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The Involvement Of Polyol Pathway In Hypgerglycemia And Cadmium Toxicity In The Establishment Of Diabetic Neprhopathy

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THE INVOLVEMENT OF POLYOL PATHWAY IN HYPGERGLYCEMIA AND CADMIUM TOXICITY IN THE ESTABLISHMENT OF DIABETIC NEPRHOPATHY

by

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This dissertation, submitted by Bethany Davis in partial fulfillment of the requirements for the Degree of Doctor of Philosophy from the University of North Dakota, has been read by the Faculty Advisory Committee under whom the work has been done and is hereby approved.

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ABSTRACT

Diabetic nephropathy (DN) is a major cause of end-stage renal disease (ESRD), where prolonged exposure to hyperglycemia induces damage to proximal tubule cells of the kidney. Since progression to ESRD correlates to pathological changes in the tubular segments of the kidney, the effects of hyperglycemia in the PT portion of the nephron may be particularly relevant to the progression of DN. Development of this disease also is likely to occur in the context of exposure to other renal toxins, and the heavy metal cadmium (Cd^{2+}) may be the most relevant due to the accumulation of this metal in the major cell type involved in glucose reabsorption: proximal tubule cells. Preliminary microarray analysis has shown human proximal tubule (HPT) cells exposed acutely and chronically to Cd^{2+} have an increased expression of an aldose reductase (AR) isoform, AKR1B10. This isoform along with AKR1B1 and sorbitol dehydrogenase (SORD) are involved in glucose metabolism under hyperglycemic conditions via the polyol pathway. The goal of this study was to verify and extend these observations in culture of HPT cells. For this purpose, HPT cells were exposed to one of the three following treatments; 5.5 (control), 7.5, 11, or 16 mM glucose concentrations for 8 days; 9, 27, 45 μM Cd^{2+} for 24 hours (acute), or 4.5, 9, 27 μM Cd^{2+} for 13 days (chronic). Real-time PCR was used to measure the expression level of these enzymes. Exposures to either hyperglycemia or Cd^{2+} stimulated a significant induction of AKR1B10 in HPT cells; however, exposure to

these renal toxins had no effect on AKR1B1 or SORD expression. We also observed glucose-induced loss of epithelial morphology that correlated to an induction of N-Cadherin (CDH2), a mesenchymal marker. These results are suggestive of potential synergistic effects of Cd^{2+} and hyperglycemia in the toxic responses of the proximal tubules during the development of DN.

CHAPTER I.I

INTRODUCTION

Impact

Diabetes mellitus is one of the most prevailing pathologies in today's society affecting more than 25.8 million people in the United States, making it the 7th leading cause of death (American Diabetes Association; Yu & Bonventre, 2018). Diabetes mellitus is characterized by chronic hyperglycemia and microvascular complications in the renal glomerulus (nephropathy), retina (retinopathy), peripheral nerve (neuropathy) (M Brownlee, 2001; Sheetz & King, 2002). Diabetes mellitus is a complicated disease that affects not only the kidneys but is also known to induce vascular problems in other regions of the body that can lead to limb amputations, cardiovascular disease, blindness, etc. (American Diabetes Association ; Das & Srivastava, 1985). A high interest in mechanistic research into diabetes mellitus is sparked by the awareness of the health problems associated with this disease.

Diabetic nephropathy (DN) is one of the most serious complications of diabetes mellitus (Edwards & Prozialeck, 2009; Suarez, Thomas, Barisoni, & Fornoni, 2013; Tervaert et al., 2010). Diabetic nephropathy is a microvascular disease where prolonged exposure to hyperglycemia induces damage to various cells of the kidney, ultimately developing into chronic kidney disease (CKD) (Kanwar, Sun, Xie, Liu, & Chen, 2011). Some of the renal morphological changes that occur in diabetic nephropathy are

basement membrane thickness, accumulation of extracellular matrix proteins, glomerular and renal hypertrophy, fibrosis, a decline in glomerular filtration rate, etc. (Kanwar et al., 2011; Macisaac, Ekinci, & Jerums, 2014; Schrijvers, De Vriese, & Flyvbjerg, 2004). Once there is a detection of proteinuria, end-stage renal disease quickly follows. The mechanism(s) of diabetic nephropathy are highly complicated and poorly understood, making the disease more difficult to treat.

Some known risk factors that contribute to diabetic nephropathy have been identified such as hyperglycemia and diet. Exposure to prolonged hyperglycemic conditions can have detrimental effects on the kidney by activating metabolic and signaling pathways where downstream targets result in morphological and functional changes. These detrimental effects can lead to acute kidney injury and if not treated it can progress into a more damaging and irreversible state. Although there are several known contributing risk factors for diabetic nephropathy, there still may be unidentified environmental factors that can make an individual more susceptible to hyperglycemic damage or cause the disease to progress into a more advanced, damaging state.

With regards to environmental factors, there is a growing body of evidence to support Cd^{2+} exposure contributes to the development of chronic kidney disease but also increases the severity of hyperglycemic damage on the kidneys (Edwards & Prozialeck, 2009; Gobe & Crane, 2010; Klaassen, Liu, & Diwan, 2009; Prozialeck & Edwards, 2012; Thévenod, 2003). Therefore to properly understand diabetic nephropathy and the

mechanisms behind the disease progression, it's important to understand how these toxicants induce irreversible damage in diabetic nephropathy. Also, understanding the pathological mechanisms behind diabetic nephropathy can provide health professionals the means for an early detection and prevention of the disease, as well as its progression.

Acute and Chronic Kidney Disease

Development of kidney diseases can occur rapidly (acute) or over long periods of time (chronic). Acute kidney injury is a reversible, sudden, rapid loss of the kidneys' excretory functions that is clinically seen by the accumulation of urea (BUN) or creatinine in the blood, decreased urine output, or a combination of both which normally dissipates within a week of initial diagnosis (Basile, Anderson, & Sutton, 2012; Bellomo, Kellum, & Ronco, 2012; Levey, Levin, & Kellum, 2013). Acute kidney injury is caused by several independent events such as dehydration, surgery, use of medicines, or overnight hospital stays (Chawla, Eggers, Star, & Kimmel, 2014). Although acute kidney injury is an independent disease, however if there is not a full recovery from the acute kidney injury it will make the patient more susceptible to disease progression to chronic kidney disease.

Chronic kidney disease is defined as an irreversible, gradual loss of the kidneys' excretory functions with the presence of kidney damage (i.e. albuminuria) which is evident for three months or longer (Levey & Coresh, 2012). Renal damage that is associated with chronic kidney disease is caused by the loss of viable nephrons. Researchers have intensely studied glomerular damage in chronic kidney disease

research, because not only is this the site of glomerular filtration, but also glomerular damage is a characteristic of chronic disease. However recent studies have suggested tubular damage is also a hallmark of chronic kidney disease and an indicator of disease progression and severity (Han, Ly, Tesch, Poronnik, & Nikolic-Paterson, 2018; Schelling, 2016).

The presence of albumin in the urine is a hallmark of both acute and chronic kidney disease and can be used to determine if the state of the disease will progress to end-stage kidney disease (Astor et al., 2011; Looker et al., 2015). Early stages of chronic kidney disease show an increase of albuminuria and a decreased glomerular filtration rate (GFR) (Coresh, Astor, Greene, Eknoyan, & Levey, 2003) whereas evidence of glomerulosclerosis and renal interstitial fibrosis is an indication of severe chronic kidney disease that leads to end-stage renal disease (T. Lee, Shah, Leonard, Parikh, & Thakar, 2018). In cases of evident glomerulosclerosis, there is an increase of endothelial damage and dysfunction, activation and proliferation of smooth muscle or mesangial cells, and injury to the podocytes (A Meguid El Nahas & Bello, 2005). Communication between cells via cytokines or growth factors leads to the increased deposition of extracellular matrix that leads to scarring of the glomerulus.

Damage to the proximal tubules is also seen in chronic kidney disease. Damage to the proximal tubules is independent of the damage that occurs in the glomerulus. The proximal tubules are subjected to irreversible damage and undergo morphologic changes, such as loss of brush border and apical mitochondria followed by complete loss of tubular

epithelial cells, or whole tubules that occurs with interstitial fibrosis (Risdon, Sloper, & De Wardener, 1968; Rosenbaum, Mikail, & Wiedmann, 1967; Schelling, 2016).

As scientists are becoming more aware of the damaging effects that are associated with chronic kidney disease, there is not only a growing need to investigate the complications associated with this disease to find a treatment to halt the disease progression but also because of the increased incidence of patients inflicted with chronic kidney disease.

The number of patients diagnosed with chronic kidney disease is growing at an alarming rate and is a major concern for not only health-care professionals but also the diagnosed individuals and their care-takers. Due to the fact of the development of chronic kidney disease in diabetic patients significantly increases the mortality and morbidity rates along with health care costs world- wide (Roshan & Stanton, 2013). The health care costs are incurred to detect and manage chronic kidney disease, end-stage renal disease, and simultaneous management of comorbid conditions such as diabetes, congestive heart failure, and hypertension (V. Wang, Vilme, Maciejewski, & Boulware, 2016). Epidemiologically, there are several causes of chronic kidney disease with diabetes mellitus being the greatest contributor(Thakar, Christianson, Himmelfarb, & Leonard, 2011). Although diabetes mellitus is heavily studied, the mechanism(s) behind the disease progression to a chronic kidney disease and ultimately end-stage renal disease (ESRD) remains least understood.

Diabetic Nephropathy

Diabetic nephropathy is a serious microvascular complication of diabetes mellitus and is the leading cause of chronic kidney disease (Kanwar et al., 2011; Suarez et al., 2013; Tervaert et al., 2010). Diabetic nephropathy is characterized by the early detection of albuminuria after diagnosis of diabetes mellitus (Suarez et al., 2013). However pathophysiological changes occur after prolonged exposure, such as hypertrophied glomeruli and tubules that is associated with increased thickening of the glomerular and tubular basement membranes that results from the excessive deposition of extracellular matrix (ECM) (Fioretto & Mauer, 2007; Kanwar et al., 2011). Renal injuries that arise from diabetes mellitus whether its type 1 or type 2 result in a similar pathophysiological outcome (Fioretto & Mauer, 2007). Diabetic nephropathy is caused by prolonged exposure to hyperglycemia.

Glucose Handling by Proximal Tubule Cells

The kidney's main function is to reabsorb and transport nutrients from the glomerular filtrate back into the blood to be used by other systems. Since the proximal tubule cells are responsible for reabsorbing roughly 90% of the nutrients such as glucose, amino acids, or salts in the ultra-filtrate, it's important to understand how these cells respond to an increased level of glucose.

The proximal tubule cell reabsorbs glucose via two mechanisms; sodium-glucose linked transporter (SGLT) and facilitated glucose transporter (GLUT) (Blantz & Singh,

2014; Y. J. Lee, Lee, & Han, 2007; Vallon & Thomson, 2012). Sodium-glucose linked transporter transports glucose in an insulin independent manner and is driven by a high-flux low-affinity of glucose at the brush border of the apical membrane (Mather & Pollock, 2011). Facilitated glucose transporters are found at the apical and basolateral regions of the cell and can bi-directionally transport glucose across the cell membrane (Y. J. Lee et al., 2007) Similar to SGLTs, GLUTs are driven by the high-flux low affinity of glucose (Alsahli & Gerich, 2017; Y. J. Lee et al., 2007). Since the transport of glucose is insulin-independent, the proximal tubule cells are more susceptible to damage caused by high levels glucose, hyperglycemia.

When glucose in the filtrate reaches approximately 10 mmol/L, the SGLTs and GLUTs become saturated and the maximal reabsorption rate has been met (Poudel, 2013). Under these conditions, glucose in the urine can be detected. If the problem persists, damage to the proximal tubule cells will occur.

Mechanisms of Hyperglycemia Damage in Proximal Tubule Cells

There are several hypothesis of hyperglycemia induced damaged that have been intensely studied, as well been the main focus in clinical trials. There are several hypotheses about the mechanism as to how complications associated with diabetic nephropathy arise. One possible mechanism, activation of the polyol pathway, was shown to be the major player in complications associated with diabetic retinopathy (VAN HEYNINGEN, 1959). Several studies following the initial discovery of the role of the polyol pathway's involvement in cataract formation in diabetic retinopathy have targeted

aldose reductase activity as a means of treatment. Since microvascular complications induced by diabetes mellitus are pathophysiologically similar in the retina and kidney, investigation into how activation of the polyol pathway leads to complications in diabetic nephropathy has been intensely studied (Das & Srivastava, 1985; Fowler, 2008).

Once inside the proximal tubule cell, glucose can be metabolized by two different enzymes 1) hexokinase to form glucose-6-phosphate or 2) aldose reductase (ARK1B1) to form sorbitol (Kinoshita, 1990; Yabe-Nishimura, 1998). However hexokinase will become saturated as the glucose influx increases rendering aldose reductase the main means of glucose metabolism. This increased activity of aldose reductase leads to complications such as osmotic and oxidative stress (Michael Brownlee, 2005).

Aldose reductase is the first and rate-limiting enzyme of the polyol pathway (Hodgkinson et al., 2001). Aldose reductase belongs to a family of NADPH oxidoreductases where there are three isoforms of the AKR1B in humans; AKR1B1, AKR1B10, and AKR1B15 (Cao, Fan, & Chung, 1998; Giménez-Dejoz et al., 2017; Taskoparan et al., 2017). Aldose reductase is a cytosolic, monomeric NAPDH-dependent oxidoreductase that metabolizes a wide variety of carbonyl molecules such as sugars, aldehyde metabolites, and ketones (Bohren, Bullock, Wermuth, & Gabbay, 1989). These enzymes are more efficient at reducing aromatic and aliphatic aldehydes but in hyperglycemic conditions will metabolize glucose to sorbitol (Cao et al., 1998; Zopf et al., 2009). Sorbitol dehydrogenase (SORD) is the second enzyme of the polyol pathway

and it oxidizes sorbitol to fructose using NAD+ as a cofactor (Gabbay, 1973; Schrijvers et al., 2004).

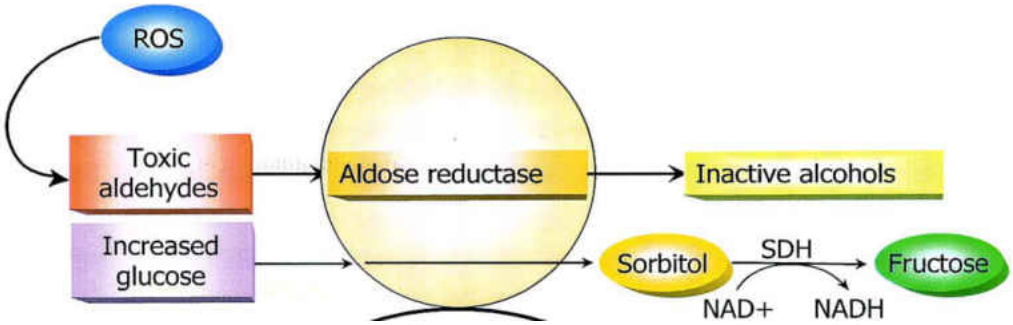

Figure 1.1 Polyol Pathway. Adapted from (Brownlee. 2004).

Evidence for the Role of the Polyol Pathway in Diabetic Complications

Since sorbitol is unable to freely cross the cell membrane it accumulates within the cell leading to complications associated with microvascular diseases such as osmotic damage. The role of aldose reductase isoform, AKR1B1, in microvascular complications with diabetes was first found out in the mid- 1950's. The early work of Dr. Van Heyningen set the stage of the role of the polyol pathway in microvascular complications associated with diabetes.

In 1974, a group from London was trying to determine why genetically diabetic mice did not experience cataracts. It was concluded that these mice (and several other mice of different backgrounds) had a low level of aldose reductase activity in the lens, but when the activity was looked at in rats, there was an appreciable high amount of aldose reductase activity in a diabetic state (Varma, Mikuni, & Kinoshita, 1975), suggesting these rats experienced cataract formation when they were clinically diagnosed

as having diabetes. After several years of intensive investigations, researchers concluded the polyol pathway activation has some role in the onset of complications associated with hyperglycemia, the generations of inhibitors targeted at aldose reductase were in popular demand.

However it wouldn't be until a decade later that researchers studied the role of the polyol pathway in complications associated with diabetic nephropathy. Researchers concluded in diabetic induced mice treated with an aldose reductase inhibitor showed decreased levels of proteinuria (Beyer-Mears, Ku, & Cohen, n.d.; Cohen, 1986). These studies were the first to relate complications of diabetic nephropathy with activation of the polyol pathway.

Shortly after Beyer-Mears study, another study looked directly at human proximal tubule cells response to hyperglycemia and if the polyol pathway was involved. This group of researchers concluded that human proximal tubule cells exposed to high levels of glucose (27.5 mM) for short periods of time had an increased accumulation of sorbitol and increased aldose reductase activity while no change in sorbitol dehydrogenase activity (Bylander & Sens, 1990). When the cells were treated with an aldose reductase inhibitor, sorbinil, there were diminished levels of sorbitol accumulation and aldose reductase activity suggesting the polyol pathway has a role in complications associated with diabetic nephropathy via intracellular accumulation of sorbitol.

Since then there have been several studies supporting aldose reductase increased activation in hyperglycemic conditions and its role in diabetic mellitus complications

(Hodgkinson et al., 2001; Rusak, Misztal, Rusak, Branska-Januszewska, & Tomasiak, 2017; Shah, Dorin, Sun, Braun, & Zager, 1997; Shukla, Pal, Sonowal, Srivastava, & Ramana, 2017).

At the time of the initial studies of the polyol pathway and hyperglycemia, little was known about the polyol pathway enzymes and their roles in diabetic complications. Researchers knew the production of sorbitol was a driving force for microvascular complications in diabetes and when aldose reductase activity was inhibited complicated ceased. Since these first investigations, there have been several other aldose reductase isoforms discovered. However, their roles in diabetic complications are least understood.

While the biological role of AKR1B10 is unknown however it has been shown to be involved in a wide variety of metabolic pathways such as retinoic acid (Gallego et al., 2006) and is over expressed in several different types of cancer such as breast, endometrial, and or lung (Huang et al., 2016; Reddy et al., 2017).

In 2014, the first study to investigate AKR1B10 involvement in complications of diabetes nephropathy was published. These researchers investigated the role of AKR1B10 in patients with type 1 diabetes mellitus but no signs of nephropathy, versus type 1diabetics with nephropathy, and healthy with no signs of type 1 or 2 diabetes. Under high glucose (20 mM) conditions there was a significant induction of AKR1B10 protein levels in samples collected from patients with diabetic nephropathy in comparison to no induction in samples collected from healthy patients (Shaw, Yang, Millward, Demaine, & Hodgkinson, 2014).

Although the work by Shaw et al., 2014 were the first to demonstrate AKR1B10 is induced by high glucose; no further research has been conducted on this enzyme's expression in the kidney of diabetic nephropathy.

Treatment for Diabetic Nephropathy

In the initial discovery of aldose reductase in the microvascular complications associated with diabetes mellitus, there was a mass generation of inhibitors as a possible treatment. These inhibitors were either the competitive or noncompetitive type. Initial studies on aldose reductase biological properties demonstrated it was sensitive to long-chain fatty acids which led to the development of the first aldose reductase inhibitor, 3,3-tetramethyleneglutaric acid (TMG) (Jedziniak & Kinoshita, 1971). TMG was shown to inhibit aldose reductase in diabetic-induced rat lens and in the process cataract formation decreased. A couple years following, the establishment of a more potent aldose reductase inhibitor was produced; alrestatin (Gabbay, Spack, Loo, Hirsch, & Ackil, 1979) In 1981, the first clinical trial was conducted with diabetic neuropathy patients but was halted due to toxic effects of the inhibitor (Tsai & Burnakis, 1993).

Several aldose reductase inhibitors have been produced and have shown to be an effective treatment in improving the complications of diabetes mellitus however due to adverse side-effects in clinical trials all but one aldose reductase inhibitor were discontinued from further trials. Epalrestat is the only aldose reductase inhibitor currently commercially available for treating complications associated with diabetes mellitus ((Grewal, Bhardwaj, Pandita, Lather, & Sekhon, 2016).

Other Contributing Factors and Diabetic Nephropathy

There are several risk factors that can lead to the disease progression of diabetes to diabetic nephropathy such as lifestyle, genetics, and environmental factors. Although several risk factors have been identified as playing a role in the disease progression to diabetic nephropathy, there are still several unidentified environmental risk factors that may also play a role in this disease progression. Up until recently, there has been more support that cadmium may have a role in the disease progression of diabetes to diabetic nephropathy.

