptosis is related to the protective effect in the kidney (Gupta et al., 2007), we examined the effect of APC on active caspase levels in the lung. At the 22hr time point, we observed very few apoptotic cells in the lung (20-30 caspase positive cells per section), making it unlikely that apoptosis is playing a significant role in the degree of lung inflammation and thus in the protection with APC treatment.
Treatment with APC increases lung ACE-2 and decreases AngII and PAI-1. As indicated above, low PC was significantly associated with a reduction in the ACE-2 and increased AngII. Therefore, we examined the effect of infusing rat APC on both of these factors. As shown in Fig. 10A, treatment of animals at 10 hrs post CLP with APC resulted in a significant increase in the level of ACE-2, while at the same time resulting in a significant decrease in the level of pulmonary AngII peptide (Fig. 10B).

Several studies have shown an association between elevated AngII and increased PAI-1. Similar to AngII levels, there was a significant rise in active PAI-1 in the lungs of the vehicle-treated animals, however, those treated with APC were completely protected (Fig. 10C). Overall, the data show that APC treatment significantly reduces pulmonary pathology and markers of injury, at the same time enhancing the protective ACE-2 pathway.

Suppression of ACE-2 in lung endothelium by inflammatory mediators and protection with APC. We examined whether or not ACE-2 expression could be inhibited in vitro, using human cultured pulmonary endothelial cells. We found that a cytokine mix (IL1, TNFα, IFNγ) significantly reduced ACE-2 expression (Fig. 11). Moreover, treatment of the pulmonary endothelial cells with MIP1α dramatically reduced ACE-2 protein levels. As also shown, treatment of cells with APC in the absence of the inflammatory mediators had no effect on expression of ACE-2 protein, however, it significantly blocked the down-reduction by the inflammatory mediators.
Discussion

We have examined the role of the PC pathway in lung injury using a rat model of polymicrobial sepsis, where like in humans, PC deficiency can develop following systemic inflammatory response. We found that acquired PC deficiency was associated with adverse pathology and showed increased level of known markers of pulmonary dysfunction and inflammation including increased level of TARC, MIP chemokines, PAI-1 and iNOS. Moreover, acquired PC deficiency was associated with suppression of the protective factor ACE-2, with associated elevation in AngII peptide. The data supported the hypothesis that animals with low PC had a compromised ability to generate their own APC resulting in increased sensitivity to tissue injury. To provide evidence for this, we administered recombinant rat APC and found significantly improved pulmonary pathology, decreased iNOS/chemokine response, and a recovery of ACE-2 with concomitant decrease in pulmonary AngII levels.

Acquired PC deficiency was associated with a significant elevation of the MIP-family chemokine response, which could be reversed by APC treatment. The chemokine response appears to be very important driver of pulmonary injury and is critical for amplification of the host response during infection. Sun et al., (Sun et al., 2006) demonstrated that MIP-1β and associated T cells response were found to be in close association in pulmonary lesions. Using microarray studies, Bonville et al., (Bonville et al., 2006) identified expression profiles of the proinflammatory mediators MIP-1α and MIP-2 that correlated with persistent respiratory dysfunction in a murine model of pneumonia. In our studies, the increase in MIP chemokines was negatively correlated with PC level but highly correlated with the level of iNOS. Kim et al. (Kim et al., 2003) have suggested that iNOS may regulate chemokine response during injury, which correlates with the degree of tissue inflammatory response (reviewed in Gupta et al., 2007).
Several reports have demonstrated an increase in pulmonary NO production in response to sepsis. Fujii et al., have shown an enhanced production of pulmonary NO in septic animals, which was contributed by macrophage mediated iNOS expression (Fujii et al., 1998). In addition, iNOS-deficient mice exhibit reduced pulmonary albumin vascular leakage during endotoxemia (Wang et al., 2002). These findings suggest that iNOS plays a detrimental role in the pathogenesis of sepsis induced lung dysfunction. In the present study, we observed increased iNOS expression in lungs of PC-deficient animals, largely the result of increased margination of iNOS-positive leukocytes. While there were MPO-stained cells in the lung, most of the marginating leukocytes appeared to be mononuclear in origin, as indicated by the ED-1 and MIP1α staining and by histopathological determination, and were substantially reduced following APC treatment. Nick et al., have shown reduction in neutrophil accumulation into the airways in a localized pulmonary inflammation model induced by bacterial pneumonia following APC treatment (Nick et al., 2004), and MPO staining was reduced in our study (data not shown). Thus, it appears that both neutrophil and iNOS positive mononuclear cell infiltration is suppressed by APC in the lung. In terms of other cell types in the lung, recent studies have demonstrated the important role of mast cells and IL-15 in the CLP model (Orinska et al., 2007), however, we found no effect of APC on either IL-15 or the number of T-blue staining cells in the lung, suggesting that the protective effect does not involve mast cells.

