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Graduate Studies

College of Pharmacy

STANDARDIZATION OF AN *IN VITRO* MODEL OF DIABETIC NEPHROPATHY IN RENAL TUBULAR CELLS AND INVESTIGATION OF THE ROLE OF ALDOSE REDUCTASE PATHWAY IN HIGH GLUCOSE-INDUCED RENAL CELL INJURY

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Abstract

Diabetic nephropathy (DN) is the leading cause of end stage renal disease, and one of the most serious microvascular complications of diabetes mellitus. Increase in the shift of glucose into the aldose reductase pathway during diabetes leads to accumulation of sorbitol and fructose in the cells, and causes an imbalance in the associated cofactors, which in turn cause deleterious events such as oxidative stress, endoplasmic reticulum (ER) stress and cell death in the kidney. The objective of this study was to investigate the effects of high glucose on renal tubular cells cultured *in vitro* and evaluate the protective effects of inhibiting the aldose reductase pathway. Normal rat kidney (NRK-52E) cells were exposed to high glucose (30 mM) or normal glucose (5 mM) media for 24 to 72 hours, and then assessed for changes in cell viability using MTT assay. The expression of aldose reductase, markers of ER stress such as GRP78 and CHOP, and activation of Akt and ERK1/2 signaling has been measured using western blotting. In addition, mitochondrial membrane potential has been assessed using JC-1 assay. Exposing NRK-52E cells to 30 mM glucose containing media decreased cell viability after 48 h (84%) with further decline in viability at 72 h (64%). Aldose reductase expression was increased at 48 h, and this was associated with slight depolarization in mitochondrial membrane potential. In addition, high glucose exposure caused acute activation of both Akt and ERK pathways. In contrast, no induction of the ER stress markers has been identified in the model. Interestingly, co-incubating cells with 1 µM of an aldose reductase inhibitor epalrestat has reversed the cellular injury and signaling changes induced by high glucose. These findings suggest that high glucose conditions trigger cell death and depolarize

mitochondrial membrane in renal tubular cells. Moreover, hyperglycemia was able to induce Akt and ERK pathways, which are actively involved in the mechanisms contributing to DN. Inhibition of the aldose reductase pathway has reversed the hyperglycemia-induced deleterious effects on renal cells, and hence, represents a potential therapeutic strategy to improve renal cell function during diabetes.

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Abbreviations

4E-BP1, Eukaryotic translation initiation factor 4E-binding protein 1

AGE, advanced glycation end products

AKR, aldo-keto reductase superfamily

AR, aldose reductase,

ARI, aldose reductase inhibitors

ASK-1, apoptosis signal-regulating kinase 1

ATF-6, activating transcription factor-6

BAD, Bcl-2-associated death promoter

CDK, cyclin dependent kinase

CHOP, C/EBP homologous protein (CHOP)

DAG, diacyl glycerol

DCF, 2′,7′-Dichlorofluorescein diacetate

DM, diabetes mellitus

DMEM, Dulbecco's Modified Eagle's Medium

DN, diabetic nephropathy

ECM, extracellular matrix

eIF2α , translation initiation factor 2α

eIF4E, eukaryotic initiation factor 4E

eNOS, endothelial nitric oxide synthetase

EPG, epidermal growth factor

ER, endoplasmic reticulum

ERK, extracellular signal-regulated kinase

ESRD, end stage renal disease

ET-1, endothellin-1

GADD34, growth arrest and DNA damage-inducible 34

GBM, glomerular basement membrane

GFR, glomerular filtration rate

GLUT4, glucose transporter type 4

GRP78, glucose-regulated protein 78

GSK-3, glycogen synthase kinase-3

HNE, 4-hydroxynonenal

HYOU1, hypoxia up-regulated protein 1

IRE, inositol-requiring protein 1

IRS 1 and 2, insulin receptor substrate 1 and 2

JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3’ tetraethylbenzimidazolylcarbocyanine iodide

JNK, c-Jun N-terminal kinase

LDH, lactate dehydrogenase

MAPK, Mitogen activated protein kinase

mTOR, mammalian target of rapamycin

MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADP, Nicotinamide adenine dinucleotide phosphate

NF-κB, Nuclear factor kappa B

PDI, protein disulfide isomerase

PERK, double-stranded RNA-activated (PKR)-like endoplasmic reticulum kinase

PI(3,4,5)P3, phosphatidylinositol (3,4,5) triphosphate

PI(4,5)P2, phosphatidylinositol (4,5) bisphosphate

PI3K, phosphatidylinositol 3-kinase

PKA, protein kinase A

PKB, protein kinase B

PKC, protein kinase C

RAGE, receptor for advanced glycation end products

ROS, reactive oxygen species

S1P, site-1 protease

S2P, site-2 protease

SAPK, Stress activated protein kinase

SDH, sorbitol dehydrogenase

SERCA, sarco(endo)plasmic reticulum Ca^{2+} ATPase

STZ, streptozotocin

TBM, tubular basement membrane

TGF-β1, transforming growth factor beta 1

TRAF-2, tumor necrosis factor receptor-associated factor 2

UAER, urinary albumin excretion rate

UPR, unfolded protein response

VEGF, vascular endothelial growth factor

WRS, Wolcott-Rallison syndrome

XBP1, X-box binding protein 1

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Dedication

To my loving family:

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Chapter 1: Introduction

1.1 Diabetes

Diabetes mellitus (DM) prevalence has been increasing tremendously worldwide over the past few decades. The prevalence of DM is higher in developed countries; however there is a rapid increase in some developing countries as well such as Qatar, Saudi Arabia, United Arab Emirates and Oman. This increase is due to rapid urbanization which is usually associated with obesity, low physical activity, rapid population growth and longer survival rates (1). It is predicted the greatest relative increase in diabetes over the following years will be in the Middle Eastern Crescent and Sub-Saharan Africa (2). According to the International Diabetes Federation (IDF) in their 2014 update, 387 million people around the world are diagnosed with diabetes and it is expected that by 2035 this number will increase to reach 592 million. Additionally, 4.9 million people died because of diabetes in 2014; which means that a person dies of diabetes every 7 seconds (3). The World Health Organization (WHO) also anticipates that by 2030 diabetes will be ranked as the 7^{th} leading cause of death (4). The prevalence of diabetes in Qatar was found to be moderately high (16.7%) and it is expected to increase even more over the coming few years due to high percentage of pre-diabetes Qatari adults (1). Diabetes is a metabolic disorder characterized by chronic hyperglycemia due to insufficient insulin production or resistance to its actions (5). The major two types of DM classified based on etiology are; type 1, also known as insulin-dependent diabetes mellitus (IDDM), which is caused by autoimmune destruction of beta cells of the pancreas leading to decreased

insulin secretion and type 2, also known as non insulin-dependent diabetes mellitus (NIDDM), which is mainly caused due to resistance to insulin actions, however many other factors could also contribute to the hyperglycemic state observed in type 2 DM like inadequate insulin secretion and increased hepatic glucose production (5, 6). The prolonged exposure to high glucose concentrations in diabetic patients progressively leads to the development of long term complications in specific tissues like retina, kidneys, neurons and arteries. Therefore, DM is usually associated with serious microvascular complications like retinopathy, nephropathy and neuropathy as well as macrovascular complications like strokes and myocardial infarctions (5). Diabetic nephropathy (DN) which is the kidney dysfunction caused by DM is the leading cause of end stage renal disease (ESRD) accounting for 44% of all new cases in 2011 (7). Around 50% of patients that progress to ESRD need kidney replacement therapies (e.g. dialysis and kidney transplantation) (8). The prevalence of diabetic complications in Qatar has been examined in a recent study and it was found that that the prevalence of diabetic nephropathy and retinopathy was 12.4% and 12.5% respectively while neuropathy was found to be 9.5%. It was concluded that diabetes is considered to be a burden on Qatar and that better diagnosis and treatment strategies are required to face the increase in the prevalence of diabetic complications and the associated morbidity and mortality (9).

1.2 Diabetic nephropathy

1.2.1 Pathogenesis of DN

Diabetic nephropathy, which is also known as Kimmelstiel-Wilson syndrome or intercapillary glomerulonephritis or nodular diabetic glomerulosclerosis, was first described in 1936 by Clifford Wilson (1906-1997) and Paul Kimmelstiel (1900-1970) (8). Both physicians were the first to describe the nodular glomerulosclerosis lesions in diabetic patients having proteinuria and hypertension (5). DN is the progressive kidney damage caused by chronic hyperglycemia in diabetic patients and characterized by three main findings (8), 1) Persistent albuminuria (more than 300 mg/day or more than 200 µg/min) that is confirmed at two visits 3-6 months apart, 2) Progressive and irreversible decrease in glomerular filtration rate (GFR), 3) Increase in arterial blood pressure.

1.2.1.1 Albuminuria:

The renal glomeruli capillaries are remarkably leaky and act as a unique biological sieve. The glomerular filtration membrane which consists of endothelial cells, glomerular basement membrane and podocytes allow the passage of water, mineral ions and small molecules while holding back cells and plasma proteins (8). However, increased intracellular glucose concentrations in diabetic patients lead to irregular blood flow patterns and increases vascular permeability and consequently more plasma proteins start leaking in urine (10). Measuring urinary albumin excretion rate (UAER) is usually one of the first tests conducted to diagnose and assess the stage of DN (8). Albuminuria is considered a prognostic index for DN and its presence is an indication of declined renal

function and the increased possibility of ESRD (11). Microalbuminuria also known as incipient nephropathy is defined as more than 30 mg and less than 300 mg of albumin in urine per day while macroalbuminuria also known as overt or clinical nephropathy is defined as more than 300 mg albumin in urine per day (5, 12). Factors that affect albuminuria levels upon diagnosis differ between individuals according to type of diabetes, duration of diabetes, glycemic and hypertensive control but generally microalbuminuria develops after a long time of the onset of diabetes (5). Around 80% of type 1 diabetes patients and microalbuminuria progress to overt nephropathy if left untreated while 20 – 40% of type 2 diabetes patients develop overt nephropathy over a 15 year period (13).

1.2.1.2 Glomerular filtration rate (GFR)

At early stages of DN the kidneys increase in size and the glomeruli undergo hyperfiltration therefore the GFR initially increases (8). As the disease progresses and the UAER exceeds overt nephropathy threshold, a steady and progressive decline in GFR is observed. The rate of GFR decrease depends mainly on systemic blood pressure and is different from one person to another. Controlling hypertension can effectively delay the progression of ESRD. For instance, in patients with type 1 and type 2 diabetes with untreated hypertension, there was an average decrease in GFR by 10 ml/min per year which means that ESRD could occur within 7 to 10 years; however by controlling blood pressure the decline in GFR was at much lower rate (4 ml/min per year) thus delaying the

development of ESRD by 10 years (5). The rate of decrease of the GFR in patients with type 1 and type 2 diabetes is illustrated in the table 1.1 (8).

Table 1.1 The rate of decrease of the GFR in patients with type 1 and type 2 diabetes

	Decline in glomerular filtration rate (ml/min/year)	
Urine Albumin Status	**Type 1 Diabetes**	**Type 2 Diabetes**
Normoalbuminuria	1.2-3.6	0.96
Microalbuminuria (Incipient nephropathy)	1.2-3.6	2.4
Macroalbuminuria (Overt nephropathy)	9.6-12	5.4-7.2

1.2.1.3 Arterial blood pressure

Increased systolic blood pressure even in the prehypertensive range is a main factor in the progression of the disease (13). Patients with type 1 diabetes and incipient nephropathy have higher blood pressure when compared to patients with normoalbuminuria. In addition, most patients with overt nephropathy whether type 1 or type 2 diabetes are hypertensive (5). Furthermore, 50% of patients with nephropathy and hypertension in type 1 diabetes patients are expected to develop ESRD within 10 year

period (13). Therefore, controlling hypertension is very essential to delay the progression of DN in diabetic patients.

The progression of diabetic nephropathy varies between individuals depending on many factors but generally it can be classified into 5 main stages: (8)

- **Stage 1:** In this stage albumin levels are within range or slightly increased. The kidneys increase in size by 20% and the blood flow to the kidneys increase by 10-15%. The glomeruli become hypertrophic and undergo hyperfiltration: therefore the GFR is either normal or increased. Blood pressure is usually within normal range.
- **Stage 2** is also known as the quiet or silent stage. In this stage, pathological changes start to appear in the kidneys like thickening of the basement membrane and mesangial expansion. However, no clinical signs are detectable at this stage. Albumin levels in urine are normal in type1 diabetes or increased in type2 diabetes but less than 30-300 mg/day. GFR values return to normal. Blood pressure is usually normal at this stage in type 1 diabetes patients while type 2 patients tend to be hypertensive. Many patients remain at this stage for long period of time and not progress to other stages.
- **Stage 3** is also called the microalbuminuria or incipient nephropathy or initial nephropathy stage. In this stage glomerular damage reach a stage where plasma proteins start leaking in urine and can be clinically detected. It is characterized by microalbuminuria where levels of albumin are between 30-300 mg/day. GFR

starts to decline. Blood pressure is either increased or normal. Around 40% of patients progress to this stage.

- **Stage 4** is also called overt nephropathy stage. Once the kidneys reach this stage the damage is irreversible and patients develop chronic kidney failure. Albumin levels in the urine exceed 300 mg/day (macroalbuminuria) and GFR declines to levels below 60 ml/min/1.73 m^2. Blood pressure values increases above normal.
- **Stage 5** is also called uremia or terminal kidney failure stage. Albumin levels in the urine decrease and the GFR decreases tremendously to less than 15 ml/min/1.73 m^2. Patients are hypertensive and around 50% will eventually require kidney replacement therapy.

The stages of DN are demonstrated in **(Fig. 1.1)** (14)

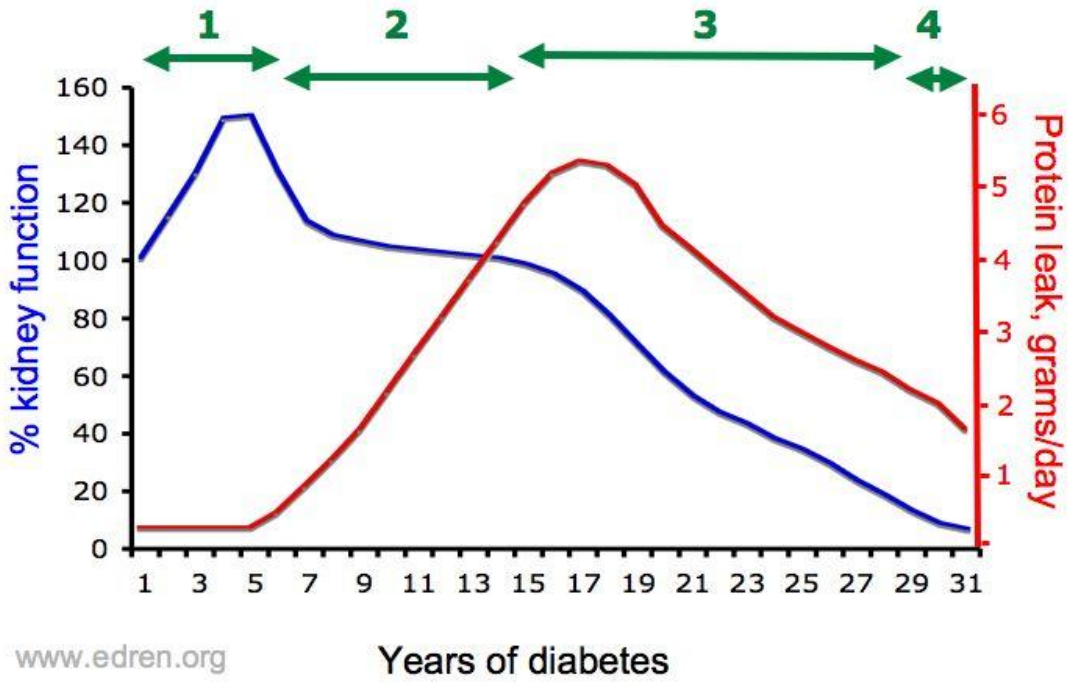


Fig. 1.1 A diagram showing changes in the percent of kidney function (blue) and protein leak in urine (red) since the onset of diabetes and over a period of approximately 30 years. Arrows in green show 4 of DN stages discussed above.

Source: http://www.edren.org/pages/edreninfo/diabetic-kidney-disease.php (14)

1.2.2 Pathology of DN

Pathological changes appear in the glomeruli and tubules of diabetic patients even before the development of microalbuminuria. The renal lesions are similar in both types of diabetes with more heterogeneity in type 2 diabetes. Chronic hyperglycemia brings about structural changes in the kidneys, which can be divided into early adaptive changes, which then progress to major pathological alterations as DN progresses. In the glomeruli, this early adaptive changes is illustrated in glomerular hypertrophy and

thickening of the glomerular basement membrane (GBM) and as the disease progresses the mesangium matrix begins to expand and mesangial cells increase in size (15). These changes in the mesangial matrix and cells leads to glomerulosclerosis and are sometimes associated with the characteristic DN nodular lesions (Kimmelstiel-Wilson nodules) which are areas depicting expansion in the mesangium and appear as large fibrotic rounded mesangial areas forming a nodule surrounded on the periphery by mesangial nuclei while the associated glomerular capillaries appear compressed (16). Similarly in the tubules there is tubular hypertrophy and thickening of the tubular basement membrane (TBM) which then progress to tubulointerstitial fibrosis and inflammation (15). The mesangial expansion and thickening of the glomerular and tubular basement membranes can be attributed to the accumulation of normal extracellular matrix (ECM) components of types IV and VI collagen, fibronectin and laminin as chronic hyperglycemia leads to increase in their production or inhibition of their degradation pathways or both (16). DN was traditionally linked to the decline in glomerular function and the degree of glomerular injury however; there is increasing evidence about the strong correlation between the degree of renal tubulointerstitial fibrosis and the rate of deterioration in renal function in DN as well (15).

1.2.3 Role of proximal tubular cells in DN

The proximal tubule plays an essential role in the pathogenesis of DN. The uptake of glucose by the proximal tubule cells is insulin independent which makes these cells susceptible to the harmful effects of chronic hyperglycemia in diabetic patients (11). The

main type of cells in the normal renal interstitium is the proximal tubular cells beside which lie a relatively few interstitial fibroblasts. Exposure to high levels of glucose increases these fibroblasts and activates them to myofibroblasts, which contributes to extracellular matrix expansion. The origin of myofibroblasts is unknown but one of the mechanisms postulated is the transdifferentiation of proximal tubule cells (transdifferentiation is defined as the change of the phenotype of cells from one type to another) however; evidence supporting this hypothesis is still inconclusive (15). It was also shown that tubule cells especially proximal tubular cells contribute to the initiation and progression of DN. At early stages tubular hypertrophy leads to hyper reabsorption, which in turn causes glomerular hyperfiltration that is observed at early stages in the disease. Later when tubulointerstitial fibrosis develops, it results in the occlusion of post glomerular capillaries leading to ischemic renal injury, and contributes to renal tubular atrophy that is observed in the advanced stage of the disease. Decreased reabsorption of sodium by atrophic proximal tubules results in reduced GFR due to tubulo-glomerular feedback and that explains the fall in GFR at later stages in DN after the initial rise (11).

1.3 Aldose reductase

1.3.1 Aldose reductase enzyme structure

Aldose reductase (ALR2; AR; EC 1.1.1.21) is a member of the aldo-keto reductase superfamily (AKR) (17). The AKR constitutes of more than 100 structurally related proteins most of which are catalytically active. The enzymes in this family catalyze and reduce a vast number of carbonyls to primary and secondary alcohols (18).

The structure of AR enzyme has been described using site directed mutagenesis and AR-ligand crystallized complexes (17). AR is a single polypeptide (monomeric) protein that consists of 315 amino acid residues (19). It is folded into a β/α barrel structure, which consists of eight parallel β strands linked together by eight marginal α helical segments. The active site has extended conformation for the binding of NADPH cofactor. The pyridine cofactor binds to AR in an atypical manner that resembles FAD more than NAD(P) dependent oxidoreductase. It is sequestered in the active site by what is called "the safety belt", which is a loop of residues (Gly213- Ser226) that is positioned between the strand and helix 7 of the β/α barrel structure. This safety belt affects the binding and release of the NADPH cofactor (20). There are three different binding pockets that have been proposed in the active site: 1) anion binding pocket, which is rigid in conformation and made up of Tyr48, His110, Trp111 and Trp20 in addition to the positively charged nicotinamide moiety of the cofactor NADP+, 2) specificity pocket, which is more flexible and is hydrophobic in nature comprising residues Thr113, Phe115, Phe122, Cys303 and Tyr309. The specificity pocket shows a great deal of flexibility and changes conformation with different ligands indicating a remarkable induced fit of the active site, and 3) another hydrophobic pocket made up of Val297-Leu300 residues which have the ability to bind to different ligands (17). In addition, AR can be oxidized at the cysteine residue located in the active site, which leads to decreased affinity to inhibitors.

1.3.2 Localization of aldose reductase in the kidneys

Reports regarding aldose reductase localization in the kidneys are highly variable which might be attributed to AR sensitivity to fixation and different antibodies and techniques used to detect the enzyme in different studies (21, 22). Despite this inconsistency, it can be concluded from several studies that AR is localized more in the medulla when compared to the cortex (22-24). This observation was reported by one of the earliest studies that was conducted by Corder et al. in 1977 to investigate the localization of polyol pathway enzymes (AR and SDH) in the kidneys of diabetic and normal rats (23). More recently, a study using normal human kidney tissues demonstrated similar distribution pattern of AR across the nephrons (more localization in medulla compared to cortex) and they concluded that rat model is a good representative for the polyol pathway in humans (22). In another study, a group of researchers measured postmortem AR activity in microdissected glomeruli taken from normal and diabetic human kidneys observed higher AR glomerular expression in patients with diabetic nephropathy when compared to glomerular expression in non-diabetic individuals which support the hypothesis of enhanced AR enzyme activity in diabetes (25).

1.3.3 The broad substrate specificity of aldose reductase and its role in detoxification

Many studies have shown that aldose reductase can catalyze a wide range of substrates; both hydrophilic and hydrophobic aldehydes (19). Aldose reductase catalyzes the reduction of biogenic aldehydes that is produced during the catabolism of

catecholamines and serotonin by monoamine oxidases which suggests that AR has role in the metabolism of biogenic amines in the central nervous system. Aldose reductase is also involved in the metabolism of steroids as it can catalyze the reduction of isocorticosteroids (intermediates produced in corticosteroid hormone metabolism), isocaproaldehyde (an intermediate in cholesterol metabolism) and 17α-hydroxyprogesterone (a major precursor for androgens, estrogens and glucocorticoids) which signifies the role of AR in the adrenal glands and reproductive organs (19, 26). Isocorticosteroids and isocaproaldehyde are considered from the best physiological substrates of AR as their Km values are less than 1 μM (19). Aldose reductase also plays an essential role in the detoxification of toxic aldehydes generated during lipid peroxidation, glycation and amino acid oxidation (26). This is demonstrated in the ability of AR to catalyze the reduction of the unsaturated toxic aldehyde 4-hydroxynonenal (HNE) (generated from the oxidative damage of unsaturated fatty acids) and its glutathione conjugate with Km values of 20-30 μM. In addition, AR catalyzes the reduction of methylglyoxal (a strong glycating agent produced from triose phosphate non-enzymatically), 3-deoxyglucosone and acrolein (a metabolic by-product of cyclophosphamide). Therefore, aldose reductase inhibitors should be designed to inhibit the reduction of glucose by aldose reductase without affecting its role in aldehyde detoxification (19, 26).

