Role of Protein C in Renal Dysfunction after Polymicrobial Sepsis

Akanksha Gupta,* David T. Berg,* Bruce Gerlitz,* Ganesh R. Sharma,* Samreen Syed,† Mark A. Richardson,* George Sandusky,† Josef G. Heuer,* Elizabeth J. Galbreath,† and Brian W. Grinnell*

*Biotechnology Discovery Research and †Pathology, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, Indiana

Protein C (PC) plays an important role in vascular function, and acquired deficiency during sepsis is associated with increased mortality in both animal models and in clinical studies. This study explored the consequences of PC suppression on the kidney in a cecal ligation and puncture model of polymicrobial sepsis. This study shows that a rapid drop in PC after sepsis is strongly associated with an increase in blood urea nitrogen, renal pathology, and expression of known markers of renal injury, including neutrophil gelatinase–associated lipocalin, CXCL1, and CXCL2. The endothelial PC receptor, which is required for the anti-inflammatory and antiapoptotic activity of activated PC (APC), was significantly increased after cecal ligation and puncture as well as in the microvasculature of human kidneys after injury. Treatment of septic animals with APC reduced blood urea nitrogen, renal pathology, and chemokine expression and dramatically reduced the induction of inducible nitric oxide synthase and caspase-3 activation in the kidney. The data demonstrate a clear link between acquired PC deficiency and renal dysfunction in sepsis and suggest a compensatory upregulation of the signaling receptor. Moreover, these data suggest that APC treatment may be effective in reducing inflammatory and apoptotic insult during sepsis-induced acute renal failure.

Acute renal failure (ARF) is a significant and devastating disorder (1), with a prevalence varying from 5 to 7% in all hospitalized patients and 30 to 50% in patients who are admitted to the intensive care unit (2). Sepsis is a major cause of ARF in hospitalized patients and is the most important trigger for ARF in the intensive care unit (3). In patients with severe sepsis or shock, the incidence of ARF is reported to be from 23 to 51% (4–6), and the combination of sepsis and ARF is associated with mortality as high as 70% compared with 45% among patients with ARF alone (1,7). Despite advances in medical practice during the past several decades, the high mortality in patients with ARF has remained unchanged (8).

The pathophysiology of ARF involves a complex interplay among various processes, including tubular and endothelial cell injury and inflammation. Microvascular dysfunction after injury to the kidney seems to play a key role in initiating and extending tubular injury and is associated with significant inflammatory activation that contributes to the extension of renal dysfunction (reviewed in reference [9]). In particular, inflammatory chemokines, including CXCL1 (keratinocyte-derived chemokine, Gro-α) and CXCL2 (MIP-2), show a progressive induction associated with ARF in polymicrobial sepsis (10–12). Wu et al. (13) suggested that GRO chemokines are expressed coordinately by mesangial cells and inflamed glomeruli and are key mediators that amplify acute kidney injury (AKI). Moreover, CXCL1 seems to be a useful biomarker for renal injury in both rodent models and humans (14). In both humans and model systems, the accumulation of leukocytes has been well demonstrated during ARF (15,16), and activated leukocytes can initiate an inflammatory cascade that leads to endothelial cell injury and disruption of the endothelial permeability barrier, thereby contributing to renal injury (17).

Although many soluble factors change during sepsis, there is substantial evidence that a reduction in plasma level of the serine protease protein C (PC) is prognostic for sepsis and sepsis severity (reviewed in reference [18]). Studies have also suggested that PC deficiency may appear before the onset of defined clinical parameters of severe sepsis or septic shock (19). Moreover, low PC levels are predictive of early death in a rat model of polymicrobial sepsis (20) and clinically have been associated with early death resulting from refractory shock and multiple-organ failure in severe sepsis (21). These studies support the important role of the PC pathway in response to infection, which is further exemplified by the efficacy of recombinant human activated PC in the treatment of severe sepsis (22). However, the relationship of PC levels or treatment with activated PC (APC) specifically to renal function in sepsis is unclear.

