MOLECULAR MECHANISMS ASSOCIATED WITH ALL-TRANS-RETINOIC ACID-MEDIATED CYTOPROTECTION AGAINST RENAL CELL INJURY

By

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<table>
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<th>Description</th>
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<tbody>
<tr>
<td>AKI</td>
<td>Acute kidney injury</td>
</tr>
<tr>
<td>APL</td>
<td>Acute promyelocytic leukemia</td>
</tr>
<tr>
<td>ATRA</td>
<td>All-trans-retinoic acid</td>
</tr>
<tr>
<td>BSO</td>
<td>Buthionine sulfoximine</td>
</tr>
<tr>
<td>BUN</td>
<td>Blood urea nitrogen</td>
</tr>
<tr>
<td>9-cis RA</td>
<td>9-cis retinoic acid</td>
</tr>
<tr>
<td>DDM-PGE₂</td>
<td>11-deoxy 16,16-dimethyl PGE₂</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular regulated kinase</td>
</tr>
<tr>
<td>Grp78</td>
<td>Glucose regulated protein 78</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>GSK-3</td>
<td>Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>γ-GT</td>
<td>γ-glutamyl transpeptidase</td>
</tr>
<tr>
<td>HGF</td>
<td>Hepatocyte growth factor</td>
</tr>
<tr>
<td>HIF1</td>
<td>Hypoxia inducible factor 1</td>
</tr>
<tr>
<td>IDAM</td>
<td>Iodoacetamide</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor-1</td>
</tr>
<tr>
<td>IRI</td>
<td>Ischemia reperfusion injury</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun NH₂-terminal kinase</td>
</tr>
<tr>
<td>Keap1</td>
<td>Kelch-like ECH-associated protein 1</td>
</tr>
<tr>
<td>KIM-1</td>
<td>Kidney injury molecule-1</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MGHQ</td>
<td>2-(glutathion-S-yl)hydroquinone</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NAC</td>
<td>N-acetylcysteine</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear factor-kappa beta</td>
</tr>
<tr>
<td>Nrf2</td>
<td>Nuclear factor erythroid-related factor 2</td>
</tr>
<tr>
<td>PAP</td>
<td>Para-aminophenol</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
<tr>
<td>PHD</td>
<td>Prolyl-4-hydroxylase</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RAR</td>
<td>Retinoid acid receptor</td>
</tr>
<tr>
<td>RBP</td>
<td>Retinol binding protein</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid x receptor</td>
</tr>
<tr>
<td>SF</td>
<td>Sulforaphane</td>
</tr>
<tr>
<td>TGHQ</td>
<td>2,3,5-tris-(glutathion-S-yl)hydroquinone</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>pVHL</td>
<td>Von Hippel-Lindau protein</td>
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ABSTRACT

Chemical-induced nephrotoxicity is a major cause of acute kidney injury. My dissertation reveals that all-trans-retinoic acid (ATRA) affords cytoprotection against renal cell injury. Pretreatment with ATRA (25 μM, 24 hr) affords selective cytoprotection against p-aminophenol (PAP), iodoacetamide (IDAM), and 2-(glutathion-S-yl)-hydroquinone-induced necrosis. In contrast, pretreatment of cells with ATRA provides no protection against cisplatin-induced apoptosis. Inhibition of protein synthesis blunts ATRA-mediated cytoprotection, suggesting that critical cell survival signaling pathways are activated prior to toxicant exposure. Oxidative stress is a major contributor to cellular damage. To investigate the mechanism(s) by which ATRA affords cytoprotection, we determined its effects on ROS generation using a DCFDA assay. ATRA did not alter PAP or MGHQ-induced ROS levels. Moreover, ATRA had no effect on GSH levels nor Nrf2 expression, suggesting that other cytoprotective mechanisms are engaged by ATRA. Elevated ROS disrupt endoplasmic reticulum protein folding guided by the molecular chaperone Grp78. During ATRA-mediated pretreatment, the ER stress proteins Grp78 and p-eIF2α were induced (2-fold) in a time-dependent manner (24 and 4 hr respectively). In addition to influencing organelle stress proteins in the ER, ATRA rapidly (15 min) induced levels of the cellular stress kinases p-ERK and p-AKT with maximum levels achieved at 30 min. Moreover, induction of these stress kinases was observed at concentrations of ATRA (10 and 25 μM) required for cytoprotection. Inhibition of p-ERK with PD98059 reduced the ability of ATRA to provide protection against PAP toxicity, implying a role for p-ERK and downstream target genes in the protective effects of ATRA. Gene ontology analysis of a microarray experiment of cells treated with ATRA revealed that ATRA rapidly (0.5, 1 hr)
induced growth factors and genes involved in cell proliferation, with subsequent (4, 8, 12 hr) induction of genes involved in ribosome biogenesis, DNA replication and repair, and cell cycle regulation. Complementary data from a cell stress protein array and western blot analyses indicated that ATRA induced HIF1α 3-fold at 8 hr. Furthermore, the microarray data indicated the HIF1α target gene BHLHE40 (which encodes a basic-helix-loop-helix protein involved in cell differentiation) was increased 3-fold. As ATRA induced genes that were associated with cell proliferation, related assays were employed. ATRA had a small effect on cell cycle distribution demonstrated by an increase in the population of cells in the S and G2 phases between 8 and 24 hr. In addition, ATRA markedly increased total DNA content and cell number at 24 hr suggesting that mitogenic/proliferative effects contribute to ATRA cytoprotection. The present studies indicate that a signaling cascade of proteins downstream of p-ERK associated with mitogenesis work cooperatively to afford ATRA protection against renal cell injury. Understanding the mechanism of ATRA-mediated cytoprotection will provide insights into the development of novel therapeutic strategies for renal pathological conditions.
CHAPTER 1: INTRODUCTION

1.1 General Comments

Humans are exposed to a variety of chemicals from natural and synthetic sources on a daily basis. Humans benefit from these chemicals as they are used in medicinal, food and consumer products, and agriculture. However, if the dose is elevated causing too much exposure, and/or there are undesired side effects associated with consumption, toxic effects can occur.

Kidneys have a primary physiological role in the excretion of chemicals and metabolites. The kidneys receive blood from the renal arteries, are relatively small in size, and are exposed to a high concentration of chemicals, some of which are metabolized through bioactivation enzymes leading to toxic intermediates. In advanced stages, chronic kidney disease is irreversible and often requires kidney transplantation. Therefore, strategies to prevent acute toxicity from occurring are warranted.

Vitamin A participates in a variety of physiological functions in the body. Recent advances in the usage of vitamin A metabolites as cytoprotective agents may provide beneficial effects in the kidney. Investigation into the molecular mechanisms involved in mediating vitamin A cytoprotection in the kidney may provide insights into developing novel therapeutics to treat kidney disease.

1.2 Kidney Anatomy and Physiology

The kidney plays a critical role in homeostasis. It is responsible for the excretion of metabolic wastes, regulation of extracellular fluid volume, electrolyte and acid-base balance, synthesis and secretion of renin and erythropoietin, and metabolism of vitamin D to its active form (Klaassen, 2008). The kidneys are equipped with detoxication
mechanisms and regenerative capabilities. However, severe damage to the kidney can overwhelm these compensatory processes and has profound effects on total body function. Chronic renal failure can result in dialysis treatment or kidney transplantation.

The kidney is divided into three anatomic sections: the cortex, medulla, and papilla. The cortex constitutes the major portion of the kidney and receives 90% of the total blood flow (Klaassen, 2008). Thus, the cortex receives the majority of the toxicant when delivered to the kidney. However, since blood flow is much lower in the medulla and papilla, the tubular fluid is more concentrated. Toxicants that are delivered to these sections remain for a prolonged period of time.

The nephron is the functional unit of the kidney (Figure 1.1), key components being the vasculature, glomerulus, and the tubules. The renal artery supplies the interlobular arteries which gives rise to the afferent arterioles forming the glomerulus. The renal circulation develops into capillaries and blood exits the glomerulus via the efferent arteriole. A second set of capillaries, the peritubular capillaries, surrounding the proximal tubule tissue are formed. A portion of blood (~20%) entering the glomerular capillaries is filtered. In general, lower molecular weight compounds are freely filtered whereas larger molecules are restricted (Berndt, 1976; Klaassen, 2008). The filtrate then proceeds to subsequent sections of the nephron for processing. In humans, the proximal tubule consists of three discrete segments: the S₁ (pars convoluta), S₂ (transition between pars convoluta and pars recta), and S₃ (pars recta). The S₁ segment is the initial portion of the proximal convoluted tubule and contains a tall brush border membrane, many mitochondria, and a well-developed lysosome system. Progressing through the proximal tubule, the brush border shortens but further develops and the number of lysosomes and mitochondria
decrease. The loop of Henle is the segment of the nephron responsible for initiating the creation of concentrated urine. Filtrate descends into the medulla via the thin descending limb, loops around, and returns to the cortex via the thick ascending limb. Filtered Na\(^+\), K\(^+\), Cl\(^-\) and water are reabsorbed in the segments of the loop of Henle. Further reabsorption occurs in the distal tubule. The concentrated filtrate, or urine, then passes through the collecting duct on to the ureter (Klaassen, 2008).

The kidneys are an organ that is highly susceptible to damage. Being small in size, they only account for 0.5% of body weight, but receive about 25% of cardiac output (Lote et al., 1996). The proximal tubule is considered the workhorse of the nephron as it reabsorbs approximately 60-80% of glomerular filtrate (Klaassen, 2008). The proximal tubule also contains numerous transport systems for ions and metabolic substrates. Moreover, enzymes in the brush border membrane can hydrolyze peptides. As a result of these functions, the proximal tubule is the segment most prone to damage and toxicants can have pronounced effects on water and electrolyte balance, transport mechanisms, enzyme function, and whole-kidney function. One example of a class of compounds highly toxic to the proximal tubule of the kidney are glutathione (GSH)-conjugated xenobiotics or metabolites. This nephrotoxicity is a consequence of the high activity of \(\gamma\)-glutamyl transpeptidase (\(\gamma\)-GT) within the brush border membrane of epithelial cells in the S\textsubscript{3} segment of the nephron. \(\gamma\)-GT catalyzes the first step in the metabolism of GSH and its S-conjugates. Through a series of subsequent steps, these conjugates cause cytotoxicity and acute kidney failure (Monks et al., 1997).
The nephron is the functional unit of the kidney composed of three regions: the cortex, medulla, and papilla. Blood flow enters the nephron through the afferent arteriole in the glomerulus. The glomerulus is surrounded by Bowman’s capsule. Initial filtration occurs in this section and filtrate passes through the proximal convoluted tubule, through the loop of Henle, and back into the distal convoluted tubule. Water and nutrient absorption occurs in these segments to produce a concentrated filtrate (urine). Lastly, the filtrate flows through the collecting duct for storage in the bladder prior to excretion. Boundless. “Nephron: The Functional Unit of the Kidney.” Boundless Biology Boundless, 15 Aug. 2016.
1.3 Acute Kidney Injury

1.3.1 Definition and Outcomes Associated with Acute Kidney Injury

Acute kidney injury (AKI) is a common problem affecting critically ill patients and is associated with high degrees of morbidity and mortality (Chang et al., 2014). It is an independent risk factor for chronic kidney disease and end stage renal diseases (Bucaloiu et al., 2012; Coca et al., 2012). The Kidney Disease Improving Global Outcomes (KDIGO) definition of AKI indicates that 22% of adults worldwide admitted to the intensive care unit experience AKI. Over 90% of this incidence rate is found in patients admitted to the hospital for other illnesses (Susantitaphong et al., 2013). The incidence of AKI is highest among patients with cardiovascular diseases; infectious diseases; skin and soft tissue diseases; endocrine, nutritional, and metabolic diseases, and injuries and poisoning (Amin et al., 2012; Chertow et al., 2005).

The short-term effects of AKI are related to deterioration of renal function including disruptions in acid-base and electrolyte homeostasis, uraemia, and volume overload through salt and water retention (Doyle et al., 2016). If these disturbances are not corrected, mortality occurs. In addition to the clinical features associated with AKI, there is a risk for the development of further complications such as sepsis. In an observational study, of 618 critically-ill patients with AKI, 40% developed sepsis (Mehta et al., 2011). Initially, the long-term effects of AKI are assessed by mortality rate and duration of hospital stay. The economic burden for management can be considerable. Several observational studies demonstrate a deterioration in renal function in patients surviving an episode of AKI. In a single-center study of 2,010 patients, those with small elevations of serum creatinine (>26.5 μmol/L) classified as stage 1 AKI had significantly
worse survival over the 10-year study period compared to those with no AKI; the 10-year survival rate was 36.3% (Linder et al., 2014). Follow-up from a retrospective cohort in 2012 showed worsening of renal function at 90 days after the onset of AKI. Only 29.5% of the patients had preserved function and 38.5% of patients had progressed to chronic kidney disease (Lai et al., 2012). Continuous renal replacement therapy by chronic dialysis resulted in rapid progression to end stage kidney disease in critically-ill patients with a single occurrence of AKI (Triverio et al., 2009).

1.3.2 Causes of Acute Kidney Injury

Chemical-induced nephrotoxicity and ischemia-reperfusion injury (IRI) are major etiologies of AKI. In the pathogenesis leading to AKI, reactive oxygen species (ROS) are released resulting in damage to DNA, proteins, and lipids. One example of a chemical-induced nephrotoxicant is cisplatin, a widely used chemotherapeutic agent used in the treatment of a variety of tumors including head and neck, lung, testis, and ovarian cancers (Bar et al., 2016; Fayette et al., 2015; Reed et al., 1986). However, the use of cisplatin is limited by its significant side effects including nephrotoxicity. Following a once per week dose of cisplatin (70 mg/m²) for three weeks, >20% of the patients demonstrated renal damage (Hosohata et al., 2016). Cisplatin-induced nephrotoxicity is a common laboratory model used to study mechanisms of AKI. In the proximal tubule, cisplatin induces apoptosis and autophagy (Rovetta et al., 2012), alterations to cell cycle proteins (Pabla et al., 2015), activation of the mitogen activated protein kinase signaling pathway, and mitochondrial dysfunction (Kim et al., 2005), and DNA damage (Kruger et al., 2016). Cisplatin also induces the generation of ROS that can lead to the aforementioned DNA damage and lipid peroxidation.
IRI causes injury in many tissues and organs including the kidney. During kidney transplantation, renal IRI is a common cause of renal cell death, renal failure, delayed graft function, and renal graft rejection (Shokeir et al., 2015). The ischemic component arises from a disruption of blood flow and subsequent lack of oxygen and the reperfusion component consists of a restoration of blood flow and re-oxygenation (Chatterjee, 2007). IRI causes oxidative stress, inflammation, mitochondrial dysfunction, endothelial disruption, and tissue damage. ROS, reactive nitrogen species, cytokines and chemokines are generated. Leukocytes are activated and infiltrate into the tissue (Malek et al., 2015). ROS released from the damaged tissue can lead to DNA, protein, and lipid alterations (Zheng et al., 2005). In concert with elevations in ROS during IRI, the antioxidant enzymes catalase, superoxide dismutase, and glutathione peroxidase are reduced (Yun et al., 2009). Nitric oxide synthase (NOS) is increased during IRI (Kocak et al., 2016) and the active inducible NOS (iNOS) isoform is detected in the proximal tubule following IRI (Sauvant et al., 2009). Oxidative phosphorylation in the mitochondria is decreased following a lack of oxygen during the ischemic component. Thus, cellular energetics are redistributed with glycolysis being the main mechanism to maintain ATP levels in the short term. However, once reperfusion begins, initially, ATP levels slowly increase as glucose provides greater fuel efficiency under these conditions (Kashiwagi et al., 2015). Lastly, inflammation is a common manifestation in IRI. The inflammatory process is complex and involves a series of cascades that can lead to the death of renal cells. There are several inflammatory mediators in AKI including cytokines (TNFα, IL-6) chemokines and cell adhesion molecules (CXCL1, P-selectin), neutrophils, lymphocytes, natural killer cells, and macrophages. During AKI, there are morphological and functional changes to
endothelial cells in the vasculature and proximal tubule. Cytokines and chemokines are released by endothelial cells contributing to leukocyte infiltration into the damaged kidney. Inflammatory cells produce vasoconstrictors and ROS that can worsen vascular and tubular injury (Akçay et al., 2009). The Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway is one of the major pathways regulating cytokine signal transduction. The importance of JAK2 phosphorylation to AKI is emphasized by findings that inhibition of JAK2 attenuates renal IRI (Yang et al., 2008).

1.3.3 Protective Strategies Against Acute Kidney Injury

Along with the mechanisms of AKI development, a number of compounds have been explored as renal protective agents against AKI. These agents work by maintaining tubular cell function, suppressing inflammation, and preserving the vasculature, and can be classified into clinical drugs, herbs or monomers, antioxidants and mitochondrial protectants, growth factors, molecular intervention, and cell therapies (Figure 1.2). Examples of clinical drugs used to treat AKI are antirheumatics and statins. Etanercept, an antirheumatic, is a fusion protein containing the tumor necrosis factor alpha (TNFα) receptor that is used for the treatment of rheumatoid arthritis.
Insults such as IRI and nephrotoxicants induce tubular injury, inflammation, and vascular dysfunction leading to AKI. Renoprotective agents maintain tubular cell function, suppress inflammation, and preserve the vasculature (Yang et al., 2016).
Etanercept attenuates IRI by decreasing the expression of the inflammatory proteins TNF-α and monocyte chemotactic protein-1 (MCP-1) and decreasing apoptosis (Choi et al., 2009b). It is well known that statins have cholesterol lowering effects. However, the use of statin therapy extends to other conditions including AKI. In patients with acute coronary syndrome, rosuvastatin has beneficial effects on contrast-induced nephropathy (Toso et al., 2014). Mechanistically, statins have additional effects beyond their lipid lowering function. Atorvastatin protects against gentamicin-induced nephrotoxicity by ameliorating renal inflammation, endoplasmic reticulum stress, and apoptosis (Jaikumkao et al., 2016).

Resveratrol is a polyphenol present in many edible plants. It has anti-aging effects, cancer preventive functions, and antidiabetic properties (Novelle et al., 2015). In a cisplatin-induced kidney injury rat model, resveratrol attenuated damage by inhibiting apoptotic signaling (Hao et al., 2016). Sulforaphane (SF) is an organosulfur compound found in cruciferous vegetables such as broccoli. Sulforaphane also has protective effects against renal IRI through the activation of the Nrf2 pathway (Shokeir, et al., 2015).

Oxidative stress is a well-characterized factor in the development of AKI. Thus, the usage of antioxidants and compounds with antioxidant-like properties are becoming common. GSH is a major cellular antioxidant present at millimolar levels in cells. It is synthesized by the precursors glutamine, cysteine, and glycine. N-acetylcysteine (NAC) is a thiol-containing antioxidant. Logistic regression models show that NAC reduced the occurrence of contrast-induced acute AKI in patients with renal dysfunction (Heguilen et al., 2013). In a rat model of gentamicin-induced nephrotoxicity, NAC and vitamin E have protective effects and reduce the levels of blood urea nitrogen (BUN), serum creatinine, GSH, and malondialdehyde (MDA) levels (Patel Manali et al., 2011). The manipulation
of cellular antioxidant enzymes has protective effects in AKI. A deletion of superoxide dismutase 3 in mice undergoing IRI further attenuates renal blood flow, exacerbates oxidative stress, and accelerates renal cast formation compared to the wild type counterparts (Schneider et al., 2010).

Mitochondrial damage is a common occurrence in tubular cell death in AKI. The burst of ROS released during the reperfusion period of IRI leads to the opening of the mitochondrial permeability transition (MPT) pore resulting in mitochondrial depolarization, decreased ATP synthesis, and increased ROS generation. MPT is used as a target for pharmacologic intervention in IRI. Inhibiting MPT with a mitochondria-targeted peptide (SS-31) preserves mitochondrial function by accelerating ATP recovery, reducing oxidative stress, minimizing infiltration of inflammatory cells, and decreasing tubular injury (Szeto et al., 2011). In addition to decreasing mitochondrial damage, another strategy to protect mitochondrial function is to increase mitochondrial biogenesis. Agonism of the 5-hydroxytryptamine (5-HT) receptor decreases BUN and kidney injury molecule-1 (Kim-1) levels following IRI. 5-HT receptor agonism increases the expression of genes in the electron transport chain and increases mitochondrial DNA copy number; indices of improvements in mitochondrial biogenesis (Garrett et al., 2014).

Growth factors, signaling molecules that stimulate cellular growth and proliferation, have been employed as renoprotective agents. Activation of the epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and hepatocyte growth factor (HGF) all have beneficial effects against AKI. EGF attenuates mercury chloride-induced AKI by enhancing proximal tubule cell division (Yen et al., 2015). In addition, IGF-1 has protective effects against mercury chloride-induced AKI through changes in insulin
receptor signaling (Friedlaender et al., 1995). The HGF receptor, c-met, is required for renal protection after AKI. Upon generating proximal tubule specific c-met knockout mice, kidney injury is exacerbated in both cisplatin and IRI-induced AKI (Mason et al., 2014; Zhou et al., 2013).

Multiple molecular intervention-type approaches are being used as renal protective compounds. Nuclear factor kappa beta (NFκβ) is a transcription factor that promotes inflammation, cytokine and chemokine production, and immune cell infiltration. NF-κβ inhibition is associated with decreased pro-inflammatory mediators and an amelioration in cisplatin-induced AKI (Ozkok et al., 2016). Thiazolidinediones are a class of antidiabetic drugs that act as peroxisome proliferator-activated receptor γ (PPARγ) agonists. Pioglitazone is a synthetic ligand in this class used to treat type 2 diabetic patients. In a type 2 diabetic rat model, a low dose of pioglitazone ameliorates renal fibrosis and preserves renal function (Toblli et al., 2009). Pioglitazone’s beneficial effects extend beyond diabetes; in a cisplatin-induced AKI model, pioglitazone improves BUN and creatinine levels and histopathological alterations (Jesse et al., 2014).

