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High glucose increases caspase-dependent apoptosis in inner medullary collecting duct (IMCD) cells, independent of prostaglandin E_2 (PGE_2).

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Monika Dougherty

This thesis is submitted as a partial fulfillment of the M.Sc. program in Cellular and Molecular Medicine.

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Abstract

Hyperglycemia is the main determinant of diabetic nephropathy, resulting in altered renal cell growth and tubular atrophy. Additionally, prostaglandin synthesis is elevated in the diabetic kidney and may play a role in antagonizing or mediating the effects of glucose on cell growth and death. Recently, our laboratory reported that high glucose increases PGE_2 synthesis in IMCD cells, consequently, this study will determine the effect of high glucose and PGE_2 on IMCD cell growth and apoptotic death. After four days high glucose exposure, there was a decrease in DNA and protein synthesis compared to low glucose. Concomitantly, high glucose treatment increased the percentage of IMCD cells displaying fragmented DNA characteristic of apoptosis, which was significantly attenuated by treatment with a pan-caspase inhibitor. Caspase-3 and/or caspase-7 activity was also augmented after four days high glucose treatment, and there was an observed increase in the expression of Bax to Bcl-2. These effects were partially mimicked by mannitol treatment. Although PGE_2 treatment increased DNA and protein synthesis in the presence of indomethacin, neither indomethacin nor PGE_2 altered the apoptotic response of IMCD cells in low or high glucose. To our knowledge, this study is the first to show that high glucose treatment induces IMCD cell apoptosis, coincident with a decrease in DNA and protein synthesis, and independent of PGE_2.

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List of Abbreviations

AA	Arachidonic Acid
AQP2	Aquaporin 2
AVP	Arginine-vasopressin
BSA	Bovine Serum Albumin
BrdU	5-Bromo-2'-deoxy-uridine
cAMP	Cyclic Adenosine Monophosphate
CCD	Cortical Collecting Duct
CDK	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
COX	Cyclooxygenase
CPM	Counts per minute
CRE	cAMP response element
DAPI	4', 6-Diamidimo-2-phenyindole, dihydrochloride
dbcAMP	Dibutyryl cAMP
DEPC	Diethylpyrocarbonate
DMEM-F12	Dulbecco's Modified Eagles Media
DN	Diabetic nephropathy
EGF	Epidermal growth factor
ENaC	Epithelial sodium channel
EP	E prostanoid
FBS	Fetal bovine serum
GPCR	Guanine nucleotide binding protein coupled receptors
HG	High glucose
HRP	Horseradish peroxidase
IDDM	Insulin dependent diabetes
IMCD	Inner medullary collecting duct
LG	Low glucose
MA	Mannitol
MDCK	Madine Darby canine kidney
M phase	Mitosis phase
NADPH	Nicotinamide adenine dinucleotide phosphate
NIDDM	Non-insulin dependent diabetes
NSAID	Nonsteroidal anti-inflammatory drugs
OMCD	Outer medullary collecting duct
PBS	Phosphate buffered saline
PGE_2	Prostaglandin E_2
PKC	Protein kinase C
PLA_2	Phospholipase 2
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SMA	Smooth muscle actin
STZ	Streptozotocin
TBS-T	Tris buffered saline-Tween 20

TCA	Trichloroacetic acid
TGF-β	Transforming growth factor β
TUNEL	Terminal deoxyuridine triphosphate (dUTP) nick-end labeling

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I appreciate the valuable guidance, suggestions and support from my advisory committee members, Drs. Stephen Lee and Ruth Slack, and would like to thank them for their time and help.

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The endless support, generosity and encouragement provided by my amazing parents, Andy and Yvonne Sklepowicz, and my loving husband Derek, has truly made this thesis possible. Thank you from the bottom of my heart.

1.0: Introduction

1.1: Diabetes.

Diabetes mellitus is a disease hallmarked by hyperglycemia that affects more than two million Canadians, a number that is expected to rise dramatically with the aging population (Mantell, 2005). Diabetes mellitus encompasses two types of diabetes: type 1, or insulin dependent diabetes mellitus (IDDM), and type 2, or non-insulin dependent diabetes mellitus (NIDDM). The prevalent morbidity and mortality of diabetic patients is largely due to complications from the disease, such as diabetic nephropathy (DN), or kidney disease caused by diabetes (Sheetz, 2002). The clinical presentation of diabetes related kidney disease includes hypertension, proteinuria, metabolic acidosis, and hyperkalemia and the severity and prevalence of DN is clearly illustrated by the fact that it is the leading cause of chronic renal failure in western societies, accountable for over 30% of the patients on maintenance dialysis (Wolf, 1999). Extensive systematic studies have led to the understanding and characterization of stages in DN that involve discrete structural changes and a concomitant loss of renal function (Mogensen, 1983). Current therapies include the use of angiotensin converting enzyme (ACE) inhibitors, which largely act to reduce blood pressure and delay the progressive loss of renal function (Barnett, 2005). However, ACE inhibition therapy alone rarely stops renal disease progression (Hebert, 2001) indicating that there is still much that needs to be understood about the complex cellular changes that occur in DN, about the signaling molecules involved or affected, and what role they may play in the pathogenesis or treatment of diabetic renal disease.

1.2: The Kidney.

In a healthy individual, the kidney functions in part to excrete wastes from the blood into the urine and to maintain the body's water and solute balance. This is accomplished by the approximately 1 million nephrons that comprise each kidney. The nephron consists of a renal corpuscle and a continuous length of four major tubule segments: the proximal tubule, the loop of Henle, the distal tubule and the collecting duct. In the cortex, or the outer region of the kidney, the renal corpuscle contains the glomerulus which is comprised of a capillary network surrounded by a heterogenous cell population, and is responsible for filtering the blood plasma. Although Na^+, Cl^-, K^+, glucose and urea are filtered freely, red blood cells and plasma proteins remain in the blood circulation (Tortora, 2003). From the glomerulus, the filtered fluid passes into the proximal tubule where 100% of most organic solutes, like glucose, are reabsorbed. Also, the leaky epithelium and simple junctions of proximal tubule cells allows for the majority of the filtered water, Na^+, K^+, and Cl^- to be reabsorbed (Tortora, 2003). In contrast, the 'tight' epithelium of the collecting duct allows for selective reabsorption of water and solutes (Shwartz, 1990, Star, 1990), and is therefore responsible for the fine tuning of the body's fluid volume and solute concentrations.

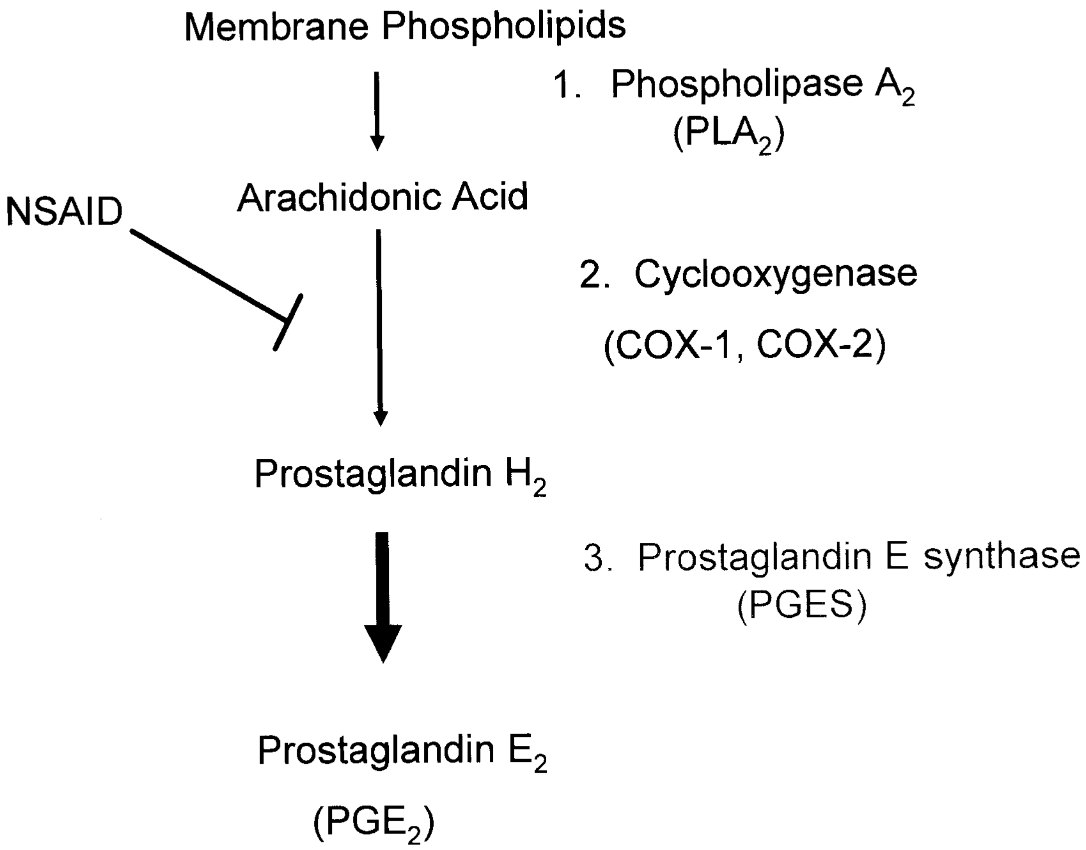
The collecting duct can be divided into three segments based on their anatomical position in the kidney. Beginning in the cortex, the cortical collecting duct (CCD) extends into the outer medulla, or the middle region of the kidney, giving rise to the outer medullary collecting duct (OMCD). The OMCD then extends into the inner medulla, or the inner region of the kidney, and is called the inner medullary collecting duct (IMCD), which is the final site for urinary composition adjustment. The IMCD is composed mainly of principal cells, responsible for water and solute transport, and less than 10% of the cells are intercalated cells, which transport H^+ and HCO_3^- to help maintain the body's acid-base balance (Zeidel, 1993). The main function of the IMCD is urea trafficking through urea transporters (Fenton, 2005), water reabsorption through arginine-vasopressin (AVP)-stimulated aquaporin 2 (AQP2) channels (Kishore, 2005), and Na^+ transport via an apical epithelial sodium channel (ENaC) and the basolateral Na^+/K^+-ATPase (Zeidel, 1993). Various hormones, in addition to AVP, play an important role in the regulation of IMCD function, including prostaglandin E_2 (PGE_2).

1.3: Prostaglandin E_2 and its role in the IMCD.

Arachidonic acid (AA) is released from cell membrane phospholipids by the enzymatic action of phospholipase A_2 (PLA_2). Subsequent metabolism by cyclooxygenase isoforms, COX-1 and COX-2, generates the intermediate, prostaglandin H_2, which is further metabolized by one of five major prostaglandin isomerases giving rise to one of five major prostanoids (PGs). As shown in **Figure 1**, PGE_2 is generated by the isomerase, prostaglandin E synthase (PGES).

Nonsteroidal antiinflammatory drugs (NSAID) are one of the most commonly used medications worldwide, inhibiting COX activity for the treatment of pain and inflammation, and inducing adverse renal complications with both acute and chronic use (Cheng, 2004). The renal effects of NSAID use encompass Na^+ retention, edema, hypertension, and hyperkalemia (Breyer, 2001). Also, the role of PGs in renal function has been assessed in the rat by in vivo indomethacin infusion (a dual COX-1/-2 inhibitor), which induced Na^+ retention, edema, hypertension, and hyperkalemia (Breyer, 2000). In part, reduced PGE_2 levels in the IMCD mediate these effects, evidenced by Na^+ and water excretion stimulated by PGE_2 treatment in the IMCD (Zeidel, 1993, Jabs, 1989, Bolterman, 1989).

Figure 1: Synthetic pathway of prostaglandin E_2 (PGE_2) through the arachidonic acid cascade. Three key enzymes, phospholipase A_2, cyclooxygenase, and prostaglandin E synthase, sequentially control PGE_2 synthesis. The widely used nonsteroidal anti-inflammatory drugs (NSAID) inhibit PGE_2 synthesis by blocking cyclooxygenase activity.


Membrane Phospholipids
1. Phospholipase A_2
(PLA_2)
NSAID
Arachidonic Acid
2. Cyclooxygenase
(COX-1, COX-2)
Prostaglandin H_2
3. Prostaglandin E synthase
(PGES)
Prostaglandin E_2
(PGE_2)

1.4: Mechanism of PGE_2 signaling.

PGE_2 signals via distinct guanine nucleotide binding protein coupled receptors (GPCR) called E-prostanoid (EP) receptors and to date, there have been four EP receptor subtypes cloned, designated EP_1, EP_2, EP_3, and EP_4 (Breyer, 2000). Upon agonist binding, each receptor activates a distinct intracellular signaling pathway to influence cellular function. Within the kidney, EP_1 messenger RNA (mRNA) is found primarily in the collecting duct, increasing from the cortex to the inner medulla (Breyer, 2000). The EP_1 receptor activates phospholipase C via the G_q-protein family and in the IMCD, PGE_2 has been shown to increase intracellular Ca^{2+} levels through this receptor (Breyer, 2000, Nasrallah, 2001). Also in the IMCD, Rouch and Kudo (2000) showed that PGE_2 inhibits water, Na^+ and urea reabsorption stimulated by AVP. The EP_1 pathway contributes to this PGE_2-dependent inhibition of solute and water reabsorption (Breyer, 2000). Additionally, in glomerular mesangial cells, PGE_2 stimulates cellular proliferation mainly through EP_1-mediated calcium mobilization (Suganami, 2001).

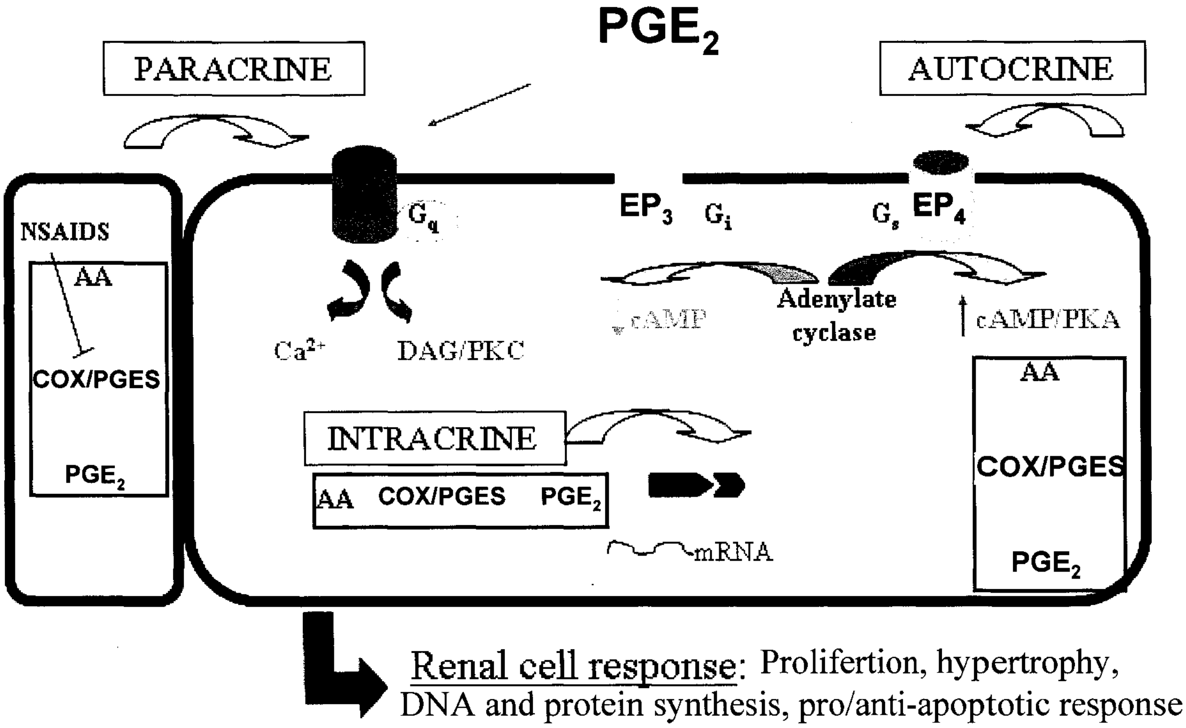
The mRNA for the EP_2 receptor has been localized to the rat inner medulla, although it is absent from healthy IMCD cells (Jensen, 2001). It is coupled to a stimulatory G_s-protein which stimulates adenylate cyclase to increase intracellular cyclic adenosine monophosphate (cAMP) and is also activated by butaprost, a selective EP_2 agonist (Breyer, 2000). In fibroblasts, PGE_2 is able to inhibit cellular proliferation through the EP_2 receptor (Moore, 2004).