PC is a member of the vitamin K–dependent family of blood coagulation proteins and circulates as an inactive zymogen. It is
converted to the activated form (APC) under conditions of thrombotic and inflammatory stress by thrombin in complex with endothelial surface thrombomodulin. APC functions as a feedback inhibitor of thrombin generation (23) and has receptor-mediated anti-inflammatory and cytoprotective effects through interaction with the endothelial PC receptor (EPCR) (24–26); recent studies have also shown that APC can inhibit leukocyte rolling and adhesion (27,28). APC plays a fundamental role in a coordinated system for controlling thrombosis, limiting inflammatory responses, and potentially decreasing endothelial cell apoptosis in response to inflammatory cytokines and ischemia (24).

In this study, we explored the role of PC in modulating renal function in a rat cecal ligation and puncture (CLP) model of polymicrobial sepsis. We demonstrate that the rapid suppression of endogenous PC after sepsis is associated with increased renal dysfunction and tissue pathology. Moreover, treatment with APC resulted in suppression of renal pathology, markers of injury and inflammation, and improved renal function. Our data further suggest that APC may exert renoprotective function in part by suppressing inducible nitric oxide synthase (iNOS) and subsequent renal apoptosis. Overall, our studies suggest that acquired PC deficiency in polymicrobial sepsis may be pathophysiologically related to compromised renal function, likely as a result of the inability to generate sufficient APC to limit inflammatory and ischemic injury. Moreover, our data suggest that APC offers a potential therapy for sepsis-induced ARF.

Materials and Methods

Anesthesia and CLP Surgery

The rat CLP model of polymicrobial sepsis was described in detail previously (20). Briefly, Sprague-Dawley rats (245 to 265 g) were purchased from Harlan (Indianapolis, IN) and allowed to acclimate for a minimum of 6 d before surgery. Rats were anesthetized with 3% isoflurane (1:1.5 with O2) and polyethylene catheters (Strategic Applications, Libertyville, IL) were implanted surgically into the femoral vein. Immediately after femoral catheterization, CLP was performed with a single puncture with a 16-G needle to obtain an expected mortality of approximately 75% by 4 d; 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). After surgery, the rats were given ketoprofen (2 mg/kg) intramuscularly for pain relief, in-
r

Plasma and Tissue Measurements

Whole blood (90 μl) was collected from the retro-orbital sinus into a tube that contained 10 μl of 3.8% sodium citrate and 500 mM benzamidine-HCl, then the mixture was centrifuged briefly to pellet cellular components and the plasma supernate was collected and stored frozen until analysis. The ELISA for measurement of PC levels was performed as described previously (20), and purified recombinant rat PC was used as a reference standard. Measurements of CXCL1 and CXCL2 were by immunoassay using the Rodent Multi-Analyte Profile (Rules Based Medicine, Austin, TX). BUN was determined using a Hitachi 911 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN). Total RNA was purified from kidney tissue samples that had been preserved in RNA-later (Ambion, Austin, TX) using the RNaseasy kit (Qiagen, Valencia, CA). RNA integrity was 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). Parallel control reactions were performed in the absence of reverse transcriptase. TaqMan Gene Expression Assays for CXCL2 and CXCL1 and for the 18S rRNA eukaryotic endogenous control kit were purchased from Applied Biosystems (ABI, Foster City, CA). QuantGene Plex (Panomics, Fremont, CA) was used for the quantification of neutrophil gelatinase-associated lipocalin (NGAL) mRNA levels according to the manufacturer’s recommendation.

Active Caspase 3 Western Analysis

Protein lysates were prepared for Western analysis using the T-PER reagent (Pierce, Rockford, IL), which contained complete protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) from kidney tissues that had been preserved in RNA-later. The protein lysates were quantified by BCA assay (Pierce), and equal concentrations of each lysate were loaded for SDS-PAGE and electrophoretic transfer. Cleaved caspase-3 was detected using the Apoptosis Marker: Cleaved Caspase-3 (Asp175) Western Detection Kit (Cell Signaling Technology, Danvers, MA). The blots were stripped and reprobed using a mAb to β-actin (Sigma, St. Louis, MO) for normalization. Levels of cleaved caspase-3 and β-actin were quantified by analysis of the pixel density of each band from scanned autoradiograms using UnScanIt software (Silk Scientific Corp., Orem, UT).

