Rolipram improves renal perfusion and function during sepsis in the mouse

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Abstract

Microcirculatory dysfunction is correlated with increased mortality among septic patients and is believed to be a major contributor to the development of acute kidney injury (AKI). Rolipram, a selective phosphodiesterase 4 (PDE4) inhibitor, has been shown to reduce microvascular permeability and in the kidney, increase renal blood flow (RBF). This led us to investigate its potential to improve the renal microcirculation and preserve renal function during sepsis using a murine cecal ligation and puncture (CLP) model to induce sepsis. Rolipram, tested at doses of 0.3 – 10 mg/kg, i.p., acutely restored capillary perfusion in a bell-shaped dose-response effect with 1 mg/kg being the lowest most efficacious dose. This dose also acutely increased RBF despite transiently decreasing mean arterial pressure. Rolipram also reduced renal microvascular permeability. Importantly, delayed treatment with rolipram at 6 h after CLP restored the renal microcirculation, reduced blood urea nitrogen and serum creatinine, and increased glomerular filtration rate at 18 h. However, delayed treatment with rolipram did not reduce serum nitrate/nitrite levels, a marker of nitric oxide production, nor reactive nitrogen species generation in renal tubules. These data show that restoring the microcirculation with rolipram, even with delayed treatment, is enough to improve renal function during sepsis despite the generation of oxidants and suggest that PDE4 inhibitors should be evaluated further for their ability to treat septic-induced AKI.
Introduction

The pathophysiology of organ dysfunction during septic shock is multifactorial and not well understood. Although systemic hemodynamic decline during sepsis can contribute to organ hypoperfusion, there is a growing appreciation of the importance of microcirculatory failure in the development of organ injury. Microvascular dysfunction is now recognized as a strong predictor of death among patients with severe sepsis (Trzeciak et al., 2007; De Backer et al., 2013).

Acute kidney injury (AKI) occurs in 20-50% of septic patients (Rangel-Frausto et al., 1995; Schrier and Wang, 2004) and approximately doubles the mortality rate to near 70% (Heemskerk et al., 2009). Rodent models of sepsis-induced AKI suggest that intrarenal microcirculatory failure is a key event leading to the development of septic-AKI (Morin and Stanboli, 1994; Wan et al., 2003; Tiwari et al., 2005; Le Dorze et al., 2009; Seely et al., 2011; Holthoff et al., 2012). The initial inflammatory response during sepsis is characterized by a robust increase in pro-inflammatory cytokines, such as TNF-α (Rackow and Astiz, 1991), which trigger an early cascade of downstream events including upregulation of inducible nitric oxide synthase (iNOS) (Wu and Mayeux, 2007; Wu et al., 2007b), the generation of reactive oxygen (ROS) (Wang et al., 2012) and nitrogen species (RNS) (Wu et al., 2007a; Holthoff et al., 2012), and increased endothelial permeability and microvascular leakage (Yasuda et al., 2006; Wang et al., 2012). Paradoxically, activation of homeostatic mechanisms to raise systemic pressure during septic shock such as activation of the renin-angiotensin system (Salgado et al., 2010) can increase renal vascular resistance and intensify the development of AKI (Cumming et al., 1988). While the effects of sepsis on renal blood flow (RBF) in humans
are still controversial, in rodent models of severe sepsis a fall in RBF (Zager et al., 2006; Brandt et al., 2009) and renal microcirculatory dysfunction (Yasuda et al., 2006; Wu et al., 2007a; Wu and Mayeux, 2007) precede the onset of AKI. We have recently demonstrated that agents which scavenge oxidants and improve the renal microcirculation improve renal function in a cecal ligation and puncture (CLP) model of murine sepsis (Holthoff et al., 2012; Wang et al., 2012). Hence, agents that act locally to improve the renal microcirculation could offer therapeutic potential to combat the development of AKI during sepsis.

