Role of mitochondrial oxidants in an in vitro model of sepsis-induced renal injury

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Non-standard abbreviations: AKI, acute kidney injury; CLP, cecal ligation and puncture; RNS, reactive nitrogen species; ROS, reactive oxygen species; MnTmPyP, Mn (III) tetrakis (1-methyl-4-pyridyl) porphyrin, tetratosylate, hydroxide; DHR, 2-(3,6-diamino-
9H-xanthen-9-yl)-benzoic acid, methyl ester; PmCTE, primary mouse cortical tubular epithelial cells; L-NMMA, L-N^G- monomethyl arginine citrate; 1400W, N[[3-(aminomethyl)phenyl]methyl]-ethanimidamide, dihydrochloride; DPI diphenyleneiodonium chloride; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide; DAR-4M AM, 3′,6′-Bis(dimethylamino)-9-[2-acetoxycarbonyl-3-amino-4-(N-methylamino)phenyl]xanthylum iodide; LDH, lactate dehydrogenase.

Recommended Section: Toxicology
Abstract

Oxidative stress has been implicated to play a major role in multiorgan
dysfunction during sepsis. To study the mechanism of oxidant generation in acute
kidney injury (AKI) during sepsis, we developed an in vitro model of sepsis using
primary cultures of mouse cortical tubular epithelial cells (PmCTE) exposed to serum
(2.5-10%) collected from mice at 4 h after induction of sepsis by cecal ligation and
puncture (CLP) or Sham (no sepsis). CLP serum produced a concentration-dependent
increase in NO (nitrate + nitrite) release at 6 h and cytotoxicity (lactate dehydrogenase
release) at 18 h compared to Sham serum treatment. Prior to cytotoxicity there was a
decrease in mitochondrial membrane potential, which was followed by an increased
superoxide and peroxynitrite levels compared to Sham serum. The role of oxidants was
evaluated using the superoxide dismutase mimetic and peroxynitrite scavenger,
manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin, tetratosylate, hydroxide
(MnTmPyP). MnTmPyP (10-100 µM) produced a concentration-dependent preservation
of ATP and protection against cytotoxicity. MnTmPyP blocked mitochondrial superoxide
and peroxynitrite generation produced by CLP serum but had no effect on NO levels.
While MnTmPyP did not block the initial CLP-serum induced fall in mitochondrial
membrane potential, it allowed mitochondrial membrane potential to recover. Data from
this in vitro model suggest a time-dependent generation of mitochondrial oxidants,
mitochondrial dysfunction and renal tubular epithelial cell injury and support the
therapeutic potential of manganese porphyrin compounds in preventing sepsis-induced
AKI.
Introduction

Acute kidney injury (AKI) is a frequent complication of sepsis that can increase mortality to as high as 70 percent (Schrier and Wang, 2004). The pathophysiology of sepsis-induced AKI is poorly understood. Consequently, treatment is mainly supportive (Russell, 2006). Oxidative stress in septic patients is thought to play an important role in the multi-organ failure associated with severe sepsis (Galley, 2010). While animal models have suggested that reactive oxygen and nitrogen species (ROS and RNS) may contribute to tubular epithelial injury during sepsis (Wu et al., 2007; Wu and Mayeux, 2007), it has been difficult to study the mechanism of injury because of the complex interactions between the systemic inflammatory response, systemic hemodynamic changes, renal microvascular failure, and peritubular capillary leakage (Yasuda et al., 2006). Nevertheless, animal models of sepsis-induced AKI have shown that the tubular epithelium is the major target in the kidney resulting in decreased renal function as sepsis progresses (Guo et al., 2004; Yasuda et al., 2006; Wu et al., 2007).

In addition to oxidative stress, it is becoming increasingly clear that mitochondrial dysfunction plays an important role in the development of multi-organ failure during sepsis (Crouser, 2004; Galley, 2010). Mitochondrial dysfunction can result in not only leakage of superoxide from the electron transport chain but also decreased ATP synthesis, both of which can lead to tubular epithelial injury (Nowak et al., 2006). Superoxide can also be generated from cytosolic sources like NADPH oxidase that could contribute to increased oxidative stress during sepsis (Wang et al., 1994). Moreover, increased generation of NO in the kidney due to induction of inducible nitric oxide synthase (iNOS) during sepsis (Heemskerk et al., 2006) can react with...
superoxide to generate the potent oxidant peroxynitrite (ONOO⁻) (Beckman, 1996). Oxidants produced during sepsis can react with cellular components like DNA, proteins, and lipids leading to their degradation and thereby accelerating the loss of cell function and damage (Galley, 2010). Indeed, nitrotyrosine protein-adducts, a marker of peroxynitrite generation (Beckman, 1996) have been localized to the damaged tubular epithelium in the kidney during sepsis (Wu et al., 2007; Wu and Mayeux, 2007; Wang et al., 2011).

