

## Activated protein C ameliorates LPS-induced acute kidney injury and downregulates renal INOS and angiotensin 2

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**Gupta A, Rhodes GJ, Berg DT, Gerlitz B, Molitoris BA, Grinnell BW.** Activated protein C ameliorates LPS-induced acute kidney injury and downregulates renal INOS and angiotensin 2. *Am J Physiol Renal Physiol* 293: F245–F254, 2007. First published April 4, 2007; doi:10.1152/ajprenal.00477.2006.—Endothelial dysfunction contributes significantly to acute renal failure (ARF) during inflammatory diseases including septic shock. Previous studies have shown that activated protein C (APC) exhibits anti-inflammatory properties and modulates endothelial function. Therefore, we investigated the effect of APC on ARF in a rat model of endotoxemia. Rats subjected to lipopolysaccharide (LPS) treatment exhibited ARF as illustrated by markedly reduced peritubular capillary flow and increased serum blood urea nitrogen (BUN) levels. Using quantitative two-photon intravital microscopy, we observed that at 3 h post-LPS treatment, rat APC (0.1 mg/kg iv bolus) significantly improved peritubular capillary flow [ $288 \pm 15 \mu\text{m/s}$  (LPS) vs.  $734 \pm 59 \mu\text{m/s}$  (LPS+APC),  $P = 0.0009$ ,  $n = 6$ ], and reduced leukocyte adhesion ( $P = 0.003$ ) and rolling ( $P = 0.01$ ) compared with the LPS-treated group. Additional experiments demonstrated that APC treatment significantly improved renal blood flow and reduced serum BUN levels compared with 24-h post-LPS treatment. Biochemical analysis revealed that APC downregulated inducible nitric oxide synthase (iNOS) mRNA levels and NO by-products in the kidney. In addition, APC modulated the renin-angiotensin system by reducing mRNA expression levels of angiotensin-converting enzyme-1 (ACE1), angiotensinogen, and increasing ACE2 mRNA levels in the kidney. Furthermore, APC significantly reduced ANG II levels in the kidney compared with the LPS-treated group. Taken together, these data suggest that APC can suppress LPS-induced ARF by modulating factors involved in vascular inflammation, including downregulation of renal iNOS and ANG II systems. Furthermore, the data suggest a potential therapeutic role for APC in the treatment of ARF.

two-photon intravital microscopy; endotoxin

ACUTE RENAL FAILURE (ARF) is a common complication in septic patients, with an incidence reported to be ~20–50% (35, 36). The mortality rate associated with ARF in septic patients is 75% compared with 45% in patients without sepsis (38). Despite advances in supportive care with appropriate antibiotic administration and maintenance of systemic hemodynamics, coupled with the lack of proven pharmacological intervention, mortality rates for ARF patients have remained unchanged (50).

During sepsis, a broad array of soluble factors are altered including the serine protease, protein C (PC). Reduction in

plasma levels of PC have been shown to be prognostic for sepsis and sepsis severity (reviewed in Ref. 11). Studies have also suggested that low PC levels are predictive of early death in a rat model of polymicrobial sepsis (14). Exogenous administration of activated PC (APC) has been shown to reduce ischemia-reperfusion-induced renal injury and to attenuate microcirculatory dysfunction (16, 26). These studies support the significance of the PC pathway in reducing organ dysfunction, which is further exemplified by the efficacy of recombinant human APC in the treatment of severe sepsis (1). However, the precise role of APC in attenuating sepsis-induced ARF is unclear.

*Escherichia coli* lipopolysaccharide (LPS) administration in rodents has been used to mimic sepsis-induced ARF (5). Although LPS challenge does not exhibit all of the manifestations of clinical sepsis, the hemodynamic changes induced by LPS result in alteration of renal blood flow and glomerular filtration rate (GFR) producing loss in renal function. The pathogenesis of ARF secondary to endotoxemia has been attributed to impaired hemodynamics caused by multiple mechanisms, including production of large amounts of nitric oxide (NO) and proinflammatory cytokines, along with enhanced renal vasoconstriction. In addition, leukocyte activation and microvascular dysfunction following kidney injury appear to play important roles in mediating renal dysfunction and ARF (28).

Substantial evidence from clinical and experimental studies demonstrates the involvement of angiotensin II (ANG II), an effector of the renin-angiotensin system (RAS), in various biological processes that lead to deterioration of normal renal function. In response to peripheral vasodilation mediated by increased NO, counterregulatory mechanisms causing RAS activation lead to increased angiotensin production via action of angiotensin-converting enzyme (ACE), which further predisposes the kidney toward enhanced injury. Although various reports suggest that APC might reduce NO by suppression of inducible NO synthase (iNOS) activation (17), its effect on renal iNOS and on the RAS system is unknown.

In the present study, we explored the role of APC in modulating the response to injury by LPS in the kidney. We demonstrate that during early endotoxemia, APC suppresses the induction of renal iNOS and ANG II, improves renal blood flow, and reduces leukocyte activation and adhesion. In addition, APC also confers renoprotective actions at late-stage

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endotoxemia as evidenced by reduced plasma blood urea nitrogen (BUN) levels and complete restoration of renal blood flow. Overall, our studies suggest a role of APC in ameliorating ARF possibly by its ability to limit both peripheral vasodilatation and paradoxical renal vasoconstriction. Moreover, our data suggest that APC offers a potential therapy for sepsis-induced ARF.

## MATERIALS AND METHODS

**Animals and surgical procedures.** Male Sprague-Dawley rats (Harrlan, IN) weighing 250–300 g were used in the study. The rats were acclimatized to the laboratory conditions for at least 7 days following their arrival. The night before surgery rats were denied access to food but had access to water. On the day of experiment, the rats were placed in an induction chamber and received a mixture of low flow oxygen and 5% halothane for induction of general anesthesia. Once induced, they were placed on a Rodent Anesthesia Circuit (Kent Scientific, Torrington, CT) and maintained on 1.5% halothane and low flow oxygen. Through a left groin incision, cutdowns were performed on the left femoral vein for catheterization with polyethylene cannulas (PE-50, internal diameter 0.58 mm, external diameter 0.965 mm, Clay Adams Division, Becton Dickinson and, Parsippany, NJ).