MicroRNAs (miRNAs) are short RNA segments produced endogenously that negatively regulate target gene expression by binding to complementary regions of mRNA and inhibiting translation. miRNAs are involved in renal physiology and pathogenesis of kidney diseases (Badal et al., 2015). Multiple miRNAs are upregulated in response to damaging insults in the kidney. For example, miRNA-155 deficient mice and mice with a systemic injection of an anti-miRNA-687 LNA oligonucleotide probe exhibit elevated levels of kidney injury markers during the development of AKI indicating a protective role
of these miRNA (Bhatt et al., 2015; Pellegrini et al., 2014). Thus, targeting specific miRNAs may provide an effective therapeutic strategy for the treatment of AKI.

The use of epigenetic regulators is a newer approach in the development of therapeutics to treat AKI. Epigenetics refers to alterations in gene expression without changing the DNA sequence. DNA methylation and histone modifications (e.g., acetylation, deacetylation) are two major epigenetic mechanisms that regulate chromatin structure and subsequently gene expression. The role of histone deacetylase (HDAC) inhibitors provided initial insights into the potential for epigenetic regulation to treat AKI; some HDAC inhibitors worsen AKI while others provide protective effects (Levine et al., 2015; Tang et al., 2014). More recently, investigations are beginning to decipher epigenetic mechanisms in AKI. In two commonly used models, IRI and endotoxin induced-injury, the expression pattern of AKI-related genes is different. Exploration into histone marks at the transcriptional start sites of these genes demonstrates diversity and involvement of multiple epigenetic pathways (Mar et al., 2015).

Stem cells and endothelial progenitor cells exhibit renoprotective effects against AKI. Mesenchymal stem cells (MSC) represent a stem cell population that resides in the bone marrow and demonstrate protective effects against AKI (Morigi et al., 2008). These cells function by releasing paracrine factors that limit injury (Tögel et al., 2007). Vascular endothelial growth factor (VEGF) is one of the paracrine factors critical in protection. Manipulation of MSCs with VEGF siRNA attenuates protection against AKI (Yuan et al., 2011). Endothelial progenitor cells (EPCs) are circulating progenitor cells derived from the bone marrow. Similar to stem cells, EPCs exert their protective functions through paracrine mechanisms and the release of growth factors (Tögel, et al., 2007). Exosomes
derived from EPCs protect the kidney from IRI-induced functional impairment and morphologic injury. Mechanistically, EPCs transfer their RNA content and specific miRNAs mediating angiogenesis are involved in reprogramming of the resident renal cells (Cantaluppi et al., 2012). EPC-derived exosomes improve kidney function during IRI. Specifically, exosomal transfer of miRNA-486-5p to endothelial cells targets the PTEN/AKT pathway reducing apoptosis during IRI (Vinas et al., 2016).

Ischemic preconditioning (IPC) is the adaptability of an organ or tissue following brief periods of ischemia. IPC-induced cytoprotection is conserved across tissues and several species (Joo et al., 2006; Kinsey et al., 2010; Murry et al., 1986). Preconditioning with hypoxia has similar effects to preconditioning with ischemia. The reduction in infarct size of hearts between preconditioned hypoxic dogs and preconditioned ischemic dogs is comparable (Shizukuda et al., 1992). Thus, when investigating effects of ischemic preconditioning in cell culture, hypoxic conditions can be used. Preconditioning is characterized by two phases of protection; an acute early phase and a late phase. The early phase begins immediately after the preconditioning begins and lasts for hours while the later phase begins at approximately 24 hours and lasts for days (Joo, et al., 2006). During these phases, signaling events occur to mediate the protection.
1.4 Para-Aminophenol

Aminophenols are components of a variety of chemicals and are generated by the biotransformation of aromatic amines (Evelo et al., 1984). Para-aminophenol (PAP) is used in hair dyes and photographic processing (Hamann et al., 2014), and is formed as a metabolite of acetaminophen (Carpenter et al., 1981; Newton et al., 1983a), phenacetin (Smith et al., 1976), and aniline (Kao et al., 1978).

PAP is an acute nephrotoxicant inducing necrosis in the pars recta region of the proximal tubule (Green et al., 1969). A number of functional changes accompany the morphological damage. In the Fisher 344 rat, a 100 mg/kg dose of PAP increases BUN levels at 24 and 48 hours. The accumulation of p-aminohippurate (PAH) and tetraethylammonium (TEA) in thin renal cortical slices is reduced following PAP exposure indicative of alterations in organic anion and cation transport (Newton et al., 1982). In addition, PAP treatment decreases glomerular filtration rate (GFR) and increases proximal intratubular pressure (Davis et al., 1983). A high resolution $^1$H-NMR analysis of urine from rats treated with increasing doses of PAP (25-100 mg/kg) demonstrated dose-related elevations in glucose, lactate, and amino acids (alanine, lysine, glutamine, valine) (Gartland et al., 1989). Strain differences in susceptibility to PAP-induced damage has been found between the Sprague-Dawley (SD) and the Fisher 344 rat. PAP-induced renal lesions in the F344 rats are more severe than the SD rats. PAP-induced functional changes in BUN and accumulation of PAH by renal cortical slices correlate with the morphological findings (Newton et al., 1983b).

Similar to acetaminophen-induced hepatotoxicity (McMurtry et al., 1978), it has been suggested that PAP-induced nephrotoxicity results from the formation of the reactive
intermediate 1,4-benzoquinoneimine which covalently binds to thiol groups of protein and GSH. In support of this suggestion, PAP reduces renal but not hepatic GSH. Moreover, PAP covalently binds to proteins in rats and kidney microsomes and the degree of covalent binding is higher in the kidney compared to the liver (Calder et al., 1979; Crowe et al., 1979).

PAP oxidation and the generation of the quinoneimine intermediate leads to nephrotoxicity and can be catalyzed by cytochrome P450, horseradish peroxidase, or prostaglandin synthase; the latter reaction leads to the formation of the \( p \)-aminophenoxo free radical. Oxidation of this radical yields \( p \)-benzoquinoneimine (Figure 1.3) (Josephy et al., 1983; Newton, et al., 1982). Quinone compounds are prone to Michael-type additions with sulphydryl groups (Li et al., 2016c). Eckert and colleagues reacted 1,4-benzoquinoneimine with GSH and identified several GSH conjugates including 4-amino-2-(glutathion-S-yl)phenol, 4-amino-3-(glutathion-S-yl)phenol, 4-amino-2,5-bis-(glutathion-S-yl)phenol, and 4-amino-2,3,6-tris-(glutathion-S-yl)phenol (Eckert et al., 1990). GSH conjugates have been demonstrated to mediate the nephrotoxicity of bromobenzene and haloalkenes (Birner et al., 1998; Monks et al., 1985). The structures of GSH conjugates amongst the bromobenzenes are similar, supporting the suggestion that PAP toxicity is dependent on GSH-mediated mechanisms. Moreover, depletion of GSH with buthionine sulfoximine (BSO) completely protected rats against PAP-induced nephrotoxicity with bile duct cannulation affording partial protection (Gartland et al., 1990). GSH S-conjugates were identified in rat bile as minor metabolites of PAP and the di and trisubstituted thio-adducts are toxic to rat kidney cortical cells (Klos et al., 1992). The 4-amino-3 (glutathion-S-yl)phenol conjugate is nephrotoxic in rats inducing necrosis
similar to PAP but at lower doses (Fowler et al., 1991). These findings suggest that the GSH conjugates may be formed in the liver, excreted in the bile, reabsorbed, and transported in the systemic circulation to reach the kidney where the toxic effects occur. However, the small level of formation of the conjugates in the bile (2-3%) and the fact that GSH conjugations of bromohydroquinone and haloalkenes occur in the kidney suggest that the formation of PAP-GSH can also occur in the kidney. Generation of PAP-GSH in the kidney may be more relevant to the renal toxicity.

The first step in the metabolism of GSH and GSH conjugates is catalyzed by γ-GT. Further metabolism by the cysteine conjugate, β-lyase, and the formation and organic ion transport of mercapturic acids may all be relevant to PAP-GSH induced nephrotoxicity (Lock et al., 1985; Stevens et al., 1985). Fowler et al (1994) conducted a rat study to gain further insights into the mechanism of PAP-GSH induced nephrotoxicity and found that co-administration of ascorbic acid with PAP-GSH ameliorated the nephrotoxicity. In addition, pretreatment of rats with acivicin, an inhibitor of γ-GT, decreased necrosis of the proximal tubules. Probenecid, an inhibitor of renal organic anion transport, and aminooxyacetic acid, an inhibitor of β-lyase, had no effect on the toxicity of PAP-GSH. Thus, the mechanism of PAP-GSH induced renal toxicity seems to involve the oxidation of PAP to a reactive quinoneimine intermediate and further processing of the conjugate by γ-GT (Fowler et al., 1994).
Figure 1.3  Mechanism of PAP toxicity through generation of quinoneimine intermediate

PAP oxidation is catalyzed by cytochrome P450 leading to formation of free radicals and redox cycling leading to toxicity (Harmon et al., 2005)
Similar studies examined the effects of unconjugated PAP and the contribution of oxidation and potential formation of a reactive metabolite, $\gamma$-GT, and organic ion transport. As observed with PAP-GSH, ascorbic acid prevented PAP nephrotoxicity and probenecid had no effect. In contrast, however, acivicin pretreatment led to a potentiation of PAP nephrotoxicity (Fowler et al., 1993). While acivicin has protective effects against PAP-GSH and other GSH conjugates (Lau et al., 1995), it does not always afford protection against the parent compound as shown with PAP and hexachlorobutadiene (Davis, 1988). Therefore, it is not clear whether GSH conjugation is critical to PAP-induced renal toxicity. It appears more likely that a step prior to the formation of a GSH conjugate contributes to the proximal tubule necrosis such as the generation of a phenoxyradical or 1,4-benzoquinoneimine.

In LLC-PK$_1$ cells, PAP induces cytotoxicity via redox cycling and oxidation to a reactive intermediate and pretreatment of cells with ascorbate or GSH protects against PAP-induced cell injury (Hallman et al., 2000; Kendig et al., 2007). Following PAP-induced generation of ROS, PAP decreases pyruvate-stimulated gluconeogenesis, GSH levels, and adenine nucleotide levels prior to an increase in LDH leakage. Moreover, PAP increases the appearance of 4-HNE-adducted proteins indicating an increase in lipid peroxidation (Harmon, et al., 2005). Multiple other investigations demonstrate functional effects of PAP on the mitochondria and endoplasmic reticulum implying that these are target organs of toxicity (Crowe et al., 1977a; Crowe et al., 1977b; Lock et al., 1993; Peyrou et al., 2007a; Peyrou et al., 2007b; Peyrou et al., 2007c; Tange et al., 1977).

From a clinical perspective, the serum biomarkers BUN and creatinine are used to determine an effect on renal function. Histopathology is the gold standard to observe a
morphological finding. There are ongoing attempts to obtain more reliable biomarkers to assess early renal damage. In addition to the known PAP-induced increases in BUN and creatinine, and the morphological features associated with proximal tubular necrosis, PAP increases urinary α-glutathione S-transferase levels, a specific indicator of proximal tubular injury. Furthermore, immunohistochemical staining of the kidney demonstrates elevated expression of vimentin, osteopontin, and Ki-67; all associated with tubular regeneration. Clusterin and Kim-1 display features associated with both renal injury and regeneration (Yang et al., 2007). Various miRNAs have also been used as biomarkers of kidney function. miRNA-21, -200c, and -423 are increased at least 10-fold in the medium of HPTEC cells following PAP exposure (Pavkovic et al., 2016).
1.5 Vitamin A and Retinoids

1.5.1 Importance of Vitamin A

Vitamin A is a fat-soluble vitamin essential for human survival. Vitamin A stimulates multiple physiological processes including those involved in cellular growth and differentiation, immune system regulation, vision, and apoptosis. In addition, vitamin A has found clinical application in the treatment of cancer and acne (Alvarez et al., 2014). These pleiotropic effects are not mediated by vitamin A itself but rather through its active metabolites all-trans-retinoic acid (ATRA) and 9-cis retinoic acid (9-cis RA). ATRA is the predominant endogenous active metabolite of vitamin A and exerts its effects through both genomic and non-genomic signaling mechanisms (Al Tanoury et al., 2013).

Deficiency of vitamin A leads to blindness, immunodeficiency, alterations in the gut microbiome, retarded growth, and iron deficiency-induced anemia (Wiseman et al., 2016). Conversely, excess consumption of dietary vitamin A can lead to toxic effects in the liver, central nervous system, musculo-skeletal system, skin, internal organs, and circulation (Blomhoff et al., 2006; Collins et al., 1999). In addition, vitamin A toxicity is associated with reduced bone mineral density and an increased risk for hip fracture and teratogenesis in the developing embryo (Collins, et al., 1999; Melhus et al., 1998).

1.5.2 Retinoid Biosynthesis, Metabolism, Transport, Target Tissue Effects

Retinoids are derivatives of retinol that cannot be synthesized de novo and must be obtained from the diet and are found in both plant and animal products. Plant products contain carotenoids, or provitamin A, that is cleaved to form retinoids whereas animals contain vitamin A. Carotenoids are the components that give rise to the yellow, red, orange, or purple color of fruits and vegetables (Blomhoff, et al., 2006). Retinoids are
absorbed in the enterocytes of the small intestine (Figure 1.4A). Retinyl esters are hydrolyzed to retinol and β-carotene is metabolized to retinaldehyde. Retinol and retinaldehyde bind to the cellular retinol-binding protein (CRBP). The formation of the retinol/retinaldehyde/CRBP complex prevents oxidation of the retinoids and serves as a substrate for the enzyme lecithin:retinol acyltransferase (LRAT) for esterification. Retinyl esters are packaged as chylomicrons which are secreted into the circulation and are delivered to hepatic parenchymal cells. In the liver, the retinyl esters are hydrolyzed to retinol which forms a complex with retinol binding protein (RBP). This complex is delivered to hepatic stellate cells where reesterification again occurs. Stellate cells are the location where over 80% of retinoids are stored (Theodosiou et al., 2010). These cells have high levels of CRBP and LRAT. Studies in CRBP and LRAT knockout mice indicate the importance of these proteins for retinyl ester storage (Batten et al., 2004; Ghyselinck et al., 1999). The remaining retinyl ester storage occurs as lipid droplets in other organs including the lung and kidney (Blomhoff, et al., 2006). The retinyl esters are hydrolyzed to retinol and bind to RBP for transport in the circulation to target tissues. As retinol is hydrophobic, it must bind to proteins for transport. Moreover, retinol/RBP binding helps prevent enzymatic degradation. The retinol/RBP complex further binds to transthyretin (TTR) in the bloodstream to prevent renal elimination (Newcomer et al., 2000).

At the target tissue, TTR dissociates from the retinol/RBP complex and holo-RBP is incorporated by the STRA6 receptor (Kawaguchi et al., 2007). In the target tissue, retinol is either converted to retinyl esters and stored or oxidized to retinaldehyde catalyzed by the retinol dehydrogenase enzymes. Subsequently, in an irreversible reaction catalyzed by the retinaldehyde dehydrogenase enzymes, retinaldehyde is oxidized to retinoic acid.
Retinoic acid is bound to the cellular retinoic acid binding protein (CRABP) and enters the nucleus to activate transcription of target genes or is transported to a target cell. CRABP has functions in delivering retinoic acid to the nucleus (Napoli, 1996). It is important to retain a balance between synthesis and degradation of retinoic acid. Catabolism of retinoic acid to oxidized metabolites occurs through the CYP26 family of enzymes.

1.5.3 Retinoid Receptor-Dependent Signaling

The biological activities of retinoids are mediated through genomic and non-genomic signaling schemes. In the nucleus, ATRA or 9-cis RA bind to the retinoid acid receptor (RAR) and retinoic x receptor (RXR) transcription factors which induce a battery of target genes (Figure 1.4B). Both RAR and RXR exist as three subtypes designated as α, β, and γ, denoting different isoforms. RAR can be activated by both ATRA and 9-cis RA whereas RXR can only be activated by 9-cis RA. A heterodimer forms between RAR and RXR for transcriptional activity. RXR homodimers and heterodimers with other nuclear receptors can also occur mediating additional signaling pathways. Similar to other nuclear receptors, the retinoid receptors possess a DNA binding domain, conferring specific DNA recognition, and a ligand binding domain allowing for ligand-dependent activation. The ligand-bound heterodimer binds to the retinoic acid response element on DNA to induce transcription of the target genes (Germain et al., 2006). Along with the dimer and ligand, multiple other RAR coregulatory molecules participate in the signaling process. These co-regulators interact with the ligand binding domain and the discrimination between coactivator and corepressor binding is governed by the presence of a ligand. In the absence of a ligand, corepressors such as the nuclear receptor corepressor (NCoR) or silencing mediator of retinoic acid and thyroid hormone receptor (SMRT), are
bound. These corepressors recruit complexes with HDAC activity to maintain the chromatin in a closed state. Upon ligand binding, the heterodimer undergoes conformational changes that cause the release of the corepressor and binding of the coactivator. Similar to the corepressors, the coactivators recruit complexes with histone acetyl transferase activity to promote chromatin configuration in an open state and thus are capable of recruiting all of the transcriptional machinery (Al Tanoury, et al., 2013).

Over the last few decades, over 500 retinoid target genes have been identified. In some cases, gene regulation is directly driven by the RAR/RXR heterodimer bound to the RARE promoter. However, in most cases, gene regulation occurs through intermediate transcription factors or associations with other proteins. Only 27 genes were unquestionably identified as direct targets and another 105 genes appeared to be possible candidates. The remaining target genes appeared to be regulated indirectly through phosphorylation and signaling schemes. With the 27 direct target genes and several others classified as likely to be direct target genes, patterns were noted in genes related to the handling, metabolism, function, or evolutionary history of retinoids as well as genes containing homeobox domains. Examples of some of these genes include RARα, RARβ, RARγ, RBP1, CRABPII, ADH1C, EDH17B2, CYP26, and NTCP; genes that contain homeobox domains include Hoxa1, HOXA4, Hoxb1, Hoxb4, Hoxd4, Cdx1, and Pit1. Hox genes are important during embryonic development (Balmer et al., 2002; Blomhoff, et al., 2006).
Figure 1.4  Retinoid Biosynthesis, Metabolism, Transport, and Signaling

(A) Retinoid biosynthesis, metabolism, and transport. Many proteins are involved in mediating retinoid biochemistry in multiple organs as explained in detail in the text.

(B) Retinoid receptor-dependent signaling. Retinoid induction of target genes is mediated through receptors and co-regulators as explained in detail in the text (Theodosiou, et al., 2010).
1.5.4 Retinoid Receptor-Independent Signaling

Non-genomic retinoid signaling is independent of RAR/RXR mediated gene transcription and occurs as a result of retinoic acid binding to retinoid receptors outside the nucleus. Non-genomic effects of retinoic acid can also occur in the absence of retinoid receptors. Indeed, in SH-SY5Y neuroblastoma cells, the RARα receptor associates with the phosphoinositol 3 kinase (PI3K) regulatory subunit p85 in the absence of retinoic acid. Ligand binding to RARα facilitates the association of the PI3kinase catalytic subunit, p110, thus promoting PI3kinase activity (Masia et al., 2007). Retinoic acid enhances acetylcholine transmitter release at developing neuromuscular synapses through a nongenomic activation of RARβ (Liou et al., 2005). Retinoic acid can also modulate the activity of several signaling proteins independently of the RAR/RXR heterodimer. For example, in human bronchial epithelial cells, retinoic acid induces cAMP response element-binding (CREB) protein independently of RAR/RXR signaling. The retinoic acid induction of CREB requires an upregulation of protein kinase C (PKC), extracellular regulated kinase (ERK), and p90 ribosomal S6 kinase (RSK) activity (Aggarwal et al., 2006). Moreover, retinoic acid modulates the activity of different kinases including PKC and ERK by direct binding and other non-genomic mechanisms (Cañón et al., 2004; Radominska-Pandya et al., 2000).

1.5.5 Uses of Retinoids

Retinoids have widespread usage in modulating disease processes. Retinoids are chemopreventive, in part due to their anti-proliferative and pro-differentiation effects. The anti-cancer effects of retinoids was first demonstrated in 1925 (Wolbach et al., 1925). The best-characterized application of retinoids in treating cancer is in acute promyelocytic
leukemia (APL). In APL, there is a chromosomal translocation t(15:17) leading to the expression of an oncoprotein consisting of the RARα receptor fused to the PML protein (Borrow et al., 1990; de The et al., 1990). Hematopoietic cells expressing the RAR-α-PML fusion protein are unable to differentiate (de The et al., 1991). Retinoids induce differentiation and reactivate the disrupted signaling pathways. The use of ATRA for APL is FDA-approved and the first successful use of ATRA to induce complete remission in patients with AML was conducted in 1987 with an even greater benefit found with combination therapy. Thus, chemotherapy with ATRA and arsenic trioxide leads to complete remission rates exceeding 93% and the 5-year survival rates approach 100% (Schenk et al., 2014).

9-cis RA has been approved by the FDA for use in the treatment of cutaneous lesions in Kaposi’s sarcoma (Baumann et al., 2005). In rodent studies, 9-cis RA has chemopreventive effects in mammary and prostate cancer (Christov et al., 2002; Wu et al., 2000). The 13-cis RA isomer (isotretinoin) has been effective in various cancers including neuroblastoma and squamous cell carcinoma. In children with high-risk neuroblastoma, 13-cis RA reduces the risk of reoccurrence and improves long-term survival (Matthay et al., 2009). In addition, 13-cis RA treatment delays the development of secondary head and neck squamous cell carcinomas (Hong et al., 1990).