While the importance of oxidative stress and mitochondrial injury during sepsis has been suggested, its role in the development of sepsis-induced AKI has not been directly studied. The goal of our study was to establish whether mitochondrial oxidant generation might be a mechanism of renal tubular epithelial injury during sepsis. To accomplish this we used a model of murine primary tubular epithelial cells exposed to serum from septic mice to mimic the tubular microenvironment during sepsis. The role of oxidants was evaluated using the superoxide dismutase (SOD) mimetic and peroxynitrite scavenger, Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin, tetratosylate, hydroxide (MnTmPyP) (Faulkner et al., 1994).
Materials and Methods

Chemicals and Reagents. Mn(III)tetrakis(1-methyl-4-pyridyl)porphyrin, tetratosylate, hydroxide (MnTmPyP) was purchased from EMD Chemicals (Gibstown, NJ). L-N\textsuperscript{G}-monomethyl arginine citrate (L-NMMA), N\,[3-(aminomethyl) phenyl] methyl]-ethanimidamide, dihydrochloride (1400W), and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanineiodide (JC-1) were purchased from Cayman Chemicals (Ann Arbor, MI). Pierce BCA Protein Assay Kit and radioimmunoprecipitation assay (RIPA) buffer were purchased from Thermo Fisher Scientific (Walthan, MA). Dulbecco’s Modified Eagle Medium (DMEM), Ham’s F12 media, Hank’s buffered salt solution, phosphate buffered saline (PBS), horse serum, 2-(3,6-diamino-9H-xanthen-9-yl)-benzoic acid, methyl ester (DHR 123) and MitoSOX\textsuperscript{TM} red were purchased from Invitrogen Molecular Probes (Eugene, OR). Collagenase type 4 was purchased from Worthington Biochemical (Freehold, NJ) and diphenyleneiodonium chloride (DPI), 3', 6'-Bis (dimethylamino)-9-[2-acetomethoxycarbonyl-3-amino-4-(N-methylamino)phenyl]xanthylium iodide (DAR 4M AM), insulin, hydrocortisone, transferrin, soybean trypsin inhibitor (SBTI) were purchased from Sigma-Aldrich (St. Louis, MO).

Collection of serum from septic mice. All animals were housed and killed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and with approval of the University of Arkansas for Medical Sciences Institutional Animal Care and Use Committee. Polymicrobial sepsis was
induced in male C57/BL6 mice at 39-40 weeks of age using cecal ligation and puncture (CLP) as described previously (Wu et al., 2007). Mice receiving sham surgery underwent the same procedure except that the cecum was neither ligated nor punctured. At 4h following CLP or sham surgery, the animals were re-anesthetized with isoflurane and exsanguinated through the vena cava. Sera from mice receiving sham surgery (Sham) or CLP were pooled (5-7 mice per group) and stored at -80°C until use. Serum was collected at 4 h because we (Pathak and Mayeux, 2010) and others (Miyaji et al., 2003) have shown that at that time levels of proinflammatory cytokines such as TNFα and IL1β are elevated. Three sets of pooled sera were required to complete the studies.

**Culture of murine cortical renal epithelial cells.** Primary murine cortical renal epithelial cell cultures (PmCTE) were established from C57/BL6 mice at 4-6 weeks of age as described previously (Sheridan et al., 1993; Seth et al., 2005). Animals are anesthetized with isoflurane, and both kidneys are removed and placed in ice-cold Hank’s balanced salt solution supplemented with antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The capsule around the kidney was removed under sterile conditions and the cortex was removed and finely minced with a sterile scalpel. Following the digestion of the pooled renal cortices from two mice with collagenase/soybean trypsin inhibitor (1 mg/ml) for 45 min at 37°C, the suspension was washed in Hank’s solution containing 10% horse serum. The large undigested pieces were allowed to sediment and the supernatant was collected and centrifuged at 50 x g for 7 min. The pellet of cells and tubule fragments was washed once with DMEM by
centrifugation and resuspended in 25 ml growth medium containing DMEM:Ham’s F12 (1:1) containing 15 mM NaHCO₃, 2 mM glutamine, 15 mM HEPES, 5 µg/ml transferrin, 5 µg/ml insulin, 50 nM hydrocortisone, 0.1 mg/ml kanamycin and 92 IU penicillin. The cell suspension was then plated onto 96-well, 48-well, 24-well, 6-well, and 60 mm x 15 mm Falcon tissue culture plates (Thermo Fisher Scientific, Waltham, MA) at approximately 0.38 mg, 0.75 mg, 1.25 mg, and 5 mg per well, respectively or 7.5 mg per 60 mm dish. The medium was changed the day after plating to remove debris and unattached nephron segments. The medium was then changed every two days thereafter. Initially, cells grow out from the nephron segments that attach to the culture dish surface. Subsequently, monolayers form and become 70-80% confluent following six to seven days in culture. When 80% confluent, PmCTE were treated with Sham-serum or CLP-serum by replacing the media with media containing Sham or CLP serum. For drug-treatment studies, PmCTE were pretreated in growth media for 1 h containing MnTmPyP at concentrations of 10 µM, 30 µM or 100 µM (Maenpaa et al., 2008) or the NADPH oxidase inhibitor DPI at concentrations of 1 µM or 10 µM (Mitchell et al., 2010; Zhao et al., 2011) or for 2 h with the nonselective iNOS inhibitor L-NMMA (1 mM) (Rees et al., 1990) or the selective iNOS inhibitor 1400W (400 µM) (Garvey et al., 1997). At the start of the experiment each drug was added to media containing CLP or Sham serum. The NOS inhibitors were re-applied every 8 h until the end of the experiment. Control cells received media vehicle for the MnTmPyP, L-NMMA and 1400W experiments or 0.8% DMSO vehicle for the DPI experiments.