Endotoxemia was induced by administration of *E. coli* LPS (10 mg/kg iv infusion for 30 min; LPS W *E. coli* 0111:B4, Sigma, St. Louis, MO) using the left femoral vein catheter as described previously (37). The animals were then randomized into two groups; sham-treated and LPS-treated animals at the time of surgery. The animals in the LPS-treated group were then further divided into vehicle- and APC-treated groups. The control group received pyrogen-free saline infusion (1 ml/kg). For treatment studies, recombinant rat APC was produced in AV12-664 cells and then activated with recombinant rat thrombomodulin/bovine thrombin complex essentially as described previously for human APC (12). In the APC-treated groups, rat APC (100 µg/kg bolus iv) was administered at the time of administration of LPS. The hemodynamic recordings were done at 3 h post-LPS administration. Animals were killed at 3-h time point for collection of kidney tissue (mRNA and protein analysis) and a blood sample was collected for analysis just before death.

For 24-h studies, rats received an intraperitoneal injection of LPS (20 mg/kg). This concentration was based on a dose response that resulted in elevated BUN and no mortality at 24 h. APC (2 mg/kg, administered at 0 and 12 h post-LPS treatment) was used to cover the entire exposure owing to its short half-life. 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.

**Intravital 2-photon imaging.** Intravital two-photon microscopy was performed as previously described (7). Live renal imaging in rats at 3 h posttreatment was performed using a Bio-Rad MRC-1024MP Laser Scanning Confocal/Multiphoton scanner (Hercules, CA) with an excitation wavelength of 800 nm through a Nikon Diaphot inverted microscope utilizing a  $\times 60$  NA 1.4 lens. Assessment of functional renal injury in the form of vascular permeability defects and disruptions in blood flow was achieved utilizing a high molecular weight dextran (HMWD) that is not filtered by the glomerulus under normal conditions (500 kDa, 7.5 mg/ml in 0.9% saline; Molecular Probes, Eugene, OR), and a low molecular weight dextran (LMWD) that is freely filterable across the glomerulus (3 kDa, 20 mg/ml in 0.9% saline; Molecular Probes). To differentiate the two dextrans, the HMWD dextran was labeled with fluorescein (Molecular Probes) while the LMWD was labeled with Texas Red (Molecular Probes). The left kidney of the anesthetized rat was imaged following exteriorization through a retroperitoneal window via a flank incision. Images were analyzed with Image J software (ImageJ, National Institutes of Health, Bethesda, MD). Approximately 10–12 images,

every 3 min, were collected for each animal examined. For studies examining leukocytes in the microvasculature, images obtained were analyzed in a  $4 \times 4$  grid. Leukocytes were identified by their characteristic uptake of the Hoechst nuclear stain (Hoechst-33342, 400 µl, 1.5 mg/ml in 0.9% saline; Molecular Probes). Leukocytes in the microvasculature were classified into three subtypes namely 1) free flowing: presence of leukocyte during real-time imaging in a grid for less than or equal to two frames, 2) static or adherent: attached to microvascular endothelium with no movement, and 3) rolling: appearance along endothelial surface for three or more frames in a grid. For quantifying microvascular leakage, images obtained were analyzed in a  $4 \times 4$  grid, and each grid section (16 per image) was scored for the presence or absence of dextran extravasation for both large and small dextrans. To avoid cross-over bias from adjacent grid images, each grid was individually scored in a random blinded fashion. Calculation of blood cell velocities as an index of blood flow was based on previously published modified line-scan method (27). Only vessels of similar diameter (8–10 µm), continuous horizontal segment length of at least 50 µm, and a minimum distance of 50 µm from an angle or tortuosity were chosen for velocity calculations as increasing diameter and tortuosity results in increased or turbulent flow under physiological conditions, respectively. Additionally, trigonometry was used to correct for vessels not running perfectly horizontally up to angles of 30°, beyond which vessels were excluded.

**Cytokine and BUN measurements.** Arterial blood was collected into EDTA tubes and the plasma supernate was collected and stored frozen until analysis. Measurements of IL-6 and IL-18 were done by immunoassay using the Rodent Multi-Analyte Profile (Rules Based Medicine; Austin, TX) as previously described (14). BUN in plasma was determined using a Hitachi 911 clinical chemistry analyzer (Roche Diagnostics, Indianapolis, IN).

**Quantitative real-time PCR.** Total RNA was purified from kidney tissue samples that had been preserved in RNA-later (Ambion, Austin, TX) using the RNeasy 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 (–RT) were performed in the absence of reverse transcriptase. Purified total RNA was analyzed by quantitative real-time PCR (qPCR) with an ABI Prism 7900HT Sequence Detection System. An internal standard curve was generated by serial dilution of an appropriate cDNA reaction and used for relative quantification. TaqMan Gene Expression Assays for iNOS, ACE1, ACE2, AT2, and angiotensinogen (Agt) and for the 18S rRNA eukaryotic endogenous control kit were purchased from Applied Biosystems (ABI, Foster City, CA). The assay for 18S rRNA was used to normalize to the gene of interest (GOI). Normalized GOI quantities were then used to calculate treatment group averages and percent expression change relative to the sham group, which was defined as 100%.

**Determination of concentration of ANG II in rat kidney tissue.** ANG II levels were measured using the kit supplied by Phoenix pharmaceuticals (Belmont, CA) following the manufacturer's instructions. To determine renal ANG II levels, tissue samples were homogenized in ice-cold methanol containing 0.1% trifluoroacetic acid (TFA). The homogenate was centrifuged at 2,000 g for 10 min at 4°C. Tissue ANG II concentration was measured after extraction through the Sep-Pak C-18 column supplied by the manufacturer (Phoenix Peptide). The peptide was eluted very slowly with 3 ml of 60% acetonitrile in 1% TFA and the eluant was collected in a polypropylene tube. The eluant was evaporated using a SpeedVac Concentrator Savant SVC 100 H (Instruments, Formingdale, NY). The residue was then dissolved in 250 µl of RIA buffer and two aliquots of 100 µl were assayed for ANG II according to manufacturer's specifications. Results were normalized to the amount of protein per sample, as determined by BCA assay (Pierce, Rockford, IL). The rat

ANG II assay has no cross-reactivity with endothelin-1, substance P, [Arg<sup>8</sup>]-vasopressin, and ANG I peptides.