Current approaches for the use of retinoids in cancer treatment are focused on combination therapies of a retinoid with a cancer preventative agent. Of note, in breast cancer, ATRA alone fails to have an anti-tumor effect (Sutton et al., 1997). However, when combined with tamoxifen, the differentiation-inducing effect of ATRA is enhanced as evidenced by an increase in NBT reduction, a functional marker of differentiation.
The combination of ATRA and chemotherapy increases response rates in patients with advanced non-small-cell lung cancer (Arrieta et al., 2010). The Biomarker-integrated Approaches of Targeted Therapy for Lung Cancer Elimination (BATTLE) trial is the first completed prospective, biopsy-mandated, biomarker-based, adaptively randomized study in pretreated lung cancer patients. One of the treatment arms incorporates bexarotene, a synthetic rexinoid, in combination with the tyrosine kinase inhibitor, erlotinib. This combination treatment produced a 50% 8-week disease control rate (Kim et al., 2011). Related findings with the bexarotene/erlotinib combination treatment were obtained in chemotherapy-refractory mutant KRAS tumors (Dragnev et al., 2011).

Commercially, retinoids are used widely in treating skin conditions. The 13-cis RA isomer (isotretinoin) is efficacious orally in treating acne as determined by the number of nodules and cysts present before and after therapy (Peck et al., 1979). Mechanistically, 13-cis RA normalizes epithelial differentiation, depresses sebum secretion, and reduces inflammation (Uray et al., 2016). Several retinoids are used in creams and ointments and are available for topical use; tretinoin and adapalene gels are used in treating acne (Millikan, 2001). Systemically, isotretinoin (as Accutane until 2009 and now Claravis) is used to treat severe, nodular acne. In an intervention study, 97.4% patients reported that their acne was improved at the 12-month follow up. Patients receiving the higher dose treatment (a cumulative dose of 220 mg/kg) had a significantly decreased risk of relapse (Blasiak et al., 2013).

In addition to acne, retinoids ameliorate other skin conditions including photodamage, psoriasis, disorders of keratinization, and precancerous skin lesions.
Retinoids are used as skin-repair agents through their ability to reduce the effects associated with collagen degradation (Varani et al., 2007). Psoriasis is a chronic disease of the immune system that impacts skin appearance. Thus, psoriasis treatment usually involves immunosuppressive agents such as cyclosporine, methotrexate, and biologic agents targeting T-cells (Uray, et al., 2016). Retinoids have been used in combination with phototherapy to manage psoriasis. In patients with chronic plaque psoriasis, the combination of isotretinoin with psoralen + sun exposure (PUVAsol) is more effective than PUVA sol alone, especially in women of child-bearing age (Gahalaut et al., 2014).

Tocoretinate, the α-tocopherol ester of ATRA, has been used to treat the skin disorders of lichen and macular amyloidosis and skin ulcers (Terao et al., 2011).

Retinoids have beneficial roles in additional conditions albeit in a preclinical setting. Retinoids may impact neurodegenerative diseases including Alzheimer’s disease (AD). The major pathological hallmarks of AD are the formation of plaques and neurofibrillary tangles. A main component in neurofibrillary tangles is the hyperphosphorylation of the tau protein. In a transgenic AD mouse model, ATRA-treated mice demonstrated fewer and smaller tau aggregates as a result of a decreased expression of kinases responsible for tau phosphorylation (Watamura et al., 2016).

Multiple studies have documented a role for retinoids in improving metabolic disease conditions. It has long been established that ATRA can activate the RAR nuclear receptor to mediate biological activities. More recent evidence demonstrates that ATRA can serve as a ligand for the nuclear receptor peroxisome proliferation-activated receptor (PPAR) β/δ (Shaw et al., 2003). ATRA is able to repress obesity and insulin resistance by
activating both the PPAR-β/δ and RAR receptors (Berry et al., 2009; Tsuchiya et al., 2012).

Finally, retinoids have shown promise in the management of several renal diseases including nephritis, nephrosis, and renal allograft rejection (Xu et al., 2004). ATRA and other RARα agonists afford protection against renal injury in the glomerular component of the nephron (Li et al., 2014; Moreno-Manzano et al., 1999). ATRA protective effects are also found in the heart through a reduction in cardiomyocyte apoptosis (Zhu et al., 2015).

In the brain, ATRA reduces cerebral ischemic injury by reducing the inflammatory markers COX-2 and C/EBP (Choi et al., 2009a). In the liver, ATRA decreases IRI through anti-inflammatory and antioxidant effects (Rao et al., 2013; Rao et al., 2010). Moreover, ATRA induces autophagy signaling as a mechanism of its protective effect against liver injury (Zhong et al., 2015). 9-cis RA is protective against renal ischemia reperfusion injury (Balasubramanian et al., 2012). While these in vivo studies demonstrate a RA-mediated protective effect, the molecular mechanisms involved are not well established.
1.6 Nrf2 Signaling and Cytoprotection

1.6.1 Nrf2 Signaling

The nuclear factor erythroid-derived-2-like 2 (Nrf2) is a member of the cap-‘n’-collar family of basic leucine zipper transcription factors. Nrf2 is tightly regulated and under basal conditions, is degraded by the ubiquitin proteasome system (Figure 1.5). Nrf2 is negatively regulated through an interaction with the Kelch-like ECH-associated protein 1 (Keap1), an adaptor protein for the cullin3 (Cul3)-based E3 ubiquitin ligase (Zhang et al., 2004). Under conditions of stress, Nrf2 levels rise and Nrf2 forms a dimer with the small Maf protein to bind to antioxidant response elements (ARE) in the DNA. ARE-responsive target genes include detoxifying enzymes, transporters, stress response proteins, and metabolic enzymes (Harder et al., 2015). Nrf2 activation leads to a reduction in ROS generation induced by cell stress and electrophiles. Upon a return to unstressed, basal conditions, Nrf2 relocates to the cytosol and binds to Keap1, maintaining cellular homeostasis.

Nrf2 is regulated through post translational mechanisms by the ubiquitin proteasome system (Sun et al., 2009). The Neh2 domain of Nrf2 is an important recognition site for Keap1 binding. The cysteine 151 residues on Keap1 are important sensors for ROS and electrophilic species and become modified ultimately inducing a conformational change in Keap1, dissociation from Nrf2, and subsequent Nrf2 activation (Zhang et al., 2003). In addition to the Neh2 domain, the Neh6 domain of Nrf2 has an important regulatory role. In a redox-insensitive manner, this domain contains serine residues that are phosphorylated by glycogen synthase kinase 3 (GSK3) creating a motif that can be recognized by an E3 ligase, signaling proteasomal degradation.
Figure 1.5. The Nrf2 signaling pathway

(A) Key domain structure of Nrf2.
(B) The canonical Nrf2 signaling pathway. Upon cellular insult, cysteine residues on Keap1 are modified leading to dissociation from Nrf2. Nrf2 is activated and translocates to the nucleus where it dimerizes with Maf and binds to the ARE promoter region to upregulate transcription of target genes (Harder, et al., 2015)
Nrf2 has additional regulatory controls beyond the ubiquitin proteasome system. For example, the p62 scaffold protein shuttles proteins destined for lysosomal degradation into autophagosomes. Disturbances in autophagy lead to an accumulation of Keap1 in autophagosomes and activation of Nrf2 since Keap1 is unavailable for binding (Lau et al., 2010). Indeed, arsenic is a human carcinogen and environmental toxicant that induces Nrf2 through this non-canonical pathway (Lau et al., 2013b).

1.6.2 Nrf2 and Cytoprotection

Numerous animal- and cell-based studies demonstrate critical roles of Nrf2 in cytoprotective processes in multiple organs and cell types including the kidney, liver, and lung (Copple et al., 2008). In renal epithelial LLC-PK₁ cells, the antioxidant dicatechol nordihydroguaiaretic acid (NDGA) affords cytoprotection against hydrogen peroxide-induced cell death by an increase in Nrf2 stability and activation. Mechanistically, NDGA activates Nrf2 through multiple signaling cascades involving cellular stress kinases and GSK3β integrates these pathways at the level of the Neh6 phosphodegron (Rojo et al., 2012). The Nrf2 inducer, bardoxolone methyl (CDDO-Me) and the Nrf2 triterpenoid activator CDDO-imidazolide protect kidneys from IRI through the increased expression of Nrf2 and its target genes (Liu et al., 2014a; Wu et al., 2011). In streptozotocin-induced diabetic nephropathy, the Nrf2 activators sulforaphane (SF) or cinnamic aldehyde (CA) improve renal damage associated with diabetes (Zheng et al., 2011). In an epithelial human hepatocyte cell line, L02, the naturally occurring flavonoid hyperoside protects L02 cells against hydrogen peroxide-induced damage also through the activation of the Nrf2 signaling pathway (Xing et al., 2015). Furthermore, the carotinoid, bixin, protects against lung injury in an Nrf2 dependent manner (Tao et al., 2016).
1.6.3 Prevalence of Nrf2 Signaling in Cancer

Many dietary compounds have chemopreventive properties with several identified as Nrf2 inducers; examples include SF (cruciferous vegetables), curcumin (spice), epigallocatechin-3-gallate (EGCG) (green tea), resveratrol (grapes), wasabi (Japanese horseradish), cafestol and kahweol (coffee), cinnamonyl-based compounds (cinnamon), zerumbone (ginger), garlic organosulfur compounds (garlic), lycopene (tomato), and carnosol (rosemary) (Lau et al., 2008). These compounds exert their chemopreventive activity by inducing Nrf2 target genes that help defend cells from carcinogens. However, several reports indicate a “dark” side to Nrf2. While Nrf2 protects normal cells from transforming into cancer cells, Nrf2 can promote the survival of cancer cells. In multiple carcinogenic cell types, Nrf2 enhances resistance of cancer cells to chemotherapeutic drugs (Jiang et al., 2010; Shim et al., 2009; Wang et al., 2008). Consequently, Nrf2 inhibitors are being developed to combat the constitutive Nrf2 activation found in cancer cells. Brusatol was the first Nrf2 inhibitor identified by Ren and colleagues in 2011. Brusatol decreases Nrf2 protein levels in cancer cells and sensitizes these cells to chemotherapeutic drugs such as cisplatin (Ren et al., 2011). Mechanistically, brusatol overcomes chemoresistance through protein translation independent of Nrf2 suggesting the need for further development of Nrf2 inhibitors to further understand the “dark” side of Nrf2 (Harder et al., 2016). Therefore, while Nrf2 clearly has beneficial effects, the role of Nrf2 as a signaling mediator is context dependent.
1.7 Glucose Regulated Protein 78 (Grp78) and Cytoprotection

1.7.1 Endoplasmic Reticulum (ER) Stress Signaling

The endoplasmic reticulum (ER) is a network of membranes and flattened sacs present in all eukaryotic cells. About one-third of newly synthesized proteins translocate to the lumen of the ER for proper folding into three-dimensional structures before being delivered to other cellular organelles or the plasma membrane. While the ER is mainly recognized as a protein folding factory, it has roles in intracellular calcium homeostasis and lipid biosynthesis (Kaufman, 1999). The ER senses cell stress conditions and the accumulation of misfolded proteins and evokes a compensatory response termed the unfolded protein response (UPR). While the initial intent of the UPR is to reestablish homeostasis, adaptive mechanisms are in place to inhibit translation of mRNAs for a brief period to reduce the influx of new proteins into the ER. If the adaptive responses fail, signal transduction events that induce apoptotic proteins are increased (Kim et al., 2008). The ER stress mediated responses are depicted in Figure 1.6.

There are three signaling pathways associated with the UPR. Under normal homeostatic conditions, the major ER chaperone and master UPR regulator glucose regulated protein 78 (Grp78) binds to the ER stress sensors inositol-requiring 1α (IRE1α), double stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK), and activating transcription factor 6 (ATF6) maintaining these proteins in an inactive configuration.
Figure 1.6  **Mammalian UPR pathways**

There are three signaling pathways associated with the UPR. Grp78, also referred to as BiP, is the master regulator and under stressful conditions, signaling in the UPR is increased to assist in correct protein folding and the return to homeostasis (Zhang *et al.*, 2008).
During ER stress, Grp78 dissociates from these sensor proteins and binds to unfolded proteins thereby activating the sensor proteins and the UPR signaling cascades. The release of Grp78 allows for the activation of PERK through autophosphorylation. Activated PERK phosphorylates and activates the elongation initiation factor eIF2α, thus reducing translation initiation. Some mRNAs, such as activating transcription factor 4 (ATF4), are translated independently of peIF2α. The dissociation of Grp78 allows IRE1α to autophosphorylate and activate its kinase and endoribonuclease activity. IRE1α serves as a splice factor to activate X-box-binding protein 1 (XBP1). Upon Grp78 dissociation, ATF6 translocates to the Golgi apparatus where it is cleaved by proteases, yielding an active fragment. ATF4, XBP1, and ATF6 all migrate to the nucleus where they induce transcription of UPR target genes (Zhang, et al., 2008).

1.7.2 Grp78 and Cytoprotection

Grp78 was originally identified as an immunoglobulin heavy chain binding protein for its role in immunoglobulins (Haas et al., 1983). Grp78 is a member of the heat shock 70 (Hsp70) family of proteins and is present in high abundance in the ER. Under homeostatic conditions, Grp78 is bound to the UPR stress sensors and interaction partners (Dudek et al., 2009). Grp78 and its interaction partners have a variety of functions in protein transport into the ER, folding and assembly of proteins, export and degradation of proteins, and signal transduction. Protein transport into the ER involves the ribosome, a signal recognition particle (SRP), and the SRP and ribosome receptors on the ER surface; collectively termed the Sec61 complex. Grp78 is involved in the insertion of precursor polypeptides into the Sec61 complex and opening and closing of the Sec61 channel (Alder et al., 2005; Dierks et al., 1996; Hamann, et al., 2014; Klappa et al., 1991). In the
assembly of proteins, heat shock protein 40 (Hsp40) co-chaperones and nucleotide exchange factors cooperate with Grp78 in immunoglobulin assembly (Shen et al., 2005). The interactions partners ERj3 and ERj5 are implicated in endoplasmic reticulum associated degradation (ERAD) (Dong et al., 2008; Ushioda et al., 2008).

Induction of ER stress chaperones, including Grp78, confers protection against cell injury. Exposure of renal LLC-PK1 cells to a variety of nephrotoxicants results in an increase in Grp78 protein levels (Ryan et al., 2005). Pretreatment with the ER stress inducer, tunicamycin, improves cell viability levels in cells exposed to iodoacetamide (IDAM) (Bedard et al., 2004). In renal IRI, pretreatment with tunicamycin or another ER stress inducer, thapsigargin, significantly ameliorates renal dysfunction suggesting a mechanism through Grp78 and the UPR (Prachasilchai et al., 2008). A chemically-synthesized molecular chaperone, 1-(3,4-dihydroxyphenyl)-2-thiocyanate-ethanone (BIX), exhibits similar protective effects against renal IRI to those of the ER stress inducers (Prachasilchais et al., 2009). A more recent investigation demonstrates that induction of ER stress induces autophagy and together induces a cytoprotective response against oxidant-induced injury both in vitro and in vivo (Chandrika et al., 2015). In addition to exogenous compounds with ER stress inducer functions, some endogenous molecules have been found to have effects on ER stress. For example, tauroursodeoxycholic acid (TUDCA) is an endogenous bile acid derivative typically used as a protective agent in cholestatic liver disease. TUDCA was found to have nephroprotective effects against IRI through the inhibition of ER stress (Gao et al., 2012). Several investigations have explored more detailed mechanisms in the protective effect. In LLC-PK1 cells, selective targeting of Grp78 with antisense strategies disrupts the ER stress response and sensitizes cells to
renal toxicants (Asmellash et al., 2005; Hung et al., 2003; Jia et al., 2004; Liu et al., 1997; Liu et al., 1998). Manipulating human endothelial cells with Grp78 siRNA demonstrates a similar effect of Grp78 being critical in protection against hydrogen peroxide-induced cell damage (Wu et al., 2009).

Grp78 has additional roles beyond the endoplasmic reticulum. For example, Grp78 is highly induced in various cancers via disruptions in glucose levels (Lee, 2007). During ER stress, Grp78 is induced in the ER and re-localized to the cell surface (Zhang et al., 2010). Thus, Grp78 has a function in cell-surface signaling. Cell-surface Grp78 expression occurs in human cancer cell lines of neuroblastoma, lung adenocarcinoma, colon adenocarcinoma, and acute lymphoblastic leukemia B-cells (Shin et al., 2003). Through the formation of complexes with other proteins on the cell surface, Grp78 mediates tumor cell signaling (Zhang, et al., 2010). Treatment of glioma cell lines with a polyclonal N-20 antibody against cell surface Grp78 suppresses cancer cell survival and growth indicating the utility of Grp78 as a biomarker and target for cancer therapy (Kang et al., 2016).
1.8  *p*-ERK and Cytoprotection

1.8.1 MAPK Signaling Overview

Proteins are considered as functional drivers in cells. Protein signaling is controlled by a variety of proteins and structural modifications. One important and common protein modification is phosphorylation and dephosphorylation. Protein kinases and phosphatases are the enzymes that control the level of protein phosphorylation. Kinases are grouped according to their substrate specificity as serine/threonine kinases and tyrosine kinases. The mitogen-activated protein kinase (MAPK) is the major family of serine/threonine kinases. The MAPK family of proteins respond to extracellular stimuli by inducing a series of intracellular phosphorylation cascades. MAPK signaling is involved in many cellular functions including cell growth, differentiation, gene transcription, cell cycle control, response to cell stress, inflammation, cell survival, and cell death. MAPKs are divided into three broad families; extracellular signal-regulated kinases (ERK), c-jun NH2-terminal kinase (JNK), and the p38 MAPK. The ERKs mediate mitogen and growth factor signaling and cell survival. The JNK and p38 families respond to cell stress and inflammation and are associated with cell death (Tian *et al.*, 2000). MAPK signaling is well characterized and extensively targeted for drug design (Bain *et al.*, 2007). The following section will focus specifically on ERK signaling as I show it to be a mediator of ATRA cytoprotection as described in chapters 3 and 4.

1.8.2 ERK Signaling and Cytoprotection

ERK is viewed as the effector protein of the Ras/Raf/MEK/ERK signaling cascade. Raf, MEK, and ERK are all kinases, each of which becomes activated sequentially (*Figure 1.7*). The Ras/Raf/MEK/ERK pathway is activated by growth factors. Through a series of
signaling events involving a membrane receptor (e.g., RTK, GPCR), an adaptor protein (Grb2), and a nucleotide exchange factor (SOS), a series of kinase events occurs that ultimately leads to increases in cell proliferation. A proteomics screen identified 270 ERK substrates supporting its wide utility (von Kriegsheim et al., 2009). ERK targets include transcription factors, adapter proteins, enzymes, and cell surface and nuclear receptors (Tian, et al., 2000). Multiple isoforms exist for each kinase in the Ras/Raf/MEK/ERK pathway. Two isoforms of ERK (ERK1 and ERK2) exist. ERK phosphorylates serine or threonine residues of substrates in the sequence PXS/TP (Buscà et al., 2016). Since the PXS/TP is a common sequence, specificity is provided by docking motifs on the kinase. There are no specific inhibitors of ERK. Thus, PD98059 and U0126 are commonly used pharmacological inhibitors of MEK, the upstream kinase, allowing for the elucidation of the physiological effects of ERK (Alessi et al., 1995; Favata et al., 1998). ERK has key functional roles in mitogenesis and cell differentiation.

Pretreatment of renal epithelial cells with ER stress inducers enhances ERK activation, leading to protection (Hung, et al., 2003). In contrast, in the brain, hypoxic preconditioning attenuates ischemic-induced damage by decreasing p-ERK expression (Zhan et al., 2013). Preadministration of human endothelial cells with the micronutrient vitamin D affords protection against injury via an upregulation of p-ERK (Polidoro et al., 2013). From a different perspective, stem cell therapy is becoming a widely used approach for organ repair due to its role in tissue regeneration. As such, preconditioning strategies in these types of models can provide more comprehensive mechanistic findings on the signaling pathways involved in protection.
Activation of membrane receptors by growth factors or mitogens leads to recruitment of the adaptor protein Grb2 and the guanine nucleotide exchange factor SOS. SOS activates the Ras/Raf/MEK/ERK kinase cascade ultimately resulting in the increase in transcription factors mediating cell proliferation (Mebratu et al., 2009).
A recent investigation in human cardiac stem cells preconditioned with a nitric oxide donor demonstrated an enhancement of cell survival through activation of a variety of survival signaling pathways. The mechanism of nitric oxide preconditioning involved the phosphorylation of ERK and AKT and the subsequent activation of the downstream targets STAT3 and NFκβ. Disruption of STAT3 or NFκβ impaired antiapoptotic gene expression indicating essential roles in cell survival (Teng et al., 2016).