3’-5’-cyclic adenosine monophosphate (cAMP) regulates vascular tone and endothelial permeability. Levels of cAMP are regulated by cyclic nucleotide phosphodiesterase enzymes (PDE), which convert cAMP into 5’-adenosine monophosphate (AMP). In various models of inflammation inhibitors of PDE reduce microvascular leakage (Miotla et al., 1998; Schick et al., 2012). At least 60 different mammalian isoforms of PDE exist and tissue-specific expression of different isoforms is thought to provide for the compartmentalization of cAMP levels (Lugnier, 2006). PDE4 is highly expressed in endothelial cells (Netherton and Maurice, 2005; Lugnier, 2006) and targeting PDE4 with inhibitors reduces vascular leakage (Miotla et al., 1998; Lin et al., 2011; Schick et al., 2012). In the kidney, several isoforms of PDE are expressed (Cheng and Grande, 2007) and inhibiting PDE4 has been shown to increase RBF by decreasing renal vascular resistance (Takahashi et al., 1999). In a lipopolysaccharide (LPS) model of sepsis in the rat, PDE4 inhibition not only increased RBF but also acutely improved glomerular filtration rate (Begany et al., 1996; Carcillo et al., 1996).
The standard of care for the septic patient is primarily supportive with administration of fluid resuscitation and inotropic agents in an attempt to maintain organ perfusion (Rivers et al., 2001; De Backer et al., 2013). Unfortunately, effective therapy in the septic patient is hampered because it is usually begun only after the onset of symptoms (Russell, 2006). Consequently, the aim of this study was to evaluate the therapeutic potential of targeting the renal microcirculation during sepsis with rolipram (4-[3-(cyclopentyloxy)-4-methoxyphenyl]-2-pyrrolidinone), a selective inhibitor of the PDE4 isoform (Frossard et al., 1981; Torphy and Cieslinski, 1990) using the CLP model of sepsis-induced AKI in aged mice receiving antibiotics and fluids, a more clinically relevant model than the LPS model, and in a clinically relevant delayed dosing paradigm.
Materials and Methods

**Chemicals and Reagents.** Rolipram was purchased from Cayman Chemicals (Ann Arbor, MI). Fluorescein isothiocyanate-dextran 500,000 DA conjugate (FITC-dextran), FITC-inulin, and Evan’s Blue dye were purchased from Sigma-Aldrich Corp. (St. Louis, MO). Dihydrorhodamine 123 (DHR-123) was purchased from Invitrogen Corp. (Eugene, OR).

**Cecal ligation and puncture (CLP) model of sepsis.** All animals were housed and handled in accordance to *National Institute of Health Guide for the Care of Laboratory Animals* with approval from an internal animal care and use committee. CLP was performed on male C57/BL6 mice (Harlan, Indianapolis, IN) ages 38-40 weeks. Mice were acclimated for 1 week with free access to food and water prior to CLP, as previously described (Wu et al., 2007a; Wang et al., 2011). Briefly, mice were anesthetized using isoflurane and the cecum was isolated via laparotomy. Approximately 1.5 cm of the cecal tip was ligated using a 4-0 silk suture. The cecum was punctured twice with a 21-gauge needle and gently squeezed to express approximately a 1 mm column of fecal material. In sham-operated mice (Sham), the cecum was isolated, but neither ligated nor punctured. Immediately following surgery all mice received 1 ml of pre-warmed normal saline i.p. and were placed in individual cages on a heating pad. Mice studied at time points longer than 6 h were given imipenem/cilastatin (14 mg/kg) and 1.5 ml normal saline (40 ml/kg, s.c.) 6 h post CLP or Sham.

**Intravital video microscopy (IVVM).** IVVM was performed as previously described (Wu et al., 2007a; Wu and Mayeux, 2007; Wang et al., 2011). After
anesthesia with isoflurane, FITC-dextran (1.4 μmol/kg) and DHR-123 (0.8 mg/kg) were administered via the penile vein (2.1 ml/kg) to visualize the capillary vascular space and detect RNS generation, respectively. The left kidney was then exposed by a flank incision and positioned on a glass stage above an inverted Zeiss Axiovert 200M fluorescent microscope equipped with an Axiocam HS camera (Zeiss, Germany). Videos of 10 s (approximately 30 frames/s) at 200X magnification were acquired from five randomly selected, non-overlapping fields of view. Body temperature was maintained at 36° - 37°C with a warming lamp or heating pad throughout the entire procedure. At the end of the experiment venous blood was collected and the right kidney was harvested and fixed in 10% buffered formalin.

**Assessment of the renal microcirculation.** Capillaries were randomly selected from each of the five 10 s videos collected during IVVM and categorized as “continuous flow” where red blood cell (RBC) movement was continuous; “intermittent flow” where RBC movement stopped or reversed; or “no flow” where no RBC movement was observed. Approximately 150 capillaries were analyzed for each animal. Data were expressed as the percentage of vessels in each of the three categories.

