Decreased kidney mitochondrial content and PGC-1α following repeated low-dose cisplatin-induced kidney injury.

Andrew Joseph Orwick
University of Louisville

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DECREASED KIDNEY MITOCHONDRIAL CONTENT AND PGC-1α FOLLOWING REPEATED LOW-DOSE CISPLATIN-INDUCED KIDNEY INJURY

By

Andrew Joseph Orwick
Pharm.D., Sullivan University College of Pharmacy, 2012

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Department of Pharmacology and Toxicology
University of Louisville
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DEDICATION

This thesis is dedicated to my wife Jenna Orwick,

my child expected in August 2021,

and my mother, who passed away from breast cancer in June 2016.
ACKNOWLEDGEMENTS

I would like to thank my mentor Dr. Leah Siskind who took a chance on me during the interview process; having no research experience, she believed in my ability and potential. I would like to thank Dr. Bradford Hill for this continuous support and time spent helping me solidify my hypothesis to bring this project together. Additionally, I would like to thank the other committee members Dr. Levi Beverly, Dr. Steven Jones, and Dr. Geoffrey Clark, for their comments and assistance. I would like to express my thanks to my wife, Jenna, for her unconditional support and encouragement throughout this process. Many thanks to my family members and in-laws, who have always supported my decision to make a career change in my early thirties.
ABSTRACT

DECREASED KIDNEY MITOCHONDRIAL CONTENT AND PGC-1α FOLLOWING REPEATED LOW-DOSE CISPLATIN-INDUCED KIDNEY INJURY

Andrew Joseph Orwick

May 4, 2021

Cisplatin is highly effective and one of the most commonly used chemotherapeutic agents in the treatment of a number of different solid organ tumors. Unfortunately, the dose-limiting nephrotoxicity occurs in up to 30% of patients, which requires alterations to treatment regimens that are often less effective. The kidney’s function is to provide fluid homeostasis, and this is an energy-intensive process. Proper renal function is dependent on functional mitochondria. PGC-1α regulates mitochondrial number, respiratory capacity, and mitochondrial proteins in proximal tubule cells. We delivered low-dose cisplatin to mice via intraperitoneal injections once a week for 4 weeks. The mice were then euthanized, and the kidneys were immediately extracted and frozen. Our data show that repeated dosing of cisplatin results in reduced renal function, increased fibrosis, reduced mitochondrial content, and reduced PGC-1α in kidney cortices. We hypothesize that increasing PGC-1α will protect against cisplatin-induced kidney injury.
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INTRODUCTION
KIDNEY PHYSIOLOGY

The kidney has numerous functions, and is involved in homeostasis, vitamin D metabolism [1], gluconeogenesis [2], and endocrine functions both locally and systemically [3-5]. The information discussed below is primarily focused on kidney’s role in maintaining fluid homeostasis. The kidney manages fluid composition by regulating the reabsorption and secretion of water, glucose, NaCl, NaHCO₃, and amino acids. The kidneys account for less than 1% of body weight but receive 20-25% of cardiac output [6, 7]. Additionally, the kidneys consume 7-10% of the total oxygen uptake to produce enough ATP to carry out the energy-intensive process of reabsorption [6, 8]. The kidneys have one of the highest resting metabolic per gram of tissue in healthy adults [9]. The high metabolic rate of the kidneys is mirrored by mitochondrial density and oxygen consumption per gram of tissue which is the second-highest in the body, preceded only by the heart [10-12]. The partial pressure of oxygen in the kidney varies greatly between regions. Blood enters into the kidney through the cortex, and only 10% of the blood vessels branch off to supply the medulla [8]. Different regions of the nephron in the cortex have varying partial pressure of oxygen ranging from 90mmHg down to 20mmHg [8, 13, 14]. The heterogeneity of the oxygen supply in the kidney makes various regions highly susceptible to hypoxia-induced damage and cell death. The corticomedullary junction is highly sensitive to hypoxia because it has minimal perfusion/oxygen supply despite being a highly energy-consuming segment.

The smallest functional unit of the kidney is the nephron, whose ultimate function is waste excretion through the concentration of filtrate into urine. Nephrons are
composed of three major sections with distinct roles; (1) the glomerulus that filters the blood, (2) the tubule that primarily reabsorbs vital nutrients and secretes solutes, and (3) the collecting duct that carries urine to the calyx, then renal pelvis, and finally exits the kidneys via the ureters [15]. There are over 26 different cell types in human adult kidneys to carry out the wide range of functions needed for proper renal function [16]. Glomerular filtration is the first step in urine production and is a passive process powered by hydrostatic pressure from the heart via glomerular capillaries. The glomerular filtration rate is the volume of fluid that is filtered per minute. The kidneys filter between 180-200 liters of blood daily, while the average human only has between 3-5 liters of circulating blood and only produces approximately 1.5 liters of urine [15, 17]. The tubule has four different segments, each with distinct absorptive properties for maintaining plasma osmolarity [15]. The renal proximal tubule epithelial cells (RPTEC) are responsible for 70% of the reabsorption and are highly enriched in mitochondria to perform this energy-intensive process [18]. RPTECs produce the majority of the required adenosine triphosphate (ATP) from β-oxidation of fatty acids in the mitochondrial matrix [19]. This process requires high oxygen levels and makes RPTECs highly susceptible to hypoxia, IRI, and vasculature damage. The majority of the energy produced in RPTECs is used for active transport by the Na⁺/K⁺ ATPase pump, which generates ion gradients across the cell membrane for reabsorption of different nutrients against concentration gradients. Over 90% of the energy production in the kidney occurs through oxidative phosphorylation, and any disruption to oxygen delivery or mitochondrial function can increase the risk of acute kidney injury (AKI) [8]. Elevated levels of glycolysis and increased mitochondrial dysfunction have been identified as hallmarks of various AKI models [20-22].
ACUTE KIDNEY INJURY

AKI is defined by a rapid loss in kidney function occurring in a period of hours to days after insult. A decline in kidney function is characterized by decreased glomerular filtration rate (GFR), decreased urine production, and increased waste product retention [23]. The loss in kidney function is measured clinically by an increase in serum creatinine (SCr) and blood urea nitrogen (BUN) [24]. Hospital admission is a risk factor for developing AKI, with incidences of 15% for adults and 25% for children after admission [25, 26]. The incidence rate of AKI for patients admitted into the intensive care unit (ICU) is above 50% [27]. Multiple studies have shown the development of AKI is also associated with an increased risk of progressing to chronic kidney disease (CKD) [26, 28-32]. Additionally, the risk of mortality is increased 5 to 6-fold higher in patients with AKI when compared to patients without AKI [33, 34].

The incidence and prevalence of AKI are difficult to track due to the different clinical markers used to diagnose and the wide range of disease classifications [35]. In 2004 the Acute Dialysis Quality Initiative proposed the Risk/Injury/ Failure/Loss/ESRD (RIFLE) classification for AKI [36]. Follow by the Acute Kidney Injury Network (AKIN) classifications in 2007 [37]. Then in 2012, Kidney Disease: Improving Global Outcomes (KDIGO) group published guidelines combining the RIFLE and AKIN definitions [38]. These guidelines were developed to provide measurable criteria to universally define the stages of kidney injury using serum creatinine and urine output. The excretion of waste products from the blood is a vital function of the kidneys, and a decline in GFR can be measured by an increase in waste products in the blood. Serum creatinine and blood urea nitrogen are two waste products that, when elevated, are used to estimate a decline in GFR. The KDIGO group’s parameters define AKI as an absolute increase in SCr $\geq 0.3$ mg/dL within 48 hours or an increase in SCr $\geq 1.5$ times within 7 days, and a
decrease in urine out of 0.5 ml/kg/hour for 6 hours [38]. The most recent estimate of the
global incidence of AKI is almost 40 cases per 1,000 people [26].

BIOMARKERS OF AKI

The clinical markers of AKI (decreased urine output, elevated SCr, and BUN) are
neither sensitive nor specific for AKI [26]. Additionally, changes in SCr do not correlate
with the severity of kidney damage nor point to the cause of the kidney damage [39, 40].
Changes in serum creatinine are delayed 24-72 hours after the renal injury has
occurred, and its levels are affected by age, race, sex, body weight, metabolism, and
protein intake [35]. BUN has similar limitations to SCr and only elevates after the renal
injury has occurred [41]. New biomarkers that are more specific and sensitive for kidney
damage have been sought after. Two of the newer biomarkers are neutrophil gelatinase-
associated lipocalin (NGAL) and kidney injury molecule 1 (Kim-1).

Neutrophil gelatinase-associated lipocalin (NGAL), 25-kDa polypeptide of the
lipocalin superfamily, was initially identified in human neutrophil gelatinase [42]. NGAL
was first discovered as a biomarker after it was elevated in multiple animal models of
AKI [43-45]. NGAL is a rapid response protein to tissue injury, and while elevated during
AKI events, NGAL is also elevated during acute and chronic inflammatory conditions
[46]. NGAL is released by activated neutrophils during bacterial infections and systemic
inflammation, making its use as a biomarker of AKI more complex [47]. NGAL can be
assayed from blood or urine, but urinary NGAL appears to carry higher specificity for AKI
[48]. NGAL is approved in Canada and Europe for diagnosing AKI [49, 50].

Kim-1 is a transmembrane cell surface receptor on epithelial and
lymphoid/myeloid cells. Kim-1 is a scavenger receptor that removes oxidized LDL
particles and interacts with phosphatidylserine to allow apoptotic cells to undergo
phagocytosis. Under normal conditions, Kim-1 expression in proximal tubule cells is
minimal, but after ischemic or nephrotoxic insult, expression of Kim-1 is significantly
upregulated in proximal tubule cells [51, 52]. Kim-1 elevation occurs within 1-3 hours of kidney injury, making it a very early signal of damage [48]. Additionally, urinary excretion of Kim-1 has been approved by the FDA and EMA for preclinical assessment of nephrotoxicity [46, 48].

The heterogeneity of the kidney in cell type, function, tissue oxygenation, and metabolism further complicates our ability to develop protective measures from AKI [8]. The pathophysiology of AKI is a complex process that is still not completely understood. The major obstacle is that AKI is a complex process of heterogenous conditions that all result in loss of kidney function as the endpoint.

**ETIOLOGY OF AKI**

The kidney encompasses multiple biologic functions, heterogenous tissue oxygenation, and metabolism, various roles in fluid homeostasis along the nephron resulting in over 26 cell types. The process of AKI varies according to the primary insult. The wide range of insults, including nephrotoxic agents, ischemia, dehydration, or sepsis, damage different regions of the kidney, but all end with reductions in kidney function. The complexity of the kidney makes the classification of AKI difficult. AKI can be categorized in multiple ways: the clinical setting, reversibility or response to therapy, the general mechanism of injury, primary affected anatomical compartment, dominant underlying pathophysiology, or traditional categories [8]. The traditional categories of AKI are Prerenal, Intrinsic Renal, and Postrenal [41, 53].

