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**MECHANISM OF DIABETIC
NEPHROPATHY: ROLE OF VEGF-A**

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“At times our own light goes out and is rekindled by a spark from another person. Each of us has cause to think with deep gratitude of those who have lighted the flame within us.” -Albert Schweitzer.

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ABSTRACT OF THE THESIS

Title: Mechanism of Diabetic Nephropathy: role of VEGF-A

Background: Microalbuminuria is the earliest clinical sign to occur in diabetic nephropathy. Podocytes-derived Vascular Endothelial Growth Factor (VEGF-A) is a potent microvascular permeability factor that has a major role in regulating renal hemodynamics. In diabetes, early increase of glomerular VEGF-A expression is associated with the onset of renal injury, however its critical role in the disease progression is still greatly controversial.

Aim: In this study, we postulated that VEGF-A mediates HG effect on podocytes injury through the involvement of NADPH oxidases.

Methods: Conditionally immortalized rat podocytes and STZ-induced type 1 diabetic rat model were used in this study. Si-VEGF-A transfected cells were treated with either 25mM glucose or 30ng/ml exogenous rat VEGF-A 164. Diabetic rats were injected with either SU-5416 or anti-VEGF neutralizing antibody to block VEGF-A signaling. Apoptosis, proteinuria, ROS production, gene and protein expression as well as enzymatic activities were assessed in cultured podocytes and renal cortices.

Results: Excessive VEGF-A expression and release induce oxidative stress and causes podocytes apoptosis in HG milieu. Blocking VEGF-A expression using siRNA ameliorates ROS production through decreasing NOX 4 expression and activity and prevents podocytes apoptosis. VEGF-A expression was also enhanced in renal cortices of STZ-induced type diabetic animals. This increase was concomitant the up regulation of oxidative stress, loss of podocytes, reduction in slit diaphragm proteins and ultimate microalbuminuria. Inhibiting VEGF-A signaling using two different inhibitory drugs (SU-5416 and anti-VEGF) was able to decrease NOX4 expression and thereby attenuate ROS production. It also reduced podocytes apoptosis, restored podocin levels and improved urinary albumin loss. We also address for the first time that VEGF-A regulates TIGAR expression in cultured podocytes and type 1 diabetic rat model.

Conclusion: Inhibiting VEGF-A signaling using genetic and pharmacological approaches proves the detrimental role of VEGF-A on glomerular injury and confirm its plausible autocrine mode of action.

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LIST OF ABBREVIATIONS

GBM:	Glomerular Basement Membrane
ECM:	Extracellular Matrix
CD2AP:	CD2 Associated Protein
CKI:	Cyclin-Dependent Kinase Inhibitor
DM:	Diabetes Mellitus
DCCT:	The Diabetes Control and Complications Trial
HbA1c:	Hemoglobin A1c
EDIC:	Epidemiology of Diabetes Interventions and Complications
UKPDS:	UK Prospective Diabetes Study
ACCORD:	Action to Control Cardiovascular Risk in Diabetes
ADVANCE:	Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation
CVD:	Cardiovascular diseases
DR:	Diabetic Retinopathy
DN:	Diabetic Nephropathy
ESRD:	End-Stage Renal Disease
UAE:	Urinary Albumin Excretion
TGB-B:	Transforming Growth Factor Beta 1
AGE/RAGE:	Advanced Glycation End products and their Receptors
NADPH:	Nicotinamide adenine dinucleotide phosphate
NO:	Nitric Oxide
NOX:	NADPH Oxidases

PPP:	Pentose Phosphate Pathway
HG:	High Glucose
TIGAR:	TP53 Induced Glycolysis and Apoptosis Regulator
DHE:	Dihydrèthidium
GAPDH:	Glyceraldehyde 3-phosphate dehydrogenase
HSC-70:	Heat Shock Cognate 71 kDa protein
VEGF:	Vascular Endothelial Growth Factor
VEGFR:	Vascular Endothelial Growth Factor Receptor
rVEGF-A:	Recombinant Rat VEGF-A 164
STZ:	Streptozotocin
ROS:	Reactive Oxygen Species

CHAPTER I

INTRODUCTION

Glomerulus is the main unit in the kidney responsible for blood filtration. In addition to the intraglomerular mesangium, the glomerulus encloses the most complex biological membrane that allows plasma filtration with total restriction to serum albumin and macromolecules. This membrane, termed the glomerular filtration barrier, is made up of three parts: the fenestrated endothelium, the glomerular basement membrane (GBM) and highly differentiated epithelial cells called podocytes.

In human, up to 180 liters of plasma are filtered each day across a surface area of 0.5–2 m². Under physiological conditions, more than 99.9% of plasma proteins are retained by the filter (Haraldsson B et al., 2008; Menzel S et al., 2011). Comprehensive understanding of the structural and functional properties of the glomerular filtration barrier components is of major importance to further elucidate the underlying mechanism of marked and persistent proteinuria associated with glomerular damage.