**Detection of renal tubule RNS generation and redox stress.** IVVM was used to measure RNS generation and redox stress as in previously described (Wu et al., 2007a; Wu and Mayeux, 2007; Wang et al., 2011). The RNS peroxynitrite preferentially oxidizes DHR-123 to fluorescent rhodamine that is visualized at 535 nm excitation and 590 nm emission (Gomes et al., 2006). Autofluorescence of NADPH can be detected at 365 nm excitation and 420 nm emission, and can be used as a marker of cellular redox stress (Paxian et al., 2004; Wunder et al., 2005; Wu et al., 2007a; Wang et al., 2012).
Still images exposed for 500 ms were captured from the same fields of view used to determine capillary perfusion. Fluorescence intensity was measured by ImageJ (National Institutes of Health, Bethesda, MD) after first subtracting background fluorescence intensity. Data were expressed as arbitrary units/μm².

Assessment of renal microvascular permeability. Renal microvascular permeability was assessed using Evans blue dye, as previously described (Wang et al., 2012). Mice were injected with Evans Blue dye (1% in 0.9% saline solution) at 2 ml/kg via the tail vein. After 30 min, mice were sacrificed and perfused using 30 mL PBS through the left ventricle until all blood was removed. The right kidney was harvested, weighed, and homogenized in 1 mL formamide, then incubated at 55°C for 18 h. The supernatant was collected after centrifugation at 12,000 × g for 30 minutes. The amount of Evans Blue dye in the supernatant was determined by measuring absorbance at 620 nm and correcting for turbidity at 740 nm. Evans Blue dye concentrations were determined from a standard curve and expressed as µg/kg kidney wet weight.

Measurement of mean arterial pressure (MAP) and heart rate in conscious mice. MAP and heart rate were monitored continuously in conscious mice using biotelemetry. Transmitters (Data Sciences International, Minneapolis MN) were implanted into the carotid artery under isoflurane anesthesia and the animals were allowed to recover for 5 days. Mice were re-anesthetized with isoflurane and received CLP or sham surgery. MAP and heart rate were recorded for 10 s every 5 min. At 5.5 h following surgery, mice were administered rolipram (1 mg/kg, i.p.) or vehicle.

Measurement of renal artery blood flow (RBF). RBF was measured using Doppler flow as previously described (Seely et al., 2011; Wang et al., 2012). Under
isoflurane anesthesia, the right kidney was exposed by flank incision and the renal artery and vein were carefully dissected from surrounding tissue using Dumont-5 forceps. The renal artery was isolated from the vein and a Transonic Systems (Ithaca, NY) 0.5 PSL renal artery Doppler flow probe was positioned around the renal artery. The probe was calibrated in water using the zero and scale settings on the TS420 flowmeter (Transonic Systems). RBF was recorded after the flow stabilized (approximately 10 min after placement of the probe) using PowerLab and LabChart software (AD Instruments, New Zealand). Rolipram (1 mg/kg, i.p.) or vehicle was administered via the penile vein. Body temperature was maintained between 36° - 37°C with a heating lamp and heating pad. Data were expressed in ml/min/g kidney weight.

**Measurement of glomerular filtration rate (GFR) in conscious mice.** GFR was measured by following the clearance of a single i.v. bolus dose of FITC-inulin as described previously (Holthoff et al., 2012). In brief, mice were injected with a 5% FITC-inulin solution in normal saline vehicle at a dose of 3.74 μL/g via the penile vein. Blood (25 μL) was collected in heparinized capillary tubes at 3, 7, 10, 15, 35, 55, 75, 90, and 120 min after injection. FITC-inulin was measured at 485 nm excitation and 538 emission, and was quantified against a standard curve. Inulin clearance was calculated using a two-phase decay nonlinear regression analysis. GFR was calculated using the fast and slow phases of inulin clearance after normalizing to the combined weight of both kidneys.

**Measurement of total serum nitric oxide levels.** Serum nitrate + nitrite levels
were determined using the Total Nitric Oxide Assay Kit (Assay Designs, Ann Arbor, MI) as directed by the manufacturer. Data were expressed as serum nitrate + nitrite concentration in μM.

**Measurement of serum creatinine and blood urea nitrogen levels.** Serum creatinine levels and blood urea nitrogen (BUN) were measured using the QuantiChrom™ Creatinine Assay kit and Urea Assay kit, respectively (BioAssay Systems, Hayward, CA). Data were expressed as serum creatinine concentration and serum BUN concentration in mg/dl.