Tissue Pathology and Immunohistochemistry

For pathology and immunohistochemistry, tissues were fixed, sectioned, and stained as described previously (30). For EPCR staining, 10 μg/ml of the anti-EPCR antibody (or isotype control antibody) followed by a biotinylated secondary antibody plus streptavidin–horseradish peroxidase kit (Dako LSAB2, Carpinteria, CA) was used along with a diaminobenzidine chromagen and peroxide substrate to detect the bound antibody complexes. For determination of localization of active caspase-3, 5 μg/ml anti–caspase-3 (Cell Signaling # 96645) was used for 6 h on Ventana Autostainer. The slides were reviewed by two investigators using light microscopy to evaluate the intensity and the localization of the staining.

Human tissue specimens were retrieved from the tissue bank of Lilly Research Laboratories. These tissues were obtained from the Cooperative Human Tissue Network using an institutional review board–approved protocol. All human samples were derived from surgical specimens that were obtained between 1996 and 1999.

Statistical Analyses

One-way ANOVA or multivariate analyses were used to determine statistical significance with JMP5.1 software (SAS Institute, Cary, NC).
Data are presented as the means ± the SEM, unless indicated otherwise. *P* < 0.05 was considered significant. Receiver operator characteristic curves that were generated from logistic regression models were performed using JMP5.1 software.

**Results**

**Endogenous PC and Renal Function**

Previous studies in the rat CLP model demonstrated that a subset of animals exhibit a rapid drop in plasma PC level and that this acquired deficiency in PC is predictive of poor outcome in this model (20). Moreover, the rapid drop in PC to a level of <60% baseline demonstrated a 100% sensitivity and specificity for early death by receiver operator characteristic analysis (31). We were interested in determining a possible relationship between renal function, which has been shown to be compromised in the CLP model (32), and low PC level. Shown in Figure 1A is the distribution of plasma PC levels at 22 h after CLP compared with surgical sham rats as a control population. Approximately half (13 of 25) of the rats had a significant reduction in PC levels, using the previously defined cutoff of <60% baseline. An analysis of BUN levels in these rats revealed a significant elevation in both the absolute level (Figure 1B) and change from baseline (Figure 1C) in rats with low PC and no change in BUN in rats that maintained PC levels within the normal range. Moreover, there was a significant negative correlation between plasma PC and BUN (r² = −0.70, *P* < 0.0003).

Recent studies suggested that Ngal, produced by neutrophils and the renal proximal tubules, is an early biomarker of acute renal injury (33) and clinically is associated with increased BUN (34). Therefore, we examined the renal expression of Ngal as a further assessment of renal injury associated with the acquired PC deficiency. As shown in Figure 1D, the level of Ngal was significantly higher in rats with low PC compared with both sham and normal PC rats. The data suggest that low PC contributes to loss in renal function as indicated by elevated BUN and Ngal levels.

**Endogenous PC and Renal Pathology**

To explore further the relationship of low PC and tissue injury, we examined the degree of corticomedullary acute tubular necrosis (ATN) 22 h after CLP. As shown in Figure 2A, rats with low PC had a significantly greater degree of kidney pathology, as evidenced by an increased degree of tubular necrosis. The mean pathology score was significantly higher in rats with low PC versus rats that remained in the normal range (Figure 2B). Moreover, there was a significant negative corre-
lation between PC level and the degree of tissue pathology ($r^2 = -0.73$, $P < 0.001$).

During polymicrobial sepsis, chemokines including CXCL1 and CXCL2 show a progressive induction that is associated with ARF (10,11). Therefore, we examined the expression of both CXCL1 and CXCL2 in the kidneys of both sham and CLP rats. As shown in Figure 2C, the degree of induction of both these chemokines was significantly higher in kidneys of rats with low PC levels. Taken together, these data suggested a strong association between the rapid drop in plasma PC and the degree of kidney damage and dysfunction in the rat CLP model.

