Mechanisms of Mitochondrial Dysfunction in Sepsis-Induced Kidney Injury

Joshua Andrew Smith

Medical University of South Carolina

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Mechanisms of Mitochondrial Dysfunction in Sepsis-Induced Acute Kidney Injury

By

Joshua Andrew Smith

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

Program in Molecular and Cellular Biology and Pathobiology

2015

Approved by:

Chairman, Advisory Committee

[Signatures]
Dedication

This dissertation is dedicated to my parents, Walter and Sandra Smith, who have provided unending support and encouragement throughout each step of my educational journey.
Acknowledgments

I would like to thank Dr. Rick Schnellmann for the tremendous mentoring and guidance he has provided over the course of my dissertation research. I also wish to acknowledge my committee members: Dr. Ken Chavin, Dr. James Chou, Dr. Zhi Zhong, and Dr. Jun Zhu for their support and advice. Finally, I am indebted to the members of Dr. Schnellmann’s laboratory for their training, technical assistance, and willingness to act as a sounding board for ideas and thoughts. Members of the lab (both past and present) who have served in this capacity are: Jenny Blakely, Pallavi Bhargava, Rob Cameron, Dr. Justin Collier, Dr. Daniel Corum, Sara Garrett, Whitney Gibbs, Dr. Sean Jesinkey, Dr. Midhun Korrapati, Janet Saunders, Dr. Jay Stallons, Dr. Lauren Wills, and Ryan Whitaker.
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<th>Description</th>
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<tbody>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>ALT</td>
<td>Alanine aminotransferase</td>
</tr>
<tr>
<td>ATN</td>
<td>Acute tubular necrosis</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPSβ</td>
<td>ATP synthase β</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
<td>CLP</td>
<td>Cecal ligation and puncture</td>
</tr>
<tr>
<td>COX1</td>
<td>Cytochrome c oxidase subunit 1</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>ERR</td>
<td>Estrogen related receptor</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>G6PDH</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
</tr>
<tr>
<td>HK</td>
<td>Hexokinase</td>
</tr>
<tr>
<td>HO-1</td>
<td>Heme oxygenase-1</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IKK</td>
<td>IκB kinase</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
</tbody>
</table>
I/R  Ischemia/reperfusion
JNK  c-Jun N-terminal kinase
KIM-1  Kidney injury molecule-1
LBP  Lipopolysaccharide binding protein
LDH  Lactate dehydrogenase
LPS  Lipopolysaccharide
MAPK  Mitogen-activated protein kinase
MAP3K  Mitogen-activated protein kinase kinase kinase
MB  Mitochondrial biogenesis
MEK  Mitogen-activated protein kinase kinase
mtDNA  Mitochondrial DNA
ND1  NADH dehydrogenase subunit 1
NDUFB8  NADH dehydrogenase 1 beta subcomplex 8
NDUFS1  NADH dehydrogenase Fe-S protein 1
NGAL  Neutrophil gelatinase-associated lipocalin
NFκB  Nuclear factor κB
NRF  Nuclear respiratory factor
PAMPs  Pathogen-associated molecular patterns
PBMCs  Peripheral blood mononuclear cells
PFK  Phosphofructokinase
PGC-1α  Peroxisome proliferator-activated receptor γ coactivator-1α
PGC-1β  Peroxisome proliferator-activated receptor γ coactivator-1β
PI3K  Phosphatidylinositol-3-kinases
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>PK</td>
<td>Pyruvate kinase</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisomal proliferator-activated receptor</td>
</tr>
<tr>
<td>PPP</td>
<td>Pentose phosphate pathway</td>
</tr>
<tr>
<td>PRC</td>
<td>PGC-1-related coactivator</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RBF</td>
<td>Renal blood flow</td>
</tr>
<tr>
<td>RNS</td>
<td>Reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPTC</td>
<td>Renal proximal tubule cell</td>
</tr>
<tr>
<td>RRT</td>
<td>Renal replacement therapy</td>
</tr>
<tr>
<td>SCr</td>
<td>Serum creatinine</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>TFAM</td>
<td>Mitochondrial transcription factor A</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TNFR1</td>
<td>Tumor necrosis factor receptor 1</td>
</tr>
<tr>
<td>TPL-2</td>
<td>Tumor progression locus-2</td>
</tr>
</tbody>
</table>
Abstract

JOSHUA ANDREW SMITH. Mechanisms of Mitochondrial Dysfunction in Sepsis-Induced Acute Kidney Injury. (Under the direction of RICK G. SCHNELLMANN).

Mitochondrial dysfunction is a well-characterized pathophysiological feature of acute kidney injury (AKI). Our laboratory has previously implicated suppression of mitochondrial biogenesis, the process by which cells generate new and functional mitochondria, as an important contributor to development of mitochondrial dysfunction in multiple experimental models of AKI. However, relatively little is known about the molecular mechanisms responsible for disruption of biogenesis in renal cells. The primary goals of this project were to define signaling pathways mediating acute suppression of renal cortical mitochondrial biogenesis and to characterize changes in glycolytic metabolism that might support both cellular and organ function following sepsis-induced AKI.

Mitochondrial biogenesis is primarily regulated by peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), which has been termed the master regulator of this process. In an endotoxin model of septic AKI in mice, we noted rapid suppression of both renal cortical PGC-1α mRNA and protein levels at 3 and 18 hours post-LPS, respectively. Endotoxin-induced loss of PGC-1α led to reduced expression of downstream regulators of mitochondrial biogenesis and electron transport chain proteins along with a decrease in mitochondrial DNA content. Using genetic and pharmacological approaches, we identified an essential role for TLR4-mediated activation of MEK/ERK signaling in acute (< 3 hr) disruption of PGC-1α expression and subsequent mitochondrial biogenesis in the renal cortex. Elucidation of this pathway
may facilitate development of novel therapeutic approaches to reverse mitochondrial dysfunction and enhance renal recovery after AKI.

We next examined changes in glycolytic metabolism that might serve as an adaptive mechanism to generate ATP and support renal function in sepsis-induced AKI. We observed a specific and rapid (< 3 hr) increase in activity of renal cortical hexokinase (HK), the first committed step of glycolysis, that was maintained up to 18 h after systemic LPS exposure. LPS-mediated HK activation was not sufficient to increase glucose flux through the glycolytic pathway as indicated by reduced or unchanged pyruvate and lactate levels in the renal cortex. Surprisingly, HK activation was closely associated with increased activity of glucose-6-phosphate dehydrogenase (G6PDH), the rate-limiting enzyme of the pentose phosphate pathway (PPP), suggesting that glucose is selectively utilized via this pathway following sepsis-induced AKI. We also demonstrated that LPS-induced HK activation occurs in an EGFR/PI3K/Akt-dependent manner in this model. Further work may lead to identification of increased glucose metabolism through the PPP as an adaptive mechanism to counteract oxidative stress in the septic kidney.

In our final study, we tested the efficacy of a potent and specific MEK/ERK inhibitor GSK1120212 in a clinically relevant model of sepsis induced by cecal ligation and puncture (CLP) in mice. Pharmacological blockade of MEK/ERK signaling partially attenuated the systemic response to CLP as indicated by reduced levels of circulating pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and GM-CSF) and restoration of core body temperature in GSK1120212-treated mice. In the kidney, GSK1120212 post-treatment reversed CLP-induced microvascular perfusion deficits and reduced expression of well-
characterized markers of proximal tubular injury. Despite these effects, MEK/ERK inhibition was not sufficient to prevent renal dysfunction as measured by BUN. Taken together, these findings suggest that MEK/ERK inhibition represents a novel approach to partially limit sepsis-induced inflammatory responses and organ injury.
Chapter 1:

Review of acute kidney injury, sepsis, mitochondrial biology, and renal proximal tubule cell metabolism

ACUTE KIDNEY INJURY

Definitions and Clinical Classification Systems

Acute kidney injury (AKI) is broadly defined as the rapid (hours to days) loss of renal function leading to subsequent disruptions in normal elimination of metabolic waste products, electrolyte and fluid balance, and/or urine output (1). Abrupt and severe loss of renal function was previously referred to as acute renal failure (ARF). However, AKI has largely replaced this terminology to better illustrate that even renal insults of lesser severity not resulting in overt organ failure may lead to clinically relevant changes in renal function. Use of ARF is now predominantly restricted to patients who suffer kidney injury which requires renal replacement therapy (RRT) or dialysis. Clinical diagnosis of AKI relies primarily on estimation of glomerular filtration rate (GFR) using serum creatinine (SCr) as well as changes in urine output. Recent efforts have led to the development of consensus criteria to stratify AKI cases by severity using these diagnostic endpoints. In particular, the RIFLE criteria (Risk, Injury, Failure, Loss of function, and End-stage kidney disease) developed by the Acute Dialysis Quality Initiative (ADQI) represents probably the most comprehensive clinical classification system for AKI (2). The RIFLE system allows for stratification of patients to the five categories listed above based on the following definitions: risk is defined as a ≥ 1.5-fold increase in SCr along with urine output < 0.5 mL/kg/h for at least 6 hours; injury is classified by a ≥ 2-fold increase in SCr and urine output < 0.5 mL/kg/h for 12 hours; failure may be defined as a ≥ 3-fold increase in SCr, SCr ≥ 4.0 mg/dL, or an acute rise in SCr ≥ 0.5 mg/dL in
combination with urine output < 0.3 mL/kg/h for 24 hours or anuria for 12 hours; Loss of function indicates the complete loss of renal function for > 4 weeks; and End-stage kidney disease is defined as sustained absence of renal function for > 3 months. A similar classification system, referred to as AKIN staging, has also been developed by the Acute Kidney Injury Network (3). An outline of criteria for both the RIFLE and AKIN classification systems is provided below (Figure 1-1). It should be noted that a number of studies have validated the utility of both the RIFLE and AKIN classification systems in predicting outcomes and mortality following AKI (4, 5). For example, a recent cohort study in the hospital setting demonstrated that patients with maximum RIFLE classes of risk, injury, and failure had mortality rates of 8.8%, 11.4%, and 26.3%, respectively (6). Thus, development of these standardized criteria has been a tremendous tool for both diagnosis and outcome prediction for AKI.
Figure 1-1: Comparison of RIFLE (Risk, Injury, Failure, Loss, and End-stage kidney disease) and AKIN (Acute Kidney Injury Network) criteria for the diagnosis and staging of AKI. sCrea = serum creatinine; RRT = renal replacement therapy. Modified from (7).
Epidemiology of AKI

Estimates of the incidence of AKI vary greatly depending on criteria used for diagnosis and characteristics of the populations studied. A recent community-based study in the US following almost four million individuals estimated incidence of non-dialysis-requiring AKI to be approximately 384 per 100,000 person-years, while incidence of dialysis-requiring AKI was 24.4 per 100,000 person-years (8). AKI occurred more often in the elderly and in males in this study population. However, this study used changes in SCr relative to baseline to define AKI rather than the standardized criteria (RIFLE, AKIN) discussed above. The occurrence of AKI in the hospital and intensive care settings is even greater with incidence in hospitalized adults suffering from acute illness predicted to be approximately 25% using the AKIN staging system (9). In addition, incidence rates as high as 65% have been reported for critically ill patients in the ICU setting (10).

An alarming trend toward increased prevalence of AKI has been observed in multiple patient populations over the last several decades. In the community-based study described above, striking increases in incidence of non-dialysis-requiring (~38%) and dialysis-requiring (~33%) AKI were reported from 1996 to 2003 (8). Similar trends have also been reported in the hospital and ICU settings, indicating that AKI is an increasingly common disorder (11, 12).

Morbidity and Mortality Associated with AKI

A strong association between development of AKI and mortality has been reported in a variety of clinical settings. A recent systematic review of large cohort studies conducted mainly in developed nations estimated that mortality resulting from all
forms of AKI is ~24% in adults and ~14% in children (9). Mortality rates in the intensive care unit are much higher, with estimates approaching 50% - 60% in critically ill patients requiring renal replacement therapy (13, 14). As discussed above, risk of mortality is strongly correlated with injury severity and exponential increases in mortality rates are apparent when stratifying patients by change in SCr after AKI (6, 15). Mortality resulting from the disease appears to be undergoing a modest downward shift, which may be partially attributed to development of better supportive care for AKI patients (16, 17). However, patients who survive an acute episode of AKI remain at higher risk of poor outcomes and long-term mortality as result of morbidity associated with the disease (18). In particular, AKI is an independent risk factor for development of chronic kidney disease (CKD) and further progression to end-stage renal disease (ESRD) leading to additional risk of mortality and poor quality of life measures (19-21).

**Financial Costs Associated with AKI**

In addition to the high risk of morbidity and mortality, AKI is also associated with a significant burden on healthcare and financial resources. Unfortunately, the majority of studies addressing overall financial impact only take into account costs associated with acute care and procedures during an episode of AKI. Given the significant risk of long-term morbidity and mortality discussed above, these calculations likely omit a significant portion of the total economic burden associated with AKI that can be attributed to long-term care and lost productivity (22). A recent study conducted in the US following approximately 20,000 new hospital admissions demonstrated that patients suffering from AKI (defined as an elevation in SCr ≥ 0.5 mg/dL) had a mean increase of 3.5 days in length of hospital stay, higher risk of mortality, and approximately $7500 in added total
hospital costs when compared to patients not developing AKI after adjusting for age, gender, and diagnosis (15). Based on extrapolation from these findings, the authors concluded that expenses directly attributable to AKI might account for as much as 5% of overall hospital costs and over $10 billion in healthcare expenses annually in the US (15). These costs are likely to be significantly higher when accounting for long-term care and lost productivity.

It is important to note here that even smaller changes in SCr (≥ 0.3 mg/dL) have been associated with increased risk of death (~70% increase) and substantially higher overall hospital costs (~$5000 after adjustment for age, gender, and diagnosis) (15). This finding further highlights the utility of the term AKI in replacing ARF to better describe a spectrum of injury where less severe insults can lead to clinically significant deterioration of renal function without overt organ failure. As with mortality, severity of injury is strongly correlated with increasing hospital costs. The figures below demonstrate effects of AKI on mortality and overall healthcare costs when stratifying based on changes in SCr from baseline (Figure 1-2A,B). Taken together, these data indicate that AKI leads to significant mortality and is associated with high financial costs.
Figure 1-2A: Effects of increasing severity of AKI determined by changes in SCr on mortality after AKI. Green bars represent unadjusted data, blue bars have been adjusted for age and gender, and grey bars are adjusted for age, gender, diagnosis-related group (DRG) weight, chronic kidney disease (CKD) status, and ICD-9-CM codes for respiratory, gastrointestinal, malignant, and infectious diseases; n = 1564, 885, 246, and 105 SCr changes of 0.3 to 0.4, 0.5 to 0.9, 1.0 to 1.9, and ≥ 2.0 mg/dl, respectively. Adapted from (15).

Figure 1-2B: Effects of increasing severity of AKI determined by changes in SCr on total hospital costs after AKI. Green bars represent unadjusted data, blue bars have been adjusted for age and gender, and grey bars are adjusted for age, gender, diagnosis-related group (DRG) weight, chronic kidney disease (CKD) status, and ICD-9-CM codes for respiratory, gastrointestinal, malignant, and infectious diseases; n = 1564, 885, 246, and 105 SCr changes of 0.3 to 0.4, 0.5 to 0.9, 1.0 to 1.9, and ≥ 2.0 mg/dl, respectively. Adapted from (15).
Clinical Management and Pharmacological Treatment of AKI

Treatment for AKI remains largely restricted to the use of supportive measures aimed at eliminating the cause of injury and preventing progressive renal dysfunction. In clinical cases where hemodynamic changes and volume depletion contribute to development of AKI, fluid resuscitation is often used in an effort to promote intravascular expansion (23). Debate remains regarding the ideal type of fluid (crystalloid or colloid) for volume replacement as well as the overall clinical utility of this approach (24). Aggressive resuscitation may lead to development of fluid overload, which is associated with poor outcomes and increased mortality after AKI (25, 26). Vasopressors (e.g. vasopressin, dopamine, norepinephrine) are often used in conjunction with fluid resuscitation to reverse hypotensive vasodilation and subsequent renal hypoperfusion. However, there is a considerable lack of evidence to suggest that vasopressor agents have beneficial effects on mortality or need for dialysis in the setting of hemodynamic shock (23).

Use of selective renal vasodilators has also been suggested as a potential therapy to prevent organ dysfunction arising from hemodynamic insufficiency. Low dose dopamine therapy promotes renal vasodilation and thus increases urine output. However, a recent systematic review concluded that low dose dopamine has a negligible effect on restoration of kidney function and mortality in patients suffering from AKI (27). Fenoldopam, a selective agonist of the dopamine receptor D1, has been employed in selected populations at risk for development of AKI. Studies to this point are largely inconclusive concerning therapeutic efficacy of fenoldopam (23). Atrial naturetic peptide (ANP) is unique among selective renal vasodilators because it promotes both vasodilation
of the afferent glomerular arteriole and vasoconstriction of the efferent arteriole leading to an increase in GFR. Although a small, open label trial suggested that ANP greatly improves GFR and reduces need for renal replacement therapy in patients experiencing AKI, subsequent multicenter, randomized trials failed to reproduce these findings (28, 29).

Laboratory evidence has also led to investigation of multiple classes of diuretics for AKI treatment. Among these, loop diuretics (such as furosemide) have been extensively studied due to potential renoprotective effects revealed in earlier mechanistic studies including: inhibition of sodium reabsorption in the medullary thick ascending limb, thereby decreasing cellular energy demand; clearance of tubular casts leading to a reduction in tubular obstruction and associated complications; and dilution of renal toxins such as myoglobin and hemoglobin in the tubular fluid limiting cellular insult in the injured kidney (30, 31). However, multiple randomized, placebo-controlled clinical trials indicate that furosemide is of little benefit in the treatment of AKI (30, 32). Mannitol, an osmotic diuretic, may have limited utility in rhabdomyolysis-induced AKI and organ transplantation, but there is no conclusive evidence for positive effects of this drug in other forms of AKI (33).

The evidence presented above highlights the dismal outlook for clinical treatment of AKI. Dialysis (or renal replacement therapy) remains the only FDA-approved intervention, further indicating the tremendous need for identification of novel therapeutic targets in this disease. However, drug development in AKI presents a number of unique challenges. Standard laboratory tests for assessment of renal function (SCr, BUN) are extremely poor indicators of early organ injury, and use of these markers to
guide treatment decisions limits effective early intervention. The pathophysiology of AKI is also extremely complex, heavily dependent on the underlying cause(s), and remains incompletely understood. However, recent studies have begun to address these deficiencies through identification of novel biomarkers and elucidation of the numerous mechanisms leading to organ dysfunction following renal injury, lending hope that new, effective therapies for AKI are on the horizon.

**Clinical Assessment of AKI: Standard Laboratory Tests and Novel Biomarkers**

*Serum Creatinine*

Serum creatinine (SCr) is the most commonly used laboratory test to measure renal function. As discussed above, changes in serum creatinine are an important component of both clinical classification systems (RIFLE and AKIN) for diagnosis and staging of AKI. Creatinine is formed via catabolism of creatine phosphate in skeletal muscle and is primarily removed from the circulation by way of glomerular filtration in the kidneys. It is often used as a clinical indicator of changes in glomerular filtration rate (GFR) for this reason, but may overestimate true GFR since creatinine undergoes modest secretion by the renal proximal tubule.

*Blood Urea Nitrogen*

Blood urea nitrogen (BUN) is another metabolic waste product that has been extensively used for diagnosis of AKI. Urea is produced in the liver as a byproduct of protein synthesis. Blood urea nitrogen is used as a general indicator of kidney function, and increases in this parameter may be suggestive of renal injury.
Problems Associated with Standard Laboratory Tests

SCr and BUN have been used extensively in detection of AKI primary due to ease of use, low cost, and the absence of better alternatives. However, both may vary due to a number of factors not directly related to renal function including gender, age, nutritional status, steroid use, gastrointestinal bleeding, muscle mass, muscle injury, and intravascular volume status. SCr and BUN are also poor indicators of early renal injury because greater than 50% loss of baseline glomerular filtration rate is required for detectable elevation (1, 34). These factors have led to extensive efforts to identify novel biomarkers for AKI that are organ-specific and have a greater dynamic range. Promising targets are discussed in further detail below.

Kidney Injury Molecule-1

Kidney injury molecule-1 (KIM-1) is a type I transmembrane glycoprotein containing both an immunoglobulin-like domain and a mucin domain within its extracellular component (35). Expression of KIM-1 has been reported in a variety of immune cells where it appears to play a role in Th2, Th1, and Th17 differentiation as well as activation of B cells, dendritic cells, and NK cells (36). Although KIM-1 is expressed at very low levels in the kidney under basal conditions, it was identified as the most highly up-regulated gene following ischemia/reperfusion (I/R) injury in rats and humans (37). Further investigation revealed that KIM-1 is shed into the urine after injury to the proximal tubule (38). A number of pre-clinical and clinical studies have validated urinary KIM-1 as an early and specific marker of AKI arising from various causes including sepsis (39-41). Based on this evidence, urinary KIM-1 was recently approved by the FDA as a biomarker for AKI in pre-clinical drug development.
Neutrophil Gelatinase-Associated Lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) was originally identified as small protein (25 kDa) localized to gelatinase-containing granules in neutrophils. NGAL appears to play an important role in innate immunity through binding and depletion of iron-carrying siderophores essential for bacterial growth (42). Experimental evidence indicates that NGAL mRNA and protein expression is rapidly (less than 3 hours) and robustly (greater than 10-fold) increased in the renal proximal tubule after I/R injury in mice (43). NGAL protein also appeared in the urine as early as 2 hours after reperfusion in this model, long before a change in SCr was detected (24 hours post-I/R) (43). Most interestingly, urinary NGAL was also elevated following milder ischemic injury in mice that was insufficient to produce an increase in SCr, indicating that urinary NGAL may have an expanded dynamic range (43). Subsequent clinical studies have supported the notion that NGAL is a reliable biomarker of AKI, and changes in NGAL may have predictive value for determining patient outcomes in this disease (44).

Cystatin C

While KIM-1 and NGAL have been identified as novel biomarkers of tubular injury, the search for a superior indicator of glomerular filtration rate (GFR) and thus renal function has led to the identification of cystatin C as a potential candidate. Cystatin C is a low molecular weight (13 kDa) protein generated by virtually all nucleated cells. Because it circulates without binding to serum proteins, cystatin C is freely filtered at the glomerulus and undergoes partial reabsorption by the renal proximal tubule. Thus, urinary cystatin C content depends on two parameters that may be disrupted in AKI: GFR and normal function of proximal tubular cells (45). Both serum and urinary cystatin C
have shown promise as early and sensitive makers of AKI in animal models and selected patient populations (46, 47). Serum cystatin C may have other distinct advantages over creatinine because circulating levels of this protein appear to be independent of gender, age, and muscle mass / injury (48).

Other Biomarkers for AKI

In addition to those discussed above, an incredible number of putative markers for AKI detection and outcome prediction are currently under investigation including interleukin-18 (IL-18), interleukin-6 (IL-6), liver-type fatty acid binding protein (L-FABP), glutathione-S-transferase (GST) and C-reactive protein (CRP) (1). Figure 1-3 below illustrates potential utility of selected biomarkers throughout the evolution of a single episode of AKI.
**Figure 1-3: Uses of standard laboratory tests and novel biomarkers throughout the clinical course of AKI.** GFR = glomerular filtration rate; NGAL = neutrophil gelatinase-associated lipocalin; Cys C = cystatin C; IL-18 = interleukin-18; KIM-1 = kidney injury molecule-1; GST = glutathione-S-transferase; L-FABP = liver-type fatty acid binding protein; IL-6 = interleukin-6; CRP = C-reactive protein. Adapted from (1).
Causes of AKI

AKI is often classified as pre-renal, intrinsic, or post-renal based on precipitating factors leading to deterioration of renal function as described below.

Prerenal Causes of AKI

Prerenal AKI may result from any condition that leads to clinically significant reduction in renal blood flow (RBF) and subsequent organ hypoperfusion. Common causes of prerenal AKI include sepsis, congestive heart failure, cardiac surgery, and cardiogenic shock (49). Sepsis is widely recognized as the most common contributing factor to the development of AKI in critically ill patients (50, 51). In addition, conditions that deplete intravascular volume (hypovolemia) such as excessive vomiting, diarrhea, inappropriate diuretic use, and poor fluid intake may also result in renal ischemia. A number of drugs inducing local changes in the renal vasculature including angiotensin-converting enzyme inhibitors, diuretics, non-steroidal anti-inflammatory drugs, and small vessel vasoconstrictors (e.g. cyclosporine, tacrolimus) may increase risk of prerenal AKI, especially in patients with pre-existing vascular disease (49). Estimates indicate that all prerenal causes account for approximately 70% of community-acquired AKI and 40% of hospital-acquired AKI (52, 53).

Intrinsic Causes of AKI

Intrinsic AKI describes direct damage to the kidney and may affect any portion of renal anatomy including the glomerulus, renal tubules, interstitium, and vessels. Tubular injury most often results from development of acute tubular necrosis (ATN) following an ischemic insult (causes discussed above). Therefore, pre-renal and intrinsic causes commonly overlap in the development of AKI, with organ hypoperfusion leading to
direct injury to renal tubules (49). Nephrotoxicants are also an important cause of tubular injury in intrinsic AKI. A number of medications have toxic effects on the tubules including aminoglycoside antibiotics, radiocontrast agents, and chemotherapeutic agents (e.g. cisplatin). Although much less common, acute interstitial nephritis (AIN) is another important contributor to cases of intrinsic AKI. Interstitial nephritis may develop secondary to use of a variety of pharmacological agents including certain antibiotics (such as penicillins and cephalosporins) and non-steroidal anti-inflammatory drugs (NSAIDs). In addition, patients with autoimmune disorders are at risk for interstitial nephritis and glomerulonephritis, both of which may lead to intrinsic renal injury. Intrinsic causes are thought to account for approximately 30% of hospital-acquired AKI (11).

Postrenal Causes of AKI

Although relatively uncommon, obstruction of the urinary tract and urine flow from the kidneys may also lead to AKI. Urinary tract obstruction contributes to renal failure by increasing intratubular hydrostatic pressure, leading to subsequent reductions in GFR and renal blood flow. The most common causes of postrenal AKI include benign prostatic hyperplasia, malignancy of the urinary tract or surrounding anatomical structures, and renal calculi (49).

The incredible number of potential causes of AKI contributes greatly to the complexity of this disorder and to challenges associated with developing effective therapeutics. Since the studies presented herein will address sepsis-induced AKI, this review will now focus on sepsis as a clinical entity with a particular emphasis on septic AKI.
SEPSIS

Definitions and Clinical Staging of Sepsis

Sepsis is an extremely complex disease that manifests by a variety of signs and symptoms depending on the etiology of infection. Recent efforts to generate a consensus definition of sepsis have described this disease as the systemic inflammatory response to known or suspected infection (54). In order to meet clinical criteria for diagnosis of sepsis, patients must display two or more signs of the systemic inflammatory response syndrome (SIRS) including: 1) body temperature > 38.3°C or < 36°C; 2) heart rate > 90 beats per minute; 3) respiratory rate > 20 breaths per minute or $\text{PaCO}_2 < 32 \text{ mmHg}$; and 4) white blood cell count > 12,000 cells per mm$^3$, < 4,000 cells per mm$^3$, or >10% immature band forms (54). The systemic inflammatory response syndrome may occur in response to a variety of conditions, but sepsis is distinguished from other causes by the presence of infection. Additional terms including “severe sepsis,” “septic shock,” and “multiple organ dysfunction syndrome” have also been developed to better describe different stages of sepsis based on disease severity. Severe sepsis refers to sepsis along with organ dysfunction, hypotension, and/or hypoperfusion (54). Clinical signs of organ dysfunction and hypoperfusion may include lactic acidosis (> 1 mmol / L), reduced urine output (< 0.5 mL/kg/hr), and/or altered mental status (55). Septic shock is defined as persistent sepsis-associated hypotension (systolic blood pressure < 90 mmHg or a decrease in systolic blood pressure > 40 mmHg from baseline) despite appropriate fluid replacement (54). Finally, multiple organ dysfunction syndrome denotes abnormal organ function in a critically ill patient such that normal homeostasis cannot be preserved without appropriate clinical management (10). This staging is particularly useful in
predicting clinical outcomes, as more severe stages are correlated with increased mortality (56). The figures provided below outline the terminology used to describe different stages of sepsis (Figure 1-4A) and expanded diagnostic criteria for this disease (Figure 1-4B).
### Figure 1-4A: Clinical terminology and staging of sepsis. Adapted from (55).

<table>
<thead>
<tr>
<th>SIRS</th>
<th>Temperature &gt;38.3°C or &lt;36°C</th>
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<tbody>
<tr>
<td></td>
<td>Heart rate &gt;90 beats/min</td>
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<tr>
<td></td>
<td>Respiratory rate &gt;20 breaths/min or PaCO₂ &lt;32 mmHg</td>
</tr>
<tr>
<td></td>
<td>White blood cell count &gt;12 × 10⁹/l or &lt;4 × 10⁹/l, or &gt;10% immature band forms</td>
</tr>
<tr>
<td>Sepsis</td>
<td>Systemic response to infection, manifested by two or more of the conditions mentioned under SIRS (SIRS + evidence of infection)</td>
</tr>
<tr>
<td>Severe sepsis</td>
<td>Sepsis associated with organ dysfunction, hyperperfusion, or hypotension including lactic acidosis, oliguria, or acute alteration in mental state</td>
</tr>
<tr>
<td>Septic shock</td>
<td>Sepsis-induced hypotension (e.g., systolic blood pressure &lt;90 mmHg or a reduction of &gt;40 mmHg from base line) despite adequate fluid resuscitation, along with the presence of perfusion abnormalities that may include lactic acidosis oliguria, or an acute alteration in mental state. Vasopressor- or inotropic-treated patients may not be hypotensive at the time of measurement</td>
</tr>
<tr>
<td>MODS</td>
<td>The presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention</td>
</tr>
</tbody>
</table>

\[ \text{PaO₂}, \text{ arterial partial pressure of carbon dioxide}; \text{ MODS, multiple organ dysfunction syndrome.} \]

### Figure 1-4B: Expanded diagnostic criteria for sepsis. Adapted from (55).

<table>
<thead>
<tr>
<th>General variables</th>
<th>Fever or hypothermia (temperature &gt;38.3°C or &lt;36°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Heart rate &gt;90 beats/min or &gt;2 SD above the normal value for age</td>
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<tr>
<td></td>
<td>Tachypnea</td>
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<td></td>
<td>Altered mental state</td>
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<td></td>
<td>Significant edema or positive fluid balance (&gt;20 ml/kg over 24 h)</td>
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<tr>
<td></td>
<td>Hyperglycemia (plasma glucose &gt;120 mg/dl or &gt;7.7 mmol/l)</td>
</tr>
<tr>
<td></td>
<td>in the absence of diabetes</td>
</tr>
<tr>
<td>Inflammatory variables</td>
<td>Leukocytosis (wbc count &gt;12,000/µl)</td>
</tr>
<tr>
<td></td>
<td>Leukopenia (wbc count &lt;4,000/µl)</td>
</tr>
<tr>
<td></td>
<td>Normal wbc count with &gt;10% immature forms</td>
</tr>
<tr>
<td></td>
<td>Plasma C-reactive protein level &gt;2 SD above the normal value</td>
</tr>
<tr>
<td></td>
<td>Plasma procalcitonin level &gt;2 SD above the normal value</td>
</tr>
<tr>
<td>Hemodynamic variables</td>
<td>Arterial hypotension (SBP &lt;90 mmHg, MAP &lt;70, or an SBP decrease &gt;40 mmHg in adults or &lt;2 SD below normal for age)</td>
</tr>
<tr>
<td></td>
<td>S₂O₂ &gt;70%</td>
</tr>
<tr>
<td></td>
<td>Cardiac index &gt;3.5 l/min × M⁻²</td>
</tr>
<tr>
<td>Organ dysfunction</td>
<td>Arterial hypoxemia (PaO₂/FiO₂ &lt;300)</td>
</tr>
<tr>
<td></td>
<td>Acute oliguria (urine output &lt;0.5 ml/kg/h or 45 mmol/l for at least 2 h)</td>
</tr>
<tr>
<td></td>
<td>Creatinine increase &gt;0.5 mg/dl</td>
</tr>
<tr>
<td></td>
<td>Coagulation abnormalities (INR &gt;1.5 or aPTT &gt;60 s)</td>
</tr>
<tr>
<td></td>
<td>Ileus (absent bowel sounds)</td>
</tr>
<tr>
<td></td>
<td>Thrombocytopenia (platelet count &lt;100,000/µl)</td>
</tr>
<tr>
<td>Tissue perfusion</td>
<td>Hyperbilirubinemia (plasma total bilirubin &gt;4 mg/dl or &gt;70 mmol/l)</td>
</tr>
<tr>
<td></td>
<td>Hyperlactatemia (&gt;1 mmol/l)</td>
</tr>
<tr>
<td></td>
<td>Decreased capillary refill</td>
</tr>
</tbody>
</table>

wbc, white blood cell; SBP, systolic blood pressure; MAP, mean arterial pressure; S₂O₂, mixed venous oxygen saturation; PaO₂, arterial partial pressure of oxygen; FiO₂, fraction of inspired oxygen; INR, international normalized ratio; aPTT, activated partial thromboplastin time.
Epidemiology of Sepsis

Much of the epidemiological data to date concerning incidence of sepsis in the general population is derived from a large observational study tracking all hospital discharges from seven US states in 1995. Of the nearly 6.7 million hospitalizations recorded in these regions throughout the calendar year, approximately 193,000 cases met the diagnostic criteria described above for severe sepsis (sepsis + organ dysfunction, hypotension, and/or hypoperfusion) (57). Using this data, the authors extrapolated a nationwide incidence of 751,000 cases of severe sepsis annually or approximately 3 cases per 1000 individuals in the general population (57). As expected, incidence of sepsis (as described above) appears to be even higher than that of severe sepsis. A study conducted in 8 academic medical centers reported 19.9 and 10.1 cases per 100 ICU admissions for sepsis and severe sepsis, respectively (58). Sepsis is also extremely common in the developing world, and current data suggests that there may be upwards of 19 million cases of sepsis and severe sepsis worldwide each year (59). Unfortunately, incidence of sepsis appears to be trending upward (60, 61).