While there are clearly beneficial cytoprotective effects associated with ERK activation, the ERK pathway is constitutively activated in many cancers. In quinol-thioether-induced tuberous sclerosis renal cell carcinoma, p-ERK levels are elevated and crosstalk with the mammalian target of rapamycin (mTOR) pathway activates cyclin D1 and tumor growth (Cohen et al., 2011). In lung cancer, a metabolic pathway inhibitor decreases cell proliferation and migration by decreasing p-ERK expression (Han et al., 2016). The high prevalence of cancers with constitutive activation of the ERK pathway has led to development of many pharmacological inhibitors targeting the ERK cascade. There was great success with the tyrosine kinase inhibitor, Imatinib, to treat chronic myeloid leukemia (CML) by blocking the kinase activity of the BCR-ABL gene with the hope that similar results would be obtained by targeting the ERK cascade. However, relapses have occurred at the receptor level (HER/Neu, PDGF, EGF-receptors), at the level of Ras, and at the level of MEK. Specific ERK inhibitors are difficult to design due to high homology between ERK and the cyclin-related kinases. However, currently, due to the resistance of inhibitors for receptor tyrosine kinases (RTKs), Raf, and ERK, ERK is being targeted itself. There are 5 clinical trials ongoing with four ERK inhibitors (MK-
8353/SCH900353, BVD 523, RG7842/GDC0994, and CC-90003). No conclusive results are known to date (Buscà, et al., 2016).
1.9  *p*-AKT and Cytoprotection

1.9.1 AKT Signaling

The phosphatidylinositol-3 kinase (PI3K)-AKT pathway promotes increased cell proliferation, loss of apoptosis, and cell survival with some overlapping physiological effects to those of the Ras/Raf/MEK/ERK pathway. The PI3K-AKT pathway is activated by ligand binding to a growth factor receptor including the epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and insulin-like growth factor receptor (IGFR) (**Figure 1.8**). In addition, cytokine receptors, G-protein coupled receptors (GPCRs), and intracellular small GTPases such as Ras induce stimulatory effects. The growth factor receptors possess receptor tyrosine kinase activity (RTK) resulting in interaction with PI3K through src-homology 2 (SH2) domains on the p85 regulatory subunit of PI3K. PI3K phosphorylates the lipid second messenger phosphatidylinositol 4,5-diphosphate (PIP2) to the active triphosphate (PIP3). PIP3 is located on the cytosolic side of the plasma membrane. Upon its activation, PIP3 is able to interact with the pleckstrin homology (PH) domain of AKT promoting AKT localization to the cell membrane. AKT, a serine/threonine kinase, subsequently becomes active through its phosphorylation by phosphoinositide-dependent kinase (PDK) 1 and PDK2. AKT phosphorylates and influences a number of effector proteins including mTOR, Iκβ kinase (IKK), mouse double minute 2 homolog (Mdm2), Bad, p27, GSK3, and the forkhead family of transcription factors (FOXO) 1,4 (Mundi *et al.*, 2016). Similarly to ERK, since AKT influences a number of proteins, it is a target for drug design.
The PI3K-AKT signaling pathway

The PI3K-AKT pathway is activated by growth factors binding to a membrane receptor that possesses RTK activity. The RTK interacts with PI3K leading to generation of the lipid second messenger signaling protein PIP3. AKT interacts with PIP3 resulting in increase in expression of genes in cell proliferation and survival (Mundi, et al., 2016).
1.9.2 AKT and Cytoprotection

AKT activation has been associated with cytoprotective mechanisms under multiple conditions. For example, the naturally occurring compound, silymarin, has protective effects against thioacetamide-induced liver and kidney injury. Mechanistically, silymarin protects against the reduction in thioacetamide-induced p-AKT (Ghosh et al., 2016). In cerebral IRI, metformin exerts protective effects through PI3K-AKT signaling (Ge et al., 2017). Metformin is primarily used as an antidiabetic drug but has shown beneficial effects in ischemic brain injury (Liu et al., 2014b). AKT activation has positive cell survival outcomes in hypoxic stem cells suggesting a cytoprotective function in stem cell therapy (Yu et al., 2016). Finally, in the context of ischemic preconditioning, in a perfused rat heart model, left ventricular pressure is improved by four short cycles of ischemia and reflow prior to global ischemia. The AKT inhibitors, wortmannin and LY294002, block this recovery of function (Tong et al., 2000). Similar findings were found in mice kidneys in which ischemic preconditioning confers resistance to injury via activation of the AKT pathway (Jang et al., 2012).

While AKT exerts protective functions under normal homeostatic conditions, overexpression of p-AKT is a common feature in many cancers. AKT is an important signaling molecule with over 100 reported AKT substrates (Manning et al., 2007). In cancer, AKT promotes cell survival downstream of growth factors and oncogenes. AKT inhibits the Bcl2 apoptotic family of proteins. In A549 lung cancer cells, nicotine induces cell survival by increasing the phosphorylation and subsequent inactivation of the apoptotic protein BAD at Ser^{112}, Ser^{136}, and Ser^{155} through activation of the AKT pathway (del Peso et al., 1997; Jin et al., 2004). In parallel, AKT stimulates an increase in cell mass in cancer
cells through activation of mammalian target of rapamycin (mTOR). AKT activation affects mTOR downstream targets including S6K1 and eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) (Manning, et al., 2007). AKT activation also stimulates proliferation of cancer cells through the impingement of cell cycle regulation. AKT mediated phosphorylation and subsequent inhibition of GSK3β favors the stability of cyclins D and E, thus favoring cell cycle progression (Diehl et al., 1998; Welcker et al., 2003). In endometrial cancer cell lines, a similar mechanism is observed since visfatin, an adipokine with metabolic associated malignancies (Tian et al., 2013), induces p-AKT expression and G1/S cell cycle progression and the consequent upregulation of cyclin D1 (Wang et al., 2016).

Pharmacologically, strategies to target AKT were first tested at the level of the growth factor-PI3K-AKT-mTOR axis. Agents that function at the level of the growth factor receptor such as trastuzumab, an inhibitor of human epidermal growth factor receptor 2 (HER2)/neu, show limited promise since there is compensatory activation of AKT through cross talk with other pathways. mTOR inhibitors allow for direct targeting of the PI3K-AKT pathway but loss of negative feedback mediated by the downstream target p70S6 can lead to increased upstream signaling and ultimately an increase in p-AKT (Manning, et al., 2007). Targeting AKT itself has been a challenge as it is unknown whether pan-AKT inhibitors or isoform-specific inhibitors should be used or whether there is a synergistic effect when AKT inhibitors are combined with chemotherapy. There are over a dozen clinical trials involving AKT inhibitors ongoing to address these questions.
1.10 HIF1α Signaling and Cytoprotection

1.10.1 HIF1α Signaling

Hypoxia inducible factor 1 (HIF1) is a heterodimeric transcription factor composed of two subunits, HIF1α and HIF1β. HIF1α is the subunit that responds to oxygen levels and it is induced under hypoxic conditions. HIF1β is constitutively expressed and can associate with the aryl hydrocarbon receptor (AhR) (Wang et al., 1995). HIF1 belongs to the basic-helix-loop-helix family of proteins and its subunits bind to the HIF1 response element (HRE) in the promoter region to induce transcription of target genes.

The HIF1α signaling pathway is regulated at multiple levels. HIF1α synthesis is initiated by signaling pathways influenced by growth factors and other molecules (Figure 1.9). HIF1α is degraded under normoxic conditions and has a short half-life of 5-10 minutes (Salceda et al., 1997). In contrast, under hypoxic conditions, several pathways mediate HIF1α stabilization and activation.

Under physiological levels of oxygen, HIF1α can be regulated by proteasomal degradation mediated by the von Hippel-Lindau protein (pVHL), a tumor suppressor protein that is recognized by an E3 ubiquitin ligase (Iwai et al., 1999). Under normoxic conditions, HIF1α is hydroxylated by the prolyl-4-hydroxylases (PHDs). The proline residues P⁴⁰² and P⁵⁶⁴ and the lysine residue K⁵³² are good substrates for the PHDs. The enzyme arrest-defective-1 (ARD-1) acetylates the lysine residue (Masoud et al., 2015). Modified HIF1α subunits are recognized by the pVHL protein to be tagged for ubiquitination and degradation (Maxwell et al., 1999). PHD and ARD-1 activities require oxygen (Epstein et al., 2001; Jeong et al., 2002); thus, under hypoxic conditions, the hydroxylation and acetylation modifications do not occur resulting in HIF1α stabilization.
Figure 1.9  HIF1α signaling pathway

Regulation of HIF1α pathway at different levels. (A) Growth factor related pathways; (B) pVHL related pathways; (C) FIH-1 pathway; (D) Mdm2-p53 mediated ubiquitination and proteasomal degradation; (E) Hsp90. HIF1α activity is regulated at the levels of protein synthesis, stability, or transactivation. Detailed descriptions of each of these pathways are provided in the text (Masoud, et al., 2015).
In addition to protein stabilization, HIF1α can be regulated through transactivation. This second oxygen-dependent pathway involves post translational modifications in the absence of the pVHL protein. Instead, the activation of HIF1α target genes occurs via the cooperative binding of the p300 co-activator to the HIF1α subunits (Masoud, et al., 2015). Under normoxia, the enzyme factor inhibiting HIF1 (FIH1) blocks the interaction of p300 with HIF1α. Under hypoxic conditions, FIH promotes protein interaction in an oxygen-dependent manner and subsequently transcription of target genes (Lando et al., 2002).

Growth factors mediating the PI3-AKT and Ras/Raf/MEK/ERK pathways can lead to activation of HIF1α. The consequence of these signaling cascades is the increased translation of HIF1α protein. Interestingly, ERK can also phosphorylate the co-activator p300 and increase HIF1α transcriptional activation. Additional HIF1α regulation involves inhibition of protein degradation regulated by the Mdm2 and heat shock protein 90 (Hsp90) proteins (Masoud, et al., 2015).

1.10.2 HIF1α and Cytoprotection

HIF1α is activated through multiple regulatory mechanisms and has a wide variety of target genes involved in increasing cell survival and repair and decreasing apoptosis and inflammation. VEGF, a growth factor mediating angiogenesis, is a common HIF1α target gene that is upregulated under hypoxic conditions. The diffusible gas carbon monoxide increases VEGF expression by increasing HIF1α protein in astrocytes. Mechanistically, carbon monoxide activates the PI3K-AKT and MEK/ERK pathways and the upregulation of the translational regulatory proteins p70S6 kinase and eIF-4E. Moreover, carbon monoxide inhibits HIF1α degradation by promoting the interaction of HIF1α with Hsp 90
(Choi et al., 2010). In addition to its physiological roles, HIF1α mediates cytoprotection against a variety of insults. In rat ventricular cells, stimulation of the muscarinic acetylcholine receptor protects against apoptosis via activation of HIF1α and the induction of the VEGF target gene (Hui et al., 2012). In renal IRI, pretreatment with a PHD inhibitor and consequently HIF1α activation leads to a reduction in serum creatinine levels and tissue damage compared to respective controls (Bernhardt et al., 2006). HIF1α heterozygote mice and rodents deficient for the VHL protein further demonstrate the importance of a HIF1α protective effect during renal IRI (Hill et al., 2008; Schley et al., 2011). In human renal biopsies, HIF1α is markedly expressed in proximal tubule cells that do not exhibit severe damage. These tubules portray normal epithelial structure, presence of microvilli, and proximal cell proliferation indicating repair and regeneration of tubules. In the rat IRI model, HIF1α is induced during the reperfusion phase when tubule repair is occurring (Bernhardt, et al., 2006). Postischemic treatment with a HIF1α hydroxylase inhibitor is similarly effective in reducing renal IRI (Jamadarkhana et al., 2012). Therefore, strategies to increase HIF1α induction are important for renal epithelial cell survival and repair in IRI.

An additional key role of HIF1α is found in many types of solid tumors where oxygen balance is impaired and cells become hypoxic. For example, in a rapidly growing tumor, HIF1α shifts the primary source of energy production to glucose metabolism from the more efficient oxidative phosphorylation accounting for the reason hypoxic cells consume more glucose to meet their energy needs. HIF1α increases the expression of its target genes in the glycolytic pathway (Denko, 2008). An additional adaptation mechanism involves HIF1α increasing the induction of target genes involved in angiogenesis, such as
VEGF, facilitating the development of new blood vessels and oxygen supply (Conway et al., 2001).

Many HIF1α inhibitors have been developed for cancer therapy purposes. Currently, there are no specific HIF1α inhibitors, thus posing challenges for managing hypoxic tumors. As discussed above, there are multiple ways to achieve HIF1α inhibition by modulating different steps in the signaling pathway. A detailed review of specific inhibitors can be found in Masoud et al (2015) with one example being the FDA-approved Bortezomib (Velcade) for multiple myeloma. This proteasome inhibitor antagonizes the p300 HIF1α interaction (Masoud, et al., 2015). YC-1 also functions as a HIF1α inhibitor used in many investigational studies originally developed as an antiplatelet aggregation agent and was later found to have HIF1α inhibitory activity (Masoud, et al., 2015). Currently, these inhibitors could perhaps serve as adjuvant therapy in combination with existing cancer treatments.
1.11 Dissertation Aims

Retinoic acids (RA) are important signaling molecules in the body that are obtained from the diet. One of the functions of RA, important in the context of this dissertation, is its role in cytoprotection. Recently, RA’s have demonstrated cytoprotective effects in animal models with the findings primarily descriptive in nature. The molecular underpinnings on how the cytoprotection occurs is not well elucidated.

The kidney is particularly susceptible to chemical-induced injury and pathological damage due to its ability to concentrate fluids especially in the inner proximal tubule region. Humans are exposed to a variety of nephrotoxicants through environmental exposure and the consumption of pharmaceuticals. PAP is a nephrotoxic metabolite of acetaminophen (Tylenol). Acetaminophen is highly toxic to the liver when taken in overdose but PAP has toxic effects in the S3 segment of the kidney. ROS generation is a key contributor to the renal toxicity of PAP. As a result of its clinical relevance, PAP is used as a model toxicant for the majority of experiments in this dissertation. LLC-PK1 cells, a porcine proximal tubule epithelial cell line, serves as a good model to conduct these studies due to its high homology to humans and its elevated expression of the enzyme γ-GT involved in renal metabolism of toxicants.

The studies described in this dissertation are designed to determine the molecular mechanisms that mediate ATRA cytoprotection in the kidney. The flow chart in figure 1.10 illustrates the aims of each chapter. Preliminary data preceding the work compiled herein provide the rationale for the use of ATRA as a cytoprotective agent. The prostaglandin derivative 11-deoxy-16,16-dimethyl prostaglandin E2 (DDM-PGE2) induces RBP, leading to the engagement of retinoid signaling. DDM-PGE2 itself has cytoprotective
effects against 2,3,5-tris(glutathion-S-yl)hydroquinone (TGHQ)-induced cytotoxicity in LLC-PK₁ cells (Weber et al., 1997). A DDM-PGE₂ pretreatment period was necessary to observe the cytoprotection suggesting the involvement of gene regulation and protein synthesis. RBP is one protein induced during the DDM-PGE₂ pretreatment period of 24 hours. In addition, ATRA signals through receptors in a genomic manner and through kinase regulation in a non-genomic manner. Thus, we hypothesized that the time-dependent nature of ATRA-mediated cytoprotection is contingent on the engagement of specific cell survival pathways.

The aim of Chapter 2 was to demonstrate that ATRA cytoprotection against PAP required a pretreatment period during which proteins are synthesized. Moreover, we evaluated ATRA-mediated protection against multiple toxicants with distinct mechanisms of toxicity to determine whether ATRA protection against renal cytotoxicity is selective or generic. Finally, we provided observational data associated with pre- and post-confluent cells and pre- and co-ATRA treatment to further support the utility of the LLC-PK₁ cell line as a useful model in ATRA cytoprotection.

After developing an appropriate cell model in Chapter 2, we then focused our investigations on determining the molecular mechanisms that mediate ATRA cytoprotection. The focus of Chapter 3 was to explore the roles of cellular stress proteins and signaling pathways involved in the cytoprotective process. The data demonstrated that p-ERK is a key stress protein involved in mediating ATRA cytoprotection. Since the ATRA-induced increase in p-ERK expression was rapid and transient, the goal of Chapter 4 was to determine p-ERK downstream target proteins and signaling pathways involved in ATRA protection.
Figure 1.10  Specific aims for each chapter

The specific aims for Chapters 2-4 are listed in this flow chart. Previous work is indicated to provide a rationale for protein synthesis being critical for ATRA protection as demonstrated in Chapter 2. In Chapter 3, cell stress proteins are explored to determine the role in cytoprotection. \( p \)-ERK downstream signaling was investigated in Chapter 4.
Chapter 2: All-Trans-Retinoic Acid-Mediated Cytoprotection Requires a 24-Hour Pretreatment Period and Protection is Dependent on the Mode of Cell Death

2.1 Introduction

RA is a fat-soluble vitamin obtained from the diet and is essential for human survival. RA stimulates multiple physiological processes, including those involved in cellular growth and differentiation, immune system regulation, vision, and apoptosis. RA has also found clinical application in the treatment of cancer and acne (Alvarez, et al., 2014). These pleiotropic effects are not mediated by retinoic acid itself but rather through its active metabolites, all-trans-retinoic acid (ATRA) and 9-cis retinoic acid (9-cis RA). ATRA is the predominant endogenous active metabolite of RA, and exerts its effects through both genomic and non-genomic signaling mechanisms (Al Tanoury, et al., 2013).

There is current interest in therapeutic strategies that utilize the pre-administration of small molecules, or short periods of ischemia prior to toxic insult in order to afford subsequent cytoprotection. For example, in mice, cycles of brief ischemia reperfusion are protective against a subsequent single, more prolonged period of ischemia that would otherwise produce renal injury (Li et al., 2016b; Nishioka et al., 2014). A variety of pharmacological agents and natural compounds have also shown protective effects in multiple organs (Lieberthal et al., 2016; Tao, et al., 2016). In particular, the RA metabolites have demonstrable cytoprotective properties, with ATRA being shown to be protective against glomerular injury by increasing the expression of podocyte differentiation markers, which improve renal function (Li, et al., 2014). In the brain, ATRA pretreatment reduces cerebral ischemic injury, presumably by reducing the inflammatory markers COX-2 and C/EBP (Choi, et al., 2009a). In the liver, ATRA decreases IRI through both anti-inflammatory and antioxidant mechanisms (Rao, et al.,
2013; Rao, et al., 2010) whereas in the heart protection occurs via a reduction in cardiomyocyte apoptosis (Zhu, et al., 2015). 9-cis RA is also protective against renal IRI (Balasubramanian, et al., 2012). Thus, in vivo studies demonstrate RA-mediated protection under a variety of conditions in multiple organs.

Pretreatment of renal proximal tubule cells (LLC-PK1) with DDM-PGE2 is protective against TGHQ-induced renal toxicity (Weber, et al., 1997). TGHQ is a nephrotoxic and nephrocarcinogenic metabolite of hydroquinone (Lau et al., 1988). A 24 hr pretreatment period is necessary for maximal DDM-PGE2-mediated protection (Weber, et al., 1997) suggesting a need to engage specific signaling pathways to afford protection. Interestingly, the cytoprotective effect of DDM-PGE2 is mediated via the thromboxane receptor coupled to the protein kinase C and NF-κB signaling pathways (Weber et al., 2000). During the 24 hr exposure time, DDM-PGE2 induces RBP (Jia, et al., 2004). The increase in RBP synthesis possibly suggests the engagement of ATRA and/or 9-cis RA during DDM-PGE2 cytoprotection, and ATRA also induces RBP (Lord, Ph.D. thesis). The focus of Chapter 2 is the development of an in vitro cell model with which to subsequently determine the molecular mediators underlying these protective effects explored (Chapters 3 and 4). The experiments described herein were conducted to demonstrate that ATRA requires a pretreatment period to afford cytoprotection against renal cell injury in LLC-PK1 cells. Moreover, several toxicants with different mechanisms of renal toxicity were used to further characterize the cytoprotective effect.

PAP is an acute nephrotoxicant that induces necrosis in the pars recta region of the proximal tubule (Green, et al., 1969). PAP induces renal toxicity via redox cycling or oxidation to a reactive intermediate. PAP-induced cell death is ROS-dependent since
pretreatment of cells with the antioxidants ascorbate or GSH protects against PAP-induced cell injury (Hallman, et al., 2000; Kendig, et al., 2007).

Iodoacetamide (IDAM) is an alkylating agent and soft electrophile that is used as a protein modifier in peptide sequencing. IDAM forms covalent bonds with cysteinyl sulfydryl groups and forms thioether adducts. IDAM depletes non-protein thiols and alkylates cell membrane proteins, cytoskeleton proteins, and thiol-dependent enzymes (Baba et al., 1986; Das et al., 1986; Johnston et al., 1985; Malm, 1984; Morgenstern et al., 1979; Roitelman et al., 1989; Varagiannis et al., 1983). IDAM induces cell death by alkylating cellular macromolecules, depleting protein thiols, lipid peroxidation, and an increase in intracellular calcium levels (Chen et al., 1991). In the context of endoplasmic reticulum signaling, IDAM can cause both necrotic and apoptotic cell death of LLC-PK1 cells depending on the experimental design and pretreatment agents used (van De Water et al., 1999).

2-(glutathion-S-yl)hydroquine (MGHQ) is a nephrotoxic metabolite of hydroquinone (Lau, et al., 1988). Similar to PAP, MGHQ induces cytotoxicity through redox cycling and the generation of ROS.

Cisplatin is a widely used chemotherapeutic agent but is frequently associated with nephrotoxicity as a side effect. Cisplatin induces renal injury by inducing DNA damage (Kruger, et al., 2016), mitochondrial dysfunction (Kim, et al., 2005), and alterations to cell cycle proteins (Pabla, et al., 2015). In LLC-PK1 cells, cisplatin induces apoptotic cell death at low concentrations and oncotic/necrotic cell death at high concentrations (Lau, 1999; Okuda et al., 2000). In our model, cisplatin (40μM, 24 hr) induced apoptotic cell death.
2.2 Materials and Methods

2.2.1 Materials

MGHQ was synthesized and purified according to established protocols (Lau, et al., 1988). PAP, IDAM, cisplatin, ATRA, 9-cis RA, and cycloheximide (CHX) were purchased from Sigma Aldrich (St. Louis, MO). HPLC-grade solvents were obtained from Sigma Aldrich (St. Louis, MO). Neutral Red was purchased from Amresco (Solon, OH). The caspase-3 antibody was acquired from Cell Signaling Technologies (Danvers, MA). GAPDH was a product of Abcam (Cambridge, MA). All secondary antibodies, were purchased from Santa Cruz (Santa Cruz, CA).

