Rolipram Improves Renal Perfusion and Function during Sepsis in the Mouse

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Received August 1, 2013; accepted September 9, 2013

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. It is noteworthy that delayed treatment with rolipram at 6 hours after CLP restored the renal microcirculation, reduced blood urea nitrogen and serum creatinine, and increased glomerular filtration rate at 18 hours. 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 proinflammatory 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 species (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 and Mayeux, 2007; Wu et al., 2007a) precede the onset of AKI. We have recently demonstrated that agents that 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.

This work was supported by the National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases [Grants F30-DK085705, R01-DK075991]; and by the American Heart Association [Grants 12PRE12040174, 10PRE4140065]. Additional support was provided by the UAMS Translational Research Institute supported by the National Institutes of Health National Center for Research Resources [Grant UL1TR000039].
dx.doi.org/10.1124/jpet.113.208520.

ABBREVIATIONS: AKI, acute kidney injury; cAMP, 3',5'-cyclic adenosine monophosphate; BUN, blood urea nitrogen; CLP, cecal ligation and puncture; DHR-123, dihydrorhodamine 123; DMSO, dimethylsulfoxide; EBD, Evans blue dye; FITC, fluorescein isothiocyanate; GFR, glomerular filtration rate; iNOS, inducible nitric-oxide synthase; IVM, intravital video microscopy; LPS, lipopolysaccharide; MAP, mean arterial pressure; PDE, phosphodiesterase enzyme; RNS, reactive nitrogen species; ROS, reactive oxygen species; RBF, renal blood flow; Sham, sham-operated mice.

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 (Tanahashi 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 Evans blue dye (EBD) were purchased from Sigma-Aldrich (St. Louis, MO). Dihydrorhodamine 123 (DHR-123) was purchased from Invitrogen (Eugene, OR).

Cecal Ligation and Puncture Model of Sepsis. All animals were housed and handled in accordance to National Institutes of Health *Guide for the Care of Laboratory Animals* with approval from an internal animal care and use committee. Cecal ligation and puncture (CLP) was performed on male C57BL/6 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). In brief, 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 after surgery, all mice received 1 ml of prewarmed normal saline intraperitoneally and were placed in individual cages on a heating pad. Mice studied at time points longer than 6 hours were given imipenem/cilastatin (14 mg/kg) and 1.5 ml of normal saline (40 ml/kg s.c.) 6 hours post-CLP or Sham.

Intravital Video Microscopy. Intravital video microscopy (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 μ mol/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 H5m camera (Zeiss, Oberkochen, Germany). Videos of 10 seconds (approximately 30 frames/seconds) at magnification $\times 200$ were acquired from five randomly selected, nonoverlapping 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-second 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 previously described (Wu and Mayeux, 2007; Wu et al., 2007a; 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 milliseconds 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 per micrometers squared.

Assessment of Renal Microvascular Permeability. Renal microvascular permeability was assessed using EBD, as previously described (Wang et al., 2012). Mice were injected with EBD (1% in 0.9% saline solution) at 2 ml/kg via the tail vein. After 30 minutes, mice were euthanized and perfused using 30 ml phosphate-buffered saline 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 hours. The supernatant was collected after centrifugation at 12,000g for 30 minutes. The amount of EBD in the supernatant was determined by measuring absorbance at 620 nm and correcting for turbidity at 740 nm. EBD concentrations were determined from a standard curve and expressed as microgram per kilogram kidney wet weight.

Measurement of Mean Arterial Pressure and Heart Rate in Conscious Mice. Mean arterial pressure (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 reanesthetized with isoflurane and received CLP or sham surgery. MAP and heart rate were recorded for 10 seconds every 5 minutes. At 5.5 hours following surgery, mice were administered rolipram (1 mg/kg i.p.) or vehicle.

Measurement of Renal Artery Blood Flow. Renal blood flow (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 minutes after placement of the probe) using PowerLab and LabChart software (AD Instruments, Dunedin, 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 milliliter per minute per gram kidney weight.