Cadmium Toxicity Causes Proximal Tubule Cell Damage

Cadmium is a heavy metal toxicant found naturally in the environment where exposures cause serious health effects in those exposed. This is a growing concern for those in the medical fields, government agencies, and the public since exposure can occur through several sources including diet and habit, burning of fossil fuels, and industrial mining to name a few (ASTDR). Individuals who smoke cigarettes obtain the majority of their cadmium exposure via tobacco smoke as this route of entry leads to an efficient absorption from the lungs, however the lungs are not the only site of Cd^{2+} insult (Garrett, Somji, Sens, Zhang, & Sens, 2011). Cadmium toxicity has also been shown to cause damaging effects in the skeletal system and kidneys.

Sources of Cadmium Exposure

The general population is exposed to Cd^{2+} through three main sources; tobacco, food, and occupation. Cadmium exposure through inhalation of tobacco smoke occurs

because cadmium accumulates in the tobacco plant, *Nicotiana tabacum* and it generates levels of .5 to 2 μg of Cd^{2+} per cigarette (Ganguly, Levänen, Palmberg, Åkesson, & Lindén, 2018). Cadmium through this route of entry is in the form of cadmium oxide where it can be deposited in the lung tissue or absorbed into the systemic blood circulation. (Yue, 1992).

Contaminated soil is the main source of Cd^{2+} exposure in the population of non-smokers. Agricultural and industrial activities have contributed to the alarming increase of contaminated soil. Therefore whole grains, leafy vegetables, root vegetables and some seeds contain high amounts Cd^{2+} and account for 80% dietary source (Ganguly et al., 2018).

The final source of Cd^{2+} toxicity is through occupational exposure. This typically occurs in industrial factories such as smelters, battery manufacturing, metal recovery factories, or production units for paint and pigment (Nair, DeGheselle, Smeets, Van Kerkhove, & Cuypers, 2013).

As researchers and health officials recognize the possible effects Cd^{2+} toxicity can have on the human body, the more researchers have studied the damaging effects of this toxicant.

Potential Mechanisms for Cadmium Cellular Damage

Cadmium enters the body through the lungs or the gastrointestinal tract through tobacco smoke or food intake, respectively. Although the exact mechanism which cells uptake Cd^{2+} is unknown, it has been theorized that Cd^{2+}uses similar transport systems as other metals such as calcium, iron, zinc, and manganese due to their similar size (Clarkson, 1993; Satarug, 2018). These transport systems include divalent metal transporter 1 (DMT1), ZIP8, and ZIP14 (Girijashanker et al., 2008; Park, Cherrington, & Klaassen, 2002). However, if there is a restriction of iron, zinc, or calcium then this will lead to a greater absorption and accumulation of Cd^{2+} (Vesey, 2010), therefore nutritional state must be taken into consideration when studying the effects of Cd^{2+} toxicity and transport.

Once Cd^{2+} is absorbed it binds to high molecular weight proteins in the blood and is transported to the liver where it binds to and stimulates metallothionein synthesis (Järup, Berglund, Elinder, Nordberg, & Vahter, 1998). Metallothioneins are low molecular weight proteins that are widely distributed in the body. These proteins have a high cysteine content which allows them to have a high affinity for metals such as zinc, cadmium, mercury, copper, lead, and iron (Klaassen et al., 2009; Sabath & Robles-Osorio, 2012) Although these metals have the capability of binding to metallothioneins, when Cd^{2+} is present it will displace other metals and bind to metallothioneins.

After Cd^{2+} is bound to the metallothionein proteins, it is then transported back into blood where it is filtered by glomerulus and reabsorbed by proximal tubule cells of

the kidney. Expression of ZIP8 transporters at the apical membrane mediates the uptake of Cd^{2+} bound metallothionein complexes (Fujishiro, Yano, Takada, Tanihara, & Himeno, 2012; Satarug, 2018; Schneider et al., 2014). Another possible mechanism of transport of Cd^{2+} bound metallothionein complexes into the kidney is by megalin whereas megalin mediates uptake of Cd^{2+} bound metallothionein via endocytosis (Wolff, Abouhamed, Verroust, & Thévenod, 2006). After the Cd^{2+} bound metallothionein complexes are within the cell, they are degraded by endosomal and lysosomal mechanisms with the release of Cd^{2+}causing programmed cell death. Since Cd^{2+} has a half-life of 10 – 30 years (Friberg, 1984; Järup et al., 2000) it easily accumulates within the proximal tubule cells rendering it more susceptible to cadmium toxicity (Edwards & Prozialeck, 2009).

The proximal tubule cells have a Cd^{2+} threshold of 150- 200 ug/g, exposure to anything of above these levels leads to polyuria, proteinuria and glucosuria (Järup et al., 2000; Satarug, Haswell-Elkins, & Moore, 2000). As Cd^{2+} overloads the proximal tubule cells system, it can exert its toxic effects by activating pathways of the inflammatory response, oxidative stress, and genotoxicity.

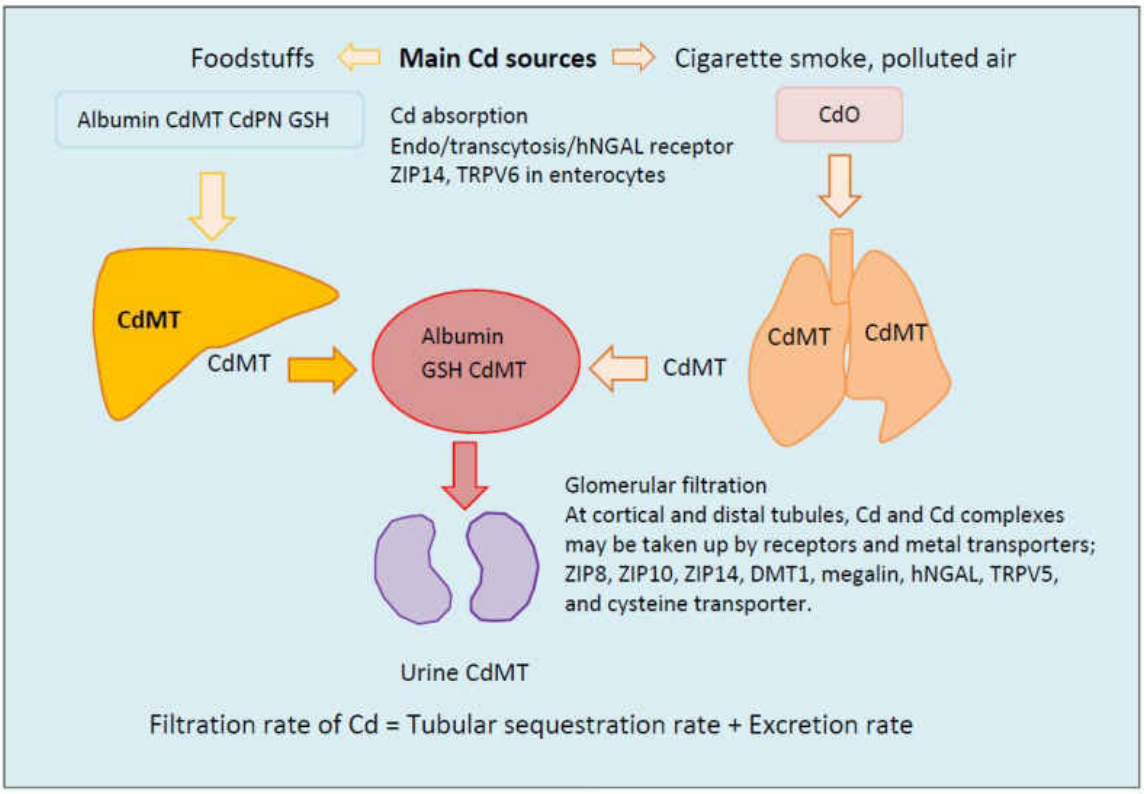

Figure 1.2 Schematic diagram of Cd^{2+} uptake, transport and urinary excretion. Adapted from (Satarug S. 2018).

The first evidence of Cd^{2+} toxicity arose in 1955 in Japan where residents were exposed to low concentrations of Cd^{2+} through ingestion of contaminated rice developed

an outbreak of Itai-Itai disease, which translates to “it hurts – it hurts”. These exposed individuals were found to have renal tubular damage, osteomalacia, anemia and malabsorption (Nordberg, 2009). In another study, conducted specifically on middle-aged women in Sweden, found a connection between Cd^{2+} accumulation and renal injury. In this study the maximal Cd^{2+} accumulation individuals experienced, impaired tubular reabsorption, as evident by the presence of proximal tubule proteins in urine i.e. N-acetyl-β-D-glucosaminidase (Akesson et al., 2005).

Due to the health risks associated with Cd^{2+} exposure, regulations were created to prevent and monitor excessive toxicant contamination and exposure.

Regulation of Cadmium Exposure

As the toxic effects of Cd^{2+} exposure are becoming more evident, guidelines were established by federal health agencies. Occupational safety and health administration (OSHA) established set limits of Cd^{2+} exposure in the workplace. According to their guidelines, the permissible exposure limit is 5 μg/ m^3 and National Institute of Occupational Safety and Health (NIOSH) has set an immediately dangerous to life and health level (IDLH) of 9mg/m^3 (OSHA, 2012; NIOSH; 2012).

Cadmium exposure standards have also been set to protect the general population by several different government agencies. The U.S. Food and Drug Administration (FDA) has a set a .0005 mg/L of Cd^{2+} in bottled water, ASTDR has a set Cd^{2+} intake of

.1 µg/kg/day, EPA has set a Cd^{2+} dose from food at .001 mg/kg/day and .0005 mg/kg/day from water (ASTDR, 2013).

When regulations fail to exist for toxicants that are known to have adverse health effects, it can lead major health problems in the general population.

Cadmium and Diabetic Nephropathy

Evidence suggests early Cd^{2+} exposure may have a contributing factor to the onset and severity of diabetes mellitus. Although, there is contradictory evidence of the relationship between cadmium exposure and the risk of developing diabetes mellitus.

Studies have shown elevated cadmium exposure was associated with a higher BMI, waist, and hip circumference in teenage females (Kim et al., 2015). These findings were supported by a study conducted on the indigenous people of the Torres Strait Islands. 32% of women between the ages of 30 and 50 had detectable levels of cadmium in their urine, 2 µg of cadmium per gram of creatinine (Haswell-Elkins et al., 2007) where normal levels are .26 µg of cadmium per gram of creatinine (ATSDR, 2013).

In 2003, Schwartz et al. analyzed data from the National Health and Nutrition Examination Survey (NHANES) III (1988-1994) that involved 8772 adults, had shown increasing urinary cadmium levels were associated with an impaired fasting glucose (defined by ≥100 and < 126 mg/dl) and increased prevalence of type II diabetes (Schwartz, Il'yasova, & Ivanova, 2003).

Similarly, Wallia et al. analyzed data from the National Health and Nutrition Examination Survey from 2005 to 2010 on 2398 patients 40 or older. Patients with a

higher exposure to cadmium were more susceptible to diabetes mellitus. Also, in individuals that were heavy smokers were more at a higher risk for diabetes mellitus (Wallia, Allen, Badon, & El Muayed, 2014).

In another study conducted on a Thailand population exposed to cadmium contaminated food sources, individuals were initially tested for renal function, hypertension, diabetes, and urinary stones with follow-up tests completed 5 years later. It was concluded there were significant increases in tubular damage (detected by β2-microglobulin urinary marker), urinary protein and serum creatinine, a decrease in glomerular filtration rate (Swaddiwudhipong et al., 2012). There were also significant increases in the prevalence of hypertension, diabetes mellitus, and urinary stones. However, there was no detection in individuals who reduced their Cd^{2+} exposure..

There have been several studies that investigated cadmium toxicity on animal models with or without diabetes mellitus. Many have supported the same findings as what is observed in humans. For example, Prozialeck et al. found in rats given subcutaneous injections of cadmium increased urinary expression of Kim-1 (marker for proximal tubule damage), protein levels, glucose, and creatinine (Prozialeck et al., 2007).

When observing toxicant damage from a diabetes mellitus stand-point, individuals diagnosed with type II diabetes mellitus were more prone to cadmium nephrotoxicity than those without diabetes mellitus suggestive of cadmium accumulation contributes to the development of diabetes mellitus (Afridi, Kazi, Brabazon, Naher, & Talpur, 2013).

All these studies support the hypothesis of individuals exposed to low levels of Cd^{2+} over a long duration of time, are more susceptible to the development of complications associated with diabetes mellitus and ultimately the disease progression to diabetic nephropathy. This could be due to the fact of both these toxicants target and induce damage to the proximal tubule cells of the kidney once an accumulation threshold is reached. When the system of the proximal tubule cell is reaching an overload of the amount of toxicant it can protect against and another toxicant is introduced, this will cause irreversible damage to the proximal tubule cell. It is because of this idea that at a given point in time the general population is exposed to more than one toxicant that researchers should investigate the damaging effects of a dual exposure on various organ systems.

Hypothesis and Rationale

This project tests the hypothesis that cadmium has a role in the onset of the complications associated with diabetic nephropathy through the activation of the aldose reductase isoform, AKR1B10. In this first study, the role of cadmium or high levels glucose exposure on the polyol pathway will be investigated in the human proximal tubule cell model system. Preliminary results from a global gene expression analysis revealed an induction of the aldose reductase isoform, AKR1B10, in human proximal tubule cells exposed acutely and chronically to cadmium (Garrett et al., 2010). The first aim of this project was to confirm AKR1B10 is induced by cadmium or hyperglycemia. Since AKR1B10 is a newly found isoform of aldose reductase family there is little data

on the physiological role of this enzyme so we needed to characterize the role of AKR1B10 in human proximal tubule cells exposed to the nephrotoxicants; cadmium or glucose. We also investigated if the other enzymes of the polyol pathway (aldose reductase isoforms, AKR1B1 and AKR1B15 or sorbitol dehydrogenase) were induced by cadmium or hyperglycemia in our human proximal tubule cell model.

To further characterize the role of the polyol pathway involvement in diabetic nephropathy, sorbitol accumulation will be measured within human proximal tubule cells exposed to cadmium or hyperglycemia. Aldose reductase metabolizes glucose to sorbitol therefore sorbitol accumulation is an indication of aldose reductase activity. Also sorbitol accumulation promotes osmotic stress therefore the accumulation of this osmolyte causes the proximal tubule cell's system to dysfunction ultimately leading to cellular death.

The second aim of this project was to investigate and characterize the effects of a dual exposure of both cadmium and hyperglycemia on human proximal tubule cells. This aim is designed to mimic the effect that would be seen in the general population since there are exposures to more than one nephrotoxicant at one time. Therefore the polyol pathway genes, as measured in the first aim, will be measured to determine if pre-exposure to cadmium toxicity will augment the effects seen in a hyperglycemia situation. Although there are several studies that explored the effects of cadmium or glucose toxicity in human diseases, no study has investigated these nephrotoxicants in an isolated situation to look at the effects of their combined exposure on the human proximal tubule cells.

The final aim of this study investigated the expression of glucose transporters and glucose utilization in the human proximal tubule cells. To ensure the current model is best suited for investigating the effects of cadmium and hyperglycemia toxicity, we characterized the uptake of glucose via glucose clearance from the media as well as measured the expression of glucose transporters in these cells.

CHAPTER I.II

METHODS

Cell Culture

There were two types of proximal tubule cells used in this study. One type was the primary proximal tubule cells or known as human proximal tubule (HPT) cells. They were isolated from healthy, normal sections of a human kidney. The other proximal cell type used was an immortalized cell called human telomerase reverse transcriptase (RPTEC/TERT1 (ATCC CRL-4031)) renal proximal tubule epithelial cells (RPTEC), referred to as RPTEC/TERT1 (ATCC CRL-4031) cells. These epithelial cells were immortalized with the human telomerase reverse transcriptase gene using the pLXSN vector, allowing these cells to have an infinite life-span.

HPT were grown using serum-free conditions and collagen coated culture flasks as described previously (Detrisac et al., 1984; Kim et al., 2002). Stock cultures were fed growth media comprised of a 1:1 mixtures of Dulbecco's modified eagle's medium (DME) and Ham's F-12 growth medium supplemented with selenium (5 ng/ml), insulin (5 µg/ml), transferrin (5 µg/ml), hydrocortisone (36 ng/ml), triiodothyronine (4 pg/ml) and epidermal growth factor (10 ng/ml). The cells were fed fresh-growth medium every 2 days and were subcultured 1:2 at confluency (~8 days post subculture) using trypsin-EDTA (.05% - .02%). RPTEC/TERT1 (ATCC CRL-4031) cells were obtained from the American Type Culture Collection, expanded according to recommended culture conditions, and aliquots were stored in liquid nitrogen.

For the glucose studies, glucose was added to growth media that contained a 1:1 mixture of a glucose free DME and Ham's-F12 such that the final glucose concentrations in the media were: 5.5, 7.5, 11, 16, 27.5, 36, 100 mM. Unless otherwise mentioned the culture media had the same concentration of all aforementioned growth factors.

RNA isolation and RT-qPCR

Total RNA was purified from cultures of HPT or RPTEC/TERT1 (ATCC CRL-4031) cells utilizing TRI Reagent (Molecular Research Center, Inc.) and quantified by spectrophotometry (Nanodrop). 100 ng of total RNA was subjected to cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad) for a final volume of 20 μL. Gene expression was measured with real-time reverse transcription polymerase chain reaction (RT-PCR) using primers of interest (see Table 1.2.1). Real time PCR was performed using SYBR Green (Bio-Rad) with 2 μL cDNA and .2 μM primers in a final volume of 20 μL in the CFX96 Touch Real-Time detection system (Bio-Rad). SYBR Green fluorescence was used to monitor the amplification of the reaction and further analyzed by interpolation from a standard curve.

Gene	Company	Catalog No.
AKR1B1	Qiagen	QT00088648
AKR1B10	Qiagen	QT000668695
SORD	Biorad	qHsaCED0044950
TXNIP	Biorad	qHsaCED0043730

Table 1.2.1 Primer Information

Protein Isolation

For the glucose studies, total protein was isolated from HPT cells using a 2% Sodium Dodecyl Sulfate (SDS) solution containing 50 mM Tris-HCL, pH 6.8 with 1% protease inhibitor cocktail (Sigma-Aldrich). Cell pellets were homogenized in the lysis buffer, boiled for 10 minutes and subjected to DNA shearing by sonication. Protein concentration was determined by BCA assay (Pierce). Stock samples were stored at -80ºC.

For the cadmium and glucose studies with RPTEC/TERT1 (ATCC CRL-4031) cells, total protein was isolated from the HPT and RPTEC/TERT1 (ATCC CRL-4031) cells using a RIPA lysis buffer containing protease inhibitors, PMSF, and sodium orthovandate (Santa Cruz). Cell pellets were homogenized in cold RIPA lysis buffer at a 1:2 w/v ratio and incubated on ice, with orbital shaking for 30 minutes. The samples were then sonicated and centrifuged at 10,000g for 10 minutes at 4°C. The supernatant was collected in cold, separate tubes and subjected to BCA assay (Pierce) for protein quantification and stored -80ºC.

Western Blot Analysis

Protein expression was measured by Western blot techniques. Twenty µg protein was separated by SDS-Page using the TGX AnyKd SDS polyacrylamide gel (Bio-Rad). Samples contained equal amounts of protein and Laemmli buffer (Bio-Rad) containing β-mercaptoethanol. The samples were then boiled for 5 minutes at 95ºC to reduce protein. Once samples were back to room temperature, they were loaded into the gel. Proteins were then transferred to a .2 µm PVDF membrane using a Trans-blot Turbo transfer apparatus (Bio-Rad). After transfer, the membranes were blocked with 5% nonfat dry milk in TBS-T for 90 minutes. Blots were incubated in 4ºC in the primary antibodies overnight on an orbital shaker. Table 1.2.1 has a list of all antibodies.

Antibody Name	Mw (kDa)	Dilution	Species	Company	Catalog No.
AKR1B10	36	1:1000	Rabbit	Abcam	Ab192865
AKR1B1	36	1:1000	Rabbit	Abcam	Ab62795
BETA ACTIN	42	1:3000	Mouse	Abcam	Ab8226
TXNIP	44	1:1000	Rabbit	Abcam	Ab188865

Table 1.2.2 Antibody source.