Outcomes Associated with Sepsis

Sepsis is as associated with significant mortality that closely correlates with disease severity. In an earlier prospective cohort study following approximately 3,700 patients, 28-day mortality rates of 7%, 16%, 20%, and 46% were observed for patients with systemic inflammatory response syndrome, sepsis, severe sepsis, and septic shock, respectively, using the consensus definitions described above (62). Similar findings have been reported in larger scale studies with estimates of mortality associated with severe
sepsis and septic shock combined ranging from 30-35% at 28 days (58). Sepsis is often listed as a top 10 cause of death in the United States (63).

In addition to early mortality, evidence indicates that survivors of an acute episode of sepsis have poorer long-term outcomes. Risk of long-term death remains elevated for at least 5 years after initial diagnosis of sepsis (64, 65). Severe sepsis is also independently associated with development of significant, persistent cognitive and functional deficits in elderly survivors (66).

**Economic Impact of Sepsis**

Sepsis places an enormous burden on healthcare and financial resources. When comparing adult sepsis admissions (1,028) to all other adult admissions (248,761) in a multi-center, academic care setting, Bates et al. reported that average length of hospital stay was approximately 2 – 3 weeks longer in septic patients (67). Nosocomial (hospital-acquired) sepsis was associated with a significantly higher mean length of stay (26.2 days adjusted for age, gender, and other factors) versus community-acquired cases (18.2 days adjusted). More importantly, total adjusted hospital charges for an acute episode of sepsis ($90,710 for nosocomial cases, $61,440 for community-acquired cases) were $44,000 - $73,000 higher than all other reasons for admission, indicating that septic patients account for substantial medical resource utilization (67). In total, healthcare costs associated with severe sepsis were estimated to be nearly $17 billion annually in the US in 2001 and are likely to be even higher today (57).

**Sepsis Management and Treatment**

Much like clinical management of AKI, sepsis care is largely limited to supportive measures to maintain organ function and limit infection. Rivers et al.
demonstrated that early, goal-directed therapy aimed at optimizing hemodynamic parameters and tissue perfusion decreased mortality in patients with severe sepsis or septic shock (68). Based on these findings, a committee of experts in critical care developed the Surviving Sepsis Campaign to standardize early interventions in septic patients (69). The Surviving Sepsis Campaign provides two “bundles” of appropriate monitoring and treatment measures to facilitate resuscitation, as well as additional treatment recommendations graded based on the strength of available clinical evidence (Figure 1-5 below). Early administration of broad-spectrum antibiotics with activity against all likely causative pathogens is suggested based on studies demonstrating a clear positive association between delay in antimicrobial therapy and mortality (70). A combination of intravenous fluids (specifically crystalloids), vasopressors (norepinephrine, epinephrine, vasopressin), positive inotropes (dobutamine), and low dose corticosteroids (hydrocortisone) are recommended as possible measures to correct hypovolemia / hypotension and improve survival (68, 69, 71). In addition, mechanical ventilation and renal replacement therapy may be necessary in patients who develop acute respiratory distress syndrome (ARDS) and AKI, respectively (69, 72). Successful compliance with the Surviving Sepsis Campaign guidelines in 165 medical centers throughout the United States, Europe, and South America resulted in a significant reduction in unadjusted mortality rates from ~37% to ~31% during a two year study period (73). Although successful implementation of these supportive measures has provided modest benefits, it is important to note that attempts to improve outcomes by directly targeting the inflammatory response have been largely unsuccessful (74). Therefore, a significant need remains for novel therapeutics to prevent mortality in sepsis.
Surviving Sepsis Campaign Bundles

**To be completed within 3 hours:**
1. Measure lactate level
2. Obtain blood cultures prior to administration of antibiotics
3. Administer broad spectrum antibiotics
4. Administer 30 mL/kg crystalloid for hypotension or lactate ≥4 mmol/L

**To be completed within 6 hours:**
5. Apply vasopressors (for hypotension that does not respond to initial fluid resuscitation) to maintain a mean arterial pressure (MAP) ≥ 65 mm Hg
6. In the event of persistent arterial hypotension despite volume resuscitation (septic shock) or initial lactate ≥4 mmol/L (36 mg/dL):
   - Measure central venous pressure (CVP)*
   - Measure central venous oxygen saturation (ScvO2)*
7. Remeasure lactate if initial lactate was elevated*

*Targets for quantitative resuscitation included in the guidelines are CVP of ≥8 mm Hg, ScvO2 of ≥70%, and normalization of lactate.

**Figure 1-5: The Surviving Sepsis Campaign care bundles.** The Surviving Sepsis Campaign provides two distinct bundles of monitoring and treatment measures to promote resuscitation in septic patients. The bundles are to be completed within 3 and 6 hours of identification of suspected sepsis cases. Modified from (69).
Causes of Sepsis

An incredible variety of infectious agents may cause sepsis including bacteria, fungi, and viruses. Gram-negative bacteria including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterobacter* species were traditionally thought to be the most likely causative organisms (75). However, data indicate a recent shift in the identity of microbes responsible for sepsis. Gram-positive bacteria now account for the majority of sepsis diagnoses, with *Staphylococcus aureus*, *Enterococcus* species, coagulase-negative *Staphylococci*, and *Streptococcus pneumoniae* being the most often observed (61, 76). In addition, fungi (especially members of the *Candida* genus) are thought to contribute to approximately 6% of cases (75, 76). The lungs, urinary tract, and peritoneal cavity are common sources of these organisms in septic patients, and the site of infection often dictates the most likely causative agent (75, 76).

Animal Models of Sepsis and Septic AKI

Much of our understanding of the pathophysiology of sepsis and sepsis-induced AKI is derived from experimental models. Animal models of sepsis are classified into three categories based on the technique used to induce a systemic inflammatory response: 1) administration of an exogenous microbial toxin; 2) administration of live pathogens; or 3) modification of the host’s protective barriers.

Administration of Exogenous Microbial Toxins

The most commonly used experimental model in this category is intravenous or intraperitoneal administration of lipopolysaccharide (LPS, also known as endotoxin), a component of the outer membrane of Gram-negative bacteria, in rodents. LPS elicits a strong systemic response by activation of Toll-like receptor 4 (TLR4) and subsequent
production of pro-inflammatory cytokines such TNF-α and IL-1β (77). There are several distinct advantages to using the LPS model in rodents including ease of the experimental technique and highly reproducible effects (77, 78). The early systemic inflammatory response to LPS also closely mimics many of the characteristics of sepsis in the clinic (78). LPS has also been shown to induce renal injury in a dose-dependent manner in rodents. In particular, administration of high doses of endotoxin results in decreased glomerular filtration rate (GFR), increased blood urea nitrogen (BUN), and elevated urinary markers of tubular injury including KIM-1 and NGAL (79). Despite the noted advantages, there are also a number of differences when comparing this model to human sepsis cases. The most important limitation of endotoxin administration is that the increase in systemic pro-inflammatory cytokines is rapid, transient, and several orders of magnitude higher when compared to the slower, more protracted, and lower grade responses in septic patients (80). In addition, high doses of LPS lead to very early development of a hypodynamic cardiovascular condition in rodents whereas humans often display an initial hyperdynamic state (77). These fundamental differences likely explain why a number of targeted anti-inflammatory agents developed based on the LPS model in rodents and primates (e.g. anti-TNF-α and IL-1) have largely failed to show efficacy in clinical trials (74). A number of other bacterial toxins and TLR agonists including synthetic lipopeptides, CpG DNA, and zymosan have also been used to model sepsis in rodents (81).

**LPS Signaling**

As discussed briefly above, LPS signals primarily through activation of TLR4 expressed in a variety of cell types including immune cells (dendritic cells, macrophages,
neutrophils, B lymphocytes) and non-immune cells such as endothelial cells and epithelial cells (including proximal tubular epithelial cells) (82). A number of accessory proteins are also involved in initiation of LPS signaling through TLR4. In its native form in vivo, LPS exists as an oligomeric micelle that is not biologically active. A soluble, circulating protein known as LPS binding protein (LBP) is required to convert endotoxin into a monomeric form that can bind its receptor (83). Monomeric LPS is then delivered to the co-receptor CD14 that is expressed in both soluble (sCD14) and glycosphatidylinositol-anchored membrane (mCD14) forms (84). CD14 then transfers LPS to the MD-2 (also known as lymphocyte antigen 96)/TLR4 receptor complex on the plasma membrane of target cells. Binding of LPS to the MD-2/TLR4 complex induces conformational changes that promote TLR4 homodimerization and subsequent activation (85). TLR4 utilizes intracellular Toll/IL-1 receptor (TIR) domains to recruit a variety of adaptor proteins that initiate signaling. TLR4 signaling is typically grouped into two broad classes based on the adaptor proteins involved: an acute MyD88 (myeloid differentiation factor 88)-dependent pathway and a delayed MyD88-independent pathway (86).

MyD88 interacts with TLR4 and another essential adaptor known as TIRAP (toll-interleukin 1 receptor (TIR) domain containing adaptor protein) via a TIR domain located near its carboxyl terminal. MyD88 also contains an N-terminal death domain (DD) that recruits other signaling proteins to the TLR4 complex. In particular, MyD88 interacts with and activates specific IL-1 receptor-associated kinases including IRAK-1 and IRAK-4. Association of active IRAK-1 with TNF receptor-associated factor 6 (TRAF6) is required for TRAF6-mediated ubiquitination and activation of TGF-β-activated kinase-
1 (TAK1), a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family. TAK1 is then responsible for initiating downstream signaling by the nuclear factor κB (NFκB) and mitogen-activated protein kinase (MAPK) pathways (86). TAK1-mediated phosphorylation of the IκB kinase (IKK) complex leads to degradation of IκB and subsequent nuclear translocation of NFκB, which then promotes transcription of a wide variety of cytokines, chemokines, cellular adhesion molecules, and other components of the innate immune response (87). The functional IKK complex also activates another MAP3K known as TPL-2 (tumor progression locus-2, also known as MAP3K8) leading to signaling through the MEK/ERK cascade (88). In addition, TAK1 phosphorylates MAP kinase kinases 7 (MKK7) and 3 (MKK3) to induce c-Jun N-terminal kinases (JNKs) and p38 MAPKs, respectively (86). Overall, the net effect of MyD88-dependent activation of NFκB and MAPK signaling pathways is a rapid pro-inflammatory response at both the transcriptional and translational levels (87, 89).

Recent evidence indicates that macrophages lacking MyD88 still respond to LPS stimulation as measured by delayed activation of NFκB and IFN-β production, suggesting that TLR4 may also signal through MyD88-independent mechanisms (90, 91). The MyD88-independent cascade was subsequently identified and begins with the interaction of active TLR4 with two adaptors known as TIR-containing adaptor molecule (TRIF) and TRIF-related adaptor molecule (TRAM) via TIR domains contained in each of these proteins. TRIF binds to and activates non-canonical IKKs including IKKe and TANK-binding kinase-1 (TBK-1). Both IKKe and TBK-1 phosphorylate the transcription factor known as interferon regulatory factor-3 (IRF-3), allowing for its homodimerization and nuclear translocation (92). Nuclear IRF-3 promotes transcription
of interferon-β (IFN-β). IFN-β in turn binds the interferon α/β receptor (IFNAR) and activates the Janus kinase (JAK)-Signal Transducer and Activator of Transcription (STAT) signaling pathway (87). In addition, TRIF possesses TRAF6-binding domains, allowing it to bind TRAF6 and initiate a delayed NFκB signaling response (93). However, it should be noted that MyD88 knockout mice are protected from endotoxin-induced elevation of pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) and lethality, suggesting that MyD88-independent pathways play only a minor role in in vivo responses to LPS (90). Recent work has also defined TLR4-independent actions of intracellular LPS including activation of the non-canonical inflammasome and caspases-1 and -11, although the overall contributions of these mechanisms to Gram-negative bacterial sepsis remain unclear (94, 95). TLR4-dependent LPS signaling and known negative regulators are summarized in Figures 1-6A and 1-6B below.
**Figure 1-6A: TLR4-dependent LPS signaling.** Binding of LPS to LBP and CD14 facilitates loading of endotoxin onto LPS receptor complex, which is composed of dimerized TLR4 receptors and two molecules of the extracellular adapter MD-2. LPS signaling leads to the early activation of NF-κB and MAPK pathways mediated by the adapters MyD88 and TIRAP (Mal). As a later response to LPS, TLR4 gives rise to the activation of TRAF6 and TBK1, an event mediated by the adapters TRIF and TRAM. ST2, SIGIRR, MyD88s, IRAK-M, Tollip, IRAK2c and IRAK2d are negative regulators. IFN = interferon; IKK = IκB kinase; IRAK = interleukin-1 receptor-associated kinase; IRF = interferon regulatory factor; ISRE = interferon-sensitive response element; LPS = lipopolysaccharide; LBP = LPS-binding protein; Mal = MyD88 adapter like (also known as TIRAP); MAPK = mitogen-activated protein kinase; MKK = mitogen-activated protein kinase kinase; MyD88 = myeloid differentiation factor 88; NF-κB = nuclear factor-κB; RIP = receptor interacting protein; SIGIRR = single immunoglobulin interleukin-1 receptor-associated molecule; TBK = TANK-binding kinase; TLR = Toll-like receptor; TRAF6 = tumor necrosis factor receptor-associated factor; TRIF = TIR-containing adapter molecule; TRAM = TRIF-related adapter molecule; Tollip = TAB, TAK-1 binding protein; TAK = transforming growth factor-β-activated kinase. Uev1a = ubiquitin-conjugating enzyme variant 1a; Ubc13 = ubiquitin-conjugating enzyme. Modified from (86).
Figure 1-6B: TLR4-dependent activation of TPL-2/MEK/ERK signaling by LPS. Stimulation of TLR4 by LPS activates the IKK complex, which phosphorylates Ser927 and Ser932 of p105 large precursor of NF-κB1. This triggers p105 ubiquitination and subsequent degradation, liberating TPL-2. In the absence of p105, TPL-2 phosphorylates and activates MEK, which in turn phosphorylates and activates ERK. In unstimulated cells, TPL-2 and p105 form a ternary complex with ABIN-2. ABIN-2 is released from p105 after LPS stimulation, but its function is currently unknown. IKK-induced p105 proteolysis also liberates the associated mature p50 NF-κB1 subunit to translocate into the nucleus and modulate target gene expression.

LPS = lipopolysaccharide; TLR4 = Toll-like receptor 4; IKK = IκB kinase complex; ABIN-2 = A20-binding inhibitor of nuclear factor-κB (NF-κB)-2; TPL-2 = tumor progression locus-2; MEK = mitogen-activated protein kinase kinase; ERK = extracellular signal-regulated kinase; TNF-α = tumor necrosis factor-α; COX-2 = cyclooxygenase-2. Adapted from (96).
Administration of Live Pathogens

Administration of live bacteria has been employed in both rodents and other larger animals (pigs, sheep, primates) as an alterative means to study sepsis. This method is particularly useful for studying unique systemic responses to a particular pathogen. However, models in this category are not well standardized across different laboratories. There is considerable variability in the route of administration (intravenous, intraperitoneal, subcutaneous, lung inoculation), bacterial species, and bacterial load (81). All of these factors may have important effects on the host response to pathogens. For example, intravenous administration of *Escherichia coli* in mice led to a profound increase in serum levels of the cytokines TNF-α, IL-1, and IL-6 whereas intraperitoneal injection of the same bacterial load resulted in only local increases in these pro-inflammatory markers (97). It has also been established that large bacterial loads commonly used in these models undergo rapid lysis via the complement cascade and are therefore unable to colonize and replicate in the host organism (98). For this reason, administration of live bacteria may better represent a model of toxicosis instead of true bacterial infection (77). Administration of live bacteria can lead to development of AKI in experimental animals depending on the experimental parameters described above. These models have been particularly useful in studying changes in renal hemodynamics in larger animals such as pigs and sheep (99, 100).

Modification of the Host’s Protective Barriers

Models in this category employ a variety of techniques to alter normal protective barriers that act to exclude potential pathogens from sterile compartments. The most commonly used methods involve induction of peritonitis via cecal ligation and puncture
CLP) or colon ascendens stent peritonitis in rodents (77). CLP is currently considered the “gold standard” of sepsis models. The CLP procedure involves midline incision, isolation of the cecum, ligation of a portion of the cecum proximal to the ileocecal valve, and perforation of the ligated cecum using a needle. This allows for expression of fecal contents into the peritoneal cavity and subsequent development of polymicrobial sepsis (101). The severity of disease can be finely tuned based on the length of cecum ligated, number of cecal punctures, and the size of the needle used (102). Unlike the models described above (LPS or bacterial administration), the systemic pro-inflammatory cytokine response following CLP is somewhat delayed, more protracted, and several orders of magnitude lower (80). CLP is also associated with an apparent immunosuppressive phase marked by lymphocyte apoptosis at later time points (103, 104). Based on these findings, CLP is thought to better mimic both the systemic inflammatory response and the compensatory anti-inflammatory syndromes (CARS) characteristic of human cases of sepsis (77, 105). CLP studies have also successfully reproduced failure of clinical trials for targeted anti-inflammatory approaches including anti-TNF-α agents, further suggesting that this may be a more clinically relevant model (106, 107). Although some reports suggested that CLP is not sufficient to produce AKI, most studies observe reproducible changes in classical markers of renal dysfunction including SCr and BUN (108-110). A significant disadvantage of the CLP model is the normal host response to isolate the infected region by creating an abscess (111). Thus, drugs that promote abscess formation may be efficacious in the CLP model but have little clinical utility for sepsis (77).
Other Factors Limiting Clinical Relevance of Animal Models of Sepsis

All of the experimental models of sepsis described above have received considerable criticism because they ignore many other clinical factors that affect outcomes in septic patients. Very few models attempt to mimic the supportive care (broad-spectrum antibiotics, fluid resuscitation, and vasopressors) administered to patients with suspected sepsis. In addition, mostly young and otherwise healthy animals are used for study. This is not truly representative of the normal population affected by sepsis, because the incidence of this disease rises with increasing age (112). In addition, many septic patients have underlying comorbidities including chronic obstructive pulmonary disease (COPD), chronic liver disease, chronic kidney disease, and diabetes that are associated with poor outcomes (57, 113). Significant efforts are now being made to address these shortcomings in experimental models of sepsis (78).

Pathophysiology of Sepsis-induced AKI

As discussed above, incidence of and mortality associated with sepsis-induced AKI remains incredibly high despite implementation of better supportive measures to resuscitate septic patients. Improved therapeutics directed towards limiting organ-specific pathophysiology in septic AKI and promoting renal recovery are essential. Key features of the pathogenesis of sepsis-induced AKI are discussed below.

Macrovascular Hemodynamic Changes in Sepsis-induced AKI

Sepsis-induced AKI has traditionally been viewed as a pre-renal form of AKI resulting from renal hypoperfusion secondary to a systemic hypodynamic state. Decreased renal blood flow may contribute to both the rapid reduction in glomerular
filtration rate as well as direct cellular injury if ischemia is prolonged (114). There are very few studies investigating changes in renal blood flow in humans with sepsis, so current understanding of hemodynamic changes in the septic kidney relies heavily on animal models. Early work by Kikeri et al. in rats demonstrated a significant decrease (~42%) in renal blood flow within 3.5 hours of intravenous administration of high dose LPS (40 mg/kg) in association with a rapid reduction in glomerular filtration rate. These findings occurred in the absence of alterations in systemic hemodynamic parameters including mean arterial pressure and cardiac output (115). Similar changes in renal blood flow have also been reported in some bacterial infusion and CLP models (116, 117).

Although early experimental findings largely confirmed the notion that hypoperfusion contributes to renal dysfunction in sepsis, new evidence has begun to challenge this canonical view. Using a thermodilution renal vein catheter, Brenner et al. reported that renal blood flow was unchanged or increased up to 90 hours after intensive care unit admission in a small cohort of seven septic patients (118). A significant reduction in glomerular filtration rate was noted in four of the seven patients followed despite relatively stable renal blood flow. Studies performed in larger animals also support the notion that renal hypoperfusion may be absent in sepsis-induced AKI. Following intravenous infusion of Escherichia coli in sheep, renal vasodilation and elevated renal blood flow was observed in conjunction with development of a hyperdynamic state (119). Although renal hyperperfusion occurred, serum creatinine increased approximately 4-fold in septic sheep when compared to controls. Subsequent studies using a similar model revealed that recovery of renal function coincided with renal vasoconstriction and normalization of renal blood flow in sheep after Escherichia
coli bacteremia (100). Taken together, these findings provide strong evidence that sepsis-induced AKI may develop independently of reduced renal blood flow.

**Microcirculatory Dysfunction in Sepsis-induced AKI**

Although changes in renal blood flow are still disputed, microcirculatory dysfunction is widely accepted as an important contributor to septic AKI. Studies in humans with sepsis have demonstrated that the density of perfused capillaries is significantly decreased in vascular networks supplying vital organs (120-122). Loss of functional capillary density may occur due to an increase in microvascular shunting. In dogs subjected to intravenous administration of LPS derived from *E. coli*, regional arterial-venous shunting was considerably increased when compared to control animals within 2 hours (15.1% vs. 4.0%) (123). The overall effect of these changes is increased heterogeneity of blood flow in renal capillaries following development of sepsis. In particular, a decrease in the number of renal peritubular capillaries with continuous perfusion in conjunction with an increase in capillaries with intermittent or no flow has been noted in both the endotoxin and CLP models of sepsis in mice (124, 125).

Other functional changes in the microcirculation may also contribute to heterogeneity in microvascular flow. Reduced deformability of circulating red blood cells (RBCs) and polymorphonuclear neutrophils (PMNs) has been observed in septic patients and may contribute to decreased capillary blood flow (126, 127). The systemic inflammatory response to sepsis also results in increased expression of cellular adhesion molecules by white blood cells (CD11b) and endothelial cells (ICAM-1, selectins). Aggregation of activated leukocytes due to interactions with the endothelium also potentially disrupts microvascular flow (127). In addition, sepsis is associated with a
hypercoagulable state and disseminated intravascular coagulation (DIC) that occlude small vessels by formation of microthrombi (127). Together, the effects described above lead to development of local regions of hypoperfusion and hypoxia that contribute to cellular injury in the septic kidney (128).

Another important component of microvascular dysfunction in sepsis-induced AKI is loss of normal endothelial barrier function. Changes in microvascular permeability may be attributed to direct structural damage to endothelial cells, cytokine-mediated disruption of endothelial tight junctions, or CD18-dependent adhesion of PMNs to the endothelium (129-131). Wang et al. demonstrated a significant increase in renal microvascular permeability as early as 6 hours after CLP in mice by measuring Evans blue dye extravasation (109). The leaky capillary endothelium contributes to disruption of capillary flow, extracellular edema formation, and increased exposure of the renal parenchyma to a variety of inflammatory mediators that may exacerbate renal injury in the setting of sepsis (discussed in further detail below) (109). In support of an important role for microvascular dysfunction in the pathophysiology of sepsis-induced AKI, a number of therapies targeted at restoring capillary flow and/or endothelial integrity have shown promise in preventing renal injury in animal models of sepsis (109, 132-135).

**Inflammatory Injury in Septic AKI**

Pathogen recognition by the innate immune system leads to activation of circulating leukocytes and subsequent production of pro-inflammatory cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α) in the setting of sepsis (136). Many of these mediators are implicated in the pathophysiology of sepsis-induced AKI. Renal TNF-α expression and content is increased in the septic
kidney, and this protein has received considerable attention for its role in promoting AKI due to sepsis and other causes (137, 138). Neutralization of TNF-α by the soluble decoy receptor TNFsRp55 prevented endotoxin-mediated reduction of glomerular filtration rate (GFR) in mice, suggesting that TNF-α is an important contributor to renal injury in septic AKI (139). LPS-induced increases in blood urea nitrogen were also attenuated after pre-treatment of mice with an anti-TNF-α antibody in a similar study (79). Cunningham et al. further demonstrated that TNF-α acts directly on tumor necrosis factor receptor 1 (TNFR1) expressed in the septic kidney to promote cellular injury (140). Decreased histological evidence of tubular injury (tubular dilatation, flattening, and vacuolization), renal dysfunction, and apoptosis were observed in global TNFR1 knockout mice after systemic LPS exposure when compared to their wild-type controls. To confirm the role of kidney-specific TNFR1 in pathogenesis of LPS-induced AKI, additional studies involving transplantation of kidneys between TNFR1+/+ and TNFR1−/− mice were performed. Blood urea nitrogen (BUN) only significantly increased in response to endotoxin when TNFR1+/+ kidneys were transplanted into TNFR1−/− mice and not when TNFR1−/− kidneys were transferred to TNFR1+/+ recipients (140). In further support of a role for TNF-α in this disease, systemic administration of recombinant TNF-α reproduces many of the effects of LPS and polymicrobial sepsis on renal function (141, 142). Taken together, these findings provide strong evidence that TNF-α is an important mediator of kidney injury in the setting of sepsis. Similar deleterious roles have been described for IL-1 and IL-6 in other forms of AKI (143, 144).

In addition to circulating pro-inflammatory cytokines, the septic kidney is also exposed to pathogen-derived components that promote inflammatory signaling. These
components are collectively referred to as pathogen-associated molecular patterns (PAMPs), and PAMPs mediate innate immune system activation through a variety of different pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) (145). Tubular and mesangial cells in the kidney have been shown to express TLRs 1-4 and 6 (146, 147) that can interact with PAMPs during sepsis. Recent work by Pierre Dagher’s laboratory indicates that TLR4 expression is increased in proximal and distal tubules, the glomerulus, and the renal vessels after induction of polymicrobial sepsis by CLP in rats (148). The same group also demonstrated that LPS, a classical PAMP, is filtered at the glomerulus, enters the tubular lumen, and interacts with TLR4 on the apical surface of proximal tubule cells in the S1 segment (149). Once LPS interacts with tubular TLR4, it undergoes fluid-phase endocytosis. Internalization in S1 tubular cells results in oxidative damage in the distal S2 portion, further indicating cross talk between segments of the proximal tubule (149). S1 cells appear to be protected from LPS-induced oxidative stress through increased expression of the antioxidant proteins heme oxygenase-1 (HO-1) and sirtuin 1 (SIRT1). Using bone marrow chimeric mice, the authors further demonstrated that LPS internalization and reactive oxygen species generation is dependent on TLR4 expressed in the kidney and not in peripheral leukocytes (149). These findings indicate an important role for renal TLR4 in pathogenesis of sepsis-induced AKI. However, an essential role for extrarenal TLR4 in endotoxin-mediated renal injury in mice has also been described (150). Pharmacological inhibition or genetic knockout of other pattern recognition receptors including TLR2 (a receptor for lipopeptides) and TLR9 (receptor for bacterial CpG DNA) also prevents
development of AKI following CLP in mice, although the issue of whether these effects are attributable to kidney-specific or systemic expression of these proteins remains unclear (151, 152). Finally, activated leukocytes and injured renal cells may produce a number of endogenous molecules known danger-associated molecular patterns (DAMPs) that activate the innate immune system and further exacerbate the inflammatory response and renal dysfunction in septic AKI (153, 154).

Tubular Injury in Sepsis-induced AKI

Development of microvascular dysfunction, local inflammatory responses, and oxidative stress in the septic kidney as described above culminates in direct injury to the renal proximal tubule (155). Unlike other forms of ischemic and toxic injury, sepsis-induced AKI is not associated with widespread acute tubular necrosis (ATN) or apoptosis (156, 157). However, Guo et al. noted an increase in renal caspase-3 activity in association with modestly increased terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive cells and DNA fragmentation after endotoxin administration in mice (158). The broad-spectrum caspase inhibitor z-VAD prevented LPS-induced elevation of blood urea nitrogen, serum creatinine, and tubular injury (158). Thus, apoptotic cell death may play a minor role in pathogenesis septic AKI.

Although a limited increase in tubular apoptosis has been observed, there is a striking discrepancy between the degree of renal cell death and the severity of renal dysfunction in the septic kidney. The histopathology of sepsis-induced AKI is predominantly characterized by heterogeneous, focal tubular injury including tubular dilatation, epithelial cell swelling, brush border flattening, and apical vacuolization (159). These sub-lethal changes are closely associated with tubular dysfunction. Endocytosis of
low molecular weight dextran, a marker of normal tubule cell function, is significantly delayed after systemic LPS administration in rats (160). In addition, endotoxin exposure also disrupts reabsorption of bicarbonate ions [\(\text{HCO}_3^-\)] by inhibiting the \(\text{Na}^+/\text{H}^+\) exchanger expressed in the basolateral membrane of medullary thick ascending limb (MTAL) cells (161). These findings indicate that sub-lethal cellular injury is an important mediator of tubular and renal dysfunction in sepsis. Of particular note in the histopathology of sepsis-induced AKI is apical vacuolization observed in the renal proximal tubule. Recent studies utilizing transmission electron microscopy have demonstrated that the apical vacuoles described above actually represent swollen, dysfunctional mitochondria (162). Thus, mitochondrial dysfunction may play an important role in tubular injury in the septic kidney. The remainder of this review will focus on normal mitochondrial biology and the pathogenic contribution of mitochondrial injury and dysfunction to septic and other forms of AKI. Figure 1-7 below summarizes the roles of microvascular dysfunction, inflammation, and tubular injury in the pathophysiology of sepsis-induced AKI as discussed above.
Figure 1-7: Pathophysiology of sepsis-induced AKI. Sepsis induces profound alterations in microcirculatory flow in the kidney. This alteration is characterized by a significant increment in the heterogeneity of flow, as well as an increase in the proportion of capillaries with intermittent or no flow (represented in the figure by darker hexagons in the peritubular capillary). These areas of sluggish flow have been shown to colocalize with expression of oxidative stress in the tubular epithelial cells, suggesting causation. In addition, immunohistological studies have shown that oxidative stress is localized to the apex of the tubular epithelial cell and that it is associated to the formation of apical vacuoles. In addition, filtered LPS is recognized by S1 tubular epithelial cells through TLR4 and is internalized via endocytosis. This event has been shown to trigger an oxidative outburst, not in the S1 segment cells, but rather in the S2 segment cells. This seems to be associated with the expression in S1, but not in S2 epithelial cells of heme oxygenase 1 (HO-1) and Sirt1, both highly protective against oxidative damage. DAMPs = damage-associated molecular patterns; PAMPs = pathogen-associated molecular patterns; TNF-α = tumor necrosis factor-α; TLR4 = Toll-like receptor 4; ICAM = intercellular adhesion molecule; VCAM = vascular cell adhesion molecule. Adapted from (155).
GENERAL MITOCHONDRIAL BIOLOGY

Mitochondrial Function

Mitochondria are subcellular organelles often viewed simply as the cellular “powerhouse” because of their essential role in ATP generation by oxidative phosphorylation. In addition to their role in energy production, it is important to note that mitochondria regulate multiple aspects of cellular metabolism and homeostasis. In particular, the mitochondrion is involved in intracellular calcium signaling and storage, fatty acid oxidation, heme and steroid biosynthesis, reactive oxygen species (ROS) generation, antioxidant defense mechanisms, apoptotic cell death, and cell cycle progression (163-168).

Mitochondrial Structure

The mitochondrion is membrane-bound organelle possessing an outer mitochondrial membrane (OMM) and an inner mitochondrial membrane (IMM) that are made up of phospholipid bilayers. The double membrane structure creates four distinct compartments within the mitochondria as follows: 1) the OMM containing various channel proteins known as porins and metabolic enzymes; 2) the IMM housing complexes I-IV of the ETC and ATP synthase; 3) the intermembrane space which is essential for maintenance of the proton gradient established by ETC activity; and 4) the mitochondrial matrix enclosed by the IMM that contains a variety of metabolic enzymes (e.g. enzymes involved in the citric acid cycle and fatty acid oxidation), the mitochondrial protein synthesis machinery, and mitochondrial DNA (mtDNA) (169). mtDNA is circular and double-stranded in structure and encodes 37 genes including 22 transfer RNAs (tRNAs), 2 ribosomal RNAs (rRNAs), and 13 electron transport chain proteins (7 in
complex I, 1 in complex III, 3 in complex IV, and 2 in ATP synthase) (170). However, the vast majority of proteins located in the mitochondria (> 1500) are encoded in the nucleus, highlighting the importance of cross-talk between the nucleus and mitochondria in maintenance of organelle function and homeostasis (171).