The lung possesses its own renin-angiotensin system that acts independently of the circulating system in terms of pulmonary function. A key element of the system is the enzyme ACE-2, which hydrolyzes AngII to the vasodilator Ang (1-7), thereby regulating the net level of AngII and facilitating the mitigation of the biological actions of AngII (Ferrario et al., 2005). Increasing ACE-2 levels by administration of recombinant protein has been shown to improve ALI associated with acid aspiration and CLP-induced sepsis (Imai et al., 2005). In the present
study, PC deficiency was associated with a marked down-regulation of ACE-2 expression coupled with elevated AngII levels and worsened lung pathology. Moreover, administration of APC restored ACE-2 expression and reduced AngII peptide. As AngII has been shown to induce neutrophil accumulation in vivo (Nabah et al., 2004), reduction of pulmonary AngII levels by APC may contribute to its ability to suppress leukocyte adhesion in the present study. In addition, reduction in AngII has been shown to improve the fibrinolytic balance by reducing plasma PAI-1 levels (Arndt et al., 2006), and local suppression of fibrinolysis as a result of elevated PAI-1 levels has been demonstrated in ALI, pneumonia and sepsis (reviewed in Schultz et al., 2006). Therefore, the reduction in AngII by APC treatment may have contributed to the observed reduction in PAI-1 levels by APC, although the ability of APC to directly inhibit PAI-1 is also likely contributing. Collectively, these data suggest a role for APC in modulating pulmonary function by altering ACE-2 expression, which may also contribute to reduction in PAI-1 and, along with the inhibition of chemokine activation, play a role in the suppression of iNOS positive leukocyte infiltration in the lung. In support of this last point, we observed a highly significant negative correlation ($r^2 = -0.8, p<0.0001$) between the level of ACE-2 and iNOS in the lung.

EPCR mediates the receptor-associated anti-inflammatory and cytoprotective effects of APC (Mosnier et al., 2007) and we now show higher level of EPCR expression in the lung post sepsis. Several studies have suggested that EPCR is suppressed during tissue injury (Dahlback and Villoutreix, 2005), although EPCR has been shown to be increased following renal injury (Gupta et al., 2007). The increase in pulmonary EPCR may be a compensatory mechanism to allow for increased protective signaling via APC. The even higher levels of EPCR in animals with low PC shown in Fig. 5, i.e. with a reduced ability to generate endogenous PC, would be consistent with this hypothesis.
Overall, our studies suggest that acquired PC deficiency in sepsis may be pathophysiologically related to compromised pulmonary function, likely due to the inability to generate sufficient APC to limit inflammation and tissue injury. In acute lung injury associated with acquired PC deficiency, APC treatment may provide a means of replacing the loss of a natural protective mechanism.
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Footnotes

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Legends for Figures

**Fig. 1.** Association of low PC and markers of lung injury in the rat CLP model of polymicrobial sepsis. (A) The levels of plasma PC were determined in the plasma of rats 22 hrs post-CLP or after sham surgery (n = 10 Sham, 25 CLP). Low PC was defined as less than 60% of baseline as described in the Methods based on ROC curve analysis. (B) PC deficiency and chemokine TARC levels. The levels of TARC expression were determined in lung tissue collected 22 hrs post-CLP or sham surgery. (C) Relationship between the acquired PC deficiently and MIP chemokines. Data are mean +/- SEM. (D) PC deficiency and PAI-1 activity in the lung. The levels of PAI-1 were determined 22 hrs post-CLP or after sham surgery using an assay that only detected active material. Values are mean +/- SEM; n= 8 sham, n=11 normal PC and n=13 low PC.

**Fig. 2.** Determination of the levels of IL6 and MCP1 as function of acquired PC deficiency. (A) IL6 and (B) MIP1α levels were determined in lung tissue by immunoassay. Values are mean +/- SEM; n= 8 sham, n=12 normal PC and n=13 low PC.

**Fig. 3.** Analysis of tissue pathology as function of acquired PC deficiency. (A) Representative histology with hematoxylin and eosin (H&E) demonstrating pulmonary congestion and edema, alveolar fluid accumulation and increased alveolar macrophages and neutrophil infiltration in the low PC animals, in contrast to the normal PC animals. (B) Immunohistochemistry using anti-ED1 demonstrating significant leukocyte margination to the vasculature (arrow) in low PC animals in contrast to normal PC animals.
Fig. 4. Inducible nitric oxide (iNOS) expression as a function of the degree of acquired PC deficiency in the rat CLP model. (A) Quantification of the level of iNOS mRNA expression in lungs as a function of PC level and (B) as a function of the % change in plasma PC from baseline. (C) Immunohistochemistry of representative animals with normal PC and low PC stained for iNOS expression 22 hrs after CLP. Increased numbers of iNOS positive cells marginating to the vessels are indicated.