1.3.4 Polyol pathway

AR is the rate-limiting enzyme in the polyol pathway, which starts with the reduction of glucose to sorbitol using NADPH as a cofactor. The second step of this pathway is the conversion of sorbitol to fructose by sorbitol dehydrogenase using NAD+ as a cofactor. At normal glucose concentrations, glucose is a poor substrate for AR due to the high Km values of AR for glucose. Glucose usually enters glycolysis and metabolized by the hexokinase enzyme. Under hyperglycemic conditions the hexokinase become saturated and there is an increase of flux of glucose through the polyol pathway. It has been reported that 33% of glucose was shifted to this pathway in rabbit lens exposed to high glucose and about 11% in human erythrocytes. This increase in flux of glucose leads to imbalances in cofactors and products that can cause several deleterious effects **(Fig. 1.2)** (27) and explained as follows:

1.3.4.1 Osmotic stress (sorbitol accumulation):

AR enzyme has been shown to have osmoregulatory role in the kidneys. It is recruited in the kidneys to balance the osmotic gap during diuresis. During hyperglycemia sorbitol starts accumulating in the cells because it cannot easily diffuse through cell membranes (26). The damage caused by accumulation of sorbitol in cells has been a matter of debate. Some studies suggest that sorbitol levels are too low to cause osmotic damage to tissues however; many studies showed that diabetic cataracts are caused mainly due to sorbitol induced osmotic stress. Sorbitol accumulation damages the

cell membrane, which results in the leakage of amino acids, myoinositol and glutathione causing cataracts and lens opacification (28).

1.3.4.2 Oxidative stress

NADPH is required as a cofactor for keeping glutathione reductase in its reduced form so; the depletion of this cofactor due to increased flux of polyol pathway increases the susceptibility of cells to oxidative stress. In addition, the conversion of sorbitol to fructose leads to the increase of NADH/NAD+ ratio. The NADH is then become oxidized by NADH oxidase forming superoxide anions (26).

1.3.4.3 Increased advanced glycation end products (AGEs)

Fructose is a more potent glycating agent than glucose due to its ability to be in an open chain form, which allows it to react non-enzymatically with the free amino groups of proteins and the subsequent formation of advanced glycation end products after a series of Maillard reactions. Additionally, increased fructose can be converted to fructose-6-phosphate by hexokinase then to fructose 1, 6-bisphosphate by phospho-fructokinase. Fructose-1, 6-bisphosphate is then fragmented to dihydroxyacetone and glyceraldehyde-3-phosphate which it turn is converted to methylglyoxal; a strong glycating agent. AGEs induced damage can be explained by three general mechanisms. 1) AGEs modify intracellular proteins by covalent bonding and subsequently affect its function 2) AGEs can also modify extracellular matrix proteins affecting their interaction with other proteins 3) the interaction of AGEs with AGE receptors (RAGE) found on endothelial cells, mesangial cells and macrophages leads to the activation of various

growth factors and cytokines (NF-κB) eventually increasing reactive oxygen species (ROS) production (10, 26).

1.3.4.4 Activation of protein kinase C (PKC)

Increased NADH/NAD+ ratio due to conversion of sorbitol to glucose leads to the conversion of dihydroxyacetone phosphate to glycerol-3-phosphate, which is a precursor of diacylglycerol (DAG). DAG then leads to the activation of PKC isoforms, which in turn contributes to numerous pathogenic effects. PKC increases the expression of TGF-β1, which causes increase production of collagen and fibronectin. The accumulation of collagen and fibronectin leads to extracellular matrix expansion and capillary occlusion. Additionally PKC causes blood flow abnormalities and affect vascular permeability by increasing vascular endothelial growth factor (VEGF) and endothelin-1 (ET-1) and decreasing endothelial nitric oxide synthetase (eNOS) (10, 26).

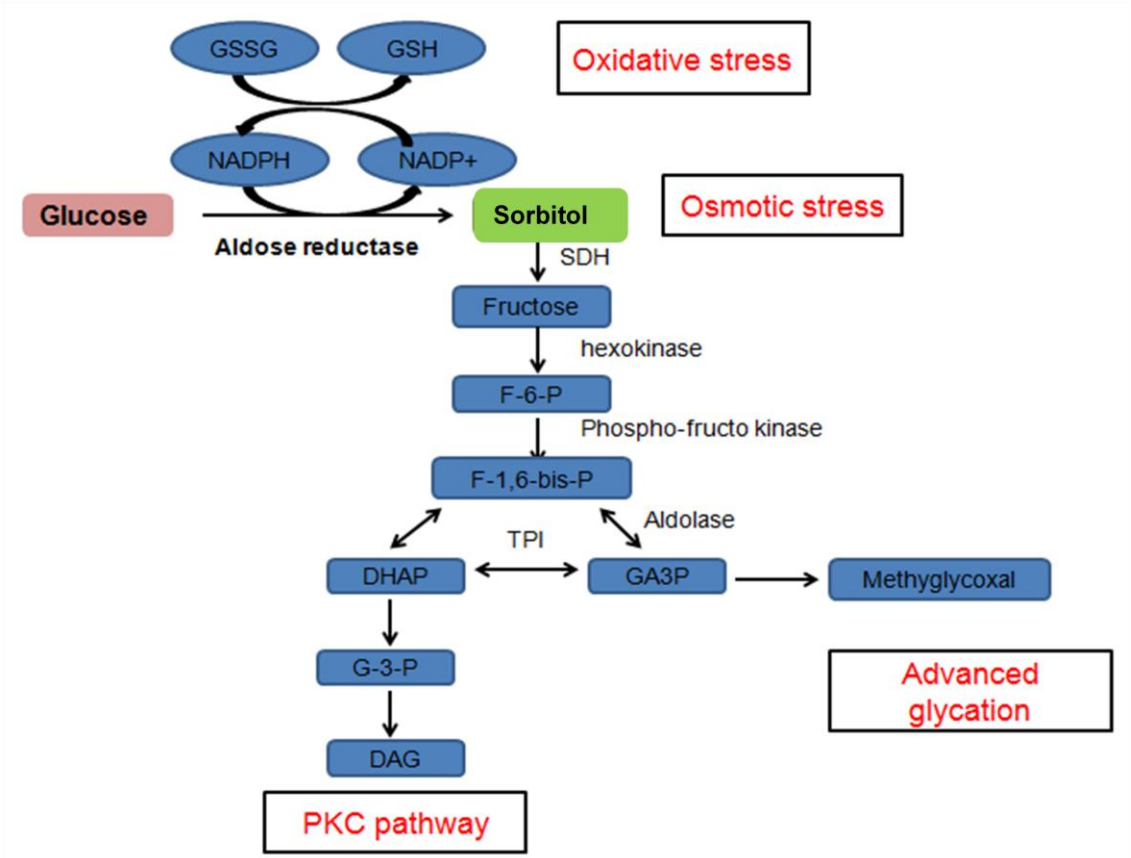


Fig. 1.2 Biochemical alterations induced by increased flux of glucose in the polyol pathway. Glucose is converted to sorbitol by AR enzyme depleting the cofactor NADPH, which is required to produce the reduced form of glutathione thus, increasing risk of oxidative stress. In addition, the accumulation of sorbitol is capable of causing osmotic stress in some tissues (such as retina). Sorbitol dehydrogenase converts sorbitol to fructose, which can then initiate AGE and PKC pathways. **SDH**, sorbitol dehydrogenase**; GSSG,** glutathione disulfide; **GSH,** glutathione; **F-6-P,** fructose 6-phosphate; **F-1,6-bis-P**, fructose 1,6-bisphosphate ; **TPI**, triosephosphate isomerase; **DHAP**, dihydroxyacetone phosphate; **GA3P,** glyceraldehyde 3-phosphate; **G-3-P**, glycerol 3-phosphate; **DAG,** diacylglycerol; **PKC,** protein kinase C**.** The figure was adopted from Tang et al. (27) (2012).

1.3.5 Aldose reductase inhibitors and diabetic nephropathy

The effectiveness of ARIs in preventing and improving diabetic complications has been investigated with most studies on retinopathy and neuropathy (24). Also, many studies have addressed the role of polyol pathway in the development of DN and evaluated the protective role of various aldose reductase inhibitors (ARIs). Generally, it can be concluded from the studies that ARIs exerts beneficial effects by correcting hemodynamic abnormalities (hyperfiltration), decreasing urinary albumin excretion (anti-albuminuric effects) and reversing extra cellular matrix accumulation (anti-fibrotic effects) while having no positive effect on blood glucose or HbA1c levels (24). *In vivo* studies using STZ diabetic rats showed improved glomerular filtration rate and blood flow after treatment with ARIs (29, 30). In addition, several studies were able to provide evidence that ARIs can significantly reduce UAER in STZ diabetic rats when compared to their respective controls (31, 32). Moreover, these finding were confirmed in some clinical trials in diabetic patients (11, 33, 34). In one study, giving ponalrestat to IDDM patients with normal albuminuria for 6 months reduced GFR thus, can be beneficial in reversing hyperfiltration observed at early stages of the disease (35). In another study, IDDM patients with diabetic nephropathy were treated with tolrestat for 6 months which was able to reduce both GFR and UAER (33). Furthermore, a clinical trial has been conducted to test long-term effects (5 years study) of aldose reductase inhibition in type 2 diabetic patients with incipient diabetic nephropathy. Cases were given 150 mg/day of epalrestat for 5 years. At the end of the study, UAE in the control group increased significantly compared to the beginning of the study while UAE in epalrestat group

remained unchanged **(Fig. 1.3)** which suggests that inhibiting AR might protect type 2 diabetic patients from developing DN (34). Studies aiming to explain the protective effects of ARIs suggested that it is probably due to its effects on VEGF. It has been shown that ARIs can lower plasma VEGF levels in diabetic rats and since VEGF plays a major role in albumin leakage, it is very likely that ARIs protective role is mediated through its interaction with this growth factor (36).

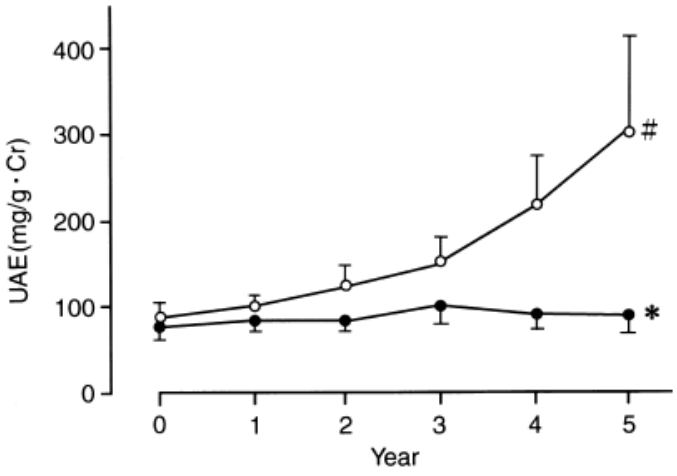


Fig. 1.3 Changes in urinary albumin excretion of microalbuminuric patients treated with (●) or without (o) epalrestat. Data are shown as mean ± SEM. $^{\#}P<0.01$ vs. 0 year. *P<0.05 vs. patients without epalrestat. Source: Iso et al. (34) (2001)

Some studies fail to see any improvement in extracellular matrix expansion in STZ diabetic rats after treatment with ARIs (Sorbinil and Statil), which might be attributed to the use of suboptimal dose or the severe form of diabetes developed in the experimental rats (37, 38). On the other hand, another study showed that using epalrestat for 6 months reduced mesangial expansion in moderately diabetic rats by 80 to 90%

when compared to control, which means that using ARIs is better to be earlier in the course of the disease (39). Many *in vitro* studies support the hypothesis of involvement of AR enzyme in ECM accumulation (40-43). It has been shown that there is a link between polyol pathway and PKC activity in mesangial cells. Inhibition of aldose reductase in mesangial cells effectively reduced high glucose induced PKC activity (40, 41). In addition, aldose reductase inhibition was able to reduce high glucose induced TGF-β1 as a consequence of PKC activity suppression in human mesangial cells (40). Furthermore, enhanced polyol pathway has been associated with the accumulation of extracellular matrix proteins in several *in vitro* studies. It has been reported that high glucose induced aldose reductase expression in human mesangial cells was associated with increase in fibronectin production (43). Another study in human renal proximal tubular cells demonstrated that high glucose induced the accumulation of collagen IV and fibronectin, which has been reversed using the aldose reductase inhibitor Sorbinil (42). Therefore, plethora of *in vitro* data supports the link between polyol pathway and PKC activity, TGF-β1 and ECM protein production, which suggest the involvement of this pathway in fibrotic changes observed in DN.

1.3.6 Challenges facing aldose reductase inhibitors

The efficacy of aldose reductase inhibitors in treating diabetic complications (in particular retinopathy and neuropathy) has been investigated since the 1970s. Although several studies demonstrated promising results in *in vitro* and *in vivo* settings, many

clinical trials had been unsuccessful (24). There are several challenges faced when targeting aldose reductase enzyme in diabetic patients.

1) The chronic nature of DM disease and the fact that symptoms appear after tissues are severely injured makes it hard to reverse structural dysfunctions. Since ARIs aim to delay and prevent deleterious effects caused by enhanced polyol pathway, using them at later stages of the disease might not be very beneficial (19).

2) Using variable endpoints in different trials (different clinical trial designs) has lead to inconsistent outcomes (19). In addition, the use of misleading biomarkers may have contributed to some of the disappointing results. For example, although it has been confirmed that sorbitol causes osmotic stress in diabetic rat lenses, lower levels of sorbitol have been detected in nerves and kidneys, which were not high enough to cause osmotic stress. Therefore, using sorbitol levels as a biomarker for pharmacological design could be misleading (24).

3) Difficulty of obtaining specific ARIs since there are several structurally related enzymes in the aldo-keto reductase family that coexist with AR in target tissues, thus quenching and reducing the inhibitor efficiency (19). For example, sorbinil has selectivity ratio for aldose reductase vs. aldehyde reductase of approximately 3 while zopolrestat is 450. Also, the ARI AL-1576 has been shown to be 13 fold more potent inhibiting aldehyde reductase compared to aldose reductase (24). Hence, using AR specific inhibitors is crucial to obtain full efficacy and avoid adverse effects.

4) The use of optimum doses for ARIs has been also challenging. For example, doses required to decrease polyol pathway metabolites in neurons are much lower than doses needed to improve nerve conduction velocity. So adjusting the dose to obtain the desired endpoint is very important (19).

5) The differences between AR enzyme from one species to another may also contribute to the ineffectiveness of ARIs after showing success in *in vivo* studies (19).

6) Efficacy of ARIs also depends on the level of expression of AR in target tissues and degree of involvement of polyol pathway in developing diabetic complications, which is different from one person to another. In addition, there are genetic variations in the AR gene, which is associated with different levels of expression of AR enzyme (Z-2 microsatellite variant) (19, 24).

1.4 Endoplasmic reticulum stress

1.4.1 Endoplasmic reticulum

Endoplasmic reticulum (ER) is a very dynamic organelle that plays an essential role in several cellular processes involved in normal cell functioning and survival (44, 45). ER is the site of lipid and sterols biosynthesis, calcium storage and homeostasis and is responsible for folding and maturation of most membrane and secreted proteins (44, 46). The highest concentration of calcium is found in the ER, which is 3 to 4 times the cytosolic calcium concentration. These high concentration of $Ca^{2+}$is attained by the sarco(endo)plasmic reticulum Ca^{2+} ATPase (SERCA), which pumps Ca^{2+} into ER against concentration gradient. Therefore, ER is involved in many calcium dependent processes

like organogenesis, apoptosis and transcription cascades (45). In addition, the abundance of calcium-dependent molecular chaperones helps in folding and stabilizing protein intermediates (44). Protein translation takes place in the ER cytosolic surface by ribosomes, and then the unfolded polypeptide is transferred into the ER lumen by sec61 complex to be folded into its native conformation (45). The ER lumen has unique environment where proteins are subjected to numerous post-translational modifications like glycosylation, lipidation, oligomerization, etc. (47). Most of the proteins in the ER are first N-glycosylated, then disulfide bonds are formed to stabilize the proteins in their secondary and tertiary structures (45). These modifications are achieved by the highly oxidative nature of the ER lumen, which catalyzes the disulfide bond formation by protein disulfide isomerase (PDI) (45, 47). Moreover, the ER is equipped by folding machinery which includes foldases (enzymes that catalyze protein folding) and molecular chaperones (GRP94, and GRP78 or BiP), which prevents protein aggregation to ensure proper folding (47). In addition to their role in protein folding and maturation, these foldases and chaperones act as a quality control system to sense the presence of any misfolded proteins and make decisions on whether the protein are of good quality to exit the ER and continues down its secretory pathways or if the proteins might need to be retained in the ER for further folding and maturation (45, 47). If the unfolded/misfolded proteins didn't meet the quality control standards, they are directed to disposal by the ER-associated degradation machine (ERAD) through cytosolic proteasomal degradation. (48). By this remarkable synchronization, the ER is able to produce properly folded proteins and get rid of improperly folded or mutant proteins.

1.4.2 Endoplasmic reticulum stress response

The ER quality control system is very sensitive to any changes in homeostasis. Some proteins fail to be folded in their final conformation due to numerous reasons such as calcium depletion, disulfide bond reduction, decreased chaperones, mutant protein formations and oxidative stress which is caused by various physiological and pathological conditions (45). The imbalance between the increased demand of protein synthesis and the capacity of ER to fold proteins and promote their maturation leads to the accumulation of misfolded proteins and the expansion of the ER causing what is collectively called ER stress (44). The ER responds to this disequilibrium by initiating a group of signal transduction pathways named the unfolded protein response (UPR) (48). UPR consists of several signaling cascades which aims to relieve ER stress, restores ER homeostasis and promotes cell survival (45). At the beginning, the ER transmits signals to decrease protein biosynthesis by transiently suppressing translation in order to reduce ER overloading and further protein misfolding. Then ER activates genes encoding ER folding machinery components and increases molecular chaperones expression to augment the ER capacity in folding proteins and prevent their accumulation. In addition, genes encoding protein degradation are also induced to get rid of irreversibly misfolded proteins and promote their degradation by proteasomes. Finally, when all attempts of the UPR to alleviate ER stress fail, a programmed cell death takes place to eliminate irreversibly damaged cells (45, 48).

1.4.3 Signaling pathways in ER stress

ER has three main transmembrane proteins which sense ER stress then become activated and respond by transmitting signals to the nucleus to induce and modulate expression of UPR target genes and proteins. These protein are A) activating transcription factor-6 (ATF-6), B) double-stranded RNA-activated (PKR)-like endoplasmic reticulum kinase (PERK), C) inositol requiring protein 1 (IRE1) (45). These 3 pathways (illustrated in **Fig. 1.4**) will be briefly reviewed.

1.4.3.1 ATF-6

ATF-6 is a resident ER-transmembrane protein with a large ER luminal domain (49). Upon activation by ER stress, ATF-6 translocate to the Golgi apparatus where it is cleaved by two proteases S1P (site-1 protease) and S2P (site-2 protease). S1P cleaves ATF-6 luminal domain, while S2P cleaves the transmembrane anchor giving rise to the cytosolic fragment of ATF-6 (46). This fragment enters the nucleus and acts as a transcription factor to activate UPR target genes involved in protein folding such as glucose-regulated protein 78 (GRP78/BiP) and -94 (GRP94). Therefore, ATF-6 protects cells by enhancing ER folding capacity (49).

1.4.3.2 PERK

The PERK is also an ER transmembrane protein that is actively involved in the UPR. When its luminal domain senses the accumulation of misfolded proteins in the ER, it oligomerizes and the kinase cytosolic domain becomes activated. The PERK then

autophosphorylate itself and then phosphorylates the ubiquitous translation initiation factor 2α (eIF2α) and inhibiting its activity. eIF2α is required for protein synthesis as it facilitates the assembly of ribosomes on the initiation codons of mRNA, therefore by inhibiting its activity protein translation is halted to reduce the flux of protein entering the ER (49). Attenuation of protein translation provides protective effect as it reduces the load on the ER and gives a chance to deal with accumulated misfolded proteins. However, attenuating protein translation for prolonged periods of time could be detrimental to cells if protein levels decreased below levels required to sustain vital functions (46). Therefore, PERK activates a protein phosphatase called GADD34, which dephosphorylates eIF2α and restores it activity and thus counteracting the PERK induced decrease in protein translation. The mechanism by which the cell balances these opposing effects is still unclear; however, they work together in harmony in order to relieve ER stress (46). Additionally, the phosphorylated eIF2α is able to translate certain proteins like activating factor 4 (ATF4), which were otherwise basally repressed in normal conditions. ATF4 then induces genes that promote apoptosis like transcription factor C/EBP homologous protein (CHOP) and growth arrest and DNA damage-inducible 34 (GADD34) (49). CHOP is not expressed under normal physiological conditions; however, it is strongly induced in ER stress. Several studies demonstrated that CHOP promotes apoptosis in response to ER stress but its downstream signaling are still being investigated (47). Therefore, the PERK branch is initially cytoprotective but it also contributes to cell death by initiating apoptosis (46).

1.4.3.3 IRE1

Due to its presence in yeast, IRE1 is the most well studied pathway involved in the UPR response (49). In response to ER stress, IRE1 oligomerizes which in turn activates unique kinase/endoribonuclease functions in its cytosolic domain. Kinase activity leads to IRE1 trans-autophosphorylation, which then activates the endoribonuclease activity and cleaves the X-box binding protein 1 (XBP1) mRNA at two sites to excise an intron. The exons are then ligated together by tRNA ligase to give rise to spliced mRNA of XBP1 which is then translated to active forms of the XBP1 transcription factor (49). The transcription factors encode proteins that are involved in lipid biosynthesis, molecular chaperones to boost ER folding capacity and proteins involved in ER-associated degradation components (46, 49). IRE1 has been shown to induce apoptosis by 2 different mechanisms. The IRE1 kinase domain binds tumor necrosis factor receptor-associated factor 2 (TRAF-2) which then activates apoptosis signal-regulating kinase 1 (ASK-1) to give rise to IRE1/TRAF-2/ASK-1 complex. This complex activates and phosphorylates c-Jun N-terminal kinase (JNK), which triggers apoptosis. The other pathway suggested is the activation of caspase 12. Caspases are a group of cysteine proteases that transmits programmed cell death signals or act as actual executors of apoptosis. Pro-caspase 12 is thought to be bound to TRAF-2 so when IRE1 binds to TRAF-2, pro-caspase 12 is converted to active caspase 12 promoting cell death. However, the role of caspase 12 in causing apoptosis in response to ER stress is still a matter of debate (46, 47).