Prerenal AKI is defined as decreased renal perfusion resulting in lower GFR. Prerenal AKI is the most common cause, with over 70% of the cases attributed to prerenal causes [53]. The most common cause of prerenal AKI is from loss of fluid volume. The loss of fluid volume can be from excessive vomiting or diarrhea or overuses of diuretic drugs. Other causes of prerenal AKI include decreased perfusion from heart
failure, systemic vasodilation from sepsis, and changes to intrarenal vasoconstriction via different medications (NSAIDs, ACE-I, ARBs, Antirejection agents) [53].

Intrinsic AKI is defined by disruption to the internal processes of the kidney. Intrinsic AKI is further divided up into categories of the primary affected anatomical compartment. These classifications are divided into glomerular damage, tubular damage, interstitial damage, and vascular damage [41]. Acute tubular necrosis is the most common cause of intrinsic AKI in hospitalized patients [41, 53]. The tubular damage is usually caused by ischemia or nephrotoxic agents. The tubule cells are highly energetic, where the majority of their energy is produced through oxidative phosphorylation, making them highly sensitive to prolonged reduction in oxygen delivery. Different exogenous chemicals, including many commonly used drugs (aminoglycosides, amphotericin B, cisplatin, radiocontrast media), damage tubule cells resulting in tubule apoptosis and necrosis.

Acute tubular necrosis-induced AKI involves several pathophysiological processes, including endothelial damage, vascular impairment, tubular cell death, and inflammatory/immune response [54]. This process has been divided into four phases, initiation, extension, maintenance, and recovery [41].

The initiation phase is characterized by functional damage to the tubule cells from ischemia or nephrotoxic agents and subsequent decreases in renal blood flow, further decreasing the oxygen levels and ATP production. These damaged cells epithelial and endothelial cells release cytokines and chemokines, which initiate the inflammatory cascade. The tubule epithelial cells responsible for most of the kidney's reabsorption function begin to undergo morphological changes and lose their brush border membranes. Loss of brush border membranes reduces their surface area and absorptive capacity. Additionally, the apoptosis or necrosis process begins, and the dead cells slough off, leading to tubular obstruction [41].
The extension phase is defined as the persistent hypoxia and inflammatory response from that initial ischemic or nephrotoxic agent. The heterogeneity of tissue oxygenation in the kidney makes certain areas more susceptible than others during this phase [8]. In the cortex, where oxygen levels have returned to near-normal levels, the tubule cells have started to recover and undergo repair. The corticomedullary junction (CMJ) and outer medullary region have lower perfusion levels, and the hypoxia leads to cell injury and ongoing apoptosis/necrosis. As cell death occurs in the CMJ and outer medulla, the GFR continues to fall. Additionally, the production and release of cytokines and chemokines continue in these regions during this phase and stimulate the inflammatory cascade [41].

The maintenance phase characterizes the beginning of repair, increased cell migration, apoptosis, and proliferation as cells attempted to maintain tubule integrity. GFR decline halts during this phase as tubule cells begin to dedifferentiate and migrate towards the damaged area and begin to reestablish tissue structure. Additionally, tissue perfusion returns to baseline during this phase [41].

Recovery is the final phase of acute tubular necrosis, and as tubule epithelial cells reestablish their polarity and structure, cellular and organ function returns to normal. Proinflammatory and profibrotic cells must be cleared for a full recovery. The ability of the kidney to fully recover from injury is dependent on many factors. Repeated insults maybe inhibit the repair process and result in chronic impairment [41].

Unlike prerenal injury, in acute tubular necrosis, removing the nephrotoxic agent or reperfusion of the kidney does not result in immediate recovery. The loss of tubular cells requires time to replenish and may require renal replacement therapy depending on the severity [41, 53].

Glomerular damage AKI is caused by acute inflammation of the blood vessel and glomeruli resulting in loss of renal function. Glomerulonephritis is commonly caused by
systemic illness, including but not limited to systemic lupus erythematosus, arthritis, viral infection, and pulmonary-renal syndromes [41, 53].

Damage to the interstitial tissue of the kidney can also result in AKI. The type of AKI is most commonly caused by allergic reactions to medications or from an infection. The acute interstitial nephritis generally resolves once the offending agent is removed [41].

The kidney accounts for less than 1% of total body weight but receives over 20% of cardiac output [6, 7]. The highly vascularized kidneys are very susceptible to vascular damage-induced AKI. Damage to the intrarenal blood vessels results in decreased perfusion and lower GFR. Common causes include arterial catheterization, hypercoagulation disorders, and vascular surgery [41, 53].

Postrenal causes of AKI are a result of obstruction to urine flow. Prostate hyperplasia is a common cause of postrenal AKI in older men. Identification and removal of the obstructing object generally result in a return to baseline [41, 53].

The current markers of AKI are only able to diagnosis after the injury has occurred and renal function has already declined. There are currently no FDA-approved agents to prevent or treat AKI. A better understanding of the disease process is needed to develop potential treatments. It was previously thought that if renal function recovered (as determined by the return of SCr and BUN to baseline levels) after an AKI incident, there would be no long-term consequences [55]. Several recent studies have indicated that patients who develop AKI are up to 10 times more likely to develop chronic kidney disease (CKD) [28-32]. Even patients that do not develop clinical AKI are at risk for long-term declines in renal function [56, 57]. In this study, the need for dialysis following an AKI event resulted in an increased risk of developing CKD and mortality [28].
CHRONIC KIDNEY DISEASE

CKD is defined as the presence of albuminuria, a marker for kidney damage or decrease in GFR, a marker for kidney function for a duration of greater than 3 months [55, 58]. Again, because of the complexity of the kidney, CKD is a heterogeneous disorder affecting the structure and function of the kidney. CKD affects over 20 million Americans, and over 500,000 have end-stage renal disease (ESRD) [59]. CKD generally coincides with interstitial fibrosis, glomerulosclerosis, and chronic inflammation [55]. The most common causes of CKD are diabetes and hypertension [59]. AKI, however, is becoming more recognized as a potential cause of CKD, even after recovery from initial nephrotoxic insult [55]. Additionally, more studies have shown that multiple nephrotoxic events leading to AKI also increase the likelihood of developing CKD [28, 60].

The most common cause of AKI is acute tubular necrosis (see above). After an AKI event, the kidney utilizes a wound healing process to recover from the insult. However, the repair process can astray if subsequent insults occur during this time resulting in maladaptive repair. Maladaptive repair during an AKI event can lead to renal fibrosis and CKD [55].

MALADAPTIVE REPAIR

Maladaptive repair is defined by incomplete recovery following an AKI event leading to abnormal kidney structure and function. Increased age and increased number of repeated nephrotoxic events increase the likelihood of maladaptive repair and development of CKD. Maladaptive repair is characterized by renal fibrosis, vascular rarefaction, tubular necrosis/apoptosis, glomerulosclerosis, and infiltrating inflammatory cells within the kidney [55]. In the maintenance phase of AKI, tubule cells begin to dedifferentiate and repopulate lost epithelium. Under normal healing conditions, these cells enter the cell cycle and proliferate to maintain tissue structure [61]. In the
maladaptive repair process, these cells undergo cell cycle arrest and become senescent. These senescent cells can adopt a secretory phenotype which is associated with the release of connective tissue growth factors (CTGF) and transforming growth factor-beta (TGF-β). CTGF and TGF-β have been associated with chronic inflammation, collagen deposition, and vascular rarefaction [55].

These secretory senescent cells affect other epithelial cells, pericytes, and the immune system. Particularly these cytokines increase the production of activated myofibroblasts [54]. These activated myofibroblasts are considered the primary source of collagen deposition and fibrotic matrix, which are hallmarks of the maladaptive repair process [55]. Activated myofibroblasts are essential to the normal healing process where they deposit extra cellular matrix (ECM), which acts as a scaffold to maintain tissue structure integrity in damaged tissue. Under maladaptive conditions, the myofibroblasts are overstimulated, leading to excessive deposition of ECM and renal fibrosis. Renal fibrosis is defined as excessive deposition and accumulation of extra cellular matrix (ECM). The accumulation of ECM in renal tissue starts to disrupt normal tissue structure, impair function, and lead to CKD [62, 63]. In the normal healing process, extra ECM is degraded, and profibrogenic myofibroblasts are removed. However, repeated nephrotoxic events or chronic injury can lead to inadequate removal of ECM, resulting in long-term organ fibrosis [63].

The injured tubule cells also secrete proinflammatory cytokines and chemokines, leading to the activation of toll-like receptors (TLR). TLRs are a class of cell-surface proteins that are responsible for activating the innate immune response. Tubule epithelial cells express TLR, and during AKI events, these receptors are activated, resulting in the production of tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein 1 (MCP-1), and interleukin 6 (IL-6). Increased cytokine production leads to immune cell recruitment to the damaged kidney [54]. These immune
cells are part of the normal healing process and help remove necrotic cells and debris. Chronic production of these cytokines can lead to overactivation of the immune cells and maladaptive repair [55].

Repeated nephrotoxic insults impair the normal healing process in the kidney and can result in maladaptive repair. The maladaptive repair process contributes to the loss of kidney function as normal tissue structure is not restored in the kidney following injury. Chronic inflammation and excessive fibrosis are characteristics of the maladaptive repair process.

CISPLATIN

cis-Diamminedichloroplatinum (II) (cisplatin) is an inorganic water-soluble planar molecule consisting of only 11 atoms. The molecular structure is a single central platinum atom surrounded by two chloride and two ammonium atoms in the cis position (Figure 1A). Cisplatin was first synthesized in 1845 by an Italian chemist, Michael Peyrone, but the structure was unknown [64]. Nearly 50 years later, in 1893, Alfred Werner published his transformative paper where he put forward his co-ordination theory of complex compounds and revealed the chemical structure of cisplatin; he would go on to receive a Noble Prize in Chemistry for this work in 1913 [65, 66]. Platinum molecules were thought to have no biological activity until the accidental discovery of cisplatin’s cytotoxicity by a biophysicist at Michigan State University, Barnett Rosenberg, in 1965 [64, 67]. Dr. Rosenberg’s laboratory was investigating the effects of electricity on bacterial cell growth, and they were using platinum electrodes to deliver the current to culture media. They discovered the addition of electrical current stopped the cell division of the bacterial cells. It would take another two years for the laboratory to discover that electricity was causing the release of platinum molecules responsible for the inhibition of bacterial cell growth [68]. The Rosenberg laboratory would go on to demonstrate the
ability of cisplatin to inhibit tumor growth of sarcoma 180 and leukemia L1210 cells, and next showed the ability of cisplatin to reduce tumor size in a rat sarcoma model [69]. By 1972 the National Cancer Institute began clinical trials using cisplatin for advanced testicular cancer, and in 1978 cisplatin was FDA approved for testicular, ovarian, and bladder cancer [67]. Cisplatin was the first inorganic heavy metal used for cancer chemotherapy and changed how inorganic molecules were viewed [70]. Testicular cancer outcomes were drastically impacted by the addition of cisplatin to the treatment regimens. Prior to the use of cisplatin, metastatic testicular cancer had a 90% mortality rate at 1 year; after the addition of cisplatin, 80% of patients were cured of metastatic testicular cancer [71].