Measurement of nitrate + nitrite in culture media. PmCTE were treated in media
without phenol red. At the end of the experiment the concentrations of nitrate + nitrite in
the culture medium, used as an indicator of NO synthesis, were estimated using the
Total Nitric Oxide Assay Kit (Assay Designs, Ann Arbor, MI). After treatment with serum
(Sham or CLP), the media was collected, centrifuged and nitrate + nitrite levels were
quantified. The total nitric oxide released by the cells was determined by subtracting
nitrate + nitrite levels initially present in the media from the levels determined after
incubation. The amount of nitrate + nitrite released by the cells was expressed as nmol
nitrite/mg protein after normalization with total protein measured using the Pierce BCA
Protein Assay Kit.

Measurement of mitochondrial membrane potential. Mitochondrial membrane
potential is an important regulator of mitochondrial function and a loss in membrane
potential is an indicator of mitochondrial dysfunction. Mitochondrial membrane potential
was measured using the lipophilic cationic probe JC-1 (Reers et al., 1995). In cells with
high mitochondrial potential, JC-1 forms aggregates, which were measured at
excitation/emission wavelengths of 560 nm/595 nm (red fluorescence). In cells with low
mitochondrial potential JC-1 remains in a monomeric form, which was measured using
at excitation/emission wavelengths of 485 nm/535 nm (green fluorescence). PmCTE
were grown in a black clear-bottom 96-well culture plate and all samples were kept in
the dark. Fluorescence was measured using a SpectraMax plate reader (Molecular
Devices, Sunnyvale, CA). The data was expressed as a ratio of red/green fluorescence
intensity compared to the untreated cells.

Measurement of ATP content. PmCTE were washed twice with ice-cold PBS,
trypsinized and centrifuged to obtain cell pellets. Cell extracts were prepared by suspending the pellet in ice cold PBS followed by sonication and centrifugation. The supernatant was removed and total ATP measurements were made on cell extracts immediately using an ATP bioluminescent assay kit (Molecular Probes, Eugene, OR, USA). Measurements were made on a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). Concentrations of ATP were calculated from a standard curve. The final concentration of ATP was normalized to protein concentration of the cell extracts.

**Assay for cell viability.** Cell viability was determined by measuring the percentage of lactate dehydrogenase (LDH) release from cells into the media using the LDH Cytotoxicity Assay Kit (Cayman Chemical, Ann Arbor, MI) as directed by the manufacturer. PmCTE were grown in 96-well culture plates. At the end of the experiment, the supernatant (100 µl) was transferred to a new 96-well plate and LDH activity was determined as described previously (Pathak and Mayeux, 2010). Samples containing MnTmPyP was diluted to avoid interference with the LDH assay. Data were expressed as the percentage of total cellular LDH released after subtracting background levels of LDH present in Sham and CLP serum.

**Fluorescence microscopy for NO generation.** PmCTE were grown in 6-well culture plates before loading with DAR-4M AM (5 µM in PBS) for 30 min at 37°C. DAR-4M AM is a cell-permeable dye used for the fluorescent detection of NO (Gomes et al., 2006). PmCTE were then washed with PBS prior to treatment. At the end of the experiment
fluorescence images were captured using a Nikon Eclipse E800 microscope equipped with a water immersion objective (60x). All images were captured using a rhodamine filter with equal exposure times. Fluorescence images from five different fields were quantified by averaging the mean fluorescence intensity from six cells selected randomly from each field using Nikon Nis Elements software.

**Quantification of superoxide generation.** Superoxide generation was measured using MitoSOX, which is selectively targeted to the mitochondria (Robinson et al., 2006). Once in the mitochondria, MitoSOX is oxidized by superoxide and exhibits red fluorescence. PmCTE were grown in 6-well culture plates and preloaded with MitoSOX (5 µM in PBS) for 15 min at 37°C. Cells were then washed with PBS to remove any free probe. At the end of the experiment, fluorescence images were captured using a Nikon Eclipse E800 microscope equipped with a rhodamine filter and water immersion objective (60x) and quantified as described above. Since fluorescence microscopy can result in overestimation of superoxide generation due to spectral overlap of MitoSOX oxidation products, a second method was used to quantify superoxide-specific MitoSOX generation (Robinson et al., 2006). PmCTE were grown in black clear-bottom 96-well culture plates and preloaded with MitoSOX as described above. Cells were then washed with PBS to remove any free probe and all plates were kept in the dark. At the end of the experiment, fluorescence (excitation/emission wavelengths of 396 nm/580 nm for superoxide-specific oxidation and 510 nm/580 nm for nonspecific oxidation) was measured using a SpectraMax plate reader (Molecular Devices, Sunnyvale, CA). The data were expressed as arbitrary fluorescence units per milligram protein.
Quantification of RNS generation. The RNS, peroxynitrite, preferentially oxidizes DHR 123 to fluorescent rhodamine 123 (Gomes et al., 2006). Two methods were used to quantify rhodamine fluorescence: fluorescence microscopy and fluorescence spectrophotometry. For fluorescence microscopy, PmCTE were grown in 6-well culture plates before loading with DHR 123 (10 µM in PBS) for 30 min at 37°C. Cells were then washed with PBS prior to treatment. At the end of the experiment, fluorescence images were captured using a Nikon Eclipse E800 microscope equipped with a fluorescein isothiocyanate filter and water immersion objective (60x) and quantified as described above. For fluorescence spectrophotometry, PmCTE were grown in 48-well culture plates and washed with PBS before loading with DHR 123 (20 µM in PBS) for 30 min in at 37°C. Cells were then washed with PBS prior to treatment. At the end of the experiment, cells were washed twice with PBS, lysed in RIPA buffer, sonicated, and then centrifuged at 2,000 x g for 10 min at 4°C as described previously (Pathak and Mayeux, 2010). Rhodamine 123 was measured (excitation/emission wavelengths of 485 nm/520 nm) in the resulting supernatants using a fluorescence plate reader (SpectraMax, Molecular Devices, Sunnyvale, CA). Data were expressed as relative fluorescence units per mg protein.