Receptor for APC Is Upregulated in Rat and Human Kidney Injury

The anti-inflammatory and cytoprotective effects of APC have been shown to require EPCR; however, little is know regarding its regulation during kidney injury (35). We examined the levels of EPCR in the kidneys after CLP and found a 4.5-fold increase in mRNA, which was significantly higher in rats with low PC (Figure 3A), and a corresponding increase in the level of protein expression by Western blot analysis (Figure 3B). Moreover, we examined the levels of EPCR in rats with acquired PC deficiency, and, as shown in Figure 3B, rats with low PC levels had significantly higher induction of renal EPCR protein. We also assessed EPCR by immunohistochemistry, and, as shown in Figure 3C, we observed staining for EPCR in the small vessels of control kidney, consistent with previous reports (36). As was observed with the mRNA and protein analyses, the level of EPCR by immunohistochemistry was significantly elevated in the rats with low PC as shown by increased staining in the tubular and glomerular microvessels.

This increase was unexpected because several studies suggested that EPCR is suppressed during tissue injury (37,38). To assess the relevance of this observation, we evaluated human kidney sections from normal and AKI by immunohistochemistry. The samples from AKI were intended for transplant but not used as a result of documented increases in BUN and/or creatinine before harvest. As was observed in the rat tissue, we observed faint staining for EPCR in the small vessels of normal human kidney but not in the microvasculature (data not shown). However, in each of the AKI samples examined ($n = 5$), we observed a marked increase in glomerular and tubular microvessel EPCR staining. Therefore, in both the rat model and human tissue, injury to the kidney seems to result in an increase in the key vascular factor that mediates the anti-inflammatory and antiapoptotic signaling of APC.

![Figure 3](image-url)

**Figure 3.** Analysis of the level of expression of endothelial PC receptor (EPCR) in the rat CLP model. (A) The mRNA levels were determined as described in Materials and Methods and are expressed in change relative to the sham rats. Data are means ± SEM ($n = 8$ sham; $n = 22$ CLP). (B) Assessment of EPCR protein expression as a function of PC level. Data are means ± SEM ($n = 8$ sham; $n = 22$ CLP). Inset is a Western blot comparing the protein levels of EPCR from sham and CLP rats (C) Examination of EPCR expression by immunohistochemistry in rat kidney as a function of PC level. Increased expression in the microvessels is noted by the brown staining.

**Treatment with Rat APC Improves Renal Function and Markers of Tissue Injury**

We speculated that the low level of circulating PC likely compromises the ability to generate APC naturally, resulting in a reduction in the endogenous mechanism to protect from vasculature injury. To test this, we examined the effect of infusing rat APC on renal function by measuring plasma BUN levels, tissue pathology, and renal chemokine response. Rats were subjected to CLP and 10 h later infused with vehicle or APC for 12 h before being killed. We observed a significant rise in BUN compared with sham rats at 10 h after CLP, before the APC or saline infusion (Figure 4A). By 22 h, the mean BUN in the group of rats that received saline remained elevated, whereas the mean BUN was significantly reduced in rats that received APC. Although there was a significant increase in BUN in the CLP rats by 10 h, we observed that this was driven largely by a subgroup of the previous population (13 of 42 rats) with markedly elevated BUN levels (defined as 50% or greater increase over sham). The mean BUN in this group was $57 ± 5$ versus $15 ± 3$ mg/dl for sham rats. An examination of the effect of APC in this subgroup with marked renal dysfunction showed a highly significant treatment effect (Figure 4B) as evidenced by an 80% reduction in BUN after the APC infusion. In this subgroup and the total population shown in Figure 4A, APC reduced plasma BUN at 22 h. The rats with elevated BUN at 10 h had significantly reduced PC levels of 42 ± 12% of normal, compared with 99 ± 8% of normal in rats without elevation in BUN, consistent with the data in Figure 1.