Cisplatin is now a generic drug, which makes tracking the current usage of cisplatin difficult. There are currently 215 active treatment trials on ClinicalTrials.gov database in the United States to highlight cisplatin's continued relevance in clinical practice. Cisplatin's mechanism of action involves the formation of DNA, RNA, and protein adducts, which in turn inhibits DNA synthesis and replication of rapidly dividing cells [72, 73]. The effectiveness of cisplatin is impaired by its dose-limiting side effect, nephrotoxicity.

Thirty percent of patients who receive cisplatin develop acute kidney injury (AKI) [74]. AKI is a life-threatening condition, and the onset of AKI from cisplatin prevents effective treatment that often requires a change in therapy or dosage reduction. Although newly developed cisplatin derivatives such as carboplatin and oxaliplatin have reduced nephrotoxicity, they are much less effective in treating many forms of cancer [75]. Cisplatin would be a much more effective cancer treatment if this toxicity could be prevented; however, there are currently no therapies approved to prevent or treat AKI. Understanding how cisplatin induces nephrotoxicity and AKI is crucial to our ability to develop preventative measures.
Figure 1. Cisplatin Chemical Structure and Mechanism of Action.

(A) Chemical structure of cisplatin. (B) Mechanism of action for cisplatin. Created with BioRender.com
CISPLATIN’S MECHANISMS OF NEPHROTOXICITY

Cisplatin’s nephrotoxicity correlates with the patient’s dose, frequency, and cumulative exposure [76]. The kidney receives between 20-25% of the cardiac output while only accounting for less than 1% of the body weight. Renal cells are exposed to high levels of cisplatin [6]. Cisplatin in the blood is uncharged and is cleared by the kidney via glomerular filtration and tubular secretion [77]. Cisplatin, once inside the cell, becomes charged and accumulates in renal epithelial cells, leading to cisplatin concentrations higher in the kidney than in the blood [74]. Additionally, cisplatin is transported intracellularly via copper transporter (Ctr1) and the organic cation transporter (OCT2), both of which are highly expressed basolaterally on proximal tubule cells, increasing their susceptibility to cisplatin toxicity [78]. Downregulating Ctr1 in kidney cells in vitro protected cisplatin toxicity by reducing uptake into the cell [79]. Additionally, the coadministration of OCT2 substrate reduced cisplatin uptake and toxicity in vitro [80, 81]. The role of OCT2 transport in cisplatin renal toxicity was further confirmed by in vivo work showing the knockout of OCT2 protected mice from cisplatin nephrotoxicity [82, 83]. Cisplatin-induced nephrotoxicity involves multiple mechanism including proximal tubule cell damage, increased oxidative stress, inflammatory response, and vascular injury [74, 78, 84].

The classical mechanism of cisplatin toxicity is through its interaction with DNA [72, 85, 86]. Once administered, cisplatin enters the bloodstream, where relatively high concentrations (100mM) of chloride limit the replacement of cisplatin’s two chloride ligands [86, 87]. However, once inside the cell, the chloride concentrations are much lower (4-20 mM); this allows cisplatin to be hydrolyzed with the chloride ligand being replaced by a water molecule (Figure 1B) [86, 87]. The newly formed hydrolyzed cisplatin is a reactive positively charged electrophile that reacts with nucleophilic sites forming adducts on DNA, RNA, and protein [85, 86]. Cisplatin binds to DNA is capable of
forming monoadducts, DNA-protein cross-links, interstrand cross-links, or the most common intrastrand cross-link adducts [88]. These DNA adducts inhibit the cell’s ability to synthesize DNA resulting in cell cycle arrest of rapidly dividing cells [72, 73]. The anti-tumor effects of cisplatin were thought to occur through this mechanism, and this was supported by the finding that cells with defective DNA repair processes were more sensitive to cisplatin-induced cell death. The idea of nuclear DNA (nDNA) being the primary target of cisplatin-induced damage and cell death has been contested. In vitro work has shown that only a small percentage of cellular platinum is bound to nDNA, and sensitivity to cisplatin cell death poorly correlates with the extent of nDNA platination [89]. Additionally, work with Chinese hamster ovary (CHO) cells showed that cisplatin adducts formed at a higher rate on the mitochondrial DNA (mtDNA) compared to the nuclear DNA (nDNA) [90]. This idea was further supported by in vitro work showing enucleated cells were sensitive to cisplatin-induced apoptosis independent of nDNA damage. Other DNA damaging agents were not able to induce this same response, indicating that cisplatin had unique cytosolic targets [91].

The idea that mitochondria, mitochondrial proteins, and mtDNA are the primary targets of cisplatin-induced toxicity has been supported by multiple lines of evidence [92]. Additionally, once intracellular cisplatin becomes a positively charged species, which preferentially accumulates in the negatively charged mitochondria (Figure 1B) [74, 93]. The density of mitochondria and the mitochondrial membrane potential have been shown to correlate with increased sensitivity to cisplatin [94, 95]. Another in vitro experiment treated cells with ethidium bromide to deplete mtDNA, which produced cells highly resistant to cisplatin toxicity [94]. Mitochondrial DNA is likely more susceptible to cisplatin-induced DNA damage than nDNA by mitochondria’s lack of effective DNA repair mechanisms [88, 96, 97]. All of this points to mtDNA as an important factor in the toxicity of cisplatin.
Renal proximal tubule epithelial cells (RPTECs) are highly enriched in mitochondria [18]; express both Crt1 and OCT2 making them very sensitive to cisplatin toxicity. RPTECs produce the majority of their energy from β-oxidation of fatty acids in the mitochondrial matrix [19]; cisplatin has been shown to disrupt mitochondrial energetics and fatty acid oxidation in RPTECs [98-100]. Defective fatty acid oxidation has been associated with a decline in renal function and the development of renal fibrosis [101-103]. Agonists of PPAR-α reduce cisplatin nephrotoxicity in vivo [99, 100]. Additionally, damage or loss of mtDNA from cisplatin leads to a loss in mitochondrial protein levels and a disruption of the mito-nuclear protein ratio leads to reduced respiratory capacity and malfunctioning RPTECs [104, 105]. Cisplatin also induces cell death in RPTECs via multiple mechanisms including the extrinsic pathway activated through death receptors, the intrinsic mitochondrial pathway, and the endoplasmic reticulum stress pathway [74]. Damage to the RPTECs and their mitochondria leading to decline in renal function are of particular relevance to this work.

Cisplatin-induced inflammation is another mechanism of nephrotoxicity that has been studied in cisplatin-induced kidney injury. Cisplatin treatment is associated with increased expression of many different proinflammatory cytokines and chemokines, including interleukin 1 beta (IL-1β), IL-6, MCP-1, and TNF-α [78, 84]. Injured RPTECs release endogenous intracellular molecules known as damage associated molecular patterns (DAMPs). DAMPs activate Toll-like receptors (TLR), which induce the expression of inflammatory genes to help repair damaged tissue. The activation of TLR by DAMPS can result in a positive feedback loop where high levels of tissue damage result in pro-inflammatory response leading to more tissue damage and chronic inflammation [106]. Cisplatin-induced inflammation is thought to occur through the activation of TLR4, leading to the production of chemokines and cytokines, including TNF-α. Zhang et. al. went on to confirm this by using TLR4 knockout mice that were
resistant to cisplatin-induced kidney injury and inflammation when compared to wild type [107]. Other groups have looked specifically at the role of TNF-α in cisplatin-induced kidney injury. Ramesh and Reeves used TNF-α knockout mouse and a pharmacological inhibitor of TNF-α; both models provided protection against cisplatin-induced kidney injury [108]. These experiments also provided data to show inhibition of TNF-α signaling reduced the production of other key pro-inflammatory mediators. Additionally, Maekawa et. al. demonstrated how cisplatin induces mitochondrial damage and leakage of mtDNA into the cytosol. Cytosolic mtDNA activates the cGAS-STING pathway leading to inflammation which contributes to the development of cisplatin-induced kidney injury [109], again highlighting the importance of mitochondria in cisplatin-induced kidney injury.

Cisplatin also damages vascular endothelial cells, which leads to vasoconstriction and reduced renal blood flow. The reduced renal blood causes a drop in GFR and creates a more hypoxic environment. The hypoxic conditions following cisplatin further contribute to tubule cell death and inflammation. The mechanism of cisplatin’s nephrotoxicity involves tubule cell death, inflammation, and vascular damage; these pathways are interconnected and show the complexity of cisplatin-induced kidney injury [84].

The nephrotoxicity of cisplatin has been a problem since it was first introduced into the clinic. Multiple strategies have been developed to prevent the toxicity. The adjunct treatment with hydration regimens and the use of diuretics such as mannitol have been tried with mixed results [110]. Additionally, reduction in dose and extension in the duration of administration time have been adopted. Unfortunately, there are no preventative measures approved for cisplatin-induced AKI. The current clinical guidelines recommend pre-hydration with normal saline and avoiding the use of diuretics for patient receiving cisplatin therapy [111].
MODELS OF CISPLATIN INJURY

Thirty percent of patients who receive cisplatin develop acute kidney injury (AKI) [74]. These patients present with increased BUN and SCr levels, and a rapid decline in renal function. Traditionally, the accepted standard model of cisplatin-induced kidney injury has been to use a single high dose (10-30 mg/kg) of cisplatin. This model results in high levels of tubular necrosis, rapid decline in renal function, and is fatal to mice within 3-4 days cisplatin [112, 113]. The single high dose model of cisplatin has allowed researchers to elucidate the mechanisms involved in the patients that develop AKI in the clinic; however, this model does not allow for long-term studies [113, 114].

In the past, it was assumed that if renal function recovered (as determined by the return of SCr and BUN to baseline levels) after an AKI incident, there would be no long-term consequences [55]. However, several recent studies indicate that patients who develop AKI are 10 times or more likely to develop chronic kidney disease (CKD) [28-32]. Even patients that do not develop clinical AKI are at risk for long-term declines in renal function [56, 57]. For the other 70% of patients who have subclinical kidney damage following cisplatin, little is known about their risk of developing CKD.