**Mitochondrial Homeostasis**

Mitochondrial homeostasis is governed by complex interplay between mitochondrial biogenesis, mitochondrial dynamics (fission/fusion), and mitochondrial quality control mechanisms (mitophagy) (172, 173). Mitochondrial biogenesis is reviewed at length below. Dynamics refers to the balance between fission and fusion, two processes that regulate mitochondrial morphology. Fission involves organelle division resulting in generation of a series of small, rod- or sphere-shaped mitochondria (174). Fission is largely mediated by interaction between the cytosolic GTPase Drp1 (dynamin-related protein 1) and Fis1 (mitochondrial fission 1 protein) located on the OMM. Fission is intimately related to induction of apoptosis in injured cells (174). In contrast, fusion creates an elongated, reticular network of mitochondria. Fusion is regulated by the activities of the mitofusins (Mfn1 and Mfn2) located in the OMM and OPA1 (optic atrophy 1) in the IMM (174). Mitophagy represents a quality control mechanism by which injured and dysfunctional mitochondrial are isolated and subsequently degraded by lysosomal digestion. The major pathway facilitating mitophagy involves PINK1 (PTEN-induced putative kinase 1) on the OMM and the cytosolic E3 ubiquitin ligase Parkin (175).
**Mitochondrial Biogenesis**

*Mitochondrial Biogenesis Definitions*

Mitochondrial biogenesis is broadly defined as the process by which cells generate new, functional mitochondria (176). Conceptually, there are at least three potential mechanisms that might lead to increased mitochondrial mass in the cell: 1) growth and division of preexisting mitochondria; 2) *de novo* production of mitochondria from cytoplasmic precursors; and 3) generation from other membrane-bound cellular components (177). There is now strong evidence that existing mitochondria are capable of growing and dividing, and this is likely the predominant process utilized for biogenesis (177). Thus, biogenesis can be viewed more precisely as the growth and division of preexisting mitochondria for the purpose of increasing mitochondrial mass and ATP production (177, 178). Biogenesis is necessary to maintain mitochondrial homeostasis under basal conditions and may be up-regulated in response to a variety of pathological states that alter cellular energy demands (178). It is important to note that the biogenic process is complex and requires orchestration of multiple biological responses as follows: 1) transcription and translation of nuclear-encoded mitochondrial targets; 2) recruitment of these nuclear-encoded products; 3) mitochondrial DNA (mtDNA) transcription and translation; and 4) assembly of both nuclear- and mitochondrial-derived proteins into a growing mitochondrial network (179).

*Regulation of Mitochondrial Biogenesis*

Mitochondrial biogenesis is largely regulated at the transcriptional level by the PGC-1 family of transcriptional co-activators, which includes peroxisomal proliferator-activated receptor γ coactivator 1α (PGC-1α), PGC-1β, and PGC-1-related coactivator
(PRC) that are responsible for coordinating both nuclear and mitochondrial responses to increase cellular mitochondrial content. PGC-1α has been termed the “master regulator” of mitochondrial biogenesis and is abundantly expressed in tissues with high energy requirements including the heart, skeletal muscle, brown adipose tissue, and the kidney (180, 181). PGC-1α was first identified as a coactivator for peroxisomal proliferator-activated receptor γ (PPARγ) in a yeast two-hybrid screen, and subsequent studies revealed that PGC-1α-mediated coactivation of PPARγ is an important determinant of adipocyte fate (white adipose versus brown adipose tissue) and adaptive thermogenesis (180, 182). PGC-1α is now recognized as a coactivator for an incredible variety of transcription factors including PPARs (PPARα, PPARδ, PPARγ), estrogen related receptors (ERRα, ERRβ, and ERRγ), nuclear respiratory factors (NRF-1 and NRF-2), and FOXO3 (forkhead box protein O3) (178, 183). PGC-1α does not directly bind DNA but instead contains an N-terminal activation domain with three LXXLL motifs that allows for direct interaction with most of the nuclear transcription factors at the promoter region of target genes (184). Once bound to its associated transcription factor, PGC-1α recruits other transcriptional coactivators including p300/CBP (CREB binding protein) and steroid receptor coactivator-1 (SRC-1) (185). Both p300/CBP and SRC-1 possess intrinsic histone acetyltransferase (HAT) activity, allowing for relaxation of the chromatin structure and increased gene expression (186, 187). In addition, active PGC-1α also associates with another multiprotein coactivator complex known as Mediator (also know as the TRAP/DRIP complex) that is essential for recruitment of the general transcription machinery (including RNA polymerase II) to the promoter region (188, 189). Transcription factors and gene targets coordinately regulated by PGC-1α are
discussed below with a particular emphasis on their relevance to mitochondrial biogenesis.

**Transcriptional Regulation of Mitochondrial Biogenesis in the Nucleus**

*Nuclear Respiratory Factors (NRF-1 and NRF-2)*

The NRFs are structurally unrelated nuclear transcription factors that modulate transcription of nuclear-encoded mitochondrial genes. Both NRF-1 and NRF-2 are ubiquitously expressed and enhance expression of target genes by interaction with NRF-1/2 binding sites in their promoter regions (190, 191). NRF-1 was first identified as a transcriptional regulator of cytochrome c, and subsequent studies have revealed an incredible array of mitochondrial genes with conserved NRF-1 binding sites (192). In particular, NRF-1 promotes transcription of genes encoding components involved in oxidative phosphorylation (e.g. subunits of ETC complexes I-V, cytochrome c), mitochondrial DNA transcription and translation (mitochondrial transcription factor A, TFAM), mitochondrial protein import and assembly (Tom20, COX17), and heme biosynthesis (5-aminolevulinate synthase) (193, 194). Similarly, NRF-2 (also known as GA binding protein, GABP) was first identified due to the presence of NRF-2 binding sites in the promoter region of the gene coding for subunit IV of cytochrome c oxidase (COXIV) (195, 196). NRF-2 promotes transcription of a smaller set of targets than NRF-1, but with similar functions including mitochondrial respiration (e.g. all nuclear-encoded subunits of cytochrome c oxidase), mitochondrial protein import (Tom20, Tom70), and transcription and translation of mitochondrial DNA (TFAM, mitochondrial transcription factor B1 and mitochondrial transcription factor B2) (193, 197). It should be noted that there are species-specific differences in the specific genes regulated by NRF-1 and NRF-
NRF-1 deficiency in mice results in embryonic lethality between embryonic days 3.5 and 6.5, owing largely to depletion of mitochondrial DNA and loss of mitochondrial membrane potential in the blastocyst stage (198). Similarly, NRF-2 knockout mice display lethality before implantation of the embryo (199). These findings provide strong evidence that the NRFs play essential roles in mitochondrial biogenesis and function.

*Estrogen-Related Receptors (ERRα, ERRβ, and ERRγ)*

The ERRs comprise a family of orphan nuclear receptors for which there is no known endogenous ligand. ERRα is the most ubiquitous and abundant of the ERR isoforms, but all three isoforms are typically expressed in tissues with high metabolic demand including the heart, kidneys, skeletal muscle, and brown adipose tissue (200). Our current understanding indicates that ERRs are primarily regulated by interactions with transcriptional coactivators (PGC-1α and SRC-1) and corepressors (receptor interacting protein 140 or RIP140) (200). Once associated with the appropriate coactivators, ERRs bind to ERR response elements (ERRE) in the promoter regions of target genes involved in multiple aspects of mitochondrial metabolism including β-oxidation of fatty acids (e.g. medium-chain acyl-CoA dehydrogenase), the citric acid cycle (fumarase), oxidative phosphorylation (cytochrome c and subunits of NADH dehydrogenase), and ATP synthesis (multiple components of ATP synthase) (200, 201). ERRs can also enhance expression of other essential factors contributing to mitochondrial DNA transcription (mitochondrial transcription factor 2B, TFB2M), protein translation (multiple mitochondrial ribosome proteins), and the transcriptional response to induce biogenesis (NRF-2 and PPARα) (200, 202). ERRs therefore play a critical role in orchestrating both nuclear and mitochondrial aspects of the biogenic program. In support
of this, ERRα-deficient mice display loss of mitochondrial mass, decreased electron transport chain activity, and reduced expression of mitochondrial genes in multiple tissue types (203-206).

Peroxisome Proliferator-Activated Receptors (PPARα, PPARγ, and PPARδ)

PPARs are nuclear transcription factors that modulate expression of genes involved in multiple aspects of lipid homeostasis including lipid synthesis, uptake, storage, and oxidation (192). PPARγ is predominantly expressed in adipose tissue and regulates adipocyte differentiation and maturation, fatty acid synthesis, and lipid transport (207, 208). PPARα and PPARδ are present in a variety of tissues including liver, heart, skeletal muscle, and brown adipose tissue (192). PPARα and PPARδ promote expression of enzymes involved in uptake and oxidation of fatty acids (192, 209). Since fatty acid oxidation generates substrates for the citric acid cycle (Acetyl-CoA) and the ETC (NADH and FADH2), PPARα and PPARδ are also important regulators of mitochondrial respiratory function. The PPARs also enhance transcription of the uncoupling proteins UCP1 (PPARγ) and UCP2 (PPARα and PPARδ) (210). UCPs dissipate the proton gradient across the IMM and thus dictate mitochondrial oxidative phosphorylation, ROS generation, and adaptive thermogenesis (210, 211). In addition, stimulation of PPARγ and PPARδ induces mitochondrial biogenesis in adipocytes and skeletal muscle myocytes, respectively (212-214). Recent studies have documented the presence of a PPAR response element (PPRE) in the promoter region of the PGC-1α gene that appears to facilitate PPARγ- and PPARδ-mediated mitochondrial biogenesis (215, 216). Since PGC-1α acts as a transcriptional coactivator for the PPARs, this PPRE permits a positive feedback mechanism by which PGC-1α may increase its own transcription and
subsequent mitochondrial biogenesis. However, evidence indicating that PPARs directly regulate expression of other components of the biogenic response such as electron transport chain proteins is lacking (192).

**Mitochondrial DNA Transcription and Replication in Mitochondrial Biogenesis**

This discussion of mitochondrial biogenesis has thus far focused on coordinated nuclear responses that increase mitochondrial mass. As discussed above, the mitochondrial genome also encodes components involved in normal mitochondrial function including 13 ETC proteins. Thus, transcription and replication of mtDNA is also necessary for mitochondrial biogenesis. Transcription of mtDNA requires association of nuclear-encoded proteins including mitochondrial RNA polymerase (POLRMT), TFAM, and a member of the mitochondrial transcription factor B family (TFB1M or TFB2M) (217). Distinct promoters located on the heavy strand (HSP1 or HSP2) or light strand (LSP) allow for POLRMT binding and transcription initiation (218). POLRMT is recruited to the appropriate promoter region by TFAM, an HMG-box protein that associates with enhancer regions upstream of HSP1/2 and LSP (219). TFAM may also promote transcription by unwinding and bending mtDNA, thus allowing better access by POLRMT (220). TFB1M and TFB2M associate with both POLRMT and TFAM and appear to increase transcription through an unknown mechanism, with TFB2M having more potent stimulatory activity than TFB1M (221, 222). POLRMT-mediated transcription initiated at HSP2 and LSP generates polygenic transcripts of the full length of mtDNA that are further processed by the activity of mitochondrial RNAse P into individual mRNAs, tRNAs, and rRNAs (223). Transcription from HSP1 produces a transcript comprising only the 12S rRNA, 16S rRNA, and a leucine-tRNA (217).
Mitochondrial-encoded proteins are then synthesized by specialized ribosomes (mitoribosomes) located in the mitochondrial matrix (224).

A number of nuclear-encoded proteins are also necessary for mtDNA replication including DNA polymerase γ (POLγ), TWINKLE helicase, mitochondrial topoisomerase I (TOP1MT), and mitochondrial single-stranded binding protein (mtSSB) (217). In addition, POLRMT and TFAM play essential roles in synthesizing an RNA primer from the LSP that is used by POLγ to initiate replication (225). POLγ contains two subunits (140 kD and 55 kD in molecular weight) and is the only DNA polymerase present in mitochondria (226). The process by which POLγ synthesizes daughter DNA from the heavy and light mtDNA strands remains unclear, but a number of different models have been proposed (217). Together, TWINKLE helicase, TOP1MT, and mtSSB are responsible for unwinding and maintenance of the mtDNA in a single-stranded state so that replication can proceed without interruption (227).

It is important to note that although PGC-1α does not directly regulate mtDNA transcription or replication, it does promote expression of factors necessary for these processes (TFAM, TFBM1, TFBM2) via coactivation of nuclear transcription factors as described above. These findings further illustrate the essential role of PGC-1α in orchestrating both nuclear and mitochondrial responses to enhance biogenesis. Genetic manipulation studies have further demonstrated the importance of PGC-1α gene targets in mtDNA transcription and replication. In particular, TFAM knockout in mice leads to lethality before embryonic day 10.5 that can be attributed to mtDNA depletion and reduced oxidative phosphorylation capacity (228).
Cellular Regulation of PGC-1α and Mitochondrial Biogenesis

Since its identification as the “master regulator” of mitochondrial biogenesis, extensive efforts have been made to characterize molecular mechanisms responsible for regulation of PGC-1α expression and activity. Transcriptional modulation of PGC-1α is the most well-characterized, although post-translational modifications also play an important role in modulating activity and stability of this protein. Major pathways regulating PGC-1α are described below. However, it is important to note that these pathways are both tissue- and context-dependent, and relatively little is known about specific regulation of PGC-1α in the kidney.

Positive Transcriptional Regulation of PGC-1α

The PGC-1α promoter contains a number of transcription factor binding sites that allow for modulation of PGC-1α transcript levels in the cell (192). The most studied positive regulator of PGC-1α is cyclic AMP response element-binding protein (CREB) (229). In skeletal muscle, exercise induces PGC-1α expression through activation of β2-adrenergic receptor (β2-AR)-cAMP-PKA-CREB signaling pathway (230). Increased intracellular Ca^{2+} levels in skeletal muscle during endurance exercise also result in calcium/calmodulin-dependent protein kinase IV (CaMKIV)-mediated activation of CREB and subsequent transcription of PGC-1α (231). Ca^{2+} also activates the myocyte enhancer factor 2 (MEF2) family of transcription factors, which are established modulators of PGC-1α expression, via the calcium-dependent phosphatase known as calcineurin A (CnA) (231).

Activating transcription factor 2 (ATF2) is another factor capable of binding to the PGC-1α promoter and modulating its transcription. p38 MAPK-dependent
phosphorylation and activation of ATF2 is a critical regulator of PGC-1α expression in brown adipose tissue (BAT) (232). In particular, the p38 MAPK-ATF2-PGC-1α signaling pathway is essential for cold-inducible adaptive thermogenesis in BAT via up-regulation of uncoupling protein 1 (UCP1) and subsequent mitochondrial heat generation (described above) (232).

Regulation of PGC-1α transcription by nuclear receptors (PPARs, ERRs) is described in earlier sections of this review. It should also be noted that there are other signaling pathways capable of inducing PGC-1α expression through as yet undefined transcriptional mechanisms. Activation of nitric oxide synthase isoforms (endothelial, eNOS; inducible, iNOS; neuronal, nNOS) and NO generation potently stimulates PGC-1α transcription in a variety of cell types via cGMP-dependent signaling (233). AMP-activated protein kinase (AMPK) also increases PGC-1α mRNA content in skeletal muscle via an unknown mechanism (234). Taken together, these findings demonstrate the incredible variety of transcriptional programs that regulate PGC-1α.

Negative Transcriptional Regulation of PGC-1α

Relatively little is known about negative transcriptional regulators of PGC-1α, although some candidates have been identified. In particular, the transcriptional corepressor receptor-interacting protein 140 (RIP140) acts to suppress PGC-1α and mitochondrial biogenesis. Similar to PGC-1α, RIP140 makes use of ten leucine-rich LxxLL motifs to interact with a number of nuclear receptors including the ERRs and PPARs (235). When associated with nuclear receptors, RIP140 recruits other corepressors to the promoter region of target genes including C-terminal binding protein 1 (CtBP1) and histone deacetylases (HDACs) (236, 237). Together, CtBP1 and HDACs
act to modulate histone methylation and acetylation in a manner that suppresses gene transcription. The net effect of RIP140 association with PPARs and ERRs is decreased expression of PGC-1α and its downstream targets (238, 239). A similar repressive role has also been described for DNA methyltransferase 3B (DNMT3B), which methylates the PGC-1α promoter leading to suppression of PGC-1α transcription and mitochondrial biogenesis (240).

A recent series of studies by Remels et al. has also identified canonical NFκB signaling as a negative regulator of PGC-1α transcription in skeletal muscle cells (241, 242). In particular, activation of NFκB following treatment with pro-inflammatory cytokines (TNF-α, IL-1β) or overexpression of IKK-β resulted in decreased mRNA content of PGC-1α and ETC subunits. These findings are in agreement with in vitro work performed in renal proximal tubule cells demonstrating TNF-α-mediated down-regulation of PGC-1α and its downstream targets (162). The PGC-1α promoter contains a putative NFκB binding site, suggesting that NFκB may negatively regulate transcription of this gene by direct association with the promoter region (243). However, further studies are necessary to demonstrate functional binding of NFκB at this site.

The MEK/ERK signaling cascade has also been implicated as a potential suppressor of PGC-1α in pathological states. In an animal model of Alzheimer’s disease (AD), intracerebroventricular injection of amyloid β (Aβ) in rats led to increased ERK1/2 activation (phosphorylation) in association with reduced expression of PGC-1α and spatial memory deficits (244). Blockade of ERK signaling using the pharmacological inhibitor U0126 reversed Aβ-induced down-regulation of PGC-1α and cognitive decline (244). In addition, in vitro treatment of skeletal muscle cells with the fatty acid palmitate
resulted in loss of PGC-1α mRNA and protein levels in a MEK/ERK-dependent manner (245). Unfortunately, the downstream mechanisms responsible for negative regulation of PGC-1α by the MEK/ERK signaling pathway have not been elucidated.

*Post-Translational Regulation of PGC-1α*

Stability and activity of the PGC-1α protein is heavily modulated by post-translational modification. PGC-1α is phosphorylated by a variety of kinases including AMPK, p38 MAPK, Akt, and glycogen synthase kinase-3β. Depending on the amino acid residue phosphorylated, these modifications can stimulate or reduce transcriptional coactivation by this protein. In response to changes in the cellular AMP:ATP ratio, AMPK phosphorylates PGC-1α on threonine-177 and serine-538 residues and promotes its activity (246). Phosphorylation by p38 MAPK at threonine-262, serine-265, and threonine-298 increases protein half-life and blocks association of PGC-1α with the transcriptional co-repressor p160MBP (247, 248). Post-translational activation of PGC-1α by both AMPK and p38 MAPK is thought to play an important role in physiological induction of mitochondrial biogenesis in skeletal muscle after endurance exercise (192). In contrast, Akt- and GSK-3β-mediated phosphorylation has inhibitory effects on PGC-1α activity (249, 250).

Acetylation represents another post-translational modification responsible for regulating PGC-1α activity. The PGC-1α protein contains at least 15 lysine residues that may be acetylated. Acetylation status is largely determined by the opposing activity of the N-acetyltransferase GCN5 (general control nonderepressible 5) and the NAD⁺-dependent deacetylase Sirtuin 1 (SIRT1) (251). GCN5 and SIRT1 are both well-characterized sensors of cellular energy status and thus modulate PGC-1α activity in...
response to changes in metabolic demand (251). In particular, deacetylation by SIRT1 activates PGC-1α, whereas GCN5-mediated acetylation has inhibitory effects (252, 253). SIRT1 activation is involved the physiologic induction of skeletal muscle PGC-1α and mitochondrial biogenesis in response to caloric restriction in mice (254). On the other hand, GCN5 expression and activity is increased in BAT and skeletal muscle of mice fed a high fat diet, leading to decreased expression of PGC-1α target genes and mtDNA content (255). In addition to phosphorylation and acetylation, the PGC-1α protein is also subject to ubiquitination, methylation, and glycosylation (O-GlcNAc modification) (251). Further work is necessary to determine the physiological relevance of these modifications. Figure 1-8 below summarizes major pathways regulating PGC-1α and downstream transcriptional responses initiated by this protein.
Figure 1-8: Regulation of PGC-1α and mitochondrial biogenesis. A summary of the pathways involved in the promotion and mediation of mitochondrial biogenesis and accompanying signaling responses is outlined above. Because of the complexity of the networks involved, many intermediates as well as feedback interactions and other targets of the transcription factors could not be included. The indicated processes may regulate PGC-1 family members at the transcriptional level, post-translational level, or both as described above. Inhibitory factors such as RIP140, GCN5, and Akt are not shown. Red boxes indicate essential PGC-1α targets required for mitochondrial biogenesis. AMPK = AMP-activated protein kinase; CAC = citric acid cycle; CamK = calcium-calmodulin-dependent protein kinase; CO = carbon monoxide; eNOS = endothelial nitric-oxide synthase; FAO = fatty acid oxidation; GABP = GA-binding protein α (also known as NRF-2); GSK-3β = glycogen synthase kinase-3β; HO-1 = heme oxygenase 1; NO = nitric oxide; OXPHOS = oxidative phosphorylation; PKA = protein kinase A; PRC = PGC-1 related coactivator; TZDs = thiazolidinediones; UCP = uncoupling protein. Modified from (178).
MITOCHONDRIAL DYSFUNCTION AND BIOGENESIS IN AKI

Renal Proximal Tubule Cell Metabolism and Bioenergetics

Maintenance of cellular ATP levels in the renal proximal tubule is essential for reabsorption of solutes from the glomerular filtrate via active transport as well as energy-dependent repair processes in the injured kidney. Thus, pathological conditions resulting in reduced ATP levels contribute to tubular de-energization and subsequent loss of renal function characteristic of AKI (256, 257). Early biochemical studies demonstrated that the proximal tubule does not rely heavily on glucose catabolism, with the lowest activity of the three major regulatory enzymes of glycolysis (hexokinase, HK; phosphofructokinase-1, PFK-1; and pyruvate kinase, PK) along the entire nephron segment (258). In fact, the renal cortex, consisting primarily of renal proximal tubule cells, contributes to de novo synthesis of glucose from lactate via gluconeogenesis that can be released into the circulation (259). Under normal conditions in vivo, the proximal tubule instead primarily utilizes mitochondrial oxidative phosphorylation to generate ATP via oxidation of alternate metabolic fuels including lactate, pyruvate, fatty acids, and amino acids (260, 261). Experimental evidence indicates that renal proximal tubule cells have extremely high mitochondrial density (258, 262). In addition, approximately 70% of oxygen consumption in the tubule is used to drive the Na⁺/K⁺-ATPase that establishes the electrochemical gradient necessary for reabsorption of a variety of solutes in the glomerular filtrate (263, 264). Thus, the structural changes described above in tubular mitochondria following sepsis-induced AKI is of particular interest since it may be a significant contributor to renal dysfunction. Evidence for alterations in mitochondrial function and biogenesis in the setting of AKI is presented below.
Evidence for Mitochondrial Dysfunction and Altered Homeostasis in Sepsis-induced AKI

There is now considerable experimental evidence identifying roles for mitochondrial dysfunction in the pathogenesis of septic AKI. Tran et al. recently demonstrated reduced expression of whole kidney cytochrome c oxidase IV (COX IV), a subunit of complex IV of the electron transport chain (ETC), in association with mitochondrial swelling following systemic endotoxin exposure in mice (162). Cytochrome c oxidase activity was also markedly reduced in the renal cortex and the outer stripe of the outer medulla (OSOM) in this model (Figure 1-9 below). Other groups have reported a rapid reduction (~2-fold) in renal ATP levels as early as five hours after LPS administration in rodents (265). LPS and its downstream effector cytokine TNF-α have also been shown to directly suppress mitochondrial oxygen consumption in renal proximal tubule cells in vitro (162, 266).
Figure 1-9: Mitochondrial dysfunction in endotoxin-induced AKI. (A) Staining (brown) for cytochrome c oxidase enzyme activity on slices from snap-frozen kidneys 18 hours after vehicle or LPS treatment. Cortex (C) and inner stripe of the outer medulla (ISOM) have the most intense staining, followed by outer stripe of the outer medulla (OSOM), with the weakest activity in the inner medulla (IM). Enzymatic activity is greatly reduced following LPS. Original magnification, ×4 (overview); ×40 (cortex, corticomedullary [CM] junction). (B) Western blot on whole kidney lysates for cytochrome c oxidase subunit IV. (C) Transmission EM of proximal tubules demonstrating swelling of mitochondria and rarefaction of cristae. n = 3–6 mice per condition. Original magnification, ×3,000 (left); ×20,000 (right). Modified from (162).
Loss of renal mitochondrial respiratory function has also been reported in a CLP model of sepsis. In particular, high-resolution respirometry revealed decreases in renal ETC complex I and complex II/III activities as early as six hours after induction of CLP in mice (267). Inactivation of ETC complexes in the kidney was accompanied by a significant decline in ATP content by 36 hours in CLP animals when compared to sham controls. The same study also demonstrated an important role for mitochondrial reactive oxygen species (ROS) generation in sepsis-induced renal injury and dysfunction in CLP mice (267). The mitochondrial-targeted antioxidant Mito-TEMPO attenuated CLP-induced changes in renal mitochondrial respiration, microcirculatory perfusion, oxidative stress, and glomerular filtration rate (267).

It should be noted that sepsis-induced mitochondrial dysfunction has also been reported in experimental models examining other organ systems including the liver, the lungs, skeletal muscle, the brain, and the heart (268-272). In addition, skeletal muscle biopsies taken from septic patients admitted to the intensive care unit revealed a significant association between morbidity/mortality and severe mitochondrial dysfunction characterized by decreased ATP content, reduced complex I activity, glutathione depletion, and elevated markers of oxidative stress (273). Taken together, these findings indicate an important role for mitochondrial dysfunction and loss of mitochondrial homeostasis in the pathophysiology of sepsis-induced organ failure including AKI.

Evidence for Mitochondrial Dysfunction and Altered Homeostasis in Other Forms of AKI

Our laboratory has previously demonstrated loss of normal mitochondrial homeostasis in multiple forms of AKI. In particular, our group has shown that a mouse
model of ischemia-reperfusion (I/R) injury results in persistent loss of electron transport chain (ETC) proteins including nuclear-encoded NADH dehydrogenase (ubiquinone) 1 beta subcomplex 8 (NDUFB8) and ATP synthase β (ATPSβ) as well as mitochondrial-encoded cytochrome c oxidase subunit I (COXI) in the renal cortex up to 6 days after injury (274). These changes likely occur in concert with depletion of total mitochondrial mass, fragmentation and swelling of mitochondria, loss of mitochondrial membrane potential, and reduced ATP generation (275-277). In addition, we have demonstrated that renal I/R in mice leads to a significant reduction in ADP-stimulated respiration (state 3 respiration) in isolated renal mitochondria, further suggesting that loss of ETC protein expression may contribute to mitochondrial dysfunction in the injured kidney (278). Similar findings have also been documented in both glycerol-induced rhabdomyolysis and folic acid models of AKI (274, 279). These data suggest that disruption of mitochondrial homeostasis and function is common to multiple forms of AKI.

Another important aspect of mitochondrial dysfunction in AKI is the development of oxidative and nitrosative stress. Mitochondria may generate ROS and reactive nitrogen species (RNS) through activity of the ETC (especially complexes I and III), mitochondria-localized dehydrogenases (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, glycerol-3-phosphate dehydrogenase), mitochondrial oxidases (NADPH oxidase 4), and enzymes involved in β-oxidation of fatty acids (167). Under normal conditions, between 0.4% and 4% of oxygen utilized by the ETC is converted to ROS via electron leak (280). ROS/RNS generation is significantly increased in the renal proximal tubule after both ischemic and toxic insult. Increased ROS/RNS in turn leads to sub-lethal cellular injury and apoptosis through lipid peroxidation, protein oxidation, and DNA
Mitochondria-derived oxidative and nitrosative stress are established contributors to tubular insult in both ischemic and toxic AKI and have been extensively studied as potential therapeutic targets for renoprotection (282, 283). In particular, another mitochondrial-targeted antioxidant, known as SkQR1 (a conjugate of plastoquinone and positively-charged rhodamine), has shown particular promise in attenuating oxidative stress and renal dysfunction as measured by BUN in both I/R and glycerol-induced rhabdomyolysis in rats (284).

Mitochondria also act as important regulators of apoptotic cell death in the setting of ischemic and nephrotoxic AKI. Activation of the intrinsic and extrinsic pathways of apoptosis after cellular insult leads to mitochondrial outer membrane permeabilization (MOMP) and subsequent release of cytochrome c by the actions of pro-apoptotic members of the Bcl-2 family including Bax, Bad, and Bid (285). Cytochrome c in turn triggers activity of initiator caspases (e.g. capase-9) that activate downstream effector caspases (e.g. caspases-3). These executioner caspases are then responsible for proteolytic cleavage of a variety of cellular substrates, eventually leading to cell death via apoptosis (285). Kelly et al. recently demonstrated increased cytochrome c release from the mitochondria after renal I/R injury in rats (286). Localization of cytochrome c to the cytosol was accompanied by DNA fragmentation and nuclear condensation, further indicating induction of apoptotic cell death in this model. In addition, treatment with the potent anti-inflammatory and anti-apoptotic agent minocycline attenuated I/R-mediated renal dysfunction and cell death (286). MOMP has also been demonstrated in the proximal tubule following cisplatin nephrotoxicity and Bax knockout mice are protected from cisplatin-induced tubular damage (287).
Mechanisms of Mitochondrial Dysfunction and Altered Homeostasis in AKI

Altered Mitochondrial Biogenesis as a Target in AKI

Despite considerable evidence linking mitochondrial dysfunction to the pathophysiology of AKI, the mechanisms responsible for these changes remain unclear. Our laboratory has begun to focus on disruption of normal mitochondrial homeostasis as a potential mediator of mitochondrial dysfunction in this setting. In particular, mitochondrial biogenesis plays an important role in regulating both mitochondrial content and function in the cell as described in previous sections. In the kidney, PGC-1α expression is localized to the proximal tubule, which relies heavily on mitochondrial ATP production to maintain its normal functions (288). We have demonstrated that loss of ETC proteins including NDUFB8, ATPSβ, and COX1 following renal I/R injury in mice (described above) is associated with an early decrease in both PGC-1α and PGC-1β expression at the mRNA level (274). Persistent suppression of PGC-1α transcript levels in the renal cortex was also noted following folic acid-induced AKI in mice. Reduced levels of PGC-1α in folic acid-treated mice correlated with decreased expression of downstream markers of mitochondrial biogenesis and homeostasis including mRNAs for mitochondrial transcription factor A (TFAM), NDUFB8, ATPSβ, and COX1 as discussed above (279). In addition, PGC-1α suppression in this model was associated with a significant and long-lasting (up to 14 days post-injury) decrease in renal cortical mtDNA copy number, a marker of cellular mitochondrial content (279). Taken together, these findings indicate that disruption of mitochondrial biogenesis may be an important mediator of mitochondrial dysfunction and tubular cell injury in AKI.
Evidence for Altered Renal Mitochondrial Biogenesis in Sepsis-induced AKI

Although mitochondrial dysfunction has been widely implicated in the pathogenesis of sepsis-induced AKI, whether suppression of renal mitochondrial biogenesis contributes to cellular injury in the septic kidney remains unclear. Tran et al. recently demonstrated that renal PGC-1α expression is decreased at the mRNA and protein levels following systemic LPS exposure and CLP in mice. PGC-1α suppression in these models was significantly correlated with both reduced expression of direct transcriptional targets of PGC-1α and renal dysfunction as indicated by BUN (162). Reduced cytochrome c oxidase activity in the renal cortex (described above) was also closely associated with acute down-regulation of PGC-1α, suggesting that suppression of renal PGC-1α may contribute to mitochondrial dysfunction in septic AKI. Restoration of normal renal function in LPS-treated mice coincided with return of PGC-1α mRNA and protein to baseline levels, further indicating a potential role for mitochondrial biogenesis in both pathophysiology of and recovery from septic AKI (162). A major limitation of this study is that it does not directly address whether mitochondrial biogenesis and total mitochondrial content is disrupted as a result of PGC-1α suppression. Thus, additional work is needed to define changes in mitochondrial biogenesis after sepsis-induced renal injury and to elucidate the mechanisms responsible for disruption of PGC-1α in this setting.