**Determination of concentration of NOx in kidney tissue.** The concentration of NO byproducts NOx (nitrate + nitrite) was determined in kidney tissue samples as previously described (13). Briefly, kidney tissue was homogenized with cold PBS on ice that inhibited the activity of NOS *ex vivo*. The homogenate was spun down (3,000 g, 5 min, Beckman) and the supernatant was collected. The NOx concentration was then determined in the supernatant using standard Griess reaction method. Absorbance was measured at 540 nm using a plate reader and converted to NOx concentration using a nitrate standard curve and expressed as micromoles per gram of protein in tissue. Protein in the supernatant obtained from each sample was determined using BCA assay (Pierce).

**Statistical analyses.** Data are presented as means  $\pm$  SE. The biochemical data were analyzed by one-way ANOVA using JMP5.1 software (SAS Institute). After obtaining a significant F value, post hoc Student *t*-test was performed for inter- and intragroup comparisons. Statistical significance was realized at  $P \leq 0.05$  to approve the null hypothesis for individual parameters.

## RESULTS

**APC improves microvascular blood flow following LPS-induced kidney injury.** Intravital two-photon microscopy was used to quantify LPS-induced functional defects on the renal vasculature and the effect of APC treatment. Administration of LPS post-3 h caused frequent occurrence of obstructed peritubular capillary flow, tubular damage, and tubular necrosis (Fig. 1B). However, following treatment with APC (0.1 mg/kg bolus), these rats exhibited rapid movement compared with the sluggish, turbulent flow that was observed following LPS administration (Fig. 1C). Please refer to supplementary material for this article to view the movies M1 (sham-treated rat), M2 (LPS-treated rat), and M3 (APC-treated rat; the online version of this article contains supplemental data). To quantify these protective effects of APC during endotoxemia, blood cell velocities were measured using the modified line-scan method (27). As shown in Fig. 2, administration of LPS significantly reduced erythrocyte flow rate by  $\sim 71\%$  compared with the sham group [ $1,023 \pm 96 \mu\text{m/s}$  (sham) vs.  $289 \pm 15 \mu\text{m/s}$  (LPS)]. Furthermore, in the LPS-treated rats, few regions of near normal blood flow were observed. On treatment with APC, rats exhibited significantly improved erythrocyte flow rates [ $288 \pm 15 \mu\text{m/s}$  (LPS) vs.  $734 \pm 59 \mu\text{m/s}$  (LPS+APC),  $P = 0.0009$ ] compared with LPS-treated rats. These data suggest that treatment with APC aids in restoration of blood flow as early as 3 h-post endotoxin administration.

**Effect of APC on leukocyte adhesion and rolling following LPS-induced kidney injury.** Since vascular endothelial dysfunction is an early event in sepsis, we quantified the effect of LPS administration on the dynamic nature of leukocyte interaction along the renal microvascular endothelium using two-

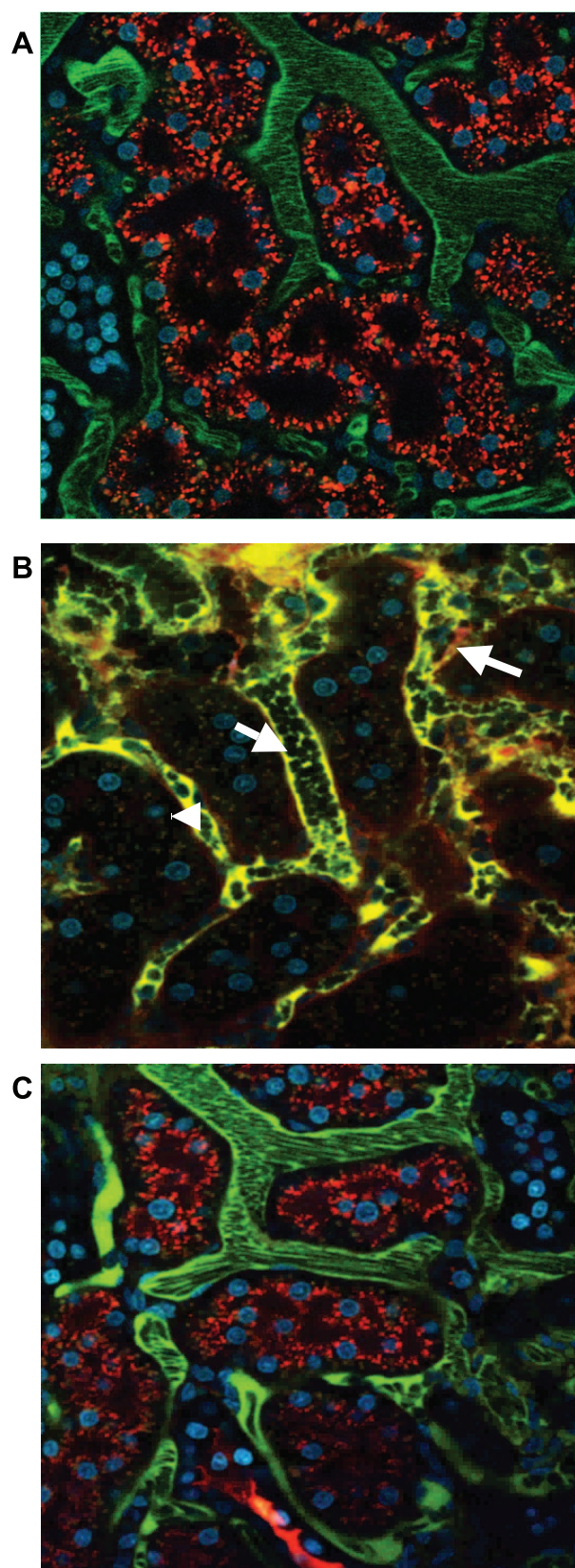


Fig. 1. Microvascular blood flow and permeability assessment using real-time intravital 2-photon microscopy. Signal void in the vasculature represents relative rapid movement of cellular structures in the vasculature (WBC, RBC) streaming compared with the acquisition speed of the image. A: sham-treated rat exhibiting normal blood flow. B: LPS-treated rats, post-3 h treatment exhibited sluggish microvascular blood flow in most areas, with evidence of rouleux formation (arrow head) in many areas. Leakage of dextrans (white arrow) indicates increased endothelial permeability. C: activated protein C (APC)-retreated rats exhibited normal rapid microvascular blood flow in most regions in the kidney without any evidence of endothelial disruption.