We also examined the effect of APC treatment on the degree of renal pathology using the scoring method described previously. As shown in Figure 5, we observed a significant reduction in the mean pathology score with APC treatment. Moreover, the number of rats that were scored as having ATN was reduced from 55 to 22% with APC treatment.
APC Treatment Reduces Inflammatory Chemokine Expression in the Kidney

As indicated in the previous section, we observed an elevation in mRNA level of chemokines CXCL1 and CXCL2 in rats with low plasma PC levels. Therefore, we also examined the expression of CXCL1 and CXCL2 in the kidney after APC treatment and found a significant reduction in the expression of both of these chemokines in the kidney (Figure 6A), as well as a reduction in the circulating levels of these two markers of acute renal damage (Figure 6B). We also analyzed the level of myeloperoxidase in the tissue as a measure of inflammation and observed a significant suppression with APC treatment in the CLP kidney and other organs (liver and lung; data not shown).

APC Suppresses iNOS and Active Caspase-3 Induction in the Kidney

Recent studies suggested that apoptosis plays an important role in acute renal injury and can be driven by the release of NO from iNOS upregulation (39,40). To assess the possibility that APC might render a protective effect in the kidney by suppression of iNOS and subsequent apoptosis, we examined the effect of APC on the induction of iNOS and caspase-3 expression. As shown in Figure 7A, there was a significant induction of iNOS after CLP, which was substantially reduced by the treatment with APC. Moreover, we found that iNOS levels correlated highly with the markers of renal injury Ngal ($r^2 = 0.94$ $P < 0.0001$), CXCL1 ($r^2 = 0.83$ $P < 0.0003$), and CXCL2 ($r^2 = 0.85$ $P < 0.0001$). In addition, the level of caspase-3 was elevated after CLP and significantly reduced by the treatment with APC. We also found a significant correlation between caspase-3 and renal pathology, with the highest levels of active caspase-3 observed in rats with the highest pathology score. We also found that CD36 levels in the kidneys, recently shown to track
with tubular apoptosis (41), were reduced by 60% after APC treatment (data not shown).

The distribution of active caspase-3 protein in kidney sections was assessed by immunohistochemistry. After CLP, we observed scattered active caspase-3–positive staining in the kidney, particularly in the glomerulus, interstitium, and tubular lumen regions (Figure 7C), and the positively stained cells exhibited morphologic changes that were associated with apoptosis (pyknotic, shrunken cell with condensed nucleus). Also, the positive caspase-3 staining in the kidney correlated with the percentage of ATN in these rats. In addition, similar to the suppression of active caspase-3 expression shown in Figure 7B, the overall number of caspase-3–positive cells was reduced by APC treatment in these rats, with only an occasional cell stained per field (Figure 7D) versus eight to 20 positive cells per field in untreated rats.

**Discussion**

The pathophysiology of sepsis-induced ARF is incompletely understood. Reports have implicated a variety of mediators, including NO, angiotensin, endothelin-1, and various cytokines/chemokines, that contribute to organ dysfunction that is associated with sepsis via changes in systemic hemodynamics, infiltration of neutrophils and other inflammatory cells, and apoptosis (42–45). We examined the role of the PC pathway in ARF after polymicrobial sepsis and found that acquired PC deficiency was associated with reduced renal function, increased renal pathology, and key mediators of renal injury such as iNOS and chemokine activation. The data suggested that rats with low PC have 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 renal pathology, decreased BUN, and decreased inflammatory and apoptotic response in the kidney.