The rat renal medulla is the principal location of EP_3 receptors, which are specifically expressed in the IMCD (Takeuchi, 1993). There are many splice variants of the EP_3 receptor, but they all appear to inhibit adenylate cyclase and cAMP generation through a pertussis toxin sensitive G_i (inhibitory)-protein (Breyer, 2000). The different splice variants can activate additional signaling mechanisms, including the small G protein *rho*, which appears to be the mechanism by which PGE_2 inhibits AVP-stimulated water absorption in the IMCD (Tamma, 2003). In a proximal tubule cell line, it was shown that inhibition of Rho proteins decreases DNA synthesis and induces apoptotic cell death (Anderson, 2000). This indicates that PGE_2 has the potential to increase DNA synthesis and protect against cell death through EP_3-mediated Rho activation.

The highest intrarenal expression of the EP_4 receptor is in the cortex (Breyer, 2000, Jensen, 2001) however, functional and molecular studies reveal that the EP_4 receptor is present in the IMCD (Nasrallah, 2001). Like the EP_2 receptor, EP_4 is coupled to a G_s-protein and increases cAMP, but is insensitive to butaprost (Breyer, 2000). The effect of PGE_2 on basolateral water absorption in the collecting duct, opposite to that mediated by EP_1/EP_3, appears to be mediated by cAMP production through the EP_4 receptor (Breyer, 1998). Although the effect of PGE_2 on IMCD growth is largely unknown, it is interesting that the augmented proliferation of renal tubule cells in polycystic kidney disease is mediated in part by PGE_2-stimulated cAMP (Belibi, 2004). However, it is also known that cAMP stimulating agents inhibit proliferation and interestingly, in mesangial cells, PGE_2 inhibits cellular proliferation in the presence of growth factors and stimulates proliferation in their absence (Mahadevan, 1996). It has also been shown that non-confluent Madin-Darby canine kidney (MDCK) type II cells (a distal tubule cell line) release much higher amounts of AA and PGE_2 than confluent MDCK cells that have formed cell-cell junctions (Bailleux, 2004). This suggests that PGE_2 may also play a role in cellular proliferation in the IMCD.

Adding to the complexity of PGE_2 signaling, it has been suggested that PGE_2 alters gene expression via intracrine signaling through nuclear EP receptors (Bhattacharya, 1998). It has also been shown that EP receptors are located on mitochondrial membranes and may play a role in the regulation of cell survival (Fang, 2004). Thus, it is apparent that PGE_2 has the capacity to exert numerous cellular effects, summarized in **Figure 2**, depending on the expression and activation of the various receptor subtypes. The opposing action mediated by the different receptor subtypes has led to the referral of PGE_2 as a physiological buffer (Breyer, 1998) enabling it to compensate for enhanced or aberrant signaling of other molecules in a disease state. However, it is also conceivable that dysregulation of one or more of the EP receptor subtypes could propagate a disease state. For example, upregulation of the EP_3 receptor in the cortex of rats with passive Heymann nephritis is proposed to play a key role in the progression of this disease (Waldner, 2003). Additionally, increased expression of, and signaling through, the EP_4 receptor in glomerular podocytes appears to prevent the morphological changes required for podocytes to adapt to mechanical stretch in vitro, which could contribute to proteinuria in hypertensive patients, for example (Martineau, 2004). In mesangial cells cultured in high glucose, Ishibashi, *et al.* (1999) demonstrated that an enhanced proliferative response to PGE_2 was attributed to attenuated EP_4-mediated cAMP production, without a change in EP_4 mRNA levels. Therefore, it is of interest to evaluate EP receptor expression and PGE_2/EP signaling in IMCD cells in the diabetic kidney to determine if changes in these components could contribute to IMCD dysfunction.

Figure 2: Multiple pathways through which PGE_2 can exert its effects in the IMCD cell. PGE_2 may be synthesized by neighbouring cells and act in a paracrine manner through specific E prostanoid receptors (EP_1, EP_3, and EP_4) identified in the IMCD. Also, PGE_2 may be synthesized and released by the IMCD cell itself acting in an autocrine fashion through the EP receptors on the cell membrane. Finally, PGE_2 may act through EP receptors on mitochondrial or nuclear membranes in an intracrine type of signaling. Depending on the predominant pathway activated, variable cellular responses can occur.



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1.5: Diabetic nephropathy and the IMCD.

In diabetes, collecting duct dysfunction could contribute to complications such as hypertension, hyperkalemia, edema, and diuresis (Koren, 2001). Moreover, in a disease with similar symptoms, ischemic acute renal failure, IMCD dysfunction is a factor in the increased sodium excretion and decreased urine osmolality (Wilson, 1988). However, most studies concentrate on glomerular and proximal tubule changes, overlooking the involvement of the IMCD in the pathogenesis and progression of DN. The earliest pathological change in DN is general renal growth, associated with an increase in the glomerular filtration rate (Bak, 2000, Wolf, 1999). It has been suggested that renal growth is an important factor in the onset of DN and both the cortex and the medulla are enlarged in diabetes (Lohr, 1991, Wolf, 1999). Although it has been shown that both glomerular and proximal tubule cells contribute to the increased size of the diabetic kidney, through hyperplasia (increased cell number with augmented DNA and protein synthesis) and hypertrophy (increased cell size and augmented protein synthesis without DNA synthesis) (Wolf, 1999), it is unclear if IMCD growth is altered in DN.

As DN progresses, the mesangial matrix expands, leading to glomerulosclerosis and proteinuria (Ziyadeh, 2004). In advanced DN, a gradual decline in renal function is associated with tubule epithelial cell degeneration, also called tubular atrophy and interstitial fibrosis (Susztak, 2005). In an animal model of type 1 diabetes, the streptozotocin (STZ)-induced diabetic rat, with poor glycemic control, i.e. no insulin treatment, it was apparent that there was distal tubule degeneration as early as two weeks after the onset of diabetes (Yong, 1986). In a similar model, Ha, *et al.* (1994), reported an increase in oxidative DNA damage in the inner medulla of diabetic rats, which was reduced by insulin treatment. Additionally, Palm, *et al.* (2004), demonstrated that cellular metabolism in the inner medulla is altered in STZ rats and is associated with increased medullary glucose concentration and activation of the polyol pathway, where aldose reductase uses nicotinamide adenine dinucleotide phosphate (NADPH) to reduce glucose to sorbitol, which is then oxidized to fructose (Sheetz, 2002). In a separate study, the same group (Palm, 2003), demonstrated that oxidative stress occurs in the inner medulla of diabetic animals and is related to hyperglycemia and the formation of reactive oxygen species (ROS). In various renal cells, high glucose, partially through the generation of ROS, is cytotoxic (Ha, 2000, Wolf, 1999). Therefore, these studies indicate that inner medullary cells are exposed to toxic conditions in DN, as a result of high glucose levels, that may result in cell death. However, tubular epithelial degeneration in the inner medulla in DN is poorly studied and investigations of the effect of high glucose on IMCD survival are lacking.

1.6: High glucose as a model of DN and its role in cellular proliferation.

Diabetic complications are determined by hyperglycemia (Nishikawa, 2000, Reichard, 1996), and in DN, many of the renal changes that occur can be prevented or delayed with tight glycemic control (reviewed in Lee and Ha, 1997). Kidney growth occurs shortly after the induction of hyperglycemia in STZ-induced diabetic rats and is related to alterations in DNA and protein synthesis (Bak, 2000). Exposure of renal cells to high glucose has been shown to alter the cell cycle, or the phases that determine cellular proliferation, known as G_0, G_1, S, G_2 and M phase (Wolf, 2000). For the purpose of this thesis, it is essential to give a brief description of the cell cycle and different types of cell growth.

Cellular proliferation is reflected by an increase in DNA and protein synthesis resulting in an increase in cell size and number; this type of growth is referred to as hyperplasia (Preisig, 1999). Under normal conditions, less than 1% of renal cells are proliferating and are considered to be resting in the G_0 phase (Wolf, 2000). Entry into G_1, where cells increase their size and protein synthesis, can be stimulated by different growth factors, and cells arrested in this phase exhibit hypertrophic growth (Shankland, 2000). Progression into the S phase occurs if certain conditions are met, and is marked by DNA synthesis (Wolf, 2000). In the G_2 phase, cells prepare for mitosis and again, if certain conditions are met, progression into the mitosis (M) phase and cell division occurs.

Progression through the mammalian cell cycle, and therefore cellular proliferation, is positively controlled by cyclin-dependent kinases (CDKs), and negatively controlled by cyclin-dependent kinase inhibitors (CKIs) (Huang, 2002). In MDCK cells, high glucose exposure for two days increases CDK activity, stimulates proliferation and increases the percentage of cells in the G_2/M phase (Yang, 1998). Also, in murine glomerular mesangial cells, 24 hrs high glucose exposure increases cellular proliferation and the percentage of cells in S/G_2 phase, and after 72 hrs high glucose, proliferation decreases and there is an increase in cellular hypertrophy and the percentage of cells in the G_1 phase (Wolf, 1992 and reviewed in Shankland, 2000).

High glucose exerts myriad effects on the renal cell cycle via numerous mechanisms, such as the polyol pathway, advanced glycation end products (AGEs), protein kinase C (PKC) generation, stimulation of growth factors, ROS generation, and osmotic effects (Sheetz, 2002). In MDCK cells, AGE administration increases transforming growth factor-β (TGF-β) expression and cellular hypertrophy (Yang, 2004). Other factors are also important mediators in the pathogenesis of DN, and are increased in response to high glucose.

1.7: The role of PGE$_2$ in high glucose.

It is well documented that COX enzymes are elevated in the diabetic kidney, particularly in the IMCD of the STZ rat (Nasrallah, 2003). Furthermore, PGE$_2$ synthesis is increased in the diabetic IMCD (Jaisser, 1989) and is increased in renal cells, particularly IMCD cells, exposed to high glucose (Nasrallah, 2003, Nesbitt, 2004). Although prostaglandins have long been implicated in the pathogenesis of DN (Pugliese, 1992), the exact role of PGE$_2$ is unclear. In the glomerulus, a harmful effect of PGE$_2$ has been suggested since it affects mesangial matrix components and stimulates mesangial cell proliferation (Mahadevan, 1996). Additionally, administration of a selective EP$_1$ antagonist, ONO-8713, prevents the progression of DN including the amelioration of renal hypertrophy, decreased mesangial expansion, and suppressed proteinuria (Makino, 2002). Furthermore, prostaglandin synthesis inhibition in type 1 diabetic women was reported to decrease the diuresis and Na$^+$ excretion without, however, altering albumin excretion (Linne, 1991). On the other hand, it has been shown that administration of ACE inhibitors to diabetic patients greatly reduces proteinuria and increases urinary PGE$_2$, thereby suggesting that enhanced renal production of PGE$_2$ may partly account for the beneficial effects of the ACE inhibitor on reducing protein excretion (Tajiri, 1990). There is also recent evidence that EP$_1$ and EP$_4$ receptor subtypes are increased in the

IMCD in the presence of high glucose (Nasrallah, 2003), indicating that an enhanced cellular response to PGE_2 may play a role in determining IMCD cell fate in high glucose.

1.8: Apoptosis in DN.

It is strongly suggested that apoptosis underlies tubular epithelial cell degeneration in DN (Kelly, 2002, Susztak, 2005, Wolf, 1999). Apoptosis is an active form of cell death, tightly regulated by distinct genes and involves cell shrinkage, chromatin condensation, DNA fragmentation and the formation of membrane bound vesicles called apoptotic bodies (Wang, 2005). There are two major signaling pathways in apoptosis (**Figure 3**), the death receptor pathway and the mitochondrial pathway (Gupta, 2003). A well known member of the death receptor-ligand family is Fas-Fas ligand, and upon ligand binding, the Fas death receptor activates a caspase cascade (Gupta, 2003). Caspases are a large family of cysteine proteases that are activated specifically in apoptotic cells (Hengartner, 2000). There are specific initiator caspases activated by the death receptor pathway, caspases-8 and -10, and by the mitochondrial pathway, caspase-9, which cleave the effector caspases caspase-3, -6, and -7 to culminate in DNA fragmentation and cytoskeleton remodeling (Gupta, 2003, Hengartner, 2000). Caspase-8 can also activate pro-apoptotic proteins involved in cytochrome c release from the mitochondria indicating the potential for cross-activation of the two apoptotic pathways (Gupta, 2003).

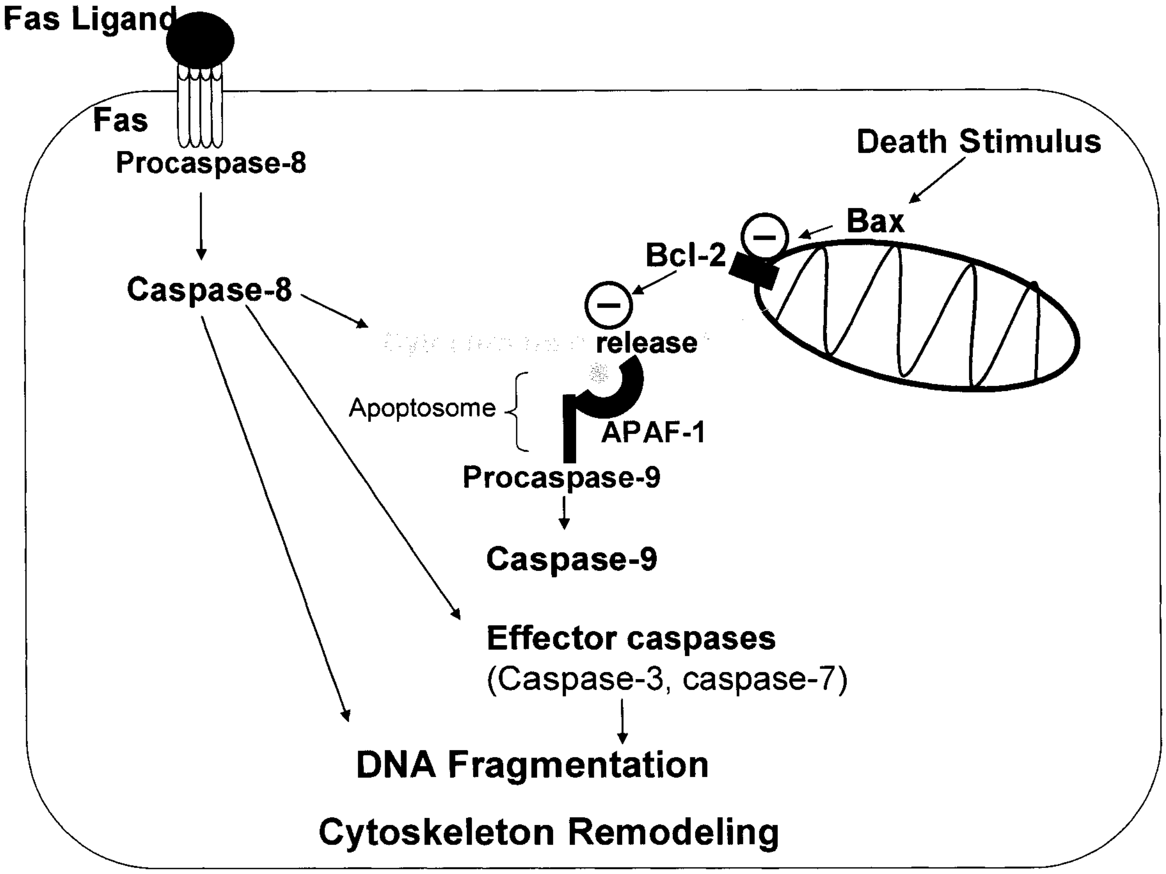
A number of stimuli, including chemotherapeutic agents, ultra violet radiation, stress molecules (for example ROS), and growth factor withdrawal appear to mediate apoptosis via the mitochondrial pathway (Gupta, 2003). An apoptotic death stimulus results in mitochondrial membrane permeability and cytochrome c release, which is controlled by a variety of members of the Bcl-2 protein family, (Joza, 2001). A pro-apoptotic Bcl-2 family member, Bax, appears to stimulate cytochrome c release by binding to and inhibiting the anti-apoptotic Bcl-2 protein at the mitochondrial membrane (Hengartner, 2000). Cytochrome c binds to an adapter molecule Apaf-1 (Apoptotic protease-activating factor) and procaspase-9, forming the apoptosome (Gupta, 2003). Subsequently, procaspase-9 is activated to caspase-9 which cleaves and activates effector caspases-3, -6, and -7 to induce apoptosis. There are also intra-mitochondrial molecules that, once released, can activate apoptosis independent of caspases (Joza, 2001).