In the clinic, patients are typically treated with periodic low doses of cisplatin over weeks to months, not a single high dose. The Siskind laboratory and others have recently developed repeated low dose cisplatin (RLDC) models that more closely mimic the dose regimen of cisplatin in the clinic and enables the study of long-term impacts on the kidney [112, 113, 115-121]. In the RLDC model, mice receive 7-9 mg/kg cisplatin once a week for four weeks. The mice treated with the RLDC regimen can survive 6 months following treatment. Published data from our laboratory indicate that the RLDC model causes a low level of repeated injury [115-120]. These data indicate that repeated low levels of cisplatin injury induce fibrosis which progresses to CKD and that the cellular processes induced in the kidney by the RLDC model differ greatly from that when a
single high dose of cisplatin is administered [115-120]. The mild injury caused by RLDC that does not meet the clinical criteria for AKI can progress to CKD [115].

The RLDC model can be broken up into two phases, the injury and progression phases. The injury phase consists of the four weeks of cisplatin treatment and is characterized by a mild decline in kidney function, ER stress, increased markers of kidney damage (Kim-1, NGAL), inflammation, and development of fibrosis. The progression phase is defined by the removal of the nephrotoxic agent and markers of AKI (Kim-1, NGAL) return to baseline; however, there is persistent inflammation, increased fibrosis, and development of CKD [115-120]. Additionally, following the injury phase, mice show mild increases in BUN; however, at the end of the progression phase (6 months post-treatment), mice have a progressive increase in BUN levels compared to vehicle-treated mice and the injury phase counterparts. This suggests that mice have progression and permanent loss of kidney function following the RLDC regimen even without elevated clinical markers of AKI during the injury phase [115]. The cellular biology involved in each phase is quite different, and the mechanisms involved in both phases of this model remain largely unknown [116]. Elucidating these mechanisms is necessary for identifying novel therapeutic targets.

MITOCHONDRIA AND PGC-1α

As previously mentioned, the kidneys have the highest mitochondrial density and oxygen consumption per gram of tissue in the body, preceded only by the heart [10, 11]. The kidney is responsible for maintaining fluid homeostasis and the renal proximal tubule epithelial cells (RPTECs) are responsible for 70% of the reabsorption; RPTECs are highly enriched in mitochondria to sustain this highly energy-intensive process [18]. RPTECs produce the majority of the required adenosine triphosphate (ATP) from fatty acid oxidation (FAO) in the mitochondrial matrix [19]. A number of inherited genetic
mitochondrial diseases coincide with renal impairment to further illiterate the connections between mitochondrial function and kidney function [122-126]. Disorders of mitochondrial function are referred to as mitochondrial cytopathies, and the most common mitochondrial diseases with kidney involvement cause tubular defects [125, 126]. Fanconi Syndrome is an example of a mitochondrial disease with tubular defects that results in electrolyte disturbances. RPTECs are highly dependent on mitochondrial function because of their high-energy demand and because they also lack the capability to synthesize ATP anaerobically from glycolysis [127].

Mitochondria are intracellular organelles with numerous essential roles, including production of ATP, co-factors, amino acids, induction of apoptosis, calcium homeostasis, and regulation of cellular redox state and innate immune signaling [128, 129]. Each mitochondrion has its own circular genome, and there are multiple copies (100-10,000) per cell [96, 97]. Mitochondria are dynamic organelles with inner and outer membranes and constantly change their shape and size through fusion and fission processes, known as mitochondrial dynamics [130]. The opposing effects of fusion and fission are interconnected and balanced to maintain the overall normal morphology of mitochondria. Excessive activation of either process can result in fragmentation (fission) or hypertubulation (fusion), both if left unchecked, can result in mitochondrial dysfunction [130]. Mitophagy is a mechanism that uses the autophagy pathway to selectively degrade damaged mitochondria. During AKI events, damaged mitochondria are cleared via mitophagy and need to be replaced with new mitochondria. New mitochondria are created from existing ones by a process called mitochondrial biogenesis. Mitochondrial biogenesis (MB) involves the production of new mitochondrial mass and replication of mtDNA. MB and replication of mtDNA occurs independent of the cell cycle unlike replication of nDNA [96]. MB is a highly regulated process, and its activation is controlled by a set of transcription factors that link environmental cues to cellular energy status and
adaptive stress response. The master regulator of MB is a transcriptional coactivator, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) [103, 131-133].

PGC-1α was identified in brown adipose tissue as a cold-inducible regulator of adaptive thermogenesis in 1998 by the Spiegelman laboratory [134]. The name is derived from the originally identified interaction between the PGC-1α protein and PPARγ (peroxisome proliferator-activated receptor-γ) protein. The Spiegelman laboratory would go on to show PGC-1α’s role in mitochondrial biogenesis. They demonstrated that PGC-1α activated the transcription factor NRF-1 (Nuclear Respiratory Factor 1), which subsequently leads to the expression of Tfam (Transcription factor A, mitochondrial) and other nuclear-encoded mitochondrial proteins [135]. The PGC-1α protein is interesting, as it does not directly bind to DNA, but it binds to other transcription factors already bound at their respective response elements. The binding of PGC-1α to the already bound transcription factors coactivates them to exert their regulatory effect [103, 131-133, 136]. PGC-1α is highly expressed in metabolically active tissues such as the heart, kidneys, liver, skeletal muscle, brain, and brown adipose tissue.

It is now understood that PGC-1α is a master transcriptional regulator of many pathways, including mitochondrial biogenesis, fatty acid oxidation, lipogenesis, thermogenesis, and glucose metabolism [103, 131-133]. The expression of PGC-1α can be induced by a number of different external stimuli, including exercise, fasting/nutrient deprivation, hypoxia, cAMP activation, and oxidant stress. The three main transcription factors that regulate PGC-1α expression are myocyte enhancer factor-2 (MEF2), activating-transcription factor 2 (ATF2), and cAMP response element-binding protein (CREB). The promotor region for PGC-1α contains binding sites for MEF2, ATF2, and CREB that increase the expression of PGC-1α. ATF2 and CREB bind to the CREB-responsive element (CRE) site, and MEF2 binds to its own specific site on the PGC-1α promotor. Additionally, PGC-1α expression can be suppressed by external stimuli,
including pro-inflammatory and pro-fibrotic mediators. The pro-inflammatory factor, TNF-α, activates NF-κB, which leads to epigenetic downregulation of PGC-1α via histone H3 deacetylation [137]. Additionally, activation of Notch signaling and its downstream target Hes1, a profibrotic transcription factor, can directly bind the PGC-1α promoter region and inhibit PGC-1α expression [138]. Of particular relevance to the RLDC, TGF-β1 stimulates activation of Smad3, which again leads to decreases PGC-1α levels through epigenetic downregulation [102]. To further add to the complexity of PGC-1α expression, Long et. al. have shown that long noncoding RNA (lncRNA) taurine-upregulated gene 1 (Tug1) interacts with PGC-1α. They demonstrated that Tug1 acts as a scaffold and allows the PGC-1α protein to bind to its own promoter and increase expression [139].

Finally, the activation of TLR by various stimuli leads to activation of the MEK1/2, ERK1/2 pathway, which then interacts with CREB leading to decreased PGC-1α expression [140].

PGC-1α is also regulated by post-translational modifications, including phosphorylation, deacetylation, acetylation, and methylation. These modifications affect the protein's activity and stability and determine which transcription factor will interact with PGC-1α. There are several kinases that can phosphorylate PGC-1α at multiple serine and threonine sites. The most well-characterized of these kinases are p38 MAPK, AKT, AMPK, S6 kinase, and GSK3β. Phosphorylation by AMPK and p38 MAPK leads to the activation of PGC-1α, whereas phosphorylation AKT inhibits activity and GSK3β increases its degradation [103, 131-133]. The half-life of the PGC-1α protein is only 2-3 hours, and the phosphorylation p38 MAPK also helps stabilize the PGC-1α protein, allowing for a longer activity window [141]. Additionally, SIRT1 activates PGC-1α by the deacetylation of the lysine residues, and PRMT1 (protein arginine methyltransferase 1) activates PGC-1α by the methylation of arginine residue [103, 131-133]. All of this together shows that PGC-1α expression and activity are finely tuned and highly
responsive to environmental cues relaying the energetic/metabolic demand of the cell to changes in gene expression.

Mitochondria have over 1,500 proteins, and these are almost entirely encoded in the nuclear genome. Mitochondrial DNA only encodes two ribosomal RNAs, 22 transfer RNAs, and 13 messenger RNAs that are all involved in the formation of supercomplexes of the electron transport chain [96, 97, 142]. Proper mitochondrial function requires coordination between nuclear-encoded mitochondrial genes and the mtDNA encoded genes. If the replication or synthesis of mtDNA is inhibited or mtDNA is damaged, the steady-state levels of the mtDNA proteins can be reduced. The significant reduction in mtDNA encoded proteins results in a mito-nuclear protein imbalance and subsequent reduction in cellular respiration [96, 97, 142]. As previously stated, PGC-1α regulates the expression of NRF-1 (Nuclear Respiratory Factor 1). NRF-1, as the name indicates, is a transcription factor involved with the transcription of nuclear-encoded electron transport chain components; β-ATP synthase, cytochrome c, and cytochrome c oxidase subunits. NRF-1 also triggers the expression of Tfam (Transcription factor A, mitochondrial). Tfam is a transcription factor that, once expressed, translocates from the nucleus to the mitochondria. Tfam fully coats mtDNA, organizes the mtDNA structure, and is essential for mtDNA maintenance [143]. Tfam binds to and activates transcription at the two major promoters of mtDNA [144]. The importance of Tfam is demonstrated in the global Tfam knockout mice, which are embryonic lethal [145] and the tubule-specific deletion of Tfam resulted in the development of renal inflammation and fibrosis by 6 weeks of age [146]. By PGC-1α regulating NRF1/2 and Tfam, it controls the expression of mitochondrial proteins encoded in both the nuclear and mitochondrial genome.

Increased mitochondrial electron transport chain activity is also associated with increased production of reactive oxygen species (ROS). Excessive ROS production can lead to DNA damage and cell death. Because of this, PGC-1α is also involved with
increasing the expression of enzymes needed for the detoxification of ROS. PGC-1α induces the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase [147, 148].