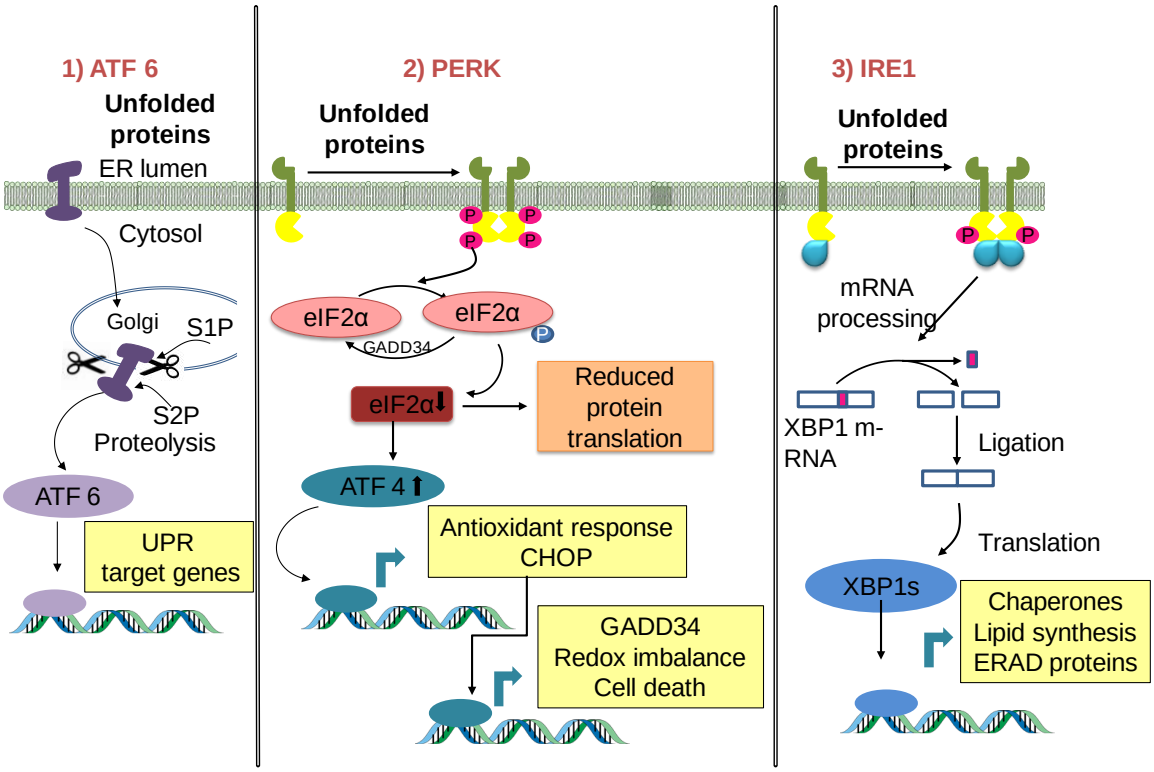


Fig. 1.4 Diagram showing the three main pathways involved in the UPR in cells experiencing ER stress. The figure was adopted from Walter et al. (49) (2011).

1.4.4 ER stress and diabetes

UPR is induced as a compensatory mechanism in an attempt to alleviate ER stress however; prolonged and inappropriate activation of UPR leads to cell death and contributes in the development of several diseases (46). Pancreatic beta cells are active secretory cells, which make it susceptible to ER stress and its deleterious consequences. The main role of pancreatic β-cells is to secrete insulin to regulate glucose metabolism. About 20% of β-cells mRNA expression encodes pro-insulin and when stimulated pro-insulin biosynthesis approaches 50% of total protein production. Insulin together with

other secretory and cell membrane proteins are translated on ribosomes found on the cytosolic surface of the ER. The newly synthesized pro-insulin which is a single molecule of 110 amino acids then enters the ER where disulfide bonds are formed and the protein is folded into its appropriate conformation. Therefore, any condition that causes increased protein synthesis in β-cells can lead to ER stress, misfolded insulin molecule, β-cells dysfunction and apoptosis (45).

One of the models that exemplify the role of ER stress in the development of diabetes mellitus is the Akita mouse model. The Akita mice carry a mutation in the insulin 2 gene (*Ins 2*) in which a highly conserved cysteine (C96Y) residue is replaced by tyrosine. This mutation disrupts one of the two disulfide bonds that are normally found in the pro-insulin resulting in abnormal pro-insulin conformation (47). The Akita mice are born with normal appearing β-cells but then develop early onset diabetes associated with β-cells destruction (48). The diabetic phenotype cannot be attributed to the mutant gene because rodents have two insulin genes (*Ins 1* and *Ins 2*), therefore the loss of *Ins 2* is unlikely to cause diabetes as insulin levels are maintained by *Ins 1* gene. Additional investigations have showed that the progressive hyperglycemia observed in the akita mice was associated with increased levels of ER stress markers like GRP78 and CHOP. Therefore, it was concluded that mutant pro-insulin molecules are accumulated in the ER of β-cells causing ER stress and subsequent cell damage. Moreover, when CHOP was knocked out from Akita mice, the onset of diabetes was delayed which suggests that ER stress plays a crucial role in the development of the diabetic phenotype however, other

mechanisms seem to be involved since the disease onset was delayed rather than prevented (47).

ER stress also plays a role in the development of diabetes in patients with rare genetic disorders such as Wolcott-Rallison syndrome (WRS) and Wolfram syndrome (46). WRS is a rare autosomal recessive disorder characterized by early onset of insulin dependent diabetes in infants associated with β-cells destruction and loss (47). WRS was found to be caused due to a mutation in EIF2AK3, which encodes PERK. The lack of PERK leads to the inability of ER to handle the increased load of protein synthesis, as the ER is incapable of attenuating protein translation to allow the folding machinery to meet the increased demand for insulin synthesis. Similarly, PERK knockout mice show clinical syndrome similar to that observed in WRS patients. Hence, PERK induced ER stress is crucial for β-cells differentiation and survival especially in neonatal life (45, 46).

Moreover, some clinical studies showed that ER stress markers are induced in the pancreatic β-cells of diabetic patients. In one study, GRP78 and CHOP expression were induced in β-cells of diabetic patients compared to non diabetics while in another study the ER size of β-cells in type 2 diabetic patients was double the size of that in non-diabetics (50, 51).

1.4.5 Endoplasmic reticulum stress and diabetic nephropathy

The initiation of the UPR differs from one tissue to another according to its function. For instance, secretory cells such as β-cells and hepatocytes are affected more rapidly than other cells by any disturbances in ER function. In addition, various metabolic conditions such as hyperlipidemia and hyperglycemia affect UPR in different cell types. Therefore, ER stress response should be viewed in a cell and tissue specific manner (52). Renal cells posses a well-developed ER as they are involved in the secretion of numerous cytokines and glycoproteins. Hence, renal cells are susceptible to ER stress induced damage especially in diabetic patients where renal cells are always exposed to metabolic imbalances (53). Several studies have demonstrated the activation of ER stress response in diabetic kidneys (53-55). In a study using streptozotocin (STZ) treated rats, after 16 weeks of induction of diabetes the rats showed features of diabetic nephropathy compared to normal rats (53). Analysis showed induction of mRNA and protein expression of ER stress markers GRP78, CHOP and caspase 12 in diabetic rats' kidneys when compared to control rats' kidneys. JNK protein expression has also been increased however; mRNA levels haven't been changed. Since CHOP, caspase 12 and JNK are markers of apoptosis, it was concluded that ER stress induced apoptosis contributes in renal cell death and that ER stress is implicated in the progression of DN. Nonetheless, the extent of renal cell damage produced by ER stress induced apoptosis in DN is still to be investigated (53). In another study, the activation of ER stress response in 9 and 22 months old STZ-treated mice was examined. Samples taken from 22 months old diabetic mice kidneys showed increased expression of GRP78 associated with elevated levels of

phosphorylated PERK, phosphorylated eIF2α and CHOP when compared to 9 months diabetic mice (56). Furthermore, STZ induced diabetic CHOP knockout mice were protected from DN and showed less proteinuria, better β-cell survival and insulin sensitivity compared to wild type. Therefore, these data demonstrates the role of ER stress and particularly CHOP in DN (56).

Hyperglycemia and proteinuria in DN causes non enzymatic protein glycosylation, free radical generation and elevated tubular epithelial proteins and membrane components turnover which puts burden on the ER in renal cells (57). Therefore, a group of researchers decided to examine the expression of ER stress markers generated in tubulointerstitial compartment of renal biopsies taken from patients with established DN secondary to proteinuria. Results showed increase in mRNA expression of XBP1, heat shock 70 kDa protein 5 (HSPA5 also known as GRP78) and hypoxia up-regulated protein 1 (HYOU1) in patients with marked DN compared to those with mild DN which suggests that ER stress response is related to the degree of tubulointerstitial damage. Moreover, protein expression ofGRP78 and HYOU1 has also been induced as illustrated by immunofluorescence. Interestingly, the pro-apoptotic markers genes have not been induced in renal biopsies. To further confirm the results, human tubular cells (HK-2) were exposed to various concentrations of albumin (50 and 100 µg/ml) and high glucose levels (30 mM) for 6 days. High glucose increased mRNA expression of the 3 ER stress markers XBP1, GRP78 and HYOU1. High glucose has also augmented albumin-induced expression of the 3 genes. Hence, concomitant existence of hyperglycemia and proteinuria in DN intensify ER stress response in renal cells. These

results suggest that adaptive and pro-survival UPR is predominant in the tubulointerstitial cells in patients with progressive DN rather than terminal apoptotic pathways (57). However, the chronic nature of DN disease may lead to that cells eventually overcome adaptive UPR and initiate apoptotic UPR causing renal tubular injury. Therefore, therapeutic intervention should reinforce protective pro-survival UPR while preventing terminal pathways (57, 58).

1.5 PI3K-Akt signaling pathway

1.5.1 Akt physiology and activation mechanism

Akt is a 57 KDa serine/threonine kinase that regulates numerous cellular functions such as transcription, translation, cell proliferation and survival (59). It is also called protein kinase B (PKB) or RAC (related to A and C) kinase due to its similarity with protein kinase A (PKA) and protein kinase C (PKC) (60). Mammalian genome contain three Akt genes encoding three abundant isoforms; Akt1, Akt2 and Akt3 (59). Sequencing the three isoforms revealed 81% amino acid sequence homology of Akt2 with Akt1 while Akt3 showed 83% homology with Akt1. Each isoform gene encodes a protein containing C-terminal regulatory domain, a central kinase domain and N-terminus containing pleckstrin homology (PH) domain. All three isoforms are expressed widely in various tissues summarized as follows (table 1.2) (59)

Table 1.2. Distribution of Akt isoforms in various tissues

Isoform	**Highly expressed**	**Low/moderately expressed**
Akt1	Brain, heart, testis, thymus	Kidney, liver, spleen
Akt2	Brown fat, heart, skeletal muscle	Brain, kidney, lung, spleen, testis
Akt3	Brain, testis	Heart, kidney, liver, lung, skeletal muscle, spleen

The activation of PI3K/Akt pathway begins by the stimulation of tyrosine kinase receptors by several stimuli such as cytokines and growth factors. Tyrosine kinase receptor then phosphorylates substrates such as insulin receptor substrate 1 and 2 (IRS1 and IRS2). IRS then binds to P85 subunit of the phosphatidylinositol 3'kinase (PI3K), which in turn activates its p110 catalytic subunit which bears a lipid kinase activity. Activated PI3K then phosphorylates the plasma membrane lipid phosphatidylinositol (4,5) bisphosphate (PI(4,5)P2) at the 3 position of the inositol ring giving rise to phosphatidylinositol (3,4,5) triphosphate (PI(3,4,5)P3). PIP3 has the ability to bind to proteins with PH domain therefore, Akt translocates to the plasma membrane and bind to PIP3 which induces conformational changes exposing the two main phosphorylation sites of Akt which are Thr308 in its central kinase domain and Ser473 in its C-terminal regulatory domain. Phosphorylation of these two sites by PKD1 and PKD2 respectively leads to the full activation of Akt and the initiation of its downstream signaling cascades (59, 60). Akt signaling pathway is illustrated in **(Fig. 1.5).**

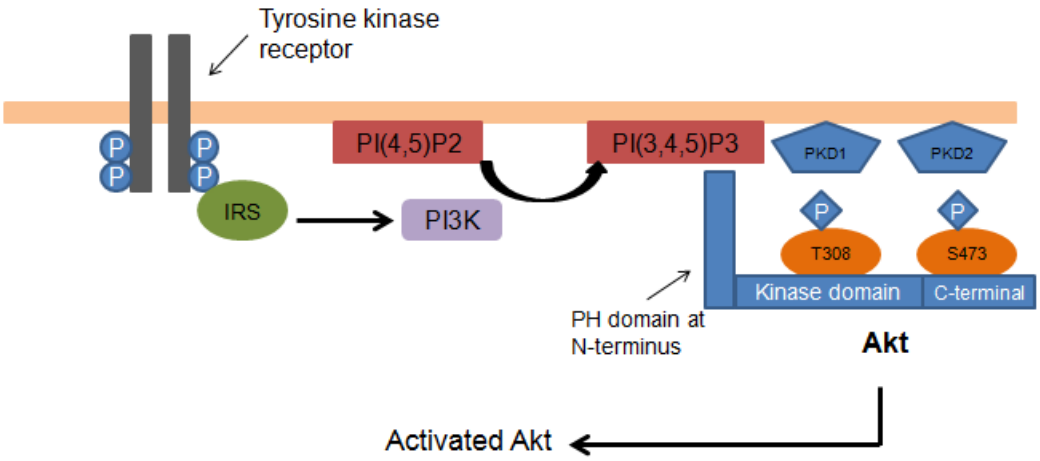


Fig. 1.5 A diagram showing PI3K/Akt signaling pathway. **IRS,** insulin receptor substrate; **PI(4,5)P2,** phosphatidylinositol (4,5) bisphosphate; **PI(3,4,5)P3,** phosphatidylinositol (3,4,5) triphosphate; **PI3K,** phosphatidylinositol 3'kinase; **PKD1,** protein kinase D1; **PKD2,** protein kinase D2. The figure was adopted from Osaki et al. (59) (2004).

1.5.2 Akt downstream targets

1.5.2.1 Role of Akt in glucose metabolism

Akt has been implicated in glucose metabolism through its effects on glucose transporter type 4 (GLUT4) and glycogen synthase kinase-3 (GSK-3) (59). It has been shown that following insulin stimulation, Akt associates with GLUT4 and translocates the transporters from intracellular stores to the plasma membrane hence, enhance glucose uptake (60). However, inhibiting Akt doesn't fully block insulin effect on glucose transport which suggests that other kinases are involved in the insulin dependent glucose uptake such as atypical members of protein kinase C family (61). In addition, Akt phosphorylates a serine residue in GSK-3 N-terminal (60). GSK-3 normally

phosphorylates and inhibit glycogen synthase enzyme, so Akt promotes glycogen synthesis by reducing GSK-3 inhibitory activity (62).

1.5.2.2 Role of Akt in cell survival and proliferation

The involvement of Akt in pro-survival and anti-apoptotic mechanisms has been widely studied due to its implication on several diseases ranging from cancer to cardiovascular diseases. Akt increases cell survival by two mechanisms; regulation of the transcription of pro-survival and anti-apoptotic genes and by directly phosphorylating factors in the apoptosis signaling cascade.

1.5.2.2.1 Transcriptional regulation of cell survival:

Generally, Akt is involved in downregulating genes that promote cell death and upregulating genes that promote cell survival.

- **The forkhead (FoxO or FH) family**

Akt can negatively regulate the four isoforms of the forkhead family of transcription factors (FKHR/FoxO1, FoxO2, FKHRL1/FoxO3, AFX/FoxO4). The fokrhead family target genes (such as Fas ligand and TRADD) promote apoptosis. Therefore, Akt promote cell survival by inhibiting the activity of forkhead family of transcription factors (63).

❖ **Necrosis factor Kappa B (NF-κB)**

NF-κB transcription factor regulates cell proliferation, apoptosis and survival. NF-κB is sequestered in the cytoplasm through binding to an inhibitory cofactor I-κB. Akt activates kinases known as IKKs, which phosphorylates and degrade I-κB thus, releasing NF-κB and promoting cell survival (60, 63).

1.5.2.2.2 Direct regulation of apoptosis

Akt can also interact directly and phosphorylate key regulators in the apoptosis cascade.

❖ **Bcl-2-associated death promoter (BAD)**

BAD is a pro-apoptotic member of the Bcl-2 family, which acts by interacting and inhibiting pro-survival members of the family such as Bcl-2 and Bcl-XL. Akt phosphorylates BAD leading to it sequestration in the cytoplasm and inhibiting its activity; therefore Akt promotes and facilitates cell survival (60, 63).

❖ **Stress activated protein kinase (SAPK)**

When cells are exposed to physical stress stimuli, they activate stress activated kinases such as JNK and p38 MAP kinase pathways which eventually induce apoptosis in cells(60). Akt phosphorylates upstream kinases such as apoptosis signal regulating kinase 1 (ASK1) thus interfering with apoptotic stress signals induced by these pathways (63).

❖ **Caspase 9**

It has been shown that Akt phosphorylates and inhibits caspase 9, which is an initiator and effector in the caspase mediated apoptosis signaling cascade (63).

1.5.2.3 Effects of Akt on cell cycle

Cell cycle is divided into 5 stages G_0, G_1, S, G_2 and M phases. In G_0 phase the cells are not dividing (quiescent) then the cells enter G1 phase (in which all cellular contents except for DNA is duplicated) and proceed to S phase where DNA replication occurs. The cells then progress to G2 phase (in which any necessary repairs are made by the cells) and finally to M phase in which mitosis occurs (60). The progression of cells through this cycle is a coordinated and synchronized process, which is regulated by positive regulatory proteins (such as cyclins and cyclin dependent kinases (CDK)) and negative regulatory proteins (CDK inhibitors such as p21 and p27). These regulators act synergistically to ensure that progress through the cycle proceeds in a well-defined order (64). In addition, they make sure that cellular machinery is ready for DNA replication and that DNA replication occurs only once before proceeding to mitosis (64).

It has been shown that different Akt isoforms have distinct and opposing effects on cell cycle. This difference in part may be attributed to the different interaction with the cyclin kinase inhibitor p21, which regulates cell cycle by regulating both DNA synthesis and CDK activity (65). In a study, it has been demonstrated that Akt 1 interaction with p21 promotes its delocalization and release of its inhibitory effect on cdk2 thus promoting cell cycle progression and proliferation while Akt 2 phosphorylates p21 at a

position, which interferes with Akt1 site of phosphorylation. Therefore, Akt2 retains p21 in the nucleus which in turn stabilizes the inhibitory complex formed with cdk2 leading to cell cycle arrest and exit at the G1 phase. Therefore, Akt effects on cell cycle progression depend on the specific isoform activated (65).

1.5.2.4 Effects of Akt on protein synthesis

Akt has been shown to stimulate protein synthesis by its action on the eukaryotic initiation factor 4E (eIF4E), which is a vital translational regulatory factor. eIF4E is rendered in active in resting cells as it is complexed with an inhibitory protein called 4E-BP1. The mammalian target of rapamycin (mTOR), which is one of the downstream targets of Akt can phosphorylate 4E-BP1 which facilitates its release and subsequent activation of eIF4E leading to increase protein translation (60, 66).

1.5.2.5 Effects of Akt on nitric oxide production

Akt is involved in endothelial NO production through its effects on eNOS enzyme. Akt phosphorylates and activates eNOS promoting its association with calmodulin and decrease its inhibitory interaction with caveolin-1 (CAV-1) (60, 67). Several factors have been shown to affect endothelial NO production through PI3K/Akt pathway such as VEGF (68), insulin (69, 70), leptin (71) and TGF-β (72). Therefore, alterations in the PI3K/Akt pathway could be observed in many conditions that are caused due to vascular dysfunction (67).

Akt downstream targets are summarized in **(Fig.1.6).**

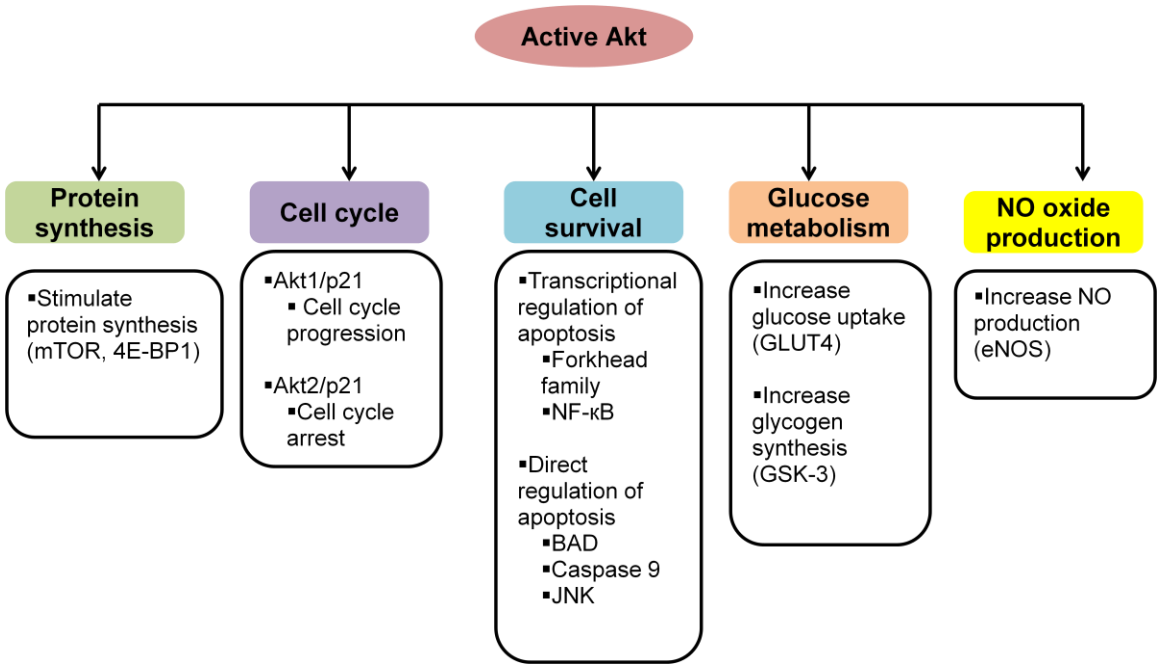


Fig. 1.6 Downstream targets of Akt. **mTOR,** mammalian target of rapamycin; **4E-BP1,** eukaryotic translation initiation factor 4E-binding protein 1; **NF-κB,** necrosis factor kappa B; **JNK,** c-Jun N-terminal kinase; **GLUT4,** glucose transporter type 4; **GSK-3,** glycogen synthase kinase-3; **eNOS,** endothelial nitric oxide synthetase. The figure was adopted from Zdychova et al. (60) (2005).