Increased apoptosis has been documented in human diabetic kidneys (Kumar, 2004a, Susztak, 2005) and in the STZ rat, specifically in medullary tubule cells, among others (Kumar, 2004b). It has also been shown that reduced Bcl-2 expression and elevated Bax expression is observed in the rodent diabetic kidney (Kikuchi, 2002, Oritz, 1997, Wolf, 1999). Renal proximal tubule cells cultured in high glucose also demonstrate augmented apoptosis associated with a decrease in the Bcl-2 to Bax ratio and an increase in ROS generation (Verzola, 2002).

In the STZ-induced diabetic rat, tubular apoptosis was associated with a decrease in epidermal growth factor (EGF) and an increase in transforming growth factor (TGF)-β (Kelly, 2002). It is suggested that EGF inhibits tubular epithelial cell apoptosis, and interestingly, it has been shown that in the IMCD, EGF signals through the production of PGE_2 (Harris, 1989). Although indomethacin did not alter the proliferative effect of EGF in IMCD cells, and the exact role of PGE_2 on IMCD survival is unknown, it is possible that PGE_2 may be involved in the anti-apoptotic effect of EGF in DN (Harris, 1989).

Additionally, in MDCK cells, it is suggested that COX metabolites function as survival factors and it was shown that PGE_2 protected against chemically induced apoptosis (Nishimura, 2004). Similarly, in bladder epithelial cells, PGE_2 and forskolin, a direct activator of adenylate cyclase, protected against chemically induced apoptosis through cAMP generation (Jabbour, 2002). Therefore, PGE_2 has the capacity to protect against apoptosis in DN, however, differential expression and stimulation of EP receptors throughout DN may control the susceptibility of renal cells to apoptotic stimuli.

Figure 3: Overview of the two major apoptotic signaling pathways in the mammalian cell: the death receptor and mitochondrial pathway. Activation of the death receptor pathway, for example by Fas ligand binding to Fas, results in procaspase-8 cleavage to active caspase-8. Caspase-8 can stimulate cytochrome c release from the mitochondria to form the apoptosome with APAF-1 and procaspase-9. Subsequent cleavage of procaspase-9 initiates the activation of effector caspases like caspase-3 and -7. These caspases cleave various substrates resulting in morphological changes characteristic of apoptosis, including DNA fragmentation and cytoskeleton remodeling. Cytochrome c release is also regulated by the anti-apoptotic Bcl-2 protein and upregulation of the pro-apoptotic Bax protein by a death stimulus results in Bcl-2 inhibition and cytochrome c release. (Based on information from the references, Hengartner, 2000 and Joza, 2001).


Fas Ligand
Fas
Procaspase-8
Death Stimulus
Bax
Bcl-2
Caspase-8
Cytochrome c release
Apoptosome
APAF-1
Procaspase-9
Caspase-9
Effector caspases
(Caspase-3, caspase-7)
DNA Fragmentation
Cytoskeleton Remodeling

1.9: Purpose.

The purpose of this study is to characterize the growth response and apoptosis in IMCD cells exposed to high glucose and PGE_2 and to determine if alterations in PGE_2 signaling in high glucose may contribute to pathological changes in the IMCD in DN.

1.10: Hypothesis and objectives.

It is hypothesized that high glucose, representative of the diabetic environment, is detrimental to IMCD cell survival and will therefore increase apoptosis. It is also hypothesized that alterations in cell growth and PGE_2 signaling in the presence of high glucose will favor IMCD cell death.

This hypothesis will be addressed by two main objectives:

OBJECTIVE #1: Determine the effect of high glucose on primary cultures of rat IMCD cells.

First Aim of Objective #1: Examine the growth response and apoptosis in IMCD cells in a diabetic environment. High extracellular glucose will be used as a model of the diabetic environment and the following are the different treatment groups:

LG (low glucose): 7.5 mM glucose

HG (high glucose): LG + 20 mM glucose

MA (Mannitol): LG + 20 mM mannitol

Also, two time points, 1 and 4 days, will be used to evaluate the effect of high glucose on cell growth. It has been shown that prolonged glucose exposure can induce a biphasic growth response with early hyperplasia and later hypertrophy in epithelial cells or sustained proliferation in renal interstitial fibroblasts (Wolf, 1999). DNA and protein synthesis will be measured by BrdU and ^{3}H-leucine incorporation, respectively. Apoptosis will be measured by three independent techniques: western blotting of Bax and Bcl-2 proteins, caspase-3/-7 activity and TUNEL labeling.

Second Aim of Objective #1: Determine the effect of HG on PGE_2/EP receptor signaling. PGE_2 stimulated cAMP accumulation and EP_4 receptor expression will be characterized in LG, HG and MA to determine if alterations in the EP_4/cAMP pathway may contribute to, or serve to antagonize, the effect of HG in the IMCD cell. EP_4 receptor mRNA will be measured by real time reverse transcriptase polymerase chain reaction (RT-PCR) and PGE_2-stimulated cAMP will be measured by a competitive binding ^{3}H-cAMP radioassay.

OBJECTIVE #2: Examine the growth and apoptotic response of IMCD cells to PGE_2 in the above-mentioned treatment groups.

Since PGE_2 is known to alter the growth response in other cell types (Nishihara, 2003), PGE_2 may play a role in antagonizing or propagating the effect of glucose through specific EP receptors in IMCD cells. Indomethacin will be used to inhibit endogenous PGE_2 synthesis and the procedures described in **OBJECTIVE #1** will be used to measure the growth response to PGE_2 and indomethacin.

1.11: Significance of study.

Renal complications in diabetes largely contribute to the morbidity and mortality of the vast number of people suffering from the disease. Although progress has been made in identifying some key mediators of DN, current therapies that intercept these mediators are ineffective in preventing progressive loss of renal function. It is apparent that there is a need for a deeper understanding of the pathogenesis and mediators of DN.

The inner medulla, particularly the IMCD, is important for urine concentrating mechanisms and the fine regulation of physiological solute and water levels. IMCD dysfunction is indicated by diabetic symptoms such as hypertension, edema and diuresis. Also, ROS generation (Palm, 2003), increased activity of the polyol pathway (Palm, 2004), and an increase in the rate of apoptosis in the diabetic inner medulla (Kumar, 2004b) have linked high glucose to the generation of a toxic IMCD environment. Since these studies allude to changes in inner medullary structure and function in DN, it is of interest to examine direct effects of high glucose on IMCD cells. Also, since PGE_2 regulates IMCD function, and is elevated in DN, it is of interest to investigate its role in mediating or antagonizing the effects of glucose.

PGE_2 has the capacity to exert numerous and opposing effects through its EP receptors, which are up-regulated in response to high glucose treatment in the IMCD (Nasrallah, 2003) Therefore, it is of interest to determine if the PGE_2/EP receptor signaling pathway is altered in the diabetic IMCD in order to evaluate it as a therapeutic target to treat and possibly prevent progressive renal failure in DN.

2.0: Materials and Methods

2.1: IMCD cell culture. The kidneys from five Sprague-Dawley rats (275-350g) were cut in half longitudinally, and the inner medulla/papilla (white region) was dissected out and placed in a petri dish on ice. The tissue was finely minced using a one-sided razor blade and transferred to a 50-ml sterile polypropylene tube containing 20 ml media [DMEM-F-12 containing 7.5 mM glucose, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Gibco), 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml selenium, 2.5 nM triiodothyronine/sodium salt, and 50 nM hydrocortisone (Sigma)] and 0.05% (wt/vol) collagenase A and 0.01% (wt/vol) DNase I (Roche). The tissue was then digested for 1.5-2 hrs at 37°C, in bubbling 5% CO_2-air. IMCD cells were isolated, as previously described (Nasrallah, 2001), briefly, the tubule suspension was centrifuged at 1100 rpm for 3 min, washed in media, described above, without collagenase and DNase, and placed in a hypoosmotic solution (1:3, media-H_2O) for 3 min, while the tube was gently, continuously inverted. The suspension was then centrifuged for 2.5 min, washed in media, and the IMCD pellet was collected after a 2 min centrifugation. IMCD cells were cultured in 45 mL media for one day at 37°C and 5% CO_2. Subsequently, the cells were grown in the above media without FBS (to minimize fibroblast growth) and with different glucose concentrations according to the following treatment groups: LG (low glucose: 7.5 mM), HG (high glucose: LG + 20 mM glucose), or MA (mannitol: LG + 20 mM mannitol to control for osmolarity). The HG media remained constantly above 25 mM until 48 hrs after initial plating, when it dropped to ~20 mM (measured using Keto-

Diastix glucose indicators). Therefore, the culture media was changed at 2 days to ensure exposure of cells to 27.5 mM glucose over the 4-day period.

2.2: IMCD cell culture characterization by immunocytochemistry. The cells were cultured on glass coverslips for five days then rinsed in phosphate buffered saline (PBS) and fixed in 100% methanol for 30 min at room temperature (RT). After rinsing 3 times in PBS the coverslips were incubated in blocking solution [2% bovine serum albumin (BSA), 0.1% Triton X-100 in PBS] for 15 min then anti-cytokeratin (Cyt) (Sigma #C9687) or anti-smooth muscle actin (SMA) (Sigma #A2547) was added at 1:100 overnight at 4°C. After a wash in PBS, anti-mouse-Ig-fluorescein was used at 1:15 in PBS for 30 min at RT in the dark. The coverslips were washed in PBS and the fluorescent nuclear stain DAPI [4', 6-Diamidimo-2-phenyindole, dihydrochloride (Sigma)] was added for 15 min at RT. The coverslips were then mounted with Vectashield fluorescent mounting media (Vector Laboratories), visualized with a fluorescent microscope (Axioskop 2) and captured with a DVC camera and analyzed using Northern Eclipse 5.0 software (©1999, Empix Imaging, Inc.).

2.3: PGE_2 treatment. To inhibit endogenous PGE_2 synthesis, IMCD cells were treated with 10^{-6} M indomethacin (Sigma) for 24 hrs, unless otherwise stated. For the PGE_2 treatment groups, a stable analogue of PGE_2 (dinoprostone, Cayman Chemicals) was added at the same time as 10^{-6} M indomethacin for a final concentration of 10^{-7} M PGE_2 for 24 hrs.

2.4: Western blotting. Protein lysates from 60 mm plates of IMCD cells, were prepared by washing with PBS and incubating plates on ice for 20 min with RIPA buffer containing 1% NP 40, 1% sodium deoxycholate, 0.1% SDS (w/v) 4.5 mM NaCl, 2.5 mM Tris (pH 7.4), 8 μM EDTA, 0.2 mM sodium phosphate (pH 7.2) and added fresh, 0.5 mM PMSF, 1:100 protease inhibitor cocktail (Sigma), 1mM sodium pyrophosphate, 10 mM sodium fluoride and 100 μM sodium orthovanadate. The cells were scraped, transferred to Eppendorf tubes, and sonicated for 3 sec using a Fisherbrand Sonic dismembrator. The cell lysates were then centrifuged at 10,700 rpm for 10 min, and the supernatant was recovered. Twenty-five micrograms of each sample were resolved by SDS-PAGE on a polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking overnight in 15% milk/TBS-T (137 mM NaCl, 20 mM Tris base, 0.1% Tween20), the membrane was incubated with anti-Bax monoclonal antibody (Santa Cruz sc-7480), 1:500 for 2.5 hrs in 5% milk/TBS-T and visualized using ECL reagent (Amersham) after a 1 hr incubation with 1:2000 horseradish peroxidase (HRP) conjugated anti-mouse antibody (Promega). The membranes were then stripped and treated as above using anti-Bcl-2 monoclonal antibody (Santa Cruz sc-7382). The cleaved caspase-3 fragment was detected using a polyclonal anti-cleaved caspase-3 antibody (Cell Signaling, #9661S) diluted 1:1500 in 10% milk/TBS-T overnight after blocking 3 hrs in 15% milk/TBS-T. COX-2 was detected with a specific polyclonal antibody (Cayman Chemical #160106) at 1:1000 in 5% milk/TBS-T after blocking 3 hrs in 10% milk/TBS-T. For both COX-2 and cleaved caspase-3, an HRP conjugated anti-rabbit antibody (Promega) was used at 1:2000 for 1 hr and the protein samples were normalized with detection of β-actin expression, 1:5000 anti-β-actin (Sigma) for 30 min, then 1:5000 HRP conjugated anti-mouse for

30 min. The densitometry analysis was carried out using Kodak Digital Science™ 1D Image Analysis software (©1995-1998, Eastman Kodak Company).

2.5: ^{3}H cAMP assay. IMCD cells were cultured in 24-well plates and treated four days in LG, HG or MA. Cells were pretreated 15 min with 0.5 mM IBMX, a cAMP phosphodiesterase inhibitor, and 10^{-5} M indomethacin in LG, HG, or MA. Then, 0.01, 0.1, and 1 µM PGE_2 and 10 µM forskolin were added for 10 min stimulations. The samples were all prepared in duplicate. To stop the reaction, 300 µl of ice-cold 10% trichloroacetic acid (TCA, v/v) was added to each well and after 30 min at 4°C, the samples in TCA were transferred to Eppendorf tubes and centrifuged for 10 min at 4000 rpm. Next, 250 µl of each sample were transferred to glass test tubes, and four ether extractions of TCA were performed by using 4× the volume of H_2O-saturated diethyl ether per extraction. By using the cAMP radioassay kit (Intermedico), cAMP levels in each sample were then measured in 100 µl of sample according to the manufacturer's instructions.

2.6: Real Time RT-PCR. RNA was isolated from 60 mm plates of IMCD cells with 600µL TRIzol reagent (Gibco) according to the manufacturer's instructions and was dissolved in a final volume of 20 µL diethylpyrocarbonate (DEPC)-treated water. The total RNA was subsequently treated with 0.3 U/µg DNase I (Invitrogen) to eliminate genomic DNA. EP receptor mRNA levels were determined by real-time RT-PCR using TaqMan One-Step RT-PCR master mix reagents (Applied Biosystems) and an ABI Prism 7000 sequence detection system. Reactions were carried out by using 50-100 ng of total IMCD RNA under the following conditions: 48°C for 30 min, 95°C for 10 min, and

40 cycles of 95°C for 15 sec and 60°C for 1 min. The probe and primers for rodent EP_4 were as follows: forward primer, 5'-TGTACGCGGGCTTCAGTTC-3'; reverse primer, 5'-CGCACACCAGCACATTGC-3'; probe, 6FAM-TTCCTCATCCTCGCCACCGTGCT-TAMRA. Primers and probes were synthesized by Sigma Genosys Canada and were verified for specificity by using BLAST to search sequences in the National Center for Biotechnology Information database. A standard curve was generated by determining the RNA concentration of a sample from the LG treatment group and performing the real-time RT-PCR reaction with serial dilutions from 100 – 12.5 ng of the sample RNA. Values were normalized to GAPDH mRNA levels in each sample as determined by a TaqMan Rodent GAPDH control reagent kit (Applied Biosystems).