**Treatment with rolipram.** Rolipram was dissolved in 100% dimethyl sulfoxide (DMSO) and stored at -20°C. Immediately prior to administration, the rolipram stock solution (5 mg/mL) was diluted serially in DMSO and then diluted 1:1 with normal saline to achieve the desired dose when administered at 2 µL/g body weight. Vehicle control animals received DMSO diluted 1:1 in normal saline at a dose of 2 µL/g body weight.

**Histological analysis of renal damage.** The periodic acid–Schiff (PAS) stained sections were scored in a blinded, semi-quantitative manner. For each mouse, at least 10 high power (400x) fields were examined. The percentage of tubules that displayed cellular necrosis, loss of brush border, cast formation, vacuolization, and tubule dilation were scored as follows: 0 = none, 1 = <10%, 2 = 11-25%, 3 = 26-45%, 4 = 46-75%, and 5 = >76%.

**Statistical analysis.** Data, presented as mean ± SEM, were analyzed using Prism 5.0 (GraphPad Software Inc., San Diego, CA). The Student’s t-test was used when two groups were compared and a one-way ANOVA followed by the Newman-
Keuls post-hoc test was used when three or more groups were compared. A $P$ value < 0.05 was considered significant. Renal tubular injury scores were analyzed by using the nonparametric Kruskal-Wallis test followed by Dunn multiple-comparisons test.
Results

Acute dose-dependent effects of rolipram. In previous studies we showed that renal cortical peritubular capillary perfusion is extremely low at 6 h following CLP (Holthoff et al., 2012; Wang et al., 2012). To evaluate the acute dose-effects of rolipram on renal microvascular perfusion during sepsis, mice received rolipram (i.p.) at 5.5 h post CLP and peritubular capillary perfusion was assessed by IVVM at 6 h. Sham and CLP control mice received vehicle at 5.5 h. Rolipram produced a bell-shaped dose-response increase in capillary perfusion (Fig. 1). Doses of 1 mg/kg and 3 mg/kg restored the percentage of capillaries with continuous perfusion at 18 h and reduced the percentage of capillaries with no flow to sham levels. Since the lowest and most efficacious dose that acutely restored peritubular capillary perfusion was 1 mg/kg, this dose was used in all subsequent experiments.

Systemic blood pressure effects of rolipram. Our CLP model is a model of severe septic shock (Holthoff et al., 2012; Wang et al., 2012). Changes in mean arterial pressure (MAP) in conscious mice during the course of sepsis are shown in Fig 2A. Data at specific time points are presented in Fig 2B. CLP produced a significant decrease in MAP at 5.5 h (75.6 ± 4.1 mm Hg for CLP versus 113.4 ± 4.7 mm Hg for baseline, n = 4-8, p < 0.05), the time of rolipram injection (1 mg/kg, i.p.). At 30 min after injection, vehicle had no effect on MAP, while rolipram significantly lowered MAP (74.6 ± 4.0 mm Hg for CLP + vehicle versus 60.3 ± 4.0 mm Hg for CLP + rolipram, p < 0.05). At 18 h post CLP MAP in both vehicle and rolipram groups were significantly lower than at baseline but were not different from each other (Fig. 2B). Heart rate is shown in Fig. 2C. CLP produced a significant decrease in heart rate at 5.5 h (361 ± 31 bpm for CLP
versus 527 ± 60 bpm for baseline, n = 4-8, *p* < 0.05), the time of rolipram injection (1 mg/kg, i.p.). At 30 min after injection, vehicle had no effect on MAP, while rolipram significantly raised heart rate (320 ± 16 bpm for CLP + vehicle versus 445 ± 26 bpm for CLP + rolipram, *p* < 0.05). At 18 h post CLP heart rate in both vehicle and rolipram groups were at baseline values.

**Effects of rolipram on renal capillary permeability.** Increased endothelial permeability is a major contributor to end-organ damage during septic shock (Lee and Slutsky, 2010) and occurs as early as 2 h after CLP (Yasuda et al., 2006; Wang et al., 2012). Because inhibitors of cyclic 3’,5’-phosphodiesterase-4 have been shown to decrease endothelial permeability in other inflammatory models (Lin et al., 2011; Schick et al., 2012), we evaluated the effects of rolipram on renal vascular permeability at 6 h following CLP using Evans Blue dye. At 6 h post-CLP, there was a significant increase in renal vascular permeability compared to Sham (0.006 µg EBD/mg kidney for Sham versus 0.026 µg EBD/mg kidney for CLP, n = 5-7, *p* < 0.05). Administration of rolipram (1 mg/kg, i.p.) at the time of CLP blocked the increase in EBD measured in the renal tissue (Fig 3A).