1.5.3 Role of PI3K/Akt pathway in diabetes

As illustrated above, the PI3-K/Akt pathway is involved in many cellular processes therefore; it is not surprising that alterations in the pathway that occurs in diabetes contribute to the disease. The activation of Akt in DM is highly variable according to the type of diabetes as well as cell type and degree of sensitivity of cells to insulin (60). Changes in Akt pathway in type 1 and type 2 DM will be briefly reviewed

1.5.3.1 Akt pathway in type 2 DM

1.5.3.1.1 Akt pathway and insulin resistance

DM type 2 is characterized by insulin resistance and since PI3-K/Akt pathway plays a major role in insulin signaling, many studies investigated the role of this pathway as a possible site of insulin resistance (60). Krook et al. studies showed impairment in insulin stimulated Akt phosphorylation in insulin resistant Goto-Kakizaki rats skeletal muscles which was further confirmed in muscle biopsies taken from type 2 diabetic patients (73, 74). In line with these findings were other studies showing impaired insulin stimulated Akt activation in rodent adipocytes (75) as well as rodent skeletal myocytes (76). In addition, some researchers have demonstrated impairments in GLUT4 translocation and protein expression along with impaired Akt activation (75, 77). On the other hand, several other studies reported normal Akt activation despite defects upstream and downstream from Akt in the insulin-signaling pathway (78-80). Therefore, evidence regarding the involvement of Akt in insulin resistance is still inconclusive. Despite contradictions reported in literature, the use of gene knockout mice shed light on the specific roles of different Akt forms in the development of diabetes (60). It was reported in a study that Akt 2 knockout mice demonstrated mild hyperglycemia which was associated glucose intolerance, increased insulin levels as well as defective glucose disposal in *in vitro* skeletal muscles (81). On the contrary, Akt 1 knockout mice didn't display any diabetic phenotype and they demonstrated mainly growth abnormalities (82). Another study that aimed to study the distinct roles of Akt isoforms reported that Akt 2 null mice exhibited hyperglycemia, dyslipidemia and hyperinsulinemia which lead to

mild growth abnormalities, age-dependent adipose tissue loss, impaired glucose tolerance and insulin resistance. In a large proportion of male mice, these symptoms worsened causing pancreatic β cell failure and loss giving rise to a severe form of diabetes while female mice just showed mild hyperglycemia and hyperinsulinemia (83). More recently, a study aiming to further clarify different roles of Akt isoforms in glucose homeostasis using knockout mice was able to confirm the diabetic phenotype that develop in Akt2 knockout mice. In addition, they showed that Akt1 knockout mice displayed an opposite phenotype with enhanced insulin sensitivity and lower blood glucose levels and they provided evidence that Akt1 is also involved in glucose homeostasis and β-cells function. They also showed that Akt3 didn't show any metabolic abnormalities (84).

1.5.3.1.2 Akt pathway and vascular complications

In theory, tissues that develop insulin resistance will have impaired Akt activation while tissues that remain sensitive to insulin will have normal or increased Akt activation. To investigate alterations in Akt pathway in endothelial cells, Jiang et al. examined aortas and microvessels from Zucker lean and obese type 2 diabetic rats. It was found that components of insulin signaling pathway downstream to Akt was reduced in obese rats and it was concluded that there is resistance to PI3-K activity in their vascular tissues (85). In addition, a study which compared aortae from diabetic type 2 mice with their matched controls showed that insulin induced endothelium dependent relaxation and NO production was reduced in diabetic rats aortae due to decrease in PI3-K/Akt activity (86). Therefore, it can be concluded that hyperglycemia and insulin resistance observed in type

II diabetes together cause defects in NO production and endothelial function due to impaired Akt activation (67).

On the other hand, some studies showed that renal tissues remain sensitive to insulin, which leads to increased activation of Akt pathway (87, 88). Feliers et al. demonstrated in their study increased insulin receptor activation and increased activities of PI3-k and Akt activities in cortical renal tissues from db/db mice in their early stage of type 2 diabetes. These changes coincided with the onset of hypertrophy and matrix accumulations in the kidneys which are characteristic to DN (87). Hypertrophy of renal cells occurs as a compensatory mechanism to increased work load on the kidneys (89). Hypertrophy could be cell cycle dependent or independent and is defined as cell enlargement due to increase in mRNA and protein production that is not associated with corresponding DNA replication (64). Several attempts were made to investigate molecular mechanisms behind hypertrophy observed in DN. It has been shown that p21 was increased in diabetic mice with glomerular hypertrophy and it was also induced in mesangial cells cultured in high glucose medium (90, 91). Since p21 is one of the downstream targets of PI3-K/Akt pathway, Chaung et al studied the expression of p21 and PI3K in LLC-PK1 to study the molecular mechanism of high glucose induced hypertrophy in tubular cells. It was shown that culturing LLC-PK1 cells in high glucose medium caused hypertrophy associated with increase in PI3-K and p21 levels (92). p21 has several proposed roles that might contribute to DN (89). p21 has been shown in a study to be a senescence inducer (93). Furthermore, p21 interaction with cyclin E kinases (which promotes cell progression to S phase) causes cell cycle arrest at G1 phase. This

cell cycle arrest causes hypertrophy because cells remains under the growth stimulatory effects (duplicating cellular components) of cyclin D kinases without replicating their DNA as they fail to progress to S phase due to cyclin E kinases inhibition by p21 (94, 95). Therefore it can be concluded that induced p21 *via* PI3-K/Akt pathway is a major player in fibrotic and hypertrophic changes observed in DN in renal tubules as showed in Chaung et al. study using LLC-PK1 cells. Further studies are required to confirm the involvement of PI3-K/Akt pathway in p21 induced changes in DN in other kidney cell types.

Another line of investigation is the altered regulation of protein translation in DN which might be due to Akt effects on 4E-BP1 phosphorylation (60). A group of researchers indirectly tested this hypothesis by studying the effects of insulin on 4E-BP1 phosphorylation in murine proximal tubular epithelial cells. They found that insulin increased 4E-BP1 phosphorylation, which was PI3-K and mTOR dependent (66). Since type 2 DM is characterized by hyperinsulinemia and given the evidence that renal tissues are sensitive to insulin actions, it is possible that insulin actions on 4E-BP1 via PI3-K/Akt pathway could lead to increased protein translation and subsequent matrix synthesis and accumulation causing DN.

1.5.3.2 Akt pathway in type 1 DM

1.5.3.2.1 Akt and insulitis

Type 1 diabetes is characterized by autoimmune destruction of beta cells of islets of Langerhans, therefore it is expected that Akt signaling pathway to be impaired due to lack of insulin (60). Since Akt pathway promotes cell survival, effects of Akt overexpression in beta cells have been investigated. Studies using transgenic mice overexpressing Akt showed that these mice displayed increased β-cells size and total mass as well as improved glucose tolerance when compared to wild type control. In addition, the mice were generally more resistant to diabetes, which suggests that targeting Akt pathway in early stages of DM type 1 may protect and delay β-cells destruction (96, 97).

1.5.3.2.2 Akt and vascular complications in type 1 DM

In contrast to type 2 DM, insulin signaling in type 1 DM is supposed to be normal or slightly reduced. As a result, Akt activation in tissues is impaired which might be associated with increased apoptosis. However, during course of treatment of the disease there are inevitable periods of hyperinsulinemia which in turn activates the Akt signaling pathway (60). Akt activation in type 1 DM reported in literature is very different. For example, it has been shown that retinal Akt activity was increased in STZ diabetic rats treated with low dose of insulin when compared to counterpart controls (98). Similarly, it has been shown that there is growth hormone induced Akt activation in hypertrophic kidneys in STZ diabetic rats (99). In contrast, myocardium from STZ diabetic rats

demonstrated diminished Akt activity after insulin administration (100). Therefore, it is very hard to predict Akt activation in various tissues in type 1 DM as it depends on insulin levels and cell type.

1.6 Mitogen activated protein kinase (MAPK) signaling pathway

1.6.1 MAPK pathway

MAPK pathways are one of the most important cell signaling cascades which transmits extracellular signals through the cells to regulate vital cellular functions such as cell proliferation, growth, migration, differentiation and apoptosis (101). Each MAPK signaling cascade consists of three main kinase components; MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK which phosphorylates and regulates several important functional proteins such as protein kinases, phosphatases and transcription factors (101). The MAPK are serine/threonine kinases which are located in the cytoplasm and they include 1) the extracellular signal-regulated kinase (ERK) family in which (ERK1-ERK8) forms have been described, 2) p38 kinase family including p38 α/β/γ/δ and 3) c-Jun N-terminal kinase family (JNK and also known as stress activated protein kinase) which includes JNK 1-3 (102). A general overview of the MAPK pathway is illustrated in **(Fig 1.7).**

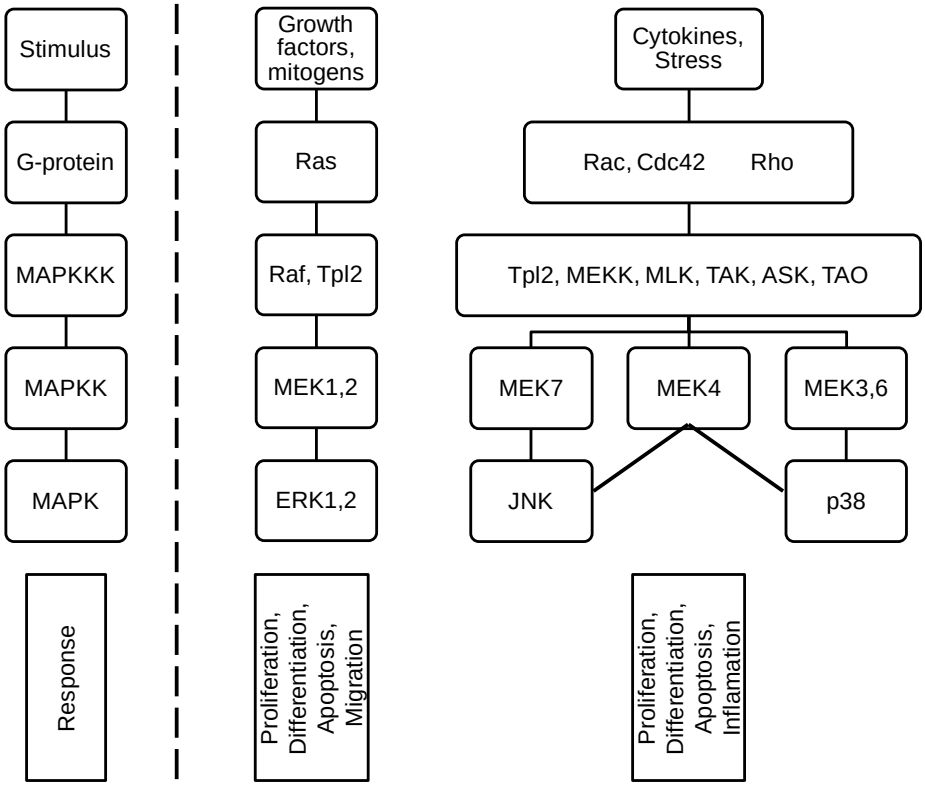


Fig. 1.7 MAPK pathways overview. **MAPK,** mitogen-activated protein kinase; **ERK1/2,** extracellular signal-regulated kinase 1/2; **Tpl2,** tumor progression locus 2; **MLK,** mixed lineage kinase; **TAK,** transforming growth factor-β-activated kinase; **ASK,** apoptosis signal-regulating; **TAO,** thousand-and-one kinase; **JNK,** c-Jun N-terminal kinase. The figure was adopted from Dhillon et al. (101) (2007).

1.6.2 The ERK1/2 signaling cascade

The signal begins by binding of various growth stimuli and cytokines to receptor tyrosine kinases (RTK). RTK then dimerize and autophosphorylates and then activates Sos1/2 (from *Drosophila* son of sevenless). Sos1/2 then promotes the conversion of Ras-GDP to Ras-GTP. The signaling cascade then proceed as active Ras-GTP phosphorylates Raf then Raf phosphorylates MEK1/2 which in turn activates and phosphorylates ERK1/2. This cascade is referred to as Ras-Raf-MEK-ERK signal transduction cascade

(101). Human ERK 1 and ERK 2 are almost identical in their functions and share 84% sequence homology therefore usually referred to as ERK 1/2 (102). ERK1/2 catalyzes the phosphorylation of numerous nuclear and cytoplasmic substrates. ERK 1/2 binds and stabilizes c-Fos and c-Jun which then translocates to the nucleus and bind to activating protein 1 (AP1) which regulate early transcriptional processes and induce genes encoding cell survival, proliferation and motility. In addition, ERK 1/2 has more than 50 cytoplasmic substrates such as ribosomal S6 kinase (RSK) family protein kinases, cytoskeletal proteins (such as palladin and myosin light chain kinase (MLCK)) as well as other regulatory and signaling molecules. Therefore, ERK pathway is involved in diverse cellular processes depending on cell type and regulates cell proliferation, migration, differentiation, angiogenesis and chromatin remodeling (102). ERK pathway is illustrated in **(Fig 1.8).**

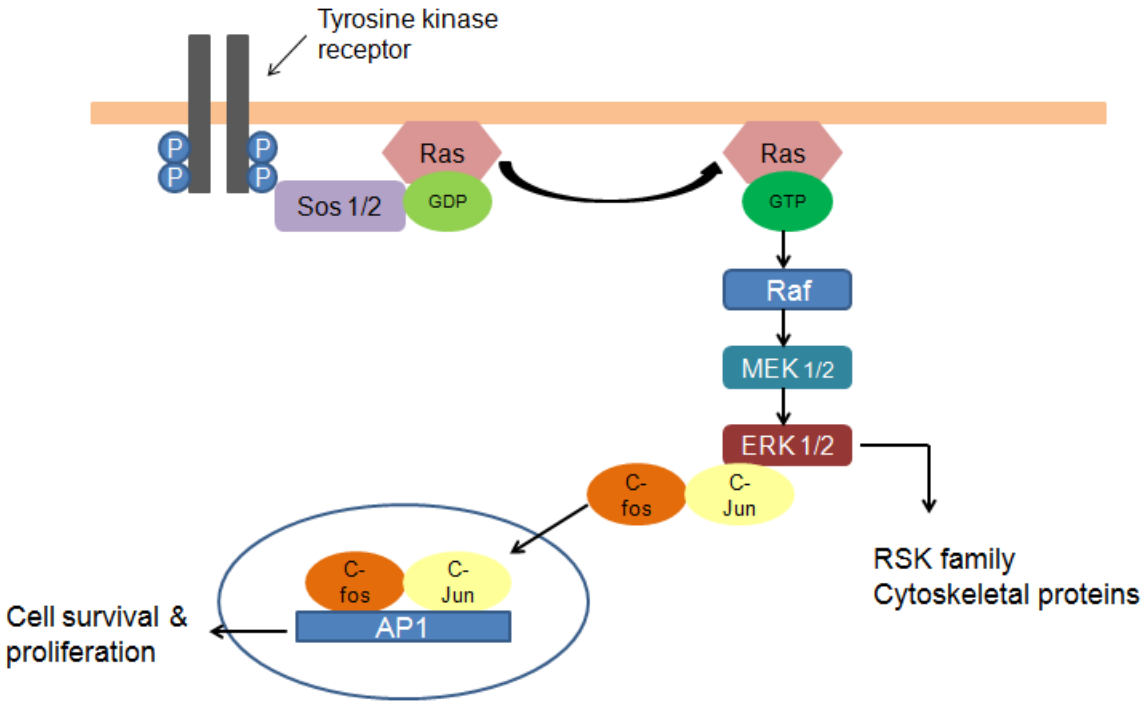


Fig 1.8 A diagram illustrating the ERK pathway. **SOS 1/2,** son of sevenless 1/2; **ERK1/2,** extracellular signal-regulated kinase 1/2; **AP1,** activator protein-1; **RSK,** ribosomal s6 kinase. The figure was adopted from Dhillon et al. (101) (2007).

1.6.3 ERK pathway and diabetic nephropathy

Since ERK pathway is a crucial signaling network involved in numerous cellular processes, research has shown that ERK pathway plays a key role in the intracellular signaling pathways implicated in DN pathogenesis. Under hyperglycemic conditions, the Ras-Raf-MEK-ERK signal transduction cascade becomes activated eventually causing activation of rennin-angiotensin-aldosterone-system (RAAS), which is known to cause renal injury by several mechanisms (103). Angiotensin II causes intrarenal hypertension

and reduced renal blood flow, which leads to systemic and glomerular hypertension as well as renal ischemic injury. In addition, Angiotensin II is linked to activating renal fibroblasts to myofibroblasts and subsequent fibrosis, inducing the profibrotic cytokine TGF-β and mesangial cells hypertrophy. Angiotensin II has also been shown to induce oxidative stress and stimulate chemokines and osteopontin causing local inflammation (104). Therefore, high glucose activation of RAAS through ERK pathway is considered a major contributor in DN. Additionally, ERK pathway causes renal cell injury through several other mechanisms. It has been shown that high glucose induces vascular endothelial growth factor (VEGF) production in podocytes through ERK and PKC dependent pathways (105). Increased VEGF levels cause albuminuria and increased filtration rate as well as hypertrophy in diabetic rats (106). It has also been demonstrated that ERK pathway participate in TGF-β signaling cascade which is a major player in kidney fibrosis (103). Furthermore, several studies showed cross talk between ERK pathway and other pathways in DN such as AGE and PKC pathways (107). Therefore, ERK pathway is considered one of the significant signaling pathways in DN. ERK pathway and its role in DN is summarized and illustrated in Figure 9 (103).

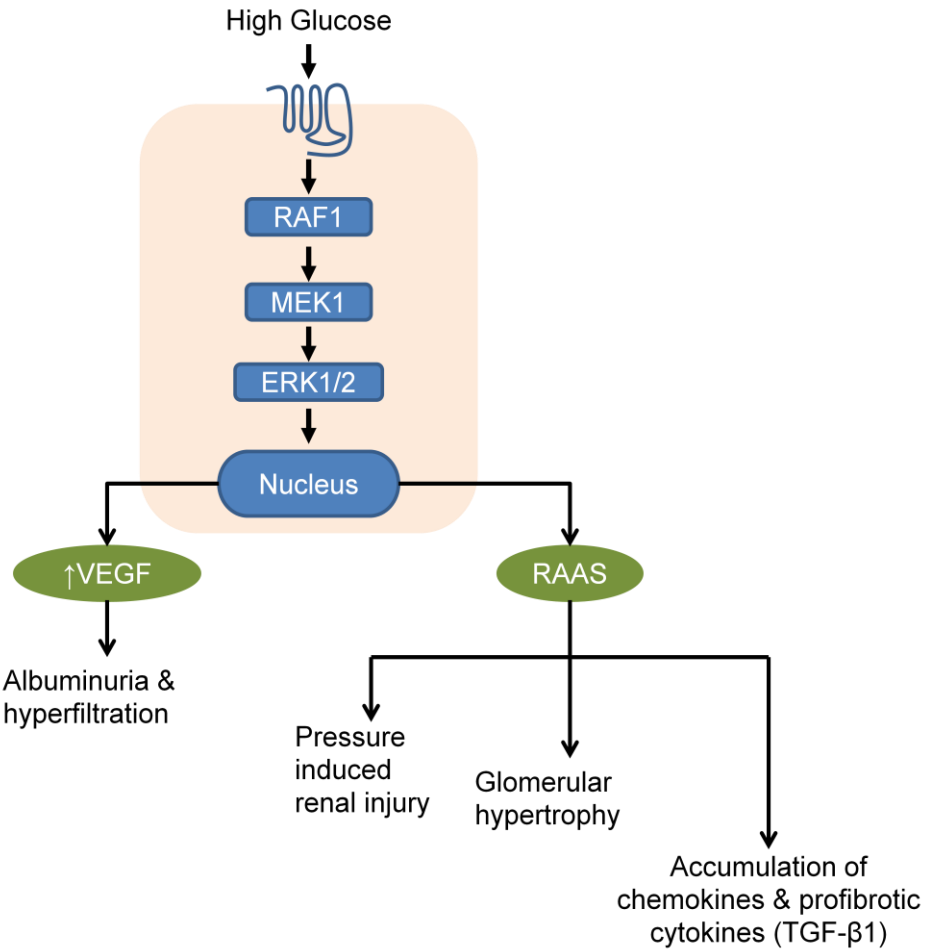


Fig. 1.9 Schematic overview for the role of ERK 1/2 pathway in the development of DN**. RAAS,** rennin-angiotensin-aldosterone system; **VEGF,** vascular endothelial growth factor; **TGF-β1,** transforming growth factor beta 1. The figure was adopted from Ni et al. (103) (2015).

1.7 Thesis objectives

Kidneys are among the main sites that are affected by chronic hyperglycemia observed in diabetic patients. Kidneys posses several pivotal physiological roles such as regulating fluid balance and blood pressure, release of erythropoietin hormone and vitamin D activation. Additionally, kidneys consist of various cell types (e.g. mesangial cells, podocytes, endothelial cells, tubular cells, *etc*..) with different roles which makes the development and progression of DN highly complex. In this study we were interested in studying the effects of high glucose concentration on renal tubular cells cultured *in vitro*. The study aimed to identify different stress markers such as cell injury and oxidative stress induced by hyperglycemia and to explore molecular pathways that can be initiated by increased glucose concentration.

As discussed earlier, one of the main pathways that contribute to the development of DN is the polyol pathway. Several *in vitro* studies that were conducted on various kidney cells have elucidated some of the molecular mechanisms by which polyol pathway contributes to the disease. In addition, *in vivo* studies demonstrated beneficial anti-albuminuric and anti-fibrotic effects after administering aldose reductase inhibitors. Therefore, we hypothesized that exposing renal tubular cells to high glucose media might induce aldose reductase (rate limiting enzyme of polyol pathway) expression and thus inhibiting the enzyme using the standard aldose reductase inhibitor epalrestat could have a potential protective effect against high glucose induced renal cell injury.

Chapter 2: Methods

To establish an *in vitro* model of DN, three treatment groups were used in all experiments as follows.