2.7: BrdU labeling and detection assay. IMCD cells were cultured on glass coverslips in duplicate in a 24-well plate and treated for the specified time in LG, HG, and MA. At 70% confluence, the cells were pulsed with BrdU (incorporated by cells synthesizing DNA) for 3 hrs and processed for immunofluorescence according to the manufacturer's instructions (Roche Diagnostics #1296736). The cells were incubated with anti-BrdU (1:15) for 1.5 hrs, washed with PBS, then incubated with anti-mouse-Ig-fluorescein (1:10) for 30 min. The cells were then washed with PBS and incubated with the DNA stain DAPI for 45 min. The coverslips were then mounted and visualized as described in section **3.2**. For each coverslip, 10 fields of view at 100x magnification were analyzed using pictures from both the DAPI filter (excitation: ~360 nm and emission: 450nm) and the green filter (excitation: ~480 nm and emission: ~540 nm). The pictures were processed by Image-Pro Plus 5.1 to minimize background and nonspecific fluorescence.

The DAPI-stained nuclei were automatically counted and a mask of their position was applied to the picture from the green filter in the same field of view to determine the number of nuclei that were labeled with anti-BrdU and anti-mouse-Ig-fluorescein. For each sample, the percent of BrdU positive nuclei was determined from a total of 20 fields of view, or two coverslips.

2.8: ^{3}H-leucine incorporation. IMCD cells were grown to 70% confluence in 24-well plates for the specified time in LG, HG, and MA. On the third day of treatment, the media was replaced and the cells were stimulated in the presence of 0.5 μCi/μL ^{3}H-leucine for 30 hrs. The cells were washed 4 times with PBS and then permeabilized with 500 μL 1N NaOH for 15 min at 37°C. The samples in NaOH were transferred to vials containing 10 mL scintillation fluid and the ^{3}H-leucine was measured in counts per minute (cpm) by a scintillation counter. Each sample was done in duplicate and the average is expressed as fold LG control.

2.9: Colorimetric caspase 3 activity assay. Lysates from 60 mm plates of IMCD cells, were prepared by transferring the media, containing floating cells, to 15 mL tubes on ice and scraping cells in cold PBS, which was added to the 15 mL tubes. They were then centrifuged for 5 min at 1100 rpm. The pelleted cells were washed in PBS and centrifuged as above. The pellet was resuspended in 200 μL cell lysis buffer provided in the CaspACE^{TM} assay kit (Promega # G7220). The pan-caspase inhibitor Z-VAD-FMK provided in the kit and was applied to the IMCD cells for the last 24 hrs of treatment, according to the instructions. In the lysis buffer, the cells were cycled through two freeze (at -20°C)-thaws to completely lyse cells, as per the manufacturer's instructions. The samples were incubated 15 min on ice; centrifuged for 20 min at 13,100 rpm and the supernatants were stored at -80°C. Twenty-five micrograms of each sample were incubated in duplicate with the colorimetric caspase-3 and caspase-7 substrate, Ac-DEVD-pNA, in a 96-well plate at RT overnight, according to the manufacturer's instructions. The amount of coloured product produced by endogenous caspase-3 and/or caspase-7 activity was measured by the absorbance (405 nm) in a 96-well plate spectrophotometer. The average absorbance of the duplicates is expressed as fold LG control.

2.10: TUNEL. The cells were grown as described in section ***2.7***, and at confluence, they were rinsed in PBS and placed for 15 min in a fixative consisting of 4% paraformaldehyde and 0.2% picric acid in 0.16 M sodium phosphate buffer at pH 6.9 (Staines, 1988). After washing three times in PBS, each coverslip was incubated with 2 μL $CoCl_2$, 5 μL 5x TDT buffer, 0.1 μL terminal transferase (all from Roche #3333566), 0.17 μL biotin-16-dUTP (Roche #1093070), and 17.73 μL H_2O for 1 hr at 37°C in a humid chamber. The coverslips were then washed in 4x SSC (0.6 M NaCl, 76 mM Na Citrate, pH 7) and blocked 30 min with 1% milk/4x SSC at room temperature. Next, streptavidin-Cy2 stock, 1.0 mg/mL (Molecular Probes #112233), was added to the coverslips at 1:1000 in blocking solution for 45 min at RT in the dark. They were then washed with PBS and incubated with DAPI for 45 min. The coverslips were then mounted, visualized and processed as described in section ***2.7***. For each sample, the percent of TUNEL positive nuclei was determined from a total of 20 fields of view, or two coverslips.

2.11: Statistics. GraphPad Prism software for Windows, version 4.02 (May 17, 2004), was used to analyze data. Results are expressed as means ± standard error of the mean (SEM). An unpaired t-test or a one sample t-test was used to assess the statistical significance between data points and a p value < 0.05 was considered statistically significant.

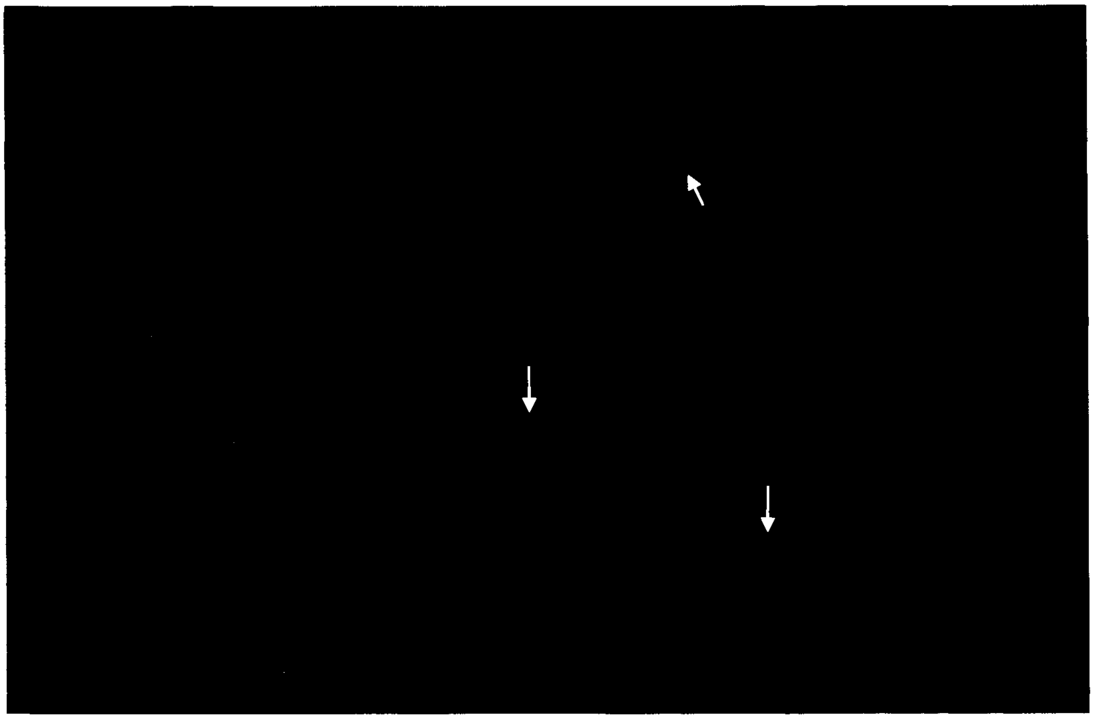
3.0: Results

3.1: Characterization of IMCD cell culture.

The cells isolated by the method described in section **2.1** were previously characterized (Nasrallah, 2001) and found to consist of intercalated and principal cells (~50% of the population). In this study, the cells were further characterized with specific antibodies to SMA and Cyt, which label myo-fibroblasts and epithelial cells, respectively. Specific staining was evaluated by immunofluorescence with the majority of cells expressing Cyt and <5% of the population expressing SMA, characteristic of IMCD tubule cells rather than interstitial cells (**Figure 4**). Additionally, the nuclear stain DAPI revealed Cyt-positive cells in various stages of mitosis confirming that the epithelial cells are proliferating after four days culture in LG.

Figure 4: Primary cultures of rat IMCD cells express cytokeratin, a marker for epithelial cells. After four days culture in LG, nuclei were stained with DAPI (blue) and most cells are labeled with an antibody to cytokeratin (green, **A**). Alternatively, few cells are labeled with an antibody to smooth muscle actin (green, **B**), expressed by myo-fibroblasts. Arrows indicate mitotic nuclei with characteristic condensed chromatin configurations. Magnification: 400x.

(A)

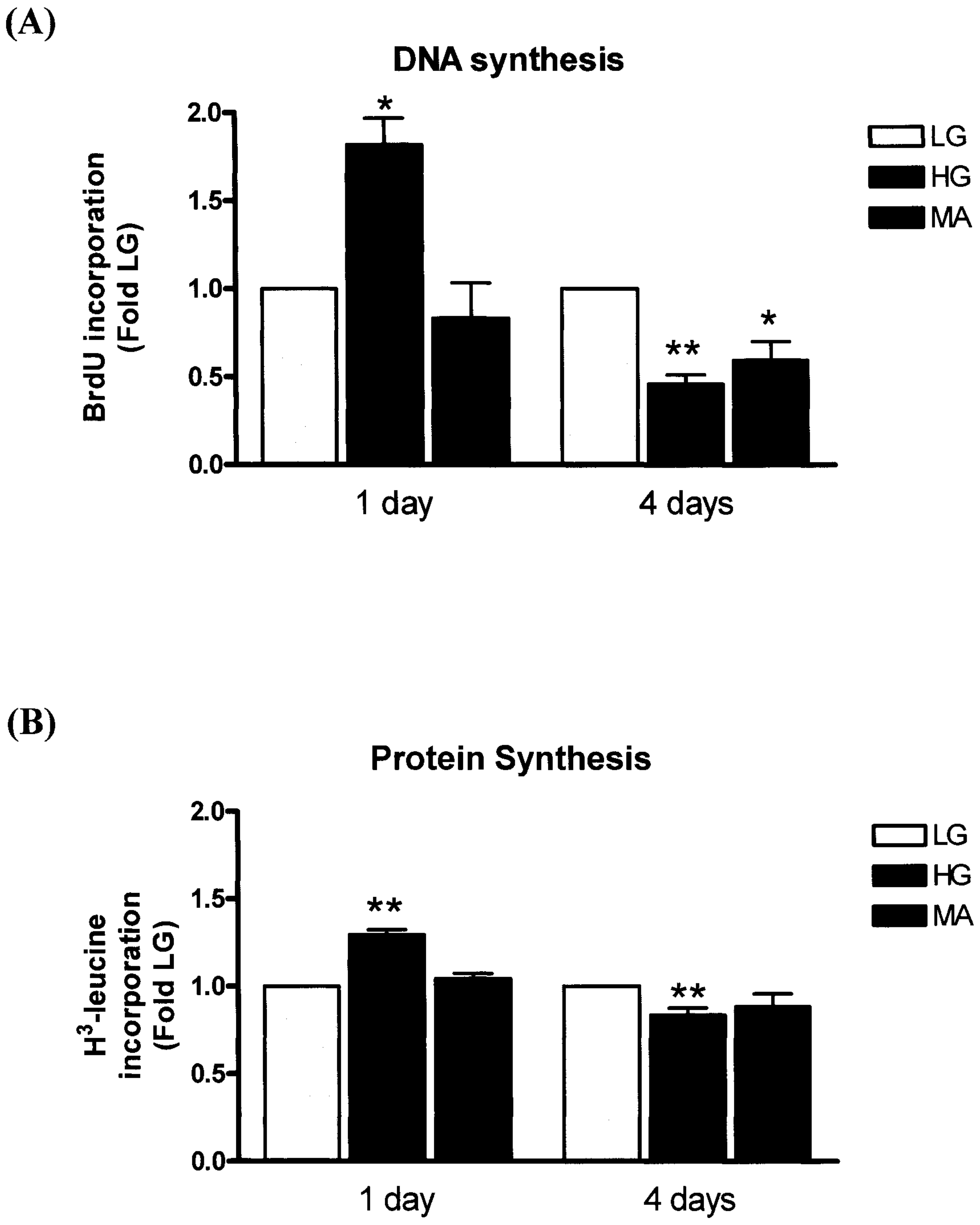


(B)

3.2: Effect of high glucose on DNA and protein synthesis.

To assess the response of IMCD cells to HG, DNA and protein synthesis were measured at after short-term exposure to HG (1 day) and long-term exposure to HG (4 days). For the 1 day time point, IMCD cells were grown three days in LG, then one more day in LG, HG, or MA; for 4 days glucose treatment, IMCD cells were grown four days in LG, HG or MA. As shown in **Figure 5 (A)** and **(B)**, after 1 day HG, BrdU and H^3-leucine incorporation were significantly 1.8 ± 0.2 and 1.3 ± 0.03 fold higher than LG, respectively, indicating that there is an increase in both DNA and protein synthesis. This response is characteristic of cell growth by hyperplasia, although we did not determine whether there was also an increase in cell number or proliferation, we will therefore refer to this result as a growth response. After 4 days HG, BrdU and H^3-leucine incorporation dropped significantly to 0.46 ± 0.05 and 0.84 ± 0.04 fold LG. This indicates that both DNA and protein synthesis decrease in IMCD cells with long-term exposure to HG, which may reflect an arrest in the cell cycle or cell death. The decrease in IMCD cell DNA and protein synthesis induced by HG was partially mimicked by MA treatment for 4 days, which dropped BrdU and H^3-leucine incorporation respectively to 0.60 ± 0.11 ($p<0.05$) and 0.88 ± 0.07(not significant) fold LG (**Figure 5 (A)** and **(B)**).

Figure 5: High glucose increases DNA and protein synthesis in rat IMCD cells after one day and decreases DNA and protein synthesis after four days treatment. To measure DNA synthesis, IMCD cells were plated on glass coverslips and cultured one or four days in LG, HG and MA. BrdU was then pulsed for 3 hrs and the percentage of DAPI labeled nuclei that were immunoreactive for anti-BrdU was determined by fluorescence microscopy. Separately, protein synthesis was measured by the addition of H^3-leucine for the last 24 hrs of treatment and the amount incorporated is expressed as fold LG. Values are means ± SEM; n=3-6. ** $p < 0.01$, *$p < 0.05$ vs. LG.


(A)
DNA synthesis
BrdU incorporation (Fold LG)
2.0
1.5
1.0
0.5
0.0
*
**
*
1 day
4 days
LG
HG
MA
(B)
Protein Synthesis
H^3-leucine incorporation (Fold LG)
2.0
1.5
1.0
0.5
0.0
**
**
1 day
4 days
LG
HG
MA

3.3: TUNEL positive nuclei increase in HG.

Since 4 days HG exposure results in decreased IMCD cell DNA and protein synthesis, it was hypothesized that this was coincident with an increase in the rate of cell death by apoptosis. This hypothesis was tested with the TUNEL reaction which labels the free ends of DNA fragments in cells undergoing apoptosis. Four days HG increased the percentage of TUNEL positive nuclei, which revealed morphologic changes characteristic of apoptotic cells, such as chromatin condensation and formation of small apoptotic bodies, as illustrated in **Figure 6**. Although the basal rate of cell death by apoptosis in LG is variable between sample groups (**Table 1**), the average increase induced by HG in each sample group is significantly 1.87 fold higher than LG (shaded region in **Table 1**). It is also noted that the effect of HG is partially mimicked by MA, indicating that the apoptotic signal induced by HG may in part be due to an osmotic effect.

To characterize the apoptotic response of IMCD cells to HG, Z-VAD-FMK, a pan-caspase inhibitor (Slee, 1996), was administered for the last 24 hrs of LG, HG, and MA treatment. In all groups, Z-VAD-FMK significantly reduced the percentage of TUNEL positive nuclei (**Table 1**), indicating that the apoptotic effect of HG is through caspase activation in these cells.

Figure 6: Apoptotic nuclei labeled by the TUNEL assay after four days HG, which exhibit distinct morphological changes characteristic of apoptosis shown by the DAPI stain. Arrows indicate condensed chromatin and apoptotic bodies that are also stained positive by the TUNEL reaction. Both pictures are from the same field of view at 400x magnification.