**Acute effects of delayed rolipram treatment on renal blood flow.** Previous studies have shown a rapid decline in RBF following CLP (Holthoff et al., 2012; Wang et al., 2012). Rolipram (Sandner et al., 1999; Tanahashi et al., 1999) and other PDE-4 inhibitors (Begany et al., 1996; Carcillo et al., 1996) have been shown to increase renal blood flow by lowering renal vascular resistance. To evaluate the effects of rolipram on RBF in our model, rolipram or vehicle was given at 5.5 h post-CLP and RBF was measured at 6 h. CLP resulted in a dramatic decline in RBF (1.5 ± 0.4) compared to
Sham (3.7 ± 0.4). Rolipram (1 mg/kg, i.p.) given at 5.5 h post-CLP was able to restore RBF to a level not significantly different from Sham at 6 h \((n = 5-6, \ p < 0.05)\) (Fig. 3B).

**Effects of delayed rolipram administration on renal cortical capillary perfusion at 18 h.** At 18 h after CLP, renal capillary perfusion remains depressed compared to sham-surgery mice (80.3 ± 1.9% continuous flow for Sham + Vehicle versus 33.4 ± 5.0% for CLP + Vehicle, \(n = 5, \ p < 0.05\)). Administration of rolipram (1 mg/kg, i.p.) at 6 h post-CLP was able to restore renal cortical capillary perfusion to Sham + Vehicle levels at 18 h post-CLP (Fig. 4A).

NAD(P)H autofluorescence can be quantified during IVVM and is considered a marker of cellular stress (Paxian et al., 2004; Wunder et al., 2005; Wu and Mayeux, 2007). CLP increased renal tubular NAD(P)H autofluorescence at 18 h after CLP compared to sham (447 ± 56 units/µm² for CLP + Vehicle versus 250 ± 21 units/µm² for Sham + Vehicle, \(n = 5, \ p < 0.05\)). Rolipram given at 6 h post-CLP significantly reduced NAD(P)H autofluorescence at 18 h (295 ± 23 units/µm², \(n = 6, \ p < 0.05\) compared to CLP + Vehicle) (Fig. 4B).

**Effects of rolipram on systemic NO generation and renal tubule RNS generation.** The systemic inflammatory response during sepsis is associated with systemic cytokine release and NO generation (Miyaji et al., 2003; Wang et al., 2011) and induction of inducible nitric oxide synthase (iNOS) in the kidney (Wu et al., 2007b). Moreover, pharmacological inhibition of iNOS has been shown to improve the renal microcirculation and lessen septic AKI (Millar and Theimermann, 1997; Wu et al., 2007b; Wang et al., 2011). To examine whether rolipram blunted the increase in nitric oxide as a potential mechanism of protection, serum levels of nitrate/nitrite were
measured. At 18 h post-CLP, rolipram had no effect on the increase in serum nitrate/nitrite levels (Fig. 5A).

Inhibiting the synthesis of or scavenging NO-derived RNS can protect the renal tubules during sepsis (Wu and Mayeux, 2007; Holthoff et al., 2012; Wang et al., 2012). To assess the effects of rolipram on RNS generation in the cortical renal tubules, oxidation of DHR-123 to rhodamine (Halliwell and Whiteman, 2004; Gomes et al., 2006) was monitored during IVVM. Rhodamine fluorescence was increased in the renal tubules at 18 h (7.7 ± 1.2 units/µm² for Sham + Vehicle versus 21.9 ± 2.8 units/µm² for CLP + Vehicle, n = 6-8, p < 0.05). Rolipram (1 mg/kg, i.p.) administered at 6 h did not affect rhodamine fluorescence at 18 h (Fig. 5B).

**Effect of rolipram on morphological changes.** At 18 h, morphological changes in the CLP group were characterized by mild brush-border loss, tubular degeneration, and vacuolization in the cortical tubules (Fig. 6A & 6B). Treatment with rolipram at 6 h blunted the development of histological damage at 18 h (Fig. 6C) and lowered the tissue injury score (Fig. 6D).