1) Control group (5 mM glucose)

2) High glucose group (30 mM glucose)

3) Osmotic control group (5 mM glucose + 25 mM mannitol)

All the three groups were exposed to the before mentioned conditions for various periods of time ranging from 10 minutes to 72 hours **(Fig 2.1).**

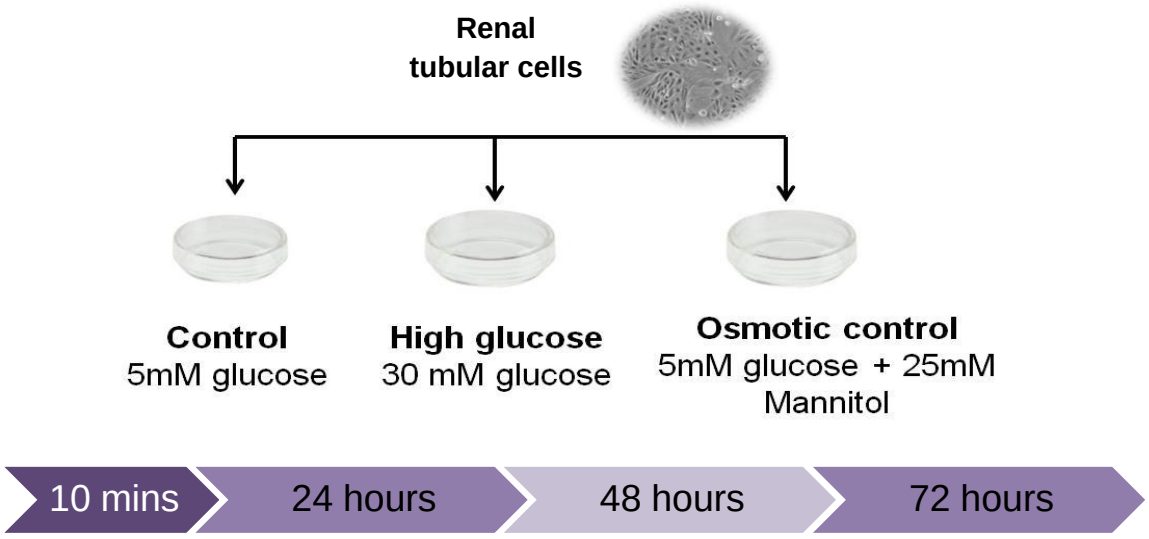


Fig. 2.1 Experimental model used to study the effects of high glucose on renal tubular cells.

2.1. Cell culture

Normal Rat Kidney cells (NRK-52E) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Life Technologies) supplemented with 10% Fetal Bovine Serum (FBS), 1% L-Glutamine and 1% Penicillin/Streptomycin. The cells were maintained at 37°C in 5% CO_2 incubator. Viability of cells was routinely checked using Trypan blue exclusion method. To obtain a model that represent the features of diabetic nephropathy (DN), cells were divided into 3 main groups; 1) control, where cells were treated with DMEM medium containing 5 mM glucose and 1% FBS, 2) high glucose treated, where cells were treated with DMEM medium containing 30 mM glucose and 1% FBS, 3) osmotic control, where cells were treated with DMEM medium containing 5 mM glucose and 25 mM mannitol to get a medium osmotically equivalent to high glucose media. Three doses of Epalrestat (0.1, 1 and 10 µM) were used as a co-treatment with these 3 main groups to investigate the effects of the aldose reductase inhibitor eplarestat on high glucose induced stress markers in NRK-52E cells and compare it to controls. The cells were exposed to the above-mentioned conditions for 24, 48 and 72 hours.

2.2 Trypan blue assay

Viability of NRK-52E cells was measured using trypan blue assay. At the endpoint, media was removed and cells washed with PBS. The cells were trypsinized and then counted using hemocytometer. Dead cells are stained blue and cell viability was calculated by taking ratio of live cells (not stained blue) to total number of cells.

2.3 Cell viability (MTT assay):

Viability of NRK-52E cells was assessed using MTT assay. The assay briefly depends on the ability of viable cells with active mitochondria to reduce the MTT reagent ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) which is yellow in color to insoluble purple formazan crystals. These crystals can then be solubilized in an organic solvent and then the color produced can be measured spectrophotometrically. NRK-52E cells were seeded in 96-well plates and then treated according to the previous mentioned protocol. At the end point, media was removed and replaced by 100 µl of fresh serum free media plus 10 µl of 5mg/ml MTT reagent (Sigma, M2128). The plates were incubated at 37°C for 3-4 hours. The media was then removed carefully in order not to disturb the formazan crystals formed, which were then solubilized using 50 µl of dimethylsulfoxide (DMSO). The absorbance was then measured at 570 nm using a microplate reader. Absorbance value from the control group was set as 100% and the values from treatment groups were expressed as a percentage of control.

2.4 Western blotting:

Western blotting was used to determine the change in expression of certain proteins following high glucose exposure and after various co-treatments with Eplarestat. NRK-52E cells were seeded in 6-well plates. At the end point, cells were lysed (60 mM Tris, pH 6.8 and 2% SDS), scrapped off the plates and collected in eppendorf tubes. Samples were sonicated briefly for 10-15 seconds and then centrifuged at 16000xg for 15 minutes at 4°C. The supernatant was then transferred to eppendorf tubes and protein concentration was quantified using bicinchonic acid (BCA) Protein Assay Kit (Pierce,

USA) according to the manufacturer's protocol. About 20 to 40 µg of protein from each group were loaded onto 10% or 12% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and resolved by electrophoresis under denaturing conditions. Running conditions were set at 80 volts for 20 minutes then 120 volts for about 2 hours. The proteins were then transferred from the gel to polyvinylidene fluoride (PVDF) membranes using wet transfer technique. The membranes were incubated in 5% skimmed milk prepared in Tris-buffered saline (TBS) for 1 hour at room temperature to block any non-specific binding. Primary antibodies diluted in TBS were added according to an optimized protocol for each antibody (as listed in the table 2.1) and the membranes were incubated overnight at 4°C. The membranes were then washed using 0.1% Tween-20 containing TBS (TBS-T) for 3 times at 10 minute intervals. Membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (1:10000; Abcam, UK) or HRP-conjugated goat anti-rabbit IgG (1:20000; Abcam, UK) secondary antibodies (diluted in TBS-T) for 1 hour at room temperature. The membranes were then washed 3 times with TBS-T at 10 minutes intervals. The immunoreactivity was then visualized using Optiblot ECL detection kit (Abcam, UK). Images were captured using Syngene (G-BOX Chemi XX6) imaging system, and analyzed using Scion Image software. The densitometry values were normalized to actin (used as loading control) and expressed as percentage of control.

Table 2.1 Primary and secondary antibodies used in the study

Protein	Primary antibody	Secondary antibody	Source
Aldose reductase	Mouse monoclonal [1E10-E8-E9] to AKR1B1 antibody; 1:1000	Goat anti-mouse antibody: HRP conjugated	Abcam (ab131182)
GRP78	Rabbit polyclonal to GRP78 BiP antibody; 1:2000	Goat anti-rabbit antibody: HRP conjugated	Abcam (ab21685)
CHOP	Mouse monoclonal [9C8] to DDIT3 antibody; 1:500	Goat anti-mouse antibody: HRP conjugated	Abcam (ab11419)
p-Akt	Rabbit monoclonal [EP2109Y] to AKT1 (phospho S473) antibody; 1:2000	Goat anti-rabbit antibody: HRP conjugated	Abcam (ab81283)
Akt	Rabbit monoclonal [Y89] to AKT1	Goat anti-rabbit antibody: HRP	Abcam

	1:2000	conjugated	(ab32505)
p-ERK 1/2	Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10) Mouse monoclonal antibody; 1:1000	Goat anti-mouse antibody: HRP conjugated	Cell signaling (#9106)
ERK 1/2	p44/42 MAPK (Erk1/2) Antibody; 1:1000	Goat anti-rabbit antibody: HRP conjugated	Cell signaling (#9102)

2.5 Assessment of mitochondrial membrane potential (JC-1 assay)

Changes in mitochondrial membrane potential in NRK-52E cells were assessed using a lipophilic cationic probe 5,5′,6,6′-tetrachloro-1,1′,3,3’ tetraethylbenzimidazolylcarbocyanine iodide; JC-1 (T-3168, Life Technologies). Cells were seeded in black 96-well plates. At the endpoint, media was removed and cells washed once with Phosphate Buffered Saline (PBS). 10 µM of the JC-1 reagent was added to 100 µl of phenol red free media and incubated at 37°C for 30 min. The dye containing media was then removed and the cells were washed twice using PBS. 100 µl

of PBS was added and fluorescence emitted was measured using a microplate reader at Ex/Em 488/529 to measure monomers fluorescence (high intensity in unhealthy cells) and Ex/Em 488/590 to measure J-aggregates fluorescence (high intensity in healthy cells). The ratio of fluorescent intensity of J-aggregates (red) to the fluorescent intensity of monomers (green) was calculated to determine changes in mitochondrial membrane potential.

For fluorescence microscopy, similar protocol was used in a 6 well plate. After staining with JC-1 dye for 30 minutes at 37°C, wells were washed twice with PBS then 1 ml of PBS was added to each well then viewed using fluorescent microscope at 20X magnification.

2.6 Production of reactive oxygen species (ROS) (DCF assay)

DCF assay was used to measure the production of ROS in NRK-52E cells after exposure to high glucose concentration. Cells were seeded in black 96 well plates and left to attach overnight. Cells were then treated with high glucose as described in cell culture. At endpoint, media was removed and cells washed once with PBS. 2′,7′-Dichlorofluorescein diacetate (DCFDA) (Sigma, 3584) reagent was prepared in 5 and 10 µM concentrations in phenol red free media then 100 µl were added to each well. Plates were then incubated at 37ºC for 30 minutes. Media containing DCF was then removed and wells washed twice with PBS. 100 µl of PBS was added to each well, and the DCF

fluorescence was measured using microplate reader (Spectramax 2) at Ex./Em. 485/535 nm.

2.7 Statistical analysis

All values were calculated as a percentage compared to control group (5 mM glucose). Values were expressed as Mean ± SEM rather than SD to maintain consistency among graphs. One-way ANOVA test was used to detect statistical differences between different groups and Tukey's post-hoc test was conducted to compare pairs of groups together. $P < 0.05$ was considered statistically significant.

Chapter 3: Effects of high glucose concentration on renal distal tubular cells using Madin-Darby Canine Kidney (MDCK) cells

3.1 Results

3.1.1 Cell viability

3.1.1.1 Effect of 25 mM glucose and 10% FBS on MDCK cells viability

MDCK cells were exposed to high glucose media (25 mM) containing 10% FBS for 24 and 48 hours. 25 mM mannitol was used as osmotic control. Cell viability was assessed using trypan blue assay. No difference in cell viability was observed in high glucose and osmotic control groups when compared to control.

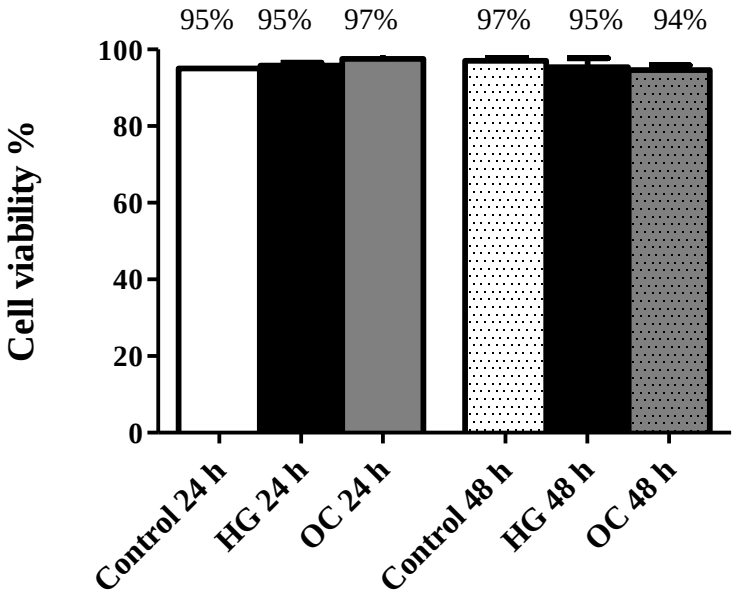


Fig. 3.1. Effect of high glucose (25 mM and 10% FBS) on cell viability in MDCK cells after 24 and 48 h as determined by Trypan blue assay. Values were expressed as Mean ± SEM. (24 hours, n=2; 48 hours, n=3)

3.1.1.2 Effect of 30 mM glucose and 10% FBS on MDCK cells viability

Glucose concentration was increased to 30 mM containing 10% FBS for 48, 72 and 96 hours and then cell viability was assessed by Trypan blue and MTT assays. 30 mM mannitol was used as osmotic control. In Trypan blue assay, no difference in cell viability was observed in high glucose and osmotic control groups when compared to control while by using MTT assay there was a slight decrease in viability observed after 48 hours but was not statistically significant.

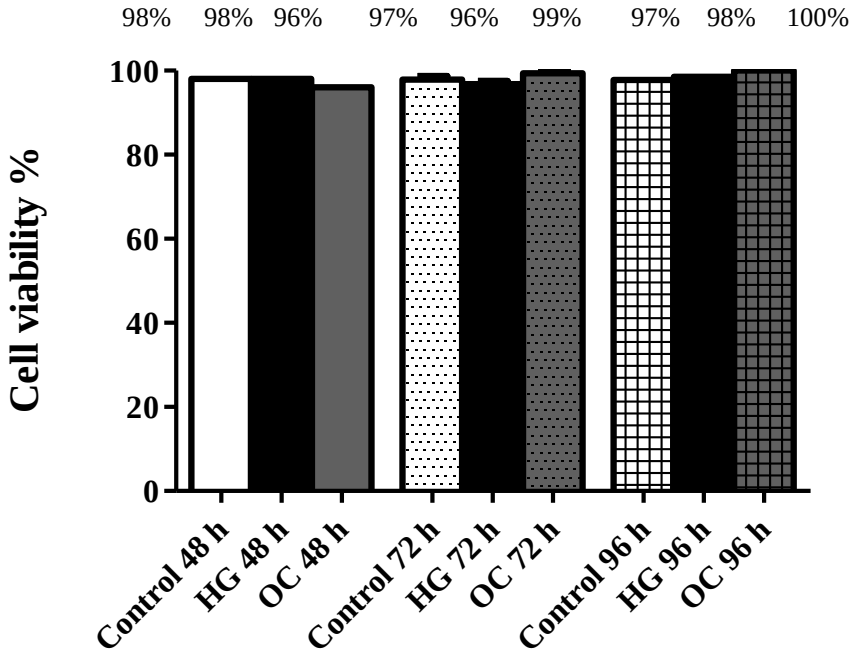


Fig. 3.2. Effect of high glucose (30 mM and 10% FBS) on cell viability in MDCK cells after 48, 72 and 96 h as determined by Trypan blue assay. Values were expressed as Mean ± SEM. (48 hours, n=1; 72 hours, n=2; 96 hours, n=1)

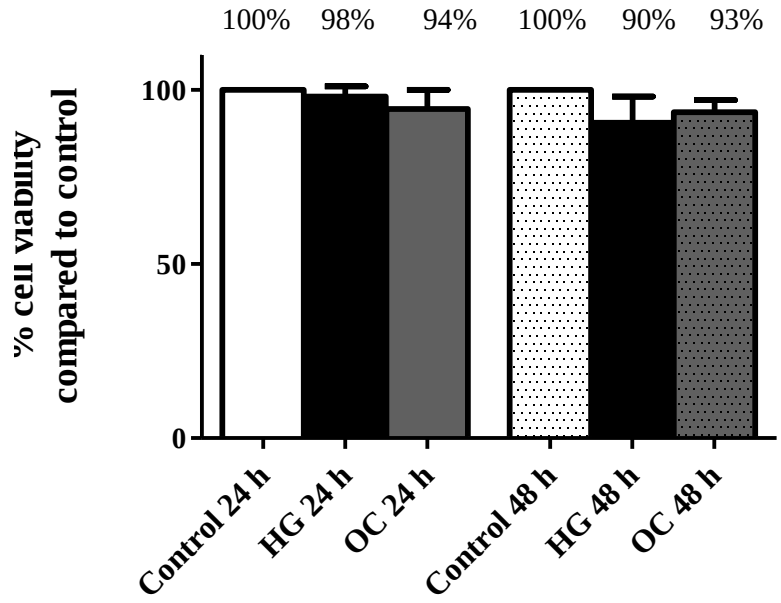


Fig. 3.3 Effect of high glucose (30 mM and 10% FBS) on cell viability in MDCK cells after 24 and 48 h as determined by MTT assay. Values were expressed as Mean ± SEM. (24 hours, n=2; 48 hours, n=2)

3.1.1.3 Effect of 30 mM glucose and 1% FBS on MDCK cells viability

MDCK cells were then exposed to high glucose media (30 mM) while decreasing FBS concentration from 10% to 1% for 24, 48 and 72 hours. 30 mM mannitol with 1% FBS was used as osmotic control. Cell viability was assessed by Trypan blue and MTT assays. Using Trypan blue assay no difference in cell viability was observed in high glucose and osmotic control groups when compared to control after 24, 48 and 72 hours. However, by using MTT assay, there was a decrease in viability after 48 hours by 17%, which was statistically significant ($P<0.05$)

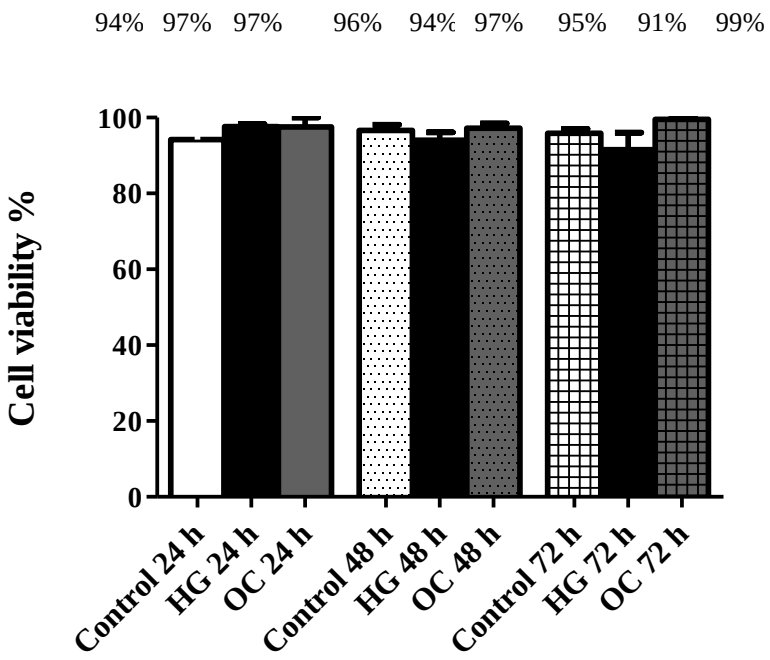


Fig. 3.4 Effect of high glucose (30 mM and 1% FBS) on cell viability in MDCK cells after 24, 48 and 72 h as determined by Trypan blue assay. Values were expressed as Mean ± SEM. (24 hours, n=2; 48 hours, n=3; 72 hours, n=2)

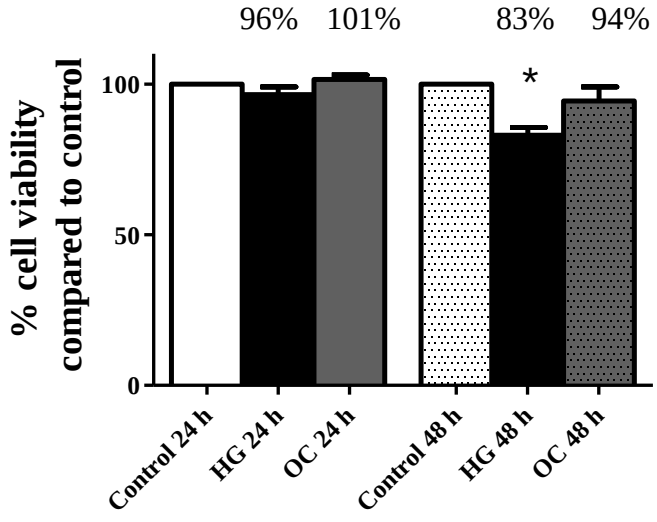


Fig. 3.5 Effect of high glucose (30mM) on cell viability in MDCK cells after 24 and 48 h as determined by MTT assay. Values were expressed as Mean ± SEM. *P < 0.05 compared to control 48 h. (24 hours, n=2 and 48 hours, n=5)

3.1.2 Effect of 30 mM glucose and 1% FBS on aldose reductase expression in MDCK cells

Western blot was used to measure aldose reductase expression in MDCK cells following exposure to high glucose medium (30 mM and 1% FBS) for 48 hours. Aldose reductase expression has increased in high glucose group when compared to controls.

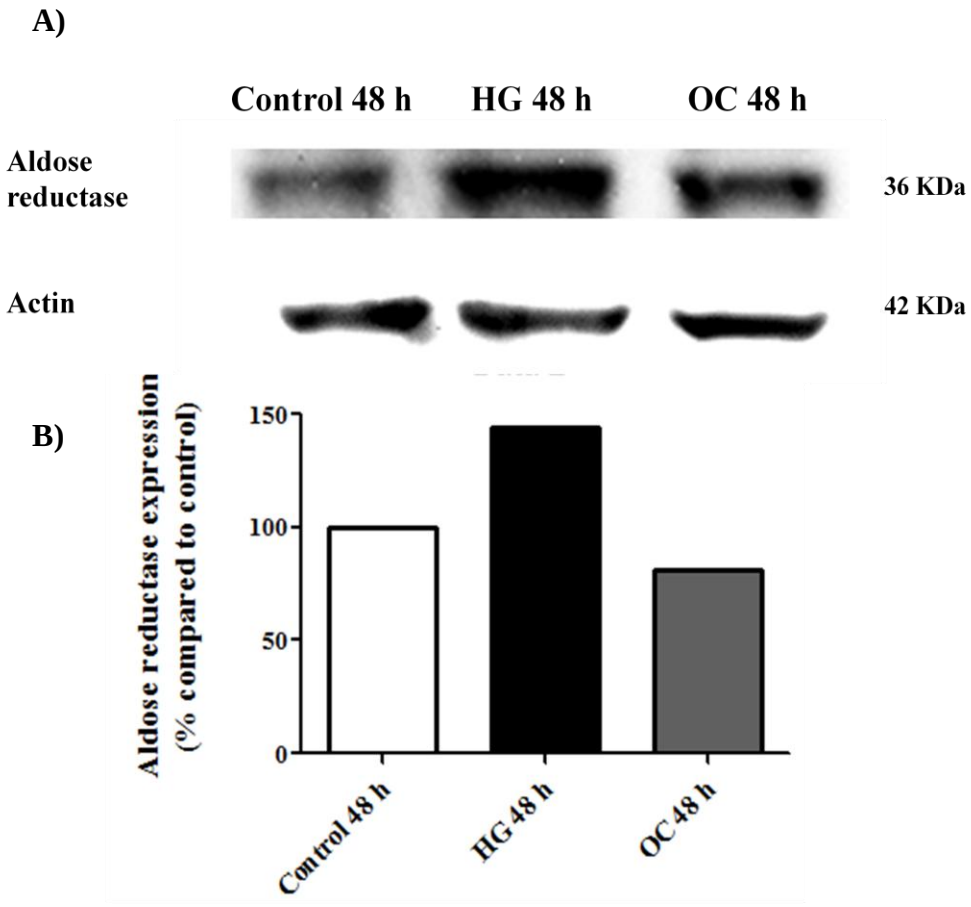
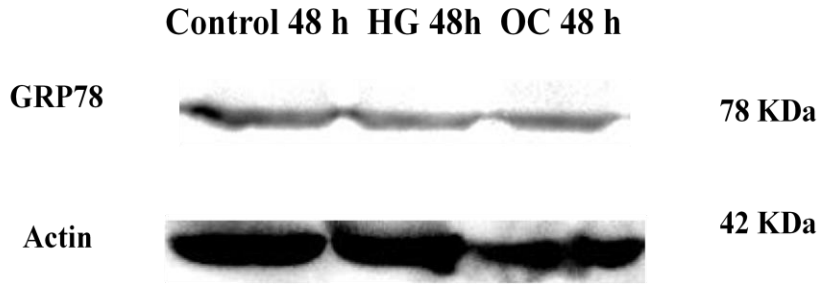


Fig. 3.6 Effect of high glucose (30 mM and 1% FBS) on aldose reductase expression in MDCK cells after 48 h as measured by western blotting. A) A representative western blot. B) Densitometric analysis. (n=1)

3.1.3 Effect of 30 mM glucose and 1% FBS on endoplasmic reticulum stress in MDCK cells

The expression of GRP78 as an indicator of ER stress was measured in MDCK cells after exposure to 48 hours of high glucose (30 mM and 1% FBS). GRP78 expression has decreased when compared to controls.