Pharmacological and Genetic Induction of PGC-1α and Mitochondrial Biogenesis in Experimental Models of AKI

Further evidence that suppression of mitochondrial biogenesis contributes to the pathophysiology of renal injury may be derived from studies that pharmacologically
target this process in experimental models of AKI. A variety of approaches have been employed to stimulate PGC-1α and mitochondrial biogenesis in the injured proximal tubule. Our laboratory previously demonstrated that adenovirus-mediated overexpression of PGC-1α in rabbit primary RPTCs 24 hours after exposure to H₂O₂ in vitro restored mitochondrial function (cellular respiration, ATP production) and expression of ETC proteins (289). In addition, PGC-1α overexpression was associated with return of sodium transport in these cells, suggesting that mitochondrial biogenesis might promote recovery of normal cellular function after injury (289). Pharmacological stimulation of SIRT1 in vivo resulted in PGC-1α deacetylation/activation and ameliorated tubular injury and mitochondrial dysfunction in rats subjected to bilateral renal I/R injury (290). Our group has also identified a number of targets/compounds including formoterol (a β₂-adrenoreceptor agonist), cGMP-specific phosphodiesterase inhibitors, and 5-HT₁F agonists that potently stimulate mitochondrial biogenesis in RPTCs in vitro and facilitate recovery after AKI (278, 291, 292). Figure 1-10 below summarizes a number of pharmacological approaches that have been used to promote mitochondrial biogenesis and prevent dysfunction in experimental models of AKI. Despite promising results, these studies have yielded only partial restoration of mitochondrial and renal function, indicating a need for further characterization of mitochondrial dysfunction associated with AKI. An alternate approach likely to generate novel therapeutic targets is identification of pathways leading to suppression of mitochondrial biogenesis in the setting of AKI.
Figure 1-10: Pharmacological targeting of mitochondrial dysfunction. Mitochondrial biogenesis (MB), the generation of new mitochondria, is a highly regulated cellular process controlled by the central mediator and transcriptional coactivator PGC-1α. PGC-1α expression and activity is regulated through diverse pathways including receptor tyrosine kinases, G-protein coupled receptors, naturetic peptide receptors and nitric oxide synthase through cGMP, as well as AMPK activation, and SIRT1-mediated deacetylation. In response to various stimuli, mitochondria can undergo the process of fusion (enlargement by combination with other mitochondria) via mitofusin proteins, such as MFN1/2 and OPA1, or fission (size reduction by budding off of mitochondrial contents) via fission proteins, such as Drp1. Damaged mitochondria are eliminated by selective degradation in lysosomes. EGF = epidermal growth factor; VEGF = vascular endothelial growth factor; RTKs = receptor tyrosine kinases; β2AR = β2-adrenergic receptor; 5HTR = serotonin receptor; GC-A/B = guanylyl cyclase A/B; eNOS = endothelial nitric oxide synthase; NO = nitric oxide; sGC = soluble guanylate cyclase; cGMP = cyclic guanosine monophosphate; cAMP = cyclic adenosine monophosphate; AMPK = AMP-activated kinase; SIRT1 = sirtuin 1; PPAR = peroxisome proliferator-activated receptor; ERR = estrogen related receptor; NRF1 = nuclear respiratory factor-1; PGC-1α = peroxisome proliferator-activated receptor gamma coactivator-1 alpha; Ac = acetyl; TCA = tricarboxylic acid cycle; OXPHOS = oxidative phosphorylation; TOM = translocase of the outer membrane; TIM = translocase of the inner membrane; MFN2 = mitofusin 2; OPA1 = optic atrophy 1; DRP1 = dynamin-related protein 1; mtDNA = mitochondrial DNA. Adapted from Whitaker et al, in press.
Stimulation of Glycolytic Metabolism in Response to Mitochondrial Dysfunction in AKI

As discussed above, renal function depends heavily on the proximal tubule for reabsorption of solutes from the glomerular filtrate via active transport. Mitochondrial injury may therefore contribute to renal dysfunction in AKI by interfering with energy production that drives this transport. Although renal proximal tubule cells have very low glycolytic capacity under normal conditions (see above), glycolysis may be induced in response to mitochondrial insult in these cells to produce ATP and preserve cellular function. The glycolytic pathway facilitates the conversion of glucose to pyruvate (or lactate under anaerobic conditions) with a net gain of 2 ATP per molecule of glucose metabolized. There are three major regulatory steps in glycolysis: 1) hexokinase (HK) catalyzes the conversion of glucose to glucose-6-phosphate, the first committed step of glycolysis; 2) phosphofructokinase (PFK) catalyzes the phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate; and 3) pyruvate kinase (PK) which is responsible for the conversion of phosphoenolpyruvate to pyruvate. Early work by Dickman and Mandel demonstrated that purified rabbit proximal tubules induce glycolytic metabolism after hypoxia or pharmacological inhibition of oxidative phosphorylation (293). In particular, exposure to hypoxia (1% O2), the complex I inhibitor rotenone, or the uncoupler FCCP stimulated anaerobic glycolysis and lactate production in isolated tubules. Further studies demonstrated that glycolytic induction restored tubular ATP content and Na+/K+-ATPase function. Inhibition of glycolytic metabolism using 2-deoxyglucose after rotenone treatment increased cell death as indicated by increased lactate dehydrogenase (LDH) release into the medium (293). These findings suggest that
induction of anaerobic glycolysis in response to suppression of mitochondrial oxidative phosphorylation may aid in preservation of tubular viability and function. Additional experimental evidence indicates that anaerobic glycolysis is increased in the proximal tubule and other nephron segments in vivo following renal ischemia or exposure to the nephrotoxicant mercuric chloride in rodents (294-296). However, whether glycolytic metabolism is increased in the renal cortex of the septic kidney to provide ATP and promote cell survival despite mitochondrial dysfunction remains unclear.

**PROJECT GOALS**

As discussed above, incidence of sepsis is increasing and sepsis is the most common contributing factor to the development of AKI in the intensive care setting. Therapy for both sepsis and AKI are primarily limited to supportive care, and mortality associated with these conditions remains extremely high, highlighting the incredible need for development of novel targets. Our laboratory and others have identified suppression of mitochondrial biogenesis as a pathogenic mechanism contributing to tubular injury and renal dysfunction after AKI. Although mitochondrial dysfunction has been widely reported in the septic kidney, it is unclear whether disruption of mitochondrial biogenesis plays a role in renal injury. Thus, the first goal of this study was to better characterize changes in PGC-1α and mitochondrial biogenesis in sepsis-induced AKI. In order to address this objective, I studied time- and dose-dependent effects of systemic LPS exposure in mice on PGC-1α, downstream markers of mitochondrial biogenesis, and mitochondrial content in the renal cortex (Chapter 2). A related aim was to define the molecular mechanisms responsible for acute suppression of PGC-1α and subsequent mitochondrial biogenesis in the septic kidney. Despite considerable evidence suggesting
that disruption of mitochondrial biogenesis is common to the pathophysiology of multiple forms of AKI in experimental models, our laboratory has not addressed how this phenomenon occurs. I hypothesized that renal cortical mitochondrial biogenesis is disrupted following LPS-induced AKI through suppression of PGC-1α expression by TLR4-dependent activation of MEK/ERK and/or NFκB signaling pathways and that this disruption contributes to loss of mitochondrial homeostasis (Chapter 2). My work focused on MEK/ERK and NFκB based on earlier studies suggesting that they negatively regulate PGC-1α (discussed above).

An additional goal of this study was to determine metabolic changes in the renal cortex that might support cellular and organ function in the face of mitochondrial dysfunction associated with sepsis-induced AKI. I concentrated on glycolysis as a potential alternative mechanism for ATP generation to support tubular cell viability and transport function based on previous work discussed above. In particular, I proposed that glycolytic metabolism is induced in the renal cortex in sepsis-induced AKI to produce ATP and preserve renal function in response to mitochondrial dysfunction. In order to complete this goal, I assessed changes in glycolytic enzyme activity and end products (lactate, pyruvate) in renal cortical tissue following systemic LPS administration in mice. I also examined other pathways of glucose metabolism (gluconeogenesis, glycogen synthesis, and the pentose phosphate shunt) in this model (Chapter 3).

The final aim of this study was to use findings generated in Chapters 2 and 3 to generate a novel therapeutic approach and test this methodology in a more clinically relevant animal model of sepsis. I demonstrate in Chapter 2 that the MEK/ERK inhibitor GSK1120212 prevents LPS-induced AKI and suppression of PGC-1α and subsequent
mitochondrial biogenesis. However, there are several limitations to the endotoxin model, and several treatments developed based on this model have failed to show efficacy in humans. I therefore determined the effects of GSK1120212 on development of AKI following cecal ligation and puncture (CLP). The CLP model shares several similarities with human cases of sepsis, making it much more clinically relevant (discussed above). Based on my earlier findings, I hypothesized that post-treatment with a MEK/ERK inhibitor would attenuate the systemic inflammatory response and renal injury following CLP in mice (Chapter 4).
Chapter 2:
Suppression of Mitochondrial Biogenesis through Toll-like Receptor 4 (TLR4)-dependent Mitogen-activated Protein Kinase Kinase (MEK) / Extracellular Signal-regulated Kinase (ERK) Signaling in Endotoxin-Induced Acute Kidney Injury

ABSTRACT

Although disruption of mitochondrial homeostasis and function is a widely accepted pathophysiological feature of sepsis-induced AKI, the molecular mechanisms responsible for this phenomenon are unknown. In this study, we examined changes in mitochondrial biogenesis and associated signaling mechanisms in a mouse model of lipopolysaccharide (LPS)-induced AKI. Down-regulation of PGC-1α, a master regulator of mitochondrial biogenesis, was noted at the mRNA level at 3 h and protein level at 18 h in the renal cortex and was associated with loss of renal function following LPS treatment. LPS-mediated suppression of PGC-1α led to reduced expression of downstream regulators of mitochondrial biogenesis and electron transport chain (ETC) proteins along with a reduction in renal cortical mitochondrial DNA content. Mechanistically, TLR4 knockout mice were protected from renal injury and disruption of mitochondrial biogenesis after LPS. Immunoblot analysis revealed activation of TPL-2/MEK/ERK signaling in the renal cortex by LPS. Pharmacological inhibition of MEK/ERK signaling attenuated renal dysfunction and loss of PGC-1α, and was associated with a reduction in pro-inflammatory cytokine (e.g. TNF-α, IL-1β) expression at 3 h post-LPS. Neutralization of TNF-α also blocked PGC-1α suppression, but not renal dysfunction, following LPS-induced AKI. Finally, systemic administration of recombinant TNF-α alone was sufficient to produce AKI and disrupt mitochondrial
homeostasis. These findings indicate an important role for the TLR4/MEK/ERK pathway in both LPS-induced renal dysfunction and suppression of MB. TLR4/MEK/ERK/TNF-α signaling may represent a novel therapeutic target to prevent mitochondrial dysfunction and AKI produced by sepsis.
INTRODUCTION

Acute kidney injury (AKI) is characterized by a rapid decrease in renal function over the course of hours to days and is associated with significant morbidity and mortality (~40%) (50). Despite recent efforts to better understand AKI, mortality associated with this clinical disorder remains unchanged over the last five decades (49, 297). Sepsis is the most common contributing factor to the development AKI, mortality resulting from AKI is almost doubled in septic patients (~70%) and treatment is limited to dialysis and supportive care (50, 51, 297-299). Taken together, these data reveal a significant need for further study of the pathophysiological mechanisms underlying renal injury with an emphasis on identifying therapeutic targets to improve clinical outcomes in septic AKI.

Much of the difficulty in developing effective therapies for sepsis-induced AKI stems from the multi-factorial nature of the disease. Septic AKI is thought to arise as a result of complex interactions involving alterations in renal hemodynamics, microvascular/endothelial cell dysfunction, and direct effects of inflammatory cells and their products (cytokines/chemokines) on the kidney (114). The degree to which changes in global renal blood flow (RBF) contribute to renal injury remains a topic of intense debate. However, it is generally accepted that microvascular dysfunction leads to sluggish capillary flow and subsequent development of local regions of hypoperfusion and hypoxia in the septic kidney (124, 125, 155). Reduced microvascular flow also amplifies injury by prolonging exposure of the renal parenchyma to inflammatory cells and various inflammatory molecules including pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and IL-6 which are primary mediators
of cellular injury in sepsis-induced AKI (134, 140, 141, 300). Data from post-mortem studies and experimental models indicate that tubular cell apoptosis and necrosis are relatively limited in the septic kidney when compared to other forms of AKI (157, 158). However, histological findings including tubular cell vacuolization, tubular dilatation, and the presence of swollen mitochondria provide strong evidence that sub-lethal injury to the proximal tubule may play an important role the development of sepsis-induced AKI (157, 162, 301).

The overt structural changes noted in tubular mitochondria following sepsis-induced AKI is of particular note given that the proximal tubule relies heavily on mitochondrial generation of ATP to drive active transport of electrolytes and fluids. Thus, mitochondrial dysfunction and/or dysregulation in the proximal tubule may contribute to loss of renal function in AKI (49, 256). Recent studies demonstrated suppression of peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) in experimental models of sepsis-induced AKI including systemic endotoxin exposure and cecal ligation and puncture (CLP) (162). PGC-1α, the so-called “master regulator of MB,” promotes transcriptional activity in both the nucleus and mitochondria to facilitate generation of new, functional mitochondria in response to a variety of physiological stimuli (302, 303). Loss of PGC-1α following sepsis-induced AKI was closely associated with renal and mitochondrial dysfunction. In addition, proximal tubule-specific PGC-1α knockout delayed recovery of renal function following saline resuscitation in mice treated with LPS (162). These findings indicate that suppression of PGC-1α and MB may play an important role in the development of renal injury. However, an important limitation of this study was that it did not fully characterize changes in MB after septic AKI. In
addition, a better understanding of the molecular alterations underlying disruption of MB may lead to development of novel therapies to restore renal function after sepsis-induced AKI.

The aim of the current study was to determine the signaling mechanisms responsible for suppression of MB in the renal cortex following endotoxic AKI. We report here that LPS exposure leads to down-regulation of PGC-1α and mitochondrial markers in the renal cortex. LPS-induced renal dysfunction and disruption of MB was dependent on TLR4/MEK/ERK and production of the pro-inflammatory cytokine TNF-α. Inhibition of the TLR4/MEK/ERK/TNF-α signaling may offer a novel therapeutic approach to reverse suppression of MB and loss of renal function in septic AKI.
METHODS

LPS Model of Sepsis-Induced AKI

Six to eight week old male C57BL/6 mice were acquired from the National Institutes of Health National Cancer Institute / Charles River Laboratories (Frederick, MD). Mice were given an intraperitoneal (i.p.) injection of 0.5, 2, or 10 mg/kg lipopolysaccharide (LPS) derived from Escherichia coli serotype O111:B4 (Sigma Aldrich, St. Louis, MO). Control mice received an i.p. injection of an equal volume of 0.9% normal saline. Mice were euthanized at 1, 3, and 18, and 42 h after LPS administration and kidneys and serum were harvested for molecular analysis. For experiments utilizing TLR4-deficient animals, TLR4KO mice on a C57BL/6 background and wild-type C57BL/6 mice were used. All studies were conducted in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal use was approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

To determine the role of MEK/ERK signaling in LPS-induced AKI, the MEK inhibitor GSK1120212 (also known as Trametinib) was obtained from Selleckchem Chemicals (Houston, TX). GSK1120212 is a potent and specific inhibitor of MEK1/2 which has been previously used in mouse models (304, 305). Mice received an i.p. injection of GSK1120212 (1 mg/kg) or vehicle control (DMSO) 1 h prior to administration of LPS.

In order to assess the effects of TNF-α on regulation of MB in this model, rat anti-TNF-α neutralizing antibody (clone MP6-XT22) and the appropriate rat IgG1 κ isotype control antibody (clone RTK2071) were purchased from BioLegend (San Diego,
Mice were randomly assigned to one of three groups: 1) control, 2) LPS + isotype control antibody (25 mg/kg), and 3) LPS + anti-TNF-α neutralizing antibody (25 mg/kg). Isotype control antibody and anti-TNF-α neutralizing antibody were administered intravenously (i.v.) 1 hr prior to LPS via tail vein injection. Control mice received an i.v. injection of vehicle control (PBS).

Recombinant mouse TNF-α was obtained from BioLegend to evaluate whether TNF-α alone reproduced LPS-mediated changes in renal function and/or MB. Wild-type C57BL/6 male mice (6 to 8 weeks in age) were treated i.v. with either vehicle control (diluent), 20 µg/kg recombinant murine TNF-α, or 50 µg/kg TNF-α via tail vein injection. Animals were euthanized at 18 h after TNF-α administration and kidneys and serum were collected for analysis.

**Human Renal Proximal Tubule Cell Culture**

Primary human renal proximal tubule cells (hRPTECs) were acquired from Lonza BioResearch (Allendale, NJ). Passage 4 – 11 hRPTECs were maintained in T-75 culture flasks and plated in 35 mm dishes for all experiments. Cells were grown in REBM basal medium supplemented with 0.5% FBS, EGF, insulin, GA-1000, hydrocortisone, transferrin, triiodothyronine, and epinephrine and maintained at 37°C in a 5% CO₂ humidified incubator as instructed by the manufacturer. Once cells reached ~90% confluence, they were treated with LPS (Sigma, described above), recombinant human TNF-α, recombinant human CD14, recombinant human LBP (all from R&D Systems, Minneapolis, MN) at various doses for 3 or 24 h as indicated.
**Blood Urea Nitrogen Measurement**

Blood urea nitrogen (BUN) was determined using the QuantiChrom Urea Assay kit (BioAssay Systems, Hayward, CA) based on the manufacturer’s directions. All values are expressed as blood urea nitrogen concentration in milligrams per deciliter.

**Quantitative Real-Time PCR Analysis of mRNA Expression**

Total RNA was isolated from renal cortical tissue with the TRIzol reagent (Life Technologies, Grand Island, NY). The iScript Advanced cDNA Synthesis Kit for RT-qPCR (Bio-Rad, Hercules, CA) was used to produce a cDNA library from 1 µg total RNA according to the manufacturer’s protocol. Quantitative real-time PCR was performed with the generated cDNA using the SsoAdvanced Universal SYBR Green Supermix reagent (Bio-Rad). Relative mRNA expression of all genes was determined by the $2^{-\Delta \Delta Ct}$ method and the 18S ribosomal RNA (18S rRNA) was used as a reference gene for normalization as previously described (306). Primer pairs used for PCR were as follows:
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Analysis of Mitochondrial DNA Content

Mitochondrial DNA content was determined by quantitative real-time PCR analysis. Total DNA was isolated from the renal cortex using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) as described in the manufacturer’s protocol. Extracted DNA was quantified and 5 ng was used for PCR. Relative mitochondrial DNA content was assessed by the mitochondrial-encoded NADH Dehydrogenase 1 (ND1) and was normalized to nuclear-encoded β-Actin. Primer sequences for ND1 and β-Actin were: ND1 sense: 5’-TAGAACGCAAAAATCTTAGGG-3’; ND1 antisense: 5’-TGCTAGTGTGAGTGATAGGG-3’; β-Actin sense: 5’-GGGATGTCTGGTCAAACCA-3’; and β-Actin antisense: 5’-GCGCTTTTGACTCAGGATTTAA-3’.

Immunoblot Analysis

Protein was extracted from renal cortex using RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 7.4) with protease inhibitor cocktail (1:100), 1 mM sodium fluoride, and 1 mM sodium orthovanadate (Sigma Aldrich). Total protein amount was determined by BCA protein assay. Equal protein quantities (50 – 100 µg) were loaded onto 4 – 15% SDS-PAGE gels (Bio-Rad). Proteins were resolved by gel electrophoresis and transferred onto nitrocellulose membranes (Life Technologies). Membranes were blocked in 2.5% BSA and incubated overnight with primary antibody at 4°C. Primary antibodies used in these studies included NGAL/Lipocalin-2 (1:1000), phospho-TPL2 (1:500), total TPL2 (1:1000, all from Abcam, Cambridge, MA), phospho-ERK1/2 (1:1000), total ERK1/2 (1:1000, both from Cell Signaling Technology, Danvers, MA), KIM1 (1:1000, from
R&D systems, Minneapolis, MN), PGC-1 (1:100, Cayman Chemical, Ann Arbor, MI), and β-Actin (1:1000, Santa Cruz Biotechnology, Dallas, TX). Membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody before visualization using enhanced chemiluminescence (Thermo Scientific, Waltham, MA) and the GE ImageQuant LAS4000 (GE Life Sciences, Pittsburgh, PA). Optical density was determined using NIH ImageJ software (version 1.46).

**Statistical Analysis**

All data are shown as mean ± S.E.M. When comparing two experimental groups, an unpaired, two-tailed t-test was used to determine statistical differences. A one-way analysis of variance (ANOVA) followed by Tukey’s post-hoc test was performed for comparisons of multiple groups. A p-value < 0.05 was considered statistically significant. All statistical tests were performed using GraphPad Prism software.
RESULTS

*Endotoxin Exposure Leads to Acute Kidney Injury in Mice*

To confirm the effects of systemic LPS exposure on renal function, we measured BUN at 3 and 18 h after administration in mice. BUN increased 1.9-fold and 3.8-fold 3 h and 18 h post-LPS, respectively, indicating a time-dependent reduction in renal function (Figure 2-1A). These findings are consistent with other studies reporting markedly reduced glomerular filtration rate (GFR) and elevated BUN with 4 h of endotoxin exposure in mice (79). To confirm proximal tubule injury in this model, we also measured protein expression of kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) in the renal cortex by immunoblot analysis at 18 h. KIM-1 and NGAL increased ~5- and ~2-fold, respectively, in mice receiving LPS when compared to vehicle-treated controls (Figure 2-1B,C). No changes were noted in KIM-1 and NGAL protein levels at 3 h post-LPS, although their mRNAs were up-regulated at this time point (data not shown). Together, these findings indicate that systemic LPS exposure results in rapid development of AKI and specifically leads to injury in the renal proximal tubule.
Figure 2-1: Systemic LPS administration induces renal dysfunction and tubular injury in mice. Six to eight week old male C57BL/6 mice were injected i.p. with lipopolysaccharide (10 mg/kg) or saline vehicle and euthanized at 3 and 18 h. Renal function at 3 and 18 h after LPS was determined by BUN (n = 7 animals / group) (A). Immunoblot analysis of renal cortical KIM-1 (B) and NGAL (C) expression was used to assess tubular injury (n = 4 animals / group). Data are shown as mean ± S.E.M. for each group. Different superscripts above bars indicate statistically significant differences (p < 0.05) when compared to time-point controls.
LPS-Induced AKI Leads to Persistent Suppression of PGC-1α

Because disruption of MB and homeostasis has been associated with loss of renal function and delayed recovery in multiple experimental models of AKI, we hypothesized that systemic endotoxin exposure would lead to disruption of PGC-1α. (162, 274, 290). PGC-1α mRNA was reduced by 70% as early as 3 h after LPS and progressively decreased at the 18 h time point to ~10% of control (Figure 2-2A). In addition, a ~70% decrease in PGC-1α protein expression was noted at 18 h after LPS exposure (Figure 2-2B). To determine whether LPS has a dose-dependent effect on renal cortical PGC-1α expression, mice were treated with 0.5 mg/kg and 2 mg/kg of LPS and PGC-1α mRNA was examined 3 h after administration. Interestingly, lower doses of LPS (0.5 mg/kg and 2 mg/kg) reduced PGC-1α transcript ~55% and ~45%, respectively, similar to the 10 mg/kg dose (~60%) (Figure 2-2C).

To further characterize the effects of endotoxin on MB, we measured mRNA expression of other members of the PGC-1 family, PGC-1β and PRC, in the renal cortex. Transcript levels of PGC-1β were decreased ~60% in LPS-treated mice at 3 and 18 h compared to vehicle-treated controls. In contrast, PRC mRNA increased 1.7-fold as early as 3 h post-administration and remained elevated at 18 h (2-fold) (Figure 2-2D). These data demonstrate that systemic LPS exposure results in suppression of PGC-1α and PGC-1β at the mRNA and/or protein levels in the renal cortex.
Figure 2-2: LPS-induced AKI is associated with suppression of PGC-1α expression in the renal cortex. Total RNA and protein were extracted from the renal cortex of vehicle- and LPS-treated mice. Time-dependent changes in PGC-1α mRNA expression at 1, 3, and 18 h after LPS exposure (10 mg/kg) were determined by qPCR analysis (n = 5 – 6 / group) (A). Protein levels of PGC-1α were assessed by Western blot (n = 4 / group) (B). To determine dose dependent effects of endotoxin on renal cortical PGC-1α expression, a separate group of mice were treated with varying doses of LPS (0.5 mg/kg, 2 mg/kg, and 10 mg/kg) and PGC-1α mRNA was measured (n = 6 / group) (C). Time-dependent changes in other members of the PGC-1 family (PGC-1β, PRC) were also determined by qPCR (n = 6 / group) (D). Values are expressed as relative expression compared to time-point controls and are presented as mean ± S.E.M. Statistically significant differences (p < 0.05) are denoted by superscripts above bars.
**PGC-1α Suppression by LPS Results in Disruption of Mitochondrial Homeostasis**

To determine whether mitochondrial homeostasis was altered in the renal cortex as a result of reduced PGC-1α expression following endotoxin administration, we assessed mRNA expression of downstream mediators of MB, (NRF-1, TFAM) as well as nuclear-encoded (NDUFS1, NDUFB8, ATPSβ) and mitochondrial-encoded (COX1, ND1) components of the electron transport chain. A ~38% decrease in NRF-1 mRNA was observed 3 h post-LPS which recovered to control levels by 18 h. Transcript levels of TFAM, NDUFS1, NDUFB8, ATPSβ, and COX1 were also decreased (~25%, 43%, 42%, 25%, and 31%, respectively) at the 18 h time-point and these decreases correspond with the reduction in PGC-1α protein (Figure 2-3A). We also assessed mitochondrial DNA copy number as a marker of mitochondrial quantity. Relative mitochondrial DNA content in the renal cortex was reduced by ~50% at 18 h after LPS and remained lower up to 42 h (Figure 2-3B). In addition, a modest, but significant reduction in protein levels of TFAM, NDUFS1, NDUFB8, and ATPSβ was noted by 42 h post-LPS (Figure 2-3C,D). These findings indicate that suppression of PGC-1α following systemic endotoxin exposure is associated with widespread disruption of transcriptional regulation of mitochondrial proteins and depletion of mitochondrial DNA content.
Figure 2-3: Disruption of mitochondrial homeostasis in endotoxic AKI. Total RNA and DNA were harvested from renal cortical tissue of mice treated with saline vehicle or LPS (10 mg/kg, i.p.) at 1, 3, 18, and 42 h. Expression of key regulators of mitochondrial biogenesis (NRF-1, TFAM), nuclear-encoded components of the ETC (NDUFS1, NDUFB8, ATPSβ), and mitochondrial-encoded respiratory proteins (COX1, ND1) were measured at the mRNA level (n = 6 / group) (A). Relative mitochondrial DNA content in the renal cortex was determined by qPCR analysis (n = 5 – 6 animals / group) (B). Protein levels of TFAM, NDUFS1, NDUFB8, and ATPSβ were determined by immunoblot analysis (C,D). Data are shown as expression relative to vehicle-treated animals (mean ± S.E.M). *p < 0.05 vs. time point controls.
TLR4 is Required for Endotoxin-Induced AKI and Disruption of Mitochondrial Homeostasis

Although LPS primarily signals through activation of Toll-like receptor 4 (TLR4), recent studies have identified TLR4-independent signaling pathways which contribute to endotoxic shock in mice (94, 95). To determine whether TLR4 signaling is responsible for both endotoxin-induced AKI and suppression of MB, we utilized TLR4 knockout mice (TLR4KO) and wild-type controls and assessed renal function and mitochondrial homeostasis 18 h post-LPS. As expected, systemic LPS exposure in wild-type mice resulted in an increase in BUN (Figure 2-4A). However, TLR4KO mice were completely protected from endotoxic AKI. These findings are in agreement with previous studies demonstrating an essential role for TLR4 in LPS-induced renal dysfunction in mice (150).

We next determined the role of TLR4 in endotoxin-mediated suppression of PGC-1α and MB. A 73% decrease in PGC-1α mRNA was noted in wild-type mice subjected to LPS while PGC-1α mRNA in TLR4KO mice was similar to controls (Figure 2-4B). COX1 mRNA and mitochondrial DNA content were also decreased (~40% and ~30%, respectively) in wild-type mice 18 h after LPS exposure and remained unchanged in TLR4KO mice (Figure 2-4C,D). Taken together, these data reveal an essential role for TLR4 in both LPS-mediated renal damage and suppression of MB.
Figure 2-4: TLR4 is necessary for development of LPS-induced AKI and suppression of mitochondrial biogenesis. Wild-type and TLR4 knockout mice were treated with LPS (10 mg/kg, i.p.) or saline vehicle and euthanized at 18 h for biochemical analysis. BUN was used to assess renal function after LPS exposure (A). Transcript levels of PGC-1α (B) and COX1 (C) as well as mitochondrial DNA copy number (D) served as markers of mitochondrial homeostasis (n = 3 – 4 / group for all analyses, p < 0.05 vs. wild-type control).
LPS-Induced AKI Leads to Activation of TPL-2/ERK Signaling in the Renal Cortex

TLR4 is known to initiate signaling by a number of different mediators, most notably NFκB and members of the mitogen-activated protein kinase (MAPK) family including extracellular signal-related kinases 1/2 (ERK1/2), p38 MAPKs, and c-Jun N-terminal kinases (JNKs) (307, 308). Previous studies showed that ERK1/2 activation may negatively regulate expression of PGC-1α in multiple organs including the brain and skeletal muscle (244, 245). In addition, TPL-2 is thought to be a critical for activation of ERK1/2 in response to LPS (309, 310). We hypothesized that the TPL-2/ERK signaling would be activated in the renal cortex and that this pathway contributes to disruption of MB in endotoxin-induced AKI. Phosphorylated TPL-2 (Thr209) increased 1.5-fold 1 h after LPS treatment and returned to control levels at 3 h (Figure 2-5). A 5-fold elevation of ERK1/2 phosphorylation was observed 1 h after LPS treatment and remained elevated at 3 h (Figure 2-5B,D). These findings indicate that TPL-2/ERK signaling is activated quickly in the renal cortex in response to systemic endotoxin exposure.
Figure 2-5: Systemic LPS exposure enhances TPL-2/ERK signaling in the renal cortex. Mice were treated with LPS (10 mg/kg, i.p.) or vehicle as described above. Immunoblot analysis revealed increased phosphorylated/activated TPL-2 (Thr209) (A) and ERK1/2 (Thr202/Tyr204) (B) in the renal cortex of LPS-treated mice at 1 h. Levels of phospho-TPL-2 and phospho-ERK1/2 were normalized to their respective total protein content and actin was used as a secondary loading control. Data are presented as phosphorylated / total ratio relative to vehicle controls (mean ± S.E.M.; n = 4 / group for all analyses). *p < 0.05 between groups.
Early ERK Activation Mediates LPS-Induced Renal Injury and Suppression of MB

To further elucidate the role of ERK1/2 signaling in LPS-induced AKI and disruption of mitochondrial homeostasis, mice were treated with the potent and selective MEK1/2 inhibitor GSK1120212 (GSK112) (1 mg/kg) 1 h prior to LPS administration. MEK1/2 are directly responsible for phosphorylation of ERKs and thus inhibition of MEK1/2 by GSK112 will prevent downstream activation of ERK1/2. In the first experiment, mice were euthanized 3 h post-LPS to determine the early effects of ERK activation on renal dysfunction and MB. Pre-treatment with GSK112 attenuated endotoxin-mediated elevation of BUN at 3 h (Figure 2-6A) and markedly reduced the increase in renal cortical KIM-1 transcript levels in LPS-treated mice (Figure 2-6B). GSK112 alone did not have an effect on either BUN or renal cortical KIM-1 (Figure 2-6A,B). Interestingly, GSK112 administration in mice receiving LPS also blocked suppression of PGC-1α mRNA expression (Figure 2-6C). Immunoblot analysis demonstrated effective blockade of ERK1/2 activation in mice treated with MEK1/2 inhibitor (Figure 2-6D). These data identify a novel role for early TLR4-mediated ERK1/2 activation in both renal dysfunction and suppression of MB in the renal cortex in LPS-induced AKI. In contrast, preliminary experiments demonstrated that multiple pharmacological inhibitors of NFκB signaling including TPCA-1, BAY11-7085, and PPM-18 had no effect on LPS-induced disruption of PGC-1α and MB (data not shown).