**Effects of rolipram on renal function.** We evaluated the ability of rolipram to improve renal function using blood urea nitrogen (BUN) and serum creatinine levels, two clinically used markers of AKI. CLP + Vehicle mice showed increased BUN (75.8 ± 6.0 mg/dl versus 33.4 ± 7.2 mg/dl) and serum creatinine at 18 h (0.75 ± 0.14 mg/dl versus 0.27 ± 0.06 mg/dl, p < 0.05, n = 6-8). Administration of rolipram (1 mg/kg, i.p.) at 6 h following CLP prevented the rise in the serum markers (Fig. 7A & 7B). Since serum creatinine is a relatively weak marker of AKI in the mouse (Doi et al., 2009), we also measured GFR using FITC-inulin clearance as a more direct measure of renal function.
In the CLP + Vehicle group GFR (0.19 ± 0.05 ml/min/g kidney) was significantly reduced at 18 h compared to the Sham + Vehicle group (1.08 ± 0.05 ml/min/g kidney, \( n = 5-6, \ p < 0.05 \)). Rolipram also significantly but not completely improved GFR (Fig. 7C).
Discussion

Microvascular dysfunction is a strong predictor of death among septic patients (Lundy and Trzeciak, 2009; De Backer et al., 2013). Early goal-directed therapy with the intent of maintaining systemic hemodynamics to preserve organ perfusion has been shown to improve patient mortality; however, mortality still approaches 30% even with adequate resuscitation (Rivers et al., 2001; Dudley, 2004) and is even much higher among septic patients with accompanying renal injury (Bagshaw and Bellomo, 2006). The effectiveness of therapy for the septic patient is limited because it is generally initiated only after the onset of symptoms (Russell, 2006). Hence, agents that are able to restore organ perfusion by improving the microcirculation, even after the onset of septic shock, could lessen organ injury and even promote recovery (Ince, 2005; Le Dorze et al., 2009).

Pretreatment with PDE inhibitors can block the fall in RBF during sepsis (Begany et al., 1996; Carcillo et al., 1996; Wang et al., 2006); however, the impact this may have on the renal microcirculation has never been examined. Lower doses of rolipram (1 mg/kg and 3 mg/kg) acutely restored renal cortical capillary perfusion; however, the higher dose (10 mg/kg) did not. Reasons for the reduced efficacy of rolipram at the high dose are unknown, but may be related to peripheral vasodilation and a worsening of septic shock. Rolipram is known to decrease MAP and increase heart rate (Tanahashi et al., 1999) and we did observe that the dose of 1 mg/kg reduced MAP following CLP even further despite acutely increasing heart rate. These finding support the notion that decreasing vascular resistance to improve the microcirculation in the septic patient may
be more important in preserving organ function than simply raising MAP (Dubin et al., 2009; De Backer et al., 2013).

CLP induced a rapid decline in MAP within the first 6 h, which approached the lower limit for renal pressure needed to maintain autoregulation of RBF and GFR in mouse (Vallon et al., 2001). Although the role of RBF in septic-AKI is not well understood, in this model RBF decreases as early as 2 h after CLP and is correlated with the decline in the renal microcirculation (Wang et al., 2012). Rolipram was able to restore RBF to sham levels within 30 minutes, paralleling the acute restoration of cortical capillary perfusion. This increase in RBF was likely due to the ability of rolipram and other PDE inhibitors to reduce renal vascular resistance since renal vascular resistance would be predicted to decrease under conditions in which MAP is reduced yet RBF is increased (Sandner et al., 1999; Tanahashi et al., 1999). Our findings are in agreement with other studies showing that selective PDE4 inhibition can improved RBF by reducing renal vascular resistance in a rat model of LPS-induced AKI (Begany et al., 1996; Carcillo et al., 1996).

Another mechanism that could contribute to restoration of the renal microcirculation during sepsis is rolipram’s ability to reduce capillary permeability. Increased microvascular permeability is a hallmark of sepsis (Lee and Slutsky, 2010) and occurs within the first few hours in the kidney following CLP in the mouse (Wang et al., 2012). PDE inhibitors have been shown to enhance the endothelial barrier by stabilizing tight junctions between endothelial cells in vitro (Liu et al., 2012) and rolipram specifically has been shown to blunt the increase in endothelial permeability in intestine and lung following ischemia/reperfusion (Souza et al., 2001). Administration of rolipram
at the time of CLP did reduce the very early increase in renal capillary permeability as anticipated. Although the initial increase in permeability may not be effectively targeted by a delayed dosing schedule because it is one of the earliest events in the kidney (Wang et al., 2012), increased renal capillary permeability persists throughout the course of sepsis (Yasuda et al., 2006; Wang et al., 2012). Consequently, interrupting renal capillary leak may be an additional mode of action to help promote recovery of the microcirculation.