**PGC-1α AND KIDNEY INJURY**

PGC-1α is highly expressed in the proximal tubule cells [149] and human biopsies from AKI/CKD patients show reduced levels of PGC-1α when compared to normal human kidney tissue [102, 150, 151]. Additionally, kidney tissue sections from patients with diabetic kidney disease had lower expression of PGC-1α and dysregulated mitochondrial function when compared to healthy controls [151]. Reduced mitochondrial bioenergetics leads to dysfunctional RPTEC and overall loss of renal function [101]. PGC-1α regulates the cell's energy metabolism and respiratory capacity. Reduced PGC-1α levels lead to reduced mitochondrial bioenergetics and a decline in renal function. The role of PGC-1α expression has been examined in multiple models of AKI and fibrosis [137, 150, 152-163]. During AKI, damaged mitochondria are removed from cells via mitophagy and need to be replaced by the generation of new mitochondria via MB. PGC-1α is the master regulator of mitochondrial biogenesis and bioenergetics; increasing PGC-1α expression has provided protection against ischemia-reperfusion injury (IRI) [150, 161-163], sepsis [158, 159], folic acid [137, 154-157], and single high dose cisplatin [152, 153, 164] induced AKI. More recently, PGC-1α has been shown to be a key mediator in renal fibrosis and CKD development [102, 103, 133, 136, 138, 151]. Additionally, the Susztak lab has shown that restoring FAO protects against folic acid-induced kidney fibrosis [102]. In mouse models of global and renal proximal tubule-specific PGC-1α knockout, both PGC-1α null mice were unable to recover from sepsis-induced AKI when compared to wild-type mice [165]. Additionally, *in vitro* work from the Schnellmann lab showed that the overexpression of PGC-1α in proximal tubular cells
produced a significant increase in mitochondria number, respiratory capacity, and intracellular ATP and improved recovery from oxidative damage. This suggests increasing PGC-1α levels after an AKI may promote repair and recovery [166].

Our laboratory has demonstrated the RLDC model of cisplatin-induced kidney injury produces mild kidney injury and development of fibrosis. Blood urea nitrogen (BUN), neutrophil gelatinase-associated lipocalin (NGAL), and urinary kidney injury molecule-1 (Kim-1) were used to assess kidney damage. Our studies indicate that following the injury phase of RLDC in C57BL/6j [121], FVB/n [115-117], and B6;129 strains of mice, there are increases in BUN, NGAL, and Kim-1. The microenvironment created by RLDC is one that is high in inflammatory (TNF-α, IL-6, NLRP3, NF-κB) and fibrotic mediators (TGF-β1) [115-120]. Increased levels of inflammatory and fibrotic mediators such as TNF-α and TGF-β1 reduce the expression of PGC-1α [102, 137, 165]. We hypothesize that repeated insult from the administration of cisplatin damages the RPTEC mitochondria and reduces PGC-1α expression needed to maintain high levels of FAO and replace damaged mitochondria leading to a decline in renal function [101-103]. In this study, we set out to examine how kidney mitochondrial and PGC-1α expression were affected following the injury phase of RLDC model. We believe PGC-1α is a unique target for the RLDC model because of its role in both AKI and the development of renal fibrosis/CKD, mirroring the injury and progression phases.
MATERIALS AND METHODS

ANIMALS

B6129SF1/J (B6;129) mice were purchased from The Jackson Laboratory. B6;129 mice are a first filial generation hybrids that are the offspring of a cross between C57BL/6J females (B6) and 129S1/SvJ males (129). Upon arrival, the mice were allowed to acclimate for 1 week prior to the initiation of the experiment. All mice were maintained on a 12-hour light/dark cycle and provided food and water ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee and followed the guidelines of the American Veterinary Medical Association.

CISPLATIN DOSING REGIMEN

Pharmaceutical grade cisplatin (NDC 16729-288-38, 1mg/ml) was purchased via the University of Louisville Outpatient Hospital Pharmacy. The repeated low dose cisplatin dosing regimen mice were administered cisplatin or vehicle via intraperitoneal (i.p.) injection once a week between 8-9 a.m. for four weeks. In the vehicle group, 400 μL normal saline was administered via i.p. injection. In the cisplatin treatment groups, 7mg/kg or 9mg/kg of cisplatin in normal saline in a total volume of 400 μL was administered via i.p. injection. All mice were administered 500 μL saline by subcutaneous injection 2 days after dose 3 and 1 day before dose 4 to prevent weight loss. All animals were injected with cisplatin at the same time of day as there are circadian influences on the response of the kidney to cisplatin. Animals were euthanized 72 hours following their final cisplatin injection. Upon euthanasia, blood was collected, and plasma prepared and frozen at -80°C; urine was collected and frozen at -80°C; and
kidneys were removed, flash-frozen in liquid nitrogen, and stored at -80°C until use. A cross-section of one kidney from each mouse was fixed in 10% neutral buffered formalin for histology.

**BLOOD UREA NITROGEN (BUN), SERUM CREATININE (SCR), AND NEUTROPHIL GELATINASE ASSOCIATED LIPOCALIN (NGAL) DETERMINATION**

BUN (AMS Diagnostics, 80146) levels were measured from plasma samples using the indicated kits following the manufactures’ instructions. ELISAs for NGAL (R&D Systems, DY1857) were performed on the urine as directed by the manufacturer.

**PROTEIN QUANTIFICATION AND WESTERN BLOT ANALYSIS**

Kidney tissues were homogenized in cell extraction buffer (Thermo Fisher Scientific) containing a Complete Protease Inhibitor Cocktail Tablet and Phosphatase Inhibitor Cocktail Tablets (Roche). Homogenates were centrifuged at 15,000 X g for 10 min at 4°C. Supernatants were removed and stored at -80°C. Protein concentrations were determined using Bradford Reagent (Bio-Rad). 40 μg of kidney homogenate protein were loaded and separated on 4–12% gradient Tris-Glycine-SDS polyacrylamide gels. Protein was then transferred to PVDF membranes that were blocked in 5% (w/v) dried milk in tris buffered saline 0.1% Tween 20 (TBST) for 1 hour. Membranes were incubated with primary antibody overnight at 4°C. The next morning, membranes were washed 3 times for 5 min each with TBST containing 5% (w/v) dried milk. Membranes were then incubated for 2 hours at room temperature with secondary antibodies conjugated with horseradish peroxidase (1:20,000) in TBST containing 1% (w/v) dried milk. Following 2 washes, 1% (w/v) dried milk and 1 wash in TBST membrane proteins were detected by chemiluminescence substrate.
ANTIBODIES

The following antibodies were purchased from Cell Signaling Technology (Beverly, MA): p44/42 MAP Kinase (Erk1/2) #4695, Phospho-p44/42 MAP Kinase (Erk1/2) (Thr202/Tyr204) #4370, Transforming growth factor-beta (TGF-β) #3711, Mothers against decapentaplegic homolog 2/3 (SMAD 2/3) #8685, Phospho-Smad2 (Ser465/467) #3108, Phospho-Smad3 (Ser423/425) #9520. The following antibodies were purchased from Abcam (Cambridge, CB2 0AX, UK): Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (PGC-1α, abcam, ab54481) and α-Smooth Muscle Actin (α-SMA, abcam, ab5694). The remaining antibodies include fibronectin (F3648, Sigma-Aldrich), and α-tubulin (SC-5286, Santa Cruz Biotechnology).

Rodent Total OXPHOS Antibody Cocktail (abcam, ab110413) contains 5 mouse mAbs, one each against Complex I subunit NDUFB8 (ab110242), Complex II-30kDa (ab14714), Complex III-Core protein 2 (ab14745), Complex IV subunit I (ab14705) and Complex V alpha subunit (ab14748) as an optimized premixed cocktail.

GENE EXPRESSION

RNA was isolated from kidney tissue using E.Z.N.A. Total RNA Kit 1 (OMEGA) per manufacturer’s protocol. cDNA was synthesized with High-Capacity cDNA Reverse Transcriptase PCR (Thermo Fisher Scientific) per manufacturer’s instructions. Gene-specific cDNA was quantified with real-time qRT-PCR using either pre-designed TAQman assays or self-designed SYBR assays. The following TAQman primers were purchased from Thermo FisherScientific: tumor necrosis factor alpha (TNF-α, Mm00443258_m1), chemokine (C-X-C Motif) ligand 1 (CXCL1, Mm04207460_m1), and the housekeeping gene beta-2-microglobulin (B2M, Mm00437762_m1). The following primers were self-designed: kidney injury molecule-1 (Kim-1, Invitrogen, forward:
AGATCCACACATGTACCAACATCAA, reverse: CAGTGCCATTCCAGTCTGGTTT),

Peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (PGC-1α, Invitrogen, forward: AACAATGAGCCTGCGAACATATT, reverse: TAGCAAGTTTG CCTCATTCTCTTC), Nuclear Respiratory Factor 1 (NRF1, Invitrogen, forward: GGATTCAGTCTCTGTGGACAAA, reverse: CCCCCGACCTGTGGAATACT), Transcription factor A, mitochondrial (TFAM, Eurofins, forward: TTTAAAGCTAAACACCGATGCA, reverse: TTCTGGTAGCTCCTCCACAG), TIMP-1, Invitrogen forward: GCAACTGGACCTGGTCATAA, reverse: TTAGTCATCTTGTATTTATACGGCTTGTA), NLRP3, Invitrogen, forward: AAGATGAAGGACCCACAGTGTAACTT, reverse: CAGATTGAAGTAAGGCCGGAATT), and Col1a1, Invitrogen, forward: CGATGGATTCCCGTTCGAGTA, reverse: GTGGACATTAGGCGCAGAA) qRT-PCR was done with either iTaq Universal Probes Supermix (172-5134, Bio-Rad) or iTaq Universal SYBR Green Supermix (172–5124, Bio-Rad).

FLOW CYTOMETRY

Whole kidneys were homogenized into single-cell suspensions via mechanical disruption and enzymatic digestion with Liberase DL Research Grade (05466202001, Millipore/Sigma). After being passed through a 40-μm filter, cells were treated with ACK Lysing Buffer (A1042-01, Life Technologies) for 2 min to remove red blood cells. Cells were then suspended in PBS with 0.5% BSA, 0.01% sodium azide, and 2 mM EDTA. CD16/32 antibody (101321, BioLegend) was used to page Fc-gamma3 receptors. Cells were then stained with 10 μg/mL of CD45-PerCP (Cat. No. 103130, BioLegend), Ly6C-APC-Cy7 (Cat. No. 560596, BD Biosciences), F4/80-BV421 (Cat. No. 565411, BD Biosciences), and 7.5 μg/mL of CD11b-BV650 (Cat. No. 563402, BD Biosciences). After being stained, cells were permeabilized with the FoxP3/Transcription Factor Staining Buffer Set (Cat. No. 00-5523-00, Invitrogen). Intracellular staining was done with 10
μg/mL of CD206-PE. Flow cytometry was done using a BD LSRFortessa flow cytometer, collecting 1 million events per sample.