A separate group of mice was pretreated with GSK112 (1 mg/kg) or vehicle control and serum and kidneys were collected at 18 h post-LPS for biochemical analysis. BUN increased 3.6-fold in LPS-treated animals and was partially attenuated by GSK112
(~2.1 fold vs. control) (Figure 2-7A). In addition, GSK112 pretreatment partially blocked LPS-induced increase in renal cortical KIM-1 mRNA expression, indicating a reduction in tubular injury (Figure 2-7B). A modest effect of GSK112 administration on transcript levels of PGC-1α and its transcriptional target NDUFS1 was also noted in LPS mice (Figure 2-7C,D). Taken together, these findings indicate that rapid activation of ERK1/2 in the renal cortex following systemic endotoxin exposure contributes to the early development of renal injury and disruption of mitochondrial homeostasis.
Figure 2-6: Inhibition of MEK/ERK signaling prevents early changes in renal function and mitochondrial biogenesis in endotoxic AKI. Mice were pre-treated with the MEK1/2 inhibitor GSK1120212 (GSK112; 1 mg/kg) 1 h prior to systemic LPS exposure. Serum and kidneys were collected at 3 h post-LPS to determine the role of MEK/ERK signaling in early pathophysiology. Renal function was assessed by changes in BUN (A). The effects of MEK/ERK inhibition on tubular injury were determined by measuring KIM-1 mRNA expression in the renal cortex (B). PGC-1α transcript levels were determined by qPCR (C). Inhibition of ERK1/2 activation was confirmed by immunoblot analysis of phosphorylated and total ERK1/2 levels in the renal cortex (D). Data are shown as mean ± S.E.M.; n = 5 – 6 / group for all analyses; p < 0.05 vs. control).
Figure 2-7: Effects of GSK1120212 on LPS-induced AKI and suppression of mitochondrial biogenesis at 18 h. Mice receiving GSK1120212 (GSK112; 1 mg/kg) 1 h prior to endotoxin administration were euthanized at 18 h post-LPS for biochemical analysis. Renal dysfunction and tubular injury were determined by BUN (A) and KIM-1 mRNA expression (B), respectively. Transcript levels of PGC-1α (C) and NDUFS1 (D) were determined by qPCR analysis. Values are presented as mean ± S.E.M. for n ≥ 6 / group. Superscripts above bars are used to denote statistically significant differences compared to control (p < 0.05).
TNF-α, but not LPS, Induces Down-regulation of PGC-1α in Renal Proximal Tubule Cells In Vitro

TLR4-mediated activation of ERK1/2 is known to induce expression of pro-inflammatory cytokines including TNF-α and IL-1β (311-313). Recent reports also indicate that these cytokines may negatively regulate PGC-1α expression and MB in a variety of cell types including renal proximal tubule cells and skeletal muscle cells (162, 242). We therefore examined whether LPS alone or the downstream cytokine target of MEK/ERK signaling, TNF-α, were necessary for suppression of PGC-1α transcription in primary human renal proximal tubular epithelial cells (hRPTECs) in vitro. Cells were treated with varying doses of LPS (1 µg/ml or 10 µg/ml) or recombinant human TNF-α (10 ng/ml or 100 ng/ml) and mRNA expression of PGC-1α was determined by qPCR at 3 and 24 h post-treatment. Cells exposed to LPS were also supplemented with necessary accessory proteins including recombinant human CD14 (100 ng/ml) and recombinant human LPS binding protein (LBP, 100 ng/ml). No changes in PGC-1α transcript levels were noted following exposure of hRPTECs to LPS at any of the doses or time points studied (Figure 2-8A,B). However, TNF-α treatment (both 1 ng/ml and 10 ng/ml) resulted in rapid and persistent suppression of PGC-1α mRNA levels by 3 h (Figure 2-8C,D). These data indicate that LPS is not capable of directly disrupting PGC-1α expression in renal proximal tubule cells in vitro. Instead, downstream effectors of LPS-induced MEK/ERK signaling including TNF-α may be required for this effect.
Figure 2-8: Effects of LPS and TNF-α on renal proximal tubule epithelial cell PGC-1α expression in vitro. Primary human RPTECs were treated with LPS (1 µg/ml or 10 µg/ml) in the presence of rhCD14 (100 ng/ml) and rhLBP (100 ng/ml) and mRNA content of PGC-1α were determined at 3 h (A) and 24 h (B) by qPCR analysis. In a separate experiment, cells were exposed to rhTNF-α (10 ng/ml or 100 ng/ml) and PGC-1α transcript levels were assessed at 3 h (C) and 24 h (D) as described above. Data are shown as mean ± S.E.M for n = 6 / group. Different superscripts above indicate statistically significant differences (p < 0.05) between groups.
Early ERK Activation Promotes Expression of Pro-Inflammatory Cytokines in the Renal Cortex After LPS Administration

Since TNF-α induced direct suppression of PGC-1α in renal proximal tubule cells in vitro, we next examined ERK1/2 signaling in the expression of TNF-α and IL-1β in the renal cortex following systemic endotoxin administration in mice. LPS administration resulted in a robust increase in TNF-α (~12 fold) and IL-1β (~3.3 fold) mRNA at 3 h and GSK112 inhibition of MEK/ERK partially attenuated the early increase in mRNA expression of both pro-inflammatory cytokines (Figure 2-9A). However, pretreatment with GSK112 had no effect on expression of either TNF-α or IL-β at 18 h post-LPS (Figure 2-9B). These findings reveal an important role for TLR4-induced ERK signaling in early induction of pro-inflammatory cytokines in the renal cortex after systemic endotoxin administration.
Figure 2-9: MEK/ERK signaling controls early renal expression of pro-inflammatory cytokines in response to endotoxin. Inflammatory changes were evaluated in the renal cortex of mice treated with LPS and GSK1120212 (GSK112; 1 mg/kg) as described above. qPCR was used to determine mRNA levels of TNF-α and IL-1β at both 3 h (A) and 18 h (B) post-LPS. Values are expressed as relative expression vs. control (mean ± S.E.M.) for n ≥ 6 / group (p < 0.05 vs control).
Involvement of TNF-α in LPS-Mediated Suppression of PGC-1α

Because pro-inflammatory cytokines have been implicated as a potential mechanism for suppression of PGC-1α in renal proximal tubules and ERK signaling promoted TNF-α expression in the kidney cortex, we utilized a neutralizing antibody directed against murine TNF-α to further elucidate its role in this process. Mice were treated i.v. with either TNF-α neutralizing antibody (25 mg/kg) or the appropriate isotype control antibody (25 mg/kg) 1 h prior to LPS. TNF-α neutralization had no effect on the LPS-induced increase in BUN at 18 h (Figure 2-10A). However, suppression of PGC-1α mRNA levels was partially attenuated in mice receiving the anti-TNF-α antibody (Figure 2-10B).

Surprisingly, TNF-α neutralization had no effect on the observed decreases in NDUFB8, NDUFS1, ATPSβ, and COX1 transcripts, and mitochondrial DNA content (Figure 2-10C,D). Taken together, these data indicate that the TLR4/ERK/TNF-α signaling axis may act as a critical mechanism for suppression of MB in the setting of endotoxic AKI.
Figure 2-10: TNF-α mediates suppression of PGC-1α in the renal cortex of LPS-treated mice. Mice were treated with anti-TNF-α neutralizing antibody or the appropriate isotype control antibody 1 h prior to systemic LPS exposure and renal function and mitochondrial homeostasis were evaluated at 18 h. Renal dysfunction was determined by measuring BUN (A). Transcript levels of PGC-1α (B) and mitochondrial biogenesis markers (C) were assessed. qPCR analysis was used to determine relative mitochondrial DNA content (D). Data are mean ± S.E.M. (n = 4 / group; different superscripts indicate statistically significant differences (p < 0.05)).
Recombinant TNF-α is Sufficient to Produce Kidney Injury and Disrupt MB in the Renal Cortex

To further characterize the effects of TNF-α on renal dysfunction and mitochondrial homeostasis, mice received i.v. injections of low dose recombinant murine TNF-α (20 µg/kg), high dose TNF-α (50 µg/kg), or the appropriate vehicle. TNF-α dosing was based on preliminary experiments and previous reports to determine the appropriate doses which have no effect on renal function (low dose) or produce renal injury (high dose) (141). As expected, an increase in BUN was noted only in mice receiving high dose TNF-α (Figure 2-11A). Renal dysfunction in these animals was associated with a 55% decrease in renal cortical PGC-1α mRNA (Figure 2-11B). Both low and high dose TNF-α suppressed transcript levels of both nuclear-encoded (NDUFS1) and mitochondrial-encoded (ND1, COX1) respiratory proteins (Figure 2-11C). A modest, but not statistically significant, decrease in mitochondrial DNA content was observed in animals treated with high dose TNF-α (Figure 2-11D). These findings indicate that recombinant TNF-α is sufficient to cause both renal injury and suppression of MB in the renal cortex.
Figure 2-11: Recombinant TNF-α induces AKI and renal mitochondrial dysfunction in mice. Mice were treated with recombinant TNF-α at variable doses (20 µg/kg or 50 µg/kg) or the appropriate diluent. Serum was collected at 18 h and BUN was determined (A). Total RNA and DNA were extracted from the renal cortex to assess expression of PGC-1α (B), downstream targets (C) and relative mitochondrial DNA content (D) by qPCR. Values are mean ± S.E.M. from n = 6 animals/group (p < 0.05 where denoted by different subscripts).
DISCUSSION

Disruption of mitochondrial homeostasis is an important contributor to initiation and progression of both tubular injury and renal dysfunction in AKI (162, 170, 274). Mitochondrial turnover is regulated by a complex interplay between fission/fusion, autophagy/mitophagy, and MB (172, 173). Our laboratory and others have recently demonstrated alterations in MB in experimental models of AKI (178, 274, 278, 279). MB is tightly controlled by the PGC-1 family of transcriptional co-activators including PGC-1α, PGC-1β, and PRC which coordinate both nuclear and mitochondrial responses to increase cellular mitochondrial content. PGC-1α is considered a “master regulator” of MB in organs with high energy demand including the kidney (180, 181) PGC-1α expression is localized to renal proximal tubule cells which rely on mitochondrial ATP production to facilitate active transport of solutes, indicating that MB may be essential for tubular function (288).

Recent studies characterized mitochondrial dysfunction in sepsis-induced AKI (162, 267). Tran et al. demonstrated that mitochondrial dysfunction was associated with acute down-regulation of PGC-1α in the kidney following endotoxin exposure or cecal ligation and puncture (CLP) in mice (162). PGC-1α suppression in these models was correlated with both reduced expression of transcriptional targets of PGC-1α and renal dysfunction. Restoration of normal renal function in LPS-treated mice given saline resuscitation at the 18 h time point coincided with return of PGC-1α mRNA and protein to baseline levels, further indicating a potential role for MB in both pathogenesis of and recovery from septic AKI. Proximal tubule-specific PGC-1α knockout in mice resulted in prolonged renal dysfunction in response to systemic LPS exposure. Together, these
findings provide strong evidence that PGC-1α and MB may be viable therapeutic targets in sepsis-induced AKI.

Systemic endotoxin exposure in mice led to rapid deterioration of renal function as measured by BUN at 3 h, and further increases in BUN and renal cortical KIM-1 and NGAL at 18 h. Despite relative minimal renal histological findings, these data provide strong molecular evidence for involvement of tubular insult in the pathophysiology of sepsis-induced AKI.

Progressive renal dysfunction and tubular cell injury were closely associated with a reduction in PGC-1α mRNA and protein in the renal cortex after endotoxin administration. These findings are similar to previous studies reporting negative correlations between BUN and PGC-1α transcript levels in mice receiving LPS or CLP (162). Interestingly, lower doses of LPS that did not induce renal dysfunction acutely had a similar effect on PGC-1α mRNA and PGC-1α protein levels did not change at the 3 h time point when renal and tubular injury were first observed as indicated by increased BUN and KIM-1 mRNA expression. These findings indicate that suppression of PGC-1α and subsequent MB is unlikely to be the initial causative factor and alone is not sufficient for the development of sepsis-induced AKI.

Early increases in circulating pro-inflammatory cytokines and renal microvascular dysfunction are also important mediators of renal injury in mice following systemic LPS exposure (124, 140). However, suppression of renal cortical PGC-1α and MB likely contribute to disease progression through disruption of energy-dependent tubular transport and repair processes (49, 256, 314). This idea is supported by earlier studies
demonstrating that both global and proximal-tubule specific PGC-1α knockout leads to persistent renal injury in LPS-induced AKI in mice (162).

We also measured transcript levels of PGC-1β and PRC. PGC-1β expression was decreased, but not as markedly as PGC-1α. In contrast, PRC mRNA levels rose after LPS treatment similar to findings reported in I/R- and glycerol-induced AKI (274). Given the relative lack of knowledge regarding the role of PGC-1β and PRC in renal mitochondrial homeostasis, it is difficult to interpret these findings and further work is warranted.

Previous studies showed that TLR4 is required for LPS-induced renal dysfunction (150). However, a number of TLR4-independent actions of LPS have been described (94, 95). As predicted, TLR4-deficient mice did not develop renal injury and markers of MB and mitochondrial DNA content did not decrease following endotoxin exposure. These findings demonstrate that TLR4 is required for suppression of renal PGC-1α and MB in LPS-induced AKI. It is important to note that the MB response to sepsis is tissue-specific. Up-regulation of hepatic PGC-1α and PGC-1β is markedly enhanced in TLR4-deficient mice after sepsis, suggesting that TLR4 may be an important negative regulator of MB (315). Further studies are needed to evaluate the possibility that TLR4 activation is a common mechanism underlying suppression of MB in multiple forms of AKI.

Binding of LPS to TLR4 activates a number of downstream signaling mediators, most notably nuclear factor kappa B (NFκB) and mitogen-activated protein kinases (MAPKs) including ERK1/2, p38 MAPKs, and JNKs (307, 308). We observed rapid (1 h) activation of TPL-2/MEK/ERK signaling in the renal cortex. These findings are similar to previous studies demonstrating TLR4-dependent increases in ERK1/2
phosphorylation after LPS exposure in the medullary thick ascending limb of the kidney (161). Although TPL-2 is known to be required for LPS-induced ERK1/2 activation in macrophages, this is the first study to demonstrate increased TPL-2 phosphorylation in the renal cortex in response to systemic endotoxin exposure (309, 310). A recent study did reveal that TPL-2 knockout mice are protected from kidney I/R, although this effect was not attributed to the MAPK function of TPL-2 (316).

MEK/ERK signaling has been implicated as a negative regulator of both PGC-1α and MB. Pharmacological inhibition of MEK reversed reductions in protein levels of PGC-1α and downstream regulators of MB (NRF-1, TFAM) in the hippocampi of rats receiving intracerebroventricular injections of amyloid β (244). In addition, blockade of ERK activation prevented palmitate-induced down-regulation of PGC-1α in skeletal muscle myotubes in vitro (245). The MEK1/2 inhibitor GSK1120212 (GSK112) blocked ERK1/2 activation and reduced early renal dysfunction and tubular injury (3 h) in mice subjected to systemic LPS. The protective effects of GSK112 were associated with restoration of renal cortical PGC-1α expression. These findings indicate an important role for MEK/ERK signaling in LPS-induced renal injury and suppression of MB. In mice followed to 18 h post-LPS, GSK112 partially attenuated loss of renal function and injury to the proximal tubule. However, only modest effects were noted on PGC-1α expression and mitochondrial DNA content. This may indicate that the TPL-2/MEK/ERK pathway is essential for early suppression of PGC-1α in the renal cortex after endotoxic AKI, but other signaling mechanisms contribute to late-phase disruption of MB and renal dysfunction. Among these, NFκB has been identified as a potential
regulator of PGC-1α transcriptional activity (242, 317). Further work is necessary to address the role of NFκB in LPS-induced mitochondrial dysfunction.

MEK/ERK activation downstream of TLR4 results in activation of a transcriptional program to increase cytokine expression in response to LPS (309, 318). In addition, pro-inflammatory cytokines have been implicated in regulation of PGC-1α and MB (162, 242). Administration of exogenous TNF-α reduced expression of PGC-1α and its transcriptional targets in primary human renal epithelial cells *in vitro* (162). In addition, TNF-α and IL-1β induced disruption of MB in skeletal muscle cells (242). As expected, inhibition of MEK/ERK signaling attenuated early up-regulation of both TNF-α and IL-1β in the renal cortex of LPS-treated mice. Mice pretreated with an anti-TNF-α neutralizing antibody and exposed to LPS exhibited less PGC-1α mRNA loss. The partial restoration of PGC-1α following TNF-α neutralization may indicate that multiple pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) signal through similar mechanisms to suppress PGC-1α in the setting of sepsis-induced AKI. To our knowledge, this is the first study to report the effects of TNF-α neutralization on PGC-1α and subsequent MB *in vivo*. We did not detect an effect of anti-TNF-α on BUN 18 h post-LPS. However, an earlier study using the same TNF-α neutralizing antibody before endotoxin administration noted a significant reduction in BUN at the 4 h time point (79). However, we were able to reproduce the effects of LPS on renal dysfunction and suppression of PGC-1α expression by administration of recombinant TNF-α in naïve mice. Together, these findings strongly implicate TNF-α as a downstream mediator of TLR4/MEK/ERK signaling in LPS-induced AKI and PGC-1α and MB suppression.
A significant decrease in cortical peritubular capillary flow has been noted as early as 2 h post-LPS exposure in mice indicating that LPS has rapid effects on renal hemodynamics (124). A limitation of the current study is that it does not address whether modulation of TLR4/MEK/ERK and TNF-α signaling might improve microvascular function and thereby restore glomerular filtration in response to LPS. TNF-α neutralization using the soluble decoy receptor TNFsRp55 has been shown to restore both glomerular filtration and renal plasma flow after systemic exposure to a lower dose of LPS (5 mg/kg) (139). Future studies using conditional, tissue-specific knockout TLR4 and downstream signaling targets in the tubular epithelium, vascular endothelium, and vascular smooth muscle may address whether the renal protective effects observed in this study are due to direct actions of pharmacological agents on the tubular epithelium or indirect actions on the renal vasculature.

The current study identifies a novel mechanism mediating injury and suppression of MB in a mouse model of septic AKI. In particular, the TLR4/TPL-2/MEK/ERK/TNF-α signaling axis was required for early renal dysfunction and disruption of mitochondrial homeostasis in LPS-induced AKI. This pathway may represent a viable therapeutic target in the treatment of sepsis-associated AKI. Further studies are warranted to determine whether this mechanism is common to other forms of AKI and to identify the downstream targets of ERK and inflammatory cytokines in this process.
Chapter 3:
Renal cortical hexokinase activation through the EGFR/PI3K/Akt signaling pathway in endotoxin-induced acute kidney injury

ABSTRACT

While disruption of energy production is an important contributor to renal injury, metabolic alterations in sepsis-induced renal injury remain understudied. In this study, we assessed changes in glycolytic metabolism in the renal cortex in a mouse model of sepsis-induced AKI. A specific and rapid increase in hexokinase (HK) activity (~2 fold) was observed 3 hr after lipopolysaccharide (LPS) exposure and maintained up to 18 h in association with a decline in renal function as measured by BUN. LPS-induced hexokinase activation occurred independently of changes in expression of high affinity hexokinase isoforms or mitochondrial localization indicating post-translational regulation of hexokinase activity in this model. No other changes in glycolytic enzymes were observed. LPS-mediated hexokinase activation was not sufficient to increase glycolytic flux as indicated by reduced or unchanged pyruvate and lactate levels in the renal cortex. In addition, HK activation did not lead to increased glycogen production in the renal cortex. LPS-induced hexokinase activation was associated with an increase in glucose-6-phosphate dehydrogenase activity, suggesting that hexokinase activation may facilitate pentose phosphate pathway flux for NADPH. Mechanistically, LPS-induced HK activation was attenuated by pharmacological inhibitors of EGFR and Akt, suggesting that EGFR/PI3K/Akt signaling may be involved in this phenomenon. Our findings indicate that LPS rapidly increases renal cortical HK activity in an EGFR- and Akt-dependent manner and that HK activation is associated with an increase in glucose metabolism through the pentose phosphate pathway and not glycolysis.
INTRODUCTION

Acute kidney injury (AKI) is defined as an abrupt decline in renal function which occurs over a period of hours to days (1). A number of factors may lead to the development of AKI including sepsis, ischemia/reperfusion (I/R) injury, trauma, or exposure to nephrotoxic agents. Among the leading causes of AKI, sepsis is thought to be the most common contributor to AKI (approximately 50% of all cases) in the intensive care unit (ICU) setting (50). Despite increased understanding of the pathophysiology underlying AKI, mortality from the disease remains near 40% and has not changed over several decades (49, 297, 319). AKI in the setting of sepsis increases this mortality by almost 2-fold (297, 298). Unfortunately, currently available treatments for sepsis-induced AKI include supportive care and renal replacement therapy. A better understanding of the mechanisms leading to renal damage as well as recovery in septic AKI is essential for development of therapeutic strategies to improve outcomes in this disease.

The pathophysiology of sepsis-induced AKI is widely recognized as multifactorial, involving microvascular, immunological, and tubular components that contribute to renal dysfunction. The microvascular component is characterized by reduced capillary flow leading to local areas of hypoperfusion (135, 320, 321). Infiltration of both macrophages and neutrophils also exposes the septic kidney to a diverse array of pro-inflammatory factors (322, 323). Finally, tubular damage characterized by tubular cell vacuolization, mild tubular dilatation, and mitochondrial swelling has been noted in septic AKI (162, 301). Although histopathology in sepsis-induced AKI appears modest, preservation of renal function depends heavily on the
proximal tubule for reabsorption and secretion of solutes including sodium, glucose, and amino acids from the glomerular filtrate via active transport processes. Therefore, any pathological condition resulting in reduced ATP levels may contribute to tubular de-energization and subsequent loss of renal function characteristic of AKI (256, 257).

Our laboratory demonstrated that I/R and glycerol-induced AKI leads to rapid and sustained mitochondrial dysfunction in the renal cortex characterized by suppressed expression of mitochondrial biogenesis markers and electron transport chain components at the mRNA and protein levels (274). Disruption of normal mitochondrial homeostasis in these models was closely associated with proximal tubule cell injury and loss of renal function (274). Tran et al. recently reported similar findings following systemic exposure to lipopolysaccharide (LPS; a component of gram-negative bacterial cell walls), a well-established model of sepsis-induced AKI. In particular, LPS administration resulted in a marked reduction in cytochrome c oxidase activity and protein levels as well as down-regulation of a number of mitochondrial genes in the renal cortex. Suppression of mitochondrial mRNAs correlated significantly with renal function as measured by blood urea nitrogen (BUN) (162). LPS-induced mitochondrial dysfunction results in a significant decline (~50%) in renal ATP content within 5 hr. of exposure in rodents, likely contributing to changes in renal function in this model of septic AKI (265). Disruption of renal mitochondrial function and ATP production has also been reported in the cecal ligation and puncture model (CLP) of sepsis (324). What remains understudied are metabolic alterations which may facilitate both proximal tubule cell energy production and transport function in the presence of mitochondrial suppression.
Under normal conditions *in vivo*, renal proximal tubule cells primarily utilize oxidative phosphorylation via mitochondria to generate ATP and have very low glycolytic capacity. Early work characterizing renal metabolism demonstrated the proximal tubules possess the lowest activities of glycolytic enzymes (including hexokinase [HK], phosphofructokinase [PFK], and pyruvate kinase [PK] – the three major regulatory steps) along the entire nephron segment (258, 325). These findings have led to considerable debate concerning the ability of the proximal tubule to induce glycolytic metabolism as an alternate means of ATP generation. However, both *in vitro* and *in vivo* work indicates that a variety of stressors including ischemia/hypoxia, nephrotoxicants, elevated intracellular calcium, and inhibition of mitochondrial respiration may increase glycolytic flux in the proximal tubule (293, 296, 326). Glycolytic induction appears to play an important role in maintaining tubular cell viability, producing ATP, and preserving transport processes (293, 295, 322). In addition, recent studies have demonstrated that glycolytic metabolism is rapidly increased in the cortex in response to renal ischemia *in vivo* (327). These findings indicate that glycolysis may provide an alternate means of energy generation in response to tubular insult.

We hypothesized that glycolysis would be rapidly induced in the renal cortex in response to LPS-induced renal injury. To test this hypothesis, an extensive characterization of renal cortical glycolytic enzyme activity and expression after LPS challenge was performed. We report herein that LPS exposure results in a rapid (within 3 hr) and specific induction of HK activity mediated by EGFR/PI3K/Akt signaling.
pathway. Surprisingly, HK activation by LPS was not sufficient to enhance glycolysis in the renal cortex.
METHODS

Animal Model

Male C57BL6 mice (6 to 8 weeks of age, 20 – 25 g body weight) were obtained from Charles River. Mice received an intraperitoneal injection of 10 mg/kg LPS from *Escherichia coli* O111:B4 (Sigma Aldrich Catalog No. L4130, Lot No. 052M4016V) as previously described.(162) Control mice were injected i.p. with an equal volume of 0.9% saline vehicle. Mice were euthanized at 3, 6, and 18 h after LPS injection and serum and kidneys were collected for biochemical analysis. All experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal use was approved by the Institutional Animal Care and Use Committee at the Medical University of South Carolina.

Administration of Gefitinib and MK-2206

For studies examining the role of EGFR and PI3K/Akt signaling in LPS-induced renal injury, the EGFR inhibitor gefitinib and the Akt inhibitor MK-2206 were obtained from Selleckchem Chemicals (Houston, TX).(328, 329) Mice were randomly assigned to three groups: 1) LPS + vehicle control (5% DMSO, 40% 2-hydroxypropyl-β-cyclodextrin), 2) LPS + gefitinib (100 mg/kg), or 3) LPS + MK-2206 (100 mg/kg). One hour prior to LPS administration, mice were given an intraperitoneal injection as described above. Mice were sacrificed at 3 h after LPS injection and serum and kidneys harvested for analysis. Appropriate dosing of gefitinib was based on previous studies demonstrating inhibition of EGFR activity in mouse models of both acute kidney injury and renal fibrosis.(330, 331)
**Blood Urea Nitrogen Measurement**

Blood urea nitrogen (BUN) was measured using a QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer’s protocol. Values are expressed as blood urea nitrogen concentration in milligrams per deciliter.

**Hexokinase Activity Assay**

Renal cortical hexokinase activity was determined using a Hexokinase Colorimetric Assay Kit (BioVision Incorporated, Milpitas, CA). Briefly, renal cortical tissue was homogenized in ice-cold Hexokinase Assay Buffer. Homogenized tissue was then centrifuged at 12,000 r.p.m. for 10 mins to clear debris. The resulting supernatant was collected and 15 µL samples were loaded into a 96-well plate and adjusted to a final volume of 50 µL. After addition of HK reaction mix to each well, absorbance at 450 nm was measured in kinetic mode using an Infinite M200 PRO spectrophotometer (Tecan Systems, Inc., San Jose, CA). NADH generation as a result of conversion of glucose to glucose-6-phosphate by hexokinase was calibrated to a standard curve using the provided NADH standard. Background samples without reaction mix were also included to correct for endogenous NADH levels. Protein content for each sample was determined by the BCA protein assay and values are expressed as milliunits of hexokinase activity per mg protein content.

**Phosphofructokinase Activity Assay**

Phosphofructokinase (PFK) activity in the renal cortex was assessed using the Phosphofructokinase Activity Colorimetric Assay Kit (BioVision Incorporated). Renal cortical tissue was extracted in ice-cold PFK Assay Buffer and centrifuged at 12,000 r.p.m. for 10 mins to remove cellular debris. A portion of the sample was aliquoted for
determination of protein concentration by the BCA protein assay. Interfering small molecules were removed using 10 kD molecular weight cutoff centrifugal filters (VWR, Radnor, PA). The concentrated protein samples were then diluted and PFK activity was determined based on the manufacturer’s protocol using an Infinite M200 PRO spectrophotometer in kinetic mode (Tecan Systems).

**Pyruvate Kinase Activity Assay**

Cortical pyruvate kinase (PK) activity was determined using the Pyruvate Kinase Activity Colorimetric Assay Kit (BioVision Incorporated). Renal cortical tissue was homogenized in cold Pyruvate Kinase Assay Buffer and cleared of debris by centrifugation at 12,000 r.p.m. for 10 mins. Samples were then assayed for pyruvate kinase activity as directed by the manufacturer by determining changes in optical density at 570 nm using an Infinite M200 PRO spectrophotometer in kinetic mode (Tecan Systems). A portion of the sample was also used for determination of protein content via the BCA protein assay.

**Analysis of mRNA Expression**

Total RNA was isolated from renal cortex samples using the TRIzol reagent (Life Technologies, Grand Island, NY). A cDNA library was generated from 2 µg RNA by reverse transcription reaction using the iScript Advanced cDNA Synthesis Kit for RT-qPCR according to the manufacturer’s protocol (Bio-Rad, Hercules, CA). Quantitative real-time PCR was then performed with cDNA using the SsoAdvanced SYBR Green Supermix (Bio-Rad). mRNA expression of genes of interest was calculated by the 2^-ΔΔCt method normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT) as
previously described (306). Primer sequences used for mRNA analysis were as described below:
### Table 3-1: Primer sequences used for qPCR.

<table>
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<th>Gene</th>
<th>Primer Sequence</th>
<th>Accession No.</th>
</tr>
</thead>
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<tr>
<td>HPRT</td>
<td>Sense 5'-GCTTACCTCAGCTTCCG-3'</td>
<td>NM_013556</td>
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<tr>
<td></td>
<td>Antisense 5'-ATCATCGCTAATCAGACGC-3'</td>
<td></td>
</tr>
<tr>
<td>HK1</td>
<td>Sense 5'-GGGACTATGACGCTAACATT-3'</td>
<td>NM_001146100</td>
</tr>
<tr>
<td></td>
<td>Antisense 5'-CCAGTGCCAATGATCAGG-3'</td>
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</tr>
<tr>
<td>HK2</td>
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<td>NM_013820</td>
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<td></td>
<td>Antisense 5'-CTCTGGTTATGCATCTACGC-3'</td>
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<td>Antisense 5'-AGAACACAGCATTGATACCC-3'</td>
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<td>NM_011400</td>
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<td>Antisense 5'-AGAACACAGCATTGATACCC-3'</td>
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<td>NM_172665</td>
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<tr>
<td></td>
<td>Antisense 5'-CCGCCTAGCCTTCATAGC-3'</td>
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**Immunoblot Analysis**

Tissue samples from the renal cortex were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 7.4) containing protease inhibitor cocktail (1:100), 1 mM sodium orthovanadate, and 10 mM sodium fluoride (Sigma Aldrich). Total protein content was determined by BCA protein assay. Equal amounts of protein (50 – 100 µg) were resolved on 4 – 15% Mini-PROTEAN TGX SDS-PAGE gels (Bio-Rad). Proteins were then transferred to nitrocellulose membranes and blocked in 2.5% BSA for 1 hr. Membranes were incubated with primary antibody overnight at 4°C. Primary antibodies included HK1, HK2, PFKP, PKM1/2, PDH, phospho-Akt (Ser 473), Total Akt, phospho-p42/p44 MAPK (Thr202/Tyr204; p-ERK1/2), and Total p42/p44 MAPK (all from Cell Signaling Technology, Danvers, MA; 1:1,000 dilution), VDAC/Porin (Abcam, Cambridge, MA; 1:1,000), and β-Actin (Santa Cruz Biotechnology, Dallas, TX; 1:10,000). Membranes were then incubated in horseradish peroxidase (HRP)-conjugated secondary for 1 hr at room temperature. Proteins were visualized using enhanced chemiluminescence reagents (Thermo Scientific, Waltham, MA) and a GE ImageQuant LAS4000 digital imaging system. NIH ImageJ Software (v 1.46) was used to measure optical density of immunoblots.

**Mitochondrial Fractionation**

Mitochondria were isolated from whole kidneys by differential centrifugation as previously described (332). Whole kidneys were washed and finely minced in isolation buffer (250 mM sucrose, 1 mM EGTA, 10 mM HEPES, 1 mg/ml fatty acid free BSA, pH 7.4). After mincing, tissue was homogenized using a grooved Teflon homogenizer.
Nuclei and cellular debris were pelleted by centrifugation at 1,000g for 10 mins. The remaining supernatant was centrifuged at 10,000g for 5 mins. to produce a mitochondrial pellet. The mitochondrial fraction was washed once in isolation buffer and re-pelleted by centrifugation. The resulting mitochondrial pellet was resuspended in RIPA buffer and mitochondrial localization of HK1 and HK2 was determined by immunoblot analysis as indicated above.

**Assessment of Renal Cortical Glucose, Pyruvate, and Lactate Levels**

Levels of glucose, pyruvate, and lactate in the renal cortex were determined using enzymatic assay kits (Glucose Fluorometric Assay Kit, Pyruvate Fluorometric Assay Kit, and Lactate Fluorometric Assay Kit; BioVision Incorporated). Renal cortical tissue was extracted in ice-cold Glucose, Pyruvate, or Lactate Assay Buffer and centrifuged at 10,000g for 10 min to remove cellular debris. A portion of the sample was used for determination of protein content by the BCA protein assay. The remaining sample was deproteinized by centrifugation through 10 kD cutoff centrifugal filters (VWR) to prevent enzymatic degradation of metabolites. After deproteinization, levels of pyruvate and lactate were determined according to the manufacturer’s instructions using an Infinite 200 PRO fluorometer at 535/587 nm (excitation/emission).

**Determination of Glycogen Content**

Renal cortical glycogen content was determined using a Glycogen Fluorometric Assay Kit (BioVision Incorporated). Tissue samples were extracted in ice-cold 1X PBS and immediately boiled to inactivate enzymes responsible for glycogen degradation. Samples were centrifuged at 14,000g for 15 mins. to clear debris and then assayed for
glycogen content based on the manufacturer’s protocol using an Infinite 200 PRO fluorometer at 535/587 nm (excitation/emission).

**Glucose-6-phosphate Dehydrogenase Activity Assay**

Glucose-6-phosphate dehydrogenase (G6PDH) activity was determined using the Glucose-6-phosphate Dehydrogenase Activity Colorimetric Assay kit (Biovision Inc.). Renal cortical tissues were homogenized in ice-cold phosphate-buffered saline (PBS) and centrifuged at 10,000g to remove any residual cellular debris. Twenty-five (25) µL samples were loaded in duplicate into a 96-well plate and brought to 50 µL in the G6PDH assay buffer provided. A portion of the homogenized sample was aliquoted for protein estimation by the BCA assay. G6PDH activity was then determined based on the manufacturer’s instructions using an Infinite M200 PRO spectrophotometer in kinetic mode (Tecan Systems). Data are shown as milliunits of G6PDH activity per gram of protein.