A)



B)

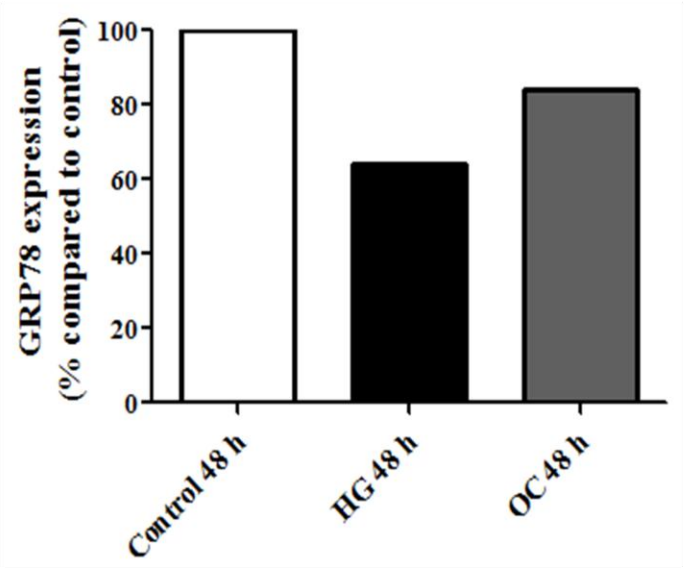


Fig. 3.7 Effect of high glucose (30 mM and 1% FBS) on GRP78 expression in MDCK cells after 48 h as measured by western blotting. A) A representative western blot. B) Densitometric analysis. (n=1)

3.2 Discussion

MDCK are epithelial cells obtained from distal tubules of dogs. For the optimization of an *in vitro* DN model using tubular cells, MDCK cells were exposed to different glucose and FBS concentrations. When cells were treated with 25 and 30 mM with 10% FBS for 24, 48 and 72 hours, no changes in cell viability were observed in high glucose-treated groups when compared to respective controls. Whereas, when cells were challenged with 30 mM glucose and at a reduced serum concentration (1% FBS), there was a decrease in cell viability after 48 hours (83%); however, results from 72 hours were inconsistent.

Other markers explored in the model were aldose reductase and GRP78 protein expression (as an ER stress marker). It has been observed that aldose reductase was slightly increased after 48 hours of exposure while GRP78 expression has decreased. However, these results were from only one trial. Some difficulties were faced during working with MDCK cells. MDCK cells being from canine origin placed some obstacles to find optimum growth conditions and reproducible results. Also, antibodies specific for canine species are not as widely available as for other species. Therefore, the effects of high glucose on renal tubular cells were tested on NRK-52E cells to determine if it could serve as a better candidate for DN model.

Chapter 4: Effects of high glucose concentration on renal proximal tubular cells using Normal Rat Kidney (NRK-52E) cells

4.1 Results

4.1.1 Effect of 30 mM glucose and 1% FBS on NRK-52E cells viability

NRK-52E cells were exposed to high glucose media (30 mM) and 1% FBS for 24, 48 and 72 hours. 30 mM mannitol with 1% FBS was used as osmotic control. Cell viability was assessed by MTT assay. It was observed when using cells at low passage number (P2 to P6), cell viability was not affected after 24 h of high glucose exposure, while there was a decrease in viability after 48 hours to 84% which further declined to 68% after 72 h. Decrease in viability was statistically significant when compared to respective controls. However, decrease in viability after 48 and 72 h was not reproduced when using cells at higher passage numbers (more than P7)

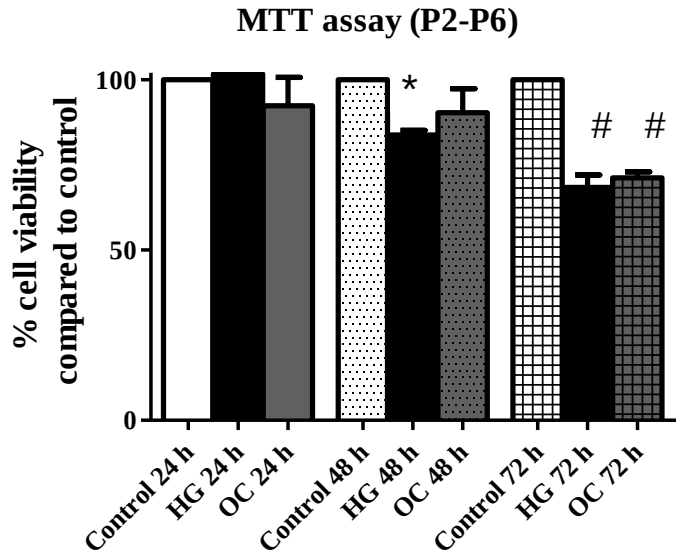


Fig. 4.1 Effect of high glucose (30 mM) on cell viability in NRK-52E (using low passage number P2-P6) cells after 24 h, 48 h and 72 h as determined by MTT assay. Values were expressed as Mean ± SEM. *P < 0.05 compared to control 48h. $^{\#}$P < 0.05 compared to control 72 h (24 h, n=3; 48 h, n=5, and 72 h, n=5)

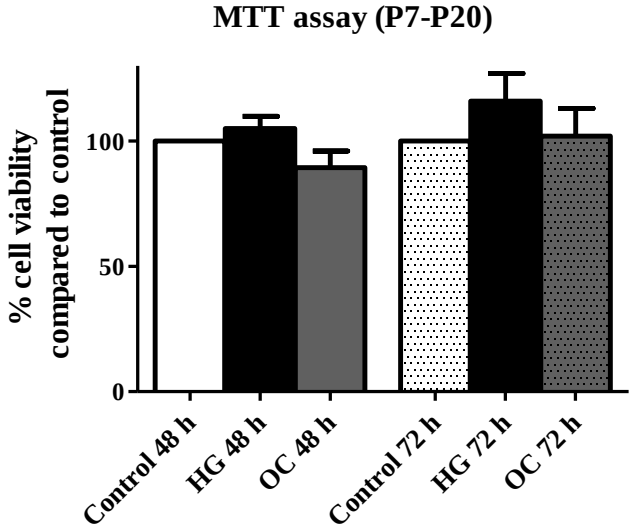


Fig. 4.2 Effect of high glucose (30 mM) and 1% FBS on cell viability in NRK-52E cells (using high passage number P7-P20) after 48 h and 72 h as determined by MTT assay. Values were expressed as Mean ± SEM. (48 h, n=5 and 72 h, n=2)

4.1.2 Endoplasmic reticulum stress

4.1.2.1 Effect of 30 mM glucose and 1% FBS on GRP78 protein expression in NRK-52E cells

When cells experience ER stress they respond by initiating the UPR signaling pathways. GRP78 is a chaperone, which is used by the ER machinery to help in proper folding of misfolded proteins and alleviate the load on ER. Therefore to examine if cells are experiencing endoplasmic reticulum stress upon exposure to high glucose media, GRP78 protein expression was measured after exposing cells to 30 mM glucose and 1% FBS for 24 and 48 h using tunicamycin (1 µg/ml for 24 h) as a positive control. No induction of GRP78 protein expression was observed when compared to control.

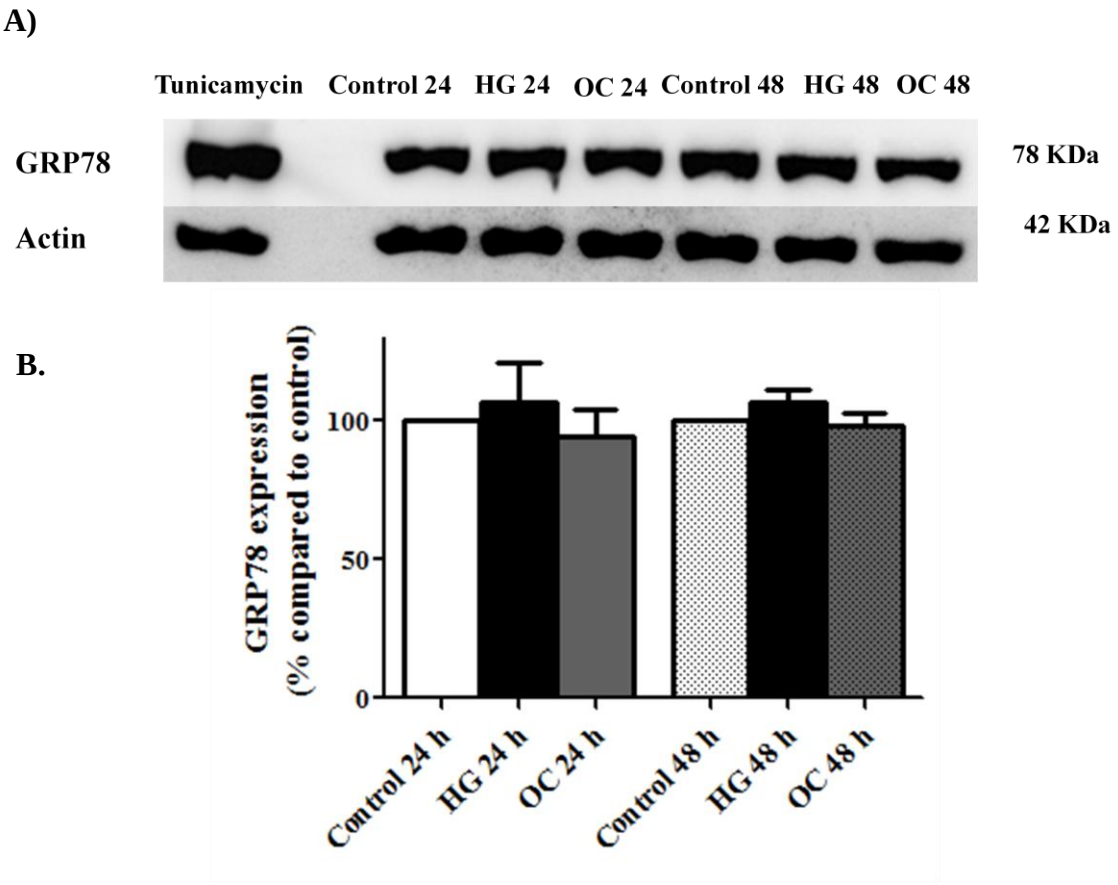


Fig. 4.3 Effect of high glucose (30 mM and 1% FBS) on GRP78 expression in NRK-52E cells after 24 h and 48 h as measured by western blotting. A) A representative western blot. B) Densitometric analysis. Values were expressed as Mean ± SEM (n=3)

4.1.2.2 *Effect of 30 mM glucose and 1% FBS on CHOP protein expression in NRK-52E cells*

To confirm that ER stress is not induced in the model, CHOP protein expression was measured as another marker of ER stress. CHOP directs the cells towards apoptotic pathways when the ER is no longer able to handle the load of misfolded protein. CHOP expression was measured after 24 and 48 h of high glucose exposure using tunicamycin

(1 µg/ml for 24 h) as positive control. CHOP was not induced in any of the samples tested except for tunicamycin.

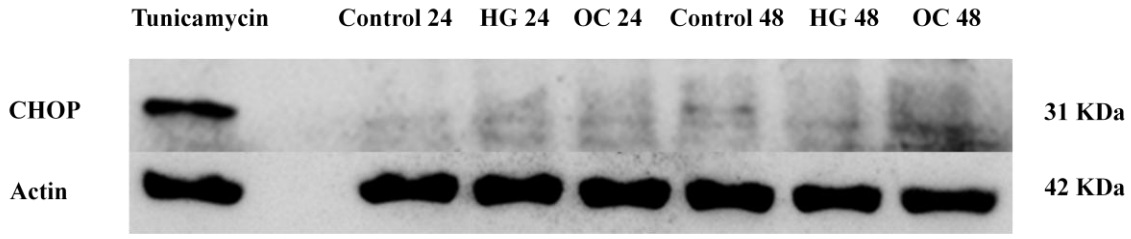


Fig. 4.4 A representative blot for effect of high glucose (30 mM and 1% FBS) on CHOP expression in NRK-52E cells after 24 and 48 h as measured by western blotting. (n=3)

4.1.3 Effect of 30 mM glucose and 1% FBS on mitochondrial membrane potential

The relative mitochondrial membrane potential (Δψm) in NRK -52E cells after being exposed to high glucose concentration was assessed using JC-1 dye. JC-1 dye is a cationic dye, which at high Δψm forms J-aggregate with red fluorescence, while at low Δψm it remains as monomers with green fluorescence. Therefore, the ratio of red to green fluorescence is indicative of the polarization status of the mitochondria membrane. When NRK-52E cells were exposed to 30 mM glucose, mitochondrial membrane was depolarized (the red to green fluorescence ratio of high glucose group was 81% as compared to control, which is set at 100%). Although the mitochondrial depolarization was sustained after 48 h of high glucose exposure, it was not statistically significant.

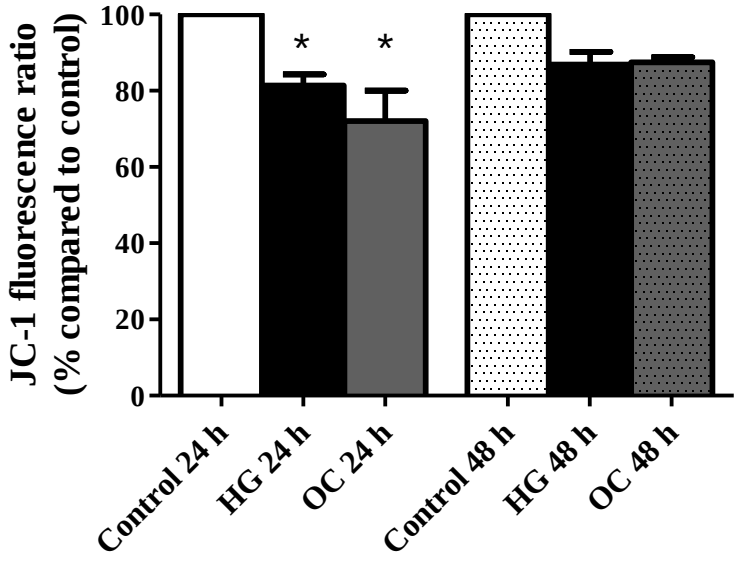


Fig. 4.5 Effect of high glucose concentration on relative mitochondrial membrane potential in NRK-52E cells after 24 and 48 h using JC-1 staining, Fluorescence values are expressed as J-Aggregate (Ex /Em 488/529) to Monomer (Ex /Em 488/590) ratio compared to control ± SEM (n = 3). *P < 0.05 compared to control 24 h.

4.1.4 Effect of high glucose (30 mM and 1% FBS) on Akt and ERK signaling pathways

Akt and ERK signaling pathways were investigated in the model to study the effects of high glucose on activation of these pathways in renal tubular cells. NRK-52E cells were exposed to high glucose for 10 minutes, 30 minutes, 1 hour, 2 hours and 4 hours. In both Akt and ERK pathways there was acute induction of P-Akt and P-ERK after 10 minutes of high glucose exposure, which then subsided at further time points tested.

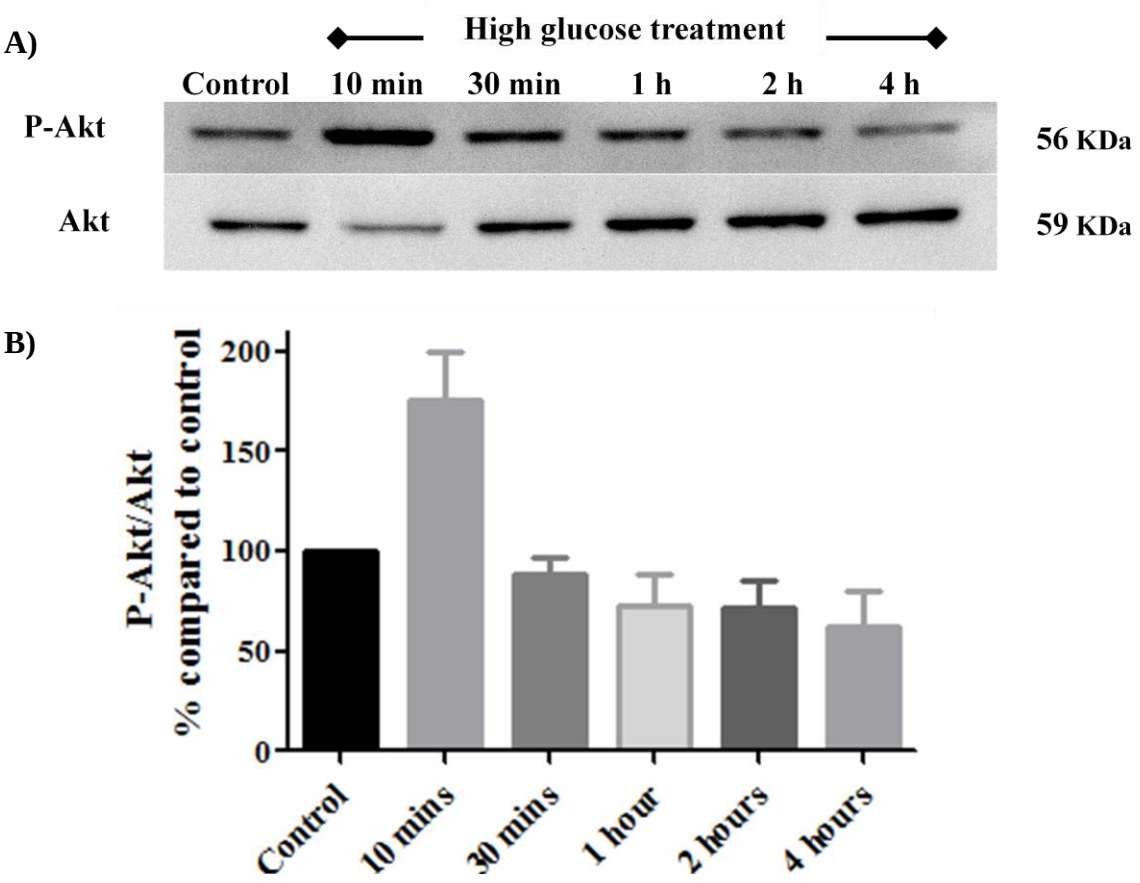


Fig. 4.6 Activation of Akt pathway was assessed after exposing NRK-52E cells to high glucose medium (30 mM and 1% FBS) for 10 minutes, 30 minutes, 1 hour, 2 hours and 4 hours. P-Akt expression was normalized to total Akt expression. A) A representative western blot. B) Densitometric analysis. Values were expressed as percentage relative to control. Data is shown as Mean ± SEM (n=3)

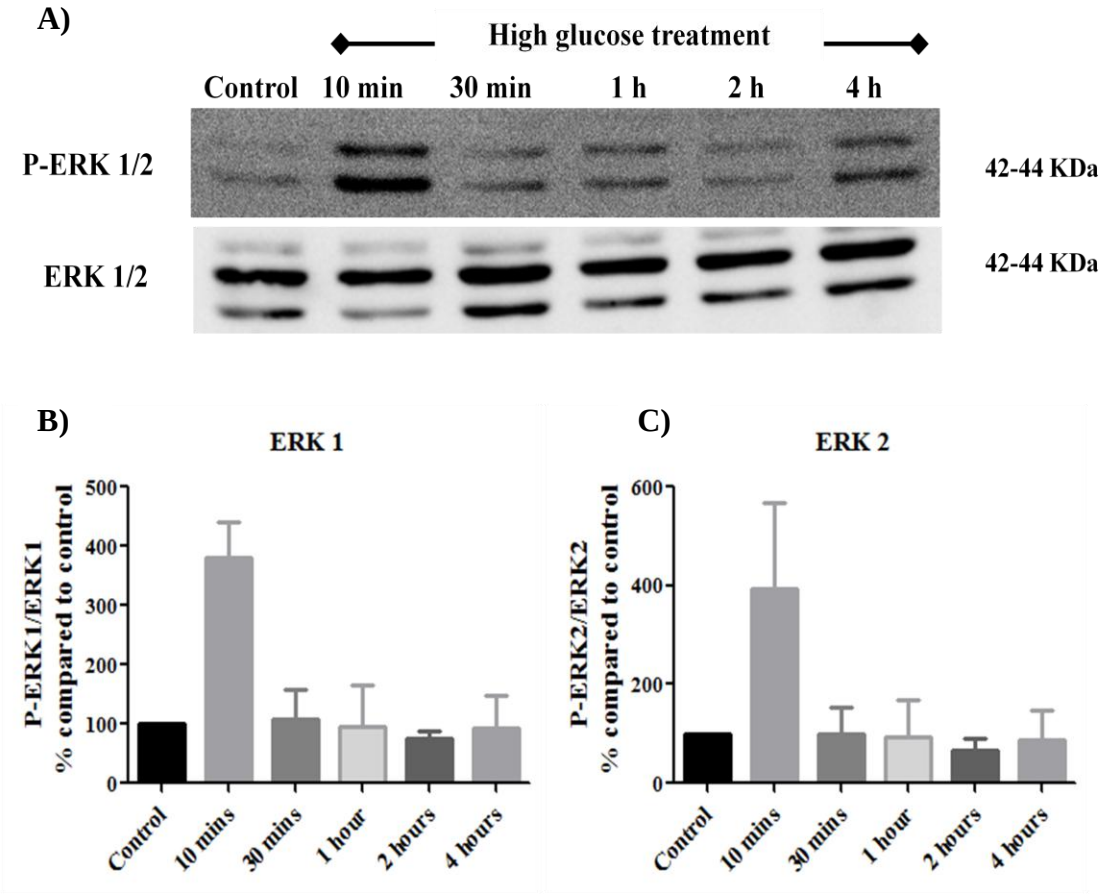


Fig. 4.7 Activation of ERK signaling pathway was assessed after exposing NRK-52E cells to high glucose medium (30 mM and 1% FBS) for 10 minutes, 30 minutes, 1 hour, 2 hours and 4 hours. A) A representative western blot for P-ERK1/2 and ERK1/2. B) Densitometric analysis for ERK1. C) Densitometric analysis for ERK2. Values were expressed as percentage relative to control. Data is shown as Mean ± SEM (n=3)

4.1.5 Effect of 30 mM glucose and 1% FBS on production of ROS

To examine if high glucose concentration causes oxidative stress in NRK-52E cells, 2',7'–dichlorofluorescein diacetate (DCFDA) staining was used to measure the production of ROS following high glucose exposure. DCFDA is a cell permeable fluorogenic probe, which becomes deacylated in cells by cellular esterase giving rise to a non-fluorescent compound. In the presence of ROS, this non-fluorescent compound is oxidized to give 2',7'–dichlorofluorescein (DCF), which is a highly fluorescent compound that can be detected using fluorescence microscopy. Intriguingly, no ROS production has been detected in cells exposed to high glucose (30 mM and 1% FBS) for 24 and 48 hours when compared to controls.