Physiological control of the renal microcirculation is complex and poorly understood (Mayeux and Macmillan-Crow, 2012). Factors such as NO, ROS, RNS, and vasoactive hormones released by the tubular epithelium and capillary endothelial cells regulate renal perfusion. Systemic and renal generation of NO and increased generation ROS and RNS by the renal tubules are early events following induction of sepsis in the mouse (Wu and Mayeux, 2007; Kalakeche et al., 2011; Holthoff et al., 2012; Wang et al., 2012). When rolipram was given at 6 h after CLP, a time when upregulation of iNOS and the formation of superoxide in the renal tubules had already begun (Wu et al., 2007a; Wu et al., 2007b; Wang et al., 2012), there was no effect on subsequent RNS levels despite improvements in capillary perfusion. While previous studies have suggested that hypoxia associated with reduced peritubular capillary perfusion facilitates in some way oxidant generation, these data suggest that oxidant generation by renal tubules is not strictly dependent on microcirculatory failure, at least not in the later stages of sepsis. However, a limitation of the IVVM studies is that it only evaluates the cortical microcirculation and associated tubules. In other region of the kidney oxidant generation may be driven by microcirculatory failure.
These studies also highlight the unique challenges associated with delayed therapy for sepsis. Increased capillary permeability, decreased RBF and GFR, iNOS induction, and oxidant generation are early events in the mouse kidney following sepsis (Wu et al., 2007b; Wang et al., 2012) and injure both the microcirculation and tubular epithelium. It has been proposed that cross-talk within the peritubular capillary microenvironment during stress may increase renal vascular resistance and oxidant generation further to hinder recovery (Venkatachalam and Weinberg, 2012). In previous studies, delayed therapy targeting oxidants also improved renal function by presumably breaking the cycle of injury and allowing recovery. Rolipram also promoted recovery but without reducing oxidant generation, indicating that restoration of the renal microcirculation is critically important for the recovery of renal function. Nevertheless, rolipram did not completely restore GFR. Additional studies are needed to evaluate whether it is the delay in therapy or the sustained oxidant generation that prevented complete restoration of GFR. Resveratrol, an agent that both decreases renal vascular resistance and RNS generation in the kidney during sepsis also failed to completely restore GFR with delayed therapy (Holthoff et al., 2012). Thus, while it may be difficulty to completely overcome the effects of initial renal injury with delayed therapy, targeting the renal microcirculation can improve renal function.

These data suggest that PDE4 inhibitors may provide a novel therapeutic option for the treatment of sepsis-induced AKI by improving renal perfusion, even after the onset of septic shock and microcirculatory dysfunction. Nevertheless, given the complexities of sepsis-induced AKI, combination therapy directed toward multiple
targets in the peritubular capillary microenvironment would likely have the greatest chance of improving outcomes in septic patients.
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Authorship Contributions

Participated in research design: Holthoff, Mayeux

Conducted experiments: Holthoff, Wang, Patil

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Wrote or contributed to the writing of the manuscript: Holthoff, Mayeux
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The natural history of the systemic inflammatory response syndrome (SIRS): a


Footnotes

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Figure Legends

**Figure 1.** Acute dose-dependent effects of rolipram on the renal microcirculation during sepsis. Rolipram at doses of 0.3, 1, 3, and 10 mg/kg or vehicle was administered i.p. at 5.5 h post CLP or sham surgery. At 6 h IVVM was used to assess cortical peritubular capillary perfusion. In the CLP + Vehicle group the percentage of capillaries with continuous flow was decreased while the percentages with intermittent and no flow were increased. Rolipram at doses of 1 mg/kg and 3 mg/kg restored perfusion to levels in the Sham + Vehicle group. *$P < 0.05$ compared to the Sham + Vehicle group. Data are mean ± SEM, $n = 4$–7 mice/group.

**Figure 2.** Effects of rolipram on systemic hemodynamics during sepsis. Changes in mean arterial pressure (MAP) in conscious mice during the course of sepsis are shown in panel A. Data at specific time points are presented in panel B. CLP produced a significant decrease in MAP at 5.5 h, the time of rolipram injection (1 mg/kg, i.p.). At 30 min later rolipram significantly decreased MAP compared to vehicle. Data on heart rate at specific time points are presented in panel C. CLP produced a significant decrease in heart rate at 5.5 h, the time of rolipram injection. Rolipram significantly raised heart rate 30 min after administration compared to vehicle. *$P < 0.05$ compared to baseline vehicle; †$P < 0.05$ compared to baseline rolipram; #$P < 0.05$ compared to 6 h vehicle. Data are mean ± SEM, $n = 4$–8 mice/group.
**Figure 3.** Effects of rolipram on capillary leakage and renal blood flow during sepsis. Rolipram (1 mg/kg, i.p.) was administered at the time of CLP and Evans Blue dye leakage was measured at 6 h (panel A). The acute effects of rolipram on renal blood flow are shown in panel B. Rolipram (1 mg/kg, i.p.) was administered at 5.5 h post CLP and renal blood flow was measured at 6 h. *$P < 0.05$ compared to Sham + Vehicle and CLP + rolipram. Data are mean ± SEM, $n =$ 5–7 mice/group.