Mode of cell death. Apoptosis and necrosis were determined with Annexin V and propidium iodide, respectively, using the Apoptosis Detection Kit (BioVision, Mountain View, CA, USA). PmCTE were scraped from 60 mm plates and washed once with binding buffer. Cells were then resuspended in binding buffer and incubated in the
presence of propidium iodide and annexin V-FITC for 15 min at room temperature according to manufacturer’s protocol. PmCTE were then washed twice with binding buffer and processed for flow cytometry. At least 10,000 cells from each experimental condition were evaluated.

**Statistical analysis.** Data were analyzed using Prism 5.0a for Mac (GraphPad Software Inc., San Diego, CA) and presented as mean ± SEM. Data with three or more groups were analyzed using a one-way followed by Newman-Keuls post-test or two-way ANOVA. A \( p \) value < 0.05 was considered significant.
Results

Concentration–dependent effects of CLP serum. Systemic induction of NO synthesis, including in the kidney, is a hallmark of the systemic inflammatory response associated with sepsis (Heemskerk et al., 2009). CLP serum produced a concentration-dependent increase in nitrate + nitrite released in the media at 6h following exposure to 5% and 10% serum (Fig. 1A; $p < 0.05$ compared to Sham). CLP serum also produced a concentration-dependent release of LDH, used as a marker of membrane damage to assess cytotoxicity (Fig. 1B). At 24 h there was a significant release of LDH at a concentration of 10% CLP serum ($p < 0.001$ compared to Sham). The increase in LDH release caused by 10% Sham serum was no different than the release caused by 10% fetal bovine serum used as a serum control (data not shown). Based on these results all subsequent experiments used CLP and Sham serum at a 10% concentration.

We next determined the time course of cytotoxicity (Fig. 1C) and found that 10% CLP serum produced a time-dependent increase in LDH release. At 18 h, CLP serum caused a significant release of LDH compared to Sham serum ($p < 0.05$). Importantly, LDH release in PmCTE exposed to Sham serum for 18 h was not different from untreated cells (growth media alone). Based on these results, subsequent studies were limited to an 18-h incubation time.

Mitochondrial membrane potential and intracellular ATP levels. Mitochondrial membrane potential is generated by the mitochondrial electron transport chain. This gradient is critical for the formation of ATP and a fall in membrane potential is an indicator of mitochondrial dysfunction (Reers et al., 1995). CLP serum produced a time-
dependent decrease in mitochondrial membrane potential, as measured by JC-1 (Fig. 2A). Mitochondrial membrane potential was unaffected by Sham serum at all time points tested. In contrast, mitochondrial membrane potential in CLP serum-treated PmCTE decreased in a time-dependent manner and was significantly lower than in Sham serum-treated cells at 10 h ($p < 0.05$) and 18 h ($p < 0.01$).

To further evaluate the effect of septic serum on mitochondrial function, we measured cellular ATP levels (Fig. 2B). Again, Sham serum was without effect over the times tested. In contrast, CLP serum caused significant fall in ATP levels at 18 h compared to Sham serum ($p < 0.05$). These data showed that the fall in mitochondrial membrane potential preceded the fall in ATP levels and cytotoxicity.

**Mitochondrial oxidant generation.** To assess the effects of Sham serum and CLP serum on mitochondrial superoxide generation, PmCTE were loaded with MitoSOX, which is targeted to the mitochondria (Robinson et al., 2006). There was a significant time-dependent increase in mitochondrial superoxide-specific oxidation of MitoSOX as measured at an excitation of 396 nm (Fig. 3A) in both CLP and Sham treatments. However, CLP serum produced a significant increase in superoxide-specific MitoSOX oxidation compared to Sham serum at 14 h ($p < 0.05$) and 18 h ($p < 0.001$). There were no differences in fluorescence between Sham serum-treated and CLP serum-treated cells at 510 nm excitation at any of the times examined (data not shown).

Co-generation of superoxide and NO leads to the generation of ONOO$^-$ (Beckman, 1996). To investigate the generation of ONOO$^-$, PmCTE were loaded with the RNS indicators DHR 123 (Gomes et al., 2006). The time course of DHR 123
oxidation (Fig. 3B) in cells exposed to CLP serum showed a significant increase in RNS generation at 14 h ($p < 0.05$) and 18 h compared to cells treated with Sham serum ($p < 0.01$).