In rats with PC deficiency, we observed elevation in CXCL1 and CXCL2 levels along with an increase in iNOS locally in the kidney. Moreover, the level of iNOS correlated negatively with plasma PC levels and was also highly correlated with the increase in both CXCL1 and CXCL2 expression. Furthermore, by exogenous administration of APC, to reduce the defect in the PC pathway, we observed a predominant suppression of iNOS expression and of these chemokines that are critical for amplification of the host response during infection (46). NO that is generated from iNOS is a key mediator of renal damage after injury (reviewed by Goligorsky et al. [47]), and its inhibition significantly improves renal functional and histologic indices (48,49). Moreover, Kim et al. (50) suggested that iNOS may regulate during injury a chemokine response that correlates with a tissue inflammatory response (51–53). Walpen et al. (54) demonstrated NO-dependent upregulation of CXCL2 in vitro in cultured mesangial cells and in vivo in a rat model of mesangiproliferative glomerulonephritis. Taken together, these data suggest that suppression of PC may contribute to progression toward sepsis-induced AKI through reduced APC generation that would normally suppress the chemokines and iNOS. In addition, our data suggest that the suppression of iNOS after APC treatment may be responsible for the observed suppression of the chemokine response.

Apoptosis in the kidney has been demonstrated in animal models of ischemia-reperfusion injury and endotoxemia-induced ARF (44,45). Caspase inhibition has been shown to protect against the decline in GFR during ischemia-reperfusion injury (44). Hence, in this study, we hypothesized that the effect of APC on ameliorating sepsis-induced ARF may be mediated in part by inhibition of caspase-3 activation. We observed a significant elevation in active caspase-3 in the kidney after CLP, which was suppressed by APC treatment. Moreover, the degree of caspase-3 activation correlated highly with the degree of renal injury, iNOS, and chemokine activation. Recent studies showed the importance of iNOS in the induction of renal apoptosis (39,40), and our results are consistent with these data. Moreover, the ability of APC to inhibit iNOS in the kidney would be consistent with its ability to suppress caspase-3. That caspase activation is downstream of NO generation is further supported by data in a mouse model of LPS-induced ARF, where caspase inhibition rendered protection against a rise in BUN levels despite any change in NO generation (55). Furthermore, Guo et al. (45) showed that the LPS-induced upregulation
of CXCL2 levels is suppressed after caspase inhibition, suggesting that the production of chemokines by the renal vasculature undergoing apoptosis may thereby cause an amplification of inflammatory response in the kidney. Therefore, APC-induced inhibition of iNOS may be central to the protective mechanism by inhibiting both the release of chemokines and the induction of apoptosis.

The receptor-mediated anti-inflammatory and cytoprotective effects of APC are mediated through EPCR (24–26), and we have observed a significantly higher level of EPCR expression in the kidney after CLP-induced sepsis, as well as in the microvasculature after human renal injury. As noted previously, this was surprising because previous studies suggested that EPCR is downregulated during inflammation (reviewed in references [37,38]). Possibly, the increase in renal EPCR is a compensatory mechanism to allow for increased protective signaling via APC. The even higher levels of EPCR in rats with low PC shown in Figure 3 (i.e., with a reduced ability to generate endogenous PC) would be consistent with this hypothesis.

Conclusion

Although the cause-and-effect relationship of low PC and clinical outcome has not been proved, the data presented here suggest that low endogenous PC levels during systemic inflammatory response may be pathophysiologically related to renal dysfunction by reducing the ability to modulate not only coagulopathic dysfunction (21) but also dampening the cascading inflammatory and apoptotic responses in the kidney after infection. The low level of PC likely compromises the ability to generate APC naturally, which results in a reduction in the natural protective mechanism of the vasculature to inflammatory and ischemic injury. Moreover, the ability to improve renal function and pathology suggests the potential for the clinical use of APC in the treatment of sepsis-induced ARF.

Acknowledgments

We gratefully acknowledge Eddie J. Stephens, Renee L. Grubbs, Kimberly C. Holmes, Kelly Fynboe, and Dominick Montani for assistance with animal care and CLP studies. We also thank Darryl Ballard for assistance with immunohistochemistry; Joe Brunson, Sherri L. Hilligoss, and Don B. McClure for assistance with rat APC cell culture; and Joe Jakubowski for helpful advice.

Disclosures

The authors are employed by Eli-Lilly and Co., who produces recombinant human APC.

References