Measurement of Glomerular Filtration Rate in Conscious Mice. Glomerular filtration rate (GFR) was measured by following the clearance of a single intravenous 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 μ l/g via the penile vein. Blood (25 μ l) was collected in heparinized capillary tubes at 3, 7, 10, 15, 35, 55, 75, 90, and 120 minutes after injection. FITC-inulin was measured at 485-nm excitation and 538-nm 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 μ M concentration.

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 milligram per deciliter.

Treatment with Rolipram. Rolipram was dissolved in 100% dimethylsulfoxide (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 μ l/g body weight. Vehicle control animals received DMSO diluted 1:1 in normal saline at a dose of 2 μ l/g body weight.

Histologic Analysis of Renal Damage. The periodic acid-Schiff (PAS)-stained sections were scored in a blinded, semiquantitative manner. For each mouse, at least 10 high power (400 $\times$) 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 $\pm$ S.E.M., 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 analysis of variance (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 hours 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 (intraperitoneally)

at 5.5 hours post-CLP and peritubular capillary perfusion was assessed by IVVM at 6 hours. Sham and CLP control mice received vehicle at 5.5 hours. Rolipram produced a bell-shaped dose-response increase in capillary perfusion (Fig. 1). Doses of 1 and 3 mg/kg restored the percentage of capillaries with continuous perfusion at 18 hours 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 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 hours (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 minutes 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 hours 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 hours (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 minutes 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 hours 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 hours after CLP (Yasuda et al.,

Fig. 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 i.p. or vehicle was administered at 5.5 hours post-CLP or sham surgery. At 6 hours 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 and 3 mg/kg restored perfusion to levels in the Sham + Vehicle group. **P* < 0.05 compared with the Sham + Vehicle group. Data are mean $\pm$ S.E.M., $n = 4-7$ mice/group.

Fig. 2. Effects of rolipram on systemic hemodynamics during sepsis. Changes in mean arterial pressure in conscious mice during the course of sepsis (A). Data at specific time points are presented (B). CLP produced a significant decrease in MAP at 5.5 hours, the time of rolipram injection (1 mg/kg i.p.). Thirty minutes later rolipram significantly decreased MAP compared with vehicle. Data on heart rate at specific time points are presented (C). CLP produced a significant decrease in heart rate at 5.5 hours, the time of rolipram injection. Rolipram significantly raised heart rate 30 minutes after administration compared with vehicle. * $P < 0.05$ compared with baseline vehicle; † $P < 0.05$ compared with baseline rolipram; # $P < 0.05$ compared with 6 hours vehicle. Data are mean $\pm$ S.E.M., $n = 4-8$ mice/group.

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 hours following CLP using EBD. At 6 hours post-CLP, there was a significant increase in

renal vascular permeability compared with Sham (0.006 μ g of EBD/mg of kidney for Sham versus 0.026 μ g of EBD/mg of 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 hours post-CLP and RBF was measured at 6 hours. CLP resulted in a dramatic decline in RBF (1.5 ± 0.4) compared with Sham (3.7 ± 0.4). Rolipram (1 mg/kg i.p.) given at 5.5 hours post-CLP was able to restore RBF to a level not significantly different from Sham at 6 hours ($n = 5-6$, $P < 0.05$) (Fig. 3B).

Effects of Delayed Rolipram Administration on Renal Cortical Capillary Perfusion at 18 Hours. At 18 hours after CLP, renal capillary perfusion remained depressed compared with sham-surgery mice ($80.3 \pm 1.9\%$ continuous flow for Sham + Vehicle versus $33.4 \pm 5.0\%$ for CLP + Vehicle, $n = 5$, $P < 0.05$). Administration of rolipram (1 mg/kg i.p.) at 6 hours post-CLP was able to restore renal cortical capillary perfusion to Sham + Vehicle levels at 18 hours post-CLP (Fig. 4A).