Glucose Utilization

Glucose utilization by HPT and RPTEC/TERT1 (ATCC CRL-4031) cells exposed to 5.5, 7.75, 11, 16mM glucose was measured. Glucose utilization was measured using a glucose colorimetric assay kit II (Biovision). Briefly, media was collected 48

hours post-feed from HPT cells fed 5.5, 7.75, 11, 16 mM of glucose and frozen in -20°C. The media was thawed at room temperature where 300 µL of the collected media was centrifuged in a 10 kDa spin cut-off filter (Millipore). The flow through was collected and used to measure glucose utilization. A standard curve was prepared using a 1 nmol/µL glucose standard to generate 0, 2, 4, 6, 8, and 10 nmol/well; bringing the final volume to 50 µL using the supplied assay buffer. A 50 µL/well glucose reaction mix was prepared with the following components: 46 µL Glucose Assay Buffer, 2 µL Glucose Enzyme, 2 µL Glucose substrate, according to supplier's protocol. Reagents and samples were added to a 96 well plate and incubated for 30 minutes at 37°C, protected from light. The absorbance was measured at 450 nm on Biotek FLx800 plate reader.

Sorbitol Assay

Sorbitol accumulation in the HPT and RPTEC/TERT1 (ATCC CRL-4031) cells was measured using a D-sorbitol colorimetric assay kit (Biovision). Briefly, HPT cells exposed to 5.5, 7.75, 11, 16 mM glucose concentrations were harvested in nuclease-free water and stored at -80°C until used. HPT cells were thawed at room temperature, subjected to brief sonication, and centrifuged at 4°C at 13,200 rpm for 10 minutes. Supernatant was used for sorbitol accumulation measurements. A standard curve was prepared using a 1 nmol/µL sorbitol standard to generate 0, 2, 4, 6, 8, and 10 nmol/well; bringing the final volume to 50 µL using the supplied assay buffer. A 50 µL/well glucose reaction mix was prepared with the following components: 36 µL sorbitol assay buffer, 2 µL sorbitol Enzyme, 2 µL sorbitol probe, 10 µL sorbitol developer, according to

supplier's protocol. Reagents and samples were added to a 96 well plate and incubated for 30 minutes at 37°C, protected from light. The absorbance was measured at 570 nm on Biotek FLx800 plate reader.

Cell Proliferation Assay

Cell viability and proliferation was measured in HPT and RPTEC/TERT1 (ATCC CRL-4031) cells exposed to 5.5, 7.75, 11, 16 mM glucose using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma Aldrich, MO). For cell proliferation, confluent cultures were split at a seeding density of ~20,000 cells per well into 6 well plates. Starting on day 2 post-seed, cells were treated with the different glucose concentrations. Day 3 post-seed, 40 µL of MTT reagent (5 mg/ml) was added to each well. One well containing no cells but media with MTT reagent was included in the analysis for the control. Plates were incubated for 3.5 hours at 37°C, followed by multiple (3x) PBS washes. 1 ml of acidic propanol was added to each well and placed on orbital shaker for 5 minutes, covered in tinfoil. Each well was mixed well then 200 µL of sample was added to a 96 well assay plate (flat bottom), done in triplicate. The optical density was immediately measured at 570 nm on a BioTek FLx800 plate reader (BioTek Instruments INC, Winooski, VT). The final optical density readings were subtracted from blank and used to calculate cell proliferation. Values were read every day for 12 days.

For cell viability measurements, confluent cultures were split at a seeding density of 300,000 cells per well into 6 well plates. Once confluent and doming, cells were then

exposed to the various concentrations of glucose. Day 2 post-treatment (24 hour glucose exposure), completed the MTT cell viability assay similar to the protocol for cell proliferation.

Transient Knockdown of AKR1B10

AKR1B10 expression was silenced in HPT and RPTEC/TERT1 (ATCC CRL-4031) using a siRNA (Qiagen) and lipofectamine methods. Cells were seeded at ~800,000 cells per well in a 6-well plate. 24 hours post-seed, a solution containing a siRNA for AKR1B10 and TransIT-X2 transfection reagent in serum- free medium was added to each well for final concentration of 20 µM and 50 nM, respectively (siRNA:TransIT complexes formed). 250 µL siRNA and transfection solution was added to each well in a drop-wise fashion. The plate was gently rocked back-and-forth and from side-to-side to evenly distribute the siRNA:TransIT complexes. The plates were incubated for 48 hours prior to treating with 5.5, 7.5, 11, or 16 mM glucose concentrations for 24 hours.

Statistical Analysis

All experiments were performed either in triplicate or duplicate and the results were expressed as the standard error of the mean. Data sets were subjected to statistical analyses using GraphPad Prism® software. Statistical analyses performed was separate

variance t-tests, ANOVA with Tukey or Dunnett's post-hoc testing. The level of significance was *p<.05, **p<.01, ***p<.001.

Microarray Analysis

Gene expression analysis was performed by Biomedical Genomics Center at the University of Minnesota (Minneapolis, MN). Isolation of total RNA was completed using a Qiagen RNeasy Mini Kit (Qiagen, CA) according to manufacturer's instructions. A total of 100 ng of RNA was used for Genome wide expression which was determined using a Human HT-12v4 Expression BeadChip array (Illumina, CA) (discontinued in 2016). Briefly, RNA was amplified and biotin-labeled using a labeling kit recommended by Illumina. The labeled RNA (cRNA) was quantitated using a Quant-iT RiboGreen RNA assay kit. cRNA was normalized and hybridized to the Illumina HT-12v4.0 BeadChips. Probes are then detected using Cy3-Streptavidin for differential detection of signals. Images from the BeadChips are then analyzed using Illumina's GenomeStudio Gene Expression Module. The data output was analyzed for differentially expressed genes by the Bioinformatics core facility at the University of North Dakota School of Medicine and Health Science. Differentially expressed probe sets (DEGs) were identified using empirical Bayes method and p-values adjusted using false discovery rate. R programming language and SAS JMPH software was used to analyze data.

CHAPTER I.III

RESULTS

Expression of AKR1B1, AKR1B10, SORD in Cd^{2+} Exposed HPT and RPTEC/TERT1 (ATCC CRL-4031) cells

Results from a global gene microarray expression analysis completed on HPT cells exposed to various concentrations of Cd^{2+} for 24 hours (acute) or 13 days (chronic) showed an induction AKR1B10. RPTEC/TERT1 (ATCC CRL-4031) were also exposed to various concentrations of Cd^{2+} for 24 hours (acute) or 13 days (chronic) and were analyzed for AKR1B1, AKR1B10, SORD expression. The results from both the HPT and RPTEC/TERT1 (ATCC CRL-4031) were compared to each other to determine if Cd^{2+} exposures have the same effect on the polyol pathway in immortalized proximal tubule cells as primary proximal tubule cells.

The expression of aldose reductase genes, AKR1B1 and AKR1B10 was measured to confirm the results of the global gene microarray of Cd^{2+} exposed HPT cells. Real-time PCR was performed in the human proximal tubule cells exposed acutely or chronically to Cd^{2+}. Both AKR1B1 and AKR1B10 expression was found to be significantly induced in HPT cells exposed to 27 and 45 μM Cd^{2+} for 24 hours (acute) (Figure 1.3.1. A, B). Acute Cd^{2+} exposures had no effect on SORD expression (Figure 1.3.2. C). Western blot analysis showed AKR1B10 protein levels were also induced by Cd^{2+} exposures whereas AKR1B1 were not (Figure 1.3.1. C, D).

Similarly, AKR1B1 and AKR1B10 expression was found to be significantly induced in HPT cells exposed to 9 and 27 μM Cd^{2+} for 13 days (chronic) (Figure 1.3.2.

A, B). Chronic Cd^{2+} exposures had no effect on SORD expression (Figure 1.3.2. C). Western blot analysis showed AKR1B10 protein levels were also induced by Cd^{2+} exposures whereas AKR1B1 were not (Figure 1.3.2. C, D).

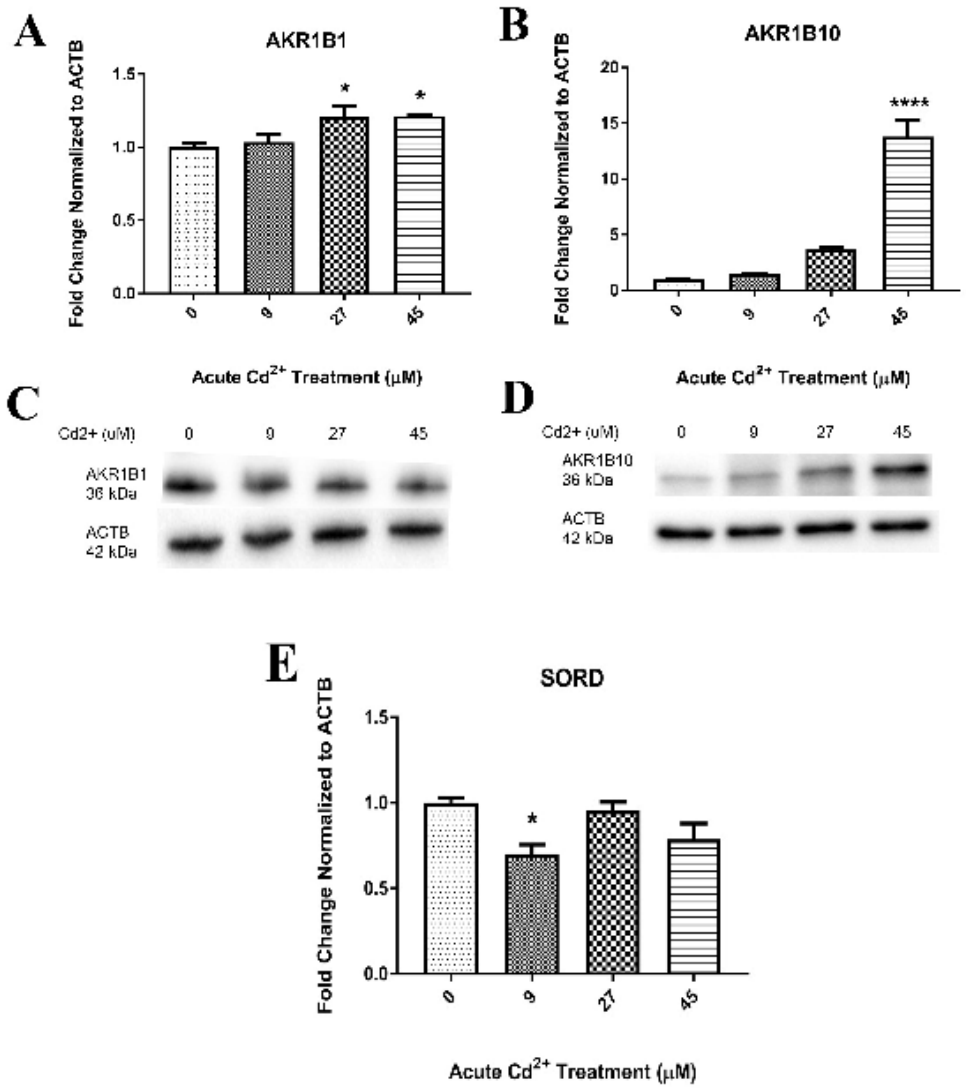

Figure 1.3.1 AKR1B1 and AKR1B10 expression is induced by acute exposures to Cd^{2+} in HPT cells. RT-PCR analysis of AKR1B1 (A), AKR1B10 (B), and SORD (E) in HPT cells exposed to Cd^{2+} for 24 hours expressed as a fold change normalized to β-actin. Western blot analysis of AKR1B1 (C) and AKR1B10 (D) proteins in HPT cells exposed to Cd^{2+} for 24 hours. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

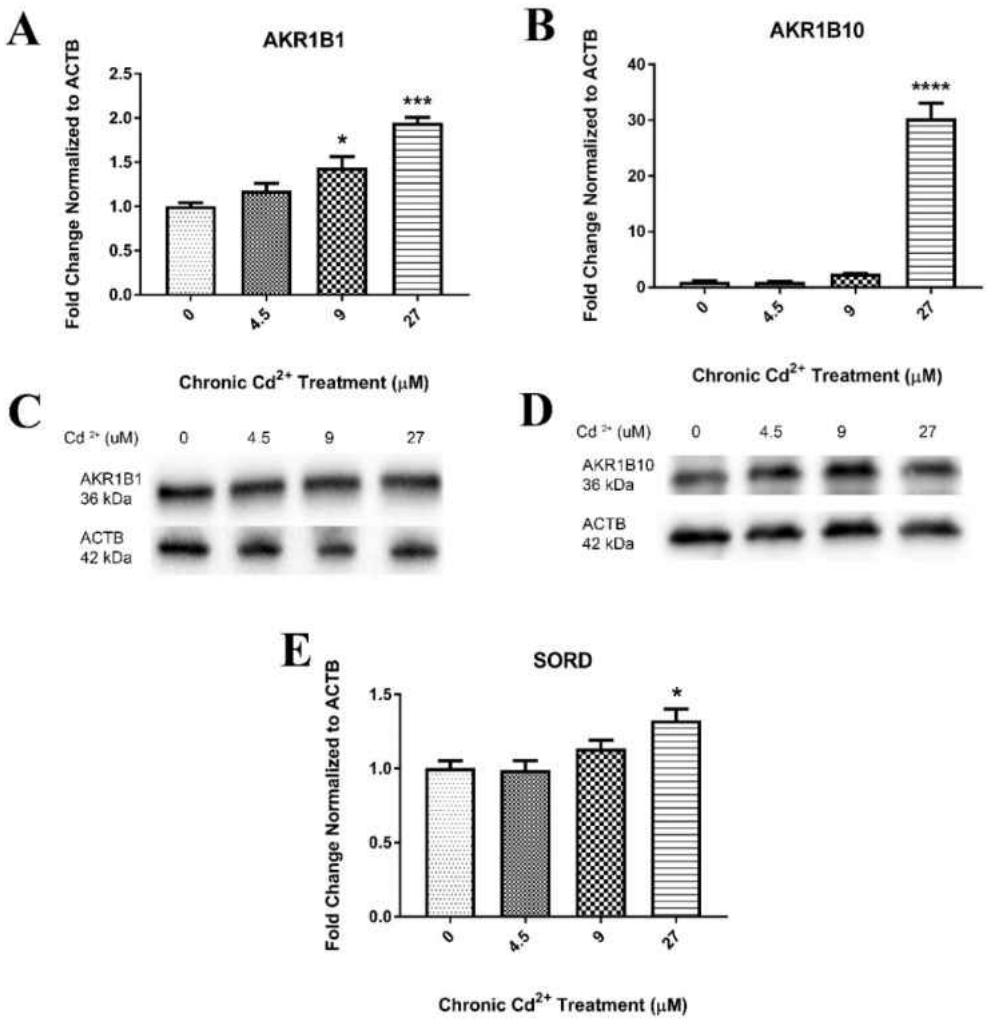

Figure 1.3.2 AKR1B1 and AKR1B10 mRNA expression is induced by chronic exposures to Cd^{2+} in HPT cells. RT-PCR analysis of AKR1B1 (A), AKR1B10 (B), and SORD (E) in HPT cells exposed to Cd^{2+} for 13 days expressed as a fold change normalized to β- actin. Western blot analysis of AKR1B1 (C), AKR1B10 (D) proteins in HPT cells exposed to Cd^{2+} for 13 days. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001.

As a means to compare and determine Cd^{2+} toxicity between a primary and an immortalized proximal tubule cell model, the expression of AKR1B1, AKR1B10, and SORD was measured in the RPTEC/TERT1 (ATCC CRL-4031) cells exposed acutely or chronically to Cd^{2+}.

Similar to the HPT cells, RPTEC/TERT1 (ATCC CRL-4031) cells showed appreciable inductions of the AKR1B10 but not AKR1B1 and SORD by Cd^{2+} toxicity (Figure 1.3.3). Cd^{2+} concentrations had to be adjusted because RPTEC/TERT1 (ATCC CRL-4031) cells were more sensitive to Cd^{2+} toxicity induced cell death.AKR1B1 and SORD expression was also unaffected by chronic exposures to Cd^{2+} toxicity but AKR1B10 is significantly induced at the mRNA levels (Figure 1.3.4 B) and there is also an increase in protein expression (Figure 1.3.4 D).

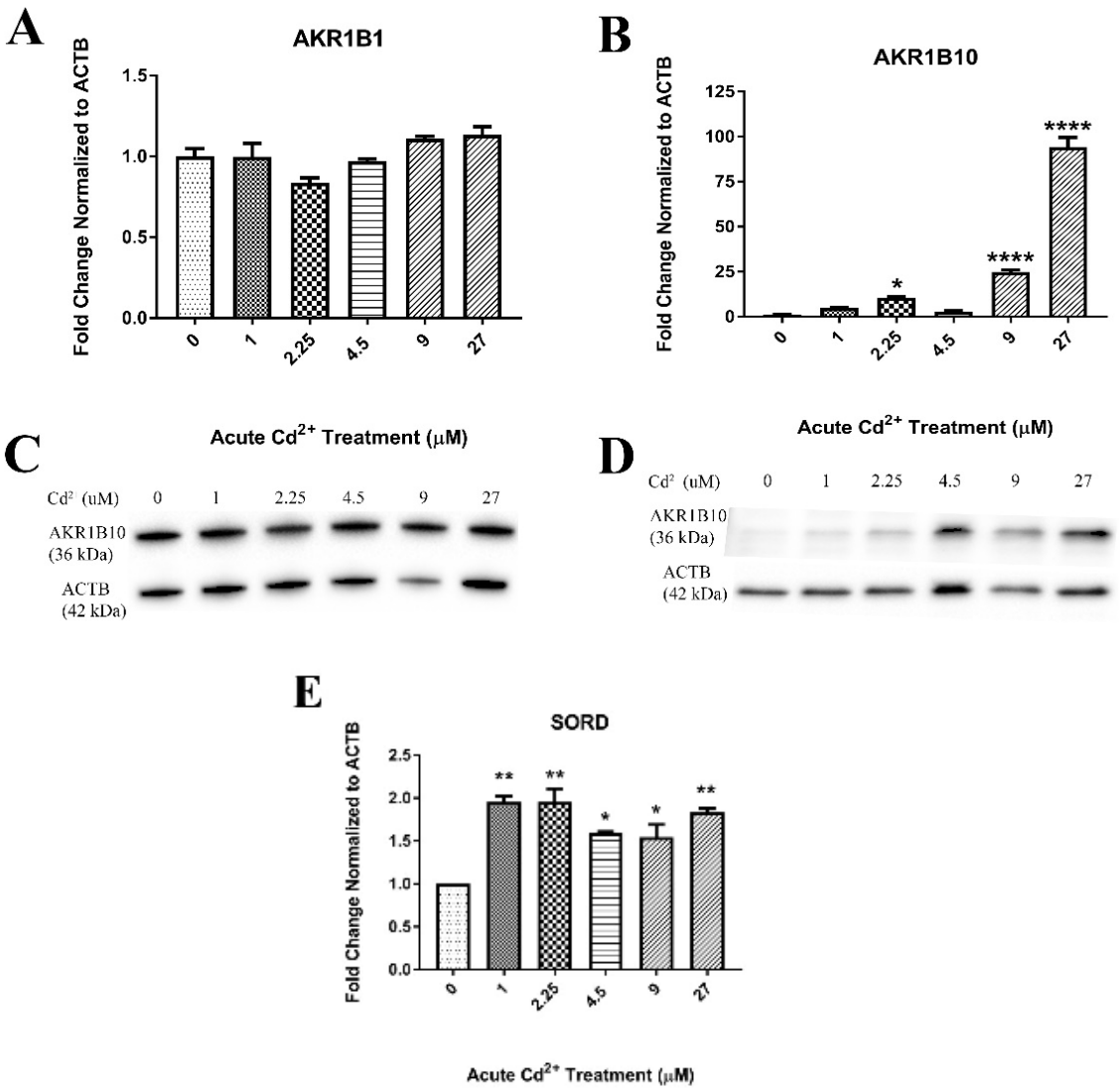

Figure 1.3.3 AKR1B1 and AKR1B10 expression is induced by acute exposures to Cd^{2+} in RPTEC/TERT1 (ATCC CRL-4031) cells. RT-PCR analysis of AKR1B1 (A), AKR1B10 (B), and SORD (C) in RPTEC/TERT1 (ATCC CRL-4031) cells exposed to Cd^{2+} for 24 hours expressed as a fold change normalized to β- actin. Western blot analysis of AKR1B1 (C) and AKR1B10 (D) in RPTEC/TERT1 (ATCC CRL-4031) cells exposed to Cd^{2+} for 24 hours. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001.