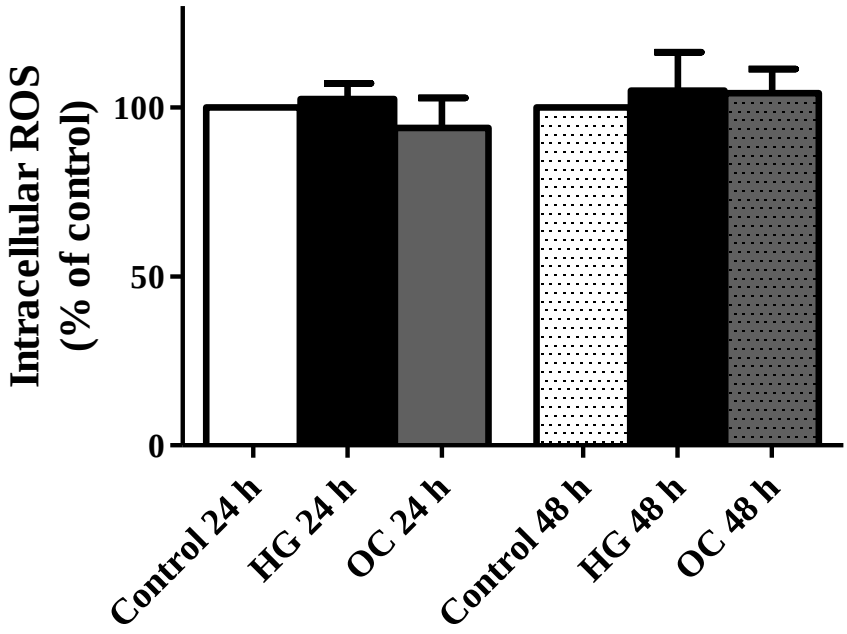


Fig. 4.8 Effect of high glucose (30 mM and 1% FBS) on levels of intracellular ROS in NRK-52E cells. Intracellular ROS production was detected by DCF fluorescence assay. Data is shown as mean ± SEM. (24 h, n=7 and 48 h, n=4)

4.2 Discussion

4.2.1 Effect of 30 mM glucose and 1% FBS on NRK-52E cells viability

It has been shown that exposing NRK-52E cells to 30 mM glucose and 1% FBS decreased cell proliferation (84% compared to control) after 48 h which further declined to 68% after 72 hours as determined using MTT assay hence, these concentrations (30 mM glucose and 1% FBS) were chosen for subsequent experiments. Similarly, the study conducted by Park et al. using primary rabbit renal proximal tubular cells challenged cells with 25 mM glucose observed inhibition of cell proliferation demonstrated in decrease of ^{3}H thymidine incorporation progressively over 24, 48 and 72 hours. In addition, they compared the effect three different glucose concentrations (10, 25 and 50 mM) on ^{3}H thymidine incorporation and found dose dependent decrease, which was further confirmed by counting cells using coulter counter. Therefore, it was concluded that high glucose induce decrease in DNA synthesis that was time and dose dependent. However, by using trypan blue exclusion method (a measure for cell viability) and lactate dehydrogenase (LDH) release assay (also a measure for cell injury) no changes have been observed in high glucose group when compared to control after using the three different concentrations (10, 25 and 50 mM) (108). This latter finding is consistent with the finding from the current study where exposing NRK-52E cells to 30 mM and 1% FBS didn't cause any changes in cell viability when compared to control by using trypan blue exclusion method. A possible explanation is that high glucose inhibits tubular cells proliferation by decreasing DNA synthesis without affecting the integrity of the cell walls

and viability of cells. On the other hand, studies reporting effects of high glucose on mesangial cells proliferation are contradicting. A study using human mesangial cells showed that there was a significant decrease in cell number (using hemocytometer for direct counting) after 8 days of exposure to 20 mM glucose and this decrease was sustained over 14 days of exposure (109). Additionally, they have also used 115 mM glucose which showed more pronounced decrease in cell number starting from day 2 and until day 14 (109). Interestingly, they didn't observe any changes in cell viability using trypan blue method with both 20 mM and 115 mM glucose concentration as in all experiments over 95% of the cells excluded the dye (109). This provides more evidence that trypan blue method is not a suitable assay for assessing high glucose induced cell injury in renal cells. In another study, Wolf et al. using murine mesangial cells reported a biphasic growth pattern of mesangial cells (initial transient replication for 24 and 48 hours followed by sustained inhibition at higher time points) when exposed to 33 mM glucose which was measured using ^{3}H thymidine incorporation assay (110). Another study showed that high glucose (25 mM) induced rat mesangial cells proliferation after 24 h, which is consistent with the study by Wolf et al., which showed initial increase in mesangial cell proliferation upon exposure to high glucose (111). The discrepancy in the literature whether high glucose induces or inhibits mesangial cells proliferation might be attributed to the difference in the species used (human, mouse and rats) and the use of different FBS concentrations, as studies that showed high glucose induced proliferation in mesangial cells (110, 111) reported using either serum-free or 0.5% FBS containing media.

4.2.2 Effects of hyperglycemia on ER stress

Investigating ER stress response in the kidneys is very challenging because kidneys consist of diverse cell types and the UPR signaling differs from one cell type to another and functions in tissue context manner. In addition, the complexity, sophistication and dynamic expression of the three arms of UPR make it hard to predict and interpret the cellular response to different stimuli (52). As discussed earlier, a study has shown that glomerular and tubular cells isolated from diabetic rats showed both enhanced pro-survival ER stress markers (GRP78) as well as pro-apoptotic markers (CHOP, JNK and caspase 12) (53). In the current study the protein expression of GRP78 was investigated as a pro-survival ER stress marker to explore the activation of ER stress in response to high glucose. GRP78 protein expression has not been induced after exposing cells to high glucose after 24 and 48 h. To further confirm that ER stress is not induced in the model, the pro-apoptotic stress marker CHOP was also investigated. Similarly, there was no induction of CHOP in response to high glucose concentration. These findings indicate that ER stress response was not activated in the current model and that cell injury observed is ER stress independent.

4.2.3 Hyperglycemia induces slight mitochondrial membrane potential depolarization

It has been shown that high glucose induces mitochondrial alterations and dysfunction, which ultimately leads to apoptosis. In addition, it has been reported that

high glucose causes remarkable depolarization in mitochondrial membrane potential and mitochondrial fragmentation in retinal pericytes, which consequently leads to apoptosis, causing prominent lesions characteristic of diabetic retinopathy (112). In the current study, the effect of high glucose on mitochondrial membrane potential was tested in NRK-52E cells using JC-1 dye. After 24 hours, there was a slight decrease (81% compared to control) in mitochondrial membrane potential, which was sustained at 48 h (86% compared to control) indicating that high glucose-induced mitochondrial membrane depolarization in the tubular cells. This finding is in line with other studies, which demonstrated that high glucose induces mitochondrial dysfunction in human kidney cells (HK-2) when exposed to high glucose medium for 72 hours (113-115). The mitochondrial depolarization was further illustrated from altered mitochondrial morphology; some mitochondria were swollen and showed dilation in their cristae, while others were angulated and attenuated along their longitudinal axis. Furthermore, the mitochondrial morphological changes were associated with depression in mitochondrial membrane potential (114). More recently, another study also demonstrated that high glucose induce mitochondrial membrane depolarization in renal proximal tubular cells. Therefore, these findings suggest that tubular cell death observed in the current model might be initiated by alterations in mitochondrial function.

4.2.4 High glucose induced activation of Akt and ERK signaling pathways

PI3K/Akt pathway is a vital pathway that is implicated in numerous cellular processes (60). Research has shown impairments in the PI3K/Akt pathway in diabetes

and signified its role in the development of diabetic complications. Activation of Akt contributes to the development of DN through several mechanisms. It has been shown that Akt regulates the production of several ECM proteins such as collagen I, collagen IV and laminin (116, 117). *In vivo* studies have demonstrated increase in S473-phosphorylated Akt in glomeruli of diabetic rats (118). To further elucidate signaling transduction pathways involved, several studies exposed mesangial cells *in vitro* to high glucose medium and observed alterations is cytokines and growth factors affecting the Akt pathway (118, 119). It has been shown that hyperglycemia induces collagen I upregulation in mesangial cells by stimulating different cytokines such as TGF-β and epidermal growth factor (EPG) through PI3k/Akt dependent pathway, which highlights the crucial role of this pathway in matrix modulation (118-120). In a similar context, the activation of PI3K/Akt pathway in renal tubular has been linked to tubular cells hypertrophy (92, 106, 121). Studies using renal tubular cells such as LLC-PK1 and HK-2 cells have demonstrated activation of PI3K/Akt pathway following high glucose exposure (92, 121). Furthermore, tubular cells exposed to high glucose have showed cell cycle-dependent hypertrophy illustrated by the increased percentage of cells in G_1 phase, indicative of cell cycle arrest at G_1 phase, i.e., increase in the cellular content without a proportional increase in DNA content. This cell cycle arrest has been shown to be mediated through induction of p21 protein (cell cycle inhibitor that binds to cyclin E and cause cell cycle arrest at G_1 phase) via PI3K/Akt pathway (92, 121). Another postulated mechanism is the involvement of the cytokine VEGF in tubular hypertrophy in a PI3k/Akt dependent manner but through the phosphorylation of another Akt downstream

target, which is 4E-BP1 (106). In this study, they showed that VEGF (which is induced in diabetes) was capable of phosphorylating 4E-BP1 in proximal tubular cells (MCT) through PI3K/Akt activation. Phosphorylation of 4E-BP1, which is a major regulator of protein translation at the initiation phase leads to increase protein translation, and subsequent increase in protein/DNA ratio in cells and hence, cells increase in size (106). Postulated mechanisms by which Akt induces hypertrophy and mesangial expansion is summarized in the following figure **(Fig 4.9):**

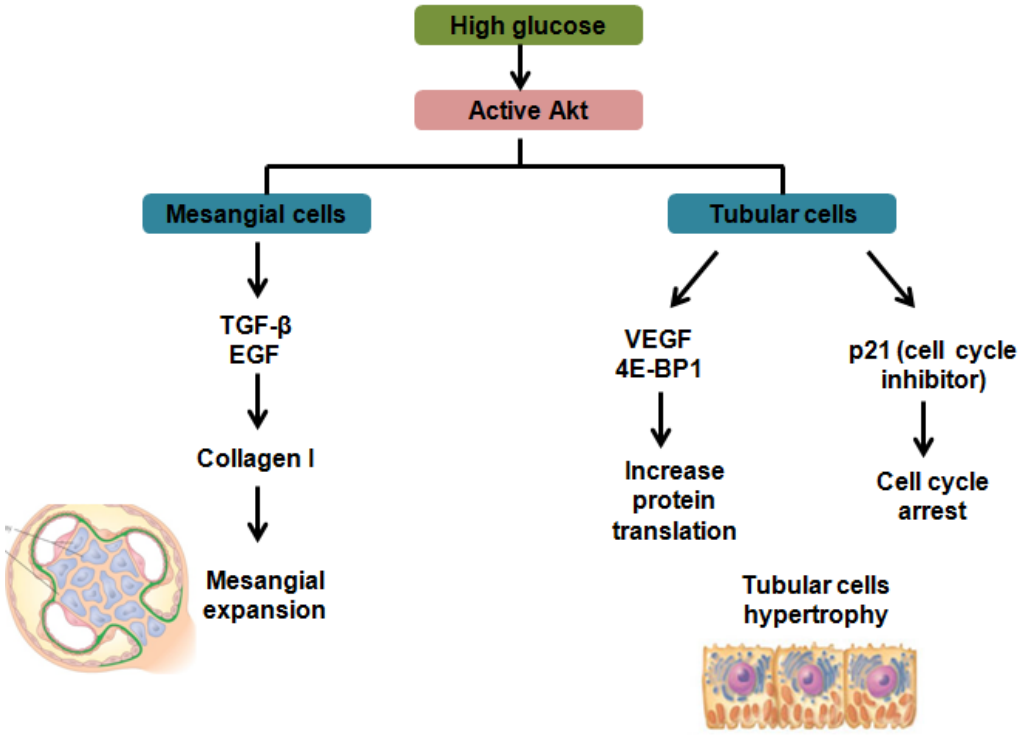


Fig. 4.9 Suggested mechanisms by which Akt activation contributes to the development of DN. In mesangial cells, Akt induces cytokines involved in upregulation of ECM proteins, while in tubular cells Akt activates pathways that ultimately causes hypertrophy. **TGF-β,** transforming growth factor beta; **EGF,** epidermal growth factor; **VEGF,** vascular endothelial growth factor; **4E-BP1,** Eukaryotic translation initiation factor 4E-binding protein 1

In this study, exposing NRK-52E cells to 30 mM glucose caused acute activation of S473-phosphorylated Akt after 10 minutes of exposure, which then subsided at higher time points (30 minutes, 1 h, 2 h and 4h). This finding was consistent with other findings previously described in literature that high glucose activate Akt pathway in renal cells.

ERK1/2 pathway is also a major pathway that is actively involved in regulation of cellular functions. It has been shown that mitogen-activated protein kinase cascade was activated in glomerular mesangial cells exposed to high glucose concentrations as well as in diabetic rats' glomeruli (122). Furthermore, inhibiting ERK 1/2 pathway in mesangial cells *in vitro* reversed high glucose induced TGF-β1 expression, α1 (I) collagen and fibronectin mRNA levels (123, 124). Therefore, it was concluded that high glucose induced ECM protein expression observed in DN involves the activation of ERK1/2 pathway. Similarly, phosphorylated ERK1/2 have been induced in renal tubules of diabetic rats (125). In addition, *in vitro* studies using renal tubular cells showed that ERK1/2 pathway is implicated in high glucose induced TGF-β1, fibronectin and collagen IV expression as well as tubular cells hypertrophy (125, 126). Moreover, a study using murine renal proximal tubular epithelial cells showed that ERK1/2 pathway mediates laminin-β1synthesis, an important component of ECM (127). Taken together, these finding provide evidence of the role of ERK1/2 pathway in the development and progression of DN. In this study, high glucose induced phosphorylation of ERK1/2 after exposing cells to high glucose conditions for 10 minutes, which is similar to Akt activation in the model.

Based on previous finding in literature, acute activation of both Akt and ERK1/2 pathway in the current model can cause hypertrophy in cells, which can be confirmed by either measuring protein to DNA ratio or cell cycle analysis. In addition, exploring the transcriptional levels of ECM proteins such as fibronectin and collage IV will give further evidence to support the involvement of Akt and ERK1/2 pathways in renal tubular cells hypertrophy and fibrosis the hallmarks of DN.

Changes in the model upon exposure to high glucose are summarized in **(Fig. 4.10).**

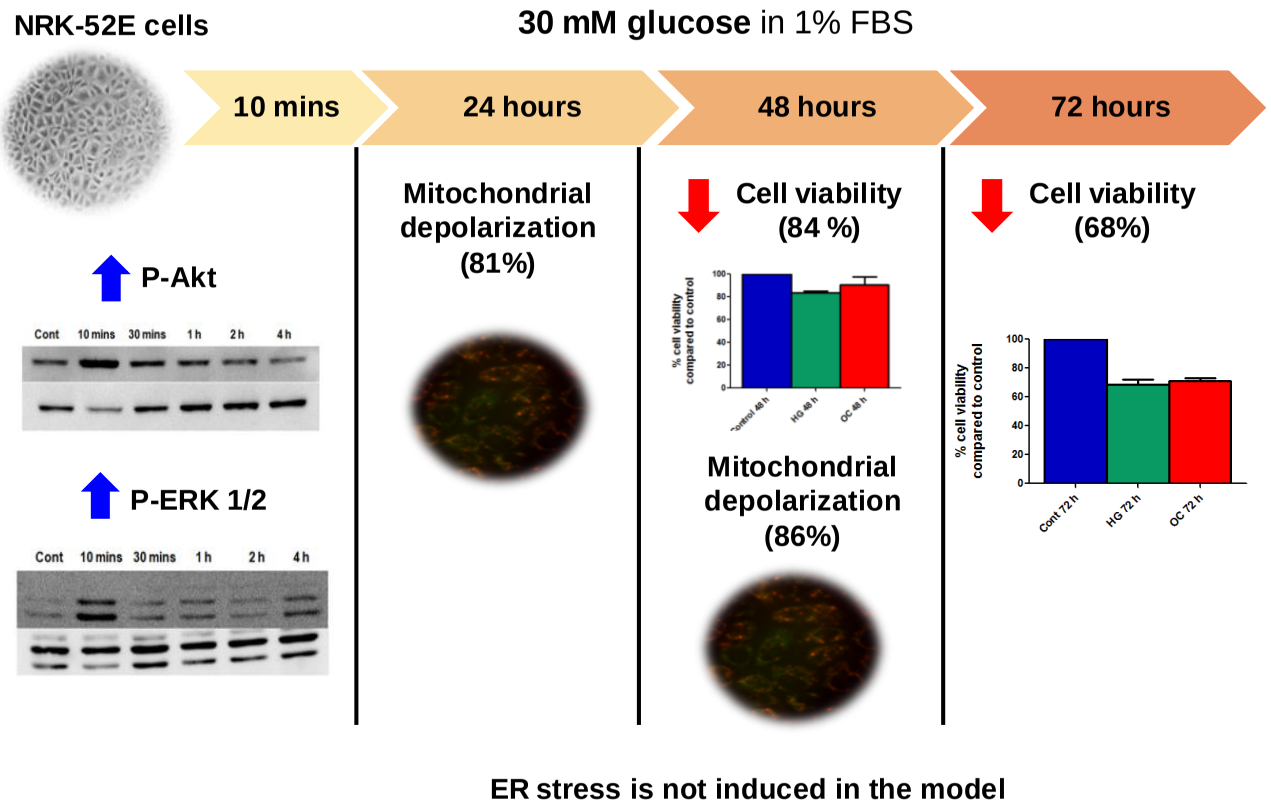


Fig. 4.10 A diagram summarizing the experimental design and findings in the present DN model in NRK-52E cells.

Chapter 5: Evaluation of the protective role of aldose reductase inhibition on high glucose induced stress in NRK-52E cells

5.1 Results

5.1.1 Effect of 30 mM glucose and 1% FBS on aldose reductase expression in NRK-52E cells

To investigate if aldose reductase was expressed in the model, aldose reductase expression was measured in NRK-52E cells after exposing them to high glucose media (30 mM and 1% FBS). AR protein expression was similar to control after 24 hours. After 48 hours AR protein expression was induced by high glucose by about 50% when compared to control and was statistically significant. Some induction of AR protein expression was also observed in cells exposed to high osmolar media (30 mM mannitol).

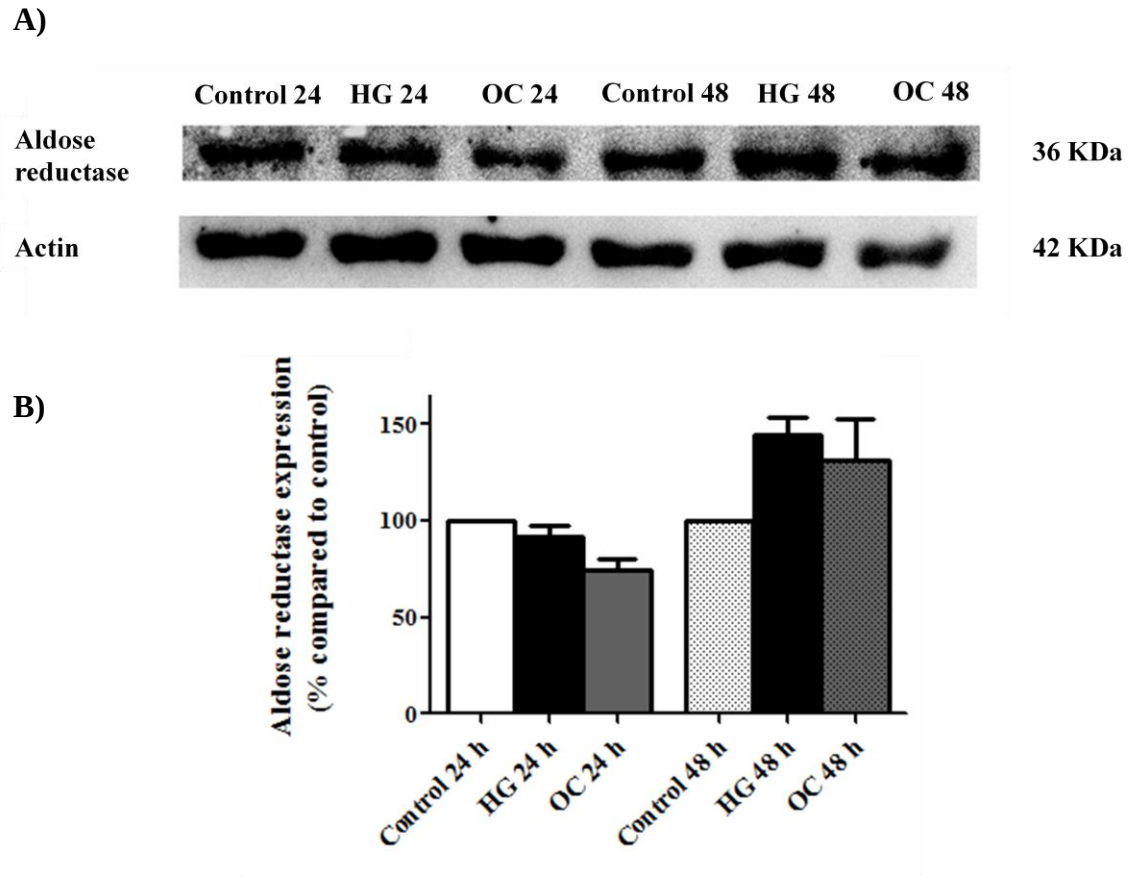


Fig. 5.1 Effect of high glucose (30 mM and 1% FBS) on aldose reductase expression in NRK-52E cells after 24 and 48 h as measured by western blotting. A) A representative western blot. B) Densitometric analysis. Values were expressed as Mean ± SEM (n=3).