2.2.2 Cell Culture and Treatment Conditions

The LLC-PK₁ renal proximal tubule epithelial cell line was obtained from the American Type Culture Collection (Rockville, MD) and is derived from the New Hampshire minipig. LLC-PK₁ cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose and supplemented with 10% fetal bovine serum (FBS) in a 37°C/5%CO₂ humidified incubator. Cells were sub-cultured every 3-4 days at 90% confluence. All assays were conducted with cells plated in multi-well plates. Unless noted for the ATRA and toxicant interaction experiments, all cells were grown to post-confluence before treatment in triplicate, and repeated at least three times. Following ATRA pretreatment, cells were subsequently washed and treated with either PAP, IDAM, or MGHQ in DMEM without FBS. Cisplatin-treated cells were exposed to DMEM with FBS due to the longer exposure time needed to observe cytotoxicity. For experiments in which ATRA was co-treated with toxicant, cells were cultured in DMEM media without FBS.
2.2.3 Neutral Red Assay

Cell viability following treatment with toxicants was measured using the neutral red lysosomal uptake assay, as previously described (Mertens et al., 1995). At the end of each experiment, cells were washed and then incubated with 0.25 mg/mL neutral red solution for 1 h at 37°C/5%CO₂. The neutral red was then removed, cells washed, and cells fixed in 1% formaldehyde/1% CaCl₂, followed by extraction of neutral red using 1% acetic acid/50% ethanol solution for 15 min at room temperature in the dark to remove excess dye. The extent of lysosomal neutral red accumulation was assessed by determining absorbance at 540 nm.

2.2.4 Western Blot Analysis

Following the various treatments, cells were washed in ice cold PBS and lysed with 1X RIPA Buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% NP-40, and 10% sodium deoxycholate. Complete protease and phosphatase inhibitor cocktail tablets (Roche, South San Francisco, CA) were added fresh to the buffer. Cell lysates were pelleted by centrifugation at 16,000g for 15 min, and supernatants, containing total protein, were collected and stored at -80°C. Protein concentrations were measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Aliquots (50-75 μg) of protein lysates were separated on 7.5 or 10% denaturing polyacrylamide gels (SDS-PAGE) and transferred to a nitrocellulose membrane for immunoblotting. The membranes were blocked in 5% non-fat dry milk in tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) for 1 hr, and then incubated with caspase 3 antibody (diluted 1:1000) overnight at 4°C in blocking solution. Secondary antibodies were diluted to 1:300 in
blocking solution and incubated with the membranes for 1 hr at room temperature. Blots were finally developed with enhanced chemiluminescence and imaged.

2.2.5 Statistical Analysis

For individual comparisons, one-way ANOVA followed by Tukey’s post hoc analysis or unpaired Student’s t test was used. All data is expressed as mean ± SE and p<0.05 was considered to be significant.
2.3 Results

2.3.1 ATRA-initiated signaling is required for cytoprotection

Cells were exposed to 25 \( \mu \text{M} \) ATRA for between 0-24 hr followed by exposure to PAP. ATRA cytoprotection became evident between 8-12 hr of pretreatment, with maximal protection occurring at 24 hr, consistent with the kinetics of DDM-PGE\(_2\) cytoprotection (Weber, et al., 1997). Co-treatment of cells with ATRA at the time of toxicant exposure failed to provide cytoprotection, implying that ATRA-initiated signaling is required for cytoprotection (Figure 2.1). Consistent with this view, addition of CHX 4 hr prior to ATRA exposure decreased ATRA-mediated protection against PAP cytotoxicity (Figure 2.2).

2.3.2 ATRA protects against necrotic but not apoptotic death of LLC-PK\(_1\) cells

PAP was the initial model toxicant used to characterize dose and time assessments with ATRA due to its high degree of clinical relevance, being a nephrotoxic metabolite of acetaminophen. To determine whether ATRA-mediated cytoprotection was specific to this toxicant, dose and time experiments with ATRA and renal toxicants were conducted in LLC-PK\(_1\) cells. After the initial characterizations to determine the concentration of toxicant that induces a 50% loss in cell viability, cells were pretreated with ATRA for 24 hours followed by exposure to multiple renal toxicants; PAP (150 \( \mu \text{M}, 3\) hr), MGHQ (300 \( \mu \text{M}, 2\) hr), IDAM (20 \( \mu \text{M}, 2\) hr), or cisplatin (40 \( \mu \text{M}, 24\) hr). ATRA protected cells against PAP, MGHQ, and IDAM induced cytotoxicity but not against cisplatin (Figure 2.3). PAP, MGHQ, and IDAM all induce oncotic/necrotic cell death, whereas cisplatin induces apoptotic cell death of LLC-PK1 cells. Consistent with these data, Western blot analysis revealed caspase-3 cleavage to active fragments occurred only in cells treated with the
apoptotic toxicant cisplatin (Figure 2.4). Collectively, the results indicate that ATRA protects against oncotic/necrotic cell death.

2.3.3 Retinoid cytoprotection is dependent on the characteristics of the confluency state of cells and the time of retinoid treatment

Initial cytoprotective experiments demonstrated that a DDM-PGE$_2$ pretreatment period of 24 hours is necessary to observe cytorprotection against TGHQ in post confluent LLC-PK$_1$ cells (Weber, et al., 1997). Jia et al. found that DDM-PGE$_2$ induces RBP during the 24 hour exposure indicating the engagement of retinoid signaling (Jia, et al., 2004). Since the current experiments involve ATRA as the cytoprotective agent and additional toxicants beyond GSH conjugates, we conducted experiments to further develop the cell culture model. Cells that were pretreated with ATRA followed by exposure to PAP in post confluent cells demonstrate protection. In contrast, no protection is found in cells co-treated with ATRA and PAP. In PAP-treated cells, regardless of the time of ATRA administration, no protection is found in pre-confluent cells. Interestingly, in pre-confluent LLC-PK$_1$ cells treated with IDAM, near-complete protection is demonstrated in both pre- and co-treated cells (Figure 2.5). To confirm the ATRA and IDAM finding, we first pretreated post confluent cells with the other biologically-derived retinoid, 9-cis RA followed by exposure to IDAM and did not observe cytoprotection indicating that in our original pretreatment post-confluent model, ATRA but not 9-cis RA is cytoprotective. When the co-treatment and pre-confluent conditions are added to the 9-cis RA experimental protocol, marked protection is noted (Figure 2.6).
Figure 2.1   Maximal ATRA cytoprotection requires a 24 hour pretreatment period

LLC-PK₁ cells were exposed to ATRA for an increasing period of time (0-24 hr) followed by PAP treatment for 3 hours. % protection was determined by a Neutral Red lysosomal uptake assay. Significantly different from vehicle treatment at time zero (*p<0.05 **p<0.01, n=3).
Figure 2.2 Cycloheximide, a protein synthesis inhibitor, blunts ATRA cytoprotection

LLC-PK₁ cells were pretreated with CHX (0.5 μM) for 4 hr followed by ATRA (25 μM, 24 hr). Cells were exposed to PAP (150 μM, 3 hr). Cell viability was assessed by lysosomal uptake of Neutral Red. Black bars, cells treated without PAP; dotted bars, cells treated with PAP (*p<0.05, **p<0.01, n=4).
Figure 2.3  ATRA affords selective protection against toxicants that induce oncosis/necrosis

LLC-PK₁ cells were exposed to PAP (150 μM, 2 hr), MGHQ (300 μM, 2 hr), IDAM (20 μM, 2 hr), or CIS (40 μM, 24 hr). Cell viability was assessed by the neutral red assay. Black bars, toxicants treatment alone; dotted bars, ATRA (25 μM, 24 hr) pretreatment. Significantly different from toxicant alone group (*p<0.05, n≥3).
Figure 2.4  Oncotic/necrotic toxicants do not induce caspase-3 cleavage

LLC-PK1 cells were lysed in RIPA buffer. The anti-caspase-3 antibody detects both full-length and a cleaved fragment of caspase-3. Activation of caspase-3 requires proteolytic processing of pro-caspase-3 to into cleaved fragments. GAPDH was used as a loading control.
Figure 2.5  ATRA and toxicant interaction in pre- and post-confluent cells

(A) LLC-PK₁ cells were either pretreated with ATRA (25 μM, 24 hr) followed by exposure to PAP (150 μM, 3 hr) or co-treated with ATRA (25 μM) and PAP (150 μM, 3 hr) in pre- or post-confluent cells. (B) LLC-PK₁ cells were either pretreated with ATRA (25 μM, 24 hr) followed by exposure to IDAM (20 μM, 2 hr) or co-treated with ATRA (25 μM) and IDAM (20 μM, 2 hr) in pre- or post-confluent cells. Cell viability was assessed by lysosomal uptake of Neutral Red. Black bars, pre-confluent cells; open bars, post-confluent cells (*p<0.05, **p<0.001, n=3).
Figure 2.6  9-cis RA and IDAM interaction in pre- and post-confluent cells

(A) LLC-PK₁ cells were pretreated with 9-cis RA (25 μM, 24 hr) followed by exposure to IDAM (20 μM, 2 hr) in post-confluent cells. (B) LLC-PK₁ cells were co-treated with 9-cis RA (25 μM) and IDAM (20 μM, 2 hr) in pre- or post-confluent cells. Cell viability was assessed by lysosomal uptake of Neutral Red. Panel (B), Black bars, pre-confluent cells; open bars, post-confluent cells (**p<0.01, n=3).
2.4 Discussion

ATRA affords cytoprotection against renal cell injury. Consistent with the kinetics of DDM-PGE$_2$ cytoprotection (Weber, et al., 1997), a 24 hr ATRA exposure is necessary for maximal protection. We extend this finding from one renal toxicant (PAP) to multiple toxicants and show that, at least in LLC-PK$_1$ renal epithelial cells, ATRA-mediated cytoprotection is specific to chemical toxicants that induce oncotic/necrotic cell death. Thus, ATRA provided protection against PAP (an oxidant), IDAM (an alkylating agent (Jiang et al., 2013)), and MGHQ (an oxidant and electrophile (Lau, et al., 1988)) induced oncotic/necrotic cell death, but not against cisplatin (DNA damaging agent and mitochondrial toxicant (Pan et al., 2015)) mediated apoptotic cell death. Indeed, ATRA was found to potentiate cisplatin-induced renal injury in a rodent model (Elsayed et al., 2016). Interestingly, DDM-PGE$_2$ is also only protective against toxicants that induce oncotic/necrotic cell death and not apoptotic cell death (Jia, et al., 2004).

Several nephrotoxicants and pathological conditions generate ROS as a component of their mechanism of nephrotoxicity. TGHQ is an oxidant and electrophile in the same GSH conjugate class with MGHQ. Exposure of renal proximal tubular cells to TGHQ results in ROS generation and DNA damage, reductions in repair, and mitochondrial abnormalities such as ATP depletion (Zhang et al., 2011). Moreover, ROS generation disrupts the actin cytoskeletal network (Lord, Ph.D. thesis) and induces lipid peroxidation and membrane instability (Jian et al., 2012, Yamamoto et al., 2011). Recently, substantial research has been conducted in the area of cytoprotection against ROS-mediated injury. The cytoprotective effects noted have primarily been achieved through genetic or pharmacologic blockade of proteins in ROS-mediated signaling pathways. The focus in
this study is on the role of retinoids in renal cytoprotection. The mechanistic contribution of ROS to ATRA’s cytoprotective effects is delineated in Chapter 3.

ER stress preconditioning is able to prevent IDAM-induced apoptosis (van De Water, et al., 1999). However, this protection is protocol-dependent as antioxidant-pretreatment was needed for the activation of apoptosis. Our findings with PAP and MGHQ indicate ATRA affords protection against oncosis/necrosis. The primary mechanism of IDAM cytotoxicity involves protein alkylation and thiol depletion with subsequent effects on lipid peroxidation (Chen, et al., 1991). Antioxidants and the iron chelator deferoxamine protect against IDAM-induced lipid peroxidation and cytotoxicity but have no effect on protein alkylation and thiol depletion (Chen, et al., 1991); the authors concluded that protein alkylation, thiol depletion, and lipid peroxidation cooperate in IDAM’s mechanism of toxicity. Thus, whether ATRA affords protection against IDAM by decreasing ROS, preventing protein alkylation, or stimulating protein repair or refolding needs further investigation.

In the model development experiments, a pretreatment period is not necessary for retinoid cytoprotection against IDAM (Figures 2.5, 2.6) perhaps due to a mechanism of protection that is a direct chemical interaction between ATRA or 9-cis RA and IDAM. Mass spectrometry or nuclear magnetic resonance (NMR) experiments can provide further insight into this potential mechanism.

Cisplatin induces apoptosis in the renal proximal tubule. Cisplatin-induced apoptosis is multifactorial involving oxidative stress and a subsequent increase in antioxidant signaling (Basu et al., 2016). We demonstrate that ATRA has no effect on cisplatin-induced renal cell injury (Figure 2.3). Several other small molecule compounds
do exert protective effects on cisplatin. Vitamin E, another lipophilic vitamin, mitigates cisplatin-induced nephrotoxicity by reducing lipid peroxidation and protecting against the depletion markers of antioxidant defense (GSH, CAT, SOD) in cisplatin-treated rats (Darwish et al., 2017). In addition, flavonoids also exert cytoprotective effects. Eriodictyol, a flavonoid present in citrus fruits, is an Nrf2 activator (Hu et al., 2012) and attenuates cisplatin nephrotoxicity by upregulating antioxidant stress proteins and the Nrf2 signaling pathway (Li et al., 2016a). Both vitamin E and flavonoids decrease toxic effects by decreasing oxidative stress. Mechanistic work in Chapter 3 indicate that neither ROS nor Nrf2 mediate ATRA’s cytoprotective process providing one possible explanation for the lack of protection against cisplatin in our model. An alternative explanation of the lack of ATRA cytoprotection against cisplatin may be related to the temporal nature of stress kinases as discussed in Chapter 3.

In summary, ATRA selectively protects against renal oncotic/necrotic agents. A 24 hr pretreatment period is necessary for protection against PAP and presumably MGHQ. ATRA co-treatment with IDAM is sufficient to observe cytoprotective effects. Understanding the mechanism of ATRA cytoprotection will further advance insights in the development of novel therapeutic strategies for renal pathological conditions such as chemical-induced toxicity, ischemia reperfusion injury, and hypoxia. This mechanism will be explored in subsequent chapters.
Chapter 3: Cellular Stress Kinase Activation Contributes to All-Trans-Retinoic Acid-Mediated Cytoprotection in Renal Epithelial Cells

3.1 Introduction

RA is a fat-soluble vitamin obtained from the diet and essential for human survival. RA stimulates multiple physiological processes, including those involved in cellular growth and differentiation, immune system regulation, vision, and apoptosis. RA has also found clinical application in the treatment of cancer and acne (Alvarez, et al., 2014). These pleiotropic effects are not mediated by RA itself but rather through its active metabolites, ATRA and 9-cis RA. ATRA is the predominant endogenous active metabolite of RA, and exerts its effects through both genomic and non-genomic signaling mechanisms (Al Tanoury, et al., 2013). ATRA localizes to the nucleus, where it binds to the RA receptor/RA X receptor (RAR/RXR) heterodimer to operate in a ligand-dependent manner and induce the transcription of RAR/RXR responsive genes. ATRA can also signal independently of the nuclear receptors via activation of the MAPK and PI3K signaling pathways in multiple cell types including neuronal, lung and embryonic stem cells (Canon et al., 2004; Garcia-Regalado et al., 2013; Persaud et al., 2013).

During conditions of cellular stress, overproduction of ROS can lead to damage to DNA, proteins, and lipids and pretreatment mechanisms in cellular protection can involve the upregulation of antioxidant genes (He et al., 2012). Growth factors are one example of a pretreatment mechanism that induces the antioxidant response. IGF1, one such growth factor, induces GSH synthesis (Takahashi et al., 2016) providing insight into the possible contribution of ATRA, as a growth factor, in inducing ROS and antioxidant signaling as a mechanism in its protection.
ER stress chaperones, including Grp78, confer protection against cell injury (Bush et al., 1999; Liu, et al., 1998). Overexpression of Grp78 prevents activation of the UPR and affords protection against cell death (Dorner et al., 1992; Morris et al., 1997). Conversely, inhibition of Grp78 sensitizes cells to oxidative stress and cytotoxicity (Koong et al., 1994; Li et al., 1992).

MAPKs participate in the regulation of a variety of cellular functions including the ERK1/2, JNK, and p38 MAPK signaling cascades. ERK1/2 is activated by growth factors and mediates cell proliferation, differentiation, and survival. JNK and p38 MAPK kinase signaling cascades are activated by cellular stress and are typically mediators of cellular toxicity (Chang et al., 2001; Tian, et al., 2000). In parallel, a number of cell survival signals are also transmitted through the PI3K-AKT cascade (Mundi, et al., 2016). Preconditioning strategies that activate ERK and AKT have protective effects in multiple organs. Pretreatment of LLC-PK1 renal epithelial cells with endoplasmic reticulum stress inducers enhances oxidative stress-induced ERK activation, leading to cytoprotection (Hung, et al., 2003). In vivo, a low priming dose of 15 mg/kg S-(1,2 dichlorovinyl)-L-cysteine (DCVC) prior to administration of a subsequent higher toxic dose of DCVC (75 mg/kg) resulted in a sustained activation of ERK and recovery from renal necrosis (Vaidya et al., 2003). AKT is a critical mediator in renal protection following brief cycles of ischemic preconditioning (Joo, et al., 2006). The EGF receptor and IGF1 demonstrate protective effects through the stimulation of ERK and AKT in multiple cell types (Niederlechner et al., 2013; Yang et al., 2013).

In the present study, we investigated whether cellular stress proteins in the antioxidant response, UPR, and MAPK and AKT signaling contribute to the cytoprotective
effects of ATRA. Our findings reveal that $p$-ERK, but not $p$-AKT nor reductions in ROS, contribute to ATRA cytoprotection.
3.2 Materials and Methods

3.2.1 Materials

MGHQ was synthesized and purified according to established protocols (Lau 1988). PAP and ATRA were purchased from Sigma Aldrich (St. Louis, MO). HPLC-grade solvents were also obtained from Sigma Aldrich (St. Louis, MO). GSH was purchased from Fisher Scientific (Pittsburgh, PA). 5,5 Dithio-bis-(2-nitrobenzoic acid) (DTNB) was a product of Sigma Aldrich (St. Louis, MO). Sulforaphane, MG132, BSO and NAC were obtained from Sigma (St. Louis, MO). PD98059 was supplied by InvivoGen (San Diego, CA) and LY24002 supplied by EMD Millipore (San Diego, CA). Neutral Red was purchased from Amresco (Solon, OH). Caspase-3, Grp78, p-eIF2α, p-ERK, ERK, p-AKT, and AKT antibodies were acquired from Cell Signaling Technologies (Danvers, MA). eIF2α was a product of Invitrogen (Carlsbad, CA). GAPDH was a product of Abcam (Cambridge, MA). Nrf2, and all secondary antibodies, were purchased from Santa Cruz (Santa Cruz, CA).

3.2.2 Cell Culture and Treatment Conditions

The LLC-PK1 renal proximal tubule epithelial cell line was obtained from the American Type Culture Collection (Rockville, MD) and is derived from the New Hampshire minipig. LLC-PK1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose and supplemented with 10% fetal bovine serum (FBS) in a 37°C/5%CO2 humidified incubator. Cells were sub-cultured every 3-4 days at 90% confluence. All assays were conducted with cells plated in multi-well plates and grown to post-confluence before treatments in triplicate, and repeated at least three times.
Following ATRA pretreatment, cells were subsequently washed and treated with the various agents in DMEM without FBS.

3.2.3 Neutral Red Assay

Cell viability following treatment with toxicants was measured using the neutral red lysosomal uptake assay, as previously described (Mertens, et al., 1995). At the end of each experiment, cells were washed and then incubated with 0.25 mg/mL neutral red solution for 1 h at 37°C/5%CO₂. The neutral red was then removed, cells washed, and cells fixed in 1% formaldehyde/1% CaCl₂, followed by extraction of neutral red using 1% acetic acid/50% ethanol solution for 15 min at room temperature in the dark to remove excess dye. The extent of lysosomal neutral red accumulation was assessed by determining absorbance at 540 nm.

3.2.4 CM-H₂DCFDA Assay

ROS generation was determined using the CM-H₂DCFDA probe (Molecular Probes, Eugene OR). Cells were grown in 96-well black walled clear-bottom plates and allowed to grow to post-confluence prior to treatments. The CM-H₂DCFDA dye was prepared according to the manufacturer’s protocol, preloaded in cells for 30 min, and following toxicant addition, fluorescence was assessed at excitation and emission wavelengths of 485/515 nm, respectively.

3.2.5 Glutathione Assay

GSH levels were measured as described (Ellman, 1959; Mitchell et al., 1973) with modifications. After treatment, cells were lysed in 8% sulfosalicylic acid and centrifuged at 8,000 x g for 10 min. The supernatant was collected and stored on ice for analysis. A standard curve for GSH was prepared at the following amounts: 0, 3.9, 7.8, 15.6, 31.25,
62.5, 125, 250, 500 μg. Standard or undiluted samples (20 μL) were pipetted into 96 well plates. DTNB (120 μL at 1 mg/ml) was subsequently added to each well and absorbance measured at 412 nm. GSH levels in each sample were determined using the GSH standard curve.

3.2.6 Western Blot Analysis

Following various treatments, cells were washed in ice cold PBS and lysed with 1X RIPA Buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% NP-40, and 10% sodium deoxycholate. Complete protease and phosphatase inhibitor cocktail tablets (Roche, South San Francisco, CA) were added fresh to the buffer. Cell lysates were pelleted by centrifugation at 16,000g for 15 min, and supernatants, containing total protein, were collected and stored at -80°C. Protein concentrations were measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Aliquots (50-75 μg) of protein lysates were separated on 7.5 or 10% denaturing polyacrylamide gels (SDS-PAGE) and transferred to a nitrocellulose membrane for immunoblotting. The membranes were blocked in 5% non-fat dry milk in tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) for 1 hr, and then incubated with primary antibodies overnight at 4°C in blocking solution. Primary antibody dilutions were either 1:500 or 1:1000. Secondary antibodies were diluted to 1:3000 in blocking solution and incubated with the membranes for 1 hr at room temperature. Blots were finally developed with enhanced chemiluminescence and imaged.