4 days HG

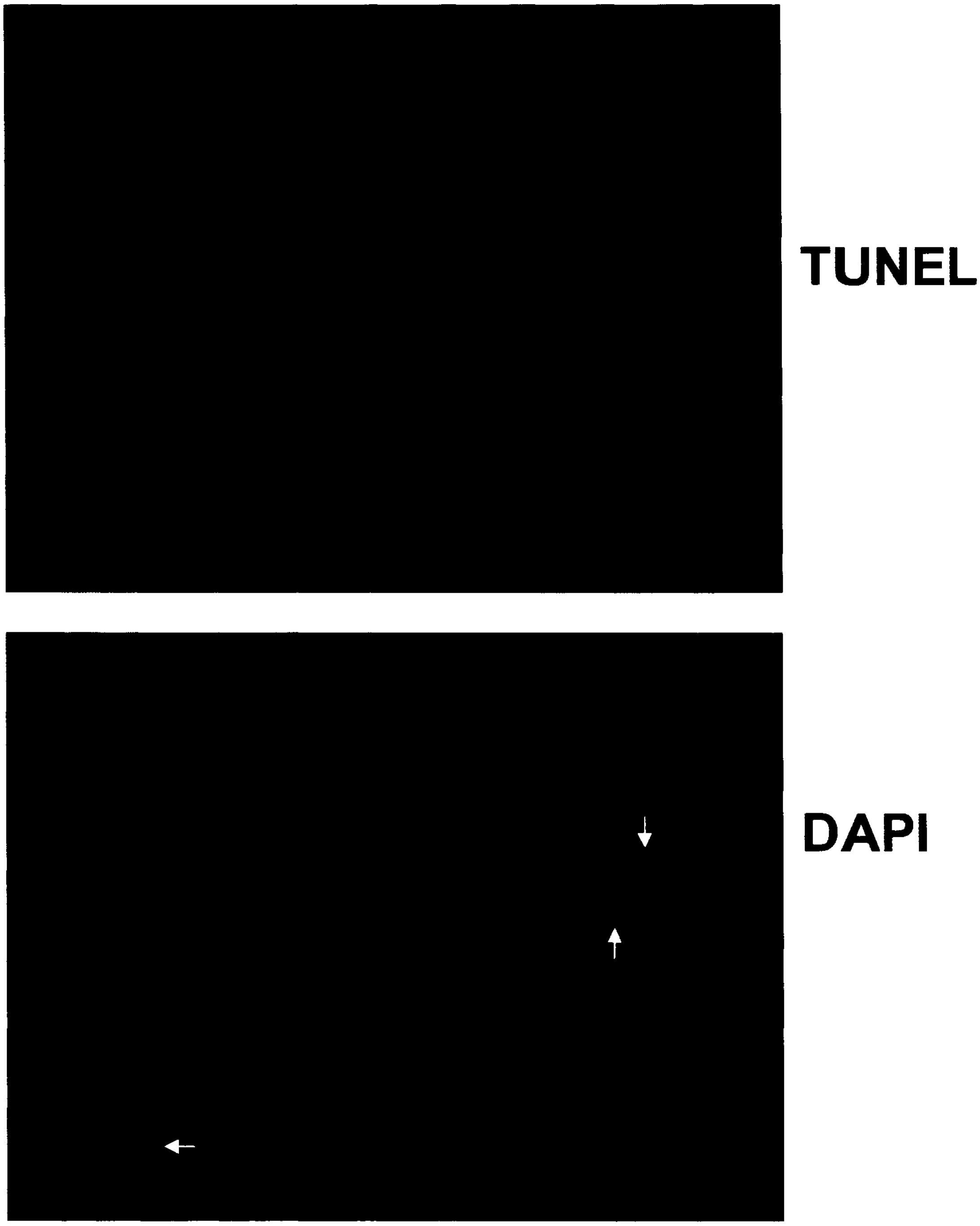


Table 1: The percentage of TUNEL positive nuclei in IMCD cells treated for four days in LG, HG and MA, also expressed as fold LG, highlighted by the shaded column. Samples 1 to 3 were treated without (-) or with (+) the pan-caspase inhibitor, Z-VAD-FMK. $*p < 0.05$ vs. LG set at 1.0.

	Percent TUNEL positive							
	LG		**HG**			**MA**		
Sample	(-)	(+)	(-)	Fold LG	(+)	(-)	Fold LG	(+)
1	7.83	1.95	12.33	1.57	4.14	7.11	0.91	2.88
2	10.38	3.15	18.96	1.82	5.66	20.01	1.92	3.89
3	8.66	1.23	12.54	1.45	0.89	9.95	1.15	2.54
4	3.28		8.61	2.62		5.23	1.59	
Average ± SEM				1.87 ± 0.26*			1.39 ± 0.23	

3.4: HG increases caspase-3 and/or -7 activity.

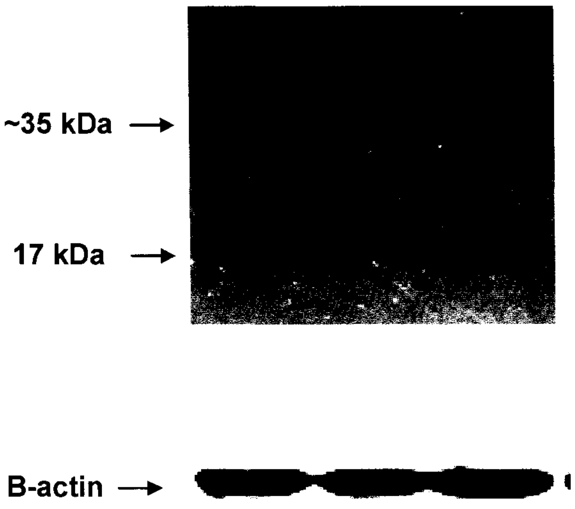
To further investigate the apoptotic response induced by HG, protein levels of caspase-3, a key enzyme in the late stage of apoptosis, was measured. IMCD cells were grown four days in LG, HG and MA, and processed for western blot. The polyclonal antibody used is specific for the large fragment of activated caspase-3 (17 kDa), and may also recognize the full length caspase-3 zymogen (~35kDa), **Figure 7(A)**. Although the active fragment was expressed in all treatment groups, the levels of cleaved caspase-3 did not significantly change. We decided to perform a caspase activity assay as a more sensitive measure of the enzymes' proteolytic activity. Treatment of IMCD cells with HG for four days increases the cleavage of the caspase-3 and -7 substrate, Ac-DEVD-pNA, which yields a coloured product. The absorbance of the coloured product from the HG sample was significantly 1.83 ± 0.26 fold higher than LG, and was not mimicked by MA, **Figure 7(B)**. The pan-caspase inhibitor Z-VAD-FMK was added for the last 24 hrs of treatment and decreased the activity of capase-3 and/or caspase-7, confirming that caspases are activated for the proteolytic cleavage of Ac-DEVD-pNA.

3.5: *HG increases the Bax/Bcl-2 ratio.*

To determine if the apoptotic response of IMCD cells grown four days in HG involved Bax and Bcl-2, their protein expression was measured using specific monoclonal antibodies. HG increases the expression ratio of Bax to Bcl-2, indicating that there is an increased probability for apoptotic signaling through the mitochondria. This effect appears to occur through a decrease in Bcl-2 as shown by a representative western blot in **Figure 8**.

Figure 7: Caspase-3 and/or -7 activity increases in IMCD cells exposed to HG for 4 days, without a change in caspase-3 protein expression. (**A**): Representative western blot using anti-cleaved caspase-3 (17 kDa), which may also detect full length caspase-3 (~35 kDa). Lanes are (from left to right): LG, HG, MA. (**B**): Expressed as fold LG, the amount of coloured product, measured at 405 nm, after IMCD cell lysates were incubated overnight with Ac-DEVD-pNA, a substrate for caspase-3 and -7. Z-VAD-FMK was added for the last 24 hrs of treatment. Values are means ± SEM; n=3-4. $^*p < 0.05$ vs. LG, ** $p < 0.01$ vs. (-) without Z-VAD-FMK.

(A)


~35 kDa →
17 kDa →
B-actin →

(B)

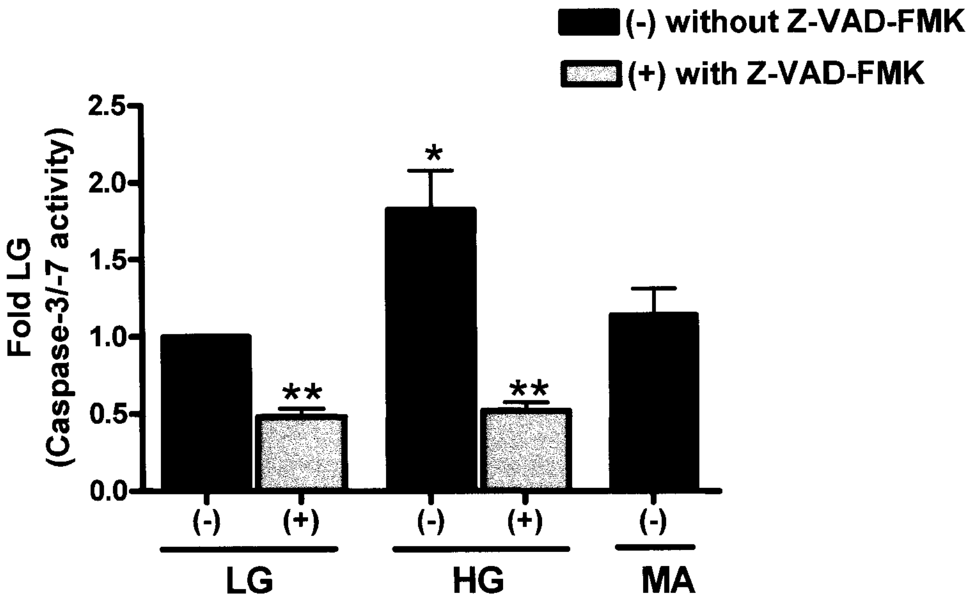
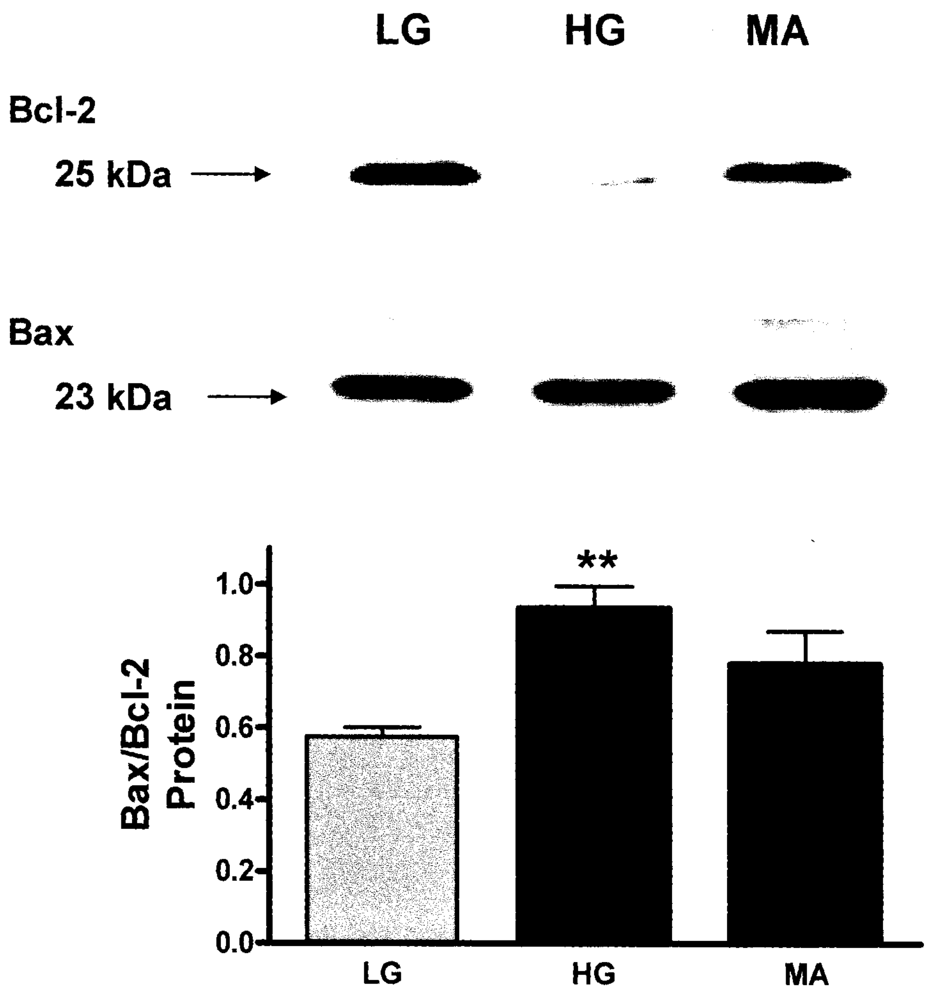

(-) without Z-VAD-FMK
(+) with Z-VAD-FMK
Fold LG
(Caspase-3/-7 activity)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
**
(-)
(+)
(-)
(+)
(-)
LG
HG
MA

Figure 8: HG increases the ratio of Bax to Bcl-2 protein expression in IMCD cells after four days treatment. A representative western blot of the bands corresponding to Bax and Bcl-2 is provided in (A) and the ratio of the densitometry of Bax/Bcl-2 is plotted in (B). Values are means ± SEM; n=3. ** $p < 0.01$.


LG
HG
MA
Bcl-2
25 kDa
Bax
23 kDa
Bax/Bcl-2 Protein
1.0
0.8
0.6
0.4
0.2
0.0
**
LG
HG
MA

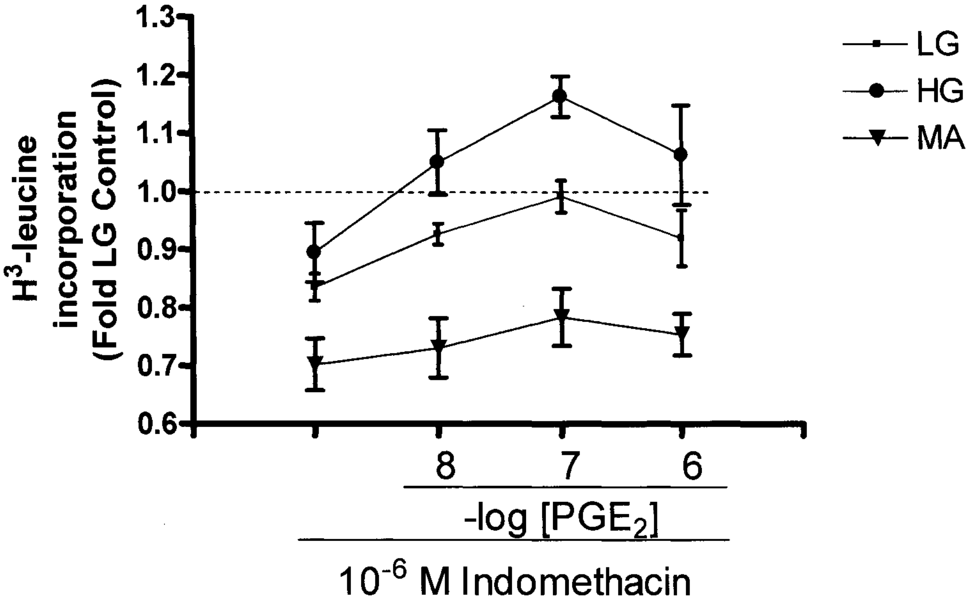
3.6: Effect of PGE_2 and indomethacin on DNA and protein synthesis.

To determine if PGE_2 may play a role in the HG-induced changes in DNA and protein synthesis in IMCD cells, the effect of PGE_2 and indomethacin on these parameters was investigated. In LG, treatment of IMCD cells with 10^{-6} M indomethacin for 24 hrs decreased H^3-leucine incorporation (0.83 ± 0.02 fold LG control, **Figure 9(A)**). Co-incubation with different PGE_2 concentrations (10^{-6}, 10^{-7}, and 10^{-8} M) increases the rate of protein synthesis to control levels. The response to PGE_2 is bell-shaped with a significant attenuation of the inhibitory effect of indomethacin on H^3-leucine incorporation with 10^{-7} M PGE_2 (0.99 ± 0.03 fold LG control, $p < 0.05$ vs. indomethacin). In 1 day HG, a similar trend is observed, with indomethacin treatment decreasing protein synthesis to levels below that of LG control, and each concentration of PGE_2 stimulates a greater maximal response than in LG. However, after four days HG, the response to PGE_2 is diminished (**Figure 9(B)**); the amount of H^3-leucine incorporation stimulated by 10^{-8} and 10^{-7} M PGE_2 in HG is significantly less than in LG ($p < 0.05$).