Fig. 5. Analysis of ACE-2 and Angiotensin-II (AngII) in the rat CLP model. (A) The level of ACE-2 expression as a function of the PC level 22 hrs post-CLP is shown relative to sham animals. (B) Assessment of AngII peptide levels in the lung of rats 22 hrs post-CLP. Values are mean +/- SEM. n= 8 Sham, n=12 normal PC and n=13 low PC.

Fig. 6. Expression of EPCR, the APC receptor, in the lungs of CLP animals. (A) Analysis of mRNA expression of EPCR as a function of PC level in rats at 22 hrs post-CLP and in surgical sham animals. Values are mean +/- SEM. n= 8 Sham, n=9 normal PC and n=7 low PC. (B) Representative Western blot showing increased EPCR protein expression. (C) Immunohistochemistry of lung tissue from representative low and normal PC animals using and anti-EPCR antibody. Intense staining of the vessels and EPCR-positive leucocytes are noted.

Fig. 7. Effect of APC treatment on the pulmonary iNOS. (A) Comparison of change in iNOS from 10 hr baseline (pretreatment) to 22 hr study endpoint with APC treatment. APC was infused starting at 10 hrs post-CLP at a dose of 200 ug/kg/hr and continued until 22 hrs post-CLP. Steady-state APC blood levels were 96 +/- 8 ng/ml. Data are the mean +/- SEM, n = 8.
sham, 14 vehicle and 12 APC- treated. (B) Immunohistochemistry of lung of a representative APC-treated animal using an anti-iNOS antibody, showing very little leukocyte margination.

**Fig. 8.** Effect of APC treatment on MIP2, MIP1α and MIP1β chemokines. The level were determined in the plasma by immunoassay. Data are the mean +/- SEM, n = 22 vehicle and 19 APC- treated.

**Fig. 9.** Effect of APC treatment on the level of IL6 and MCP1 in the lung. Levels of (A) IL6 and (B) MCP1 were determined by immunoassay and made relative to the amount of total lung protein. Data are the mean +/- SEM, n= 8 sham, n = 14 vehicle and 13 APC- treated.

**Fig. 10.** APC treatment modulates pulmonary ACE-2, AngII and PAI-1. Effect of APC treatment on (A) ACE-2, (B) AngII and (C) PAI-1 activity in lung tissue. Infusion of vehicle or recombinant rat APC was starting 10 hrs post-CLP at a dose of 200ug/kg/hr and continued until 22 hrs post-CLP and sacrifice of the animals. Data are the mean +/- SEM, n = 24 vehicle and 20 APC- treated.

**Fig. 11.** Effect of inflammatory mediators and APC on ACE-2 expression in pulmonary microvascular endothelial cells. Cell were growing in Endothelial Growth Media (Clonetics, Walkersville, MD) and treated with a cytokine mix (CM, 10 ng/ml TNFα, 10 ng/ml IL-1β and 25 ng/ml IFN-γ; R&D Systems) or with MIP1α (1ng/ml) with or without APC ( 30 nM). The level of ACE-2 determined by immunoassay. * p< 0.05.
Figure 1

(A) Protein C levels as a percentage of baseline in Sham, Low PC, and Normal PC groups, with statistical significance indicated.

(B) Lung TARC level (relative units) in Sham, Low PC, and Normal PC groups, with statistical significance indicated.

(C) Bar graph showing chemokine fold change for MIP2, MIP1α, and MIP1β in Normal PC and Low PC groups, with a p-value of <0.005.

(D) Lung Active PAI-1 level (relative units) in Sham, Low PC, and Normal PC groups, with statistical significance indicated.
Figure 2

(A) Lung IL-6 Level (pg/mg protein)

(B) Lung MCP1 Level (pg/mg protein)
Figure 4

A. Scatter plot showing lung iNOS expression (fold change from sham) with Low PC and Normal PC groups.

B. Scatter plot showing the relationship between Protein C (% of baseline) and iNOS fold change with a correlation coefficient of r = 0.94 and p < 0.0001.

C. Photomicrographs showing normal PC and low PC conditions.
Figure 5
Figure 7

(A) Pulmonary iNOS Expression (Fold Change from baseline)

Vehicle

APC

p < 0.005

(B) Photomicrograph of lung tissue stained for iNOS expression.
Figure 8

Fold Change from baseline

- **MIP2**
  - Vehicle: 4.0
  - APC: 2.0
  - p < 0.05

- **MIP1α**
  - Vehicle: 20.0
  - APC: 10.0
  - p < 0.05

- **MIP1β**
  - Vehicle: 3.0
  - APC: 2.0
  - p < 0.05
Figure 10

(A) Lung ACE-II (% of Sham Level)

(B) AngII (ng/mg protein)

(C) Lung Active PAI-1 Level (Relative Units)

P < 0.01

P < 0.05

Sham

CLP Vehicle

CLP APC
Percent change in ACE-2 (relative to control)

- APC
+ APC

Control
CM
MIP-1α

Figure 11