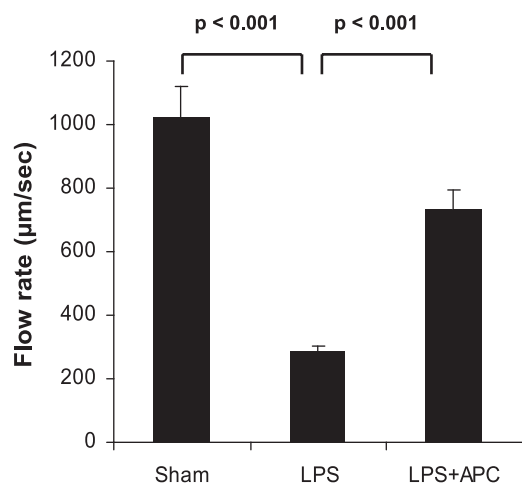


Fig. 2. Effect of APC (100 µg/kg) on mean blood cell velocity per vessel as measured by modified RBC line-scan method. Data are represented as means  $\pm$  SE,  $n = 4$  per data group.

photon intravital microscopy. In the LPS-treated rats, 3 h postadministration, there was evidence of increased leukocyte adhesiveness to the endothelium both in terms of fully adherent or static leukocytes (~6-fold increase) in the microvasculature as well as intermittent adhesion (rolling) within the microvasculature (~8-fold increase; Fig. 3). Consequently, the percentage of total free leukocytes was decreased in LPS-treated animals (~82%). In contrast, APC treatment during endotoxemia demonstrated a significant reduction in total number of static (Fig. 3A) and rolling (Fig. 3B) leukocytes. As shown in still frames from the two-photon imaging, the adherent leukocytes can be easily visualized (arrows) in the microvasculature of LPS-treated animals (Fig. 3C) and largely absent in the LPS plus APC-treated animals (Fig. 3D). Thus APC significantly suppresses LPS-induced leukocyte activation by reducing absolute adherent and rolling leukocytes.

**Effect of APC on tubular function and microvascular permeability.** LPS is known to cause a pronounced defect on endothelial permeability. Furthermore, APC has been shown to attenuate barrier disruption in cultured cells to ligands such as thrombin (10). Hence, to determine whether APC could protect endothelial barrier disruption *in vivo*, we utilized qualitative intravital microscopy and determined leakage of LMWD and HMWD as indicators of endothelial permeability. In the LPS-treated rats, we observed apparent leakage of both LMWD and the HMWD from the renal microvasculature (Fig. 1B), with leakage of LMWD (red) more pronounced than HMWD. In APC-treated rats, we observed no apparent leakage of either LMWD or HMWD in any animal (Fig. 1C).

Tubular function was also assessed by determining the time lag for the endocytic uptake of LMWD by proximal tubules. On timed analysis of the appearance of the LMWD in the proximal tubules postinjection, we observed that there was a time lag (~10 min) in the uptake of LMWD compared with sham group where the uptake was instantaneous. On APC treatment, the uptake of LMWD by the proximal tubule was restored (Fig. 4). Therefore, APC appears to protect from LPS-induced kidney injury by maintaining endothelial permeability, glomerular filtration, and proximal tubular function.

As has been reported previously in humans (44) and in model systems (17), we found that APC did prevent the

LPS-induced hypotension in our studies [mean arterial pressure (MAP) of  $96.5 \pm 2.1$  mmHg in sham,  $65.2 \pm 2.1$  mmHg in LPS, and  $98.2 \pm 3.8$  mmHg in LPS+APC]. However, volume expansion by infusion of 2 to 3.5 ml of saline resulted in an increase in MAP of  $25.6 \pm 5.6$  mmHg, similar to the APC effect, but did not significantly alter tubular dysfunction or leukocyte margination. These data suggest that the protective effects of APC were not secondary to prevention of hypotension.

**APC reduces iNOS mRNA and NOx levels.** Administration of LPS significantly increased iNOS mRNA levels in the kidney by ~16-fold compared with sham-treated group (Fig. 5A). In contrast, APC treatment in these rats significantly suppressed iNOS mRNA induction ( $P < 0.00001$ ) in the kidney. In addition, the LPS+APC group was not significantly different than the sham-treated group.

To further examine the effect of APC on LPS-induced NO activation, we measured the concentration of NOx levels in kidney tissue. Similar to the effect on iNOS mRNA induction, APC completely abolished the LPS-induced increase in NOx levels [ $147.3 \pm 29.2$  µmol/g protein (LPS) vs.  $89.5 \pm 5.6$  µmol/g protein (LPS+APC)] in Fig. 5B.

**Effect of APC on angiotensin system during endotoxemia.** Numerous studies suggest that abnormal activation of the RAS system in the kidney, leading to excess production of ANG II, results in tubulointerstitial injury and subsequent loss of renal function (24). Hence, we speculated that APC might be inhibiting the renal dysfunction associated with endotoxemia by suppressing ANG II production. As shown in Fig. 6A, LPS treatment caused a significant increase in ANG II peptide levels, which was suppressed following APC treatment in these animals. To further examine these effects, we determined the relative levels of the ANG II precursor, Agt, and the converting enzymes ACE1 and ACE2. We observed that LPS induced approximately fivefold increase in the mRNA level of the Agt (Fig. 6B), which was significantly reduced following APC treatment. LPS also increased ACE1 mRNA levels (Fig. 6C), while downregulating ACE2 (Fig. 6D). In contrast, APC treatment significantly increased ACE2 mRNA levels and suppressed the induction of ACE1. This is further evident by the fact APC treatment doubled the ACE2/ACE1 ratio compared with the LPS-treated group. These data suggest that APC suppresses LPS-induced RAS activation by blocking the induction of Agt and ACE1 and by increasing ACE2 resulting in diminished ANG II levels in the kidney.