3.2.6 Statistical Analysis

For individual comparisons, one-way ANOVA followed by Tukey’s post hoc analysis or unpaired Student’s t test was used. All data is expressed as mean ± SE and p<0.05 was considered to be significant.
3.3 Results

3.3.1 ATRA-mediated cytoprotection occurs independently of increased ROS levels and without an apparent improvement in the antioxidant response

Under certain stress conditions ATRA has the ability to engage cellular antioxidant defenses (Choudhary et al., 2008). Therefore, we investigated whether ATRA-mediated cytoprotection against renal epithelial cell injury involves modulation of ROS. Cells were pretreated with ATRA, exposed to the CM-H$_2$DCFDA dye for 30 min, and treated with PAP for 0-2 hr. Exposure of cells to PAP increased ROS levels ~6-fold, with ROS levels initially increasing at 20 min and remaining elevated until the conclusion of the experiment at 2 hr. However, ATRA had no effect on PAP-induced ROS (Figure 3.1A). To confirm the lack of effect of ATRA on toxicant-induced ROS levels, we pretreated cells with ATRA, followed by exposure to the CM-H$_2$DCFDA dye for 30 min, and treated cells with for 0-1 hr. MGHQ-exposed cells demonstrated less of an effect on ROS generation; similar to PAP, the increase was noted early and levels remained elevated until the conclusion of the experiment at 1 hr. Since the MGHQ induction of ROS was small, the influence of ATRA on ROS levels cannot be determined (Figure 3.1B).

To confirm ATRA had no effect on ROS generation, we utilized a GSH assay. Although ATRA caused a modest increase in GSH levels in cells exposed to PAP, this effect did not reach statistical significance (Figure 3.2A, B). Concomitant with the ability of PAP-induced elevations in ROS, PAP decreased GSH levels by ~80%, and ATRA had a modest, but statistically insignificant effect on preserving cellular GSH subsequent to PAP treatment (Figure 3.2B). Thus, ATRA affords cytoprotection without apparently improving the antioxidant response.
Figure 3.1 Effects of ATRA on PAP and MGHQ-induced ROS generation

(A) LLC-PK₁ were treated with ATRA (25 µM, 24 hr), loaded with CM-H₂DCFDA dye, and exposed to PAP (150 µM, 0-2 hr). (B) LLC-PK₁ cells were pretreated with ATRA (25 µM, 24 hr), loaded with CM-H₂DCFDA dye, and exposed to MGHQ (300 µM, 0-1 hr). NAC (10 mM) was co-treated with toxicant and used as a positive control. Black squares, control cells; open squares, cells treated with ATRA; open triangle, cells treated with PAP or MGHQ; black diamond, cells pretreated with ATRA followed by exposure to PAP or MGHQ; black triangle, cells treated with NAC; open diamond, cells pretreated with NAC followed by exposure to PAP or MGHQ. Control data superimposed on ATRA data. Significance determined from comparisons to time zero for ROS generation (*p<0.05, **p<0.01, n≥3).
Figure 3.2  Effects of ATRA on PAP-induced reduced GSH levels

LLC-PK1 cells were pretreated with ATRA (25μM, 24 hr) followed by treatment with PAP (150 μM) for 1(A) or 2 (B) hr. Significance determined from comparisons to vehicle-treated cells (*p<0.05, ***p<0.001, n=4).
3.3.2 ATRA-mediated cytoprotection occurs independent of the engagement of Nrf2

Since Nrf2 is a master regulator of the antioxidant stress response, we examined Nrf2 protein levels. ATRA had modest but statistically insignificant (analyzed by ANOVA) effects on Nrf2 protein levels during the 24 hr ATRA pretreatment period (Figure 3.3). When a T-test was used to determine statistical meaning, significance was found in ATRA-treated cells beginning at 1 hr. As Nrf2 is post-translationally regulated and degraded by the ubiquitin proteasome pathway under basal conditions, we stabilized Nrf2 levels with the addition of the proteasome inhibitor, MG132. As expected, basal levels of Nrf2 were enhanced, but no further significant induction of Nrf2 (analyzed by ANOVA) was observed upon ATRA treatment (Figure 3.4). When a T-test was used to determine statistical meaning, significance was found in ATRA and MG132 treated cells at 8 and 12 hr. Under conditions where cells are not pretreated with ATRA, exposure to PAP generates ROS with the subsequent engagement of the Nrf2 antioxidant response, as evidenced by increases in Nrf2 protein levels (Figure 3.5). The effects of ATRA pretreatment on the PAP-induced Nrf2 response was therefore assessed. Under these conditions, the PAP-induced elevations in Nrf2 are similar whether cells are pretreated or not with ATRA (Figure 3.5). Interestingly, even though ATRA pretreatment protects against PAP-induced cytotoxicity in the absence of a significant increase in Nrf2, pretreatment of cells with the classic Nrf2 inducer, sulforaphane, provided near-complete protection against PAP-mediated cytotoxicity (Figure 3.6A). The data reveal that LLC-PK1 cells possess multiple pathways that can be engaged to provide protection against cytotoxicants. MG132 also elevated Nrf2 levels (Figure 3.6B).
Figure 3.3  Nrf2 expression in LLC-PK₁ cells exposed to ATRA

LLC-PK₁ cells were exposed to ATRA (25 μM) for a maximum of 24 hr. Sulforaphane (5 μM, 4 hr) and MG132 (10μM, 4 hr) were used as positive controls. GAPDH was used as a loading control. Significance was determined from comparisons to vehicle-treated cells for Nrf2 induction (**p<0.001, n=4).
Figure 3.4  Nrf2 expression in LLC-PK₁ cells exposed to ATRA and MG132

LLC-PK₁ cells were exposed to ATRA (25 μM) for a maximum of 24 hr. MG132 (10 μM) was spiked into the media 4 hours prior to collection. Sulforaphane (5 μM, 4 hr) was used as a positive control. GAPDH was used as a loading control. Significance was determined from comparisons to MG132 alone-treated cells for Nrf2 induction (***p<0.001, n=3).
Figure 3.5  Nrf2 expression in LLC-PK₁ cells exposed to ATRA and PAP

LLC-PK₁ cells were pretreated with ATRA (25 μM, 24 hr) and treated with PAP (150 μM, 1 hr). Sulforaphane (5 μM, 4 hr) and MG132 (10 μM, 4 hr) were used as positive controls. GAPDH was used as a loading control. Significance was determined from comparisons to vehicle-treated cells for Nrf2 induction (*p<0.05, **p<0.01, n=5).
Figure 3.6  Cytoprotection in LLC-PK₁ cells exposed to ATRA and Nrf2 inducers

LLC-PK₁ cells were pretreated with ATRA, sulforaphane (A) or MG132 (B), or a combination of ATRA and inducer for 24 hr. ATRA (25 μM), sulforaphane (SF, 5 μM), MG132 (0.5 μM). Cell viability was determined by the Neutral Red assay. Black bars, cells treated without PAP; open bars, cells treated with PAP. Significance was determined from comparisons to the PAP alone group (*p<0.05, **p<0.01, ***p<0.001, n=3).
3.3.3 ATRA induces expression of the unfolded protein response

We have demonstrated that DDM-PGE\(_2\) induces a number of proteins including Grp78 to induce its cytoprotective effect (Jia, \textit{et al.}, 2004; Towndrow \textit{et al.}, 2003). This protection is lost in pkASgrp78 cells, a LLC-PK\(_1\) cell line in which induction of Grp78 is disrupted by stable expression of an antisense Grp78 RNA (Liu, \textit{et al.}, 1997; Liu, \textit{et al.}, 1998). Using these cells as a tool to probe downstream Grp78 signaling, we further found RBP levels to be blunted (Jia, \textit{et al.}, 2004). Since RBP is a secretory protein, it likely needs processing in the ER before secretion. Therefore, any comprised ER stress response may have an effect on RBP and associated retinoid signaling. To characterize the effect of ATRA on the ER stress response, we determined Grp78 and p-eIF2\(\alpha\) expression following ATRA treatment in LLC-PK\(_1\) cells. ATRA induced Grp78 and p-eIF2\(\alpha\) protein levels in a time-dependent manner. The Grp78 induction is sustained until 24 hr whereas the p-eIF2\(\alpha\) induction is transient with maximal expression between 8 and 12 hr followed by a return to basal levels (Figure 3.7). In addition, ATRA induced Grp78 and p-eIF2\(\alpha\) expression in a concentration-dependent manner (Figure 3.8) suggesting that these proteins may be necessary but are insufficient alone for ATRA protection.
Figure 3.7  Time-dependent induction of ATRA on unfolded protein response gene expression

LLC-PK1 cells were exposed to ATRA (25 μM) for a maximum of 24 hr. Protein extracts were analyzed for Grp78 and p-eIF2α induction using western blot analysis. Grp78 densitometric analysis was normalized to GAPDH. p-eIF2α densitometric analysis was normalized to eIF2α. Significance was determined from comparisons to vehicle-treated control cells (*p<0.05, ***p<0.001, n=4).
Figure 3.8  Dose-dependent induction of ATRA on unfolded protein response gene expression

LLC-PK1 cells were exposed to a dose range of ATRA (0-25 μM) for 12 and 24 hr for p-\(\text{eIF2α}\) and Grp78, respectively. Protein extracts were analyzed for Grp78 and p-\(\text{eIF2α}\) induction using western blot analysis. Grp78 densitometric analysis was normalized to GAPDH. p-\(\text{eIF2α}\) densitometric analysis was normalized to eIF2α. Significance was determined from comparisons to vehicle-treated control cells (*\(p<0.05\), ***\(p<0.001\), \(n=3\)).
3.3.4 ATRA increases cell survival via the MAPK-ERK pathway

NAC provides protection against the cytotoxicity of TGHQ in human proximal tubular (HK-2) cells in part via ERK1/2 activation (Zhang et al., 2011). Moreover, both the MAPK-ERK and PI3K-AKT pathways can induce cellular growth and survival pathways and have been implicated in the physiological functions of ATRA. It is important to note, however, that the contribution of these pathways in the response to ATRA is context dependent. To determine whether either of these pathways participates in ATRA-mediated cytoprotection in LLC-PK1 cells, we examined p-ERK and p-AKT expression following ATRA treatment. p-ERK and p-AKT expression were rapidly (15 min) induced after ATRA exposure. Elevations in p-ERK and p-AKT were transient, with levels returning to baseline between 60-120 min (Figure 3.9). Importantly, the concentration of ATRA required to induce p-ERK and p-AKT expression (10 and 25 μM) are consistent with the concentration required for cytoprotection (Figure 3.10).

Given that ATRA was capable of inducing p-ERK and p-AKT expression in LLC-PK1 cells, we next assessed their potential contribution to ATRA-mediated cytoprotection. Cells were treated for 30 min with either 50 μM PD98059, an inhibitor of the MAPK pathway, or 25 μM LY24002, a PI3K-AKT inhibitor, followed by addition of ATRA. Both PD98059 and LY24002 essentially inhibited their respective targets (Figure 3.11). Cells were subsequently pretreated with either the MAPK or PI3K-AKT inhibitor for 30 min followed by exposure to ATRA for 24 hr, and then PAP was added for 3 hr. In the absence of the ATRA pretreatment, PD98059 had no effect on PAP-induced cytotoxicity. However, PD98059 significantly attenuated the ability of ATRA to provide protection against PAP cytotoxicity (Figure 3.12A), suggesting that the ERK1/2 pathway participates in the cytoprotective response. In contrast, the PI3K-AKT inhibitor, LY24002,
exacerbated PAP toxicity in LLC-PK1 cells compromising our ability to assess the contribution of AKT to ATRA cytoprotection (Figure 3.12B).

AKT is a cell survival gene thus inhibition of AKT can exacerbate toxicity depending on the cell type. We lowered the concentration of LY24002 in an attempt to assess the contribution of AKT to ATRA cytoprotection without an enhancement of PAP cytotoxicity. A concentration of LY24002 of 0.5 μM was selected for ATRA protection experiments as it did not exacerbate PAP toxicity (data not shown). We subsequently treated cells for 30 min with 0.5 μM LY24002 followed by exposure to ATRA for 24 hr, and then PAP was added for 3 hr. While ATRA afforded significant protection against PAP, no effect was found in cells treated with LY24002 (Figure 3.13). To confirm that 0.5 μM LY24002 does indeed inhibit p-AKT expression, LLC-PK1 cells were treated with LY24002 (0-25 μM) for 24 hr. Although AKT inhibition with LY24002 resulted in a concentration-dependent decrease in p-AKT expression, no significant effect was found at the low concentration of 0.5 μM (Figure 3.14). Collectively, in LLC-PK1 cells, AKT is likely essential for cell survival and other cytoprotective genes will need to be investigated.
Figure 3.9  Time-dependent induction of ATRA on early stress response pathway genes in LLC-PK₁ cells

Cells were exposed to ATRA (25 μM) for a maximum of 8 hr. Protein extracts were analyzed for p-ERK and p-AKT induction. p-ERK was normalized to ERK and p-AKT was normalized to AKT. GAPDH was used as a loading control. Significance was determined from comparison to vehicle-treated control cells for p-ERK or p-AKT induction (*p<0.05, n=3).
Cells were exposed to concentrations of ATRA for 30 min. Protein extracts were analyzed for \( p\)-ERK and \( p\)-AKT induction. \( p\)-ERK was normalized to ERK and \( p\)-AKT was normalized to AKT. GAPDH was used as a loading control. Significance was determined from comparison to vehicle-treated control cells for \( p\)-ERK or \( p\)-AKT induction (*\( p < 0.05\), \( n=3\)).
Figure 3.11 Stress kinase inhibitor validation of p-ERK and p-AKT inhibition in LLC-PK1 cells

Cells were pretreated with the MEK inhibitor, PD98059 (50 μM), or the PI3 kinase inhibitor, LY24002 (25 μM), for 30 min. ATRA (25 μM) was added into each well for a maximum of 60 min. Protein extracts were analyzed for p-ERK and p-AKT induction. p-ERK was normalized to ERK and p-AKT was normalized to AKT. GAPDH was used as a loading control. Significance was determined from comparison to control cells for p-ERK or p-AKT induction (*p<0.05, **p<0.01, ***p<0.001, n=3).
Figure 3.12  Effect of stress kinase inhibitors on ATRA cytoprotection against PAP in LLC-PK₁ cells

Cells were pretreated with (A) PD98059 (50 μM) or (B) LY24002 (25 μM) for 30 min followed by ATRA (25 μM, 24 hr). Cells were exposed to PAP (150 μM, 3 hr). Cell viability was assessed by lysosomal uptake of Neutral Red. Black bars, cells treated without PAP; open bars, cells treated with PAP. Significance was determined from comparisons between PAP alone to the ATRA + PAP groups (*p<0.05, **p<0.01, ***p<0.001, n=3).
Figure 3.13  Effect of low dose LY24002 (0.5 μM) on ATRA cytoprotection against PAP

LLC-PK₁ cells were pretreated with LY24002 (0.5 μM) for 30 min followed by ATRA (25 μM, 24 hr). Cells were exposed to PAP (150 μM, 3 hr). Cell viability was assessed by lysosomal uptake of Neutral Red. Black bars, cells treated without PAP; open bars, cells treated with PAP. Significance was determined from comparisons between PAP alone to the ATRA + PAP groups (*p<0.05, n=3).
Figure 3.14  Dose-dependent inhibition of p-AKT expression in LLC-PK₁ cells

Cells were exposed to a dose range of LY24002 (0-25 μM) for 24 hr. Protein extracts were analyzed for p-AKT induction. p-AKT was normalized to AKT. GAPDH was used as a loading control. Significance was determined from comparison to vehicle-treated control cells (**p<0.01, ***p<0.001, n=3).
3.4 Discussion

Cellular stress proteins contribute to a variety of cytoprotective processes. In Chapter 2, we demonstrated that ATRA has cytoprotective effects against renal toxicants that induce oncotic/necrotic cell death. In the present chapter, we have provided mechanistic support for this protective process, specifically focusing on cellular stress proteins.

Our first approach was to assess stress proteins involved in the antioxidant response. A reduction in ROS levels via engagement of the anti-oxidant stress response mediates several cytoprotective processes, and ATRA protects against IRI, a process in which ROS play a major pathological role (Rao, et al., 2010). We therefore examined the role of ROS and antioxidant signaling in ATRA-mediated cytoprotection. ATRA-mediated cytoprotection does not appear to involve modulation of Nrf2 nor the antioxidant response, but rather involves engagement of the ERK1/2 signaling pathway. Thus, ATRA pretreatment did not decrease PAP- or MGHQ-induced ROS generation (Figure 3.1). Furthermore, ATRA had only modest effects on PAP-induced reductions in GSH levels (Figure 3.2) and Nrf2 induction (Figures 3.3, 3.4). In contrast, ATRA appears to prevent angiotensin-induced apoptosis by inhibiting ROS generation and by increasing the anti-oxidant response (Choudhary, et al., 2008). It is unclear why ATRA does not protect against cisplatin-induced apoptotic cell death as discussed in Chapter 2, but an additional consideration may be related to the temporal nature of the stress kinase responses as discussed below. Furthermore, ATRA is also protective in an in vivo model of diabetic renal injury by attenuating oxidative stress (Molina-Jijon et al., 2015) emphasizing the importance of cell context in the response to ATRA. However, the reported reductions in
Nrf2 in this model will require additional evaluation based on the reported molecular weight of Nrf2 (Lau et al., 2013a).

The apparent Nrf2-independence of the cytoprotective ATRA response is intriguing given the fact that both PAP and MGHQ generate ROS, especially since sulforaphane and MG132, classic inducers of Nrf2, are both capable of providing protection against PAP toxicity (Figure 3.6). However, the interaction between ATRA, RAR, and RXR appears complex and is also context dependent. Thus, ATRA inhibits Nrf2-targeted gene expression in culture and in rodents, apparently through activation of RARα (Wang et al., 2007), and inhibits Nrf2 in A549 cells (Jayakumar et al., 2015). Consistent with these findings, RXRα reduces Nrf2 cytoprotection via binding to the Neh2 domain in the Nrf2 gene (Wang et al., 2013). In contrast, “toxic” concentrations of ATRA activates Nrf2 and induces Nrf2 target genes (Tan et al., 2008). Of interest in this latter study is the finding that inhibition of MEK1/ERK suppressed ATRA-induced Nrf2 activation.

ATRA-mediated cytoprotection herein is associated with the rapid phosphorylation of the cytoprotective kinases ERK and AKT but only inhibition of p-ERK blocked the protective effects of ATRA (Figures 3.9, 3.12A). ATRA induced phosphorylation of ERK is rapid and transient indicating that downstream signaling from p-ERK contributes to ATRA-mediated protection. Specific p-ERK target genes and downstream signaling pathways are discussed in Chapter 4.

ERK has long been considered a key modulator of organ injury regulating survival and death. On the one hand, ERK activation is associated with protection against various insults including oxidative and heat stress (Hung, et al., 2003; Niederlechner, et al., 2013;
Ong et al., 2016) and tissue repair after renal ischemia reperfusion injury (Jang et al., 2013). However, ERK has also been found to contribute to ROS-induced cell death (Dong et al., 2004; Ramachandiran et al., 2002; Zhuang et al., 2008). The mechanisms by which ERK activation modulates cell survival or death are not well characterized and are likely to be dependent on the stimulus, duration of activation, cell type, and subcellular location. Recent studies have found ERK nuclear translocation important for access to transcription factors necessary for cell proliferation. Cytosolic retention inhibits the survival and proliferative signals and can enhance proapoptotic proteins such as death associated protein kinase (Mebratu, et al., 2009).

The contribution of p-AKT to ATRA-mediated cytoprotection could not be defined by these studies since pharmacological inhibition of p-AKT enhanced PAP cytotoxicity (Figure 3.12B). However, AKT is an important component of cell survival pathways in many cell types (Cui et al., 2016; Gusscott et al., 2016). Indeed, ATRA affords protection against cell death in SH-SY5Y cells via the AKT pathway (Cheng et al., 2013) and activation of the PI3K-AKT pathway in SH-SY5Y cells promotes cell survival signals against epoxomicin-induced apoptosis via an upregulation of anti-apoptotic factors (Zhang et al., 2016). In the studies reported in Chapter 2, ATRA selectively protected against necrotic, but not apoptotic cell death, and this difference likely distinguishes between the consequences of ATRA-mediated AKT activation in the different cell types.

In summary, we have demonstrated that ATRA possesses cytoprotective effects against renal toxicants in LLC-PK1 renal epithelial cells. We found that the protective process occurs in the absence of effects on ROS levels, but through a mechanism that involves activation of ERK, but not the Nrf2 pathway. We cannot draw any conclusions
on the involvement of AKT in the cytoprotective process since its inhibition exacerbated PAP cytotoxicity. In addition, no definitive statement on the role of Grp78 and the unfolded protein stress response can be determined since manipulation of Grp78 by knockdown or overexpression methods were not conducted. However, the contribution of Grp78 to protection is unlikely since the ATRA dose necessary for induction is many-fold lower than the dose required for cytoprotection. Since p-ERK is a key contributor to the protective process, Chapter 4 explored p-ERK downstream targets and signaling, and their contribution to the cytoprotective effects of ATRA.
Chapter 4: Profiling of \( p \)-ERK mediated signaling in All-\textit{Trans}-Retinoic Acid treated renal epithelial cells: implications for mitogenesis in cytoprotection

4.1 Introduction

RA is a fat-soluble vitamin critical to human survival. RAs are involved in a variety of physiological functions including cell growth and differentiation, immune system regulation, vision, and apoptosis. Moreover, RA has clinical usage in patients to treat cancer and acne (Alvarez, \textit{et al}., 2014). These biological functions are mediated by the active metabolites of RA; ATRA and 9-cis RA. There is current interest in RA-mediated cytoprotection as a therapeutic strategy. RA has been shown to have protective effects in a number of rodent studies. ATRA decreases renal damage by increasing the expression of podocyte markers in the glomerulus (Li, \textit{et al}., 2014). ATRA-mediated protective effects are also found in the liver, brain, and heart through anti-inflammatory and antioxidant mechanisms, a decrease in apoptotic cells, and an increase in autophagy signaling (Choi, \textit{et al}., 2009a; Rao, \textit{et al}., 2013; Rao, \textit{et al}., 2010; Zhong, \textit{et al}., 2015; Zhu, \textit{et al}., 2015). 9-cis RA has protective effects against renal IRI (Balasubramanian, \textit{et al}., 2012). Although these \textit{in vivo} studies demonstrate RA-mediated protective effects in multiple organs, the molecular mechanisms involved in mediating the protective effects are unclear.