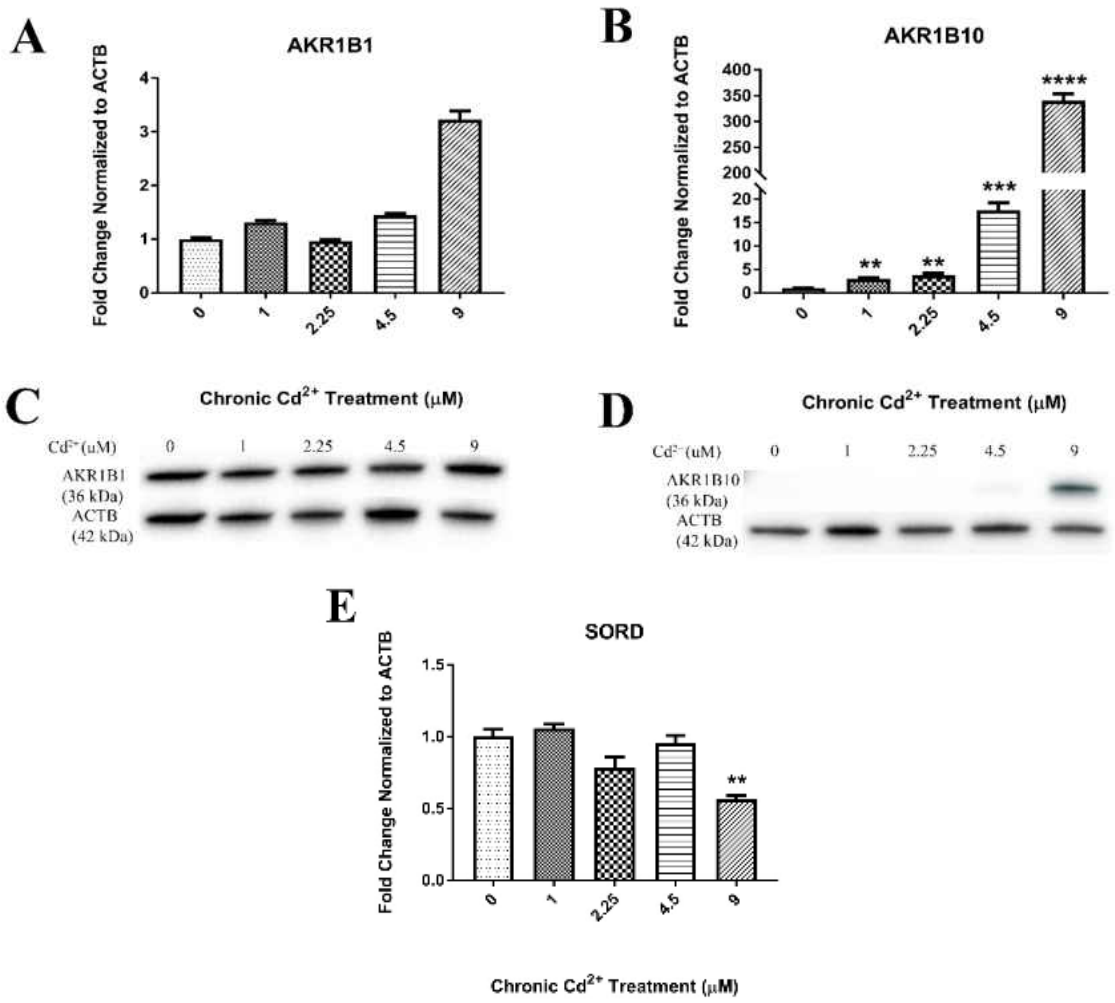

Figure 1.3.4 AKR1B1 and AKR1B10 expression is induced by chronic exposures to Cd^{2+} in RPTEC/TERT1 (ATCC CRL-4031) cells. RT-PCR analysis of AKR1B1 (A), AKR1B10 (B), and SORD (E) in RPTEC/TERT1 (ATCC CRL-4031) cells exposed to Cd^{2+} for 13 days expressed as a fold change normalized to β- actin. Western blot analysis of AKR1B1 (C) and AKR1B10 (D) proteins in RPTEC/TERT1 (ATCC CRL-4031) cells exposed to Cd^{2+} for 13 days. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001.

Since the expression of the aldose reductase enzymes was induced by Cd^{2+}, it was determined if other metals studied by this lab had an effect on these enzymes as well. Measured the expression levels of AKR1B1, AKR1B10, and SORD in HPT cells acutely exposed to 100 µM As^{3+}. Starting at 8 hours post-exposure (recovery) to 100 µM As^{3+}, there is a 2.5 fold induction of AKR1B1 and AKR1B10 with no significant induction of SORD (Figure 1.3.6 A, B, C, respectively). The induction of AKR1B1 slowly regressed back to control levels by 48 hours post-exposure. On the other hand, AKR1B10 reached maximum induction (3.5 fold induction) by 12 hours post-exposure.

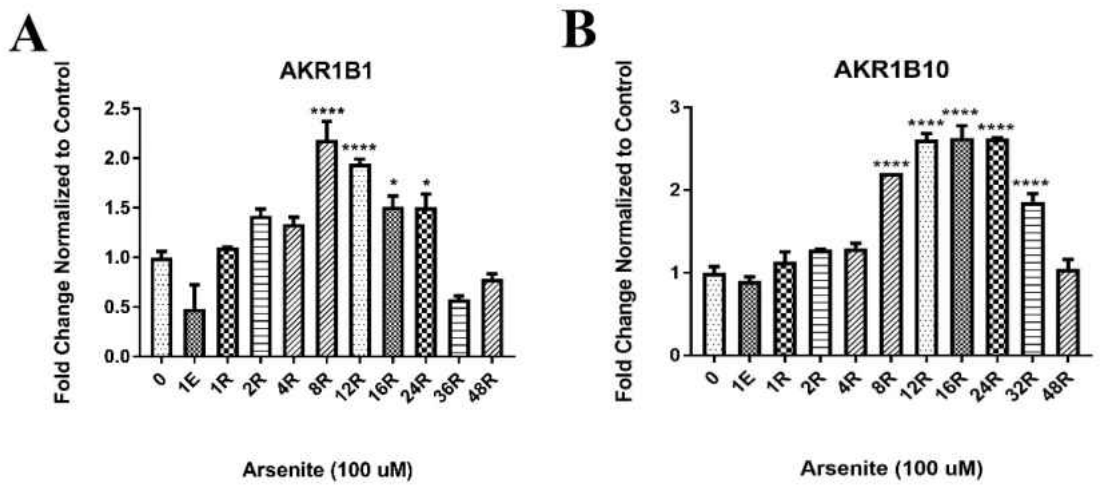

Figure 1.3.5 AKR1B10 and AKR1B10 expression is induced by acute exposures to As^{3+} in HPT cells. RT-PCR analysis of AKR1B1 (A) and AKR1B10 (B) in HPT cells exposed to As^{3+} for 4 hours expressed as a fold change normalized to β- actin. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001.

Preliminary Glucose Studies on Primary Human Proximal Tubule Cells

Since most studies investigating diabetes mellitus use non-physiological relevant concentrations of glucose in relation to the disease, a range of glucose concentrations was used from 5.5 to 100 mM. It was determined what glucose concentrations primary or immortalized proximal tubule cells can tolerate.

Initially, when trying to determine which glucose concentrations to use to study the effects of hyperglycemia on primary HPT cells a range from 7.5 to 40 mM glucose was used. Primary HPT cells exposed to the 7.5, 15, 22.5, 37.5 mM glucose for 7 days. There was no noticeable cell death when cell morphology was observed under a phase-contrast microscope. AKR1B1 expression was induced 1.4, 1.3, 2.4 fold by 15, 22.5, 37.5 mM glucose, respectively whereas AKR1B10 4.5, 2.5, 2 fold induction by 15, 22.5, 37.5 mM glucose, respectively (Figure 1.3.6 A, B). Since this was a pilot study to test the tolerance concentrations of glucose in primary HPT cells, protein analysis was not conducted.

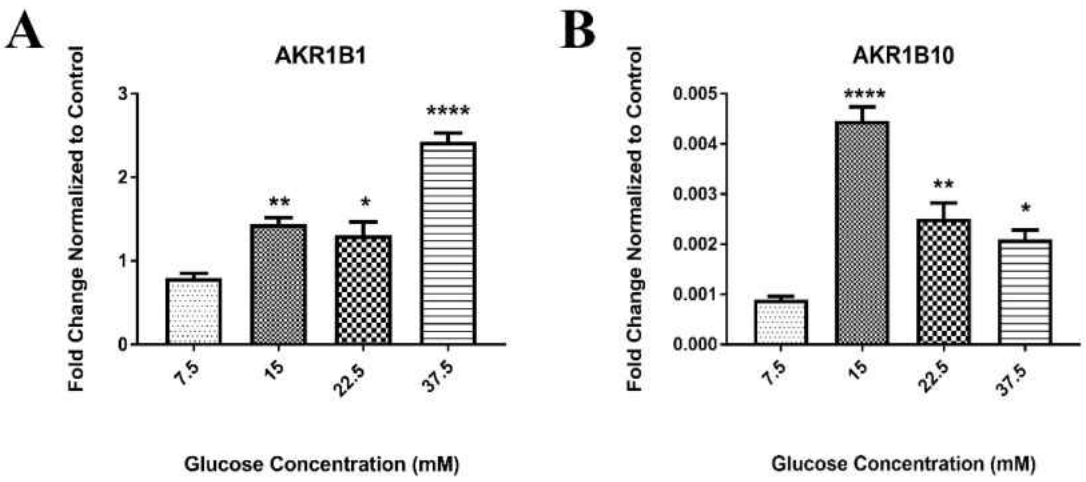

Figure 1.3.6 Preliminary results of AKR1B1 and AKR1B10 expression in HPT cells exposed to various concentrations of glucose. RT-PCR analysis of AKR1B1 (A), and AKR1B10 (B) in HPT cells exposed to 7.5, 15, 22.5, 37.5 mM glucose expressed as a fold change normalized to control. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

In another pilot study, it was determined if the feeding schedule needed to be adjusted for the optimal results. The feeding schedule of treatments is an important player in the resulting effects. Typically, cultured cells are fed every other day. It was tested how the primary or immortalized HPT cells handled a longer feeding schedule; fed every 3 days, with higher dosages of glucose.

Primary HPT cells were exposed and continuously passaged in higher glucose concentrations for 24 days. Also, the feeding schedule was changed from 2 days to 3.

Although the cells were able to handle the increased glucose concentrations, there was an increased production of lactate as seen in the color change of the media.

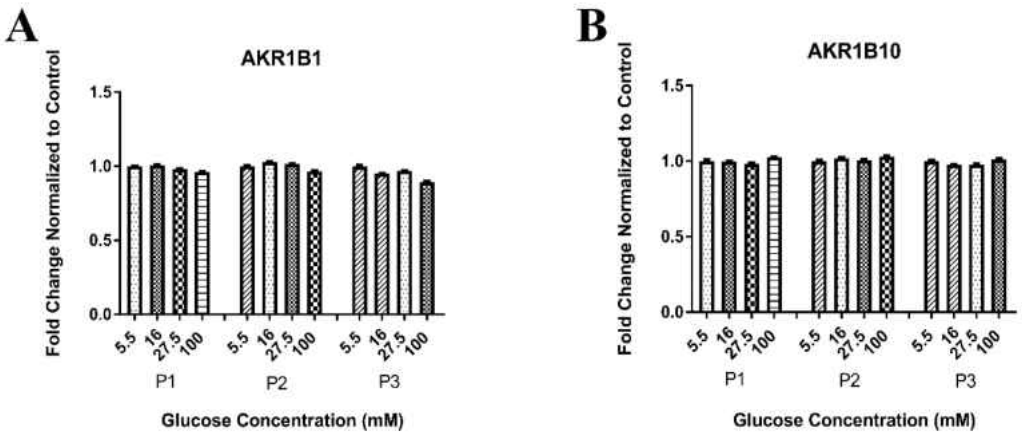

Figure 1.3.8 Preliminary results of AKR1B1 and AKR1B10 expression in RPTEC/TERT1 (ATCC CRL-4031) cells exposed to various concentrations of glucose every 3 days. RT-PCR analysis of AKR1B1 (A) and AKR1B10 (B) in RPTEC/TERT1 (ATCC CRL-4031) cells exposed to 5.5, 16, 27.5, 100 mM glucose expressed as a fold change normalized to control. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

Expression of AKR1B1, AKR1B10, SORD in High Glucose Exposed HPT and RPTEC/TERT1 (ATCC CRL-4031) cells

AKR1B10 is an isoform of the aldose reductase, AKR1B1, however the effects of hyperglycemia on this alternative isoform are not known. In order to determine if hyperglycemia induced the expression of AKR1B1, AKR1B10, and SORD in HPT and

RPTEC/TERT1 (ATCC CRL-4031) cells, confluent HPT or RPTEC/TERT1 (ATCC CRL-4031) cells were exposed to 5.5, 7.5 or 7.75, 11, or 16 mM glucose concentrations for 8 days then passaged and continuously exposed to various concentrations of glucose for another 8 days (P2). This process was repeated twice, with the cells in the final passage labeled as P3 and having a total glucose exposure of 24 days.

AKR1B1 expression is not induced by increasing glucose concentrations in primary HPT cells (Figure 1.3.9 A).

AKR1B10 expression had a 2 fold induction in 7.5 and 11 mM and a 3.5 fold inductions in HPT cells exposed to glucose for 8 days (P1). AKR1B10 expression was maximally induced in HPT cells by 11 and 16 mM glucose in a 16 day exposure (P2) and after 24 days (P3) of glucose exposure, AKR1B10 expression was only induced 2.7 fold by 16 mM glucose (Figure 1.3.9 B).

Glucose exposure had a minimal effect on SORD expression. In an 8 day (P1) exposure, 7.5, 11, 16 mM glucose induced SORD by 1.5 fold. There was no induction of SORD expression in 16 (P2) or 24 (P3) day glucose exposure (Figure 1.3.9 E).

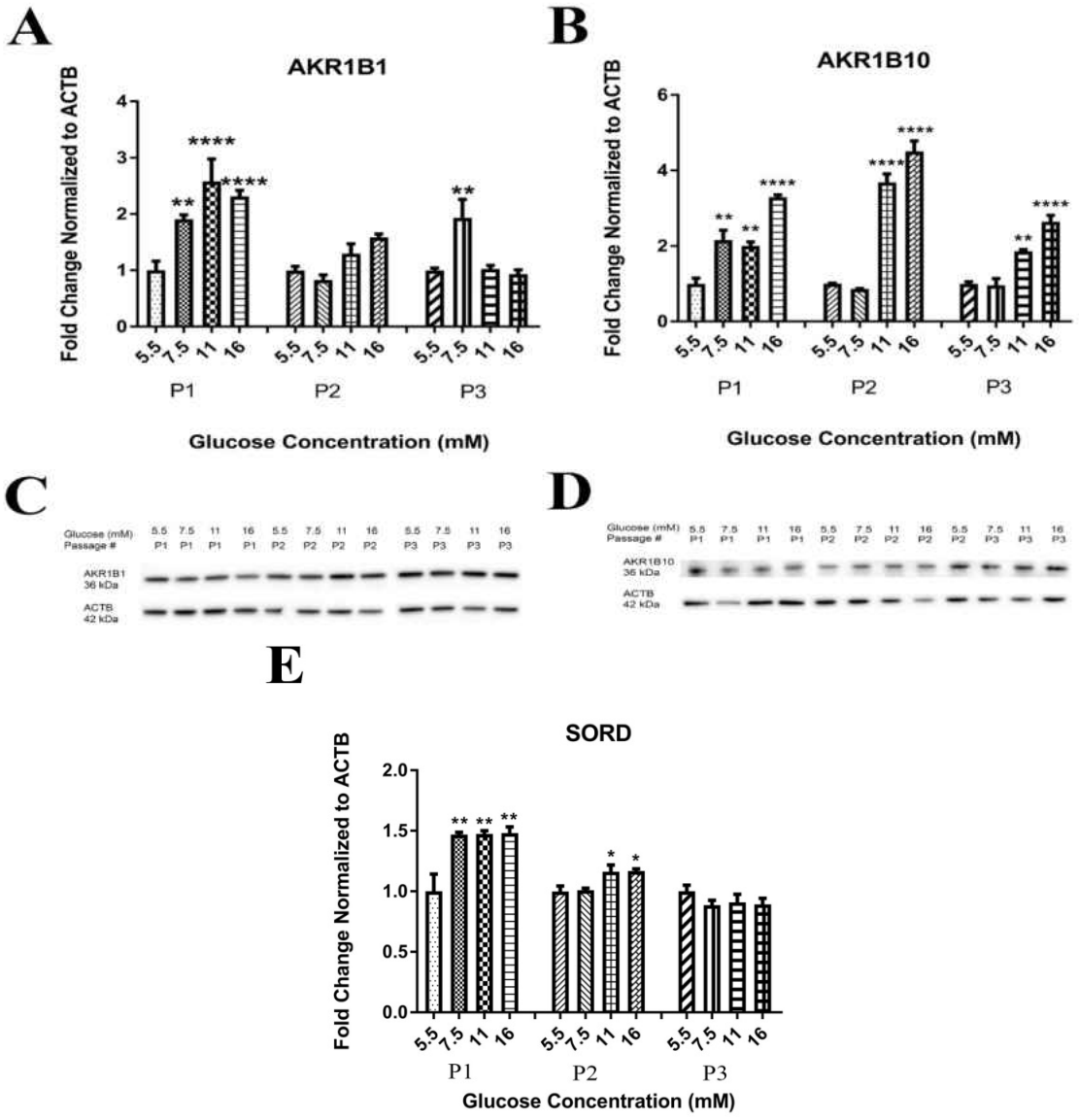

Figure 1.3.9 AKR1B1 and AKR1B10 expression is induced in HPT cells exposed and passaged in glucose. RT-PCR analysis of AKR1B1 (A), AKR1B10 (B), and SORD (C) in HPT cells exposed to 5.5, 7.5, 11, 16 mM glucose expressed as a fold change normalized to β- actin. Western blot analysis of AKR1B1 (D) and AKR1B10 (E) proteins in HPT cells exposed to glucose. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001.

The activation of the polyol pathway in response to glucose toxicity was measured and compared to the RPTEC/TERT1 (ATCC CRL-4031) cells exposed and passaged in the same concentrations of glucose as HPT cells; 5.5, 7.5, 11, 16 mM.

AKR1B1, AKR1B10, SORD expression was not induced by glucose in RPTEC/TERT1 (ATCC CRL-4031) cells (Figure 1.3.10 A, B, C).

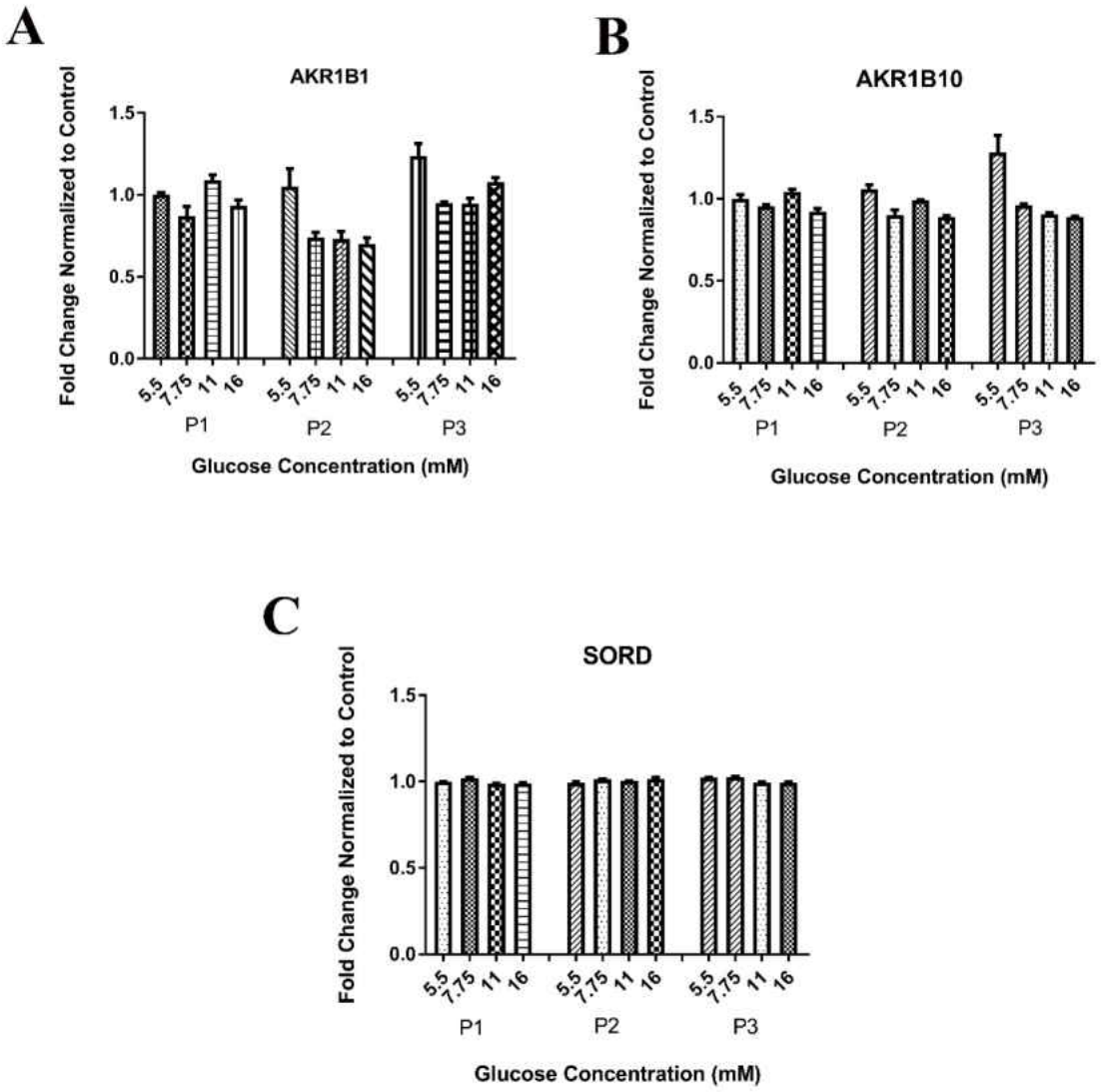

Figure 1.3.10 AKR1B1, AKR1B10, SORD expression is not induced in RPTEC/TERT1 (ATCC CRL-4031) cells exposed and passaged in glucose. RT-PCR analysis of AKR1B1 (A), AKR1B10 (B), and SORD (C) in RPTEC/TERT1 (ATCC CRL-4031) cells exposed to 5.5, 7.5, 11, 16 mM glucose expressed as a fold change normalized to control. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

Glucose utilization in HPT and RPTEC/TERT1 (ATCC CRL-4031) cells exposed to glucose

Glucose utilization was measured in HPT and RPTEC/TERT1 (ATCC CRL-4031) cells exposed to glucose for 48 hours. This measurement correlated with the glucose clearance by these cells since they were fed every other day.