Recent studies have shown that Agt is upregulated at the promoter level by IL-6 (18). Moreover, IL-6 is induced by ANG II (21) and suppressed by RAS inhibition (31). Therefore, we examined whether the ability of APC to suppress Agt might be related to an ability to suppress IL-6. We found that the level of IL-6 in the plasma was significantly increased following endotoxemia, but significantly reduced by APC treatment (~69%,  $P < 0.008$ ). Moreover, the level of IL-6 mRNA expressed in the kidney was reduced by 35% ( $P < 0.02$ ) by APC treatment.

**Effect of APC on renal function post-24 h endotoxemia.** The above data provided mechanistic understanding for the early effect of APC on LPS-induced renal dysfunction. To provide additional evidence of a sustained benefit, we examined the effect of APC at 24 h after endotoxemia. For these studies, we utilized the rat model of intraperitoneal administration of LPS

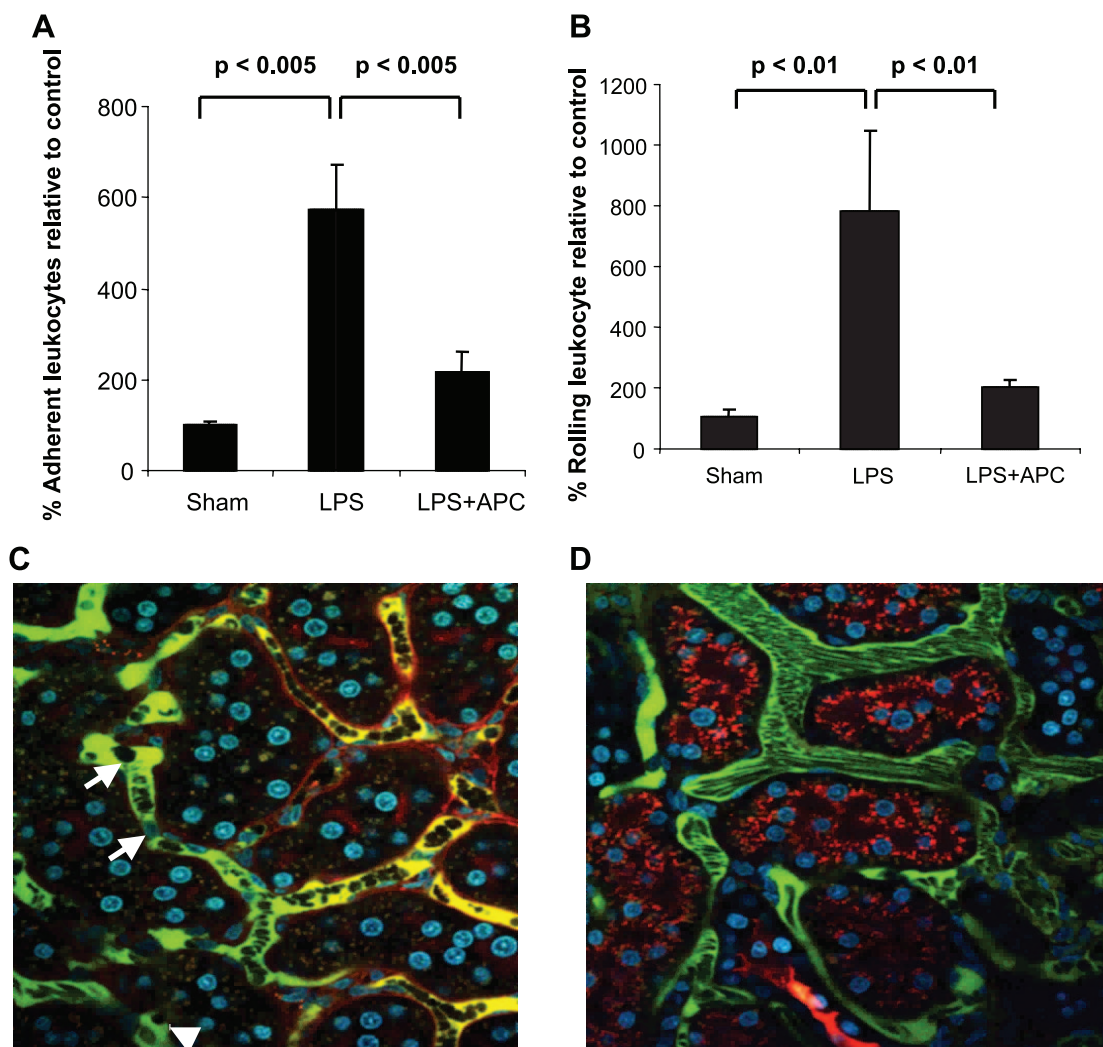


Fig. 3. Effect of APC on leukocyte adhesion (A) and rolling (B) as measured by intravital microscopy. Leukocytes counted at 3 h after treatment. Images were taken for ~30 min every 3 min accounting for ~4 s per movie image and 10 images were obtained per rat. C and D: leukocyte adhesion to endothelium in LPS- and LPS+APC-treated rats, respectively. The values are expressed as mean percentages per group. Data are represented as means  $\pm$  SE,  $n = 4$  per data group.

that induces more severe renal injury compared with intravenous infusion of LPS. Measurement of peritubular capillary flow using intravital microscopy and plasma BUN levels was used as indexes to analyze the effect of APC on LPS-induced kidney dysfunction at 24 h posttreatment. Figure 7, A and B, depicts the effect of APC on the renal vasculature as observed utilizing intravital two-photon imaging. As described in Fig. 1 for the intravital microscopy measurements at 3 h, LPS-induced sluggish erythrocyte flow rates along with a lag in endocytic uptake of the low molecular weight dextran by the proximal tubules, 24 h posttreatment. In contrast, APC-treated rats exhibited faster flow rates, as indicated by rapid streaks and improved tubular function. Quantification using the line-scan method as shown in Fig. 7C shows that APC treatment completely restored LPS-induced suppressed of renal flow ( $P = 0.001$ ). By 24 h, LPS treatment had resulted in a significant elevation in BUN (Fig. 7D), which was significantly reduced to the levels observed in sham-treated rats following APC treatment. At this time point, there was no significant reduction in MAP from the LPS treatment. These data provide evidence that the protective mechanisms observed with APC

following early endotoxemia result in improved renal function in late-stage endotoxemia.