**MnTmPyP is cytoprotective.** MnTmPyP produced a concentration-dependent (10-100 μM) restoration of ATP levels (Fig. 4A) and protected against cytotoxicity (Fig. 4B) in PmCTE treated with CLP serum. At a concentration of 100 μM, ATP levels in CLP serum treated cells were not different from levels in untreated cells (growth media) and LDH release was not different from Sham serum treated cells. In contrast, MnTmPyP had no effect on ATP levels or LDH release in cells treated with Sham serum or untreated (data not shown) cells. Based on these results, MnTmPyP was tested at a concentration of 100 μM for all subsequent experiments. To determine whether the mode of cell death in PmCTE cells exposed to Sham serum or CLP serum was altered by MnTmPyP, the percentage of apoptotic or necrotic cells was determined. At 24 h, the primary mode of cell death in both Sham serum- and CLP serum-treated PmCTE was necrosis (Fig. 4C). While, the percentage of apoptotic cells following treatment with Sham serum or CLP serum was not different, CLP serum causes a significant ($p < 0.01$) increase in the percentage of necrotic cells. MnTmPyP (100 μM) had no effect on the percentage of apoptotic cells in either Sham serum- or CLP serum-treated cells. In contrast, MnTmPyP blocked the increase in CLP serum-induced necrosis ($p < 0.001$) but had no effect on Sham serum-induced necrosis. These data support the findings with ATP and LDH in that MnTmPyP was only protective in cells treated with CLP serum.
Effects of MnTmPyP on mitochondrial membrane potential. Since a fall in mitochondrial membrane potential can cause a fall in ATP levels, the effects of MnTmPyP on mitochondrial membrane potential in PmCTE were determined. Although MnTmPyP (100 µM) did not block the initial CLP serum-induced fall in mitochondrial membrane potential at 10 h, it blocked the fall in mitochondrial membrane potential at 18 h without affecting mitochondrial membrane potential in cells treated with Sham serum (Fig. 5).

Effects of MnTmPyP on mitochondrial superoxide generation. Shown in Fig. 6A (top panel) are representative images and shown in Fig. 6B and Fig. 6C are the quantification of MitoSOX oxidation from fluorescence microscopy images and spectrophotometry, respectively. MitoSOX oxidation was intracellular and in a punctate pattern with staining of the mitochondrial network in the perinuclear zone. Both of these approaches showed that MnTmPyP (100 µM) significantly blocked the cellular fluorescence intensity and the superoxide-specific MitoSOX oxidation product in CLP-treated cells ($p < 0.001$ compared to Sham serum treatment) without affecting the levels in cells treated with Sham serum. MnTmPyP (100 µM) also significantly blocked ($p < 0.05$) the non-specific oxidation products of MitoSOX measured at excitation wavelength of 510 nm in the CLP-treated cells (data not shown).

Effects of MnTmPyP on RNS generation. Shown in Fig. 6A (bottom panel) are representative images and shown in Fig. 6D is the quantification of DHR 123 oxidation
and the effects of MnTmPyP after an 18 h of exposure of PmCTE to CLP or Sham serum. DHR 123 accumulates in the mitochondria (Tarpey et al., 2004) and, as with MitoSOX, DHR 123 oxidation was intracellular and in a punctate pattern suggesting mitochondrial localization. MnTmPyP (100 µM) significantly blocked the cellular fluorescence intensity in CLP-treated cells ($p < 0.001$ compared to Sham serum treatment) without affecting the levels in cell treated with Sham serum.

**Effects of MnTMPyP on NO generation.** Sepsis stimulates the induction of iNOS in renal tubular epithelial cells leading to increased generation of NO in the kidney (Heemskerk et al., 2006). Since the reduction in RNS by MnTmPyP could be explained by a reduction in NO in addition to superoxide, the effects of MnTmPyP on NO generation were determined. DAR-4M AM was used to directly measure intracellular NO formation (Fig. 7A). At 18 h following addition of CLP serum to PmCTE, NO levels were increased significantly compared to cells treated with Sham serum ($p < 0.001$). MnTmPyP had no effect on NO generation in cells treated with CLP or Sham serum, whereas, the selective iNOS inhibitor 1400W (Garvey et al., 1997) blocked the increase in NO caused by CLP serum. These data suggested that the increase in NO generation stimulated by CLP serum was due to iNOS induction and that MnTMPyP inhibited RNS generation by reducing superoxide levels rather than reducing NO levels.

To examine the role of NO in the cytotoxicity to CLP serum, PmCTE were treated with the nonspecific NOS blocker L-NMMA (Rees et al., 1990) or the specific iNOS blocker 1400W. LDH release from CLP serum-treated cells was significantly decreased by both L-NMMA and 1400W (Fig. 7B). Interestingly, neither the NOS inhibitors
significantly decreased LDH release from cell treated with Sham serum. These data indicated a significant role for iNOS-derived NO in the cytotoxicity caused by CLP-serum.