**Statistical Analysis**

Data are presented as mean ± S.E.M. Statistical comparisons between two groups were performed using an unpaired, two-tailed t-test. For multiple comparisons, a one-way analysis of variance (ANOVA) was performed followed by Tukey’s post-hoc test. A p-value < 0.05 was considered statistically significant. All statistical analysis was completed using GraphPad Prism software.
RESULTS

LPS Induces Specific Increases in Renal Cortical Hexokinase Activity

Because suppression of both mitochondrial function and gene expression has been reported in multiple animal models of AKI, including sepsis-induced AKI, we hypothesized that glycolytic metabolism would increase in the renal cortex following LPS exposure (162, 274). We first measured activities of the three major rate-limiting enzymes in the glycolytic pathway, hexokinase (HK), phosphofructokinase (PFK), and pyruvate kinase (PK), in the renal cortex at 3, 6, and 18 hr after LPS exposure. Renal cortical hexokinase activity increased approximately 2-fold as early as 3 hr post-LPS and remained elevated for at least 18 hr (Figure 3-1A). However, PFK and PK activities were unchanged through 18 hr (Figure 3-1C,D). BUN progressively increased from approximately 32 mg/dl in vehicle-treated mice to 61 mg/dl and 122 mg/dl at 3 hr and 18 hr after LPS administration, respectively (Figure 3-1B). Taken together, these findings indicate that HK is specifically and rapidly activated in the renal cortex of mice subjected to LPS-induced acute kidney injury.
Figure 3-1: HK activation and renal dysfunction after LPS exposure. Male C57BL/6 mice were injected with lipopolysaccharide (10 mg/kg, i.p.) or saline vehicle and euthanized at 3, 6, and 18 hr. Renal cortical tissue was isolated and activities of hexokinase [HK] (n = 3 – 11) (A), PFK [PFK] (n = 3 – 6) (C), and PK [PK] (n = 3 – 6) (D) were measured using standard biochemical assays. Decline in renal function at 3 and 18 hr post-LPS was confirmed by measuring BUN (n = 5) (B). Data are expressed as mean ± S.E.M. for each group. Different superscripts above bars indicate statistically significant differences (p < 0.05).
LPS-Induced HK Activation Occurs Independently of Changes in Isoform Expression

Because HK activity in the renal cortex increased following LPS exposure, we determined the effects of LPS on the expression of HK isoforms at both the mRNA and protein level via qPCR and immunoblotting at 3 and 18 hr post-LPS. HK1 mRNA levels did not change in response to LPS exposure at any of the time. HK2 gene expression increased ~33-fold at 3 hr in LPS-treated mice but returned to baseline levels by 18 hr post-LPS. LPS administration also resulted in increases in HK3 mRNA levels at both the 3 hr (approximately 2-fold) and 18 hr (approximately 5-fold) (Figure 3-2A,B). Although increased gene expression of HK2 and HK3 was observed in LPS-treated mice, immunoblot analysis revealed no changes in protein levels of HK1 or HK2 at 18 hr after LPS administration (Figure 3-2C,D). These data demonstrate that rapid HK activation in the renal cortex of LPS-treated mice is independent of changes in HK isoform expression, suggesting that post-translational modification by phosphorylation, which has been previously reported, may be responsible for this effect (333).
Figure 3-2: Systemic LPS exposure does not alter HK isoform expression at the protein level. RNA and protein were isolated from the renal cortex of mice treated with LPS or vehicle control. Relative expression of HK1, HK2, and HK3 at the transcript level at 3 hr (A) and 18 hr (B) after LPS administration was determined by RT-qPCR (n = 4 – 6). Immunoblot analysis revealed no changes in protein expression of HK1 or HK2 isoforms at the 18 hr time point (n = 9) (C,D). Data were normalized to fold-change in expression compared to vehicle controls and are presented as mean ± S.E.M. for each group.
LPS Exposure Does Not Alter Mitochondrial Localization of HK1 or HK2

Both HK1 and HK2 isoforms possess mitochondrial binding domains which allow for their association with the outer mitochondrial membrane (OMM) in association with the voltage-dependent anion channel (VDAC) (334, 335). Because mitochondrial binding of HK has been demonstrated in the kidney and may result in increased HK activity, we determined the effects of LPS administration on HK1 and HK2 localization to the OMM. Mitochondria were isolated by differential centrifugation from kidneys of vehicle- and LPS-treated mice at 18 h. Protein levels of HK1 and HK2 in the mitochondrial fraction were determined by immunoblot analysis using VDAC as a mitochondrial loading control. No differences were noted in the mitochondrial localization of either HK1 or HK2 following LPS administration (Figure 3-3A,C,D). Purity of mitochondrial fractions was confirmed by immunoblotting for VDAC, α-Tubulin, and Lamin B1 (Figure 3-3B). These findings demonstrate that LPS-induced increases renal cortical HK activity cannot be attributed to changes in enzyme localization.
Figure 3-3: Effects of LPS on mitochondrial localization of HK1 and HK2. Whole kidneys were harvested from mice treated as described above and the mitochondrial fraction was isolated by differential centrifugation. Immunoblot analysis was performed to examine changes in mitochondrial localization of HK isoforms. Mitochondrial VDAC was used as a loading control. Representative immunoblots demonstrating mitochondrial-associated HK1 or HK2 isoforms 18 hr after LPS exposure (A). Purity of mitochondrial (M) and cytosolic (C) fractions was assessed by immunoblotting for VDAC (mitochondrial marker), α-Tubulin (cytosolic marker), and Lamin B1 (nuclear marker) (B). Densitometric analysis revealed no change in mitochondrial localization of HK1 or HK2 (C, D) (n = 4).
LPS Administration Does Not Alter Expression of Other Glycolysis-Related Enzymes in the Renal Cortex

Because we hypothesized that glycolytic metabolism would increase in the renal cortex in LPS-induced AKI to support cellular function, we also examined expression of a number of glycolysis-related enzymes in the renal cortex of mice exposed to LPS. In particular, genes and proteins involved in cellular glucose uptake (GLUT1/Slc2a1), glycolytic metabolism (PFKP, PFKL, PFKM, PGK1, PKM), pyruvate conversion into lactate (LDHA), and pyruvate entry into the TCA cycle (PDK1, PDH). No increases were noted in gene expression of glycolysis-related enzymes at 18 hr post-LPS (Figure 3-4A). Transcript levels of the glycolytic enzymes phosphofructokinase, muscle type (PFKM) and phosphoglycerate kinase 1 (PGK1) were significantly decreased (~40%) following LPS exposure (Figure 3-4A). In addition, no changes in protein expression of glycolysis-related enzymes were observed in mice treated with LPS (Figure 3-4B,C). Taken together, these data indicate that LPS exposure does not result in changes in gene or protein expression to facilitate glycolytic metabolism in the renal cortex.
Figure 3-4: LPS administration does not increase expression of other glycolysis-related enzymes in the renal cortex. RNA and protein were harvested from the renal cortex of mice treated with saline vehicle or LPS as described above. Expression of GLUT1, PFKL, PFKM, PGK1, PKM, LDHA, and PDK1 mRNAs (A) were determined by RT-qPCR analysis (n = 6). Representative immunoblots demonstrating no change in expression of PFKP, PKM1/2, PDH, or LDHA (B, C) 18 hr after systemic LPS administration (n = 9). Data were normalized change in expression relative to control and are expressed as mean ± S.E.M. for each group.
LPS-induced HK Activation Does Not Enhance Glycolytic Flux in the Renal Cortex

To determine whether LPS-induced activation of HK was sufficient to increase glycolytic metabolism, we next measured end products of glycolysis. Mice were treated with LPS or vehicle control and kidneys were harvested at 3 hr and 18 hr after injection to determine pyruvate and lactate in renal cortical tissue. LPS exposure resulted in significant decreases in pyruvate content (~50%) at both the 3 and 18 hr time points (Figure 3-5A). Cortical lactate levels were also significantly reduced to approximately 40% of control levels at 3 hr post-LPS. However, lactate content returned to baseline levels by the 18 hr time-point (Figure 3-5B). The changes observed in both pyruvate and lactate levels did not result in an increase in the lactate-to-pyruvate ratio at 18 hr post-LPS (Figure 3-5C). These findings demonstrate that, although LPS exposure results in activation of HK in the renal cortex, increased HK activity is not adequate to increase flux of glucose through the glycolytic pathway.
Figure 3-5: LPS-induced HK activation does not increase renal cortical glycolytic flux. Lactate and pyruvate were isolated from the renal cortices of mice treated with either saline or LPS and measured by standard biochemical assays. Systemic LPS exposure resulted in a decrease in renal cortical pyruvate levels at both 3 and 18 hr time points (A). Lactate levels were lower at 3 hr post-LPS but returned to baseline levels by 18 hr (B). No significant changes were noted in the lactate:pyruvate ratio, although there was trend toward an increase at 18 hr after LPS (C). Data are presented as mean ± S.E.M. for each group (n = 4 – 5).
**Increased HK Activity after LPS Exposure Does Not Promote Glycogen Synthesis**

Because increased HK activity due to LPS exposure in the renal cortex did not result in enhanced glycolytic flux, we sought to determine whether HK activation in this setting has an alternate physiological role. Conversion of glucose to glucose-6-phosphate by hexokinase has been described as a rate-limiting step in glycogen synthesis in a variety of model systems including rodent skeletal muscle (336-338). Thus, we measured glucose and glycogen levels following LPS exposure to determine whether HK activation in this model may result in glycogenesis. Glucose levels in the renal cortex were reduced 3 hr after LPS administration (~30% reduction) and remained lower at 18 (Figure 3-6A). Measurement of glycogen content revealed no change in LPS-treated mice at 3 hr and a ~60% reduction at 18 hr (Figure 3-6B). Our results demonstrate that LPS-induced HK activation does not stimulate glycogen synthesis.

**HK Activation in the Renal Cortex is Associated with Increased Pentose Phosphate Pathway Activity**

We next questioned whether renal cortical HK activation following systemic LPS exposure might facilitate glucose flux through the pentose phosphate pathway (PPP) to produce NADPH for use as a reducing agent in glutathione regeneration. Since endotoxin exposure is known to rapidly up-regulate oxidative stress in the proximal tubule, we anticipated that any changes observed in NADPH and/or glutathione levels would be difficult to interpret as markers of PPP activity in this model (149). Instead, we measured activity of glucose-6-phosphate dehydrogenase (G6PDH) activity, the rate-limiting step of the PPP as a marker of glucose metabolism through this pathway (339). Renal cortical G6PDH activity was increased approximately 3.5 fold by 3 hr after LPS
administration (Figure 3-6C). These data indicate that HK activation following endotoxin-induced AKI is associated with an increase in glucose flux through the PPP.
Figure 3-6: LPS-induced increases in HK activity do not promote glycogen synthesis in the renal cortex but are associated with increased G6PDH activity. Glucose (A) and glycogen content (B), and G6PDH activity (C) were evaluated in the renal cortex at different time points following systemic LPS administration in mice and were normalized to wet tissue weight (glucose, glycogen) or protein content (G6PDH activity). Data are shown as mean ± S.E.M. for each group (n = 5 – 6). Different superscripts above bars indicate statistically significant differences (p < 0.05).
LPS-induced HK Activity in the Renal Cortex is Mediated by EGFR and Akt

To understand the mechanism by which LPS exposure increases HK activation, we investigated the role of the epidermal growth factor receptor (EGFR). Earlier studies from our laboratory and others have shown that EGFR ligands (EGF, HB-EGF) stimulate hexokinase activity in renal proximal tubule cells (RPTC) (340-343). LPS is also known to induce EGFR transactivation in a variety of cell types including epithelial cells and renal medullary collecting duct cells (344-348). One hour prior to LPS administration, mice were treated with the EGFR inhibitor gefitinib (100 mg/kg) or vehicle control and kidneys were harvested 3 hr post-LPS. This dose has previously been reported to inhibit EGFR signaling following AKI in mice (330). LPS-induced renal cortical HK activity was completely blocked by gefitinib, suggesting an essential role for EGFR in this process (Figure 3-7A).

Because EGFR is known to activate PI3K/Akt signaling, we determined whether Akt is responsible for EGFR-mediated HK Activation. To demonstrate Akt activation following systemic LPS exposure, mice were treated with LPS or vehicle control and kidneys harvested 1 hr later for immunoblot analysis of phosphorylated and total Akt. LPS exposure resulted in a ~1.5-fold increase in expression of phospho-Akt without a change in total Akt (Figure 3-7C,D). Based on these findings, another group of mice were pretreated with the pan-Akt inhibitor MK-2206 (100 mg/kg) 1 hr prior to LPS injection and kidneys harvested 3 hr post-LPS. MK-2206 is an allosteric inhibitor of Akt and exhibits high selectivity over other kinases because it requires the Pleckstrin homology domain for its activity (349). LPS-induced HK activity was attenuated following Akt inhibition by MK-2206 (Figure 3-7B). These data indicate that the
EGFR/PI3K/Akt signaling axis is responsible for activation of renal cortical HK in LPS-induced AKI.
Figure 3-7: HK activation following LPS exposure is mediated through EGFR/Akt signaling. One hour prior to LPS administration, as described above, mice were treated with a single dose of either gefitinib (100 mg/kg, i.p.) (A), MK-2206 (100 mg/kg) (B) or vehicle control. HK activity was determined in the renal cortex at 3 hr after systemic LPS administration (n = 5 – 12). Immunoblot analysis revealed increased activation/phosphorylation of Akt 1 hr after LPS exposure (C, D). Levels of phosphorylated Akt were normalized to total protein expression and presented as fold change versus control animals (n = 4). Data are expressed as mean ± S.E.M for each experimental group. Superscripts above bars indicate statistically significant differences between groups (p < 0.05).
DISCUSSION

Mitochondrial dysfunction is an important contributor to the pathophysiology of multiple forms of AKI (173, 274). Although histological changes in sepsis-induced AKI are limited, post-mortem and experimental studies have demonstrated mitochondrial swelling in the relative absence of overt tubular cell death (162, 301, 350). On the molecular level, sepsis-induced AKI results in suppression of mitochondrial genes which is associated with functional decline (162). Patil et al. recently demonstrated reduced renal electron transport chain complex I and complex II/III activity occurring as early as 6 h after cecal ligation and puncture in mice (324). Furthermore, a reduction of renal ATP levels has been observed in rodent models of sepsis-induced AKI which correlated well with renal dysfunction (265, 324, 351). Taken together, these data provide strong evidence that mitochondrial dysfunction plays a central role in the pathophysiology of sepsis-induced AKI. To this point, therapies that restore mitochondrial function and/or reduce oxidative stress have proven beneficial in experimental models (135, 324).

We hypothesized that renal proximal tubule cells might respond to mitochondrial dysfunction by increasing glycolytic flux to generate ATP. Although the proximal tubule has been viewed as having very low glycolytic capacity, studies have revealed that glycolysis may be induced in RPTCs in response to a cellular insult (258, 325, 352). Dickman and Mandel demonstrated that proximal tubules in vitro increase glycolytic metabolism in response to hypoxia, mitochondrial uncoupling, and inhibition of complex I of the electron transport chain (ETC) (293). Inhibition of the Na\(^+/K^+\)-ATPase and glycolysis in proximal tubules following complex I inhibition indicated that glycolytic induction serves to promote cellular function and survival (293). In vivo data also reveal
that anaerobic glycolysis is increased in the renal cortex during ischemia but rapidly returns to baseline levels after reperfusion (327). These data provide strong evidence that glycolytic metabolism can be activated for energy production in the proximal tubule.

We performed a comprehensive evaluation of the glycolytic pathway to examine activities of the major regulatory enzymes, mRNA and protein levels of key components of glucose metabolism, and end products of glycolysis (pyruvate and lactate). We observed an increase in renal cortical HK activity as early as 3 h after LPS exposure in mice that was maintained for at least 18 h without an up regulation of any other glycolytic enzymes nor an increase in flux through the glycolytic pathway as measured by renal cortical pyruvate and lactate levels.

To our knowledge, this is the first demonstration of rapid, specific activation of hexokinase in the renal cortex after systemic LPS administration. Three high-affinity HK isoforms (Km values in the micromolar range) are expressed in the mammalian kidney (HK1, HK2, and HK3) (335, 353). HK1 is constitutively expressed/active and accounts for approximately 70% of total renal HK activity under normal conditions. In contrast, HK2 appears to be regulated in response to a variety of stimuli (354-356). Little is known about the regulation of HK3 expression/activity. Together, HK2 and HK3 account for the remainder of renal HK activity (~30%) (342).

The experimental method used to measure hexokinase activity in this study does not distinguish which HK isoform is activated following systemic LPS exposure. Therefore, we measured HK isoforms at both the mRNA and protein levels in the renal cortex. Although early changes were noted in mRNA levels of HK2, no changes were seen in HK1 or HK2 isoforms at the protein level following LPS exposure. HK3 mRNA
expression increased at both 3 and 18 h time points. We were not able to measure HK3 protein due to the lack of a validated antibody with reactivity to mouse HK3. An anabolic role (PPP or glycogen synthesis) has been proposed for HK2 and HK3 since these isoforms are subjected to inhibition by glucose-6-phosphate and P_i, whereas HK1 is thought to mainly facilitate glycolytic metabolism (357).

Given that HK isoform expression did not change after LPS exposure, we investigated whether HK activation might be attributed to increased mitochondrial localization. Recent evidence revealed that mitochondrial localization of HK isoforms may serve a number of physiological roles including direct coupling of glucose phosphorylation to the intramitochondrial ATP pool, reducing feedback inhibition by G-6-P, and preventing initiation of apoptosis by pro-apoptotic members of the Bcl-2 family (334, 358-361). However, we did not observe any changes in mitochondrial localization of either HK1 or HK2 in response to systemic LPS exposure. In contrast to HK1 and HK2, HK3 does not possess a mitochondrial localization sequence and is thought to be predominantly peri-nuclear in location (362). Taken together, these data suggest that renal cortical HK activation in endotoxin-induced AKI is likely due to a post-translational modification (i.e. phosphorylation) which regulates HK activity (333, 363).

Although HK activity rapidly increased and was sustained up to 18 h after LPS exposure, we observed no changes in pyruvate and lactate content indicative of increased glycolysis. For the purposes of this study, glycolytic flux was defined as the conversion of glucose to pyruvate as well as downstream generation of lactate. Lactate:pyruvate ratios were compared under different experimental conditions as an indicator of anaerobic glycolysis. Under anaerobic conditions, LDH would be expected to convert
pyruvate to lactate, resulting in increased lactate with a corresponding equimolar reduction in pyruvate. Pyruvate content was decreased approximately 2-fold at both 3 and 18 h, whereas lactate levels trended toward a decrease at 3 h but returned to baseline levels by 18 h post-LPS. These findings are in agreement with recent reports of renal cortical pyruvate depletion in both I/R and glycerol-induced AKI in mice (327). The same study also reported glycolytic induction in the renal cortex only occurs during the ischemic period and is reversed after reperfusion (327). However, it should be noted that some of the analyses performed here, including pyruvate and lactate measurements, are not sufficiently powered to detect small changes between groups. Thus, it is possible that there are minimal changes in glycolytic flux in the renal cortex up to 18 h after LPS exposure that we were not able to distinguish.

Using a combination of micro-ultrasound and blood oxygen level-dependent MRI, Tran et al. demonstrated that although renal perfusion is markedly decreased in LPS-treated mice, there is minimal change in tissue oxygenation (162). Thus, the lack of renal hypoxia may explain why we did not detect increases in lactate following systemic LPS administration. However, the importance of hypoperfusion/hypoxia in renal pathophysiology remains unclear. Changes in renal blood flow following endotoxin exposure are local and dynamic at the dose used in this study. Wu et al. reported the disruption in cortical peritubular capillary flow at 10 h after LPS administration in mice. Peritubular capillary dysfunction observed in this study was correlated with an increase in tubular NAD(P)H autofluorescence, suggesting that local hypoxia may contribute to cellular injury after LPS (124). In light of these findings, it is important to note that our analyses are limited to only two time points (3 and 18 h) after endotoxin exposure.
Our analysis of glycolytic enzyme activities was restricted to the major rate-limiting enzymes in the glycolysis pathway (HK, PFK, PK). In addition, we measured mRNA and protein expression of other enzymes involved in glucose metabolism in the renal cortex following LPS exposure (e.g. GLUT1, PGK, LDHA, PDH, PDK1). There was no evidence of increased mRNA or protein expression of any of these components, indicating that LPS-induced AKI does not result in early activation of a transcriptional program to facilitate glycolysis in the renal cortex. We cannot rule out the possibility that activities of one or more of these enzymes are increased after endotoxin exposure without an associated increase in expression. However, the data presented here demonstrating minimal changes in the lactate:pyruvate ratio, specific HK activation and an absence of increases in expression of other glycolytic components provide considerable evidence indicating that glycolysis is minimally activated at early time points (3 and 18 h) following endotoxin-induced AKI. Further studies are necessary to determine whether glycolytic metabolism might be activated in the chronic phase.

In addition to glycolysis, G-6-P has multiple fates that include flux through the pentose phosphate pathway (PPP) to produce NADPH and nucleotide/amino acid precursors, glycogen synthesis, and the hexosamine biosynthetic pathway. Zager et al. reported that pyruvate depletion in the renal cortex following ischemic and nephrotoxic forms of AKI was partially attributed to an increase in gluconeogenesis and glycogen synthesis (327). Interestingly, generation of glucose-6-phosphate by HK is thought to be a rate-limiting step in glycogen synthesis in a number of tissues and has been associated with glycogen supercompensation in rat skeletal muscle (336-338, 364). In contrast to changes observed in other mouse models of AKI, we observed a decrease in both renal
cortical glucose and glycogen content. Together, these data indicate that gluconeogenesis and glycogen content are not up-regulated in the septic kidney. These findings are consistent with the reduction in renal glucose and gluconeogenic enzymes in the kidney after endotoxin administration in rats (365, 366).

Glucose-6-phosphate generated following HK activation in the renal cortex may also be utilized by the pentose phosphate pathway (PPP). Through the PPP, G-6-P is further metabolized by the rate-limiting enzyme glucose-6-phosphate dehydrogenase (G6PDH) to generate NADPH and biosynthetic precursors (335). Reduced NADPH levels in G6PDH deficient mice were associated with increased renal oxidative stress, inflammation, and dysfunction indicating an important role for NADPH in antioxidant defense in the kidney (367). Given that oxidative stress rapidly develops in the proximal tubule following systemic LPS exposure, these findings suggest an alternate hypothesis that HK activation may contribute to PPP activity (149). Interestingly, G6PDH activity was increased (~3.5 fold) in the renal cortex of mice exposed to LPS. These findings provide strong evidence that glucose-6-phosphate formed as a result of hexokinase activation is selectively metabolized via the PPP. The importance of the PPP in NADPH production and thus anti-oxidant defense following AKI has received little attention.

Our laboratory previously demonstrated that activation of EGFR signaling in primary rabbit proximal tubule cells lead to a rapid increase in both glycolysis and PPP activity (340). Further studies demonstrated that EGFR ligands are capable of increasing HK activity in multiple renal cell types including proximal tubule cells and mesangial cells (335, 343). A link between LPS and EGFR signaling has also been established in
studies demonstrating that TLR4 rapidly transactivates the EGFR via protease mediated EGFR ligand shedding (347, 348). In addition, EGFR activation can contribute to the pathology and recovery of multiple forms of AKI (368). Results presented here demonstrate that EGFR signaling is required for HK activation in LPS-induced AKI.

We next focused on Akt as a downstream mediator of EGFR-induced HK activation in LPS-treated mice based on extensive evidence indicating that Akt modulates both HK activity and localization through phosphorylation (363, 369). Site-directed mutagenesis has further revealed that HK2 is phosphorylated by Akt on threonine 473 within an Akt consensus sequence in cardiomyocytes in vitro (333). We examined signaling changes in the renal cortex that might precede HK activation and noted increased activation of Akt as early as 1 h after LPS administration which is consistent with other reports (370, 371). Inhibition of Akt by MK-2206 attenuated increases in HK activity following LPS exposure. Taken together, these findings provide strong evidence that the EGFR/Akt signaling pathway is responsible for LPS-mediated hexokinase activation in the renal cortex.

In conclusion, the present study reports the novel finding of rapid activation of renal cortical HK activity in a mouse model of sepsis-induced AKI. HK was activated following LPS-induced AKI via an EGFR/Akt-dependent signaling mechanism. Surprisingly, the increase in HK activity observed in this model was associated with minimal changes in glycolysis and glycogen synthesis and was strongly linked with an increase in G6PDH, the rate limiting enzyme in the PPP. The production of reducing equivalents (i.e. NADPH) may be key in preserving oxidant defense pathways.
Chapter 4:

Delayed Administration of the MEK/ERK Inhibitor GSK1120212 Attenuates Systemic Inflammatory Responses and Multi-Organ Injury Following Cecal Ligation and Puncture in Mice

ABSTRACT

Sepsis-induced organ injury is a consequence of both direct and indirect effects of innate immune system activation by infectious organisms. The MEK/ERK signaling pathway has been identified as an essential target of innate immunity necessary for mediating pro-inflammatory responses in the setting of sepsis. We previously demonstrated that a potent and specific inhibitor of MEK1/2, GSK1120212, prevents endotoxin-induced renal injury in mice, indicating that the MEK/ERK signaling cascade may represent a novel therapeutic target in sepsis-induced AKI and organ dysfunction.

We assessed efficacy of GSK1120212 in a more clinically relevant experimental model of sepsis induced by cecal ligation and puncture (CLP) in mice. GSK1120212 inhibition of MEK/ERK signaling 6 h after CLP attenuated increases in circulating pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and GM-CSF) and hypothermia at 18 h. GSK1120212 also attenuated multi-organ injury as determined by clinical markers serum creatinine, alanine aminotransferase, lactate dehydrogenase, and creatine kinase. At the organ level, GSK1120212 post-treatment completely restored peritubular capillary perfusion in the kidney. Restoration of microvascular perfusion in the renal cortex was associated with reduced mRNA expression of well-characterized markers of proximal tubule injury including KIM-1, NGAL, and HO-1. MEK/ERK blockade attenuated CLP-mediated up-regulation of cytokines (TNF-α, IL-1β, IL-6) in the renal cortex, indicating the protective effects on the proximal tubule occur primarily through modulation of the
pro-inflammatory response in sepsis. Taken together, these data reveal that the MEK/ERK inhibitor GSK1120212 attenuates systemic inflammation, and multi-organ damage in a clinically relevant model of sepsis, even with delayed administration. Because GSK1120212 has been used safely and effectively used in humans, we propose that this drug might represent a readily translatable approach to limit organ injury in septic patients.
INTRODUCTION

Sepsis is a leading cause of morbidity and mortality in the intensive care setting (56, 57, 59). The clinical course in septic patients is often complicated by dysfunction of multiple vital organs including the kidneys, lung, liver, and brain (372, 373). Acute kidney injury (AKI) develops in up to 60% of patients diagnosed with sepsis or septic shock, and septic AKI is associated with an extremely high risk of mortality (~70%) (298, 374, 375). Clinical management of sepsis remains limited to non-specific supportive care aimed at maintaining organ homeostasis and preventing further infection (376). Although recent initiatives to implement early, goal-directed measures have shown modest benefits in septic patients, mortality resulting from this condition remains unacceptably high (68, 377). Thus, development of novel therapeutics that directly target organ injury and dysfunction remains a top priority in sepsis research.

Organ insult in the setting of sepsis is initiated by activation of pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs), by pathogen-associated molecular patterns (PAMPs) derived from the causative organism(s). Activation of TLRs expressed on immune cells (including dendritic cells, macrophages, neutrophils, and B lymphocytes), endothelial cells, and epithelial cells leads to an overwhelming systemic response characterized by production of acute phase cytokines (TNF-α, IL-1β, and IL-6), chemokines, and other pro-inflammatory mediators (82). Development of AKI involves combined local and peripheral effects of these factors, which ultimately contribute to microvascular dysfunction and direct tubular injury in the septic kidney (155). The mitogen-activated protein kinase (MAPK) family, including ERK1/2, JNKs, and p38
MAPKs are a major downstream target of TLRs and other PRRs responsible for induction of inflammatory signaling (89, 378, 379).

ERK1/2 are serine/threonine kinases that regulate a variety of cellular processes including survival, differentiation, migration, proliferation, transcription, and metabolism via phosphorylation of a vast array of target proteins (380). Canonical ERK1/2 signaling is initiated by binding of growth factors (VEGF, IGF-1, EGF) to their respective receptor tyrosine kinases (RTKs) leading to downstream activation of the Ras/Raf/MEK/ERK pathway. However, more recent evidence indicates that other cellular signaling entities, including TLRs, also activate ERK1/2. TLR4 stimulation by lipopolysaccharide (LPS), a critical mediator of gram-negative sepsis, increases ERK1/2 activity in macrophages by engaging the tumor progression locus-2 (TPL-2)/mitogen activated protein kinase kinase-1/2 (MEK1/2)/ERK1/2 cascade. ERK1/2 activation in this context plays a critical role in mediating the immune response to PAMPs by increasing transcription and translation of pro-inflammatory cytokines (TNF-α, IL-1β) and chemokines (CXCL2) in multiple cell types (310-312, 381, 382). Despite considerable evidence linking MEK/ERK signaling to innate immunity, studies investigating this pathway as a potential therapeutic target for prevention of unchecked inflammation and organ dysfunction in the setting of sepsis are lacking.

We recently demonstrated that the FDA-approved MEK1/2 inhibitor GSK1120212 (also known as trametinib or JTP-74057) attenuates renal injury following systemic LPS exposure in mice (142). Pharmacological blockade of MEK/ERK signaling reversed endotoxin-induced increases in blood urea nitrogen (BUN) and mRNA expression of kidney injury molecule-1 (KIM-1), a marker of proximal tubule injury, at
both 3 and 18 h time points. Restoration of renal function following GSK1120212 administration was associated with decreased expression of TNF-α and IL-1β transcripts in the renal cortex, further suggesting that MEK/ERK signaling may regulate organ-specific inflammatory responses in this model.

A subsequent study by Shi-Lin et al. showed that GSK1120212 significantly inhibits LPS-induced TNF-α production in mouse primary bone marrow-derived macrophages and human peripheral blood mononuclear cells (PBMCs) (383). In addition, MEK/ERK inhibition prevented mortality resulting from co-administration of LPS and the sensitizing agent D-galactosamine in mice in association with decreased levels of circulating TNF-α (383). Since GSK1120212 has been safely used in the clinic, these findings suggest that targeted MEK/ERK inhibition may have translational potential as a novel therapy for sepsis-induced AKI and organ dysfunction in humans (384-386). It should be noted that clinical relevance of endotoxin models in rodents is limited due to a number of differences from human sepsis. Most importantly, systemic LPS exposure in mice leads to a massive, transient increase in serum pro-inflammatory cytokine levels (TNF-α, IL-1β, IL-6) whereas elevations in these cytokines are more protracted and several orders of magnitude lower in septic humans (387). In addition, therapies that have shown promise in experimental endotoxin models including inhibitors of TNF-α and IL-1 have largely failed to improve mortality and other secondary outcome measures in clinical trials for sepsis likely due in part to the differences noted above.

The purpose of the current study was to evaluate efficacy of the MEK/ERK inhibitor GSK1120212 in a clinically relevant model of sepsis induced by cecal ligation and puncture (CLP) in mice. We demonstrate here that post-treatment with GSK1120212
at 6 h following CLP attenuated the systemic inflammatory response and development of hypothermia. GSK1120212 administration also prevented development of AKI and multiple organ injury in CLP animals. In the kidney, MEK/ERK inhibition restored microvascular perfusion and reduced expression of the tubular injury markers including kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and heme oxygenase-1 (HO-1) at the mRNA level. The renoprotective effects of GSK1120212 were associated with decreased renal expression of pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6. Taken together, our data provide evidence that the FDA-approved MEK/ERK inhibitor GSK1120212 may be a viable drug to attenuate systemic inflammatory responses and organ injury in the setting of sepsis.
METHODS

Mouse Cecal Ligation and Puncture Model of Sepsis

Male C57BL/6 mice aged 40 weeks were obtained from Harlan Laboratories (Indianapolis, IN). Cecal ligation and puncture was performed as previously described (109, 159). Briefly, 1.5 cm of the cecum was isolated and ligated with a 4-0 silk suture under isoflurane anesthesia after midline laparotomy. The ligated cecum was then punctured with a 21-gauge needle and gently pressed to produce a ~1 mm column of fecal material from each puncture site. Sham animals underwent midline laparotomy followed by isolation of the cecum, but the cecum was not ligated or punctured. Mice were treated with imipenem/cilastatin (14 mg/kg) and 1.5 ml of warm saline (40 ml/kg) at 6 h post-CLP. Mice were euthanized 18 h after CLP and kidneys and serum were collected for analysis. All experiments were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal use was approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences.