## DISCUSSION

The pathophysiology of ARF involves a complex interplay between various processes, including tubular and endothelial cell injury and inflammation. Microvascular dysfunction following injury to the kidney appears 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 (28). In patients with sepsis, there is an increased incidence of ARF (43) and damage to the endothelium, resulting in increased vascular permeability, leukocyte adherence, and loss of normal endothelial function, has been well documented (29). We demonstrated the ability of APC to modulate these measures of endothelial dysfunction associated with ARF. We observed that APC ameliorates renal dysfunction during both early and late endotoxemia, coincident with the attenuation of NO generation and perturbation of the RAS.

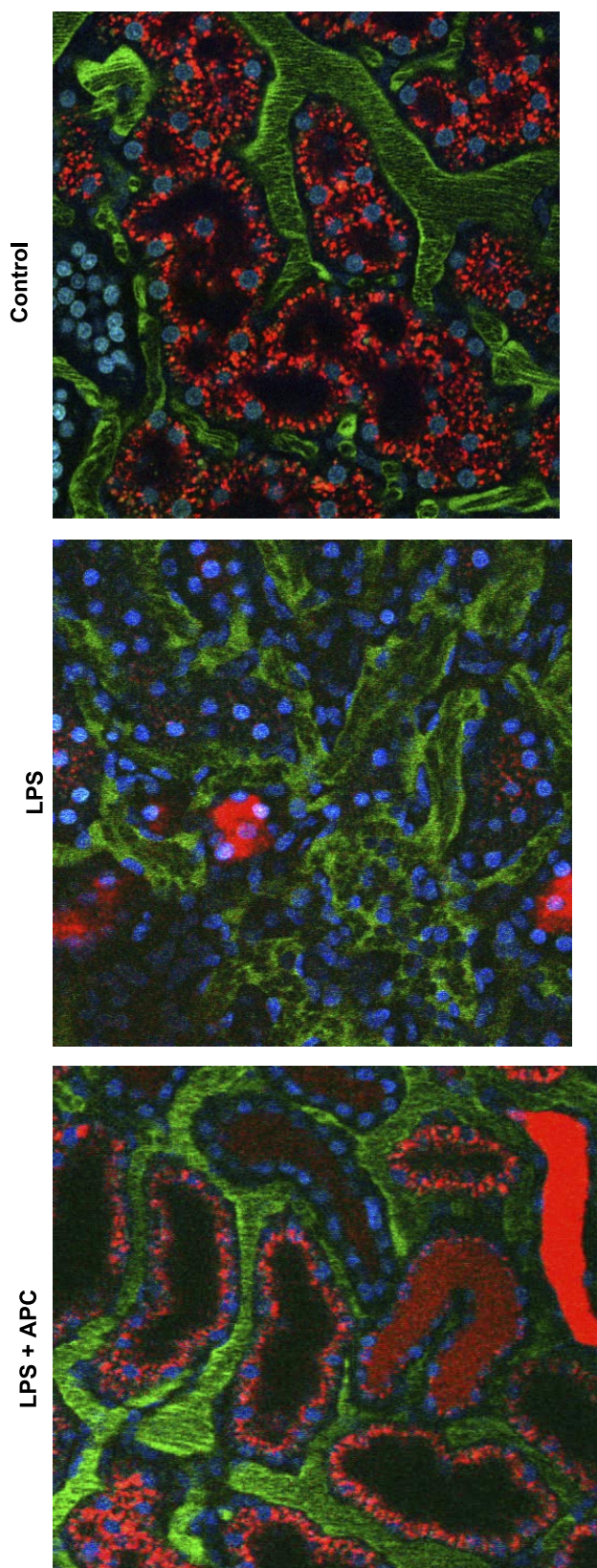


Fig. 4. Effect of APC treatment on proximal tubule function as determined by the uptake of the low molecular weight dextran by the proximal tubules, respectively. The images were taken at timed intervals postinjection of the dextrans to obtain a qualitative assessment.

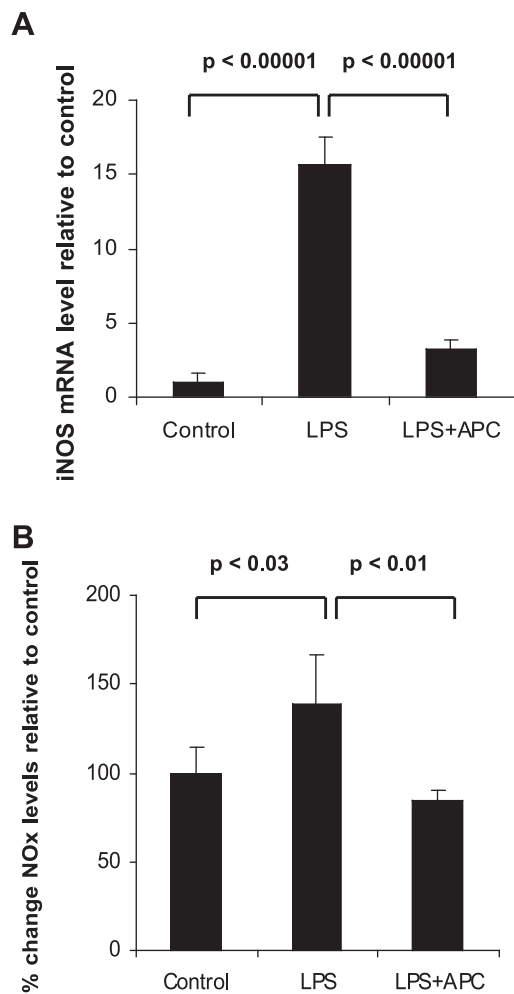


Fig. 5. Effect of APC (100  $\mu\text{g}/\text{kg}$ ) on inducible nitric oxide synthase (iNOS) mRNA level (A) and NOx levels (B) in kidney tissue obtained from sham and endotoxemic rats. Data are represented as means  $\pm$  SE,  $n = 6$  per data group.