**Effect of DPI on superoxide generation and cytotoxicity.** To evaluate the role of cytosolic NADPH oxidase as a source of superoxide, two concentrations of the non-selective NADPH oxidase inhibitor DPI were tested against MitoSox oxidation and LDH release. Treatment with DPI at 1 µM or 10 µM had no effect on MitoSox fluorescence in CLP serum-treated PmCTE (Fig. 8A), suggesting that cytosolic NADPH oxidase-derived superoxide was not contributing to MitoSox oxidation. DPI at 1 µM or 10 µM also did not alter LDH release (Fig. 8B), suggesting that cytosolic NADPH oxidase-derived superoxide was not contributing to cytotoxicity.
Discussion

The mechanism of sepsis-induced renal injury is poorly understood because the link between the systemic inflammatory response and injury to the renal tubular epithelium has not been well investigated. Studies using in vivo models of sepsis-induced AKI have shown changes in the renal tubular microenvironment, which include increases in iNOS-derived NO, cellular redox stress, and oxidant generation, all leading to renal injury (Wu et al., 2007; Wu and Mayeux, 2007). A better understanding of the cellular events leading to renal epithelial injury could uncover new potential therapeutic targets for treating or even preventing sepsis-induced AKI. Because of the complex nature of the septic syndrome, the mechanism of cellular injury is difficult to study in vivo. We therefore established an in vitro model to more directly investigate the mechanism of sepsis-induced renal tubular epithelial injury.

Cytokine release occurs within a few hours following CLP-induced sepsis (Miyaji et al., 2003; Pathak and Mayeux, 2010) and the tubular epithelium is directly exposed to serum proteins and serum factors due to the rapid increase in peritubular microvascular permeability (London et al.; Wang et al., 2002; Yasuda et al., 2006). To better model the consequences of these events in vitro, PmCTE were exposed to serum collected 4 h post CLP (or Sham), a time when cytokine release has occurred (Pathak and Mayeux, 2010). Although it is not possible to replicate in vitro all of the complex interactions in the peritubular microenvironment during sepsis, exposure of primary cultures of murine cortical renal tubular epithelial cells (PmCTE) to serum from septic mice did replicate some key features. CLP serum caused iNOS-mediated NO release and the resulting RNS-dependent tubular injury as demonstrated in the mouse kidney following CLP (Wu
Moreover, these findings correlate well with what has been observed in vivo, where RNS generation is recognized as key to the development of renal injury during sepsis initiated by CLP or lipopolysaccharide (Wang et al., 2003; Wu et al., 2007; Wu and Mayeux, 2007).

In a previous study, we used the immortal renal tubular epithelial cell line mIMCD-3 and reported that CLP serum also stimulated iNOS induction and RNS generation in these cells (Pathak and Mayeux, 2010). However, mIMCD-3 cells were less sensitive to the toxic effects of CLP serum than PmCTE, perhaps because mIMCD-3 cells are a transformed cell line. Such cell lines rely largely on glycolysis, while primary cultures obtained directly from kidney tissue are dependent on aerobic metabolism to meet cellular energy demands (Griner and Schnellmann, 1994). Hence, primary cultures may be more appropriate to study mechanisms of cell injury related to mitochondrial dysfunction. Moreover, primary cell cultures of the renal tubular epithelium maintain more characteristics of freshly isolated tubule cells than transformed cell lines (Sheridan et al., 1993; Lash, 1998) and hence, may more closely simulate the response of the tubular epithelium to serum factors during sepsis. These studies with PmCTE extended those with mIMCD-3 cells by investigating the role of mitochondrial injury.

CLP serum produced a concentration-dependent increase in NO release and cell-death indicating the presence of relevant factors in the septic serum that contribute to increased renal cell toxicity. Renal tubular epithelial cells are rich in active mitochondria, which can be a potential source of ROS production generated by an uncoupling of the electron transport chain due to mitochondrial damage during sepsis (Galley, 2010). To explore the mechanism of toxicity two approaches were used. The
first approach was to determine the time courses for loss of mitochondrial membrane potential, cellular ATP, mitochondrial superoxide generation, peroxynitrite formation, and cytotoxicity. Although cause-effect relationships cannot be established by time course studies alone, the data suggest that exposure to septic serum resulted in increased generation of superoxide in the mitochondria and a fall in cellular ATP levels, likely initiated by a fall in mitochondrial membrane potential. These events were also associated with increase in peroxynitrite formation all of which ultimately lead to loss of cell viability. Decreases in ATP due to uncoupling of oxidative phosphorylation has been linked to the collapse of the electrochemical gradient across the mitochondrial membrane and release of ROS (Reid et al., 2005), as well as mitochondrial swelling and membrane damage (Crouser et al., 2002; Crouser et al., 2004). Oxidant injury in primary cultures of renal proximal tubular epithelial cells has also been linked to mitochondrial membrane depolarization and changes in mitochondrial respiratory complex activity (Nowak et al., 2006). The second approach was a pharmacological one to investigate the source and role of superoxide using MnTmPyP, an SOD mimic and peroxynitrite scavenger, and DPI, a non-selective NADPH oxidase inhibitor. MnTmPyP but not DPI blocked CLP serum-mediated mitochondrial superoxide and cell death.

MnTmPyP at identical concentrations used in the present study, inhibited superoxide generation and cytotoxicity in ATP-depleted LLC-PK1 cells (Maenpaa et al., 2008). While MnTmPyP is not specifically targeted to the mitochondria, it has been shown to protect against the loss of mitochondrial complex activities (Ferrer-Sueta et al., 2006; Nilakantan et al., 2010), block mitochondrial superoxide generation in smooth muscle cells (Zhao et al., 2011), and reduce tubular epithelial injury caused by oxidants
during renal ischemic injury (Liang et al., 2009). Also, similar manganese porphyrin compounds, known to accumulate in mitochondria, decrease oxidative damage, protect the electron transport chain (Ferrer-Sueta et al., 2006; Spasojevic et al., 2007; Kos et al., 2009), and reduce tubular epithelial injury caused by oxidants in animal models of renal ischemic injury (Saba et al., 2007).