Media was collected on every feed day from HPT cells exposed to 5.5, 7.5, 11, 16 mM glucose for 8 days. Figure 1.3.11 represents two different measurements of glucose utilization by the HPT cells. D2 and D7 stands for day 2 and 7 of feeding, respectively. 48 hours post feed, media was collected from the flask with HPT cells and subjected to glucose analysis by the glucose assay. These measurements are critical not only to ensure the HPT cells are metabolizing and transporting the glucose but also to estimate the amount of glucose being metabolized.

In both D2 and D7, HPT cells metabolized all of the glucose in 5.5 and 7.75 mM, 97% of the glucose in 11 mM, 87% of the glucose in 16 mM media (Figure 1.3.11). Since the earliest measurement of glucose utilization was 48 hours post-feed, a similar experiment was conducted. HPT cells were exposed to 5.5, 7.75, 11, 16, 27.5, 100 mM glucose for 24 or 48 hours and glucose utilization was measured. At both time points, nearly all the glucose had been metabolized (Figure 1.3.12 A and B).

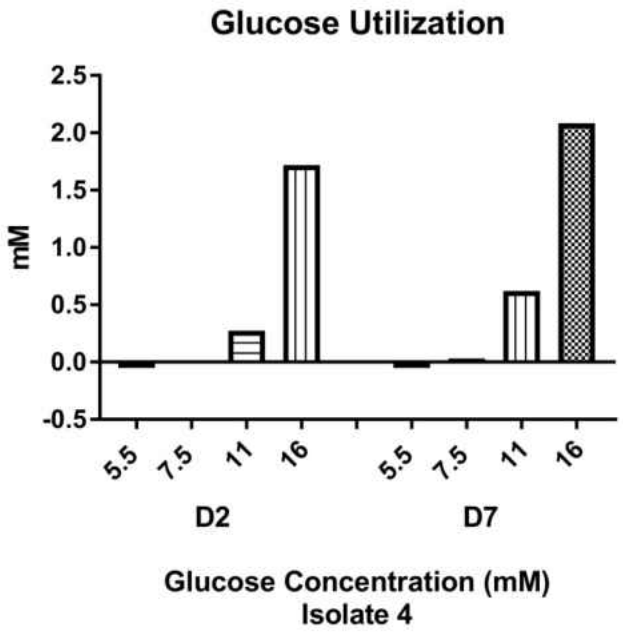

Figure 1.3.11 Glucose utilization by HPT cells exposed to glucose for 8 days. Glucose utilization in HPT cells exposed to 5.5, 7.5, 11, or 16 mM glucose for 8 days expressed as mM. Results represent ±SEM of triplicate.

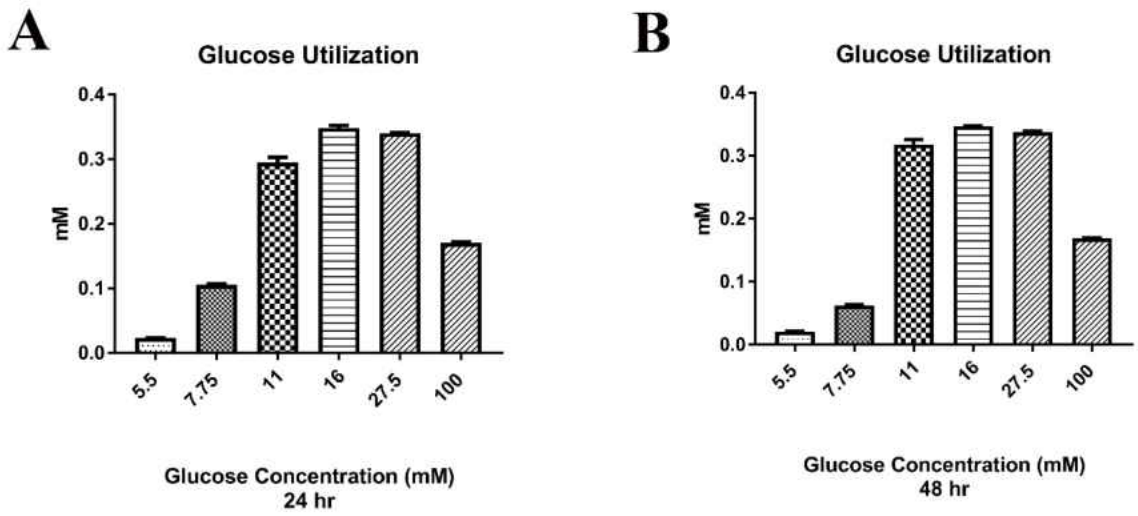

Figure 1.3.12 Glucose utilization by HPT cells exposed to glucose for 24 or 48 hours. Glucose utilization by HPT cells exposed to 5.5, 7.5, 11, 16, 27.5, 100 mM glucose for 24 (A) or 48 (B) hours expressed as mM. Results represent ±SEM of triplicate.

Intracellular sorbitol accumulation in HPT and RPTEC/TERT1 (ATCC CRL-4031) cells exposed to glucose

In a state of hyperglycemia, activation of the aldose reductase enzymes of the polyol pathway leads to the production of increased intracellular sorbitol accumulation. mRNA and protein levels showed an increased expression levels of AKR1B1 and AKR1B10, intracellular sorbitol measurements were made as a means to determine enzyme activity.

Intracellular sorbitol measurements were made in primary HPT cells exposed to 5.5, 7.75, 11, 16, 27.5, 100 mM glucose for 8 days and fed every other day. There is a 3-

fold induction of intracellular sorbitol accumulation when HPT cells are exposed to 7.75 mM glucose for 8 days as compared to the control (5.5 mM) and a 10 to 25-fold induction as the glucose concentration is increased.

Sorbitol accumulation can be seen as early as 24 hours in HPT cells exposed to various concentrations of glucose. At a 24-hour exposure, HPT have a significant accumulation of sorbitol from 11 to 100 mM glucose (Figure 1.3.12 A). Similarly, at 48-hour glucose exposure, there is a significant accumulation of sorbitol starting at 11 mM glucose (Figure 1.3.12 B)

When measurements for sorbitol accumulation were taken in RPTEC/TERT1 (ATCC CRL-4031) cells exposed to the same conditions as the HPT cells, there were no sorbitol accumulation (data not shown).

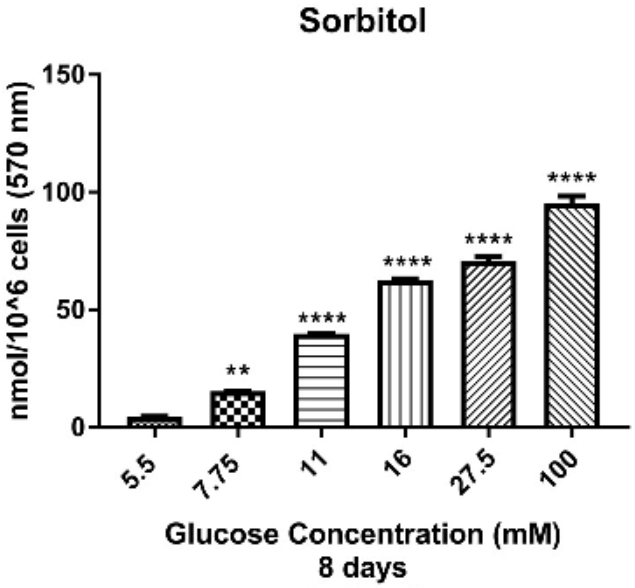

Figure 1.3.13 Intracellular sorbitol accumulation is increased in HPT cells with increased glucose concentration in an 8-day exposure. Intracellular sorbitol measurements in HPT cells exposed to 5.5, 7.5, 11, 16, 27.5, 100 mM glucose for 8 days expressed as nmol/10^6 cells. Results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

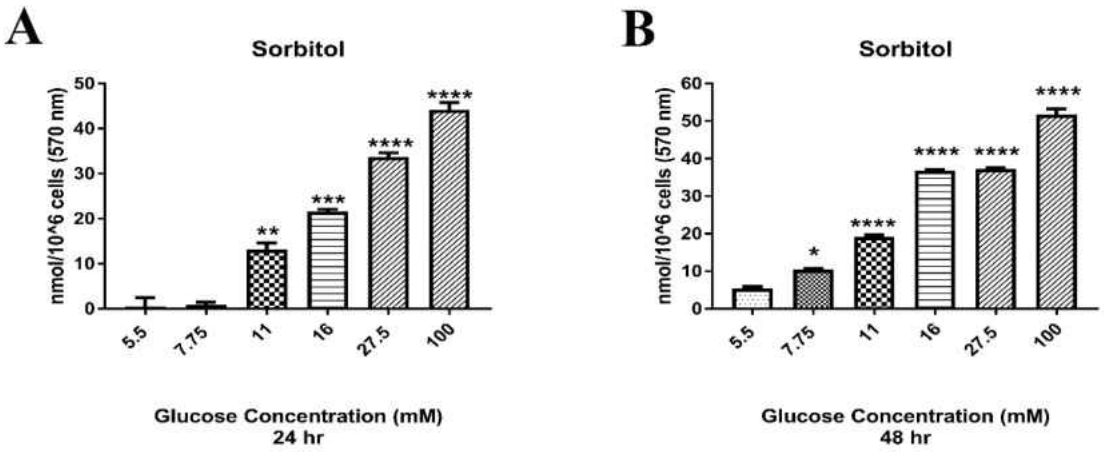

Figure 1.3.14 Intracellular sorbitol accumulation is increased in HPT cells with increased glucose concentration in 24 or 48-hour exposure. Intracellular sorbitol measurements in HPT cells exposed to 5.5, 7.5, 11, 16, 27.5, 100 mM glucose for 24 (A) or 48 (B) hours expressed as nmol/10^6 cells. Results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

Knock-down of AKR1B10 Attenuates Sorbitol Accumulation in HPT Cells Exposed to Glucose

Since AKR1B10 was more sensitive to glucose toxicity, it was determined if a transient knock-down of AKR1B10 would attenuate sorbitol accumulation seen in HPT cells exposed to glucose. Previous publications from this lab has showed knock-down of AKR1B1 by an inhibitor also showed a reduction of sorbitol accumulation but since little was known of AKR1B10 isoform and glucose toxicity, it needed to be investigated.

In HPT cells that were transiently transfected with a siRNA targeted for AKR1B10 and exposed to 5.5, 7.75, 11, 16 mM glucose for 24 hours showed a significant reduction in sorbitol accumulation (Figure 1.3.15 B).

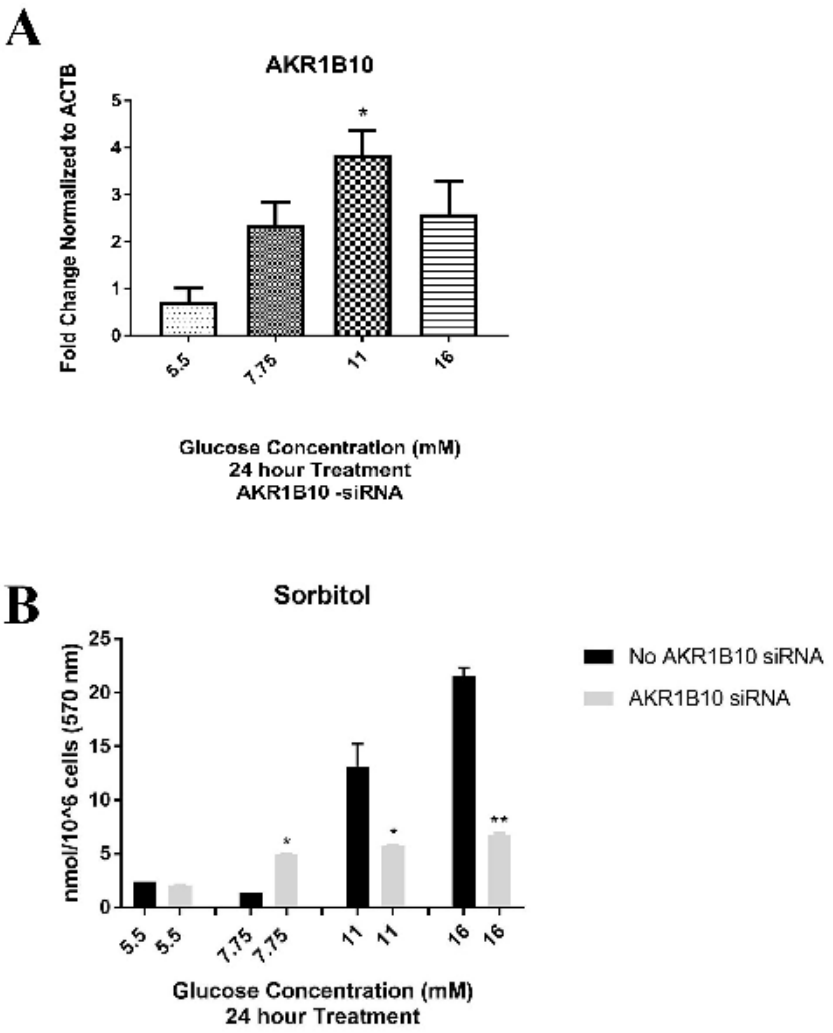

Figure 1.3.15 Intracellular sorbitol accumulation is decreased in HPT cells with AKR1B10 knock-down. RT-PCR analysis of AKR1B10 in AKR1B10 knock-down HPT cells (A) exposed to 5.5, 7.5, 11, 16 mM glucose for 24 hours expressed as a fold change normalized to ACTB. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01,

***p<0.001. Intracellular sorbitol measurements in AKR1B10 knock down HPT cells (B) exposed to 5.5, 7.5, 11, 16 mM glucose for 24 hours expressed as nmol/10^6 cells. Results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

Exposures to Hyperglycemic Conditions had no Effect on HPT Cell Proliferation or Viability

Hyperglycemia conditions had no effect on both the cell viability and proliferation. For cell proliferation, cells were seeded at a small percentage (1:100) such that they wouldn't reach a growth plateau until 8 days or later. The growing cells exposed to 5.5, 7.5, 11, 16 mM glucose had no decrease in cell growth (Figure 1.3.16). For cell viability, confluent cultures (~8 days post-seed) were treated with the same concentrations of glucose previously mentioned. There was no cell death in response to the hyperglycemic conditions (Figure 1.3.17).

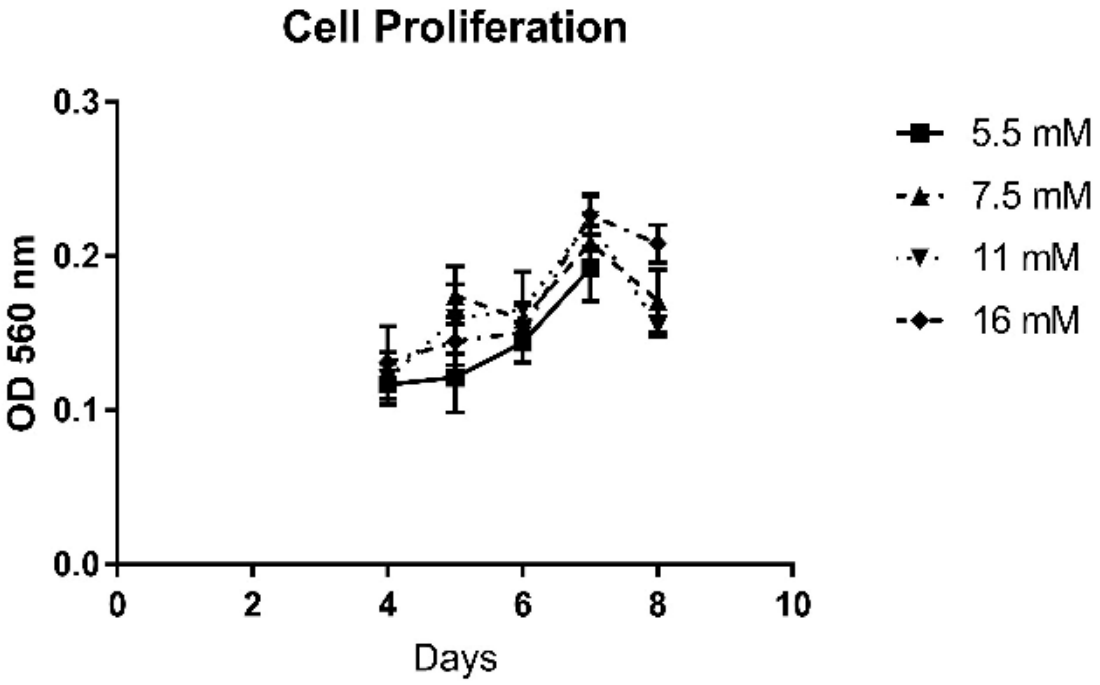

Figure 1.3.16 Cell proliferation in HPT cells exposed to hyperglycemia. Cell proliferation curves for HPT cells exposed to 5.5, 7.5, 11, 16 mM glucose for 6 days (measurements were taken starting day 4 post-seed). The glucose treatments had no effect on HPT cell proliferation.

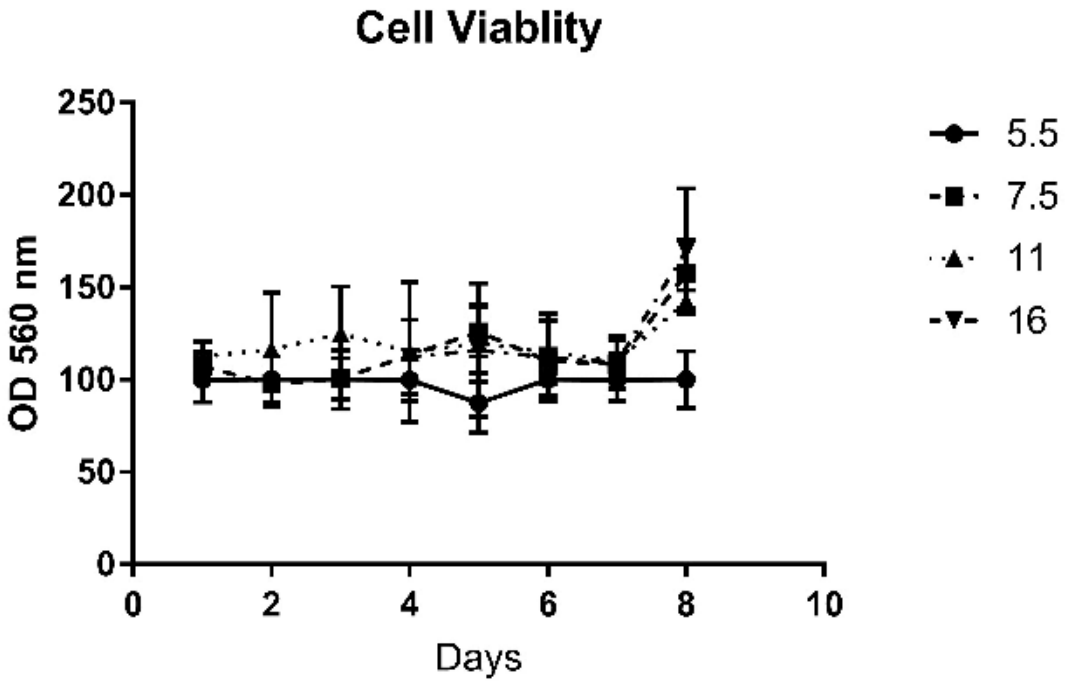

Figure 1.3.17 Cell viability in HPT cells exposed to hyperglycemia. HPT cells exposed to 5.5, 7.5, 11, or 16 mM glucose over a 8 day period had no changes in cell viability.