Since 10^{-7} M PGE_2 stimulated the greatest increase in protein synthesis in LG, this concentration was used to analyze DNA synthesis. Similar to its effect in LG, treatment of IMCD cells with 10^{-6} M indomethacin for 24 hrs significantly decreased BrdU incorporation ($p < 0.05$ vs. LG control) and coincubation with 10^{-7} M PGE_2 increased BrdU incorporation to control levels ($p < 0.05$ vs. 10^{-6} M indomethacin). This response to PGE_2 is diminished in four days HG and also in four days MA (**Figure 10**). It is apparent that in LG, PGE_2 promotes IMCD cell growth, attenuating the inhibitory effect of indomethacin. With short-term HG treatment this response is amplified, but long-term HG treatment diminished the response.

Figure 9: PGE_2 ameliorates the inhibitory effect of indomethacin on protein synthesis, an effect that is diminished in four days HG. IMCD cells were cultured either one day **(A)** or four days **(B)** in LG, HG or MA and were treated for the last 24 hrs with H^3-leucine and 10^{-6} M indomethacin alone, or in the presence of 10^{-6}, 10^{-7}, and 10^{-8} M PGE_2. The amount of H^3-leucine incorporated is expressed as fold LG control which is represented by the dashed line. Values are means ± SEM; n = 3-4.

(A)


H^3-leucine incorporation (Fold LG Control)
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
8
7
6
-log [PGE_2]
10^{-6} M Indomethacin
LG
HG
MA

(B)

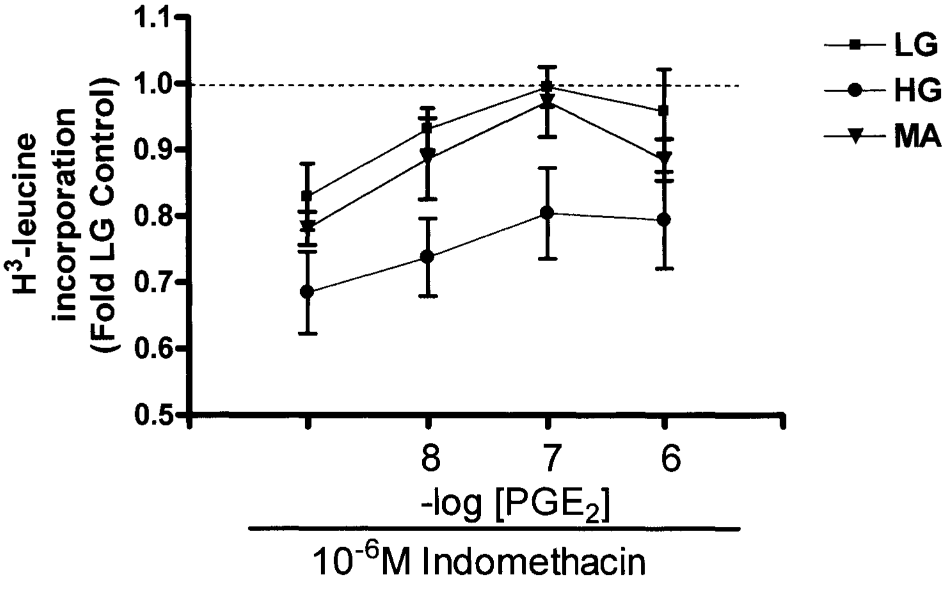
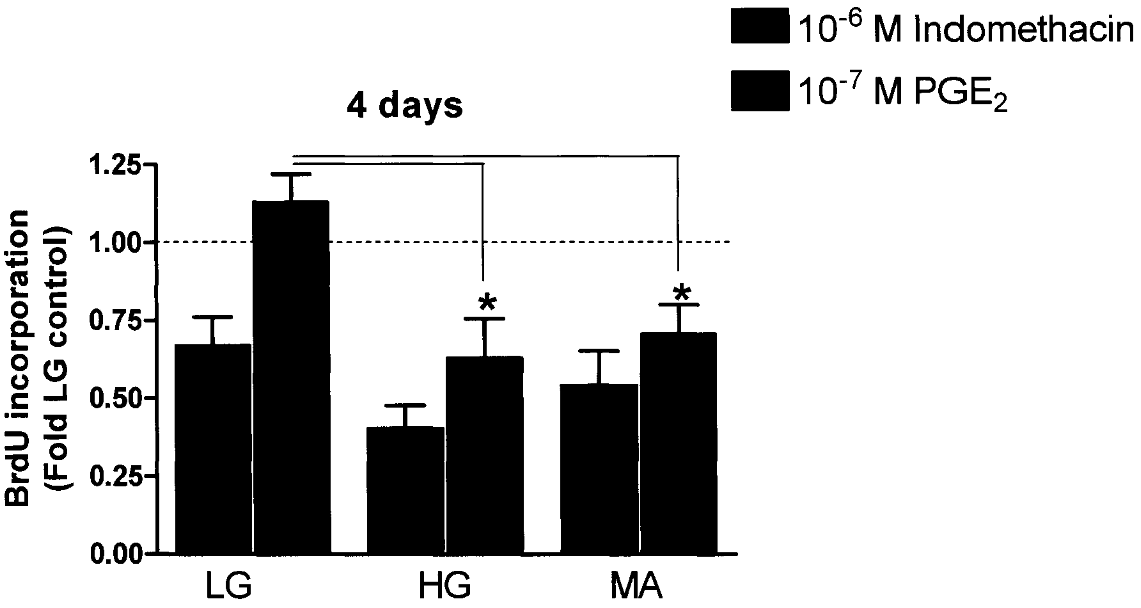

H^3-leucine incorporation (Fold LG Control)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
8
7
6
-log [PGE_2]
10^{-6}M Indomethacin
LG
HG
MA

Figure 10: PGE_2 stimulates DNA synthesis in the presence of indomethacin, and four days HG treatment diminishes this response. IMCD cells were plated on glass coverslips and cultured four days in LG, HG and MA. 10^{-6} M indomethacin alone, or in the presence of 10^{-7} M PGE_2, was added for the last 24 hrs. BrdU was then pulsed for 3 hrs and the percentage of DAPI labeled nuclei that were immunoreactive for anti-BrdU was determined by fluorescence microscopy and is expressed as fold LG control, represented by the dashed line. Values are means ± SEM; $n = 4$. $*p < 0.05$.


10^{-6} M Indomethacin
10^{-7} M PGE$_2$
4 days
BrdU incorporation (Fold LG control)
1.25
1.00
0.75
0.50
0.25
0.00
*
*
LG
HG
MA

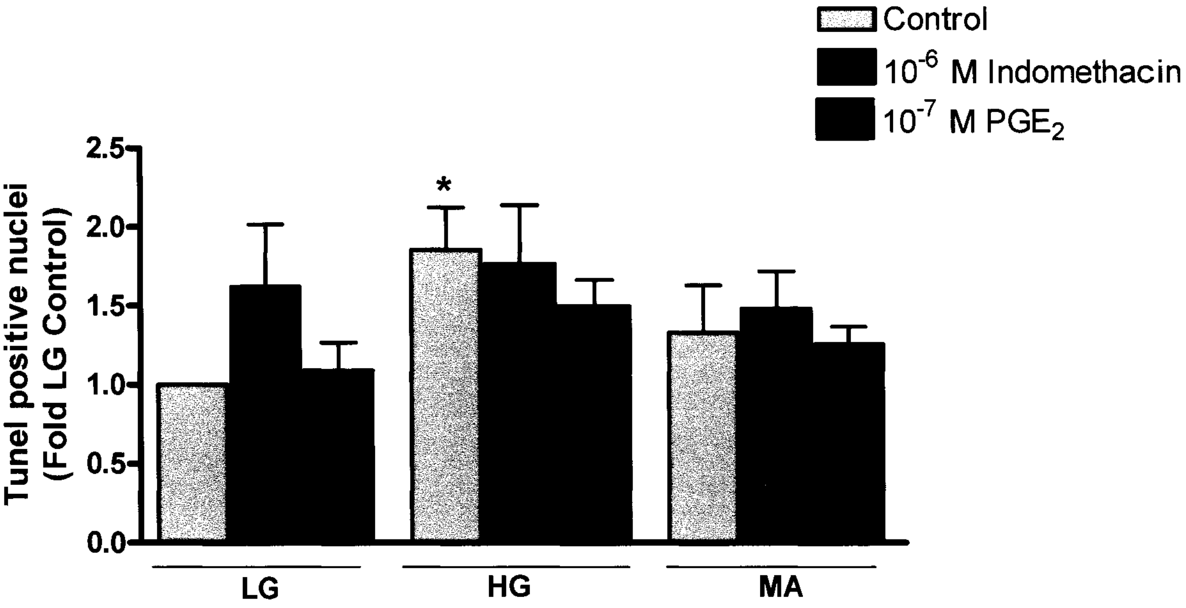
3.7: Effect of PGE_2 and indomethacin on the apoptotic response of IMCD cells.

Since indomethacin decreases DNA and protein synthesis in IMCD cells, and co-incubation with PGE_2 prevents this effect, it was hypothesized that indomethacin may increase the rate of apoptosis in IMCD cells and PGE_2 treatment would reverse it. Also, it was hypothesized that the diminished growth response to PGE_2 with long-term HG exposure may reflect a diminished protective effect of PGE_2 on IMCD apoptosis.

IMCD cells were treated 24 hrs with 10^{-6} M indomethacin alone, or in the presence of 10^{-7} M PGE_2 and as shown in **Figure 11**, neither indomethacin nor PGE_2 significantly altered the percentage of TUNEL positive cells in LG. Additionally, neither indomethacin nor PGE_2 affected the apoptotic response induced by four days HG.

To support these results, we also evaluated the effect of indomethacin and PGE_2 on caspase-3/-7 activity in LG, HG, and MA, which produced a similar result as described above (data not shown). Therefore, decreased DNA and protein synthesis induced by indomethacin is not concomitant with an increase in IMCD cell death by apoptosis and PGE_2 does not influence the apoptotic response of IMCD cells in LG, HG, or MA.

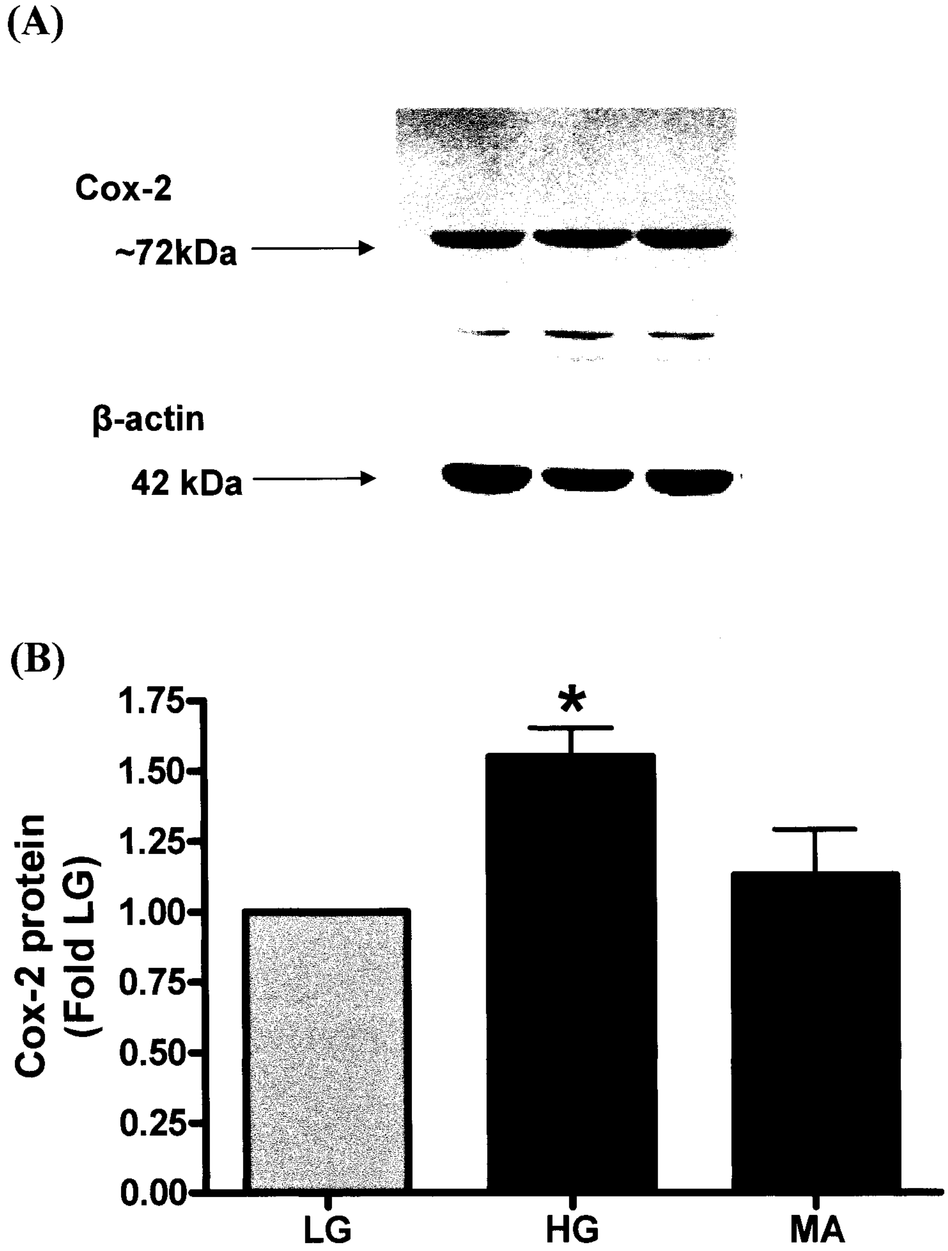
Figure 11: Indomethacin and PGE_2 do not alter the apoptotic response of IMCD cells treated for four days in LG, HG, or MA. During the last 24 hrs of treatment, 10^{-6} M indomethacin was added alone or in the presence of 10^{-7} M PGE_2. The cells were then fixed and incubated with terminal-deoxy-transferase and biotin labeled dUTP for the TUNEL reaction. After incubation with streptavidin-Cy2, the percentage of DAPI stained nuclei that were TUNEL positive was determined by fluorescence microscopy and is expressed as fold LG control. Values are means ± SEM; n=4. $*p < 0.05$.


Control
10^-6 M Indomethacin
10^-7 M PGE2
Tunel positive nuclei (Fold LG Control)
2.5
2.0
1.5
1.0
0.5
0.0
*
LG
HG
MA

3.8: COX-2 levels are increased in high glucose.

In an attempt to elucidate the mechanism by which HG diminishes the growth response induced by PGE_2, we measured the expression of the inducible form of the COX enzyme, COX-2. In a previous study from our laboratory, Nasrallah (2003) showed an increase in the production of PGE_2 after 4 days in the presence of 37.5mM glucose (compared to a control level of 17.5mM), associated with an increase in COX-2 protein expression. Since the current study uses different glucose concentrations, which are closer to blood glucose levels in some rodent models of diabetes, it is necessary to test our previous results. Western blot was performed on IMCD cell protein cultured 4 days in LG, HG, and MA using a murine COX-2 antibody. **Figure 12** illustrates that in HG, COX-2 expression is significantly 1.55 ± 0.10 fold higher than in LG, comparable to our previous results. This suggests that PGE_2 production is also increased in IMCD cells cultured at the glucose concentrations used in the current study.

Figure 12: COX-2 protein is increased in rat IMCD exposed to high glucose for four days. (A): representative western blot of COX-2 (~72 kDa) and β-actin (42 kDa). Lanes (left to right): LG, HG, MA. (B): Densitometric analysis of COX-2/β-actin expressed as fold LG. Values are means ± SEM; n=3. *$p < 0.05$.