MITOCHONDRIAL DNA TO NUCLEAR DNA ASSAY

DNA was isolated from kidney tissue using E.Z.N.A. Tissue DNA Kit 1 (OMEGA) per the manufacturer’s protocol. DNA was quantified using Nanodrop, and 5 ng/μl solutions were prepared for each sample. 20ng of DNA was loaded into each well, followed by quantitative PCR using primers designed for specific amplification of particular mtDNA encoded and nuclear-encoded fragments. Primers were designed to evaluate the relative copy number of mtDNA and nDNA. The mitochondrially encoded genes selected were 16S rRNA and ND1, and the nuclear-encoded gene was Hexokinase 2 (HK2) along with the adaptation of the protocol according to previously published work [167]. Forward and reverse primers 16S rRNA, Invitrogen, forward: CCGCAAGGGAAAGATGAAAGAC, reverse: TCGTTTGGTTTCGGGGTTTC, ND1, Invitrogen, forward: CTAGCAGAAACAAACCGGGC, reverse: CCGGCTGCGTATTCTACGTT, HK2, Invitrogen, forward: GCCAGCCTCTCCTGATTTTAGGT, reverse GGAACACAAAGACCTTCTTCTGG. qPCR was done with iTaq Universal SYBR Green Supermix (172–5124, Bio-Rad).

CITRATE SYNTHASE ASSAY

Kidney tissues were homogenized in CHAPS solution (1% CHAPS, 150 mM NaCl, 50 mM Tris, 5 mM ETDA) containing a Complete Protease Inhibitor Cocktail Tablet (Roche). Homogenates were centrifuged at 15,000 X g for 10 min at 4° C. Supernatants were removed and stored at -80°C. Protein concentrations were determined using Bradford Reagent (Bio-Rad). Citrate Synthase Assay Kit (Sigma-Aldrich, #:CS0720) was used to determine relative amounts of citrate synthase per kidney cortices. Citrate
synthase is the initial enzyme of the tricarboxylic acid (TCA) cycle. The enzyme catalyzes the reaction of acetyl CoA with oxaloacetate to form the citrate. This enzyme is an exclusive marker of the mitochondrial matrix and is used as a proxy for mitochondrial content. The hydrolysis of the thioester of acetyl CoA results in the formation of CoA with a thiol group (CoA-SH). The thiol reacts with the DTNB in the reaction mixture to form 5-thio-2-nitrobenzoic acid (TNB). This yellow product (TNB) is observed spectrophotometrically by measuring absorbance at 412 nm. Eight (8) μg of kidney protein was loaded in triplicate per sample, and a change in absorbance was recorded. The amount of citrate synthase activity was determined using the manufacturers’ instructions.

HISTOLOGY

Following formalin fixation, kidney tissue was processed and embedded in paraffin. Kidney sections (5 μm) were stained with hematoxylin and eosin (H&E) and periodic acid schiff (PAS). The degree of morphologic changes was determined by light microscopy. The following measures were assessed as an indication of morphologic damage to the kidney after drug treatment: proximal tubule degradation, loss of brush border, tubular casts, proximal tubule dilation, proximal tubule necrosis, presence of inflammatory cells, and interstitial fibrosis.

SIRIUS RED/FAST GREEN STAINING

Kidney sections (5 μm thick) were rehydrated in Histoclear followed by an ethanol gradient. Slides were then dipped in PBS with 0.1% Tween 20 and incubated for 5 minutes. Slides were washed with distilled water twice for 5 minutes each and then incubated in 1.2% (w/v) saturated picric acid (#5860-32, Ricca Chemicals) containing 0.1% sirius red/direct red 80 (#365548, Sigma) and 0.1% fast green FCF (#F7258,
Sigma). Slides were then washed with 5% glacial acetic water until the water ran clear. Tissue samples were then dehydrated and fixed using Permount (#17986-01, Electron Microscopy Sciences).

**IMMUNOHISTOCHEMISTRY**

Kidney sections (5 μm thick) were rehydrated in Histoclear followed by an ethanol gradient. Antigen retrieval was performed in citric acid buffer (pH 6.0) at 95°C in a steamer for 30 min. Endogenous peroxidases were inhibited with 3% hydrogen peroxide and dual endogenous enzyme blocker (Dako) for 10 min, followed by two 5-min PBS washes. Slides were then blocked with avidin for 10 min followed by a PBS wash and then biotin for 10 min followed by a wash in PBS (Dako). Slides were further blocked with 5% normal goat serum in 0.1% TBST for 1 h at room temperature. α-SMA primary rabbit antibody (Abcam) was added to slides at a concentration of 0.5 μg/ml and allowed to incubate at 4°C overnight. Slides were rinsed with PBS for 5 min, three times. Biotinylated goat anti-rabbit IgG antibody (1: 25,000, BA-1000, Vector Laboratories) was added to each section and incubated for 30 min at room temperature. Slides were rinsed twice with PBS (5 min each). Vector ABC reagent (PK-7100, Vector Laboratories) was added to each section and incubated for 30 min at room temperature. Slides were rinsed two times with PBS followed by the addition of 100 μl of DAB substrate for 5-7 min to detect horseradish peroxidase (SK-4800, Vector Laboratories). Slides were rinsed in distilled water for 5 min, counterstained with modified Mayer's hematoxylin (no. 72804, Thermo Scientific), and then dehydrated in an ethanol gradient to Histoclear followed by mounting with Permount (SP15, Fisher Scientific). Positive staining for α-SMA indicates the presence of myofibroblasts.
STATISTICAL ANALYSIS DATA

Data are expressed as means ± SEM for all experiments. Multiple comparisons of normally distributed continuous data were analyzed by one-way ANOVA, and group means were compared using Tukey post-tests. Nonparametric continuous data were analyzed with a Kruskal-Wallis test followed by a Dunn’s multiple comparison test. Nominal data from pathology scoring were analyzed by individual Chi-squared tests. The criterion for statistical differences was p < 0.05 for all comparisons.
RESULTS

Repeated low-dose cisplatin (RLDC) induces kidney injury in B6;129 mice.

We selected the B6129SF1/J (B6;129) mouse strain because our lab is developing a syngeneic lung tumor model to investigate the nephrotoxicity of cisplatin with the co-morbidity of cancer. Our lab has observed inter-strain variance [121], and the dose of cisplatin required to induce kidney and fibrosis often varies. To optimize the dose of cisplatin for the B6;129 mouse strain, we performed an initial cisplatin dose response experiment in B6;129 mice. The mice were injected (i.p.) with pharmaceutical grade cisplatin 7mg/kg, 9 mg/kg, or saline (vehicle) once a week for four weeks (Figure 2B.). All mice were administered 500 μL saline by subcutaneous injection 2 days after dose 3, and 1 day before dose 4 to prevent dehydration. Mice were sacrificed 72 hours following the last dose. Blood urea nitrogen (BUN), neutrophil gelatinase-associated lipocalin (NGAL), and urinary kidney injury molecule-1 (Kim-1) were used to assess kidney damage. Following RLDC, we observed an increase in BUN (Figure 3A), NGAL (Figure 3B), and Kim-1 (Figure 3C) levels between vehicle and cisplatin-treated groups. The weights of the mice were monitored throughout the RLDC regimen as a measure of overt toxicity. We saw a significant loss of body weight between the vehicle and cisplatin-treated groups and a significant difference between the 7mg/kg and 9mg/kg cisplatin groups (Figure 3D). Renal histological changes were assessed on 5 μm thick H&E and PAS stained sections. The PAS-stained sections show a loss of brush border in the proximal tubule cells from the cisplatin-treated groups (Figure 4). The H&E stained sections show evidence of tubular necrosis, proximal tubule cast formation, inflammatory cells, tubule degeneration, and tubule dilation in the cisplatin groups (Figure 5). These
findings suggest that repeated low-dose model of cisplatin increases markers of kidney damage and decreases renal function. Additionally, the kidney function and injury markers together with the histological changes suggest a dose-response to cisplatin-induced kidney injury with higher levels of loss of function (BUN), weight, kidney injury (Kim-1 and NGAL), loss of proximal tubule brush border, increased cast formation, increased tubule degeneration, and increased tubule dilation following the injury phase of the repeated low-dose model of cisplatin-induced kidney injury in the 9 mg/ Kg dose group than the 7 mg/ Kg dose group.
Figure 2. RLDC Experiment Design.

(A) Experiment schematic for RLDC model. (B) Experiment design for the study conducted, B6;129 mice were injected I.P. with 7-9mg/kg cisplatin or normal saline once a week for four weeks. Mice were euthanized three days following the last dose.
Figure 3. RLDC induced kidney injury.

(A) BUN levels from serum and (B) NGAL levels from urine following 4 doses of cisplatin. (C) Kim-1 mRNA expression from the kidney cortex was measured using qRT-PCR. (D) Percent change in body weight was recorded throughout RLDC regimen as a marker of overt toxicity. Data are expressed as means ± SEM; n=5–10. Statistical significance was determined by 1-way ANOVA followed by Tukey posttest. *p < 0.05, ***p < 0.001, ****p < 0.0001
Figure 4. PAS Stain

Renal histological changes were assessed with PAS-stained 5 μm thick sections. PAS is used to identify brush borders of proximal tubule cells. (A) Vehicle Control, (B) 7mg/kg, (C) 9mg/kg. Representative images of renal histology at 40X magnification.
Figure 5. H&E Stain

Renal histological changes were assessed with H&E stain on 5 μm thick sections. H&E stain is used to identify tubular necrosis, proximal tubule cast formation, inflammatory cells, tubule degeneration, and tubule dilation. (A) Vehicle Control, (B) 7mg/kg, (C) 9mg/kg. Representative images of renal histology at 40X magnification.
**RLDC induces renal fibrosis.** Fibrosis was evaluated via multiple approaches, including histopathology, immunohistochemistry (IHC), western analysis, and real-time qRT-PCR analysis. Sirius red fast green (SRFG) staining indicated collagen accumulation (Figure 6.) and α-smooth muscle actin (α-SMA) IHC (Figure 7.). Positive α-SMA indicates an increased number of myofibroblasts in the kidney following the RLDC regimen and is an indicator of increased fibrosis. Western analysis indicated increased protein levels of fibronectin and α-SMA (Figure 8A) between the vehicle and cisplatin-treated groups. The mRNA expression of markers of fibrosis (TIMP-1, Col1a1) showed a significant increase in the cisplatin-treated groups (Figure 8B, 8C). These findings suggest that there is a significant development of fibrosis following the injury phase of the repeated low-dose model of cisplatin-induced kidney injury.
Figure 6. SRFG Stain

Renal histological changes were assessed with SRFG stain on 5 μm thick sections. SRFG stains collagen red and is a marker for interstitial fibrosis. (A) Vehicle Control, (B) 7mg/kg, (C) 9mg/kg. Representative images of renal histology at 20X magnification.
Fig. 7. IHC for α-SMA

Renal histological changes were assessed with IHC for α-SMA on 5 μm thick sections. α-SMA IHC indicates an increased number of myofibroblasts in the kidney and is a sign of increased interstitial fibrosis. (A) Vehicle Control, (B) 7mg/kg, (C) 9mg/kg. Representative images of renal histology at 40X magnification.
Figure 8. RLDC increases protein and mRNA markers of fibrosis

(A) Western blot for Fibronectin, α-SMA, TGF-β1, and α-Tubulin. mRNA expression via qRT-PCR of TIMP-1 (B) and Col1a1 (C) normalized to B2M. Data are expressed as means ± SEM; n=5–10. Statistical significance was determined by 1-way ANOVA followed by Tukey posttest. *p < 0.05, **p<0.01, ***p<0.001.
**RLDC induces renal inflammation.** Inflammation was assessed using real-time qRT-PCR and flow cytometry. The mRNA expression of inflammatory markers, TNF-α (Figure 9A), CXCL-1 (Figure 9B), and NLRP3 (Figure 9C) showed a significant increase in the cisplatin-treated groups. We found a significant increase in the percentage of CD45+ immune cells (Figure 10A) in the kidney of cisplatin-treated mice compared to vehicle control kidneys. We observed significantly increased populations of F4/80 hi and F4/80 lo macrophages (Figure 10B, 10C). These findings suggest that there is a significant immune response and infiltration to the kidney following cisplatin-induced renal damage in the repeated low-dose model.
Figure 9. RLDC induced kidney inflammation.