Glucose Transporters absent in primary and immortalized kidney cell model systems

There have been reports of increased glucose transporter expression when exposed to high glucose concentrations therefore the expression levels of the sodium coupled glucose and facilitated glucose transporters based on the effects of glucose exposures in HPT and RPTEC/TERT1 (ATCC CRL-4031) cells was measured (Rahmoune et al., 2005; Umino et al., 2018).

In both HPT and RPTEC/TERT1 (ATCC CRL-4031) cells exposed and passaged in 5.5, 7.5/7.75, 11, and 16 mM glucose showed very little to no expression of SGLT1

(Figure 1.3.18 A), SGLT2 (Figure 1.3.18 B), and GLUT2. Other kidney cell models; RPTEC/TERT1 (ATCC CRL-4031), HK2, HK2-MT3, HPT-3, HPT-4, HPT-5, and whole kidney systems were tested for the expression of these transporters. Whole kidney sample had the highest expression of all SLC5A1 and SLC5A2 transporters (Figure 1.3.18 A, B).

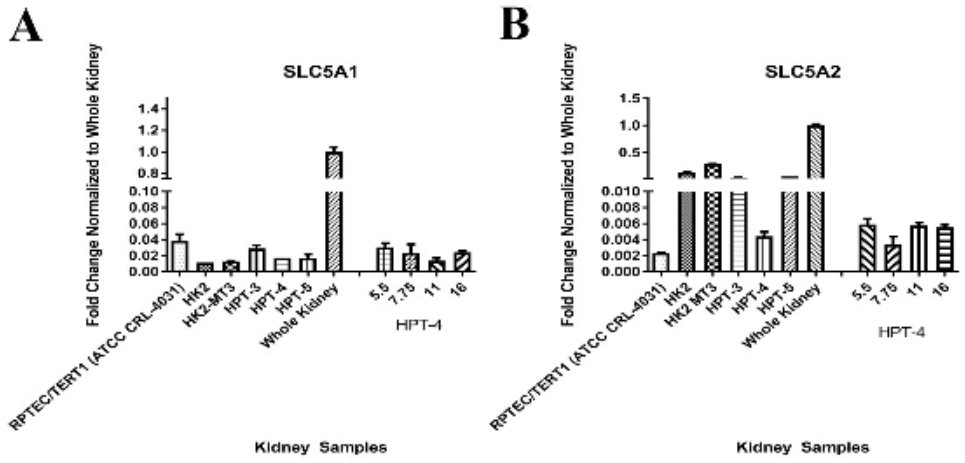

Figure 1.3.18 SLC5A1 and SLC5A2 expression in various kidney samples. RT-PCR analysis of SLC5A1 (A) and SLC5A2 (B) in various kidney samples grown in 2012 or HPT-4 cells exposed to 5.5, 7.75, 11, 16 mM glucose for 8 days. Data expressed as fold change normalized to whole kidney

The expression of GLUT2 in the same set of kidney samples as in Figure 1.3.18 is not shown because the expression was so low or not detected in some of the samples.

HPT Cells do not Elicit an Additive Response to the Combine Glucose and Cd^{2+} Exposure

Using in vitro model systems to study diseases helps to understand an isolated response that may occur due to exposures to a single toxicant or drug. Since the general population is exposed to more than one toxicant at time and there has been more support of cadmium toxicity having a role in diabetic nephropathy, the effects of a combined glucose and cadmium exposure was determined on HPT cells. RPTEC/TERT1 (ATCC CRL-4031) cells were not used for this part of the study since they didn't respond to the glucose toxicity.

HPT cells were exposed to low levels of Cd^{2+} (4.5 or 9 μM) for 72 hours then 5.5, 7.75, 11, or 16 mM glucose was added for a combined exposure of 48 hours. There are significant inductions of both AKR1B1 and AKR1B10 in HPT cells exposed separately to glucose or Cd^{2+}only. Also both AKR1B1 and AKR1B10 are induced by the combined exposure of Cd^{2+} and glucose however the response in not additive to what is seen by separate toxicant exposure (Figure 1.3.19 A and B).

Intracellular sorbitol accumulation was also measured in HPT cells exposed to both cadmium and hyperglycemia. Although there is a significant induction of sorbitol accumulation in HPT exposed to hyperglycemic conditions for 48 hours, there is not an increased accumulation of sorbitol when exposed to both cadmium and hyperglycemic conditions (Figure 1.3.20).

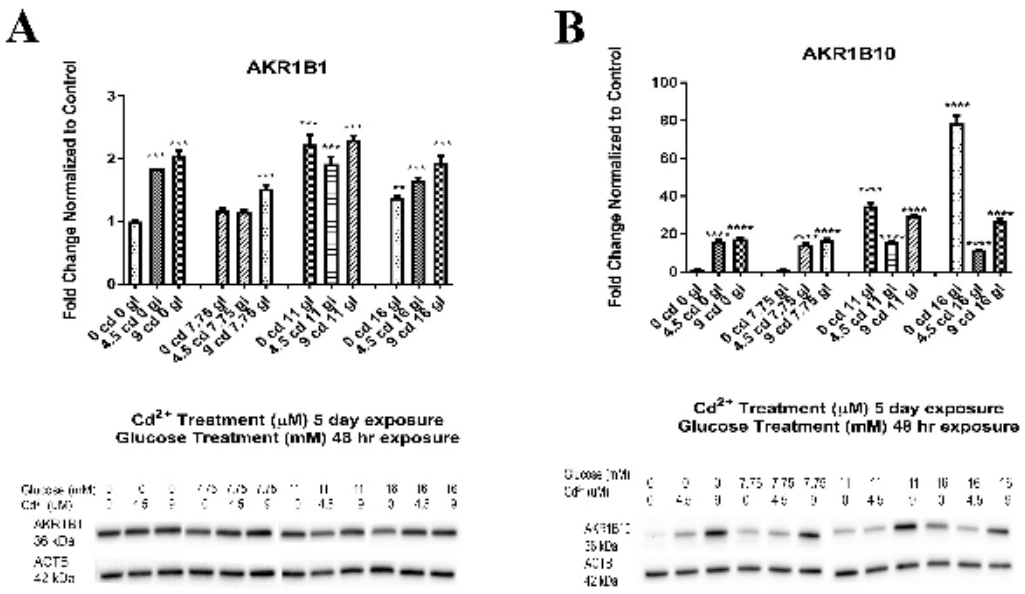

Figure 1.3.19 AKR1B1 and AKR1B10 expression is induced by combined exposures to Cd^{2+} and Glucose in HPT cells. RT-PCR analysis of AKR1B1 (A) and AKR1B10 (B) in HPT cells exposed to 4.5 or 9 µM Cd^{2+} for 72 hours then added 5.5, 7.5, 11, 16 mM glucose for a combined exposure for 48 hours. Data expressed as a fold change normalized to control. Western blot analysis of AKR1B1 (D) and AKR1B10 (E) proteins in HPT cells exposed to 4.5 or 9 µM Cd^{2+} for 72 hours then added 5.5, 7.5, 11, 16 mM glucose for a combined exposure for 48 hours. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

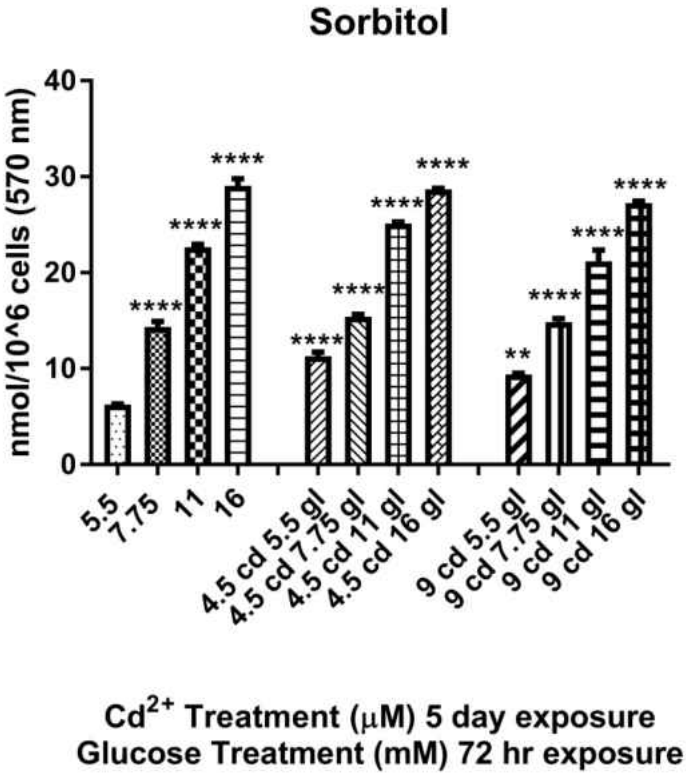

Figure 1.3.20 Intracellular sorbitol accumulation is increased in HPT cells in a combined exposure to Cd^{2+} and Glucose. Intracellular sorbitol measurements in HPT cells HPT cells exposed to 4.5 or 9 μM Cd^{2+} for 72 hours then added 5.5, 7.5, 11, 16 mM glucose for a combined exposure for 48 hours. Data expressed as nmol/10^6 cells. Results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

Global Gene Analysis on Primary Human Proximal Tubule Cells Exposed to High Glucose concentrations for 24 days (P3)

The disease etiology of diabetic nephropathy is highly complicated and not subjected to a single cause. There are numerous contributors to the onset and progression

of the disease. With this in mind, to investigate what other pathways or genes that may be activated in response to glucose toxicity in HPT cells, HPT cells that were passaged and exposed to glucose for 24 days (P3) was subjected to global gene analysis.

Results from the global microarray had shown little gene induction although, there was one gene significantly induced in the 16 mM glucose compared to 5.5 mM (control). This gene was thioredoxin-interacting protein (TXNIP). TXNIP is an oxidative stress marker that binds and inhibits thioredoxin protein promoting oxidative stress.

To confirm the microarray data, TXNIP expression was measured in HPT cells exposed and passaged in 5.5, 7.5, 11, 16 mM glucose. In the first passage, there is a 50-fold induction of TXNIP expression at the mRNA level in HPT cells exposed to 16 mM glucose for 8 days. In the later passages, TXNIP is still significantly induced by the glucose exposures just not as significant as the first passage (Figure 1.3.21).

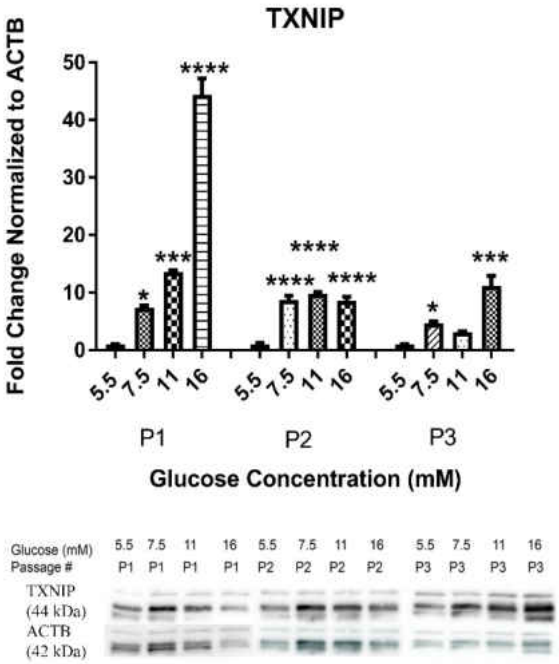

Figure 1.3.21 TXNIP expression is induced in HPT cells exposed and passaged in glucose. RT-PCRand protein analysis of TXNIP in HPT cells exposed and passaged in 5.5, 7.5, 11, 06 16 mM glucose. Data expressed as a fold change normalized to ACTB. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

CHAPTER I.IV

DISCUSSION

Establishing a Proper Model for Investigating Diabetic Nephropathy

Diabetic nephropathy is a major complication in individuals stricken with diabetes mellitus. Although there have been several pathophysiological insights there still remains a successful treatment for inhibiting the disease progression to the onset of more severe complications such as fibrosis. This is partly due to complicated nature of the disease with several contributing factors that have a role in the disease etiology. The main new findings of this study that will contribute to the diabetic nephropathy field are the roles of AKR1B10 in glucose toxicity but the role of cadmium induced toxicity in contributing to the disease onset. With the exception of one publication from Shaw et al.2014 AKR1B10 hasn't been studied in the context of diabetes nephropathy or cadmium induced diseases therefore this study will help in the development of new treatments to target AKR1B10 for both diseases. This study is also the first to investigate the effects the combined effects of cadmium and glucose toxicity and the usage of a human primary cell model system to characterize complications of diabetic nephropathy.

Most studies investigating or characterizing the pathophysiology of cell model systems in hyperglycemic conditions as a means to better understand complications associated with diabetes mellitus have used non-physiological relevant concentrations of glucose. Therefore to properly investigate the glucose handling by proximal tubule cells

of the kidney and how hyperglycemia induces damage, we had to test multiple glucose concentrations and conditions for this study.

Since diabetic nephropathy is a progressive disease, kidney cells are continuously exposed to hyperglycemic conditions for long periods of time. Therefore the duration of the hyperglycemic exposure was also addressed and manipulated such that it would properly elicit a response seen in diabetic nephropathy. With these conditions in mind, the first goal was to determine how well the HPT cells responded to a longer exposure period and also if they could tolerate being passaged in hyperglycemic conditions.

Both HPT and RPTEC/TERT1 (ATCC CRL-4031) cells were able to tolerate exposures to and passage in the hyperglycemic conditions with no cell death evident of the cell viability assay. However, prolonged exposures to the hyperglycemic conditions underwent a morphology change. These cells were more elongated and less cuboidal in shape as seen in the earlier passages. When processes like this occur, it's commonly associated with an epithelial to mesenchymal transition that is known to have a role in the onset of fibrosis that occurs in the severe disease states of diabetic nephropathy (Carew, Wang, & Kantharidis, 2012b; Grabias & Konstantopoulos, 2014). This phenomenon is addressed in more detail in the second chapter.

Although the cell model systems utilized in this study were able to tolerate the hyperglycemic conditions, they are missing or have low expression of key glucose

transporters. The proximal tubule cell is well- known of its reabsorbing and transporting properties however through the culturing process of these cells they lose their brush border. Sodium-coupled transporters which is the primary source of glucose re-uptake by these cells is located in the brush border which could explain the lack of expression in the proximal tubule cell model systems. However the lack of these transporters in proximal tubule cell model systems is not addressed in literature.

Since all proximal tubule cell model systems utilized by this lab lack these transporters, to get a better understanding of complications induced by hyperglycemic exposures, the primary HPT cells would be better model system to use. The HPT cells were more sensitive to the different glucose concentrations in comparison to the lack of response by the immortalized RPTEC/TERT1 (ATCC CRL-4031) cell model system. Also when investigating human diseases, it is ideal to use human model systems because they will elicit the closest pathophysiological response as the seen in the organ.

To have the best model to use for studying diabetic nephropathy, it would need all its physiological properties. As a future direction for this study, the HPT cells should be transfected with a sodium- coupled transporter and this study re-investigated.

Involvement of AKR1B10 in Hyperglycemic Conditions

As mentioned in the introduction, the polyol pathway has been heavily studied since its first discoveries and role in diabetic cataracts (Das & Srivastava, 1985; Shaw et

al., 2014; VAN HEYNINGEN, 1959). Findings from these earlier studies eluded to sorbitol accumulation being the driving factor for complications associated with diabetes cataracts and inhibition of aldose reductase eliminates such complications. These findings set the stage for future investigations of complications of diabetic mellitus.

Previous studies from this lab have investigated the response of HPT cells exposed to hyperglycemic conditions through the polyol pathway. Initial studies have shown HPT cells exposed to glucose concentration ≥11 mM for 24 hours have a 2-fold induction of aldose reductase, AKR1B1, activity and induction of sorbitol accumulation that is reduced to normal levels when AKR1B1 is inhibited (Bylander & Sens, 1990)The early studies and evidence for the involvement of the polyol pathway in diabetes mellitus complications became the new target for the establishment of pharmaceutical inhibitors and treatments for this disease (Dvornik et al., 1973; Hotta et al., 2006; Srivastava, Ramana, & Bhatnagar, 2005). The fact that AKR1B1 expression in HPT cells is induced within the first 8 days of exposure to hyperglycemic conditions but not the latter passages is highly suggestive of the initial involvement of this isoform in contributing to sorbitol accumulation. Whereas long-term damage that is associated prolong exposures to hyperglycemic conditions is contributed to by this other aldose reductase isoform, AKR1B10.

AKR1B1 and AKR1B10 belong to same aldose reductase family of the polyol pathway (Petrash, Flath, Sens, & Bylander, 1992). These enzymes are responsible for

metabolizing intracellular toxic carbonyl compounds in physiological conditions however in a state of hyperglycemia metabolizes the extra glucose to sorbitol rendering the system highly susceptible to damage by reactive oxygen species (Michael Brownlee, 2005; Crosas et al., 2003; Huang et al., 2016).

The results from these studies have demonstrated that prolong exposures to hyperglycemia induce the expression of AKR1B10 and knock down of this isoform significantly decreases the amount of intracellular accumulated sorbitol. Therefore, AKR1B10 has a role in the extra glucose handling associated with diabetes mellitus and could also serve as target for drug treatments. The consequences of activation of AKR1B10 hasn't been fully established in diabetic nephropathy however one can postulate there would be an increase in cellular damage due to oxidative and osmotic stress that contributes to the progression of diabetic nephropathy.

Exposure to Cadmium Toxicity Induced AKR1B10 Expression

AKR1B10 is the least understood and studied aldose reductase isoform such that there could be other factors that may lead to the activation of this enzyme. Results from a global microarray analysis conducted on HPT cells exposed acutely and chronically to cadmium showed an induction of AKR1B10 (Garrett et al., 2013, 2011). Results from the present study have confirmed the findings from the global microarray data that acute and chronic exposures to cadmium toxicity significantly induced AKR1B10 expression more so than AKR1B1 expression. Prolong exposures to cadmium toxicity is capable of

inducing several detrimental health effects. This is due to the fact cadmium is a nephrotoxicant and has a long-half such that it easily accumulates within the proximal tubule cell promoting damage, a hallmark signature of cadmium induced toxicity (Garrett et al., 2011; Satarug, Garrett, Sens, & Sens, 2009).

Although there are little to no studies that can support the findings of activation of the polyol pathway by cadmium toxicity, this study can bring forth a new area of research for understanding the involvement of environmental toxicants in contributing to the disease progression of diabetic nephropathy.

Cadmium Toxicity has a Role in Progression of Diabetes Mellitus

Epidemiological studies involving the long-term effects of cadmium exposures in the human population have educated the not only researchers and health professionals but also the general public about the adverse health effects that could occur if cadmium exposure is not limited. Some early studies from Japan recognized a correlation between cadmium exposures and increased incidence of kidney diseases and osteomalacia known as “itai-itai” diseases (AOSHIMA, 2017; EMMERSON, 1970)

There have been other epidemiological studies that showed exposures to cadmium toxicity increased the risk for diabetes mellitus however if the exposure was limited or completely eliminated that risk for the onset of diabetes mellitus was minute ((Swaddiwudhipong et al., 2012, 2015; Tinkov et al., 2017).