Tubular epithelial cell function and renal blood flow play a significant role in regulating renal function by modulating GFR (46). Using two-photon imaging, the present study revealed a reduction in cortical peritubular capillary flow 3 h post-LPS administration. Similar results have been reported using laser doppler studies in rats following endotoxemia (25). Besides diminished flow, we observed defective tubular function as revealed by reduced uptake of LMWD by the epithelial cells of the proximal tubules. However, administering a single bolus injection of APC improved renal function by increasing peritubular capillary flow as well as tubular function in these endotoxemic rats as early as 3 h posttreatment. In addition, APC also suppressed renal expression of the proinflammatory cytokine IL-6 and the circulating levels of both IL-6 and IL-18 (data not shown). Detection of biomarkers such as proinflammatory cytokines released in response to renal injury have been suggested to provide more compelling evidence of kidney injury at the early phase of disease progression (33, 40), e.g., more so than serum creatinine, which exhibits a time delay for a detectable increase postinjury (32). Therefore, these data suggest that APC can alter renal injury by modulating microvascular flow and inflammatory stimuli and change early plasma markers suggestive of renal injury.

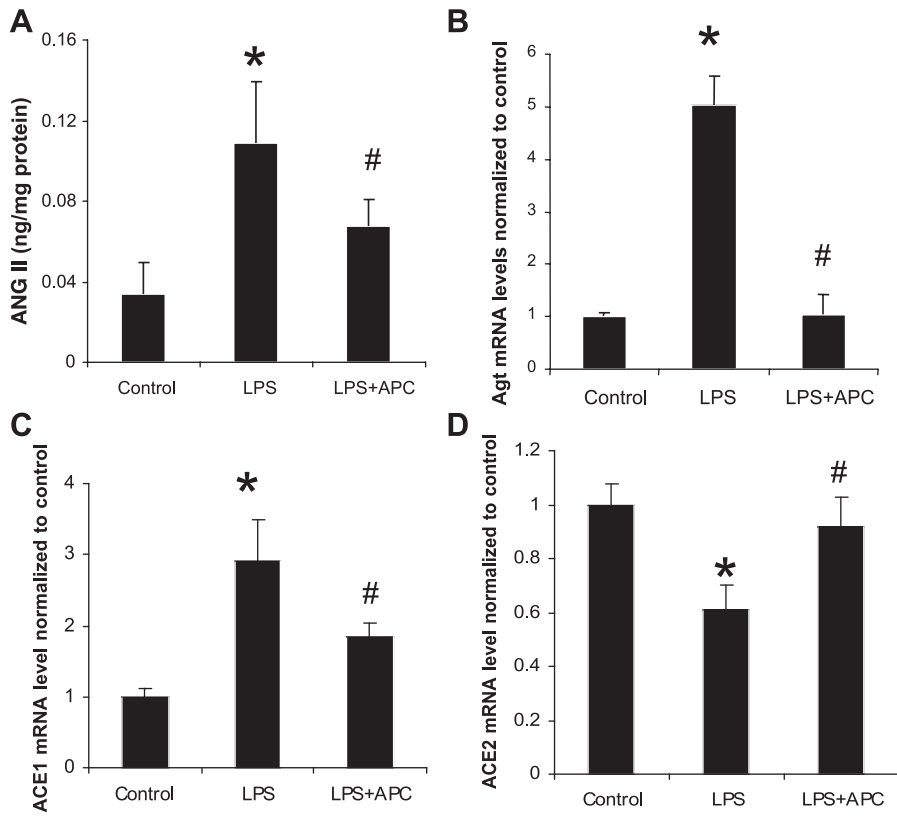


Fig. 6. Effect of APC on the renin-angiotensin system. APC (100  $\mu\text{g}/\text{kg}$ ) effect on concentration of ANG II (A), Agt (B), ACE1 (C), and ACE2 (D) in kidney tissue obtained from sham and endotoxemic rats. Data are represented as means  $\pm$  SE,  $n = 6$  per data group. \* $P \leq 0.05$  compared with vehicle treatment. # $P \leq 0.05$  compared with LPS treatment.

APC functions both as a feedback inhibitor of thrombin generation (8) and as an anti-inflammatory and cytoprotective agent via EPCR/PAR-1 receptor signaling (19). In our studies, LPS administration caused coagulation activation at the 3-h

time point as indicated by reduced plasma fibrinogen levels. However, at the relatively low dose used, APC treatment did not prevent the reduction in fibrinogen levels by LPS. Similarly, in a human study of recombinant APC in a model of