(A) qRT-PCR of TNF-α, CXCL-1 (B), and NLRP3 (C) normalized to B2M. Data are expressed as means ± SEM; n=5–10. Statistical significance was determined by 1-way ANOVA followed by Tukey posttest. ***<0.001, ****p < 0.0001.
Figure 10. Immune Cell Infiltration

Flow cytometric analysis of renal immune cells. Whole kidneys were homogenized and ~1 million cells were stained. One million events were collected from each sample. Analysis identified CD45+ immune cells (A), CD11b+ F4/80 hi resident macrophages (B), CD11b+ F4/80 lo infiltrating macrophages (C). Populations are expressed as a percentage of the number of single cells counted. Data are expressed as means ± SEM; n=5–10. Statistical significance was determined by 1-way ANOVA followed by Tukey posttest. *p < 0.05, **p<0.01 ***<0.001, ****p < 0.0001.
RLDC reduces total kidney mitochondrial content. As previously mentioned, the high metabolic activity and density of mitochondria in the kidney led us to investigate the effects of the RLDC model on kidney mitochondrial content. We performed three different assays to evaluate mitochondrial content in the kidneys following RLDC. First, we quantified the ratio of mtDNA to nDNA as described [167]. RLDC resulted in a significant reduction in the mtDNA/nDNA ratio (Figure 11A), indicating a loss of mtDNA and suggesting a loss of mitochondrial mass. We also evaluated citrate synthase activity on kidney cortex homogenate. Data indicate a significant decrease in citrate synthase activity following RLDC treatment (Figure 11B). Finally, we performed western analysis of the electron transport chain (ETC) complex proteins. Data indicate a decrease in complexes 1-5 of ETC subunits (Figure 11C). These results suggest that following the injury phase of the RLDC model, there is a significant reduction in kidney mitochondria.
Figure 11. RLDC reduces kidney mitochondrial content.

(A) qPCR on nuclear-encoded Hexokinase 2 (HK2) and mitochondrial encoded 16S were used to calculate the ratio between mtDNA to nDNA as a marker for mitochondrial content, normalized to vehicle-treated kidneys. (B) Citrate Synthase Activity measured using Sigma-Aldrich kit (CS0720). (C) Western blot for electron transport chain (ETC) subunits of super-complex proteins: Complex 1 (NDUFB8), Complex 2 (SDHB), Complex 3 (UQCRC2), Complex 4 (MTCO1), and Complex 5 (ATP5a). Data are expressed as means ± SEM; n=5–10. Statistical significance was determined by 1-way ANOVA followed by Tukey posttest. *p< 0.05, **p<0.01, and ****p < 0.0001.
**RLDC reduces the expression of PGC-1α and its downstream targets.** The previous results lead me to investigate possible causes of the reduced mitochondrial content. The RLDC models causes a significant reduction in PGC-1α protein evaluated by western analysis (Figure 12A) and mRNA expression evaluated by real-time qRT-PCR (Figure 12B). Data indicate decreased levels of PGC-1α at the mRNA and protein level. I next evaluated genes whose expression is regulated by PGC-1α. Nuclear respiratory factor 1 (Nrf1) expression is increased by PGC-1α, and it triggers the activation of multiple mitochondrial genes encoded in the nucleus [131]. Nrf1 expression is significantly reduced following RLDC (Figure 13A). One gene whose expression is regulated by Nrf1 is Tfam (transcription factor A, mitochondrial), and its expression is significantly reduced in the RLDC model (Figure 13B). Tfam binds to and activates transcription at the two major promoters of mitochondrial DNA (mtDNA) [144]. Tfam is responsible for organizing and maintaining mtDNA structure [143], and loss of Tfam in a mouse model resulted in the development of renal inflammation and fibrosis by 6 weeks of age [146]. Reduced Tfam results may be related to reduced mtDNA levels as shown in Figure 11A. mtDNA has a less efficient repair process compared to nuclear DNA (nDNA), making mtDNA highly susceptible to cisplatin-induced damage [168, 169].
Figure 12. RLDC reduces the expression of PGC-1α.

(A) Western blot for PGC-1α and α-Tubulin. (B) qRT-PCR of PGC-1α normalized to B2M. Data are expressed as means ± SEM; n=5–10. Statistical significance was determined by 1-way ANOVA followed by Tukey posttest. ****p < 0.0001.
Figure 13. RLDC reduces the expression of PGC-1α’s downstream targets.

qRT-PCR of NRF1 (A), Tfam (B) normalized to B2M. Data are expressed as means ± SEM; n=5–10. Statistical significance was determined by 1-way ANOVA followed by Tukey posttest. ****p < 0.0001.
**RLDC increases the expression of inhibitory pathways that regulate PGC-1α expression.**

The previous results lead me to investigate possible causes of the reduced PGC-1α protein and mRNA expression. As previously stated, PGC-1α expression and activity are finely tuned and highly responsive to changes in the microenvironment. PGC-1α is negatively regulated by pro-inflammatory and profibrotic cytokines. We looked at profibrotic factor TGF-β1 and phosphorylation of downstream target Smad 2 and Smad 3 by western analysis (Figure 14A). We looked at pro-inflammatory cytokine TNF-α mRNA expression by real-time qRT-PCR (Figure 14B). As mentioned previously, activation of TLR4 has been seen in cisplatin models of injury. We looked at phosphorylation of ERK1/2, which is a downstream target of TLR4 by western analysis (Figure 14A). Data indicate increased levels of multiple inhibitors of PGC-1α expression and activity following the injury phase of the RLDC model.
Figure 14. RLDC increases the expression of inhibitory pathways that regulate PGC-1α expression

(A) Western blot for TGF-β1, p-Smad2, p-Smad3, total Smad 2/3, p-ERK1/2, total ERK1/2, and α-Tubulin. (B) qRT-PCR of TNF-α normalized to B2M. Data are expressed as means ± SEM; n=5–10. Statistical significance was determined by 1-way ANOVA followed by Tukey posttest. ****p < 0.0001
DISCUSSION

Cisplatin is a highly effective first-line chemotherapeutic agent, but its usage is limited by its nephrotoxicity. Cisplatin is commonly used to treat many solid-organ cancers, including head, neck, testicular, breast, ovary, and lung [74]. The effectiveness of cisplatin can be seen in the remission rates of testicular cancer that went from 5% to 80% when cisplatin was approved for its treatment [114]. Thirty percent of cancer patients treated with cisplatin develop AKI [74]. AKI is a life-threatening condition defined as a rapid decrease in renal function and is diagnosed clinically by an increase in serum creatinine (SCr) and blood urea nitrogen (BUN) [24]. The onset of AKI from cisplatin prevents effective treatment and often requires a change in therapy or dosage reduction. Although newly developed cisplatin derivatives such as carboplatin and oxaliplatin have reduced nephrotoxicity, they are much less effective in treating many forms of cancer [75]. Cisplatin would be a much more effective cancer treatment if this toxicity could be prevented; however, there are currently no therapies approved to prevent or treat AKI.

Despite the high rate of AKI development, 10-20% of all cancer patients are prescribed cisplatin as part of their treatment regimen [67]. In the past, it was assumed that if renal function recovered (as determined by the return of SCr and BUN to baseline levels) after an AKI incident, there would be no long-term consequences [55]. However, several recent studies indicate that patients who develop AKI 10 times or more likely to develop chronic kidney disease (CKD) [28-32]. Even patients that do not develop clinical AKI are at risk for long-term declines in renal function [56, 57]. As we improve diagnosis and treatment of cancer, the number of cancer survivors is increasing. In 2016, there were 15.5 million cancer survivors in the U.S., and that number is estimated to increase
to 20.3 million by 2026 [170]. AKI and CKD incidences have also been increasing [29, 171, 172]. Thus, cisplatin-induced kidney injury (CDDP-KI) is an important unmet medical need and places a large burden on patient quality of life as well as our healthcare system.

There is a great need to develop therapeutic strategies to protect from CDDP-KI in order to increase cisplatin’s therapeutic potential and decrease the rate of CKD development in cancer survivors. All clinical trials on nephroprotective agents have failed, and we believe this is in part due to a lack of clinically relevant animal models.

Traditionally, the accepted standard model of CDDP-KI has been to use a single high dose of cisplatin. This model results in high levels of tubular necrosis, rapid decline in renal function, and is fatal to mice within 3-4 days cisplatin [112, 113]. In the clinic, patients are typically treated with periodic low doses of cisplatin over weeks to months, not a single high dose. The Siskind laboratory and others have recently developed repeated low dose cisplatin (RLDC) models that more closely mimic the dose regimen of cisplatin in the clinic and enables the study of long-term impacts on the kidney [112, 113, 115-121]. The mechanism of RLDC leading to CKD is poorly understood and appears to be vastly different from the acute model. Unpublished data from our lab has shown agents that are protective in the acute model provide no benefit in the chronic RLDC model. Other models of CKD and fibrosis have shed light on possible mechanisms, but it is still unclear how RLDC leads to a decline in renal function and fibrosis. This model allows us to study the pathology of CDDP-KI in a way that more closely mirrors the pathology of patients receiving CDDP in the clinic.

The results from RLDC model indicates that repeated low levels of cisplatin injury induce fibrosis which progresses to CKD and that the cellular processes induced in the kidney by the RLDC model differ greatly from that when a single high dose of cisplatin is administered [115-120]. The mild injury caused by RLDC that does not meet the clinical
criteria for AKI can progress to CKD [115] and better represent the 70% of patients that receive cisplatin without clinical markers of AKI. Furthermore, the RLDC model can be broken up into two phases, the injury and progression phases. The injury phase, which consists of the four weeks of cisplatin treatment and is characterized by a mild decline in kidney function, ER stress, increased markers of kidney damage (Kim-1, NGAL), inflammation, and development of fibrosis. The progression phase is defined by removal of the nephrotoxic agent and markers of AKI (Kim-1, NGAL) return to baseline; however, there is persistent inflammation, increased fibrosis, and development of CKD [115-120]. The cellular biology involved in each phase is quite different, and the mechanisms involved in both phases of this model remain largely unknown [116]. Elucidating these mechanisms is necessary for identifying novel therapeutic targets.