MAPKs are signal transducing enzymes that participate in the regulation of a variety of cellular functions and include ERK1/2, (JNK), and the p38 MAPK signaling cascades. The ERK cascade is the most well characterized (Chang, \textit{et al}., 2001). ERK1/2 is activated by growth factors and mediates cell proliferation, differentiation, and survival (Tian, \textit{et al}., 2000). ERK increases cell proliferation by stimulating DNA synthesis through phosphorylation of carbamoyl phosphate synthetase, the rate-limiting enzyme in
pyrimidine nucleotide synthesis (Graves et al., 2000). In addition, ERK has indirect effects on cell proliferation via cell cycle regulation. ERK activation is important for the induction of cyclin D1 expression and its entry into the cell cycle and is important in regulating the formation of cyclin E/cyclin D kinase complexes (Chambard et al., 2007).

The HIF1α pathway is activated through multiple regulatory mechanisms and has a wide variety of target genes that are involved in cell survival and repair. HIF1α is degraded under normoxic conditions, but under hypoxia and other insults, several pathways mediate HIF1α stabilization and activation (Kapitsinou et al., 2015; Salceda, et al., 1997). One such level of regulation is through growth factor activation of the Ras/Raf/MEK/ERK pathway and the resulting increased translation of HIF1α protein (Masoud, et al., 2015). HIF1α has been shown to mediate cytoprotection against a variety of insults. In rat ventricular cells, HIF1α mediates a cardio-apoptotic effect upon muscarinic acetylcholine receptor stimulation (Hui, et al., 2012). HIF1α activation has tremendous benefit during IRI regulating a tissue’s ability to adapt to low oxygen, as evidenced in mice with partial deficiency of HIF1α and a loss of cardioprotection (Cai et al., 2008). In renal IRI, pretreatment with a prolyl hydroxylation domain protein (PHD) inhibitor, and consequently HIF1α activation, leads to a reduction in serum creatinine levels and tissue damage compared to wild type counterparts (Bernhardt, et al., 2006). Studies with HIF1α heterozygote mice and rodents deficient for the VHL protein further demonstrate the protective effect of HIF1α in renal IRI (Hill, et al., 2008; Schley, et al., 2011).

Preconditioning strategies that activate ERK also have protective effects. LLC-PK1 cells primed with endoplasmic reticulum stress inducers enhance ERK activation leading to cytoprotection against oxidant-induced injury (Hung, et al., 2003). In addition, we have
recently demonstrated that ERK activation is a mechanism involved in ATRA-mediated cytoprotection. ATRA induces a rapid activation of ERK (15 min) followed by a return to baseline at 24 hr suggesting that p-ERK downstream signaling is essential during the 24 hr pretreatment period (Sapiro et al, submitted AJP Renal Physiol, In Revision). ERK activation induces the phosphorylation and activation of many proteins including HIF1α (Du et al., 2011; Richard et al., 1999). Furthermore, hypoxic preconditioning of cardiomyocytes affords cytoprotection through an increase in ERK-mediated induction of HIF1α (Liu et al., 2003). In the present study, we profiled p-ERK-mediated signaling and downstream mitogenic effects in ATRA-mediated cytoprotection against renal injury. Our findings reveal that ATRA increases the expression of mitogenic related genes and the p-ERK-mediated induction of HIF1α in its contribution to ATRA cytoprotection.
4.2 Materials and Methods

4.2.1 Materials

PAP, ATRA, and MG132 were purchased from Sigma Aldrich (St. Louis, MO). PD98059 was supplied by InvivoGen (San Diego, CA). Neutral Red was purchased from Amresco (Solon, OH). \( p \)-ERK and ERK antibodies were acquired from Cell Signaling Technologies (Danvers, MA). HIF1\( \alpha \) was bought from Novus Biologicals (Littleton, CO). GAPDH was a product of Abcam (Cambridge, MA). All secondary antibodies, were purchased from Santa Cruz (Santa Cruz, CA).

4.2.2 Cell Culture and Treatment Conditions

The LLC-PK1 renal proximal tubule epithelial cell line was obtained from the American Type Culture Collection (Rockville, MD) and is derived from the New Hampshire minipig. LLC-PK1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose and supplemented with 10% fetal bovine serum (FBS) in a 37°C/5%CO\(_2\) humidified incubator. Cells were passaged every 3-4 days at 90% confluence. All assays were conducted with cells plated in multi-well plates and grown to post-confluence. Cells were subsequently washed and treated with ATRA. PAP exposure to cells was in DMEM without FBS.

4.2.3 Microarray Analysis

Cells were grown in 6-well plates and exposed to ATRA for different time periods. Cell lysis was accomplished and total cellular RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. RNA concentration and quality were determined using absorbance values at A260 and A280 on an Agilent Nanodrop machine (Agilent, Santa Clara, California). Total RNA (500 ng) was labeled,
hybridized to GeneChip Porcine Gene 1.0 ST Arrays (Affymetrix, Santa Clara, CA), and read using an Affymetrix scanner at the University of Arizona Cancer Center Genomics Shared Service. Data was analyzed in collaboration with the Wayne State University Applied Genomics Center. Intensity data was normalized using RMA (Bolstad et al., 2003). Differential expression of probes was determined relative to the time zero time point using ImFit and eBayes methods from limma (Ritchie et al., 2015; Smyth, 2005).

4.2.4 qRT-PCR Analysis

Cells were grown in 6-well plates and exposed to ATRA for different time periods. Cells were lysed and total RNA was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. RNA concentration and quality were determined using absorbance values at A260 and A280 using the Take3 application part of the Gen5 quantification program (BioTek Instruments, Winooski, VT). Total RNA (1 μg) was reverse transcribed to cDNA using Applied Biosystems High-Capacity RNA-to-cDNA™ Kit following the manufacturer’s protocol. Primer sets and probes using Applied Biosytems TaqMan Gene Expression Arrays were specific for selected genes that encompassed ATRA functions spanning the entire time period (Table 4.1). RT-PCR was conducted using Taqman Applied Biosystems TaqMan® Gene Expression Master Mix qPCR Master Mix (Foster City, CA) using 20 ng cDNA for each reaction. qRT-PCR was performed using the following amplification conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 sec 60°C for 1 min. RT-PCR reactions were run on the QuantStudio 12K Flex Real-Time PCR System (Life Technologies, Carlsbad, CA) at the Applied Genomics Technology Center Wayne State University, School of Medicine. Gene specific products were normalized to ribosomal protein L5 (RPL5) and quantified using
the comparative Ct method. Fold expression changes were calculated using the $2^{-\text{ddCT}}$ method.

### 4.2.5 Proteome Cell Stress Array

The expression profile of cell stress related proteins was analyzed using the Proteome Profiler Human Cell Stress Array (R&D Systems). Cells were grown in 6-well plates and exposed to ATRA for different time periods. Lysis of cells was accomplished using the lysis buffer from the manufacturer with the addition of protease and phosphate inhibitors. Three wells per treatment were used and pooled together at the time of collection. The protein concentration of each sample was determined using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA). The assay was conducted following the manufacturer’s protocol. Cell lysates (600 μg) were mixed with a cocktail of biotinylated detection antibodies and applied to an array consisting of a nitrocellulose membrane spotted with 26 cell stress associated capture antibodies. Following an overnight incubation, the membranes were washed and incubated with streptavidin-conjugated horseradish peroxidase. The signal was detected using chemiluminescent detection reagents on an Odyssey FC (Licor Technologies, Lincoln, NE) imager. Pixel densities of the spots were determined directly with quantitation in the imager.
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Table 4.1  Probe sets for qRT-PCR candidate genes

FOS, FBJ murine osteosarcoma viral oncogene homolog; EGR2, early growth response 2; BHLHE40, basic helix-loop-helix family member e40; CCNE2, cyclin E2; CYP26A1, cytochrome P450, family 26, subfamily A, polypeptide 1; RPL5, ribosomal protein L5.
4.2.6 Western Blot Analysis

Following the various treatments, cells were washed in ice cold PBS and lysed with 1X RIPA Buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% NP-40, and 10% sodium deoxycholate. Complete protease and phosphatase inhibitor cocktail tablets (Roche, South San Francsico, CA) were added fresh to the buffer. MG132 was added to the buffer for the HIF1α experiments. Cell lysates were pelleted by centrifugation at maximal speed for 15 min, and supernatants, containing total protein, were collected and stored at -80°C. Protein concentrations were measured using the Bio-Rad Protein Assay Dye Reagent (Bio-Rad Laboratories, Hercules, CA). Aliquots of protein lysates were separated on 7.5% denaturing polyacrylamide gels (SDS-PAGE) and transferred to a nitrocellulose membrane for immunoblotting. The membranes were blocked in 5% non-fat dry milk in tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) for 1 hr, and then incubated with primary antibodies overnight at 4°C in blocking solution. Primary antibody dilutions were 1:500 (HIF1α), 1:1000 (p-ERK), 1:2000 (ERK), or 1:15000 (GAPDH). Secondary antibodies were diluted to 1:3000 in blocking solution and incubated with the membranes for 1 hr at room temperature. Blots were finally developed with enhanced chemiluminescence and imaged.

4.2.7 Cell Cycle Analysis

Following exposure of LLC-PK₁ cells to ATRA for different time periods, cells were washed and a combination of pepsin and trypsin were used to detach cells from the wells. Cells were pelleted by centrifugation for 10 min at 1000 rpm. Media was removed, samples were fixed by resuspending the pellet in 70% ethanol, and stored overnight at -20°C. Cells were pelleted by centrifugation for 15 min at 2000 rpm and resuspended in 500
μL ice cold PBS and transferred to a flow cytometry tube. 25 μl (1/20 vol) of 10 mg/ml RNAse A and 12.5 μl (1/40 vol) of 1.0 mg/mL propidium iodide were added and tubes were incubated at 37 °C for 30 min. Flow cytometry was conducted on a BD LSRII instrument (BD Biosciences, San Jose, CA) at the Microscopy, Imaging and Cytometry Resources Core at Wayne State University, School of Medicine. The data was analyzed via ModFit LT v3.0 (Verity Software House, Topsham, ME).

4.2.8 DNA Isolation

Cells were seeded in 6-well plates and grown to post-confluency. Following ATRA exposure, media was removed, cells were washed with PBS, and 1 mL DNAzol reagent (Thermo Fisher Scientific) was added to lyse the cells. Lysates were pelleted by centrifugation and supernatant was collected. DNA was precipitated with the addition of 100% ethanol. The resultant precipitate was centrifuged, the pelleted DNA was washed with 75% ethanol, and resuspended in 8mM NaOH. DNA concentration was measured at 260/280 nm using Take3 application part of the Gen5 quantification program (BioTek Instruments, Winooski, VT).

4.2.9 Trypan Blue Assay

Cells were seeded in 6-well plates and grown to post-confluency. After ATRA exposure, cells were washed and a combination of pepsin and trypsin were used for detachment. Cells were mixed with an equal volume of media and trypan blue solution (Thermo Fisher Scientific) and counted on a hemocytometer.

4.2.10 Neutral Red Assay

The neutral red lysosomal uptake assay was used to measure cell viability following treatment with toxicants, as previously described (Mertens, et al., 1995). Briefly, cells
were grown in 96-well plates and allowed to grow to post-confluence prior to treatment. At the end of each experiment, cells were washed and then incubated with 0.25 mg/mL neutral red solution for 1 h at 37°C/5% CO₂. The neutral red was then removed, cells washed, and cells fixed in 1% formaldehyde/1% CaCl₂, followed by extraction of neutral red using 1% acetic acid/50% ethanol solution for 15 min at room temperature in the dark to remove excess dye. The extent of lysosomal neutral red accumulation was assessed by determining absorbance at 540 nm.

4.2.11 Statistical Analysis

With the exception of the microarray analysis, for individual comparisons, one-way ANOVA followed by Tukey’s post hoc analysis or unpaired Student’s t test was used. All data is expressed as mean ± SE and p<0.05 were considered to be significant. For the microarray data, the adjusted P-value and FDR p-value were used for the fold change descriptions and gene ontology analysis, respectively. P<0.05 was considered to be significant.
4.3 Results

4.3.1 Gene expression profiling analysis in ATRA-treated LLC-PK1 cells

Total RNA from LLC-PK1 cells treated with ATRA for 0, 0.5, 1, 4, 8, or 12 hr were collected for microarray analysis. The total number of upregulated genes (fold changes ≥2) are as follows: 0.5 hr- 21 genes, 1 hr- 61 genes, 4 hr- 81 genes, 8 hr- 51 genes, 12 hr- 26 genes (Table 4.2). At the early time points (0.5, 1 hr), we found the most significantly upregulated genes were FOS, EGR1, EGR2, EGR3, CYR61, PLAT, DUSP5, ATF3, CXCL2, and ZFP36 suggesting cell proliferative and differentiation effects. Beginning at 4 hr, target genes of the transcription factors from the earlier time points associated with ribosome biogenesis, DNA replication, DNA repair, and the cell cycle were significantly expressed. PLAT and CXCL2 levels remained elevated multifold. A representation of ATRA upregulated genes at each time point is displayed in a heat map (Figure 4.1). Only a small number of down-regulated were detected at any of the time points and were therefore not further investigated.

4.3.2 Gene ontology analysis

When compared to the time zero vehicle control group, significant enrichment analysis of GO function was found in ATRA-treated cells. The top 10 enrichment processes are listed in (Table 4.3) for each time point unless there were fewer than 10 significant changes. At 1 hr, differentially expressed genes were significantly enriched in the regulation of biological, cellular, and metabolic processes with approximately 40 genes contained in each cluster. In general, there were fewer genes clustered together at the other time points but more specific physiological functions were detected (ribosome biogenesis, DNA replication).
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**Table 4.2**  Upregulated genes in LLC-PK1 cells following ATRA exposure

Total number of upregulated genes with significant fold increases following ATRA (25 μM) treatment at 0, 0.5, 1, 4, 8, and 12 hours. Significance was defined as p<0.05 with a fold change ≥2.
Figure 4.1  Heat map of ATRA-target genes during the 12 hr exposure period

Selected target genes to represent each time point are depicted. The colors represent mean fold changes between ATRA and the time zero control with the brighter the red, the more enhancement of target gene.
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Table 4.3  The top ten enrichment genes (if applicable) in each ontology term
4.3.3 Validation of gene expression results with qRT-PCR

Five highly differentially expressed genes including FOS, EGR2, CYP26A1, BHLHE40, and CCNE2 were selected for quantitative RT-PCR analysis. The mRNA expression levels of all five genes were significantly upregulated in ATRA-treated cells. Consistent with the microarray analysis, the kinetics of FOS and EGR2 induction portrayed a rapid (0.5 hr) and transient induction with elevated expression levels of ~8-12 fold. In addition, the gene expression level of BHLHE40 more subtly, yet still statistically significant, increased with a maximal increase in expression at 4 hours (~3-fold). Moreover, the p-ERK downstream target gene CCNE2 increased ~2-fold in ATRA-exposed cells at 8 and 12 hours. The results for CYP26A1 expression were below the significance level by microarray analysis but the ATRA-induced increase in expression beginning at 4 hours was confirmed by qRT-PCR (Figure 4.2). ATRA-induced elevations in CYP26A1 gene expression provide a proof-of-concept for this experiment since CYP26A1 is a signaling protein in retinoid metabolism.

4.3.4 Profiling of cell stress protein expression in LLC-PK1 cells

To gain deeper insights into specific stress proteins downstream of p-ERK induction, proteome profiling in LLC-PK1 cells treated with ATRA was conducted. ATRA significantly increased HIF1α expression at 4 and 8 hr. Moreover, ATRA markedly increased p-JNK expression at 1 hr although the finding did not reach statistical significance. Additional proteins detected in LLC-PK1 cells were a disintegrin and metalloproteinase with thrombospondin motifs 1 (ADAMTS1), heat shock protein 60 (HSP60), and heat shock protein 70 (HSP70), but none were influenced by ATRA (Figure 4.3).
Western blot analyses were performed to confirm the induction of HIF1α levels. Consistent with the cell stress array observations, ATRA induced HIF1α early, with a maximal increase at 8 hr, and levels returning to baseline by 24 hr (Figure 4.4).

4.3.5 **HIF1α is an ATRA-induced p-ERK downstream signaling protein**

ATRA increases HIF1α expression in renal proximal tubular cells (Fernandez-Martinez et al., 2012; Fernandez-Martinez et al., 2011). HIF1α is activated by ERK phosphorylation (Du, et al., 2011). Thus, we determined whether HIF1α is a downstream target of ATRA-induced p-ERK in LLC-PK1 cells. HIF1α is degraded under normoxic conditions (Kapitsinou, et al., 2015). To stabilize HIF1α, we added MG132 both to the culture media 4 hr prior to harvest and in the lysis buffer. Both p-ERK and HIF1α expression were induced following ATRA exposure periods of 0.5 and 8 hr, respectively. Cells were treated for 30 min with 50 μM PD98059, a MAPK inhibitor, and, as expected, p-ERK expression was completely inhibited. Importantly, when cells were treated with PD98059 for 30 min followed by ATRA exposure, both p-ERK and HIF1α expression were blunted suggesting HIF1α is a downstream target gene of ATRA-induced p-ERK (Figure 4.5).
Figure 4.2 RT-PCR validation of select ATRA upregulated genes in LLC-PK₁ cells following ATRA treatment

RT-PCR analysis of FOS, EGR2, BHLHE40, CCNE2, and CYP26A1. LLC-PK₁ cells were exposed to ATRA (25 μM) for a maximum of 12 hr. Cells were lysed, RNA was extracted and reverse transcribed to cDNA. RT-PCR was performed using Applied Biosystems Taqman Gene Expression Assays. Gene specific products were normalized to ribosomal protein 5 (RPL5) and quantified using the comparative Ct method. Fold changes were calculated using the 2-ΔΔCt method. Significance was determined from comparisons to vehicle-treated control cells (*p<0.05, **p<0.01, ***p<0.001, n=4).
Figure 4.3  Cell stress profile array of ATRA-treated LLC-PK₁ cells

Proteome profiling of stress proteins modified by ATRA. Equal amounts of protein from control and ATRA-treated cells were applied to an array spotted with 26 stress antibodies. Only proteins with marked changes are noted. HIF1α, hypoxia inducible factor 1 alpha. Significance determined from comparisons to vehicle-treated control cells. (*p<0.05, n=4).
Figure 4.4  Time-dependent induction of ATRA on HIF1α protein expression in LLC-PK1 cells

Cells were exposed to ATRA (25 μM) for a maximum of 24 hr. MG132 (10 μM) was added in 4 hr prior to harvest. Protein extracts were analyzed for HIF1α induction using western blot analysis. HIF1α densitometric analysis was normalized to GAPDH. Significance was determined from comparison to vehicle-treated control cells (*p<0.05, n=4).
Cells were pretreated with the MEK inhibitor, PD98059 (50 μM for 30 min. ATRA (25 μM) was spiked into each well for 0.5 hr or 8 hr for p-ERK and HIF1α detection, respectively. For HIF1α lysate, cells were treated with MG132 (10 μM) for 4 hr prior to harvest. Protein extracts were analyzed for p-ERK and HIF1α expression. p-ERK was normalized to ERK and HIF1α was normalized to GAPDH. Significance was determined from ATRA alone or ATRA + PD98059 groups (***p<0.001, n=4).
4.3.6. ATRA-mediated cytoprotection does not involve changes in cell cycle distribution

Results from the microarray study indicated that ATRA induces genes involved in DNA replication and cell cycle. Thus, we determined the effect of ATRA on cell cycle progression. LLC-PK1 cells were treated with ATRA for 1, 8, 16, or 24 hr to span the entire 24 hr pretreatment period necessary to observe protective effects. While ATRA stimulated an increase in the population of cells in the S phase (~2-fold increase) beginning at 8 hr and G2 phase (~3-fold increase) at 16 and 24 hr, the magnitude of these changes, although significant, was small (Figure 4.6).

4.3.7 ATRA preconditioning increases cell proliferation and cytoprotection

The microarray findings indicated ATRA increases growth-related genes (Figure 1). We therefore measured cell growth by assessing total DNA content and cell number in ATRA-treated cells. Following a 24 hr exposure period, ATRA-treated cells demonstrated significant increases in DNA content (2.4-fold) and overall cell number (1.4-fold) (Figure 7). Previously, we demonstrated that ATRA affords cytoprotection against p-aminophenol (PAP)-induced cytotoxicity (PAP 150 μM, 3 hr) and a 24-hr ATRA pretreatment period is necessary for maximal cytoprotection (Chapter 2). Since ATRA stimulated a 25% increase in cell number, we extended the protection experiment to confirm that the observations are a result of the engagement of critical cytoprotective cell survival signaling proteins and not a false measurement based solely on an increase in cell growth. In LLC-PK1 cells exposed to PAP (150 μM, 3 hr), a ~60% decline in cell viability was found. In cells pretreated with ATRA (25 μM, 24 hr), the concentration of PAP was adjusted to 165 μM to equalize dose per cell number. ATRA-pretreated cells demonstrated ~50% increase in cell viability when
administered PAP (150 μM). A slight but insignificant decrease in protection was noted in cells treated with the higher dose of PAP (Figure 4.8). Taken together, ATRA increases the synthesis of mitogenic-related proteins that may contribute to its cytoprotection against renal cell damage.
Figure 4.6  Effect of ATRA on cell cycle distribution in LLC-PK1 cells

Cells were treated with ATRA (25 μM) for a maximum of 24 hr. Cell pellets were incubated with propidium iodide and cell cycle was conducted by flow cytometry analysis. Significance was determined from comparisons to vehicle-treated cells (**p<0.01, ***p<0.001, n=4).
Cells were treated with ATRA (25 μM) for 24 hr. (A) Cell number was determined by trypan blue staining and (B) DNA content was measured absorbance readings. Significance was determined from comparisons to vehicle-treated cells (*p<0.05, **p<0.01, n=3).