In light of the increasing evidence and support for the role of cadmium toxicity and the onset or progression of diabetic mellitus this current study also supports this link. Results have shown cadmium toxicity induces AKR1B10 expression and the same trend is seen in a combined exposure of both cadmium and hyperglycemia. Although the effects are not additive, both cadmium and hyperglycemia are inducing damage synergistically through the activation of AKR1B10. Therefore it can be postulated that AKR1B10 can be a marker for cadmium induced toxicity in relation to the disease progression of diabetic nephropathy.

Role of Oxidative Stress in Diabetic Nephropathy

Activation of the polyol pathway by hyperglycemic events has been shown to cause oxidative and osmotic stress that leads to the onset of complications of diabetic nephropathy. When the polyol pathway is activated it promotes to oxidative and osmotic stress in three ways; 1) decreased availability of NADPH for usage by glutathione reductase which reduces glutathione disulfide to glutathionine protecting against reactive oxygen species, 2) increased accumulation of intracellular sorbitol and 3) increased production of reactive oxygen species (M Brownlee, 2001; Burg, 1988; Srivastava et al., 2005; Tang & Lai, 2012). However results from this study also show hyperglycemic conditions induce the expression of TXNIP.

TXNIP contributes to oxidative stress and damage by inihibiting the activity of thioredoxin (Singh, 2013). Thioredoxin acts as an antioxidant utilizing NADPH to reduce

reactive oxygen species in physiological conditions. However in state of hyperglycemia thioredoxin is inhibited in two ways; 1) binding of TXNIP to the catalytic site and 2) limited availability of NADPH. Results from the global microarray analysis on HPT cells exposed and passaged in glucose for 24 days (P3) showed induction of TXNIP where expression levels were confirmed by qPCR and western blot techniques. Since the aldose reductase enzymes utilize NADPH for their reducing properties it could be argued that activation of the aldose reductase contributes to inhibition of thioredoxin promoting an increased accumulation of reactive oxygen species.

Chapter V

Conclusions/ Future Directions

Diabetic nephropathy is a complicated disease where current treatments aim to prevent the disease progression into renal fibrosis but are unable to do so. This study has shown the complexities of the disease in primary and immortalized proximal tubule cell model systems. Primary and immortalized proximal tubule cell model systems have shown to have different biological responses to exposures to hyperglycemia.

The primary or immortalized cell model system's response to hyperglycemia has some variation as evident in the data of the polyol pathway enzymes. Primary HPT cells have shown an induction of aldose reductase enzymes, AKR1B1 and AKR1B10, whereas the RPTEC/TERT1 (ATCC CRL-4031) cells have shown a little to no induction of the same enzymes. Activation of the aldose reductase enzymes have also led to an intracellular sorbitol accumulation in primary HPT cells but not in RPTEC/TERT1 (ATCC CRL-4031), another major difference between these cell model systems.

When attempting to understand human diseases it is best to start with the best model system and correct exposures of the toxicants being studied. For this project, having access to primary kidney cells have set this study apart from those that do not have access. This is because the elicited response should be almost similar to what is seen

in the whole organ. However it also best to ensure the cells used maintain their physiological properties.

The proximal tubule cells utilized in this study did not express key glucose transporters. Therefore, these glucose transporters should be transfected in the kidney cells and the study be reinvestigated although the lack of expression of these transporters did not have an effect on the glucose utilization by these kidney cells.

All in all this study sheds some light into possible avenues that could be investigated further to determine if the alternative isoform of aldose reductase, AKR1B10 can also serve as a biomarker for the disease progression of diabetic nephropathy.

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CHAPTER II

Abstract

Renal fibrosis is a major complication associated with the disease progression of diabetic nephropathy (DN) to end-stage renal disease (ESRD), where prolong exposure to hyperglycemia induces damage to proximal tubule cells of the kidney. Since progression to ESRD correlates to pathological changes in the tubular segments of the kidney, the effects of hyperglycemia in the proximal tubule portion of the nephron may be particularly relevant to the progression of DN. Epithelial to mesenchymal transition (EMT) of proximal tubule cells may play a role in the disease manifestation and progression. The goal of this study was to characterize the pathological changes that occur in human proximal tubule (HPT) cells exposed to hyperglycemia. For this purpose, cells were exposed to one of the following treatments; 5.5 (control), 7.5, 11, or 16 mM glucose concentrations for 8 days then consecutively subcultured for two more passages. Real-time PCR and western blot analysis was used to measure the expression levels of CDH2 and CDH1 at the mRNA and protein levels, respectively. Exposures to hyperglycemia induced morphological changes and stimulated a significant induction of CDH2 in HPT cells; however, exposure had no effect on CDH1 expression. Hyperglycemia also induced the expression of SNAI1 at the mRNA level. The data from the current study suggests hyperglycemic induced damage of the proximal tubule in the kidney during the development of DN occurs through an EMT phenomenon.

Introduction

There are several complications associated with diabetic nephropathy. One process that seems to be the most daunting is the onset of fibrosis. Renal fibrosis is an inevitable consequence that is seen in progressive chronic kidney diseases which occurs when the kidney is no longer able to regenerate to heal itself. Evidence of fibrosis (or scarring) leads to the loss of renal function eventually progressing into end-stage renal disease. At this point, the only form of treatment is to have the patient undergo renal dialysis or transplantation which is a major cost burden (Zeisberg & Kalluri, 2004)

Renal fibrosis is characterized as an excessive deposition of extracellular matrix (ECM) in the tubulointerstitium, glomerulus, and vasculature (Zhou & Liu, n.d.). Although the mechanism behind the irreversible damage is least understood, a common denominator exists regardless of the primary cause which is the activation of myofibroblasts. Activation of myofibroblasts occurs when the wound healing process is initially turned on in response to mediators such as cytokines or growth factors produced by injured resident epithelial cells (Efstratiadis, Divani, Katsioulis, & Vergoulas, 2009) however fibrosis occurs when this process is continuous for long periods of time.

Early studies regarding renal fibrosis has been extensively focused on glomerular cells (Danne, Spiro, & Spiro, 1993; D. Wang et al., 2015) because it was believed damage to this region of the kidney would be the driving force for other complications associated with diabetic nephropathy. However within the past decade of kidney fibrosis

investigations, researchers have begun to appreciate tubulointerstitium damage also contributes to the severity of the disease but also serve as a predictor of progression.

There are various cellular components contributing to the onset of renal fibrosis. All cell types within the kidney are known to participate in the pathogenesis of renal fibrosis however it is widely accepted the fibroblasts/myofibroblasts are the main cell type responsible for accumulation of interstitial extra-cellular matrix (Carew, Wang, & Kantharidis, 2012a; Efstratiadis et al., 2009; A.M. El Nahas, Muchaneta-Kubara, Essaway, & Soylemezoglu, 1997). Activated fibroblasts are recognized by their production of α smooth muscle actin (αSMA) (Hewitson, 2012; Zhou & Liu, n.d.), however the source behind fibroblast activation or the origin of these cells is least understood.

After several theories and speculations, a study characterizing the differences between fibroblasts and epithelium found one gene is active in the promoter region of fibroblasts but not epithelium. This gene came to be known as fibroblast specific protein 1 (FSP1) and is observed in injured regions of the tubular sections of the kidney undergoing fibrosis, indicating tubular epithelial cells were converting to fibroblast in response to injury (Strutz et al., 1995). This process of resident epithelial cells converting into cells with characteristics of fibroblasts is known as epithelial to mesenchymal transition (EMT).

There are three types of epithelial to mesenchymal transition processes. Type 1 epithelial to mesenchymal transition occurs during organogenesis where immature mesenchymal cells give rise to the mature epithelium. Type 2 epithelial to mesenchymal transition occurs during the tissue repair process such as fibrosis, resulting in myofibroblasts formation from resident epithelium to heal the injured regions of the tissue. Type 3 epithelial to mesenchymal transition results in malignant cells having the ability to metastasize and invade nearby tissue (Figure 2.1.1) (Tennakoon, Izawa, Kuwamura, & Yamate, 2015).

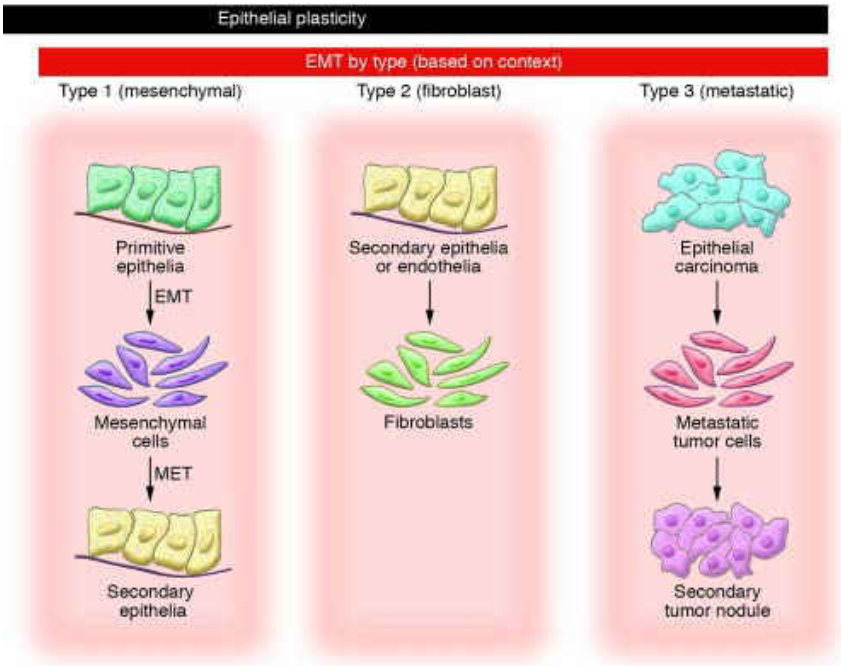

Figure 2.1. Types of epithelial to mesenchymal transition processes. Adapted from Zeisberg and Neilson, 2009.

Tubular epithelial to mesenchymal transition is defined as the process where resident renal epithelial cells lose their epithelial characteristics and obtain features of mesenchyme (Zhou & Liu, n.d.). Epithelial cells are derived from the metanephric mesenchyme in the reverse process of epithelial to mesenchymal transition, mesenchymal to epithelial transition (MET) (Hay & Zuk, 1995). During homeostasis, renal epithelial cells have distinct features that allow them to maintain the tissue's organization. For instance, renal epithelium has apical and basolateral surfaces that are easily distinguishable from each other. The apical membrane is lined with a brush border that helps to facilitate the maximal reabsorption of nutrients. The polarity of the epithelium is regulated by adherens junctions and tight junctions.

Adherens junctions are composed primarily of E-cadherin (CDH1) and maintain cell integrity by interacting with other E-cadherin proteins on neighboring cells (Gumbiner, 2005; Yonemura, Itoh, Nagafuchi, & Tsukita, 1995). Tight junctions have two primary proteins, occludins and claudins with each having separate roles in maintaining the tight junction between cells (Hartsock & Nelson, 2008). All these proteins have a profound role in maintaining organization of the cell but when there is damage to these proteins, the epithelial cell organization is compromised.

The hallmark of epithelial to mesenchymal transition is the loss of E-cadherin from the adherens junctions resulting in a dramatic remodeling of the cytoskeleton resulting in a loss of adhesion and polarity and an increase in N-cadherin (CDH2)

expression, a phenomenon called the cadherin switch (Tennakoon et al., 2015). Along with the induction of cytoskeletal markers such as α-SMA, vimentin, fibroblast specific protein -1 and β-catenin, these markers have been studied in context of Type 2 epithelial to mesenchymal transition occurring in injured renal epithelial cells (Zeisberg & Neilson, 2009).

Hypothesis/ Rationale

Primary and immortalized proximal tubule cells continuously exposed and passaged in hyperglycemia experienced a morphology change from a more cuboidal to an elongated shape. Because this characteristic is evident of an EMT process, it would be suggested EMT markers such as N/E-Cadherin, vimentin, fibronectin to name a few will be induced in HPT cells in response to exposure and passaging in hyperglycemic conditions.

CHAPTER II.II

Methods

RNA isolation and RT-qPCR

Total RNA was purified from cultures of HPT or RPTEC/TERT1 (ATCC CRL-4031) cells utilizing TRI Reagent (Molecular Research Center, Inc.) and quantified by spectrophotometry (Nanodrop). 100 ng of total RNA was subjected to cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad) for a final volume of 20 µL. Gene expression was measured with real-time reverse transcription polymerase chain reaction (RT-PCR) using primers of interest (see Table 1.2.1). Real time PCR was performed using SYBR Green (Bio-Rad) with 2 µL cDNA and .2 µM primers in a final volume of 20 µL in the CFX96 Touch Real-Time detection system (Bio-Rad). SYBR Green fluorescence was used to monitor the amplification of the reaction and further analyzed by interpolation from a standard curve.

Gene	Company	Catalog No.
ACTA2	BioRad	qHsaCID0013300
CDH1	BioRad	qHsaCED0042076
CDH2	BioRad	qHsaCID0015189
OCLN	BioRad	qHsaCED0038290
Snail1	BioRad	qHsaCED0038290
TGFB1	BIORAD	qHsaCID0017026
Twist	BioRad	qHsaCED0043959
Vimentin	BioRad	qHsaCID0012604

Table 2.2.1 Primer Information

Protein Isolation

For the glucose studies, total protein was isolated from HPT cells using a 2% Sodium Dodecyl Sulfate (SDS) lysis buffer containing 50 mM Tris-HCL, pH 6.8 with 1% protease inhibitor cocktail (Sigma-Aldrich). Cell pellets were homogenized in the lysis buffer, boiled for 10 minutes and subjected to DNA shearing by sonication. Protein concentration was determined by BCA assay (Pierce). Samples were stored at -80ºC in 100 mM dithiothreitol.

For the cadmium and glucose studies with RPTEC/TERT1 (ATCC CRL-4031) cells, total protein was isolated from the HPT and RPTEC/TERT1 (ATCC CRL-4031) cells using a RIPA lysis buffer containing protease inhibitors, PMSF, and sodium orthovandate (Santa Cruz). Cell pellets were homogenized in cold RIPA lysis buffer at a 1:2 w/v ratio and incubated on ice, with orbital shaking for 30 minutes. The samples were then sonicated and centrifuged at 10,000g for 10 minutes at 4°C. The supernatant was collected in cold, separate tubes and subjected to BCA assay (Pierce) for protein quantification and stored -80ºC.

Western Blot Analysis

Protein expression was measured by western blot techniques. Twenty µg protein was separated by SDS-Page using the TGX AnyKd SDS polyacrylamide gel (Bio-Rad).

Samples contained equal amounts of protein and Laemmli buffer (Bio-Rad) containing β-mercaptoethanol. The samples were then boiled for 5 minutes at 95ºC to reduce protein. Once samples were back to room temperature, they were loaded into the gel. Proteins were then transferred to a .2 µm PVDF membrane using a Trans-blot Turbo transfer apparatus (Bio-Rad). After transfer, the membranes were blocked with 5% nonfat dry milk in TBS-T for 90 minutes. Blots were incubated in 4ºC in the primary antibodies overnight on an orbital shaker. Table 1.2.1 has a list of all antibodies.

Antibody Name	Mw (kDa)	Dilution	Species	Company	Catalog No.
ACTA2	42	1:1000	Rabbit	Abcam	Ab5694
ACTB	42	1:3000	Mouse	Abcam	Ab8226
CDH1	120		Rabbit	Santa-Cruz	Sc-7870
CDH2	137	1:1000	Mouse	Invitrogen	333900

Table 2.2.2 Antibody source.

Transepithelial Resistance

Transepithelial resistance was measured in HPT or RPTEC/TERT1 (ATCC CRL-4031) cells exposed to hyperglycemia. HPT or RPTEC/TERT1 (ATCC CRL-4031) cells were seeded at a 2:1 ratio in triplicate onto a 24 mm-diameter cellulose ester membrane inserts (Corning). The inserts were then placed into six-well plates. On the third day post-seed, HPT or RPTEC/TERT1 (ATCC CRL-4031) cells started 5.5, 7.5, 11, 16 mM glucose treatments. On the first day of the glucose treatments, transepithelial resistance (TER) was measured every day for twelve days with the EVOM Epithelial Voltohmmeter

(World Precision Instruments, Sarasota, FL) with a STX2 electrode according to manufacturer's instructions. The resistance of a filter containing control medium but with no cells was subtracted from filters containing a monolayer of cells. The corrected resistance values were multiplied by the surface area of the filter (available for growth) to get a final unit of area resistance. Three sets of four readings were taken at four different quadrants on each filter. The morphology of the monolayer was monitored for dome formation in a separate 6-well plate without inserts. The experiment was repeated twice and the final replicate recordings were reported as the ±SEM.

CHAPTER II.III

Results

HPT Cells Passaged in Glucose Exhibit a Morphology Change

HPT cells continuously exposed and passaged in glucose for 24 days (P3) had showed signs of morphology change. These changes were captured using a light/dark phase contrast microscope. In the first 8 days (P1) of exposure, the HPT cells are still forming domes (outlined regions) and are cuboidal in shape (Figure 2.3.1). However, as these cells are continuously exposed and passaged in hyperglycemic conditions, they undergo a morphology change. These cells become more elongated (Figure 2.3.2) and not as cuboidal in shape as seen in the first passage (Figure 2.3.1).

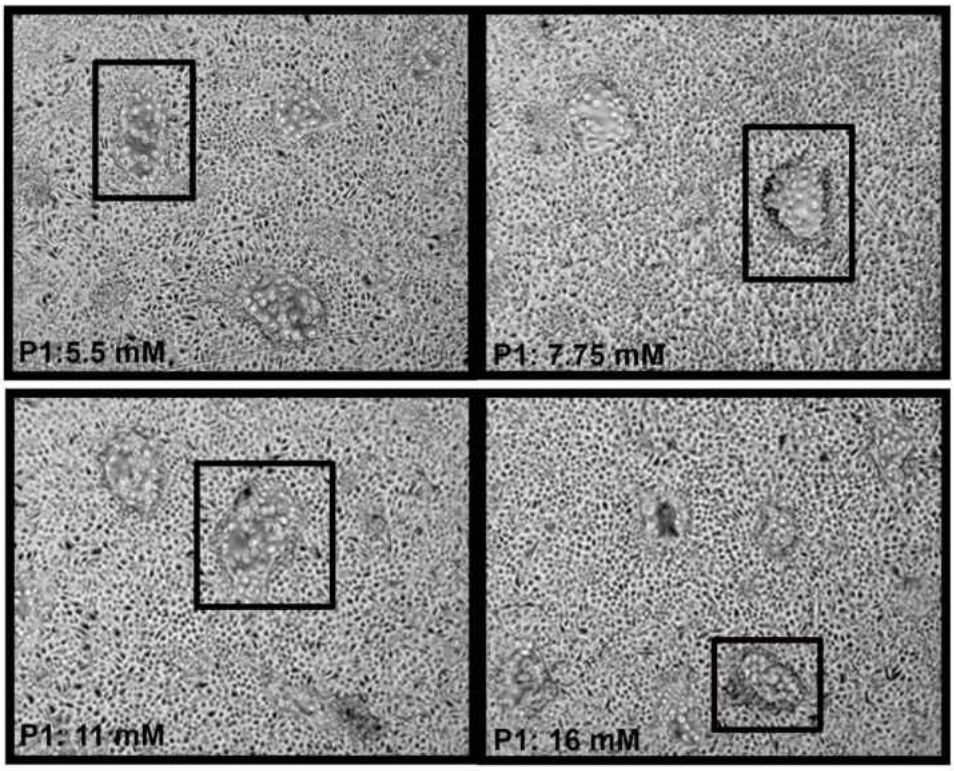

Figure 2.3.1 Light Level Morphology of HPT cells Exposed to Glucose for 8-Days. Human proximal tubule cells exposed to 5.5 mM (control), 7.5 mM, 11 mM, and 16 mM glucose for 8 days. Highlighted boxed represent regions of dome formation.