To better understand the mechanism involved in the RLDC, this study evaluated the effects of repeated low-dose cisplatin (RLDC) on kidney mitochondria content and kidney PGC-1α expression. The RLDC model results in kidney injury and the development of fibrosis, along with reduced mitochondrial content and PGC-1α expression. Reduced PGC-1α is seen in human renal biopsies from AKI/CKD patients when compared to normal human kidney tissue [102, 150, 151]. Increasing PGC-1α expression has provided protection in multiple models of AKI, including IRI [150, 161-163], sepsis [158, 159], folic acid [137, 154-157], and single high dose cisplatin [152, 153, 164]. Additionally, increasing PGC-1α in other cell types besides RPTEC may not be protective, as shown by Li and colleagues, where podocyte-specific inducible overexpression of PGC-1α actually accelerated progression toward end-stage renal disease by inducing collapsing focal and segmental glomerulosclerosis [173].

Cisplatin has been shown to preferentially accumulate in mitochondria [74, 93], and sensitivity to cisplatin correlates with mitochondria density and membrane potential [94, 95]. Furthermore, mtDNA is more susceptible to cisplatin-induced DNA damage
than nDNA because of the mitochondria's lack of effective DNA repair mechanisms [88, 96, 97]. Damage or loss of mtDNA from cisplatin leads to a loss in mitochondrial protein levels, and a disruption of the mito-nuclear protein ratio leads to reduced respiratory capacity and malfunctioning RPTECs [104, 105]. Additionally, multiple studies have demonstrated that damaged mtDNA is degraded to counteract ineffective mtDNA repair processes and prevent the accumulation of damaged mtDNA [174]. Degradation of damaged mtDNA leads to the release of fragmented mtDNA in the cytosol and activation of the innate immune response [96, 174]. Activation of the innate immune response by cytosolic mtDNA has been shown to be directly involved with the development of AKI and renal fibrosis [109, 146] and sheds light on a possible mechanism of the RLDC induced model of kidney injury (Figure 15).

The RPTEC are responsible for 70% of the reabsorption, are highly enriched in mitochondria [18], and rely on FAO to sustain adequate ATP levels [19]. We hypothesize that repeated insults from cisplatin preferentially targets kidney mitochondria resulting in mild RPTEC death, clearance of damaged mitochondria, initiation of the inflammatory cascade, which all coalesce to a decline in renal function. The microenvironment created by RLDC is proinflammatory (TNF-α) and profibrotic (TGF-β), both of which suppress the activity and expression of PGC-1α. The reduced PGC-1α and subsequent reduced mitochondrial content, functionality, and intracellular ATP results in dysfunctional RPTEC. Inherited genetic mitochondrial diseases coincide with renal impairment [122-126], and the most common of these genetic diseases lead to tubular defects [125, 126], demonstrating the importance of RPTEC in proper renal function. The repeated insult of cisplatin impairs RPTEC's ability to recover which, results in a maladaptive repair process leading to fibrosis and development of CKD. We believe increasing PGC-1α expression following administration of cisplatin will restore lost FAO activity,
mitochondria content, increase antioxidant enzymes and intracellular ATP to RPTEC, leading to increased kidney repair processes and kidney function.

Future studies are needed to examine the relationship between PGC-1α expression and the development of fibrosis/CKD in both phases of the RLDC model. We believe that increasing PGC-1α expression during the injury and progression phases of RLDC will protect against kidney injury and prevent progression to CKD, respectively. Experiments utilizing pharmacological inhibitors and inducers of PGC-1α, as well as transgenic mice overexpressing PGC-1α or with PGC-1α deletion, are required to determine the role of PGC-1α in the different stages and whether it is a potential therapeutic target. The results from these proposed experiments will help answer if changing PGC-1α expression during the injury phase of RLDC will provide any protection. This information will be very useful in developing future therapeutic agents for clinic practice.

The progression phase of the RLDC has its own distinct cellular biology, and the role of PGC-1α needs to be investigated separately. PGC-1α has been shown to be a key mediator in renal fibrosis and CKD development [102, 103, 133, 136, 138, 151]. We propose carrying out the same proximal tubule-specific overexpression and knockout studies but starting the intervention only after the four doses of cisplatin have been administered. For the 70% of patients that receive cisplatin with no clinical markers of AKI, it is currently not known if later presentation of kidney damage can be reversed. Studying the progression phase of the RLDC will help to uncover the mechanisms involved with this process. Additionally, the proposed studies will allow us to intervene after subclinical damage has occurred and will provide useful information about the ability to reverse subclinical kidney damage and prevent progression to CKD. This information will fill key knowledge gaps, providing scientific advances for the field.
The mouse models used to study cisplatin-induced AKI are not fully representative of patients who receive multiple low doses of cisplatin in the clinic. The current models lack solid organ tumors, and our preliminary unpublished data suggest the co-morbidity of lung cancer potentiates the nephrotoxicity of cisplatin. We have developed a subcutaneous transplanted syngeneic lung cancer model to investigate the interaction between the co-morbidity of cancer and cisplatin nephrotoxicity. Understanding the role of solid organ tumors in cisplatin-induced nephrotoxicity may provide mechanistic insights and novel therapeutic targets for the prevention and effective treatment of cisplatin-induced AKI-CKD. Lastly, any protective measure developed to limit the nephrotoxicity of cisplatin must also consider its effect on cancer viability.
Figure 15. Graphical Abstract of RLDC Model

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SUMMARY

Cisplatin is a first-line chemotherapeutic for many solid organ cancer types, but its usages are limited by its nephrotoxicity. Thirty percent of patients who receive cisplatin develop acute kidney injury (AKI), which increases the risk of chronic kidney disease (CKD) and mortality. There are currently no treatment options to prevent or treat cisplatin-induced kidney injury (CDDP-KI).

Cisplatin-induced kidney injury (CDDP-KI) has been extensively investigated in the past by our lab and many other labs using a single, high-dose model. However, patients are typically treated with periodic low doses of cisplatin, not a single high dose. Our lab and others have recently developed a potentially more clinically relevant model utilizing repeated low-dose cisplatin (RLDC) treatment. In this new model, mice receive 4 weekly doses of low dose cisplatin and are able to survive more than 6-months post-treatment. The RLDC model can be broken up into two phases. The repeated low-level injury phase, which consists of the four weeks of cisplatin treatment and is characterized by a mild decline in kidney function, insignificant levels of tubule cell death, inflammation, and development of fibrosis. After the injury phase, there is a progression phase that is characterized by persistent inflammation, exacerbated fibrosis, and the development of CKD. The mechanisms involved in both phases of this model remain largely unknown.

The kidneys have the highest mitochondrial density and oxygen consumption per gram of tissue in the body, preceded only by the heart. The majority of the reabsorption performed occurs in the renal proximal tubule epithelial cells (RPTECs). These cells are highly enriched in mitochondria and rely on fatty acid oxidation (FAO) as their many energy source. Cisplatin has been shown to disrupt FAO, and defective FAO in RPTEC
is seen in other models of fibrosis. Our data suggest that the RLDC model causes a
decrease in renal function, mitochondrial content, and PGC-1α. PGC-1α is a master
transcriptional regulator of mitochondrial biogenesis, fatty acid oxidation, lipogenesis,
thermogenesis, and glucose metabolism. Future studies are needed to examine the
relationship between PGC-1α expression and the development of fibrosis/CKD in both
phases of the RLDC model. We believe that increasing PGC-1α expression during the
injury and/or progression phases of RLDC will protect against kidney injury and prevent
progression to CKD, respectively.
REFERENCES


CURRICULUM VITAE

Andrew Orwick, Pharm.D.
University of Louisville School of Medicine
Department of Pharmacology & Toxicology
505 South Hancock Street (CTRB 252 G)
Louisville, KY 40202
Phone: 812-267-4777
E-mail: andrew.orwick@louisville.edu

Positions Held
August 2019-Current, Graduate Fellowship
University of Louisville

July 2016-July 2019, Vice-President of Operations
Precision Compounding Pharmacy
- Calculate monthly sales and profits for pharmacy
- Formulary Review, Monthly, Quarterly, Yearly
- Manage Sales Team
  - Facilitate weekly team meetings, assist with future/current client meetings, determine contract, bonus, and the total cost of the individual sale employee. Educate sales team on current and new product lines
- Manage daily operations of the pharmacy
- Monthly sales reports on specific disease states and specific products
- Proficiency in Pharmacy Benefit Managers Formularies and coverage

August 2014-July 2016, Operations Manager
Precision Compounding Pharmacy
- Calculate monthly sales report for pharmacy
- Formulary Review, Monthly, Quarterly, Yearly
- Manage Sales Team
  - Facilitate weekly team meetings, assist with future/current client meetings, determine contract and bonus. Educate sales team on current and new product lines
- Manage daily operations of the pharmacy
- Proficiency in Pharmacy Benefit Managers Formularies and coverage

June 2012-August 2014, Compounding Pharmacist
Precision Compounding Pharmacy
- Proficiency in Pharmacy Benefit Managers Formularies and coverage
- Manage daily operations of the pharmacy
- Manage daily operations of the compounding lab
- Calculate monthly sales report for pharmacy
- PK software advanced user
May 2009–May 2012, Compounding Tech/Pharmacy Intern
Precision Compounding Pharmacy
  Compounding prescriptions
  Maintenance and cleaning in compounding lab.

May 2004-August 2005, Clerk
Davis Drug Store, INC
  Cashier

Education
June 2012, Doctor of Pharmacy, Sullivan University College of Pharmacy
May 2009, Biology Minor, IUPUI

Educational Conferences
IACP Compounders on Capitol Hill, Washington, D.C., June 2018
PCCA International Seminar, Houston, TX, November 2014
PCCA International Seminar, Houston, TX, October 2013
PCCA Comprehensive Compounding Course, Houston, TX, September 2013
PCCA PK Software Advanced Users Course, Houston, TX, April 2013
PCCA Aseptic Technique Compounding Course, Houston, TX, January 2013
PCCA Marketing-Sales-Business Course, Austin, TX, April 2012
PCCA Sports Medicine/Pain Management Symposium, Colorado Springs, CO, August 2011
PCCA Student Boot Camp, Houston, TX, June 2011

Skills
  Leadership
  Problem Solver
  Time Management
  Public Speaking
  Teamwork
  Adaptability/Flexibility
  Interpersonal Skills

Licenses and Certificates
State of Indiana Pharmacist License

References available upon request