Figure 4.7  Effect of ATRA on cell number and DNA content in LLC-PK1 cells
Figure 4.8  Effect of alteration of PAP dose per cell number on ATRA cytoprotection

Cells were pretreated with (25 μM, 24 hr) followed by exposure to PAP (150 μM or 165 μM, 3 hr). Cell viability was assessed by lysosomal uptake of Neutral Red. Significance was determined from comparisons between PAP alone to the ATRA + PAP groups (*p<0.05, n=3).
4.4 Discussion

ERK1/2 is a key signaling and cytoprotective protein induced by a variety of stimulants including growth factors. In LLC-PK₁ renal epithelial cells, ATRA-mediated cytoprotection involves ERK activation (Chapter 3). The current study explored ERK downstream signaling processes. The microarray data indicated that ATRA induced several genes associated with cellular growth and differentiation processes, consistent with ATRA’s physiological roles. A total of 27,574 probes were detected. At 0.5, 1, 4, 8, and 12 hr following ATRA treatment, 80, 242, 347, 154, and 92 genes were up-regulated (fold change ≥2) respectively, with a variety of genes portraying altered expression levels following ATRA exposure. ATRA induced EGR expression several fold at the 0.5 and 1 hr time points, consistent with other investigations with its role as an early response mediator of mitogenic effects (Abbott et al., 2005; Lahaye et al., 1998). Moreover, ATRA induced changes in gene expression included FOS, CYR61, ELF3, PLAT, and BHLHE40 which are additional immediate response factors to stimuli (Andreoli et al., 1997; Huang et al., 2016; Muhammad et al., 2016; Welling et al., 1996). TAGLN is an actin binding-associated protein (Prinjha et al., 1994) and crosslinking activity has implications in cell proliferation and differentiation (Chen et al., 1999). These genes are induced early within 1 hr, primarily in a transient manner. Beginning at 4 hr, ATRA induces genes associated with specific cellular functions in growth and differentiation. For example, at 4 hr, NOC4L and GNL3 are induced 2-fold. GNL3 is involved in the processing of pre-rRNA and NOC4L is part of a subcomplex of proteins that shuttles pre-rRNA factors to the nucleolus for ribosome biosynthesis (Lin et al., 2014; Warda et al., 2016). The expression of a variety of genes associated with DNA replication, cell cycle progression, and DNA repair
are enhanced by ATRA at 8 and 12 hr (POLE2, CCNE2, CDC6, XRCC2, DTL, CHEK1, MCM4, RAD51) consistent with the findings of other mitogens (Damera et al., 2009; Ikari et al., 2008).

According to the GO term enrichment analysis, ATRA might regulate the expression of genes in several biological processes. At 0.5 and 1 hr, enriched genes are clustered into the regulation of biological, cellular, and metabolic processes as well as cell migration and death. While the regulation of cell death and apoptotic processes was detected as a GO category at 0.5 hr, it is unlikely that ATRA induces apoptosis in our cell model as ATRA did not afford cytoprotection against cisplatin-induced cytotoxicity (Chapter 2). The 4 hr findings indicate that ATRA treatment stimulated the clustering of genes into several ribosome biogenesis-related processes confirming the specific fold-change increases of individual genes detected by the microarray analysis. By 8 and 12 hr, overall, there was less clustering of genes into biological functions but greater fold enrichment. ATRA altered the biological process of DNA replication consistent with the upregulated genes. Although the regulation of the MAPK cascade (GO: 0043408, FDR: 0.00000483) and regulation of ERK1 and ERK2 cascade (GO: 0070372, FDR: 0.000223) are not in the top 10 enrichment processes, we remain interested in these processes since p-ERK is a critical stress response protein mediating ATRA’s cytoprotective effects.

The protein stress array and western blot data indicate ATRA induces HIF1α expression at 4 and 8 hr (Figures 4.3, 4.4) which follows the increase in p-ERK expression (0.5 hr). Inhibition of p-ERK leads to inhibition of HIF1α expression indicating HIF1α is a p-ERK downstream target gene in our model (Figure 4.5). However, similar to the kinetic pattern of p-ERK induction, HIF1α induction is transient with levels returning to
basal conditions by 24 hr suggesting further downstream HIF1α signaling contributes to ATRA-mediated protection. Of note, ATRA increased BHLHE40 expression (Figures 4.1, 4.2), a HIF1α target gene (Miyazaki et al., 2002), throughout the 12 hr time frame. The signaling events between HIF1α induction and cell proliferation at 24 hr in ATRA-mediated protection is unknown. Therefore, the contribution of BHLHE40 to the mechanism of ATRA-mediated cytoprotection may provide additional insights.

The ATRA-stimulated increases in cell proliferation and differentiation gene expression (Figures 4.1, 4.2), the small increase in population of cells in the S and G2 phases of the cell cycle (Figure 4.6), and enhanced DNA content and cell number (Figure 4.7) collectively suggest that mitogenic processes appear to be a contributor to ATRA-mediated cytoprotection. Other mitogens are known to possess cytoprotective properties (Niederlechner, et al., 2013; Yang, et al., 2013), and mesenchymal stem cell-based keratinocyte growth factor gene delivery attenuates lipopolysaccharide-induced acute lung injury through an enhancement of epithelial cell proliferation (Chen et al., 2013). We demonstrated that no significant decrease in ATRA-mediated cytoprotection was observed upon altering the dose of toxicant to correlate with an increase in ATRA-stimulated cell number (Figure 4.8) furthering indicating a mitogenic contribution and the requirement of a pretreatment time with the induction of proteins critical to cell survival.

Prostaglandins PG, not typically thought of as potent mitogenic compounds, play important diverse roles in mammalian cell signaling participating in proliferation and differentiation and stress response pathways among other functions (Schlondorff, 1986). Pretreatment of LLC-PK1 cells with either PGE2 itself or DDM-PGE2 affords cytoprotection against TGHQ-induced renal toxicity (Weber, et al., 1997). In agreement
with ATRA-mediated signaling, a 24 hr pretreatment time is critical for maximal DDM-PGE₂-mediated protection (Weber, et al., 1997). DDM-PGE₂ induces a number of specific proteins and total DNA synthesis. Mitogenic-related proteins induced by DDM-PGE₂ included elongation factors and proteins associated with the cytoskeleton (Person et al., 2003). While the direct proteins induced by between ATRA and DDM-PGE₂ do not overlap, they share similarities as correct cytoskeletal organization is necessary for cellular growth.

The contribution of mitogenesis to renal protection is context dependent with a beneficial role in acute conditions (<24 hr) but with an exacerbation of chronic conditions. For example, HGF is protective against acute IRI decreasing BUN and creatinine levels and increasing the mitogenic index and incorporation of 5-bromo-2’-deoxyuridine (BrdU) into cortical tubular epithelia (Miller et al., 1994). Moreover, in the post-ischemic kidney, proliferating cell nuclear antigen (PCNA) and vimentin are present in the S3 segment of proximal tubule cells (Witzgall et al., 1994). In contrast, however, in chronic kidney disease which is characterized by progressive fibrosis, mitogenic factors potentiate toxicity as transforming growth factor-β1 induces cell proliferation and collagen production (Chen et al., 2011). In kidney transplantation, EGF is induced contributing to the pathogenesis of chronic allograft injury (Rintala et al., 2014).

In summary, ATRA-mediated cytoprotection involves p-ERK activation and subsequent downstream signaling proteins involved in cellular growth and differentiation and DNA replication and repair. Furthermore, HIF1α is a p-ERK target gene and its contribution to the cytoprotective processes warrants future investigation. Understanding the mechanism of ATRA-mediated cytoprotection will advance insights into the
development of novel therapeutics for renal pathological conditions including chemical-induced toxicity, ischemia reperfusion injury, and hypoxia.
Chapter 5: Concluding Remarks and Future Directions

5.1 Summary

RA is a fat-soluble vitamin obtained from the diet with roles in a variety of functions including cell growth and differentiation, immune system regulation, vision, apoptosis, cancer, and acne (Alvarez, et al., 2014). The biologically active forms of RA, ATRA and 9-cis-RA, mediate these pleiotropic effects through genomic and non-genomic signaling pathways (Al Tanoury, et al., 2013). Within the last decade, RA in particular, ATRA have demonstrated cytoprotective effects. These studies were primarily conducted in rodents in which ATRA afforded protection in multiple organs (Choi, et al., 2009a; Li, et al., 2014; Rao, et al., 2013; Rao, et al., 2010; Zhu, et al., 2015). However, the mechanism(s) of ATRA-mediated cytoprotection have not been well characterized. The experiments described in this dissertation were designed to investigate the molecular mechanisms of ATRA-mediated cytoprotection in the well-established proximal tubule cell line, LLC-PK1 cells.

Proteins orchestrate cellular functions. Previous work in our laboratory found that DDM-PGE\textsubscript{2} is cytoprotective against TGHQ-induced renal cell death (Weber, et al., 1997). The protection is mediated by a thromboxane receptor coupled to NFκβ signaling (Weber, et al., 2000). The cytoprotective effect was not immediate and required a 24 hr DDM-PGE\textsubscript{2} pretreatment period, suggesting the need for induction of proteins that mediate the cytoprotective process. One such protein induced by DDM-PGE\textsubscript{2} was RBP. Thus, we hypothesized in a manner similar to DDM-PGE\textsubscript{2}, there would be a time-dependent nature of ATRA-mediated cytoprotection contingent on protein synthesis and the engagement of cell survival pathways.
The aim of chapter 2 was to develop an *in vitro* model characterizing ATRA cytoprotection in LLC-PK1 cells. Consistent with the effects of DDM-PGE2, a 24 hr pretreatment time is needed to observe ATRA cytoprotection. Moreover, upon inhibiting protein synthesis with CHX, ATRA protection is blocked indicating that protein synthesis is critical for cytoprotection. These findings were observed with the renal toxicant, PAP. Upon extending the studies to include multiple renal toxicants, ATRA-mediated cytoprotection was found to be specific to toxicants that induce oncotic/necrotic cell death indicating ATRA cytoprotection is cell death form specific. Tissue damage is a common feature of necrotic-induced cell death during chemical-induced renal injury, IRI, and hypoxia suggesting that the findings from this study can provide insights into the development of novel therapeutic strategies for kidney diseases. Interestingly, in our model development experiments characterizing pretreatment time, pretreatment was not necessary for retinoid protection against IDAM and that a retinoid/IDAM interaction may be occurring.

The mechanisms mediating ATRA cytoprotection against renal cytotoxicity are delineated in Chapters 4 and 5. We chose to focus our investigations on stress response proteins as they mediate cell survival pathways. ATRA protects against IRI in which ROS play an important role (Rao, *et al.*, 2010). Intriguingly, ATRA did not have an effect on ROS levels nor the Nrf2 signaling pathway but rather involved the engagement of the ERK1/2 signaling cascade. Upon treatment with the MEK inhibitor, PD98059, LLC-PK1 cells exposed to ATRA followed by PAP demonstrated a marked reduction in cell viability indicating *p*-ERK is critical for ATRA-mediated cytoprotection.
ERK activation is typically associated with protection against various insults including oxidative and tissue repair after renal IRI (Hung, et al., 2003; Jang, et al., 2013; Ong, et al., 2016). In contrast, ERK has also been found to contribute to ROS-induced cell death (Ramachandiran, et al., 2002). The mechanisms by which ERK activation mediates cell survival or death are unknown and possibly dependent on stimulus and duration of activation. Results from these experiments indicate growth factor (ATRA) induction of p-ERK consistent with a role in cell proliferation. Ramachandiran et al, however, found that p-ERK mediates toxicant (TGHQ)-induced cell death. In both studies, ERK activation portrayed a transient response indicating that the proliferative effects (survival) or severity of the stress response (death) depend on p-ERK downstream signaling. Our data indicates a role for p-ERK in mediating ATRA cytoprotection in a ROS independent manner as no effect was found when ROS levels and Nrf2 expression were measured. ROS are required for both ERK and p38 MAPK activation but also in TGHQ-induced cell death (Ramachandiran, et al., 2002). Although ERKs, JNKs, and p38 MAPK’s are activated by stresses such as ROS and DNA damage (Holbrook et al., 1996), the role of ERK in the stress response still remains ambiguous. Nonetheless, since p-ERK levels were induced by ATRA early and in a transient manner in our study, p-ERK downstream signaling proteins and resultant functions were explored in Chapter 5.

The microarray and qRT-PCR data indicate that ATRA induces genes associated with cell growth and differentiation, DNA replication and repair, and cell cycle regulation; all downstream signaling events of ERK activation. Moreover, ATRA increased DNA content and total cell number suggesting mitogenesis may contribute to ATRA cytoprotection. Additional mitogens are known to possess cytoprotective properties
(Niederlechner, et al., 2013; Yang, et al., 2013) consistent with a role of mitogenesis in mediating cytoprotective processes.

In addition to the mitogenic effects induced by ATRA, inhibition of p-ERK decreased ATRA-induced HIF1α expression indicating HIF1α as a p-ERK target gene. Of note, the kinetic pattern of HIF1α induction is transient with maximal expression at 8 hr and a return to baseline by 24 hr. The signaling events between HIF1α induction and cell proliferation at 24 hr in ATRA-mediated protection is unknown. Moreover, the role of HIF1α in the mechanism of ATRA cytoprotection is unknown and warrants further investigation. The mechanism of ATRA cytoprotection delineated by the experiments described in this dissertation is shown in Figure 5.1.
Figure 5.1  Proposed cytoprotective mechanism mediated by ATRA

DDM-PGE$_2$ engages retinoid signaling. ATRA stimulates protein synthesis including the cell stress kinase p-ERK. ERK activation stimulates HIF1$\alpha$ and mitogenesis contributing to cytoprotection. ATRA’s cytoprotective effects occur independently of ROS generation and antioxidant signaling.
5.2 Future Directions

5.2.1 Identification of HIF1α target genes and the relationship to cell proliferation

*p-ERK* and downstream mitogenic signaling are critical aspects to ATRA’s cytoprotective effects (Chapters 3, 4). The microarray data reveal that ATRA increases the expression of genes related to DNA replication, DNA repair, and cell cycle at 8 and 12 hr. The cell cycle analysis demonstrates that, although there is not a marked change in cell cycle distribution, there is a small increase in the percentage of cells in the S and G2 growth phases beginning at 8 hr. Furthermore, the cell number data indicates a 25% increase in total cell number in ATRA-treated cells by 24 hr. HIF1α is one protein activated by ERK phosphorylation (Du, et al., 2011) and our data provide support that ATRA activates HIF1α and inhibition of *p-ERK* blunts HIF1α induction. While we have identified a *p-ERK* downstream target, the kinetics of HIF1α induction are transient suggesting additional downstream signaling proteins are involved. Currently, there is a gap in our knowledge of the kinetics between HIF1α induction (4-8 hr) and the increase in cell proliferation at 24 hr. Although we obtained insights into the mitogenic proteins noted above, a link between HIF1α and these processes is unknown. Thus, identification of HIF1α target genes and their connection to cell proliferation will provide further insights into the mechanism of ATRA-mediated cytoprotection.

The BHLHE40 transcription factor and BLM protein are HIF1α-associated target genes (Miyazaki, et al., 2002). Both of these genes were induced 2-3 fold in our microarray analysis. BLM encodes a RecQ helicase that mediates DNA repair. BHLHE40, a transcription factor, encodes a basic-helix-loop-helix protein involved in cell differentiation; thus providing possible support for a connection between HIF1α and
mitogenesis. Therefore, western blot analysis can be used to determine protein expression of these two proteins following collection of whole lysates from ATRA-treated cells. Subsequently, the expression levels of BHLHE40 and BLM will be determined following p-ERK inhibition.

We anticipate that ATRA will increase the protein expression of BHLHE40 and BLM beyond 8 hr since the mRNA expression of these proteins is elevated at 8 and 12 hr suggesting a higher correlation with the time needed for maximal cytoprotection. In the event that ATRA does not increase the expression of these proteins or the induction is transient, HIF1α may not be an important protein in the cytoprotective mechanism.

5.2.2 Development of an acute rodent model to assess ATRA protection

The data in this dissertation describe a mechanism involved in ATRA-mediated cytoprotection against cell injury in LLC-PK₁ cells. Both ATRA and 9-cis-RA exhibit protective effects in multiple organs in animal models (Balasubramanian, et al., 2012; Choi, et al., 2009a; Li, et al., 2014; Rao, et al., 2013; Rao, et al., 2010; Zhu, et al., 2015) but the molecular mechanisms underpinning protection is not well known. In addition, 9-cis-RA is the RA metabolite that has demonstrated protection in the kidney; the ATRA investigations being focused on other organs. Therefore, future studies will need to be conducted to develop an in vivo ATRA protection model against renal injury.

Similar to the in vitro studies, PAP will be the model toxicant due to its ability to target the S₃ segment of the proximal tubule and induce toxic effects. The Fisher 344 rat should be utilized as the rodent species as this rat is more sensitive to PAP-induced renal lesions (Newton, et al., 1983b). A PAP acute single dose response experiment will need to be conducted to determine the optimal dose that induces toxicity. Serum and urinary
analyses and histological markers in the proximal tubule will be assessed. An effective ATRA dosing paradigm and vehicle for solubilization will need to be determined. mRNA and protein analysis of kidney sections will be conducted for \( p\)-ERK target genes identified in the experiments described in future direction 5.2.1. Finally, as our mechanistic work has demonstrated a critical involvement of \( p\)-ERK in mediating ATRA protection against renal injury, experiments can be designed to explore \( p\)-ERK inhibition using the PD98059 inhibitor in this rodent model.

We anticipate that ATRA will afford protection against PAP-induced renal damage in a rat model and that our \textit{in vitro} mechanistic findings will translate to the \textit{in vivo} setting. We have been challenged to demonstrate ATRA protection in a rodent model but that was under the assumption that ATRA afforded protective effects through a reduction in ROS and engagement of antioxidant signaling. We have since shown \textit{in vitro} that ATRA has no effect on ROS levels and Nrf2 signaling. Furthermore, we are designing a repeat dose experiment to account for the short life of ATRA (Le Doze et al., 2000; Lee et al., 1993; Saadeddin et al., 2004). If ATRA protection is not observed, experiments that involve a combination therapy of ATRA with another drug should be considered as described below.

5.2.3 Combination protection against renal injury

ATRA has approved clinical usage for cancer treatment. Current investigations in cancer treatment involve a combination therapy of retinoids with a cancer preventative agent (Adachi, \textit{et al}., 2016; Arrieta, \textit{et al}., 2010). An investigational study in a mouse model of renal failure indicates that a combination treatment with a retinoid and roflumilast, a phosphodiesterase 4 inhibitor, possesses additional protective effects in combination than either compound alone. Mechanistically, the protection involved an
increase in intracellular cAMP levels in which roflumilast has positive stimulatory effects. The addition of roflumilast did not decrease the several day pretreatment period of retinoid but the daily dosage of retinoid is markedly lower in the microgram range (Zhong et al., 2012). Resveratrol is an ERK activator and has notable protective effects against kidney damage (Hao, et al., 2016; Rossler et al., 2015). Thus, screening experiments with a combination of ATRA and resveratrol can be conducted. Initially, these dose-response experiments will be performed in LLC-PK₁ cells with the goal of lowering the concentration of ATRA required to observe cytoprotection.

Based on the known cytoprotective effects of ATRA and resveratrol when administered alone, it is suspected that additional protective effects will be found. It is unclear, though, the extent of the magnitude in protection. If the combination protective effect is markedly substantial, then mechanistic experiments can be conducted and perhaps, an in vivo model similar to that described in future direction 5.2.2 will be developed.

5.2.4 Retinoid screening for protection in multiple kidney cell lines

One of the main limitations of this work is that it was conducted in LLC-PK₁ cells; a well-characterized cell line derived from the proximal tubule of a pig with strong gene homology to that of humans. Findings from the HK2 cell line indicate less of a protective effect compared to the LLC-PK₁ cell line. Additional preliminary data from our laboratory indicate a similar finding in HKC5 and HKC8 cells, however, characterization of these two cell lines is less clear. Thus, to provide validity for the data from the LLC-PK₁ cells, screening assays in several kidney cells models with ATRA, 9-cis RA, and a variety of synthetic retinoids should be performed. Synthetic retinoids are developed with the objective of improving retinoid bioavailability to increase their effectiveness.
The following cell models could be employed: LLC-PK₁, HK2, RPTEC/TER1, NRK-52E, MDCK, and primary cells. Hundreds of synthetic retinoids have been tested; the ones selected for this study have been tested for therapy in both benign and cancerous conditions. The following retinoids will be assessed for protection in these cell lines: ATRA, 9-cis RA, 13-cis RA, AGN190168 (Tazarotene), LGD1069 (Bexarotene), and Etretinate. These synthetic retinoids have been tested in several different cancers and etretinate is highly lipophilic, released slowly from the adipose tissue, and is therefore present at detectable levels in plasma for 2-3 years (Uray, et al., 2016). Dose response experiments will be conducted with each of the synthetic derivatives in each cell line to determine the lowest concentration that provides protection.

It is anticipated that protection will be noted in some of the cell lines with some of the retinoids. The data from ATRA-treated cells will provide support for our chosen LLC-PK₁ cell model. The remaining results will provide guidance for which retinoid(s) can be potentially used as an investigative tool for comparisons with ATRA.


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