Treatment of PmCTE with MnTmPyP did not block the initial fall in mitochondrial membrane potential but decreased mitochondrial superoxide generation and allowed the mitochondrial membrane potential to recover. MnTmPyP also increased ATP levels, decreased RNS generation, and decreased cell death. These data support the time course studies and the notion that mitochondrial dysfunction and mitochondrial, rather than cytosolic superoxide generation link increased NO synthesis, RNS generation and cytotoxicity as proposed in Fig. 8. Moreover, the data suggest that tubular epithelial mitochondrial dysfunction may be an initiating event in vivo. The results with MnTmPyP also suggested that NO-derived RNS rather than NO itself is the toxic species. These findings also help to explain how iNOS inhibitors as well as other manganese porphyrin SOD mimetics/peroxynitrite scavengers can protect the kidney during sepsis (Wang et al., 2003; Tiwari et al., 2005; Wu et al., 2007; Wu and Mayeux, 2007).

In summary, the model of PmCTE exposed to septic serum displays a number of key features of sepsis-induced renal tubular epithelial injury in vivo. The data suggest a mechanism of toxicity dependent on mitochondrial oxidant generation and mitochondrial dysfunction. Hence, this model can be valuable in investigating mechanisms of renal tubular epithelial cells injury under “septic” conditions. Also, the protective effects of MnTmPyP support the therapeutic potential of manganese porphyrin compounds and
other mitochondrial protective agents in preventing sepsis-induced AKI.
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None
Authorship Contribution

*Participated in research design:* Pathak, MacMillan-Crow, and Mayeux

*Conducted experiments:* Pathak

*Performed data analysis:* Pathak and Mayeux

*Wrote or contributed to the writing of the manuscript:* Pathak, MacMillan-Crow, and Mayeux
References


Footnotes:

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Figure Legends

Fig. 1. Concentration-dependent effects of CLP serum. Total NO generation (nitrate + nitrite) in PmCTE was measured at 6 h following treatment with 2.5%, 5%, and 10% Sham or CLP serum (panel A). Data are mean ± SEM (n = 5-6; *p < 0.05 compared to Sham serum). Cytotoxicity (LDH) release was measured at 24 h following treatment with 2.5%, 5%, and 10% Sham or CLP serum (panel B). Data are mean ± SEM (n = 3-8; ***p < 0.001 compared to Sham serum). The time courses of cytotoxicity for 10% serum is presented in panel C. Data are mean ± SEM (n = 3-8). There was a time-dependent release of LDH from both CLP and Sham serum treated cells (p < 0.001 by two-way ANOVA). However, CLP serum increased the release of LDH compared to Sham serum (*p < 0.05 compared to Sham serum at 18 h, ***p < 0.001 compared to Sham serum at 24 h, #p < 0.01 compared to untreated).

Fig. 2. Time course of changes in mitochondrial membrane potential and ATP levels. PmCTE cells were treated with 10% Sham or CLP serum for 6, 10, and 18 h. JC-1 was used to assess mitochondrial membrane potential (panel A). Data are mean ± SEM (n = 4-5). A two-way ANOVA indicated a significant (p < 0.01) time-dependent change in CLP serum treatment. CLP serum decreased the red:green fluorescence ratio compared to Sham serum (*p < 0.05 and **p < 0.01 compared to Sham serum treatment). ATP levels (panel B) were measured at 6, 10, and 18 h and reported as the percentage compared to untreated cells. Data are expressed mean ± SEM (n = 4; *p < 0.05 compared to Sham serum treatment).
**Fig. 3. Time course of mitochondrial oxidant generation.** The time course for mitochondrial superoxide generation (MitoSOX fluorescence at 396 nm) and RNS generation (rhodamine fluorescence) in PmCTE exposed to 10% Sham serum or CLP serum are presented in panels A and B, respectively. Data for panels A and B were obtained using a fluorescence spectrophotometer and are expressed as arbitrary fluorescence units/mg protein (mean ± SEM, n = 4-7). A two-way ANOVA indicated a significant ($p < 0.001$) time-dependent change in MitoSOX fluorescence for CLP serum and Sham serum treatments. CLP serum significantly increased MitoSOX fluorescence compared to Sham serum at 14 h ($p < 0.05$) and 18 h ($**p < 0.001$). A two-way ANOVA indicated a significant ($p < 0.05$) time-dependent increase in rhodamine fluorescence for the CLP serum treatment. CLP serum also significantly increased rhodamine fluorescence compared to Sham serum at 14 h ($p < 0.05$) and 18 h ($**p < 0.01$).