(A)
Cox-2
~72kDa
β-actin
42 kDa
(B)
Cox-2 protein
(Fold LG)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
*
LG
HG
MA

3.9: High glucose decreases EP_4 mRNA but does not alter PGE_2-stimulated cAMP.

Since HG may decrease PGE_2 signaling through one of its EP receptors, resulting in a diminished growth response, we investigated the effect of four days HG on EP_4 receptor expression and PGE_2-stimulated cAMP generation in the current study. EP_4 mRNA levels from IMCD cells grown for four days in LG, HG, or MA were measured by real time RT-PCR. HG significantly reduced EP_4 mRNA, which was not mimicked by MA (**Figure 13**). These results are consistent with a previous report from our laboratory which demonstrated that alterations in COX levels resulted in a change in EP receptor RNA expression (Nasrallah, 2001).

To determine if PGE_2 signaling via EP_4 is altered by HG, cAMP levels were measured in response to 10 min stimulations with 10^{-6}, 10^{-7}, and 10^{-8} M PGE_2 in LG, HG, and MA. Although basal cAMP levels were significantly attenuated with four days HG treatment, the levels of PGE_2-stimulated cAMP in HG were not significantly different from those in LG, **Figure 14**. This finding indicates that PGE_2 signaling through the EP_4/cAMP pathway is intact in HG, despite a decrease in EP_4 RNA.

Figure 13: High glucose decreases EP_4 RNA in rat IMCD cells. Real time RT-PCR was performed on DNase treated RNA from IMCD cells grown four days in LG, HG, and MA. EP_4 expression was normalized to GAPDH levels in each RNA sample. Values are expressed as means ± SEM; n=5 for LG and HG, n=4 for MA.*$p<0.05$

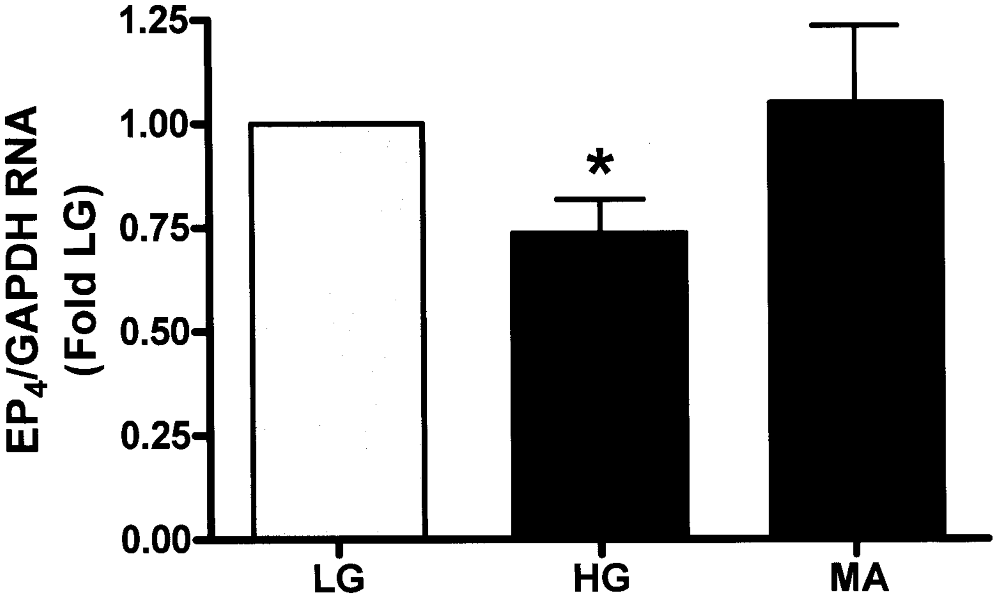
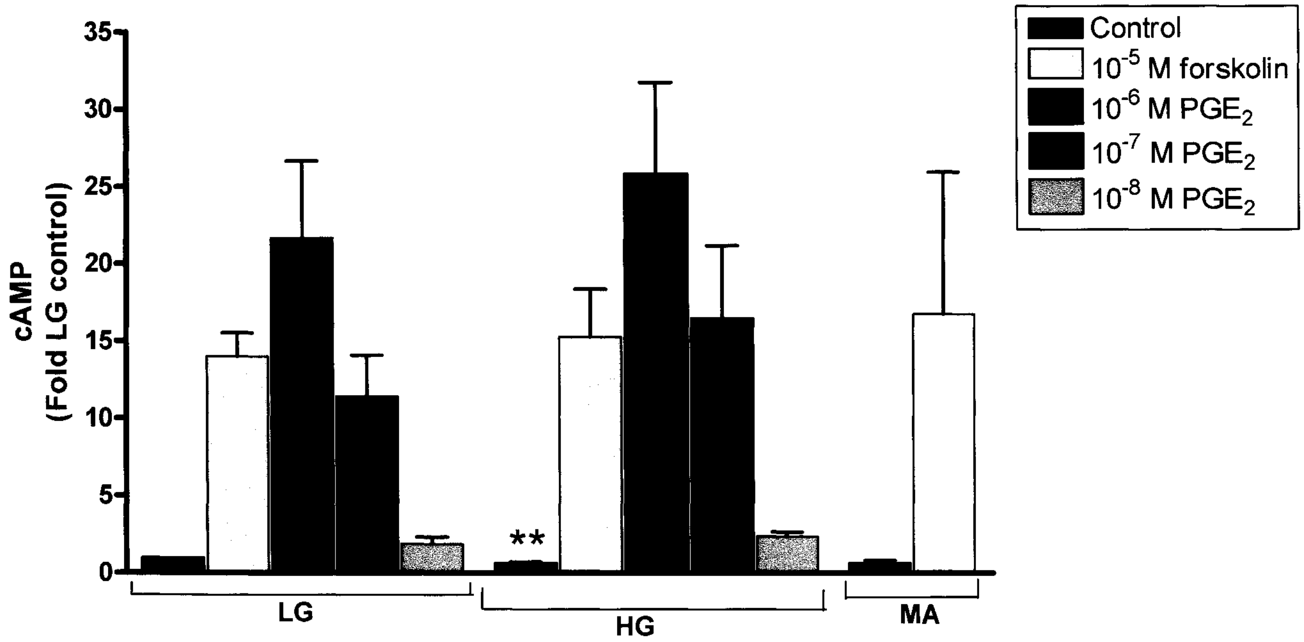

EP4/GAPDH RNA
(Fold LG)
1.25
1.00
0.75
0.50
0.25
0.00
*
LG
HG
MA

Figure 14: Basal levels of intracellular cAMP are decreased in rat IMCD cells exposed to high glucose for four days, however cAMP levels stimulated by PGE_2 are unaltered. Cellular cAMP levels were determined by a ^3H cAMP competitive binding radioassay and are expressed as fold LG control. Values are means ± SEM; n = 3-9. **p <0.01 vs. LG control.


cAMP
(Fold LG control)
35
30
25
20
15
10
5
0
**
LG
HG
MA
Control
10^-5 M forskolin
10^-6 M PGE2
10^-7 M PGE2
10^-8 M PGE2

3.10: Exogenous cAMP does not alter protein synthesis or caspase activity.

In order to investigate the signaling mechanism of PGE_2-stimulated protein synthesis, IMCD cells were treated 24 hrs with 500 μM dibutyryl cAMP (dbcAMP), a cell permeable cAMP analog, in the presence of indomethacin. Also, we assessed the effect of dbcAMP on caspase-3/-7 activity in the presence of indomethacin for 24 hrs. As shown in Table 2, dbcAMP did not significantly alter protein synthesis or caspase-3/-7 activity. It appears that the effect of PGE_2 on protein synthesis occurs through a cAMP-independent pathway. Furthermore, cAMP does not appear to alter the apoptotic response of IMCD cells, however, it should be noted that we did not test different concentrations of dbcAMP or incubation times which may affect our results.

Table 2: DbcAMP, a cell permeable analog of cAMP, does not alter protein synthesis or caspase-3/-7 activity in the presence of indomethacin in rat IMCD cells. Each stimulant was applied for 24 hrs and PGE_2 and dbcAMP were in the presence of 10^{-6} M indomethacin. Protein synthesis was measured by H^3-leucine incorporation and the colorimetric caspase-3/-7 assay was performed as described previously. The results are expressed as fold LG control. $*p < 0.05$ vs. LG control, $^{\neq}p < 0.05$ vs. 10^{-6} M Indomethacin.

Stimulant	Protein Synthesis, Fold LG control (n=4)	Caspase-3/-7 Activity, Fold LG control (n=3)
10^{-6} M Indomethacin	0.83 ± 0.05 *	1.31 ± 0.16
10^{-7} M PGE_2	1.03 ± 0.04 ≠	1.09 ± 0.13
500 μM dbcAMP	0.76 ± 0.07 *	1.10 ± 0.09

4.0: Discussion

4.1: Growth response of IMCD cells to high glucose treatment.

The pathophysiology of DN is characterized by alterations in renal cell growth, and in this thesis we provide results indicating that HG alters the growth of first passage rat IMCD cells. Specifically, HG treatment induces a biphasic growth response in IMCD cells with an increase in DNA and protein synthesis after 1 day HG and a decrease in DNA and protein synthesis after 4 days HG. This may indicate that there is an increase in the number of proliferating cells with short-term high glucose treatment, however, we did not directly measure IMCD cell proliferation. An increase in DNA content and cellular proliferation induced by 24 hrs high glucose has been shown in mesangial cells (Wolf, 1992), and in MDCK cells Yang, *et al.* (1998) showed that after a minimum of two days high glucose, cellular proliferation increases and is associated with an increase in DNA synthesis and protein content. Therefore, it is likely that the augmented IMCD cell DNA and protein synthesis observed in this thesis is associated with an increase in the number of proliferating cells with short-term HG exposure.

After 4 days HG exposure, we observed a decrease in the rate of DNA and protein synthesis which could be related to an arrest in the cell cycle, and/or an increase in cell death (Rocha, 2001). Our results indicate that the latter occurs, and will be discussed in the next section. Although we did not focus on cell cycle arrest in IMCD cells, the observed decrease in protein synthesis contradicts the possibility of cellular arrest in G_1 and hypertrophic growth, which are associated with an increase in protein synthesis (Shankland, 2000). Our results are consistent with a study by Yancey, *et al.* (1990), who demonstrated that 17 mM – 50 mM glucose treatment for 14 days dose-dependently decreased cellular survival and growth of a rabbit renal inner medulla cell line (GRB-PAP1). However, in renal mesangial cells, a decrease in cellular proliferation associated with an increase in hypertrophy was observed after 72 hrs high glucose (Wolf, 1992), and in MDCK cells, it was shown that cellular proliferation is sustained after three days high glucose (Yang, 1998). This may indicate a difference between renal cell types since mesangial cells are a type of smooth muscle cell, and the MDCK cell line is an immortalized type of renal distal tubule cells. Additionally, an advantage to our cell model is that they have not been immortalized, which has been shown to alter IMCD proliferation and osmotic tolerance in culture (Zhang, 2002).

An important implication of our study is that short-term high glucose increases and long-term high glucose decreases IMCD proliferation in conditions that mimic hyperglycemia, like diabetes, yet glucose-induced alterations of IMCD proliferation have yet to be investigated in vivo. This could be accomplished by staining for proliferating cells in the inner medullary region of the kidney in the early and late stages of diabetes and control for the effect of hyperglycemia with insulin treatment. According to the results in this thesis, it would be hypothesized that early stage diabetes would increase the number of proliferating IMCD cells and there would be a decrease in the later stages of diabetes. Insulin treatment, to control blood glucose levels during diabetes, would be expected to prevent any alterations in IMCD cell proliferation.

Early in diabetes, the observed increase in renal mass could be mediated in part by high glucose-induced IMCD proliferation. Renal growth is proposed to precede any change in renal function and as reviewed in a recent publication (Vallon, 2003), an increase in tubular mass will increase the amount it transports and alter physiological solute levels and glomerular feedback mechanisms which control glomerular filtration rates. Although the contribution of IMCD growth to altered solute and water excretion in the diabetic kidney has not been investigated, the importance of the IMCD in the fine-tuning of urine concentration and physiological solute and water levels warrants attention. Additionally, it has been shown that, in certain physiological conditions, DNA damage is high in normal mouse inner medullary cells in vivo and low rates of cellular proliferation is thought to promote cell survival by minimizing the replication of damaged DNA (Zhang, 2004). Therefore, an increase in IMCD DNA and protein synthesis by high glucose may increase the replication and translation of damaged DNA in these conditions and increase the production of dysfunctional IMCD proteins. This indicates a means by which IMCD function could be altered in the early stages of diabetes.

4.2: Proapoptotic response of IMCD cells exposed to high glucose.

Prolonged exposure to high glucose could attenuate any further alteration in IMCD dysfunction by decreasing IMCD cell proliferation. In the present study, we generated results indicating that a decrease in the IMCD cell growth response is concomitant with an increase in the apoptotic response of IMCD cells after four days high glucose. In the kidney, apoptosis is a regulated mechanism of cell death to get rid of cells that are in excess, in the way, or potentially dangerous (Hengartner, 2000). Although increased apoptosis in the IMCD may attenuate harmful effects caused by short-term high glucose, it has been demonstrated that even small levels of apoptosis over time can contribute to tubular atrophy and the progression of the late stages of DN (Hauser, 2002).

The detrimental effect of high glucose on renal cells is well documented and has been shown to induce apoptosis in glomerular mesangial cells (Mishra, 2005), proximal tubule cells (Allen, 2003), and specifically, chronic exposure to high glucose induces apoptosis in an endothelial cell line (Varma, 2005). Similarly, our results show that with four days high glucose exposure, concomitant with a decrease in cellular proliferation, there is an increase in the rate of apoptosis in IMCD cells through a caspase dependent pathway. It is also likely that the effector caspases-3 and/ or -7 play an important role since we measured an increase in their activity.

Although we did not focus on elucidating which apoptotic pathway is activated by high glucose exposure, it is likely that high glucose acts as a death stimulus through the mitochondrial pathway, since an increase in the Bax to Bcl-2 ratio was shown in this thesis. This result is in contrast to a previous study that did not detect Bcl-2 protein expression in freshly isolated IMCD tubules exposed to high glucose for 3 hrs (Kikuchi, 2002). This discrepancy is likely caused by a difference in antibodies indicating that the anti-Bcl-2 antibody used in this thesis is more sensitive and can detect Bcl-2 expression. This theory is supported by a study that detected Bcl-2 protein expression in the mIMCD-3 cell line (Michea, 2002). Alternatively, our results may indicate a difference between cell culture and in vivo conditions, such that Bcl-2 protein expression may be induced or unconstrained in culture. Despite this difference, the increased Bax:Bcl-2 in HG is thought to alter mitochondrial function, releasing proapoptotic molecules that activate downstream caspases culminating in IMCD cell death by apoptosis (Michea, 2002).

Another important implication of our study is that IMCD cell apoptosis could lead to inner medulla injury in the kidney in diabetes. A recent study by Kumar, *et al.* (2004b), showed TUNEL positive tubular cells in the inner medulla of STZ-diabetic rats, that was absent in insulin treated animals. Additionally, Bamri-Ezzine, *et al.* (2003) revealed that caspase-3 activation was apparent in the renal distal tubules of the STZ-diabetic rat. A recent study showed that proximal tubule cell apoptosis in human diabetic kidneys was involved in tubular epithelial cell degeneration, and was marked by caspase-3 activation (Susztak, 2005). These results provide evidence that high glucose can induce IMCD apoptosis in vivo, which could result in tubule injury and a decline in IMCD function.

4.3: IMCD cell apoptosis is unaltered by PGE$_2$.