Fig. 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 EBD leakage was measured at 6 hours (A). The acute effects of rolipram on renal blood flow are shown (B). Rolipram (1 mg/kg i.p.) was administered at 5.5 hours post-CLP and renal blood flow was measured at 6 hours. * $P < 0.05$ compared with Sham + Vehicle and CLP + rolipram. Data are mean $\pm$ S.E.M., $n = 5-7$ mice/group.

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

NADPH 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 NADPH autofluorescence at 18 hours after CLP compared with Sham (447 ± 56 units/ μm^2 for CLP + Vehicle versus 250 ± 21 units/ μm^2 for Sham + Vehicle, $n = 5$, $P < 0.05$). Rolipram given at 6 hours post-CLP significantly reduced NADPH autofluorescence at 18 hours (295 ± 23 units/ μm^2 , $n = 6$, $P < 0.05$ compared with 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 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 Thiemermann, 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 hours 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

Fig. 5. Effects of rolipram on serum nitrate/nitrite levels and tubular RNS generation. At 18 hours after CLP serum levels of nitrate/nitrite were elevated (A) and rhodamine fluorescence was increased in the cortical tubules (B) compared with the Sham group. Administration of rolipram (1 mg/kg i.p.) at 6 hours post-CLP did not affect serum nitrate/nitrite or rhodamine fluorescence levels. * $P < 0.05$ compared with Sham + Vehicle and CLP + Vehicle. Data are mean $\pm$ S.E.M., $n = 6-8$ mice/group.

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 hours (7.7 ± 1.2 units/ μm^2 for Sham + Vehicle versus 21.9 ± 2.8 units/ μm^2 for CLP + Vehicle, $n = 6-8$, $P < 0.05$). Rolipram (1 mg/kg i.p.) administered at 6 hours did not affect rhodamine fluorescence at 18 hours (Fig. 5B).

Effect of Rolipram on Morphologic Changes. At 18 hours, morphologic changes in the CLP group were characterized by mild brush-border loss, tubular degeneration, and vacuolization in the cortical tubules (Fig. 6, A and B). Treatment with rolipram at 6 hours blunted the development of histologic damage at 18 hours (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 and serum creatinine levels, two clinically used markers of AKI. CLP + Vehicle mice showed increased BUN (75.8 ± 6.0 versus 33.4 ± 7.2 mg/dl) and serum creatinine at 18 hours (0.75 ± 0.14 versus 0.27 ± 0.06 mg/dl, $P < 0.05$, $n = 6-8$). Administration of rolipram (1 mg/kg i.p.) at 6 hours following CLP prevented the rise in the serum markers (Fig. 7, A and B). 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 per gram kidney) was significantly reduced

Fig. 6. Effects of rolipram on renal morphology. Representative images from PAS-stained tissue from the Sham + Vehicle (A), CLP + Vehicle (B), and CLP + Rolipram (1 mg/kg i.p.) (C) groups. Arrows point to tubules with mild morphologic changes at 18 hours, including loss of brush border, vacuolization, and tubular degeneration. Rolipram administered at 6 hours post-CLP blunted the modest increase in morphologic damage at 18 hours (D). * $P < 0.05$ compared with Sham + Vehicle and CLP + Rolipram.

at 18 hours compared with the Sham + Vehicle group (1.08 ± 0.05 ml/min per gram 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 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 findings 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).

Fig. 7. Effects of rolipram on renal function. At 18 hours after CLP, BUN (A) and serum creatinine (B) were elevated and GFR was decreased (C). Rolipram reduced BUN and serum creatinine levels while improving GFR (A-C). * $P < 0.05$ compared with Sham + Vehicle and CLP + Vehicle. Data are mean $\pm$ S.E.M., $n = 6-8$ mice/group for BUN and serum creatinine and $n = 5-6$ mice/group for GFR.

CLP induced a rapid decline in MAP within the first 6 hours, 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 hours 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 improve 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.

Physiologic 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 of 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 hours 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 regions 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 difficult 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.

Acknowledgments

The authors thank Dr. Shi Liu, director of the Biotelemetry and Ultrasound core at UAMS for assistance with the biotelemetry experiments.

Authorship Contributions

Participated in research design: Holthoff, Mayeux.

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