Administration of GSK1120212

GSK1120212 (Selleckchem, Houston, TX) was formulated in vehicle solution containing 1% DMSO in Neobee M-5 (Spectrum Chemical, New Brunswick, NJ) (388). GSK1120212 is a specific and potent allosteric inhibitor of MEK1/2 signaling that has been used in animal models and humans (304, 305, 389, 390). Mice were treated with 1 mg/kg GSK1120212 or an equal volume of vehicle solution via intraperitoneal (i.p.) injection at 6 h after CLP.
Determination of Circulating Cytokine Levels

Measurement of serum levels of TNF-α, IL-1β, IL-6 and GM-CSF was performed using the Inflammatory Cytokine Mouse Magnetic 4-Plex Panel for the Luminex platform (Life Technologies, Grand Island, NY). Detection of cytokines was performed using a Bio-Rad Bio-Plex 200 system (Bio-Rad Laboratories, Hercules, CA) and quantified using a standard curve and protein standards supplied in the kit. For values that fell below the lower limit of detection of the assay in sham animals, values were recorded as the lower limit (TNF-α – 22.4 pg/ml; IL-1β – 28.1 pg/ml; IL-6 – 9.8 pg/ml; GM-CSF – 16.06 pg/ml).

Intravital Microscopy and Assessment of Peritubular Capillary Perfusion

To assess changes in renal microcirculation after CLP, intravital video microscopy was performed as previously described (109, 135). Mice were anesthetized with isoflurane and FITC-labeled dextran (2 μmol/kg in 3 ml/kg normal saline) was injected via the penile vein to visualize microvascular flow. After 10 minutes, the left kidney was exposed by a flank incision and placed on a glass stage above an inverted Zeiss Axiovert 200M fluorescent microscope with an Axiocam HSm camera (Zeiss, Jena, Germany). Ten second videos were acquired at ~30 frames per second from five randomly selected, non-overlapping fields for each animal. Core body temperature was maintained at 35-37°C with a heating lamp. Roughly 150 capillaries from each animal were selected for analysis and categorized into three categories based on the degree of perfusion as follows: “continuous flow” where red blood cell movement was uninterrupted throughout the video; “intermittent flow” where red blood cell movement stopped or reversed during the course of the video; or “no flow” where no red blood cell
movement was observed throughout the video. Data are shown as percentage (mean ± S.E.M.) of vessels in each category.

**Serum Chemistries**

Blood urea nitrogen (BUN) was measured using the QuantiChrom Urea Assay kit as directed by the manufacturer (BioAssay Systems, Hayward, CA). Serum creatinine (SCr) was assessed using the Creatinine Enzymatic Reagent Set based on the provided protocol (Pointe Scientific, Canton, MI). Alanine aminotransferase (ALT), creatine kinase (CK), and lactate dehydrogenase (LDH) were measured using standard biochemical assay kits from BioVision (Milpitas, CA) as directed.

**Quantitative Real-Time PCR Analysis of Gene Expression**

Total RNA was extracted from renal cortex tissue samples using the TRIzol reagent (Life Technologies). One microgram of total RNA was reversed transcribed into a cDNA library using the iScript Advanced cDNA Synthesis Kit for quantitative real-time polymerase chain reaction (qRT-PCR) based on the manufacturer’s protocol (Bio-Rad). qRT-PCR was then performed with the synthesized cDNA using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Relative mRNA content for all genes of interest was calculated by the $2^{\Delta\Delta Ct}$ method as previously described using β-actin as a reference for normalization (306). Primer sequences used for qRT-PCR were as follows:
<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse ICAM-1</td>
<td>Sense: 5'-CACGTGCTGTATGGTCCTCG-3'</td>
<td>NM_010493</td>
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<tr>
<td></td>
<td>Antisense: 5'-TGGAGATGGGTCCCCCAG-3'</td>
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<td>Mouse E-Selectin</td>
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Immunoblot Analysis

Protein was isolated from the renal cortex in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, pH 7.4) containing protease inhibitor cocktail (1:100), 1 mM sodium fluoride, and 1 mM sodium orthovanadate (Sigma-Aldrich, St. Louis, MO). Protein quantification was performed using the BCA protein assay. Equal quantities of protein were loaded onto 4-15% SDS-PAGE gels (Bio-Rad) and resolved by gel electrophoresis. Primary antibodies used for this study included phospho-ERK1/2 (Thr202/Tyr204, 1:1000) and total ERK1/2 (1:1000, Cell Signaling Technology, Danvers, MA), and β-Actin (1:1000, Santa Cruz Biotechnology, Dallas TX). Proteins of interest were imaged using enhanced chemiluminescence (ECL) reagents and the GE ImageQuant LAS4000 digital imaging system (GE Life Sciences, Pittsburgh, PA). Optical densitometry was performed using NIH ImageJ software (version 1.46).

Statistical Analysis

All data are presented as mean ± S.E.M. A one-way analysis of variance (ANOVA) followed by Neuman-Keuls post hoc test was performed for statistical comparisons. A P value less than 0.05 was considered statistically significant. All statistical tests were completed using GraphPad Prism software (GraphPad Software, San Diego, CA).
RESULTS

GSK1120212 Reverses CLP-induced Systemic Inflammatory Cytokine Production and Hypothermia

Because MEK/ERK signaling is thought to be necessary for generation of pro-inflammatory cytokines in response to innate immune system activation in sepsis, we first determined effects of GSK1120212 on the systemic inflammatory response following CLP in mice using the Luminex platform to measure serum TNF-α, IL-1β, IL-6, and GM-CSF (383). Serum levels of TNF-α were below the lower limit of detection (22.4 pg/ml) in sham animals, but rose after CLP (~400 pg/ml). Interestingly, post-treatment with GSK1120212 at 6 h after CLP induction significantly attenuated (~50%) the increase in TNF-α (Figure 4-1A). In a similar manner, serum levels of IL-1β, IL-6, and GM-CSF were elevated in mice subjected to CLP. Delayed MEK/ERK inhibition by GSK1120212 partially reduced these circulating pro-inflammatory cytokines at 18 h post-CLP (Figure 4-1B,C,D).

In addition, we measured core body temperature as a secondary index of the systemic response. Development of hypothermia is strongly correlated with physiological dysfunction and mortality in the CLP model (391, 392). At 6 hours after CLP, both CLP + vehicle and CLP + GSK1120212 animals had markedly reduced core body temperatures (~7°C decrease) in comparison to vehicle-treated sham controls (Figure 4-2). GSK1120212 did not have a significant effect on body temperature at 18 h in sham animals. However, post-treatment with this drug completely attenuated CLP-induced hypothermia (Figure 4-2). Taken together, these data indicate that GSK1120212 prevents the early inflammatory response following CLP in mice.
Figure 4-1: Delayed MEK/ERK inhibition partially attenuates pro-inflammatory cytokine production after CLP. Male C57BL/6 mice were treated with the MEK/ERK inhibitor GSK1120212 (1 mg/kg, i.p.) at 6 h post-CLP or sham surgery and euthanized 18 hr after CLP. Serum was collected and circulating levels of the pro-inflammatory cytokines TNF-α (A), IL-1β (B), IL-6 (C), and GM-CSF (D) were determined using a multiplex Luminex assay (n ≥ 6 per group). For animals that fell below the lower limit of detection of the assay in sham animals, values were recorded as equal to the lower limit (TNF-α – 22.4 pg/ml; IL-1β – 28.1 pg/ml; IL-6 – 9.8 pg/ml; GM-CSF – 16.06 pg/ml). Data are expressed as mean ± S.E.M. for each group. Different superscripts above bars indicate statistically significant differences (p < 0.05).
Figure 4-2: GSK1120212 post-treatment reverses CLP-induced hypothermia. Core body temperature in CLP mice receiving either GSK1120212 (1 mg/kg, i.p.) or the appropriate vehicle was monitored via a rectal temperature probe. Data are presented as mean ± S.E.M. for each group (n ≥ 6 per group). *p < 0.05 vs. time-point sham control; #p < 0.05 vs. CLP + vehicle at 18 h.
GSK1120212 Post-treatment Partially Attenuates AKI and Multiple Organ Injury After CLP

Given that MEK/ERK inhibition effectively blocked the systemic inflammatory response, we assessed the effects of GSK1120212 on organ/cellular damage following CLP. SCr and BUN, used as measures of renal dysfunction, increased 2-fold and 4-fold, respectively 18 h post-CLP when compared to sham controls (Figure 4-3A,B). Post-treatment with GSK1120212 at 6 h completely reversed the CLP-induced rise in SCr, but had no effect on BUN (Figure 4-3A,B). In addition, we measured serum markers of hepatic (ALT), skeletal muscle/myocardial (CK), and general cellular damage (LDH) to further evaluate the efficacy of GSK1120212 in sepsis. CLP resulted in elevations in ALT (~5.4-fold), CK (~6.7-fold), and LDH (~4-fold) at 18 h (Figure 4-3C,D,E). These findings are consistent with previous reports of multi-organ injury following CLP in mice (104, 159). Importantly, delayed GSK1120212 administration partially attenuated increases in hepatic (ALT), skeletal muscle/myocardial (CK), and cellular damage (LDH) after CLP (Figure 4-3C,D,E). These findings reveal that MEK/ERK blockade decreases injury to multiple organs in the setting of sepsis.
Figure 4-3: GSK1120212 post-treatment decreases CLP-induced organ injury. Serum creatinine (A), blood urea nitrogen (B), alanine aminotransferase (C), creatine kinase (D), and lactate dehydrogenase (E) were assessed at 18 h after CLP or sham surgery as indicators of organ and cellular injury. Data are shown as mean ± S.E.M. for each group (n ≥ 5 animals per group). Different superscripts above bars indicate significant differences between groups (p < 0.05).
Effects of Delayed GSK1120212 Administration on Renal Peritubular Capillary Perfusion Following CLP

Microvascular dysfunction is a common contributor to sepsis-induced organ injury, including AKI, and is thought to occur secondary to cytokine-induced disruption of endothelial cell integrity (393-395). In particular, a marked reduction in renal peritubular capillary perfusion has been noted in multiple experimental models of sepsis in association with development of local areas of hypoxia and oxidative stress (135, 393, 396). We next determined the effects of GSK1120212 on microvascular perfusion in the kidney following CLP using intravital video microscopy. A significant decrease in the portion (~50%) of peritubular capillaries with continuous red blood cell flow was noted in mice subjected to CLP along with a concomitant increase in capillaries classified by continuous (~10%) or no flow (~30%) (Figure 4-4). Interestingly, GSK1120212 administration at 6 h post-CLP completely reversed the deficiencies in peritubular capillary perfusion described above. These data indicate that MEK/ERK inhibition by GSK1120212 may protect the renal microvasculature and improve perfusion after CLP and in mice.
Figure 4-4: Effects of GSK1120212 on alterations in peritubular capillary perfusion following CLP. Peritubular capillary perfusion was assessed by intravital video microscopy (IVVM) in sham and CLP mice treated with GSK1120212 or vehicle as described above. The observed microvasculature was classified based on degree of perfusion as having “continuous,” “intermittent,” or “no flow.” A significant reduction in the percentage of peritubular capillaries with continuous flow, along with an increase in vessels with intermittent or no flow was observed after CLP. GSK1120212 post-treatment completely reversed CLP-induced deficiencies in peritubular capillary perfusion. Data are expressed as percentage of peritubular capillaries in each category (mean ± S.E.M.). Different superscripts above bars indicate statistically significant differences (p < 0.05).
GSK1120212 Partially Prevents Endothelial Cell Activation After CLP

Endothelial cell activation and subsequent expression of adhesion molecules plays an important role in microvascular dysfunction and immune cell infiltration in the septic kidney (322). Since GSK1120212 prevented CLP-induced disruption of microvascular flow in the kidney, we next questioned whether MEK/ERK inhibition alters endothelial cell activation in this model. To address this question, we measured expression of endothelial cell adhesion molecules including intracellular adhesion molecule-1 (ICAM-1) and E-selectin at the mRNA level by qPCR. Transcript levels of ICAM-1 and E-selectin increased approximately 8-fold and 22-fold, respectively, at 18 h post-CLP (Figure 4-5A,B). GSK1120212 administration did not alter expression of either of these markers, although there was a non-significant trend toward increased E-selectin in the CLP + GSK1120212 group (Figure 4-5A,B).

In order to further assess the effects of GSK1120212 on endothelial cell activation, we also measured protein levels of ICAM-1 in renal cortical tissue by immunoblot analysis. A modest, but significant increase (~1.5-fold) in ICAM-1 was observed in vehicle-treated CLP mice at 18 h (Figure 4-5C). Interestingly, ICAM-1 protein expression returned to control levels in CLP animals treated with GSK1120212 (Figure 4-5C). These data indicate that blockade of MEK/ERK signaling may partially prevent renal endothelial cell activation following sepsis-induced AKI.
Figure 4-5: Pharmacological blockade of MEK/ERK has modest effects on endothelial cell activation in the renal cortex. Total RNA and protein were isolated from renal cortical tissues at 18 h after CLP in order to analyze markers of endothelial cell activation. mRNA expression of ICAM-1 (A) and E-selectin (B) were determined by RT-qPCR. Immunoblot analysis of ICAM-1 protein levels (C and D) revealed minor effects of GSK1120212 on endothelial cell activation after CLP. Data are shown as fold-change relative to sham + vehicle controls (mean ± S.E.M.; n ≥ 6 per group). Different superscripts above bars indicate statistically significant differences (p < 0.05).
Effects of GSK1120212 Post-treatment on CLP-induced Renal Proximal Tubular Injury

Given that inhibition of the MEK/ERK pathway by GSK1120212 restored renal microvascular perfusion after CLP induction and that microvascular dysfunction plays an essential role in development of AKI in the setting of sepsis, we next investigated the effects of this pharmacological intervention on renal proximal tubule cell injury. Expression of well-characterized markers of tubular insult including kidney injury molecule-1 (KIM-1), neutrophil gelatinase-associated lipocalin (NGAL), and heme oxygenase-1 (HO-1) were first assessed in the renal cortex by qPCR (37, 43, 397). When compared to sham controls, a robust increase in KIM-1 mRNA content (~83-fold) was observed in the renal cortex following CLP (Figure 4-6A). Treatment of CLP animals with GSK1120212 reduced KIM-1 transcript levels by approximately 52% (Figure 4-6A). Likewise, delayed MEK/ERK inhibition significantly reduced CLP-mediated induction of NGAL (~44% decrease vs. CLP + vehicle group) and HO-1 (~54% decrease) (Figure 4-6B,C). Although GSK1120212 had significant effects on transcriptional up-regulation of tubular injury markers, no changes were noted in renal cortical KIM-1 and NGAL protein levels in drug-treated animals at 18 h post-CLP when assessed by immunoblot analysis (Figure 4-7A,B). It should be noted, however, that there was considerable variability in KIM-1 and NGAL proteins in both CLP + vehicle and CLP + GSK1120212 treatment groups. Taken together, these findings suggest that pharmacological targeting of the MEK/ERK signaling cascade may partially ameliorate renal proximal tubular injury following sepsis-induced AKI.
Figure 4-6: Delayed GSK1120212 administration attenuates transcriptional up-regulation of tubular injury markers in the renal cortex after CLP. Total RNA was isolated from the renal cortex of mice treated with GSK1120212 or vehicle control after CLP or sham surgery. Transcript levels of markers of proximal tubular injury including KIM-1 (A), NGAL (B), and HO-1 (C) were determined by qPCR analysis. Data are shown as fold change relative to sham + vehicle animals (mean ± S.E.M.; n ≥ 5 mice per experimental group. Superscripts above bars indicate statistically significant differences (p < 0.05).
Figure 4-7: MEK/ERK inhibition by GSK1120212 has no effect on renal cortical KIM-1 and NGAL protein content. Protein levels KIM-1 (A) and NGAL (B) in the renal cortex were determined by immunoblot analysis at 18 h in sham and CLP animals. β-actin was used as a loading control for normalization of densitometry. Data are presented as mean ± S.E.M. for each group (fold change relative to sham control; n = 6 mice per group). Different superscripts above bars indicate statistically significant differences (p < 0.05).
MEK/ERK Inhibition Does Not Alter ROS/RNS Generation in the Proximal Tubule Following CLP

As shown above, treatment with GSK1120212 restored renal peritubular capillary perfusion and partially attenuated proximal tubular injury following CLP in mice. In order to further elucidate the mechanisms by which MEK/ERK inhibition protects the proximal tubule, we next assessed oxidative stress in the renal cortex using intravital video microscopy to detect oxidation of dihydrorhodamine-123 to rhodamine as an index of ROS/RNS generation. Faint rhodamine fluorescence was observed in sham animals (Figure 4-8). Fluorescence intensity was significantly elevated (~6-fold) at 18 h following CLP, indicating an increase in oxidative stress in the proximal tubule. However, contrary to our expectations, GSK1120212 administration at 6 h post-CLP had no effect on rhodamine fluorescence (Figure 4-8). Thus, GSK1120212-mediated protection of the proximal tubule after CLP is not associated with a reduction in tubular oxidative stress.
Figure 4-8: GSK1120212 post-treatment does not attenuate CLP-induced oxidative stress in the renal proximal tubule. CLP mice were treated with either vehicle or GSK1120212 at 6 h post-CLP as described above. Oxidative stress was assessed using intravital video microscopy to quantify oxidation of dihydrorhodamine-123 to rhodamine as an index of tubular ROS/RNS generation. Data are expressed as arbitrary units (A.U.) of rhodamine fluorescence per µm² (mean ± S.E.M.; n ≥ 5 per group). Superscripts above bars indicate statistically significant differences between groups (p < 0.05).
GSK1120212 Attenuates Up-regulation of Pro-inflammatory Cytokines in the Renal Cortex After CLP

In addition to oxidative stress, direct effects of pro-inflammatory cytokines (TNF-α, IL-1β) on the proximal tubule also play an important role in development of sepsis-induced AKI (134, 140, 142). Given that pharmacological blockade of MEK/ERK had no effect on oxidative stress generation in the proximal tubule, we next questioned whether GSK1120212-mediated attenuation of tubular injury in the CLP model might be associated with a decrease in inflammatory changes in the renal cortex. In order to answer this question, we measured renal cortical mRNA expression of pro-inflammatory cytokines by qPCR analysis. Transcript levels of TNF-α, IL-1β, and IL-6 increased ~3.4-, 3.5-, and 74-fold, respectively, in the renal cortex in CLP animals when compared to their sham controls (Figure 4-9A,B,C). Delayed treatment with GSK1120212 at 6 h post-CLP either fully (TNF-α) or partially (IL-1β, IL-6) attenuated these changes (Figure 4-9A,B,C). Taken together, these data indicate that MEK/ERK signaling is essential for up-regulation of pro-inflammatory cytokines in the renal cortex following CLP. In addition, inhibition of MEK/ERK-mediated renal cortical inflammatory changes is associated with reduced proximal tubule injury in this model.
Figure 4-9: MEK/ERK signaling mediates renal expression of pro-inflammatory cytokines after CLP. Inflammatory changes were evaluated in the renal cortex of CLP mice treated with GSK1120212 (1 mg/kg, i.p.) as described above. qPCR was used to determine mRNA levels of TNF-α (A), IL-1β (B) and IL-6 (C) at 18 h post-CLP. Values are expressed as relative expression vs. control (mean ± S.E.M.) for n ≥ 6 / group (p < 0.05 vs control).
MEK/ERK Inhibition Does Not Reverse Suppression of Renal PGC-1α and Subsequent Mitochondrial Biogenesis Following CLP

Our laboratory and others have previously demonstrated suppression of PGC-1α and subsequent mitochondrial biogenesis in multiple experimental models of AKI (274, 279). In addition, we recently showed that MEK/ERK signaling is required for acute (< 3 h) disruption of PGC-1α expression and mitochondrial biogenesis in the kidney following systemic exposure to lipopolysaccharide (LPS), another model of sepsis-induced AKI. Thus, we next investigated whether reduced tubular injury following GSK1120212 administration might be associated with restoration of renal proximal tubule cell mitochondrial biogenesis in the CLP model. Renal cortical mRNA expression of PGC-1α was significantly reduced (~80%) at 18 h in mice subjected to CLP compared to sham controls (Figure 4-10A). In addition, transcription of downstream targets of PGC-1α including nuclear-encoded components of the ETC (NDUFS1) and mitochondrial-encoded ETC proteins (ND1) were decreased following CLP (Figure 4-10C,E). CLP did not have an effect on TFAM or COX1 mRNAs in vehicle-treated mice (Figure 4-10B,D). Interestingly, GSK1120212 treatment did not restore expression of PGC-1α or its downstream targets. In fact, transcript levels of TFAM, COX1, and ND1 were significantly lower in the CLP + GSK1120212 group when compared to CLP + vehicle animals (Figure 4-10B,D,E). These findings provide strong evidence that delayed MEK/ERK blockade by GSK1120212 is insufficient to reverse suppression of PGC-1α and mitochondrial biogenesis in the CLP model.
Figure 4-10: Delayed GSK1120212 therapy has no effect on CLP-mediated suppression of PGC-1α and mitochondrial biogenesis. Sham and CLP mice were treated with GSK1120212 or the appropriate vehicle at 6 h as described above. Transcript levels of PGC-1α (A) and downstream targets in mitochondrial biogenesis including TFAM (B), NDUFS1 (C), COX1 (D), and ND1 (E) were determined in renal cortical samples using qPCR analysis. Data are mean ± S.E.M. (n ≥ 6 per experimental group). Different superscripts indicate statistically significant differences (p < 0.05).
DISCUSSION

Sepsis is defined as an unchecked inflammatory response resulting from activation of the innate immune system in the presence of infection (136). Clinically, sepsis is associated with significant mortality and morbidity characterized by injury to multiple organ systems (104, 372, 396). Innate immune system activation in the setting of sepsis is largely mediated through activation of PRRs by microbial products collectively referred to as pathogen-associated molecular patterns (PAMPs). Stimulation of PRRs initiates signaling through a variety of downstream targets that act to promote the pro-inflammatory response including multiple MAPK signaling pathways involving MEK/ERK, JNK, and p38 MAPKs (378, 379).

Although members of the JNK and p38 MAPK families have classically been implicated in the host response to infection, more recent evidence suggests that MEK/ERK signaling may control multiple aspects of the pro-inflammatory state associated with sepsis. Exposure of macrophages to lipopolysaccharide (LPS), a classical TLR4 agonist, has been shown to activate the MEK/ERK cascade in vitro (381, 383). MEK/ERK signaling is required for transcriptional up-regulation of TNF-α and IL-1β in certain populations of LPS-treated macrophages (381, 383, 398). In addition, ERK activation in response to LPS in macrophages promotes nucleocytoplasmic transport and subsequent translation of TNF-α mRNA, indicating that the MEK/ERK signaling pathway may facilitate TNF-α biosynthesis by both transcriptional and post-transcriptional mechanisms (310). The molecular machinery necessary for stimulation of the MEK/ERK cascade in response to LPS includes TLR4 and its downstream effector TPL-2, a MAP kinase kinase kinase (MAP3K) that directly phosphorylates and activates
MEK. Interestingly, TPL-2 is essential for endotoxin-induced TNF-α production in macrophages, and TPL-2-deficient mice are significantly protected against lethality following systemic administration of LPS and D-galactosamine (310). These data suggest that the MEK/ERK signaling pathway represents a novel and viable therapeutic target for the treatment of sepsis and sepsis-induced organ dysfunction. Unfortunately, studies that directly target MEK/ERK in animal models of sepsis are lacking.

We recently used the potent and specific MEK1/2 inhibitor GSK1120212 in a mouse model of endotoxin-induced AKI. Administration of GSK1120212 (1 mg/kg, i.p.) inhibited LPS-induced phosphorylation of ERK1/2 in the renal cortex, indicating that in vivo administration of low dose GSK1120212 is sufficient to block this signaling cascade in the LPS model (142). GSK1120212 pre-treatment at 1 h before systemic LPS exposure in mice attenuated early (< 3 h) development of renal dysfunction as measured by BUN. Preservation of renal function by MEK/ERK blockade was associated with reduced expression of KIM-1, a well-characterized marker of proximal tubule injury, and pro-inflammatory cytokines (TNF-α, IL-1β) in the renal cortex of LPS-treated mice (142). A subsequent study demonstrated that MEK/ERK inhibition by GSK1120212 also blocked TNF-α production in macrophage cell lines and isolated human PBMCs exposed to LPS in vitro (383). GSK1120212 treatment also prevented death of mice exposed to LPS and D-galactosamine in association with a reduction in circulating levels of TNF-α (383).

GSK1120212 is a highly potent allosteric inhibitor with IC₅₀ values of 0.92 nM and 1.8 nM for MEK1 and MEK2, respectively in an in vitro protein kinase assay (304). This compound also displays high specificity for MEK1/2, with no significant inhibitory
activity seen on a panel of over 100 kinases including other members of the MAPK family (304). GSK1120212 has been safely and effectively used in humans and recently received FDA approval as a chemotherapeutic agent for use in BRAF-mutant melanoma (384-386). Thus, this drug has a number of favorable properties that make it an ideal candidate for clinical use in sepsis. However, the endotoxin models used to date to evaluate efficacy of GSK1120212 suffer from a number of severe limitations. Studies delivered this compound either before or concurrent with LPS administration which would not be feasible in the clinical setting. In addition, neither study employed supportive measures commonly used in the intensive care unit such as fluid replacement, broad-spectrum antibiotics, and/or vasopressor agents (78). It should also be noted that the inflammatory response to LPS in mice is much more rapid and of a higher magnitude than that observed in septic humans (387). These fundamental differences likely explain why therapies developed using endotoxin models (such as anti-TNF-α agents) have largely failed to improve outcomes in septic patients (399). Thus, we sought to determine the efficacy of GSK1120212 in attenuating systemic inflammatory responses and organ injury in a more clinically relevant model of sepsis.

We employed a well-established sepsis model induced by cecal ligation and puncture (CLP) in mice. CLP is widely considered the “gold standard” of sepsis models because it produces a much lower grade and sustained pro-inflammatory response when compared to LPS models (77). The model used here also incorporates supportive measures commonly used in the clinic including a broad-spectrum β-lactam antibiotic (imipenem/cilastatin) and fluid resuscitation with saline in aged mice to better reflect the elderly population which is most commonly affected by sepsis (57, 78). In addition, we
chose to delay GSK1120212 administration until 6 h post-CLP, a time when organ injury and microvascular dysfunction is already established (104, 135, 159).

Delayed inhibition of MEK/ERK signaling attenuated CLP-induced increases in all pro-inflammatory cytokines measured including TNF-α, IL-1β, IL-6, and GM-CSF. Among these, the most significant inhibitory effect was observed on TNF-α. These findings are consistent with earlier studies demonstrating that MEK/ERK signaling is required for maximal induction of TNF-α synthesis in immune cells exposed to TLR agonists (310, 318, 378, 383, 400). Interestingly, serum levels of TNF-α, IL-1β, and IL-6 may all serve as reliable predictors of mortality resulting from sepsis in the clinical setting, and IL-6 appears to be the single best predictor of poor outcomes in septic patients (401, 402). In addition, IL-6 has been validated as a marker of disease burden following CLP in mice (102). The observed decreases in circulating pro-inflammatory cytokines in CLP animals treated with GSK1120212 likely indicate reduced disease severity. These findings suggest that MEK/ERK inhibition may prevent CLP-induced mortality, although we did not assess this outcome. In support of this, GSK1120212 also reversed development of hypothermia, a widely used indicator of physiologic dysfunction and impending death in septic mice (391, 392).

Reducing systemic pro-inflammatory cytokines alone may have significant effects on organ dysfunction in the setting of sepsis. Cunningham et al. elegantly demonstrated that LPS-induced AKI is mediated by TNF-α acting on TNF receptor 1 (TNFR1) expressed on renal cells (140). Administration of a single dose of recombinant TNF-α is sufficient to cause AKI and subsequent renal dysfunction in rodents (141, 142). Similar effects of pro-inflammatory cytokines have also been described in other organ systems.
that commonly become dysfunctional in sepsis including the liver and the lung (403, 404). Thus, it is important to note that the systemic effects of MEK/ERK inhibition may be beneficial in for preventing injury and dysfunction in multiple organ systems.

As discussed above, multiple organ dysfunction syndrome (MODS) is a common consequence of sepsis, and injury to multiple organs including the kidney, lungs, liver, and skeletal/cardiac muscle contributes to morbidity and mortality resulting from this disease. Our previous study demonstrated that GSK1120212 attenuates early changes in renal dysfunction and proximal tubular injury after endotoxin administration in mice. We therefore examined organ/cellular injury in the CLP model to better demonstrate efficacy of this drug in sepsis. Delayed GSK1120212 administration reversed renal dysfunction as assessed by changes in SCr after CLP. However, there was no change in BUN in GSK1120212-treated animals. Reasons for the observed discrepancy in SCr and BUN are unclear. It should be noted that BUN can be altered by a number of extrarenal factors in sepsis including increased protein catabolism, glucocorticoid production, and extracellular fluid volume (405). In addition, the change in BUN after CLP may also reflect increased tubular reabsorption of urea rather than glomerular filtration rate (GFR). GSK1120212 also had protective effects on clinical markers of injury to the liver (ALT), skeletal/cardiac muscle (CK), and general cellular injury (LDH). Taken together, these findings indicate that MEK/ERK inhibition by GSK1120212 is effective in preventing multiple organ system injury in sepsis.

For the remainder of our studies, we focused on CLP-induced AKI. The pathophysiology of sepsis-induced AKI and is complex and multi-factorial in nature. Increased endothelial cell permeability in the renal microvasculature leads to sluggish
peritubular capillary flow, development of local areas of hypoxia and hypoperfusion, and increased exposure of the renal parenchyma to circulating pro-inflammatory molecules due to reduced flow (124, 125, 155). There is now strong experimental evidence indicating that therapeutic targeting of renal microvascular dysfunction may reverse sepsis-induced AKI (109, 132, 406). As expected, CLP reduced the percentage of peritubular capillaries with continuous flow, and this phenomenon occurred in conjunction with an increase in the proportion of vessels characterized by intermittent or no flow. Treatment with GSK1120212 completely restored renal microvascular perfusion after CLP. These findings are in agreement with earlier studies demonstrating that MEK/ERK signaling plays in an important role in development of increased endothelial cell permeability and dysfunction in response to inflammatory insult \textit{in vitro} (407, 408).

Since pharmacological blockade of the MEK/ERK cascade restored renal microvascular perfusion after CLP, we also wondered whether this approach might alter vascular endothelial cell activation. Activated endothelial cells in the kidney contribute to sepsis-induced AKI by expressing chemokines and a variety of cellular adhesion molecules including ICAM-1 and E-selectin, which together promote infiltration of immune cells into renal parenchyma (409). Mice deficient in either ICAM-1 or E-selectin are protected from development of renal dysfunction in experimental models of sepsis (322, 410). In order to assess endothelial cell activation, we measured changes in renal cortical expression of ICAM-1 and E-selectin after CLP. MEK/ERK inhibition by GSK1120212 had no effect on the robust increases in ICAM-1 and E-selectin transcript levels observed at 18 h after CLP. However, there was a modest, but significant decrease
in ICAM-1 protein levels in CLP mice treated with GSK1120212. Given the relatively minor (~25%) nature of this reduction in ICAM-1 protein expression, the physiological relevance of this finding is likely limited. There remains considerable debate concerning the role of MEK/ERK and other MAPK signaling cascades in regulation of cellular adhesion molecule expression in response to inflammatory stimuli (411, 412). However, our findings provide strong evidence indicating that ERK signaling plays a relatively insignificant role in renal endothelial cell activation in the CLP model of sepsis.

Restoration of renal microvascular perfusion following treatment with GSK1120212 was associated with a reduction in transcriptional up-regulation of multiple markers of tubular injury. In particular, we observed substantial increases in mRNA expression of KIM-1, NGAL, and HO-1 in renal cortical tissue at 18 h after CLP, which is consistent with previous reports (413). GSK1120212 treatment partially attenuated changes in each of these markers of tubular injury. These findings are in agreement with our previous studies employing MEK/ERK inhibition in the LPS model and suggest that inhibition of MEK/ERK signaling may partially prevent insult to the renal proximal tubule following sepsis-induced AKI (142). However, we did not detect changes in renal cortical protein levels of KIM-1 or NGAL at 18 h after CLP. Interpretation of these data is difficult given that GSK1120212 was administered only 12 h prior to analysis of tubular injury. It is not clear whether this short time frame would be sufficient to allow for the transcriptional changes described above to result in appreciable changes in protein levels of KIM-1 and NGAL. Further studies are necessary to determine whether MEK/ERK inhibition might decrease protein expression of KIM-1 and NGAL at later time points.
We next focused on potential mechanisms responsible for the protective effects GSK1120212 on proximal tubule injury following CLP. Given that this drug restored microvascular perfusion, we anticipated that MEK/ERK inhibition would attenuate CLP-induced ROS/RNS generation and oxidative stress in the proximal tubule. Previous studies demonstrated that multiple pharmacological approaches that improve peritubular capillary blood flow prevent oxidative injury to the proximal tubule (134, 406). CLP robustly increased oxidation of dihydrorhodamine-123 to rhodamine in the proximal tubule, indicating that renal oxidative stress was elevated in this model. However, contrary to our expectations, GSK1120212 had no effect on CLP-mediated ROS/RNS production. These findings suggest that the sepsis-induced increase in proximal tubular oxidative stress may occur independently of deficits in microvascular function and perfusion. It should be noted that this phenomenon has been previously described (132).