**Fig. 4. Cytoprotective role of MnTmPyP.** The concentration-dependent effects of MnTmPyP on cellular ATP levels at 18 h are presented in panel A. PmCTE cells were exposed to 10% Sham serum or CLP serum in the absence or presence of 10, 30, and 100 µM MnTmPyP. Data are mean ± SEM (n = 4; $#p < 0.05$ compared to untreated and Sham serum treatment; $*p < 0.05$ and $***p < 0.001$ compared to CLP serum). The concentration-dependent effects of MnTmPyP on cytotoxicity (LDH release) at 24 h are presented in panel B. LDH data for Sham serum and CLP serum control groups are replotted from Fig. 1B as the zero concentration for reference. Data are mean ± SEM (n = 3-8; $#p < 0.05$ compared to Sham; $*p < 0.05$ and $***p < 0.001$ compared to CLP in the
absence of MnTmPyP). The mode of cell death at 24 h in PmCTE cells exposed to 10% Sham serum or CLP serum in the absence or presence of 100 µM MnTmPyP are presented in panel C. Data are mean ± SEM (n = 3; ##p < 0.01 compared to Sham serum treatment; ***p < 0.001 compared to CLP serum in the absence of MnTmPyP).

**Fig. 5. Effect of MnTmPyP on mitochondrial membrane potential.** PmCTE cells were exposed to 10% Sham serum or CLP serum in the absence or presence of 100 µM MnTmPyP. At 10h and 18 h, JC-1 was used to measure mitochondrial membrane potential. Data are mean ± SEM (n = 4-5; *p <0.05 compared to Sham at 10 h; **p < 0.01 compared to all other groups).

**Fig. 6. Effects of MnTmPyP on mitochondrial superoxide and RNS generation.** Panel A shows representative images (60X) of MitoSOX fluorescence microscopy (top) and rhodamine 123 fluorescence (bottom) in PmCTE cells treated with 10% Sham serum (Sham), 10% CLP-serum (CLP), 10% Sham serum and MnTmPyP (100 µM) (Sham + MnTmPyP), 10% CLP serum and MnTmPyP (100 µM) (CLP + MnTmPyP). Mean intensity of MitoSOX fluorescence (n = 4) and rhodamine 123 fluorescence (n = 3) were quantified from images using Nikon Nis Elements software are presented in panel B and panel D, respectively. The quantitation of superoxide-specific oxidation of MitoSOX (n = 5-6) obtained using a fluorescence spectrophotometer is presented in panel C and are expressed as arbitrary fluorescence units/mg protein. Data are mean ± SEM (***p < 0.001 compared to all other treatments).
Fig. 7. Effects of NO generation on cytotoxicity. To determine whether MnTmPyP altered NO generation, PmCTE cells were incubated with MnTmPyP (100 µM) or the iNOS inhibitor 1400W (400 µM) for 18 h. Fluorescence microscopy with DAR-4M was used to measure NO generation (panel A). Mean fluorescence intensity was quantified from images using Nikon Nis Elements software. Data are mean ± SEM (n = 4; ***p < 0.001 compared to 10% Sham serum treatment and #p < 0.05 compared to 10% CLP serum treatment). The effects of the nonselective iNOS blocker L-NMMA (1 mM) and the selective iNOS blocker 1400W (400 µM) on LDH release in PmCTE are presented in panel B. LDH data for Sham serum and CLP serum control groups are re-plotted from Fig. 1B for reference. Data are mean ± SEM (n = 3-8; ***p < 0.001 compared to all other groups).

Fig. 8. Effects of DPI on mitochondrial superoxide and cytotoxicity. To determine whether cytosolic NADPH oxidase-derived superoxide plays a role in this model, PmCTE cells were incubated with DPI (1µM or 10 µM) and MitoSox oxidation was measured at 396 nm at 18 h (panel A) and in separate experiments, LDH release was measured at 24 h (panel B). Sham serum and CLP serum control groups received DPI vehicle (0.8% DMSO). Data are mean ± SEM (n = 3-4; ***p < 0.001 compared to Sham serum treatment).

Fig. 9. Proposed pathway of cytotoxicity in renal tubular epithelial cells exposed to septic serum. Exposure of renal tubular epithelial cells to septic serum results in induction of iNOS and an initial fall in mitochondrial membrane potential. This is...
followed by mitochondrial superoxide generation and the formation of peroxynitrite, which act to sustain mitochondrial dysfunction (dashed arrows) leading to ATP depletion and cell death. By scavenging superoxide and peroxynitrite, MnTmPyP was able to break the cycle of mitochondrial injury and protect the renal epithelium.
Figure 4

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)
Figure 5

Mitochondrial membrane potential (% untreated)

Sham  CLP  Sham  CLP  Sham  CLP  Sham  CLP

10h  18h  +MnTmPyP  +MnTmPyP

*  *  **  **
Figure 7

Panel A: Mean DAR fluorescence

Panel B: Percent LDH release

Legend:
- Sham
- CLP
- + MnTmPyP
- + 1400W
- #
- + L-NMMA
Figure 8

A

MitoSOX Fluorescence (396 nm) Arbitrary units/mg protein

Sham  CLP  Sham  CLP  Sham  CLP

1 μM DPI  10 μM DPI

B

Percent LDH release

Sham  CLP  Sham  CLP  Sham  CLP

1 μM DPI  10 μM DPI
Figure 9

Septic Serum

iNOS induction

Fall in mitochondrial membrane potential

NO

O$_2^-$

ONOO$^-$

MnTmPyP

ATP depletion

Cell Death

Cell Survival

tubular epithelium