**Figure 4.** Effects of rolipram on the renal microcirculation and renal cortical cell stress during sepsis. At 18 h following CLP the percentage of cortical peritubular capillaries vessels with continuous flow was reduced (panel A) and renal cortical NAD(P)H autofluorescence was increased (panel B). Rolipram (1 mg/kg, i.p.) administered at 6 h post CLP reversed the decline in peritubular capillary perfusion and lowered NAD(P)H autofluorescence. *$P < 0.05$ compared to Sham + Vehicle and CLP + Vehicle. Data are mean ± SEM, $n =$ 6 mice/group.

**Figure 5.** Effects of rolipram on serum nitrate/nitrite levels and tubular RNS generation. At 18 h after CLP serum levels of nitrate/nitrite were elevated (panel A) and rhodamine fluorescence was increased in the cortical tubules (panel B) compared to the sham group. Administration of rolipram (1 mg/kg, i.p.) at 6 h post CLP did not affect serum nitrate/nitrite or rhodamine fluorescence levels. *$P < 0.05$ compared to Sham + Vehicle and CLP + Vehicle. Data are mean ± SEM, $n =$ 6-8 mice/group.
Figure 6. Effects of rolipram on renal morphology. Shown are representative images from PAS-stained tissue from the Sham + Vehicle (panel A), CLP + Vehicle (panel B) and CLP + Rolipram (1 mg/kg, i.p.) (panel C) groups. Arrows point to tubules with mild morphological changes at 18 h including loss of brush border, vacuolization, and tubular degeneration. Rolipram administered at 6 h post CLP blunted the modest increase in morphological damage at 18 h (panel D). *P < 0.05 compared to Sham + Vehicle and CLP + Rolipram.

Figure 7. Effects of rolipram on renal function. At 18 h after CLP, BUN (panel A) and serum creatinine (panel B) were elevated and GFR was decreased (panel C). Rolipram reduced BUN and serum creatinine levels, while improving GFR (panels A-C). *P < 0.05 compared to Sham + Vehicle and CLP + Vehicle. Data are mean ± SEM, n = 6-8 mice/group for BUN and serum creatinine and n = 5-6 mice/group for GFR.
Fig. 2

A

MAP (mm Hg)

0  25  50  75  100  125  150

Hours

CLP

CLP + Vehicle

CLP + Rolipram

Rolipram

B

MAP (mm Hg)

Baseline  5.5 h  6 h  18 h

CLP + Vehicle

CLP + Rolipram

C

Heart Rate (bpm)

Baseline  5.5 h  6 h  18 h

CLP + Vehicle

CLP + Rolipram

*  †  #
Figure 3

A. Evans Blue Dye Leakage (µg/mg kidney weight)

- Sham + Vehicle
- CLP + Vehicle
- CLP + Rolipram

B. Renal Blood Flow (ml/min/g kidney weight)

- Sham + Vehicle
- CLP + Vehicle
- CLP + Rolipram

* indicates a significant difference.
A
Peritubular Capillary Perfusion (% cortical vessels)

Continuous | Intermittent | No Flow

Sham + Vehicle
CLP + Vehicle
CLP + Rolipram

B
NAD(P)H Autofluorescence (arbitrary units/µm²)

Sham + Vehicle
CLP + Vehicle
CLP + Rolipram

* indicates significant difference.
Serum Nitrate + Nitrite Concentration (µM)

Sham + Vehicle
CLP + Vehicle
CLP + Rolipram

Rhodamine Fluorescence (arbitrary units/µm²)

Sham + Vehicle
CLP + Vehicle
CLP + Rolipram
Fig. 7

A

Serum Creatinine (mg/dl)

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B

Blood Urea Nitrogen (mg/dl)

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C

Glomerular Filtration Rate (ml/min per kidney weight)

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* Significant difference compared to Sham group
† Significant difference compared to CLP group