Since MEK/ERK inhibition had negligible effects on oxidant generation in the proximal tubule following CLP, we explored other potential mechanisms underlying GSK1120212-mediated protection of the proximal tubule. In our previous study, pre-treatment with GSK1120212 partially attenuated local increases in inflammatory cytokine (TNF-α, IL-1β) production in the renal cortex at early time points (3 h) after systemic endotoxin exposure in mice (142). Given the well-characterized roles of pro-inflammatory cytokines in sepsis-induced tubular insult, we hypothesized that the protective effects of MEK/ERK inhibition might be associated with reduced production of these mediators in the kidney. GSK1120212 post-treatment fully attenuated CLP-induced up-regulation of TNF-α in the renal cortex. Similar effects, though less pronounced, were also noted on renal transcript levels of IL-1β and IL-6. Taken together,
these data suggest that MEK/ERK inhibition prevents local, organ-specific inflammatory responses in the setting of sepsis. In support of this, previous reports have observed a reduction in tissue levels of TNF-α, IL-1β, and IL-6 in the lung and liver in CLP mice following treatment with the MEK1 inhibitor PD98059 (414). It is likely that attenuation of both systemic and local cytokine production contributes to GSK1120212-mediated protection of the proximal tubule after sepsis-induced AKI. Indeed, treatments aimed at reducing renal inflammation have proven beneficial in multiple experimental models of AKI (415, 416).

The MEK/ERK signaling pathway has been identified as a potential negative regulator of mitochondrial biogenesis in multiple cell types (244, 245). In addition, early (< 3 h) improvement in renal function following endotoxin-induced AKI in mice treated with GSK1120212 was closely associated with restoration of PGC-1α and subsequent mitochondrial biogenesis in the renal cortex (142). Interestingly, MEK/ERK inhibition in the CLP model did not promote renal cortical expression of PGC-1α or its downstream targets involved in regulation of mitochondrial biogenesis and electron transport chain function. This result is not entirely unexpected given that GSK1120212 had only modest effects on PGC-1α and mitochondrial biogenesis at later time points (18 h) after systemic LPS exposure in our earlier work (142). Further studies are necessary to determine whether ERK activation plays a similar role in acute suppression of renal mitochondrial biogenesis after CLP induction. However, the data presented here do allow us to rule out stimulation of mitochondrial biogenesis as a contributor to GSK1120212-mediated renoprotection in the CLP model.
In summary, we have demonstrated here that a potent, specific allosteric inhibitor of MEK1/2, GSK1120212, reduces the systemic inflammatory response and prevents AKI and other organ injury in a clinically relevant model of sepsis induced by CLP in mice. Given the favorable pharmacological properties of GSK1120212 and its recent FDA approval, we believe that MEK/ERK inhibition by GSK1120212 may represent a readily translatable approach to limit organ injury in the setting of sepsis.
Chapter 5:
Summary, Contributions, and Future Directions

SUMMARY OF CURRENT LITERATURE

Disruption of Mitochondrial Biogenesis in AKI

AKI arises from a variety of insults including sepsis, ischemia/reperfusion injury, and nephrotoxicant exposure. Mitochondrial dysfunction is now recognized as a critical contributor to the development of renal and proximal tubular dysfunction after multiple forms of AKI. Thus, restoration of normal mitochondrial function may represent a novel therapeutic approach to prevent renal injury and facilitate repair after AKI. Unfortunately, relatively little is known about the mechanisms responsible for inducing mitochondrial dysfunction in the setting of AKI.

Our laboratory and others have focused on disruption of mitochondrial homeostasis and dynamics in the proximal tubule as a potential contributor to renal dysfunction. In particular, we have demonstrated that mitochondrial biogenesis, the process by which a cell generates new, functional mitochondria, is suppressed in multiple experimental models of AKI. Funk et al. demonstrated that expression of PGC-1α, the so-called “master regulator of mitochondrial biogenesis,” is decreased in the renal cortex following I/R injury in mice (274). Reduced PGC-1α levels in I/R animals were closely associated with persistent down-regulation of electron transport chain proteins including nuclear-encoded NDUFB8 and ATP synthase β (ATPSβ) and mitochondrial-encoded cytochrome c oxidase subunit I (COXI) in the renal cortex up to 144 hours, indicating that mitochondrial biogenesis is persistently disrupted after AKI (274). Further studies by our laboratory also demonstrated that loss of PGC-1α and ETC proteins following I/R
leads to a significant decrease in ADP-stimulated respiration in isolated renal mitochondria, further revealing an important role for disruption of biogenesis in mitochondrial dysfunction (278). We have also observed similar changes in renal mitochondrial biogenesis in mouse models of glycerol-induced rhabdomyolysis and folic acid-induced AKI (274, 279). These data suggest that disruption of mitochondrial biogenesis and function is common to multiple forms of AKI.

Based on the findings described above, our group has identified a number of pharmacological compounds that stimulate mitochondrial biogenesis in the kidney and promote recovery of renal function in experimental models of AKI. For example, we found that administration of formoterol, a long-acting β2-adrenoreceptor agonist, after I/R injury in mice restores ETC protein expression, mitochondrial respiration, and renal function (278). The cGMP-specific phosphodiesterase 5 (PDE5) inhibitor sildenafil also increased mitochondrial biogenesis and mtDNA content and reduced tubular injury as measured by KIM-1 protein expression following folic acid-induced AKI in mice (291). In addition, we have previously shown that overexpression of PGC-1α in primary renal proximal tubule cells (RPTCs) following oxidative insult with tert-Butyl hydroperoxide (TBHP) promotes recovery of mitochondrial function and active Na⁺ transport (289).

Taken together, these findings suggest that pharmacological targeting of mitochondrial biogenesis represents a feasible approach to prevent both mitochondrial dysfunction and renal insult following AKI. However, very little is known about the molecular mechanisms underlying suppression of PGC-1α and subsequent mitochondrial biogenesis in the setting of AKI. Thus, one goal of the work presented here was to characterize signaling mediators responsible for this phenomenon in a mouse model of
sepsis-induced AKI. We propose that a better understanding of these mechanisms may lead to development of novel therapeutic approaches to limit mitochondrial dysfunction following renal injury.

**Changes in Glycolytic Metabolism in the Renal Cortex Following AKI**

Renal function depends heavily on oxidative phosphorylation in the proximal tubule to facilitate active reabsorption of solutes from the glomerular filtrate. Disruption of normal mitochondrial function may thus contribute to renal dysfunction by reducing energy production that drives active transport in the tubule. Early work by Guder and Ross demonstrated that renal proximal tubule cells have the lowest glycolytic capacity along the entire nephron segment (258). However, it is possible that the proximal tubule responds to disruption of mitochondrial function and biogenesis by increasing glycolytic metabolism to provide ATP in support of normal cellular function. In isolated rabbit proximal tubules *in vitro*, previous studies demonstrated that inhibition of ETC function by hypoxia (1% O₂), rotenone (a complex I inhibitor), or FCCP (an ETC uncoupler), resulted in a significant increase in lactate production via anaerobic glycolysis (293). Induction of anaerobic glycolysis was closely associated with preservation of cellular ATP content and Na⁺/K⁺-ATPase function. In addition, 2-deoxyglucose, an inhibitor of glycolysis, increased injury in proximal tubules exposed to rotenone as indicated by increased cellular release of lactate dehydrogenase (LDH) (293). These findings support the notion that induction of glycolytic metabolism may serve a protective role in the face of mitochondrial dysfunction in the proximal tubule by facilitating energy generation.

Despite the *in vitro* evidence described above, there have been relatively few studies investigating changes in glycolytic metabolism in the proximal tubule following
AKI *in vivo*. A recent study by Zager et al. demonstrated that increases in renal cortical lactate content occur with as little as 15 minutes of ischemia in mice, indicating a shift toward anaerobic glycolysis in the ischemic kidney (294). However, lactate production rapidly returned to baseline levels after restoration of vascular flow (294). The authors therefore suggested that physiologically meaningful induction of glycolytic metabolism in the proximal tubule only occurs during overt renal ischemia. In contrast, earlier work by Ash and Cuppage demonstrated that renal glucose uptake and lactate production are increased following administration of the nephrotoxicant mercuric chloride in rats despite an absence of changes in renal blood flow (296). However, these data should be interpreted with caution because the increase in renal glycolytic metabolism occurs at a much later time point (5 – 10 days) after mercuric chloride-induced AKI and glycolytic parameters were measured for the whole kidney rather than more specifically in the renal cortex or proximal tubule.

Evidence for induction of glycolysis in the septic kidney is lacking. Given that sepsis-induced AKI is associated with substantial mitochondrial dysfunction in the proximal tubule, we wondered if glucose utilization through the glycolytic pathway might be increased to support cellular and renal function. Thus, another goal of the work presented here was to better characterize time-dependent changes in glycolytic metabolism following LPS-induced AKI in mice.

**MEK/ERK Signaling as a Therapeutic Target in Sepsis and Sepsis-induced AKI**

The MEK/ERK signaling pathway is an important downstream target of pattern recognition receptors (PPRs) expressed on a variety of cell types including immune cells (dendritic cells, macrophages, neutrophils, B lymphocytes) and non-immune cells such as
endothelial cells and epithelial cells (including proximal tubular epithelial cells). Activation of PRRs including the Toll-like receptors (TLRs) by bacterial products known as pathogen associated molecular patterns (PAMPs) is responsible for the massive inflammatory response observed in sepsis and subsequent sepsis-induced organ dysfunction. Previous studies demonstrated that MEK/ERK signaling is involved in both transcriptional and post-transcriptional regulation of pro-inflammatory cytokines including TNF-α and IL-1β following cellular exposure to TLR agonists (309, 310, 318, 378, 379, 382, 398).

There has been surprisingly little interest in targeting MEK/ERK in animal models of sepsis, probably due in part to the lack of potent, specific, and safe pharmacological inhibitors of this pathway. However, a new generation of allosteric inhibitors of MEK with more favorable pharmacological properties has been developed for use in chemotherapy regimens for cancer. In particular, GSK1120212 (also known as trametinib) is a potent allosteric inhibitor of MEK1 (IC₅₀ = 0.92 nM) and MEK2 (IC₅₀ = 1.8 nM) that showed no inhibitory activity on a panel of over 100 off-target kinases (304). GSK1120212 has been safely and successfully used in a number of clinical trials and gained FDA approval for use in unresectable metastatic melanoma with specific BRAF⁷⁶⁰E or BRAF⁷⁶⁰K mutations in 2013 (417-419). In our earlier work, we demonstrated that GSK1120212 pre-treatment prevents acute LPS-induced renal dysfunction and tubular injury in mice, suggesting that this drug may be efficacious in the treatment of sepsis-induced AKI. However, the clinical relevance of these findings was limited due to the model and pre-treatment protocol used. Thus, the final aim of this work was to determine the potential efficacy of GSK1120212 in a more clinically
relevant model of sepsis and septic AKI induced by cecal ligation and puncture (CLP) in mice.

**CONTRIBUTIONS TO THE FIELD**

**Mechanisms Underlying Suppression of Mitochondrial Biogenesis in Septic AKI**

Although our lab and others have reported suppression of renal mitochondrial biogenesis in the setting of AKI, there have been very few studies aimed at determining the molecular mechanisms mediating this effect. I decided to address this unanswered question in a mouse model of sepsis-induced AKI. I hypothesized that renal mitochondrial biogenesis is disrupted following LPS-induced AKI through suppression of PGC-1α expression by TLR4-dependent activation of MAP kinase and/or NFκB signaling pathways and this disruption contributes to loss of mitochondrial homeostasis. PGC-1α expression was rapidly decreased at both the mRNA (3 h) and protein (18 h) level the renal cortex following systemic exposure to LPS in mice. Loss of renal cortical PGC-1α was closely associated with increased BUN and up-regulation of KIM-1 and NGAL, proteins that served as markers of injury to the proximal tubule. Taken together, these findings suggested that development of AKI occurs simultaneously with suppression of PGC-1α following endotoxin administration.

Since a significant reduction in renal cortical PGC-1α content was observed in the LPS model, I next sought to determine whether this resulted in disruption of normal mitochondrial biogenesis in the septic kidney. LPS-induced PGC-1α suppression was associated with reduced transcript (at 18 h) and protein (at 42 h) expression of essential regulators of mitochondrial biogenesis (TFAM), nuclear-encoded ETC components (NDUFS1, NDUFB8, and ATPSβ), and mitochondrial-encoded ETC proteins (COXI).
In addition, I observed significant reductions in renal cortical mtDNA, an indicator of cellular mitochondrial content, at both 18 and 42 h post-LPS. The results generated from these experiments provide strong evidence that suppression of PGC-1α is closely associated with loss of normal mitochondrial biogenesis following LPS-induced AKI.

Once I established that disruption of PGC-1α and subsequent mitochondrial biogenesis in the LPS model is consistent with other experimental models of AKI, I began to focus on the molecular mechanisms underlying the observed changes. LPS primarily signals through TLR4-mediated activation of NFκB and MAPK pathways (86, 87, 347). However, TLR4-independent effects of LPS have also been described in some models of septic shock (94, 95). Using TLR4-deficient mice, I asked whether TLR4 was required for disruption of mitochondrial biogenesis in endotoxin-induced AKI. TLR4KO mice were completely protected from LPS-induced disruption of PGC-1α and its downstream targets. In addition, TLR4 deficiency blocked development of renal dysfunction in endotoxin-treated mice. Although the requirement for TLR4 in sepsis-induced AKI has been previously reported, this is the first study to demonstrate that TLR4 is also essential for suppression of renal cortical PGC-1α and mitochondrial biogenesis in LPS-induced AKI (150).

I next focused on downstream targets of TLR4 that might mediate mitochondrial dysfunction in sepsis-induced AKI. There is some evidence suggesting that cytokine-induced NFκB signaling may down-regulate PGC-1α and mitochondrial biogenesis in skeletal muscle cells in vitro (241, 242). Despite testing multiple pharmacological inhibitors of the NFκB pathway including TPCA-1, BAY11-7085, and PPM-18, I was unable to detect an effect of NFκB blockade on renal cortical mitochondrial biogenesis in
the LPS model (see Figure 5-2 in the following section). Given these results, I turned my attention to MAPK signaling pathways downstream of TLR4. I noted rapid phosphorylation (and thus activation) of ERK1/2 and TPL-2, a MAP3K responsible for TLR4-mediated stimulation of the MEK/ERK signaling, in the renal cortex within 1 h of systemic LPS exposure.

Pre-treatment with the MEK1/2 inhibitor GSK1120212 renal cortical blocked ERK1/2 activation and attenuated early (3 h) LPS-induced renal dysfunction and tubular injury in mice. Most interestingly, the protective effects of this compound were associated with restoration of renal cortical PGC-1α expression at the 3 h time point. These findings indicate an important role for MEK/ERK signaling in LPS-induced renal injury and disruption of PGC-1α and mitochondrial biogenesis. However, it should be noted that blockade of MEK/ERK had only modest effects on renal cortical expression of PGC-1α and downstream targets at later time points (18 h). This likely indicates that the TPL-2/MEK/ERK pathway mediates acute suppression of PGC-1α in the renal cortex after endotoxic AKI, but other signaling mechanisms contribute to late-phase disruption of mitochondrial biogenesis. Despite the modest effects of GSK1120212 on mitochondrial homeostasis at 18 h post-LPS, this drug maintained significant protective effects on late-phase elevation of BUN and renal cortical KIM-1 expression in this model, indicating further therapeutic potential for pharmacological inhibition of MEK/ERK in sepsis-induced AKI. To our knowledge, this is the first study to reveal an important role for MEK/ERK in regulation of mitochondrial biogenesis in response to renal injury.
After determining that MEK/ERK signaling was critical for LPS-induced suppression of PGC-1α, I focused on downstream effectors of this pathway. In particular, I noted that GSK1120212 pre-treatment significantly reduced transcriptional up-regulation of the pro-inflammatory cytokines TNF-α and IL-1β in the renal cortex after systemic LPS administration in mice. Based on previous studies and our own preliminary data indicating that TNF-α is a negative regulator of mitochondrial biogenesis in renal proximal tubule cells in vitro, I hypothesized that a reduction in TNF-α production might be responsible for the effects of MEK/ERK inhibition. Indeed, mice receiving an anti-TNF-α neutralizing antibody prior to systemic LPS exhibited partial restoration of renal cortical PGC-1α mRNA and mtDNA content. The incomplete return of PGC-1α after TNF-α neutralization suggests that multiple pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) signal through similar mechanisms to suppress PGC-1α in sepsis-induced AKI. In an additional set of experiments, I also demonstrated that in vivo administration of high dose (50 µg/kg) recombinant murine TNF-α is sufficient to disrupt mitochondrial homeostasis and renal function in mice. Taken together, the studies describe above have identified a novel role for the TLR4/TPL-2/MEK/ERK/TNF-α signaling axis in mediating early renal injury and suppression of mitochondrial biogenesis in a mouse model of sepsis-induced AKI. These findings may be useful in developing new therapeutic approaches to reverse mitochondrial and renal dysfunction in AKI.

**Changes in Glucose Utilization in the Renal Cortex Following AKI**

As described above, mitochondrial homeostasis and function is significantly disrupted in the renal proximal tubule following sepsis-induced AKI. However, little is
known regarding how glucose metabolism through glycolysis changes in response to this mitochondrial dysfunction. I hypothesized that glycolytic metabolism is activated to provide ATP, support cellular function, and limit renal dysfunction in the septic kidney.

In order to assess changes in glucose flux through glycolysis, I first measured time-dependent changes in the activities of the 3 major regulatory enzymes of this pathway (hexokinase [HK], phosphofructokinase [PFK], and pyruvate kinase [PK]) in the renal cortex after systemic LPS administration in mice. I observed a rapid increase in renal cortical HK activity as early as 3 h after LPS exposure in mice that was maintained for at least 18 h without changes in PFK or PK activity at any of the time points examined. Analysis of mRNA and protein expression of other glycolytic enzymes further confirmed that the effects of LPS were specific to HK. Since I observed a sustained increase in HK activity, I next questioned whether this change led to an overall increase glycolytic metabolism. Using standard biochemical assays, I measured pyruvate and lactate content in the renal cortex of mice subjected to endotoxin. Contrary to our original hypothesis, I was unable to detect increases in renal cortical pyruvate, lactate, or the lactate:pyruvate ratio, an index of anaerobic glycolysis. Together, these findings indicated that LPS-mediated HK activation in the renal cortex was not sufficient to increase glucose utilization through glycolysis.

Because glycolytic metabolism was not increased in the septic kidney, I next sought to determine the physiological relevance of HK activation in the LPS model by assessing alternative fates of glucose-6-phosphate produced by this enzyme including gluconeogenesis, glycogen synthesis, and the pentose phosphate pathway (PPP). Previous studies reported a significant increase in both gluconeogenesis and glycogenesis
in the renal cortex after I/R injury (294). In contrast to changes observed in the I/R model, I observed a decrease in both glucose and glycogen content in response to systemic endotoxin exposure. Instead, activity of glucose-6-phosphate dehydrogenase (G6PDH), the rate-limiting step of the PPP was significantly increased in the renal cortex of mice exposed to LPS. To our knowledge, this is the first study to demonstrate increases in both HK and G6PDH in the setting of sepsis-induced AKI. These findings provide evidence that increased glucose phosphorylation by activated HK may be necessary to supply substrates for the PPP. These findings are consistent with earlier reports of increased renal cortical glucose phosphorylation and PPP activity following mercuric chloride-induced injury in rats (296). The physiological relevance of PPP induction in this setting remains unclear. However, it should be noted that this pathway is essential for generation of NADPH and thus replenishment of cellular glutathione and antioxidant defense. Since oxidative stress is an important contributor to tubular insult following systemic LPS exposure, these findings suggest an alternate view that HK and G6PDH activation may be an adaptive response to limit cellular injury (339). Interestingly, reduced NADPH levels in G6PDH-deficient mice are associated with increased renal oxidative stress, inflammation, and dysfunction, indicating an important role for NADPH in antioxidant defense in the kidney (367). Further studies are warranted to confirm the physiological significance of increased PPP activity following LPS-induced AKI in mice.

I also investigated the mechanisms responsible for HK activation in this model. LPS-induced increases in renal cortical HK activity occurred independently of changes in HK isoform exposure or subcellular localization. These findings suggested that HK
activity might be regulated by post-translational modifications after endotoxin-induced AKI. In particular, Akt is known to modulate both HK activity and subcellular localization via phosphorylation (363, 369). Using a series of pharmacological inhibitors, I demonstrated that LPS-induced HK activation in the renal cortex occurs via an EGFR/PI3K/Akt-dependent mechanism. These findings are consistent with previous reports from our laboratory and others demonstrating induction of HK and/or PPP activity in response to EGFR stimulation in a variety of renal cell types in vitro (335, 340, 343). In addition, TLR4 agonism by LPS has been shown to rapidly transactivate EGFR via protease-mediated EGFR ligand shedding in epithelial cells (347, 348).

**MEK/ERK Inhibition in a Clinically Relevant CLP Model of Sepsis**

As discussed previously, the potent and specific MEK1/2 inhibitor GSK1120212 prevented early renal dysfunction following systemic LPS exposure in mice. Given the well-characterized limitations of endotoxin models in rodent, I decided to test the efficacy of this drug in the “gold standard” model of sepsis induced by CLP in mice. The specific model used in these studies also implemented supportive measures commonly used in clinical management of sepsis including a broad-spectrum β-lactam antibiotic (imipenem/cilastatin) and aggressive fluid resuscitation with saline. In contrast to our earlier studies conducted in the LPS model that utilized a pre-treatment protocol, GSK1120212 administration was also delayed to 6 h post-CLP, a time point when renal injury is already established. I anticipated that MEK/ERK inhibition by GSK1120212 would prevent CLP-induced systemic inflammation as well as development of AKI.

In order to assess the systemic response to sepsis induction, I first measured serum levels of acute phase pro-inflammatory cytokines including TNF-α, IL-1β, IL-6,
and GM-CSF. All of the cytokines measured increased markedly at 18 h after CLP in mice. In agreement with our hypothesis, GSK1120212 treatment significantly attenuated CLP-induced increases in these pro-inflammatory molecules. I also evaluated the development of hypothermia as a secondary measure of physiologic dysfunction. Delayed drug administration completely reversed the decrease in core body temperature following CLP in mice. These findings provided encouraging evidence that MEK/ERK signaling is an important mediator of the systemic inflammatory response. To our knowledge, this is the first study to demonstrate that inhibition of MEK1/2 may represent a novel therapeutic approach to limit deleterious inflammation in the CLP model.

Once I established the effects of GSK1120212 on systemic parameters, I turned my focus to CLP-mediated AKI. Microvascular dysfunction is a well-characterized mediator of renal injury, resulting in sluggish capillary blood flow and development of local regions of hypoxia and hypoperfusion in the septic kidney. We utilized intravital microscopy to assess changes in microvascular perfusion after CLP. MEK/ERK inhibition completely restored peritubular capillary flow to sham levels at the 18 h time point. However, I noted only modest effects of GSK1120212 on cellular adhesion markers expressed by the vascular endothelium including ICAM-1 and E-selectin that facilitate immune cell infiltration, indicating that this drug does not fully prevent microvascular dysfunction in the septic kidney.

Since pharmacological blockade of MEK/ERK reversed peritubular capillary perfusion deficits after CLP, I expected to see a reduction in injury to the renal proximal tubule. At 18 h post-CLP, markers of tubular including KIM-1, NGAL, and HO-1 were markedly increased at the mRNA level in renal cortical. GSK1120212 post-treatment
partially attenuated the observed transcriptional up-regulation of all three markers. When examining potential mechanisms for GSK1120212-mediated tubular protection, I noted that renal cortical pro-inflammatory cytokine (TNF-α, IL-1β, IL-6) expression was decreased in CLP mice treated with this drug. These findings are consistent with our earlier studies in LPS-induced AKI (142). However, delayed MEK/ERK inhibition had no effect on either oxidative stress or renal cortical PGC-1α/mitochondrial biogenesis. It is not entirely surprising that mitochondrial biogenesis remained unaltered after GSK1120212 administration, because we previously noted that MEK/ERK signaling was only necessary for acute (< 3) suppression of PGC-1α in LPS-induced AKI. Together, these findings suggest that GSK1120212 exerts protective effects against renal injury primarily through modulation of circulating cytokine production, microvascular dysfunction, and local, organ-specific inflammatory responses in the septic kidney at later time points.

Given the beneficial effects of MEK/ERK inhibition described above, I was optimistic that this therapeutic approach would reverse CLP-mediated renal dysfunction. However, to my surprise, I was unable to detect differences in BUN when comparing the CLP + vehicle and CLP + GSK1120212 groups. There are a number of potential explanations for this finding. BUN is affected by changes in intravascular fluid status and nutrition, both of which may be significantly altered in the setting of sepsis (420-422). It is therefore likely that the observed change in BUN reflects more than simply renal dysfunction in the CLP model. A more direct assessment of renal function (e.g. glomerular filtration rate) would be useful to address this issue. However, it is also possible that delayed administration of GSK1120212 at this late time point is not
sufficient to reverse CLP-mediated renal dysfunction. Although this may suggest limited utility of MEK/ERK inhibition in sepsis-induced AKI, it is important to note that we did see a number of beneficial effects of this intervention including reduced systemic inflammation, improved renal microvascular perfusion, and decreased proximal tubule injury. Given that GSK1120212 has already been FDA approved, we believe we have provided sufficient evidence to suggest that this drug may represent a rapidly translatable and targeted approach to improve outcomes associated with sepsis and sepsis-induced organ injury.

**FUTURE DIRECTIONS**

**Mechanisms Underlying Suppression of Mitochondrial Biogenesis in AKI**

The findings presented above raise a number of new and exciting questions that warrant further attention. In an endotoxin model of sepsis-induced AKI, I identified TLR4-dependent activation of MEK/ERK as an essential component of acute suppression of PGC-1α and mitochondrial biogenesis in the renal cortex. A question I have been particularly interested in is whether this pathway might be a common mediator of mitochondrial dysfunction in multiple forms of AKI. Interestingly, a number of endogenous agonists of TLR4, referred to as danger-associated molecular patterns (DAMPs), are elevated after renal I/R including hyaluronan, high-mobility group box 1 (HMGB1), and biglycan (423, 424). TLR4 deficiency in mice protects against development of AKI in the I/R model and this appears to be dependent on local TLR4 signaling in the kidney, further indicating that TLR4 activation may be a common mediator of renal injury in multiple experimental models (423). Given these results, I was optimistic that we would be able to implicate TLR4 in disruption of renal
mitochondrial homeostasis after I/R injury. To address this hypothesis, we subjected both wild-type and TLR4KO to bilateral renal pedicle clamping for 15 min before reperfusion as previously described (425). In our hands, TLR4 deficiency had no effect on acute (< 3 hr) development of renal dysfunction as measured by BUN. Suppression of renal cortical PGC-1α and its downstream targets (TFAM, NDUFS1, and COX1) at 3 h post-I/R was similar in both wild-type and TLR4KO mice, suggesting that TLR4 does not mediate acute disruption of mitochondrial biogenesis in this model (Figure 5-1). This does not rule out the possibility that TLR4 activation contributes to the persistent loss of mitochondrial homeostasis that we have previously demonstrated following renal I/R injury (274). However, it should be noted that pre-treatment with the MEK/ERK inhibitor GSK1120212 (1 mg/kg, i.p.) does restore renal cortical PGC-1α mRNA levels at 3 h, but not 24 h, after I/R AKI (data not shown). Taken together, these findings suggest that MEK/ERK signaling is a common contributor to acute down-regulation of mitochondrial biogenesis in multiple forms of AKI, although the upstream signals for activation may differ. Further work using both genetic and pharmacological approaches is warranted to determine whether these effects can be attributed solely to MEK/ERK-induced TNF-α production or modulation of other downstream targets of this pathway.
Figure 5-1: TLR4 deficiency does not alter suppression of mitochondrial biogenesis following I/R-induced AKI in mice. Wild-type and TLR4 knockout (TLR4KO) mice on a C57BL/6 background were subjected to bilateral renal ischemia for 15 min prior to reperfusion. Kidneys were collected for biochemical analysis at 3 h post-I/R and total RNA was isolated from the renal cortex. mRNA expression of mitochondrial biogenesis markers including PGC-1α (A), TFAM (B), NDUFS1 (C), and COX1 (D) were determined by RT-qPCR. Disruption of mitochondrial homeostasis was not different when comparing wild-type and TLR4KO animals subjected to I/R injury. Data are presented as fold change relative to wild-type sham (mean ± S.E.M.; n = 3 animals per group). Different superscripts above bars indicate statistically significant differences (p < 0.05).
Studies conducted in both the LPS and I/R models now indicate that MEK/ERK inhibition is only sufficient to reverse suppression of PGC-1α in the early time period (< 3 h) after development of AKI. An issue of particular interest to our laboratory is defining the molecular mechanisms that maintain disruption of mitochondrial biogenesis at later time points. We were particularly interested in the NFκB proteins given recent evidence suggesting that this family of transcription factors acts as a negative regulator of PGC-1α transcription in skeletal muscle cells and identification of a putative NFκB binding site in the PGC-1α promoter (241, 242). I was unable to show any significant effects of pharmacological inhibition of NFκB signaling on PGC-1α at 3 h after LPS-induced AKI using a diverse set of compounds including TPCA-1, BAY11-7085, and PPM-18 (Figure 5-2). However, it should be noted that immunoblot analysis of phosphorylated p65 NFκB and IκBα in the renal cortex as an indicator of NFκB activation did not conclusively demonstrate efficacy of any of the drugs tested. This suggests alternative approaches may be necessary to probe the role of NFκB in late-phase dysregulation of PGC-1α following AKI. Although global knockout of most components of the NFκB signaling pathway is embryonic lethal in mice, recent studies have employed cell-type specific ablation of the NFκB essential modulator (NEMO, required for IKK-mediated phosphorylation and degradation of IκB) or expression of a degradation-resistant, dominant negative IκBα to genetically target NFκB (426). It would be interesting to generate mice expressing these alleles under the control of proximal tubule-specific promoters (e.g. γ-glutamyl transferase) to further elucidate the role of NFκB signaling in transcriptional regulation of PGC-1α after AKI. Further examination of
other well-characterized negative regulators of mitochondrial biogenesis such as RIP140 is also warranted in the LPS model.
Figure 5-2: Pharmacological blockade of NFκB by diverse chemical inhibitors does not restore acute loss of PGC-1α following systemic LPS exposure in mice. Wild-type, male C57BL/6 mice were treated with inhibitors of the NFκB signaling pathway including TPCA-1 (20 mg/kg, i.p.), BAY11-7085 (20 mg/kg, i.p.), or PPM18 (5 mg/kg or 15 mg/kg, i.p.) 1 h prior to administration of LPS (10 mg/kg, i.p.) Kidneys were collected for biochemical analysis at 3 h post-LPS and total RNA was isolated from the renal cortex. RT-qPCR analysis revealed no changes in transcript levels of PGC-1α following pre-treatment with TPCA-1 (A), BAY11-7085 (B), or PPM18 (C). Data are presented as fold change relative to control mice (mean ± S.E.M.; n ≥ 5 animals per group). Different superscripts above bars indicate statistically significant differences (p < 0.05). Note: TPCA-1 and BAY11-7085 were used in the same experiment, so control and LPS + vehicle animals/values are identical.
Hexokinase and PPP Activation in Sepsis-induced AKI

I was surprised to observe increased activity of glucose-6-phosphate dehydrogenase in association with HK activation in the renal cortex after systemic LPS exposure in mice. Since this was not in agreement with our original hypothesis that induction of HK would facilitate increased glucose flux through the glycolytic pathway, I did not further pursue the physiological relevance of PPP activation. However, it is possible that this shift in glucose metabolism in the renal cortex provides NADPH for glutathione regeneration and thus cellular antioxidant defense in the septic kidney. G6PDH knockout mice were previously generated and are viable to adulthood (367). It would be interesting to expose these mice to LPS and determine whether renal oxidative stress (and thus renal injury) increases. In addition, we could employ transgenic overexpression of G6PDH in the renal proximal tubule to further elucidate the role of this pathway in antioxidant defense. These studies could potentially identify a novel metabolic adaptation to protect renal cells after AKI and lead to interest in developing compounds that stimulate G6PDH/PPP activity for treatment of this disease.

MEK/ERK Inhibition in the CLP Model

My studies using GSK1120212 in the CLP model were largely focused on renal injury and dysfunction. Since this project was intended to address the therapeutic potential of MEK/ERK blockade in sepsis, it would be beneficial to further evaluate the effects of GSK1120212 on CLP-induced dysfunction of other organ systems. In particular, there is evidence of injury to the liver, lungs, pancreas, and skeletal muscle in the same CLP model used for our studies (159). Many of these parameters are easily assessed by serum markers commonly used in the clinic including aspartate transaminase (AST) and alanine transaminase (ALT) for the liver, amylase for the pancreas, and
creatine kinase (CK) for skeletal muscle. Unfortunately, there was not sufficient serum from the mice used in these experiments to measure all of these endpoints. It would also be interesting to conduct an additional study determining the effects of GSK1120212 on mortality in the CLP model. Together, these results could provide even stronger support for MEK/ERK inhibition as a viable therapeutic option for treatment of sepsis and associated organ dysfunction.
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