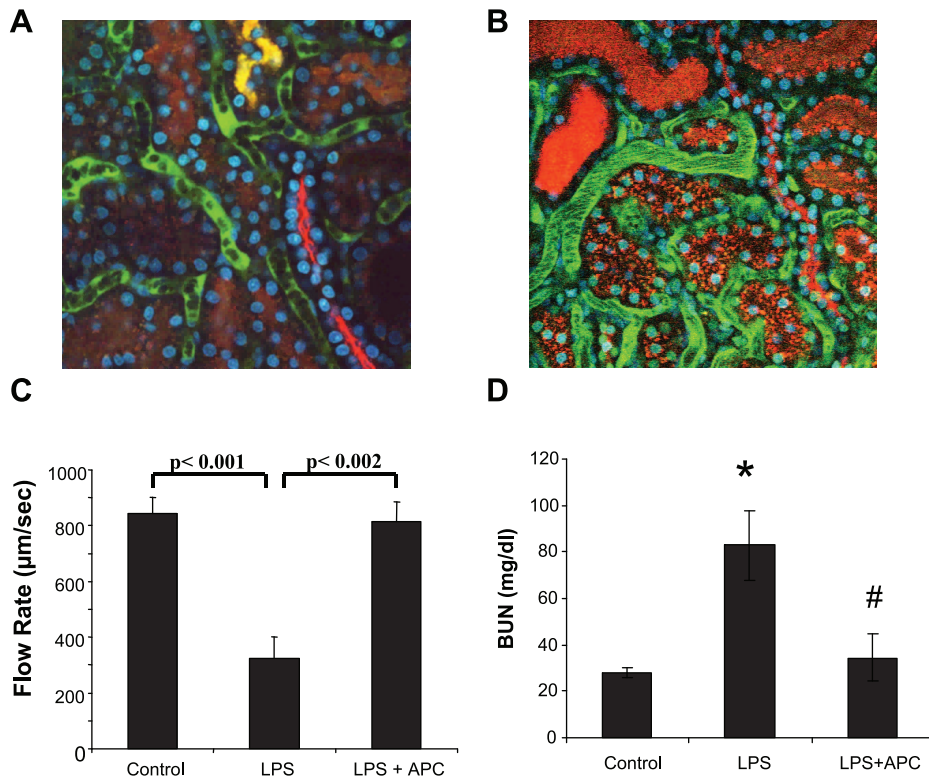


Fig. 7. Effect of APC on renal function at 24 h postendotoxemia. A and B: representative of microvascular blood flow assessed using real-time intravital 2-photon microscopy. Signal void in the vasculature represents relative rapid movement of cellular structures in the vasculature (WBC, RBC) streaming compared with the acquisition speed of the image. C: APC (2 mg/kg, administered at 0 and 12 h post-LPS treatment) restores renal blood flow as quantified by modified RBC line-scan method. D: effect of APC treatment on renal function as determined by measurement of plasma blood urea nitrogen (BUN) levels. Data are represented as means  $\pm$  SE,  $n = 4$  per data group. \* $P \leq 0.05$  compared with vehicle treatment. # $P \leq 0.05$  compared with LPS treatment.

endotoxemia, little effect on coagulation markers was observed (6). These authors further speculated that compared with other anticoagulants, the effect of APC may be less driven by its anticoagulant activity and more by its role as an endothelial function modulator through EPCR/PAR-1. In addition, Hoffmann et al. (15) demonstrated that thrombin inhibition alone could not reduce endotoxin-mediated leukocyte adhesion and rolling. Overall, the data suggest that the anti-inflammatory properties of APC may be more important than the anticoagulant properties for the observed protection against LPS-induced renal injury.

The pathogenesis of sepsis-induced ARF is incompletely understood but involves systemic vasodilation leading to reduced systemic vascular resistance, mediated by iNOS activation (20). The impact of NO on tubular function and GFR is well established (25, 41). Furthermore, inhibition of iNOS by L-NIL has been shown to preserve renal cortical perfusion during endotoxemia (42). The relevance of the NO pathway in the pathogenesis of sepsis is further supported by the fact that iNOS knockout mice do not exhibit hypotension on LPS treatment and also do not increase NO synthesis following a septic insult in these knockout mice (23, 45). In the present study, APC treatment downregulated LPS-induced increased renal iNOS mRNA and NO<sub>x</sub> levels along with restoring tubular function and renal blood flow. Therefore, downregulation of iNOS-derived NO by APC may account for improved peritubular capillary flow and function.

The kidney possesses its own RAS system that acts independently of the circulating system and is pivotal in regulation of renal function (2). A key element of the RAS system is the enzyme ACE1 that cleaves the inactive precursor peptide ANG I to active ANG II that induces vasoconstriction. Recently, ACE2 which is a homolog of ACE1 has been identified that hydrolyzes ANG II to the vasodilator Ang (1–7), thus regulating the net level of ANG II and facilitating the mitigation of the biological actions of ANG II (9). As a counterregulatory measure to peripheral vasodilation, subsequent RAS activation culminates to maintain hemodynamic stability, inducing renal vasoconstriction that in turn further contributes to the pathogenesis of sepsis-induced ARF (4). This unique vasoconstrictor response of the renal vasculature contrasts with the observed vasodilation of the mesenteric, intestinal, hepatic, splenic, and non-splanchnic vascular beds during sepsis and LPS administration (39). Studies in both humans and animals suggest that the observed renal vasoconstriction may result from impaired endothelium dependent vasodilation (3, 34). In the present study, LPS administration produced increased renal vasoconstriction observed by two-photon imaging and as demonstrated by elevated renal ANG II levels. This increase in ANG II levels by LPS was also accompanied by elevated Agt and ACE1 but diminished ACE2 expression. These results are in agreement with Yamaguchi et al. (48) where they demonstrated elevated ANG II and ACE levels post-3 h endotoxemia. Interestingly, treatment of these endotoxemic animals with APC blocked the LPS-induced activation of the renal RAS components. We speculate that the ability of APC to suppress the local synthesis of ANG II in the kidney by upregulating ACE2 and inhibition of Agt and ACE1, and also by feedback inhibition of Agt, may contrib-

ute to the observed improvement in renal function during endotoxemia. Similar observations were made by Ye et al. (49) where they suggested that increased ACE2 expression coupled with low ACE activity may be renoprotective in diabetes. Also, ACE inhibitor captopril has been shown to prevent LPS-induced decline in renal blood flow and GFR (22). In addition, since ANG II has been shown to induce neutrophil accumulation in vivo (30), reduction of renal ANG II levels by APC may contribute to its ability to suppress leukocyte adhesion in the present study. Collectively, these data suggest a role of APC in modulating the renal RAS system to regulate renal function in vivo.

Peritubular capillary dysfunction during LPS-induced injury is a progressive phenomenon that has been shown to precede the development of ARF (47). We observed peritubular capillary dysfunction following LPS treatment as early as 3 h along with induction of IL-6 and IL-18 levels but no detectable increases in BUN levels. However, at late stage of endotoxemia (24 h), we observed elevation in plasma BUN levels along with diminished renal blood flow. These observations suggest that peritubular dysfunction following LPS induction early on can contribute to loss of renal function evident at late-stage endotoxemia. Interestingly, APC treatment mitigated the peritubular dysfunction during both early- and late-stage endotoxemia. Since, LPS administration does not reproduce the hyperdynamic stage of sepsis; further studies may be required to elucidate the role of APC in improving peritubular capillary flow in a more relevant sepsis animal model such as CLP.

Overall, the data presented in the current study demonstrate the ability of APC to protect the renal microvasculature from injury. APC may improve renal function during endotoxemia by restoring impaired renal hemodynamics possibly by suppressing local NO and the ANG II system, as well as inhibiting leukocyte-endothelial cell interactions. In view of the important role of endothelial dysfunction in acute renal injury, our data suggest the potential for the clinical use of APC in the treatment of sepsis-induced ARF.

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#### DISCLOSURE

The authors disclose that A. Gupta, D. T. Berg, B. Gerlitz, and B. W. Grinnel are employed by Eli Lilly and Co. which produces recombinant human APC.

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