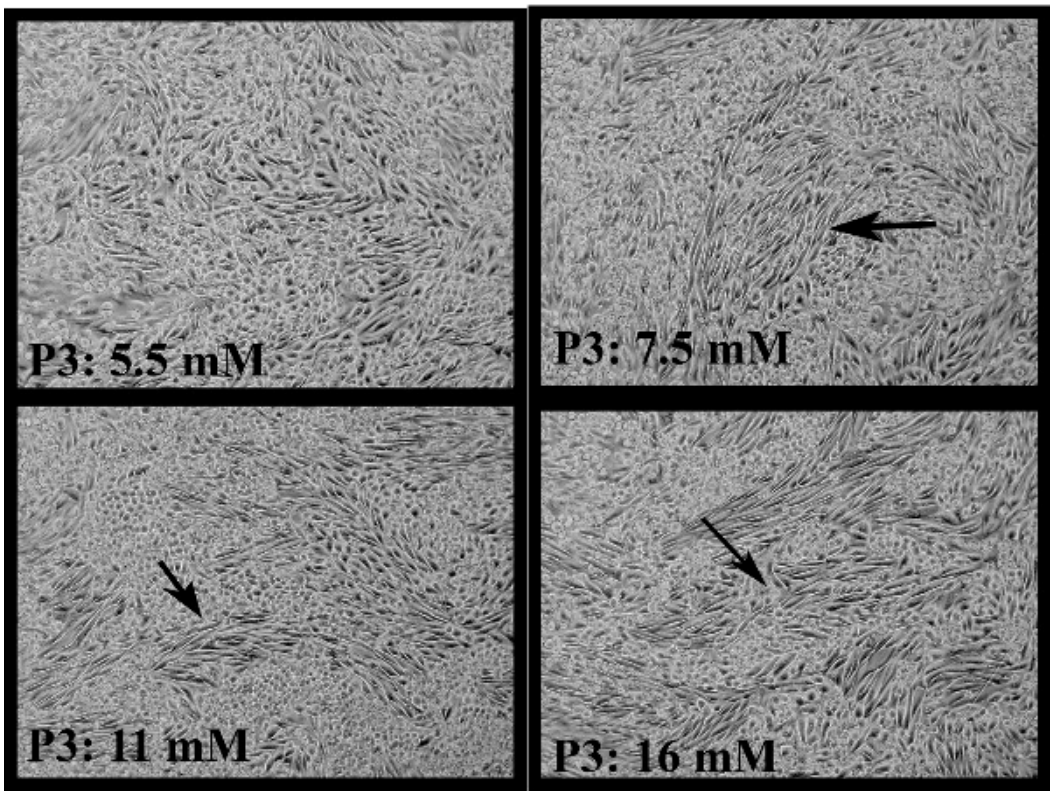

Figure 2.3.2 Light level morphology of HPT cells exposed and passaged in Glucose for 24-days. Human proximal tubule cells exposed and passaged in 5.5 mM (control), 7.5 mM, 11 mM, or 16 mM glucose for 24 days (P3). Black arrows represent regions of irregular morphology.

CDH2 Expression is Induced in HPT Cell Passaged in Glucose

Evidence from the morphology changes in response to being exposed and passaged in high concentrations of glucose is suggestive of an EMT process. This process is characterized by the E-Cadherin (CDH1) and N-Cadherin (CDH2) switch however there are other markers that when induced could indicate an EMT process occurring.

These other markers are: fibronectin (FN1), vimentin (VIM), smooth-muscle actin (ACTA2) and transcription factors: snail (SNAI1), slug (SNAI2), twist (1/2). Measured the expression of CDH1 and CDH2 in HPT cells exposed and passaged in glucose.

The expression of CDH1 is induced in the first 8 days of exposure by 16 mM glucose but then no induction in 16 day (P2) exposure and is back to being induced in 24 days (P3) exposure by 16 mM (Figure 2.3.3 A). On the other hand, CDH2 expression in continuously induced by 16 mM glucose exposure across all three passages, with a 6 to 7-fold induction in the 24 day (P3) exposure (Figure 2.3.3 B). The induced expression is translated into the protein analysis as well.

ACTA2 expression levels were shown to be affected by the hyperglycemic conditions. In the first 8 days (P1) of exposure, there is a 2-fold induction of ACTA2 by 16 mM glucose to a 10 to 15- fold induction by 16 days of exposure (P2). After 24 days (P3) of being in hyperglycemic conditions, there is steady and significant induction of ACTA2 (Figure 2.3.4). However both VIM and FN1 expression were not induced in HPT cells exposed and passaged in 5.5, 7.5, 11, 16 mM glucose (Figure 2.3.5 A, B).

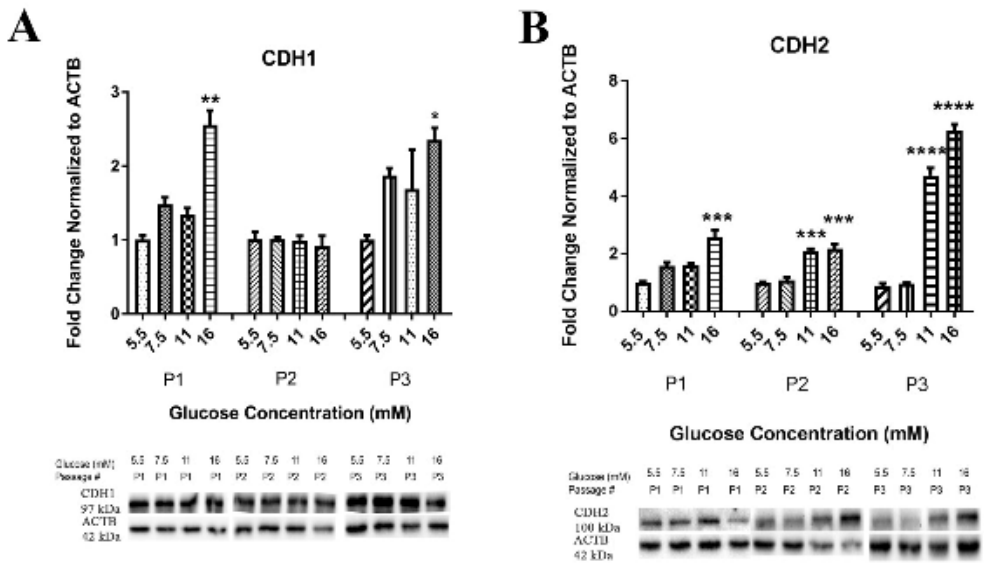

Figure 2.3.3 CDH1 and CDH2 expression in HPT cells exposed and passaged in glucose. RT-PCR and protein analysis of CDH1 (A) and CDH2 (B) in HPT cells exposed and passaged in 5.5, 7.5, 11, 16 mM glucose. Data expressed as a fold change normalized to ACTB. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

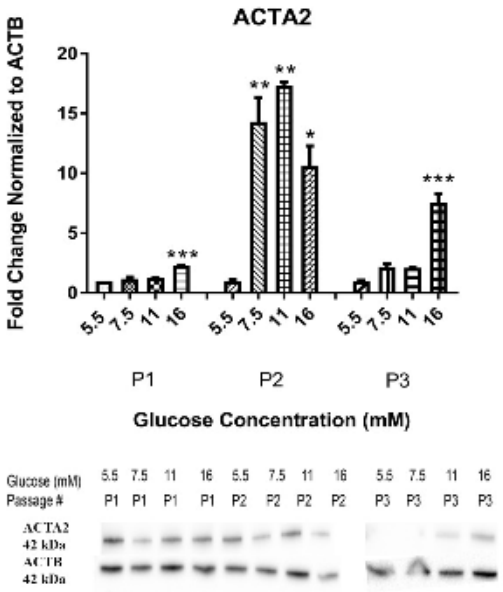

Figure 2.3.4 ACTA2 mRNA expression in HPT cells exposed and passaged in glucose. RT-PCR and protein analysis of ACTA2 in HPT cells exposed and passaged in 5.5, 7.5, 11, 16 mM glucose. Data expressed as a fold change normalized to ACTB. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001Junctions Proteins are not Affected by Glucose Toxicity

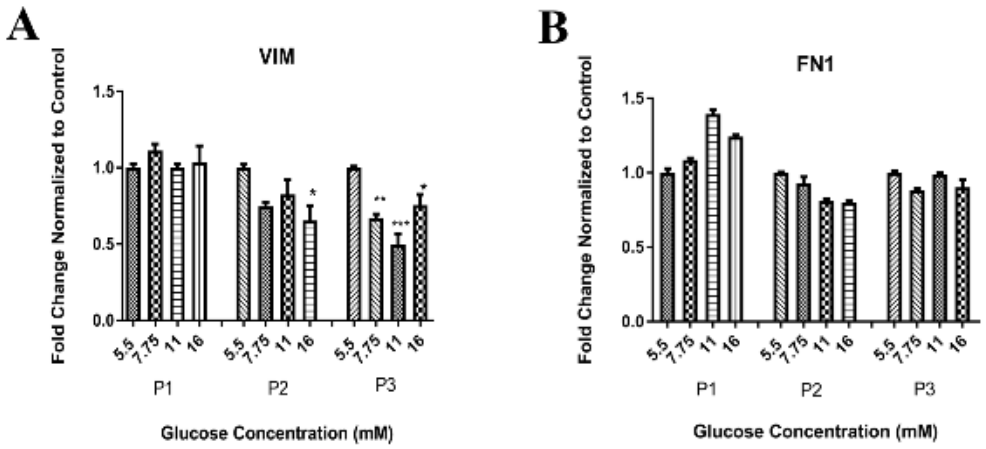

Figure 2.3.5 VIM and FN1 mRNA expression in HPT cells exposed and passaged in glucose. RT-PCR and protein analysis of VIM (A) and FN1 (B) in HPT cells exposed and passaged in 5.5, 7.5, 11, 16 mM glucose. Data expressed as a fold change normalized to control. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.00

All of aforementioned genes are turned on by certain transcription factors. SNAI1 and TWIST1 are common transcription factors that will induced an EMT response. Expressions of both SNAI1 and TWIST1 were measured in HPT cells exposed and passaged in glucose. SNAI1 expression is induced ~3-fold in the first 8 day (P1) exposure to 16 mM glucose (Figure 2.3.6 A). On the other hand, TWIST1 expression is induced 10 - 20–fold in HPT cells exposed to hyperglycemic condition for 16 days (P2) or longer (P3) (mRNA data only) (Figure 2.3.6 B). There is no western analysis for TWIST1 protein expression due to antibody problems.

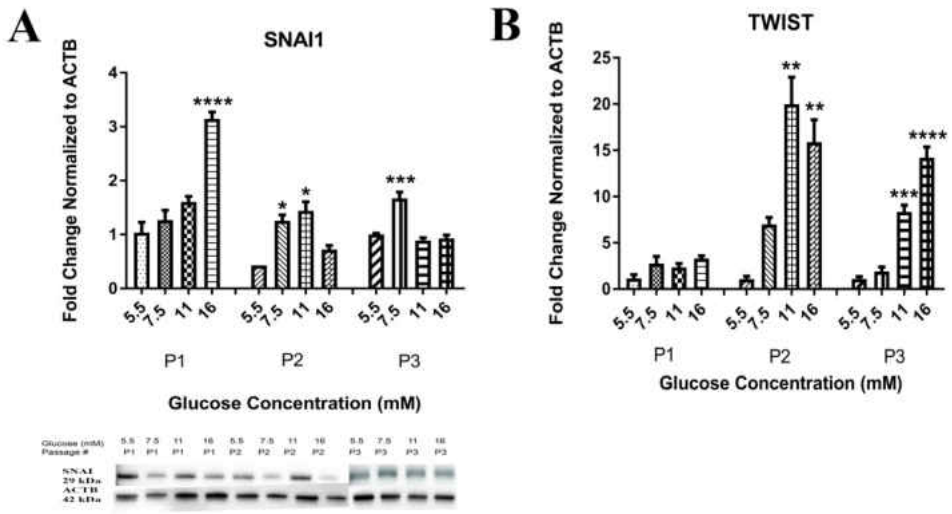

Figure 2.3.6 SNAI1 and TWIST expression in HPT cells exposed and passaged in glucose. RT-PCR and protein analysis of SNAI1 (A) and TWIST (B) in HPT cells exposed and passaged in 5.5, 7.5, 11, 16 mM glucose. Data expressed as a fold change normalized to ACTB. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

Junctional Proteins are not Affected by Exposures to Hyperglycemia

Claudin and occludin proteins are found at the intercellular junctional complexes. These proteins help to establish tight junctions and maintain the stability and barrier properties of tight junctions. Since passaging of HPT cells in high levels of glucose causes morphology changes, it would be possible that glucose could affect the expression of these tight junction proteins. However data from this study has shown HPT cells exposed and passaged in glucose had no effect on the following tight junction proteins; claudins-1/2, (Figure 2.3.7 A, B) and occludin-1 (Figure 2.3.8).

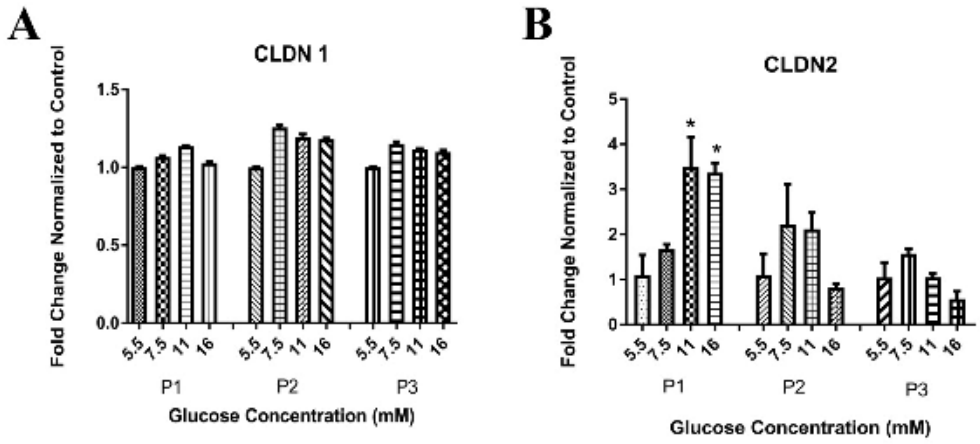

Figure 2.3.7 CLDN1 and CLDN2 mRNA expression in HPT cells exposed and passaged in glucose. RT-PCR and protein analysis of CLDN1 (A) and CLDN2 (B) in HPT cells exposed and passaged in 5.5, 7.5, 11, 16 mM glucose. Data expressed as a fold change normalized to control. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

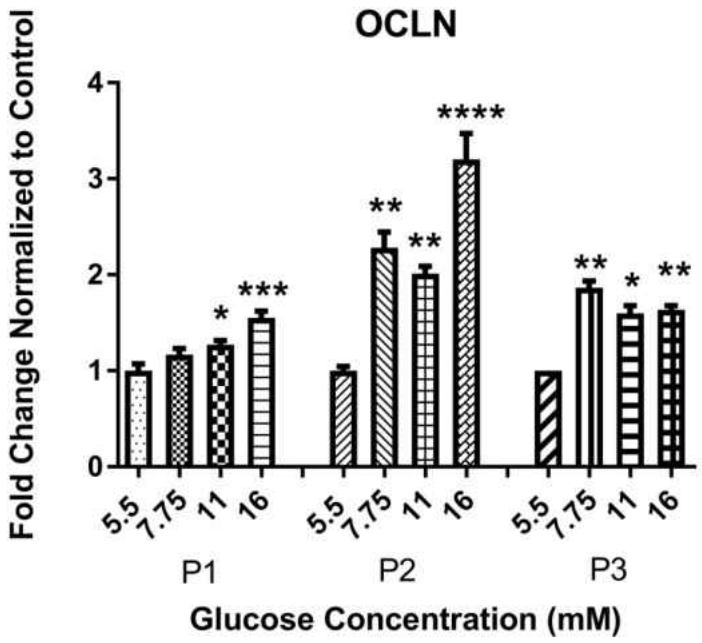

Figure 2.3.8 OCLN1 mRNA expression in HPT cells exposed and passaged in glucose. RT-PCR and protein analysis of OCLN1 in HPT cells exposed and passaged in 5.5, 7.5, 11, 16 mM glucose. Data expressed as a fold change normalized to control. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

Gap junction proteins, GJB1 and GJB2, expression was also measured to determine if hyperglycemia had altered their expression. Within the first passage in glucose, both GJB1 and GJB2 expression was induced in response to increasing glucose concentrations (Figure 2.3.9 A,B). GJB1 expression was also induced in HPT cells exposed and passaged in glucose for 24 days (P3) however (Figure 2.3.9 A), GJB2 was not induced in the latter passages (Figure 2.3.9 B).

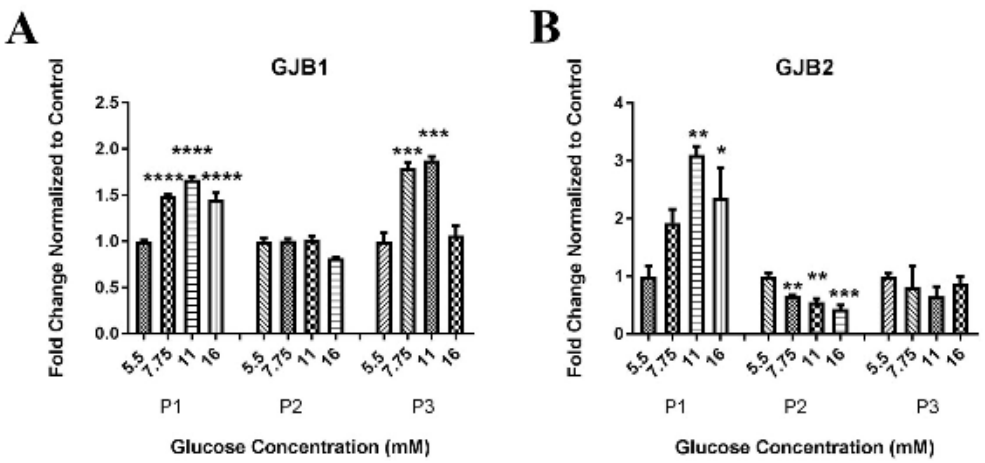

Figure 2.3.9 GJB1 and GJB2 mRNA expression in HPT cells exposed and passaged in glucose. RT-PCR and protein analysis of GJB1 (A) and GJB2 (B) in HPT cells exposed and passaged in 5.5, 7.5, 11, 16 mM glucose. Data expressed as a fold change normalized to control. RT-PCR results represent ±SEM of triplicate. *p<0.05, **p<0.01, ***p<0.001

CHAPTER II.IV

Discussion

The present study establishes that HPT cells in a prolonged hyperglycemic environment undergo a partial EMT process. Chronic kidney disease is a global epidemic and becoming a worldwide healthcare burden not only to the health care physicians but more importantly to the patient. Progressive injury to the kidneys results in renal fibrosis which is the final pathway for all chronic kidney diseases and the point at which irreversible damage is prevalent. At this state, there are no therapeutic treatments other than kidney transplantation or life-long dialysis treatments therefore understanding the mechanisms behind the development of renal fibrosis will help to develop therapeutic targets for inhibiting the disease progression of diabetic nephropathy into renal fibrotic state.

The present study provides an insight into a potential mechanism of the gradual damaging process that may occur during the disease transition of diabetic nephropathy to a fibrotic state. Several studies have suggested dedifferentiation of renal epithelial cells through type 2- EMT process has a role in the onset of fibrosis. EMT is a biological process that has a major role in developmental and pathological properties such as the loss of apical-basolateral polarity, loss of cell to cell contact, and reorganization of cytoskeletal actin (Expósito-Villén, E. Aránega, & Franco, 2018; Kizu, Medici, & Kalluri, 2009). These genetic alterations promotes the transition of epithelial cells into cells with mesenchymal features which is commonly characterized by an induction of N-

cadherin and a repression of E-cadherin (Iwano et al., 2002; Lovisa et al., 2015; Zeisberg & Kalluri, 2013; Zeisberg & Neilson, 2009).

Results from this study suggest there is an EMT process occurring through the activation of CDH2, SNAI1, ACTA2, TWIST genes in response to exposures to hyperglycemica. Although there were several epithelial markers that had no change in expression such as CDH1, Claudin-1/2, occludin, GJB-1/2, these cells may have been caught in the early stages of initiating a mesenchymal state. This partial transition is also depicted in the morphology images HPT cells exposed and passaged in hyperglycemia where some cells appear to be "elongated" in shape whereas others are more "cuboidal".

There is a lack of support in the literature for the evidence of the EMT phenomenon occurring in only some of the cells because researchers in this area of the field believe the EMT process is an all-or-none phenomenon. In a study conducted by Grande et al., 2015, had shown activation of SNAI1 in mouse renal epithelial cells promotes signals for myofibroblast differentiation without inducing the myofibroblast population (Grande et al., 2015) supporting the idea of a "partial" EMT process. In another study where immortalized proximal tubule cells were exposed to 25 mM glucose for 48 hours, showed appreciable high expression of vimentin and α-smooth muscle actin and repressed expression of e-cadherin (Liu et al., 2016).

Although there have been several studies investigating EMT processes and its role in renal fibrosis, there are still remains several controversies regarding if EMT is the

driving force for the development of fibroblasts. However, as researchers continue to investigate the relationship between EMT and renal fibrosis, there will be a possible therapeutic approach to prevent renal fibrosis.

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