5.1.2 Tolerability of NRK-52E cells to Epalrestat

NRK-52E cells were treated with three different concentrations (0.1, 1 and 10 µM) of epalrestat for 48 hours, and compared against vehicle-treated control (0.1% DMSO). All three concentrations were well tolerated by NRK cells, and there was no significant difference in cell viability when compared to vehicle-treated control.

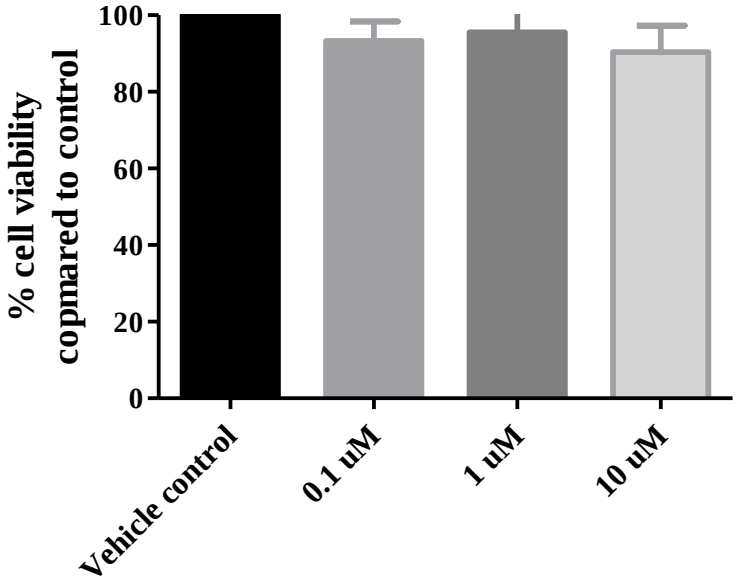


Fig. 5.2 Effect of different concentration of epalrestat on the viability of NRK-52E cells after co-incubation for 48 hours.

5.1.3 Effect of epalrestat on high glucose induced cell injury after 48 hours

To examine the protective effect of aldose reductase inhibition on high glucose induced cell death, cells were treated with 1 µM Epalrestat. After 48 hours, the cell viability declined to 84% ($p < 0.05$) when compared to control. Epalrestat (1 µM) was co-incubated with high glucose media NRK-52E cells for 48 h then cell viability was measured using MTT assay. Epalrestat at 1 µM was able to improve high glucose induce cell death.

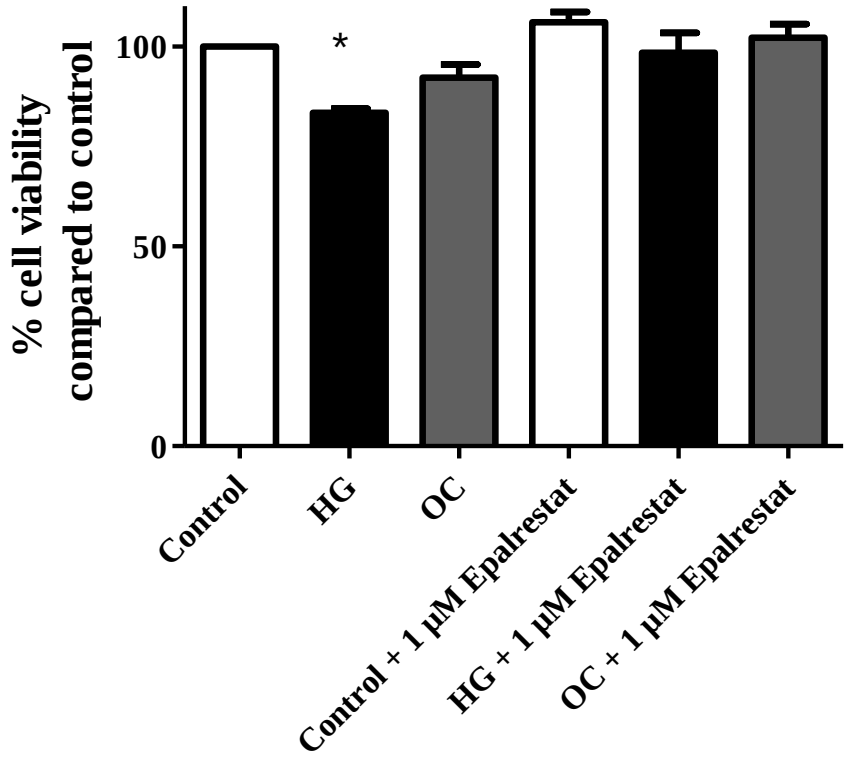


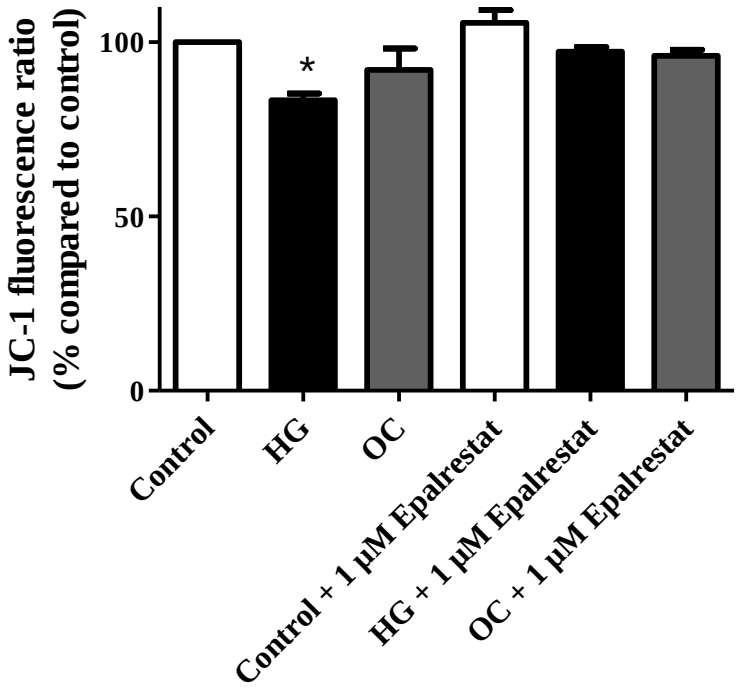
Fig. 5.3 Cell viability of NRK-52E cells after exposure to high glucose with and without 1 µM Epalrestat for 48 h as determined by MTT assay. Values were expressed as Mean ± SEM. *P< 0.05 compared to control

5.1.4 Effect of epalrestat on mitochondrial membrane potential depolarization

The effect of Epalrestat on the mitochondrial membrane potential was assessed using JC-1 dye after 24 hours of high glucose exposure. Depolarization observed with high glucose after 24 h of treatment was reversed after co-incubating high glucose treated cells with 1 µM Epalrestat when fluorescence signal was measured using microplate reader. Images taken by fluorescent microscopy further confirmed this finding showing a slight green staining in high glucose treated cells mitochondria. The green staining has

been reversed to staining similar to control cells (mixture of red and green stain) when high glucose cells were co-incubated with the aldose reductase inhibitor.

A)



B)

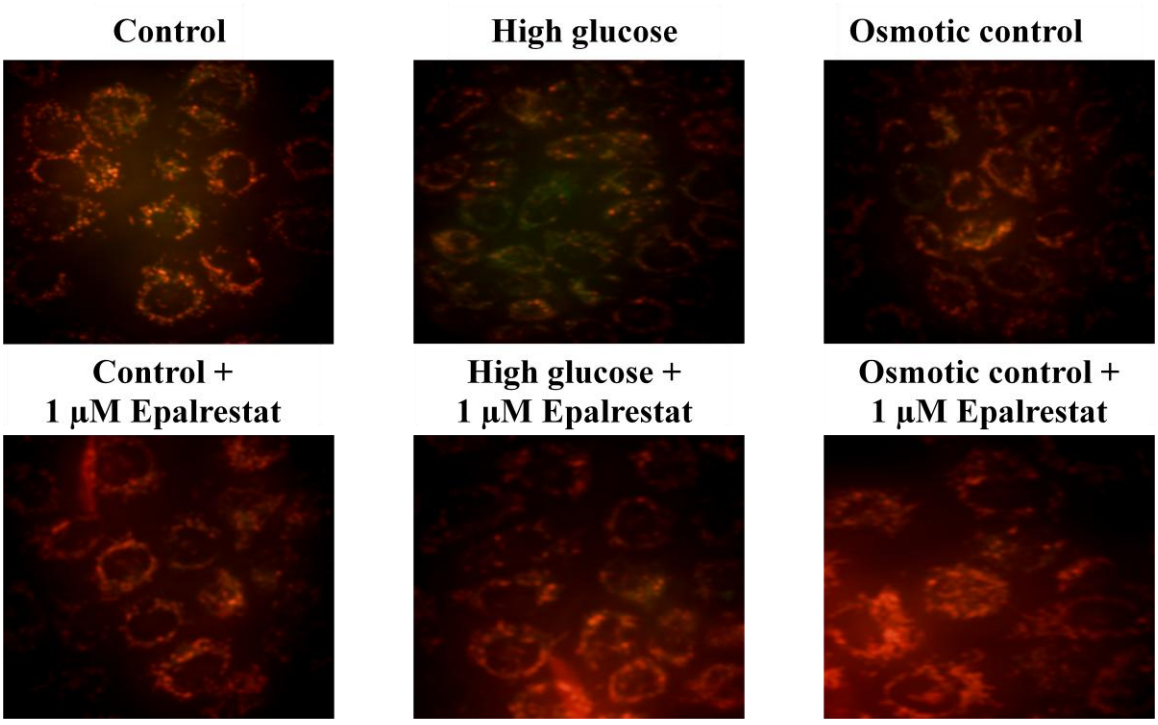


Fig. 5.4 Assessment of relative mitochondrial membrane potential in NRK-52E cells A) Fluorescence values after 24 h of high glucose exposure with and without 1 µM epalrestat using JC-1 staining, values are expressed as J-Aggregate (Ex /Em 488/529) to Monomer (Ex /Em 488/590) ratio compared to control ± SEM. *P<0.05 compared to control (n=4). B) Representative images for NRK-52E cells treatment groups after staining with JC-1 dye using fluorescence microscopy.

5.2 Discussion

It has been shown that activation of polyol pathway due to hyperglycemia in renal cells plays a pivotal role in the development of DN (128). Increased flux of glucose in this pathway causes imbalances in associated cofactors and cellular redox state, and

activates several other pathways and cytokines such as AGE pathway, PKC and TGF-β1 (10). Therefore, it is hypothesized that inhibiting aldose reductase enzyme, which is the rate-limiting enzyme in the polyol pathway, can protect the cells against high glucose induced cell injury in renal tubular cells. Activation of polyol pathway in response to high glucose has been reported to cause a seven-fold increase in sorbitol production in renal proximal tubules (129). In the current model, aldose reductase protein expression in NRK-52E cells has been measured after exposing cells to high glucose media for 24 and 48 hours. After 24 hours, there was no protein induction, while after 48 hours aldose reductase expression has been increased by about 50% when compared to respective control. Similar induction of protein expression has been reported in studies using mesangial cells cultured under high glucose conditions (43, 128).

To evaluate the protective role of aldose reductase inhibition in the model, epalrestat (a standard aldose reductase inhibitor) has been used. Three different concentrations of epalrestat have been used based on what has been reported in literature, which are 0.1, 1 and 10 µM (40). The three concentrations were tolerated well by the cells at 48 and 72 hours of co-incubation, and 1 µM epalrestat was then chosen to be used in subsequent experiments. Co-incubating high glucose treated NRK-52E cells with 1 µM epalrestat for 48 hours was able to reverse high glucose induced cell death and values obtained was similar to control. In addition, co-incubating cells with 1 µM epalrestat was able to normalize mitochondrial membrane depolarization which was observed after exposing cells to high glucose for 24 hours. These two findings suggest that inhibiting aldose

reductase enzyme in renal tubular cells can protect cells against high glucose induced insults and improve cell health.

In support to our findings, inhibiting aldose reductase has been shown to reverse high glucose induced production of ECM proteins such as fibronectin (43). Additionally, *in vitro* studies reported amelioration of high glucose stimulated production of TGF-β1 and PKC after using aldose reductase inhibitors which provides more evidence for the potential protective effects produced by inhibiting polyol pathway (40). However, as discussed earlier aldose reductase inhibition is very challenging due to great homology with other aldo-keto reductase family members, which makes it hard to find specific inhibitors without blocking the protective physiological detoxification role of the enzyme (19). Hence, suggestions to validate the beneficial effects of inhibiting aldose reductase pathway in the current model are as follows:

- Quantify aldose reductase activity and mRNA expression in the cells and correlate the levels with time course of protein expression
- Measure cellular hypertrophic markers and evaluate their degree of alteration with aldose reductase inhibition
- Evaluate the effect of aldose reductase inhibition on ROS production

Chapter 6: Discussion and conclusion

6.1 Discussion

Several *in vitro* models for kidney disorders are available for drug screening and mechanistic studies (130). Currently available cell culture systems can be divided into two categories: 1) primary cell cultures isolated from glomerular compartment (mesangial and epithelial cells) and tubular segment (epithelial cells obtained from various regions across the nephron), and 2) immortalized renal cell lines, which have unlimited proliferative potential (130). Both systems have their advantages and disadvantages, which are summarized in table 6.1 (130)

Table 6.1 List of advantages and disadvantages of primary cell cultures versus renal cell lines

Type	Advantages	Disadvantages	Examples
Primary cell cultures	• Cells not influenced by other biological regulatory systems • Cell polarity, drug metabolism and transport are maintained • Close representation for *in vivo* settings	• Requirement of an animal or human donor • Difficulty of maintenance • Limited lifetime compared to cell lines • Challenges in obtaining homogenous culture due to the	• Mesangial and renal tubular cells isolated from humans, rats and rabbits

		presence of 15-20 cell types in the kidneys	
Cell lines	• Reproducibility • Lack of time consuming isolation procedures • Unlimited life span (immortalization) • Exposure to predefined and controlled environment • Easy to subculture and transfect	• Lack of some differentiated function from their *in vivo* ancestor • Often of ill-defined function	• MDCK (canine origin) • LLC-PK1 (Porcine origin) • NRK-52E cells (rat tubular cells)

Exposure of renal cells to hyperglycemic conditions causes structural and functional changes to all types of cells in the glomeruli and tubules with different degrees of severity. These abnormalities are summarized in table 6.2 with some markers that can be measured *in vitro* (131).

Table 6.2 Mechanisms of cell injury in different types of kidney cells

Site of injury	Effect	Underlying mechanism	Markers
Glomerular hemodynamics	Glomerular hyperfiltration	Afferent arteriole vasodilation, Efferent arteriole vasoconstriction, Increased glomerular capillary pressure	Angiotensin II, VEGF
Glomerular endothelial cells	1) Cell injury	High glucose, advanced glycation end products, reactive oxygen species	Glomerular endothelial cell death, ROS production
	2) Decreased endothelial glycocalyx	Endothelial cell injury and enzymatic cleavage	Glycocalyx
Glomerular basement membrane	1) Irregular thickening	Increased production and decreased degradation of extracellular matrix (ECM) proteins	Increased fibronectin, collagen I and IV, decreased metalloproteinases (matrix-degrading

			enzymes), increased in metalloproteinase inhibitors
	2) Decrease negative charge	Decreased production or increased degradation of heparan sulfate proteoglycan (HSPG)	HSPG
Podocytes	1) Podocytopenia	Apoptosis, detachment and decreased proliferation	Apoptosis markers (e.g. caspases), proliferation assays, reduced expression of the α3β1 Integrin (which tethers podocytes to GBM)
	2) Slit diaphragm disintegration	Decrease or alteration in nephrin localization	Nephrin
	3) Foot process widening and effacement	Altered cytoskeleton, Slit diaphragm disintegration, impaired	α-actinin 4

		interaction between podocytes and GBM	
Mesangial cells	Mesangial cells hypertrophy	Increase protein synthesis, cell cycle arrest	Protein/DNA ratio, cell cycle analysis (increased percentage of cells at G1 phase)
Mesangium	Mesangium expansion	Increased production and decreased degradation of extracellular matrix (ECM) proteins	Increased fibronectin, collagen I and IV, decreased metalloproteinases (matrix-degrading enzymes), increased in metalloproteinases inhibitors
Tubular cells	1) Hypertrophy	Hyperglycemia, increase protein synthesis	Protein/DNA ratio, cell cycle analysis (increased percentage of cells at G1 phase

	2) Tubulointerstitium fibrosis and inflammation	Activation of RAS system, epithelial-to-mesenchymal transformation, ROS and inflammatory mediators production	Angiotensin II, α smooth muscle actin, interleukins, TGF-β, PDGF-B

The tubulointerstitium, which consists of interstitium, vascular structures and tubular epithelium and accounts for more than 90% of the kidney volume, has been a major focus of study in diabetic kidney disease (132). Moreover, the degree of tubulointerstitium injury has been correlated to deterioration of kidney function in diabetic nephropathy. The renal tubular cells are exposed to various pathogenetic influences due to its position in the nephron and its role in reabsorption (132). Challenges that face using renal tubular immortalized cell lines are their susceptibility to abnormal growth patterns and loss of their phenotypic features. Phenotypic characteristics include polarized morphology, distinctive tubule transport system (Na^+/glucose co-transport) and apical membrane proteins (108).

In the present study, NRK-52E cells, which are an immortalized cell line from rat proximal tubular epithelial cells, were exposed to high glucose concentrations and several stress markers related to DN have been investigated. During the process of standardizing conditions that best recapitulates diabetic milieu, different glucose concentrations (25, 30

and 35 mM) with different FBS concentration (1, 5 and 10%) over different incubation periods of time (24, 48 and 72 hours) have been used.

The current model showed that culturing NRK-52E cells under high glucose conditions (30 mM) and reducing FBS concentration to 1% caused decrease in cell viability after 48 hours to 84% which further declined to 68% after 72 hours. In addition, high glucose caused slight mitochondrial membrane depolarization after 24 hours which was sustained at 48 hours as well. Moreover, Aldose reductase protein expression has been slightly induced after 48 hours. No inductions of ER stress markers or ROS species have been identified in the model. Finally, high glucose caused acute activation to both Akt and ERK1/2 pathways after 10 minutes of exposure. Inhibiting aldose reductase enzyme with the standard inhibitor epalrestat was able to normalize and reverse high glucose induced cell death and mitochondrial membrane potential depolarization.

It can be observed in the model that exposing tubular cells to high glucose concentration *in vitro* induced low levels of stress and injury. This is expected given the complexity of mechanisms causing diabetic complications which is difficult to maintain in an *in vitro* setting. In addition, although *in vivo* models using streptozotocin (beta cell toxin) injections leads to early structural changes in kidneys such as mesangial expansion, glomerular basement membrane thickening, increased GFR and albuminuria, animals fail to progress to advanced stages of the disease seen in humans that is characterized by decline in GFR and overt nephropathy. Difficulty in obtaining representative experimental models of diabetic nephropathy is due numerous factors and

features observed in the chronic disease. For instance, diabetic patients are not only exposed to hyperglycemic conditions, they are also subjected to episodes of hypoglycemia and variable glucose concentration due to impaired insulin signaling, which has been shown to increase diabetic complications susceptibility and progression. Additionally, variable glucose concentration causes imbalances in energy production in cells. Furthermore, diabetic patients usually have other risk factors such as obesity, dyslipidemia and blood pressure abnormalities and subsequently redox imbalances, inflammation and altered protein folding and turnover in the cells (133). Therefore, establishing an *in vitro* model of DN is extremely challenging.

The chronic nature of the disease and the fact that morphological, structural and molecular changes require time to develop makes it hard to attain a representative model *in vitro*. When cells incubated for longer time intervals with high glucose, they reach confluency and require frequent passaging, which might introduce variability and inconsistency in the experimental design. To establish an *in vitro* model of DN within a reasonable and convenient time frame, high glucose concentration (although concentrations greater than 30 mM might not be physiologically relevant) can be used (108, 109). Alternatively, cells might be transfected with DN markers like angiotensin II and TGF-β1 to acquire DN features *in vitro*. Additionally, albumin can be used with high glucose to attain a proteinuric environment (57). FBS concentration is also a crucial factor during the development of an *in vitro* DN design as some studies reported serum starving renal cells overnight before adding high glucose media while others (especially

using mesangial cells) sustained the serum deprivation throughout the period of high glucose treatment.

In our model, serum deprivation (no FBS) has greatly affected the growth and morphology of the NRK-52E cells, and the best results were attained when cells were exposed to 30 mM glucose in 1% FBS containing media for 24 and 48 hours. Despite the loss of cell viability and decreased mitochondrial membrane potential, there was no induction of ER stress markers or production of ROS detected in our model. Thus, suggestions to improve the current model would include:

- Addition of aggravating factors such as albumin, angiotensin-II and TGF-β1 or transfecting cells with other DN stimuli such as TGF-β1 and ET-1.
- Use alternative methods for detection of ROS production such as flow cytometry and electron spin resonance.
- Although it has been shown that NRK-52E cells provide valid and reproducible results even at high passage numbers (more than P20), it was noted that the decrease in viability with high glucose exposure was observed only with cells at low passage number (up to P6); the results were not reproducible in cells at higher passage numbers (more than P7). Therefore, it is advisable to use lower passage numbers (less than P7) for DN studies.
- Investigating fibrotic markers such as fibronectin and collagen IV, and measuring cell hypertrophic markers and cell cycle analysis.

6.2 Conclusions

It can be concluded from this study that *in vitro* exposure to high glucose acutely activates Akt and ERK pathways, and induces pathologic changes such as decrease in cell viability and depolarization of mitochondrial membrane potential, in renal tubular cells. The molecular mechanisms and alterations initiated by hyperglycemia could potentially contribute to high glucose-induced complications. It can also be concluded that polyol pathway plays a crucial role in development of renal tubular cells injury during diabetes, and inhibiting its rate-limiting enzyme aldose reductase (AR) demonstrates nephroprotective potential against DN. However, further studies *in vitro* and *in vivo* are required to ascertain their therapeutic potential of AR inhibitors to prevent and/or treat DN.

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