In the kidney, PGE$_2$ protects against cell death through a number of proposed mechanisms including changes in toxicant metabolism, regulation of tight junctions, increased membrane stability, inhibition of spontaneous apoptosis, and enhanced tissue regenerative capacity (Jia, 2004). In the present study, we found that neither inhibition of PGE$_2$ synthesis, through the administration of indomethacin, nor the exposure of IMCD cells to PGE$_2$ in the presence of indomethacin, altered the apoptotic response of IMCD cells in LG or HG. These results suggest that PGE$_2$ does not alter spontaneous apoptosis in LG, nor does it protect against HG induced apoptosis. This is consistent with a study in a proximal tubule cell line where PGE$_2$ protected against agents that induced oncotic/necrotic cell death (where cells swell and burst), but did not protect against apoptotic cell death (Jia, 2004). Additionally, it was shown that chronic COX-2 inhibition did not increase apoptosis in the rat IMCD under normal conditions, but did increase IMCD apoptosis after the rat was water deprived (Neuhofer, 2004). This indicates that COX-2 metabolites can protect against osmotic stress-induced apoptosis in vivo.

Another possibility that may account for the ineffectiveness of PGE_2 to alter the apoptotic response induced by HG is that PGE_2-signaling is altered in IMCD cells chronically exposed to HG, thereby preventing an anti-apoptotic effect of PGE_2. This theory could be tested by assessing the protective effect of PGE_2 on IMCD cells exposed to other apoptotic agents in LG and HG. If PGE_2 did not warrant any protection against chemically induced apoptosis, it would appear that PGE_2 does not play any role in IMCD cell apoptosis.

4.4: Growth response of IMCD cells to PGE_2 in the presence of indomethacin.

From our results, PGE_2 appears to have a protective effect on IMCD cell growth in the presence of indomethacin in LG. It is possible that indomethacin is blocking the endogenous action of PGE_2 on IMCD growth and when PGE_2 is added to the media, this growth response is replenished. However, this role for PGE_2 has not been thoroughly investigated in the IMCD in vivo. The only study, to our knowledge, that addresses this issue was by Harris (1989), who showed that EGF stimulated PGE_2 synthesis and DNA synthesis in primary cultures of rat IMCD cells and indomethacin administration did not alter the effect of EGF on proliferation. This is in contrast to our results however the stimulatory effect of EGF on IMCD DNA synthesis may have masked any effect by PGE_2.

Although previous studies have reported that indomethacin treatment does not reduce IMCD cell number (Cai, 2003), our study employs a more sensitive or immediate method by measuring DNA and protein synthesis. Also, indomethacin has been shown to have a toxic effect on IMCD cells, reducing cell number independent of COX inhibition (Cai, 2003), and in oral epithelial cancer cells, COX-2 inhibition also decreases proliferation, without altering apoptosis, by a PGE_2-dependent and independent mechanism (Minter, 2003). In this thesis, PGE_2 may be protecting against a toxic insult on the IMCD cells by indomethacin and an implication from these results is that PGE_2 has the capacity to protect against nephrotoxic injury in the IMCD, similar to its actions in other tubule segments, like the proximal tubule (Jia, 2004) and the distal tubule (Nishimura, 2004).

Growth inhibition induced by indomethacin has been shown to be accompanied by arrest in the G_0/G_1 phase of the cell cycle (Minter, 2003), and up-regulation of the CKI $p27^{Kip1}$ (Huang, 2002). In this thesis, indomethacin may cause arrest in G_0 since it decreases both DNA and protein synthesis in the IMCD cells. Both calcium and cAMP, stimulated by PGE_2, play a pivotal role as mediators of cell cycle progression. The calcium signaling system is believed to activate the immediate-early genes responsible for inducing resting cells to reenter the cell cycle and, on the other hand, the contribution of the cAMP signaling pathway to cell growth seems to be dependent on both cell type and cell cycle status (Sanchez, 2003).

4.5: PGE$_2$-stimulated proliferation is diminished in HG.

In the presence of indomethacin, and after four days HG treatment, DNA and protein synthesis stimulated by PGE$_2$ in IMCD cells is significantly lower than in LG. The effect of PGE$_2$ is diminished with chronic exposure to HG and this may indicate a means by which glucose exerts its detrimental effect on IMCD survival. Since PGE$_2$ is known to be a cytoprotective factor in the kidney, specifically in collecting duct cells (Nishimura, 2004), a defect in PGE$_2$-mediated protection could leave the cell susceptible to injury. This could explain why PGE$_2$ is unable to protect against HG-induced apoptosis, however, as explained in section **4.3**, more experiments are needed to test this hypothesis.

A mechanism by which HG treatment may decrease the effect of PGE$_2$ on proliferation is through a change in the PGE$_2$/EP receptor signaling system. Since the cAMP signaling pathway is linked to many downstream events, like gene expression, inhibition or stimulation of cellular proliferation (Yamaguchi, 2004), and apoptosis (Nishihara, 2003), a demise or enhancement of this pathway may suggest a possible mechanism by which chronic HG treatment diminishes PGE$_2$-stimulated proliferation and increases IMCD cell susceptibility to apoptotic death. However, despite a reduction in EP$_4$ mRNA expression, we did not observe any change in PGE$_2$-stimulated cAMP levels after four days chronic HG treatment. The decrease in EP$_4$ mRNA expression is opposite to what we observed in a previous study using 37.5 mM glucose for four days (Nasrallah, 2003), however this result may be attributable to a difference in analytical techniques (real time RT-PCR vs. Northern blot analysis) or a difference in the glucose concentrations used for low (7.5 vs. 17.5 mM) and high (27.5 mM and 37.5 mM) glucose treatment. Consistent with our previous results using 37.5 mM glucose for four days, we observed an increase in COX-2 expression, which is associated with increased PGE_2 synthesis (Nasrallah, 2003). Therefore, down-regulation of EP_4 receptor expression represents a putative mechanism to compensate for augmented PGE_2 synthesis in HG.

4.6: Possible mechanism of PGE_2-induced proliferation in IMCD cells.

In a previous study in mesangial cells, it was shown that high glucose augmented PGE_2-stimulated mesangial cell proliferation by decreasing PGE_2-stimulated cAMP levels, without a change in EP_4 receptor expression. This may indicate that cAMP has different effects on proliferation in mesangial and IMCD cells, since it would be expected that the diminished proliferative response to PGE_2 in HG would be associated with an enhanced cAMP response to PGE_2. Although one study alludes to a beneficial effect of dbcAMP, added for the duration of culture, on the growth of primary cultures of rat IMCD cells (Maric, 1998), the effect of cAMP and cAMP-stimulating agents on IMCD cell proliferation and survival is largely unknown. However, our results provide evidence that an increase in intracellular cAMP levels, through PGE_2 stimulation and through dbcAMP treatment, does not stimulate DNA or protein synthesis, nor protects against apoptosis induced by chronic HG.

It is possible that the effect of PGE_2 on DNA and protein synthesis is mediated through either the EP_1 and/or EP_3 receptor subtypes and an attenuation of either of these signaling pathways in HG may account for the diminished response to PGE_2. Both the EP_1 and EP_3 receptor are expressed and are functional in rat IMCD cell (Nasrallah, 2001) and the PGE_2-induced proliferation in rat hepatocytes is mediated through the EP_3

receptor (Hashimoto, 1997). Similarly, in a fibroblast cell line PGE_2-induced proliferation is mediated in part by the EP_1 receptor (Sanchez, 2002). It has also been shown in gastric epithelial cells, that PGE_2 can transactivate the EGF receptor through the cellular release of an EGF ligand, which contributes to PGE_2-induced DNA synthesis in these cells and in the gastric mucosa in vivo (Pai, 2002). Also, the EP_1 agonist, ONO-DI-004, has been shown to transactivate the EGF receptor in a human cholangiocarcinoma cell line promoting cell growth (Han, 2005). Since EGF binding sites are apparent in the IMCD (Kim, 1992) and EGF receptors have been shown to be transactivated by GPCRs in mIMCD3 cells (Zhao, 2003), it is possible that PGE_2 via EP_1 transactivates the EGF receptor to stimulate IMCD cell DNA and protein synthesis. Downregulation of any component of this signaling pathway with chronic HG treatment may diminish the proliferative effect of PGE_2 in IMCD cells, as reported in this thesis.

4.7: The detrimental effect of HG on IMCD cell survival.

Although an alteration in PGE_2 signaling in IMCD cells chronically exposed to HG may contribute to the HG-induced apoptotic response, it is more likely that other factors or mediators account for the increase in apoptosis. An important observation in our study was that MA treatment partially mimicked the apoptotic effect of HG on IMCD cells, implicating an osmotic mechanism of cell death. Mannitol does not permeate the cell nor is it metabolized, however it can alter intracellular signaling pathways (Malek, 1998). Interestingly, in rat aortic smooth muscle cells, glucose concentrations above 22 mM activated p38 mitogen-activated protein kinase in a similar fashion to mannitol, suggesting that at these concentrations, glucose activates an osmotic pathway that could alter cell survival (Igarashi, 1999). Although primary inner medullary cells can tolerate a wide range of osmotic changes and are less susceptible to cell death caused by acute osmotic changes (Zhang, 2002), it is possible that chronic exposure to elevated osmolytes, like glucose and mannitol, could induce cell damage leading to apoptotic cell death. However, according to our results, there are also glucose specific mechanisms, which are not mimicked by mannitol, that contribute to the HG induced IMCD cell apoptosis.

Increased glucose metabolism in diabetes is associated with the intracellular accumulation of glucose metabolites like lactate and sorbitol. In IMCD cells exposed to 30 mM glucose for 3 hrs, there was an accumulation of lactate, which can decrease cellular pH, and an increase in sorbitol (Willi, 1989), which has been linked to a decrease in inner medullary cell growth and survival (Yancey, 1990). Inhibitors of the enzyme involved in glucose metabolism to sorbitol, aldose reductase inhibitors, have proven beneficial in ameliorating the adverse effects of high glucose in the inner medulla, however, their use in vivo may interfere with kidney osmoregulation in an anti-diuretic state (Yancey, 1990, Palm 2004). To further investigate the mechanism of HG actions in this study, we could use an aldose reductase inhibitor to determine if this glucose metabolism pathway plays a role in the effects of HG on IMCD cell growth and apoptosis.

It has also been demonstrated that an increase in ROS in the inner medulla of the kidney is linked to high glucose levels (Palm, 2003). ROS may act as signaling molecules in IMCD cells in this thesis, mediating the effects of HG on cell growth and apoptosis, similar to its actions in mesangial cells (reviewed by Ha, 2000). However, it has been suggested that inner medullary cells are normally exposed to ROS in vivo

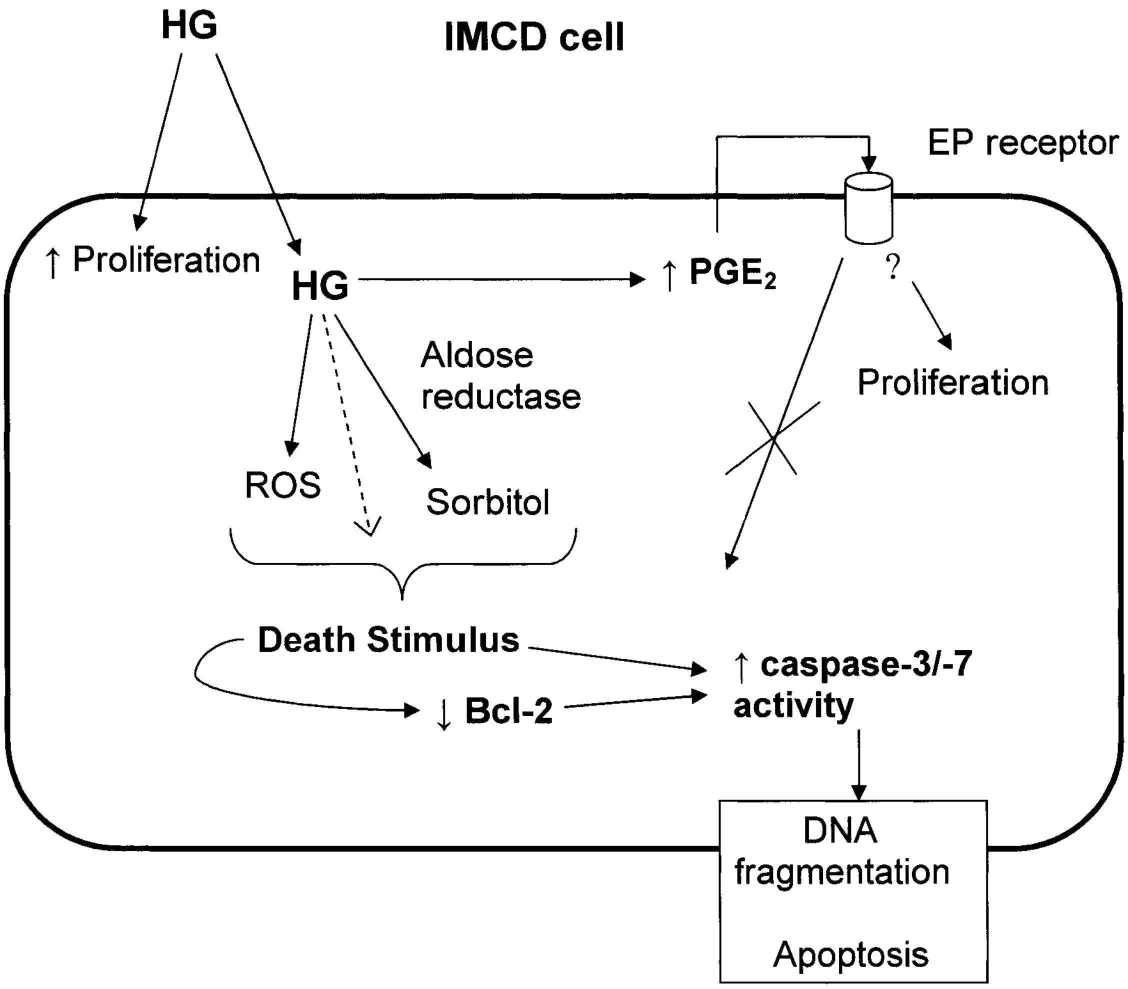
(Zhang, 2004), therefore, IMCD susceptibility to the detrimental effects of ROS stimulated by high glucose is questionable.

4.8: Summary.

It is apparent that high glucose may activate multiple pathways in IMCD cells to alter IMCD cell proliferation and induce apoptosis, as summarized in **Figure 15**. The importance of our results is evidenced by the lack of studies that investigate the pathogenesis of DN in the inner medulla. We have shown that acute high glucose exposure increases IMCD proliferation, which may prove to be detrimental to IMCD function in vivo. With chronic exposure to high glucose, IMCD cell proliferation decreases and there is a concomitant increase in apoptosis. Over time, the diabetic environment may reduce IMCD cell number culminating in IMCD tubular atrophy and loss of IMCD function.

Although PGE_2 synthesis is increased by high glucose in IMCD cells (Nasrallah, 2003) and in the diabetic kidney (Hommel, 1987), it appears that PGE_2 does not play a role in the apoptotic response of IMCD cells to high glucose. It is unclear if PGE_2 plays a protective role in the IMCD, however, this study demonstrates that PGE_2 does reverse the inhibition of DNA and protein synthesis caused by indomethacin. Also, the effect of PGE_2 on levels of DNA and protein synthesis in the IMCD is diminished after chronic high glucose exposure, independent of any change in PGE_2-stimulated cAMP levels. This indicates that there may be an alteration in the PGE_2/EP receptor signaling pathway that could contribute to a high glucose induced alteration in renal function. Our results warrant investigation into the in vivo survival and function of IMCD cells in diabetes and the role of an altered PGE_2 induced response in the IMCD.

Figure 15: Proposed role of HG in IMCD proliferation and apoptosis. Short-term HG exposure increases IMCD proliferation while long-term HG exposure acts as a death stimulus, possibly through increased ROS and/or sorbitol accumulation. Long-term HG exposure also increases PGE_2 synthesis which increases proliferation in the IMCD by an unknown mechanism, but does not alter the detrimental effect of HG on the IMCD cell.


HG
IMCD cell
EP receptor
↑ Proliferation
HG
↑ PGE_2
?
Aldose reductase
Proliferation
ROS
Sorbitol
Death Stimulus
↑ caspase-3/-7 activity
↓ Bcl-2
DNA fragmentation
Apoptosis

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