A- Components of Glomerular Filtration Barrier

1- Fenestrated Endothelium:

The fenestrated endothelium constitutes the first filtration barrier in the glomerulus. It is made up of endothelial cells that line up the innermost side the glomerular capillary. The fenestrated area occupies 20%-50% of total endothelium surface area (Bulger RE et al., 1983). Endothelial cells are covered by endothelial cell surface layer facing the luminal side of the capillaries (Haraldsson B et al., 2008). This layer is composed of glycocalyx, a network of negatively charged glycoproteins, glycosaminoglycans and proteoglycans that fills the fenestrae and highly contributes to the endothelium permselectivity.

2- Glomerular Basement Membrane

The glomerular Basement membrane (GBM) is made up of meshwork of extracellular proteins including Laminin (mainly Laminin 11), Type IV Collagen (collagen $\alpha 3$, $\alpha 4$, and $\alpha 5$ chains), nidogen and heparan sulfate proteoglycan. Most of extracellular matrix (ECM) components of GBM are synthesized by the podocytes and the endothelial cells (Pavenstädt H et al., 2003; St John PL et al., 2001) and secreted into the extracellular space between them. With its specialized properties, GBM in turn provides a scaffold for podocytes and endothelial cells. Primary changes in GBM components are shown to affect the function of glomerular cells and vice-versa.

GBM plays a major role in glomerular permselectivity by being size- and charge-selective. Alport syndrome and Pierson syndrome are two genetic diseases result from mutations in ECM proteins, Collagen IV and Laminin β 2, respectively. Studies using murine models of Pierson and Alport syndromes have revealed that defective GBM becomes more permeable to macromolecules, suggesting the essential function of GBM in permselectivity (Suh JH et al., 2013; Miner JH et al., 2012). The failure of proper matrix protein assembly is also associated with several structural and functional injuries leading to ultimate microalbuminuria (Sung S et al., 2006).

3- Glomerular Epithelial Cells or Podocytes:

Glomerular epithelial cells or Podocytes are terminally differentiated cells with unique cytoarchitecture. Podocytes comprise the outermost epithelium that lines the GBM and form the final filtration barrier within the glomerular capillaries. They are highly polarized cells having their outer membrane covered with a negatively charged coat that is rich in glycocalyx such as podocalyxin (Pavenstädt H et al., 2003). This coat plays a role in maintaining podocytes architecture through augmenting physical separation (Reiser J et al., 2016).

Podocytes have voluminous cell body rich in endoplasmic reticulum and mitochondria indicating its high levels of metabolic activity (Pavenstädt H et al., 2003). They bear major processes that extended to form foot processes. Podocytes processes are found embedded into the GBM. Their cytoskeleton contains a dense network of actin filaments, intermediate filaments and microtubules. The foot processes of two adjacent podocytes interdigitate with each other to form the filtration slit which is bridged by intercellular

junction called “slit diaphragm”. The slit diaphragm proteins such as nephrin, ZO-1, podocin, CD2AP, etc. are shown to induce signal transduction within podocytes cytoskeleton (Tufro A et al., 2012).

Podocytes are associated to GBM through two major transmembrane matrix receptors that are present on the foot processes and bind to their ligands in GBM. Firstly, the heterodimeric complex, $\alpha3\beta1$ -integrins, which binds to each of collagen IV, fibronectin, laminin, and

nidogen in the GBM. Secondly, 2- dystroglycan complex that provides a link between matrix and the actin network (**Figure 1**). The interaction between podocytes cytoskeleton and matrix components allows the conduction of mechanical force from the GBM into the foot processes and from the primary processes into the cell body. Thus, due to this complex architecture and extremely complicated cytoskeleton, podocytes are considered as an early player in renal injury.

Podocytes start to establish their characteristics during glomerular capillary loop stage at which foot processes and filtration slit are formed. This phenotype conversion is associated with the expression of slit diaphragm proteins and other proteins as actin-associated synaptopodin and podocalyxin. During this stage, podocytes lose its mitotic activity and become highly differentiated. The general paradigm behind the fact that podocytes don't undergo proliferation under normal conditions in mature glomerulus is due to the up regulation of cell cycle inhibitors such as CKI p21, p27 and p57 (Combs HL et al., 1998, Nagata M et al, 1998).

Studies have shown that the disruption of podocytes cell cycle control is detrimental causing glomerular destruction. The increase in cyclin kinase inhibitors enhances

podocytes loss. Nagata M et al. have shown that podocytes undergo mitosis but not cytokinesis in glomerular diseases. These observations were associated with hypertrophy and foot processes effacement (Nagata M et al., 1995). Once foot processes lose their integrity, podocytes start to detach leaving bare areas on GBM surface. These areas interact with parietal epithelial cells in the Bowman's capsule forming synechia. These sequential events eventually lead to the development of more lesion and progress to glomerulosclerosis (Matovinović MS et al., 2009; Reiser J et al., 2016).

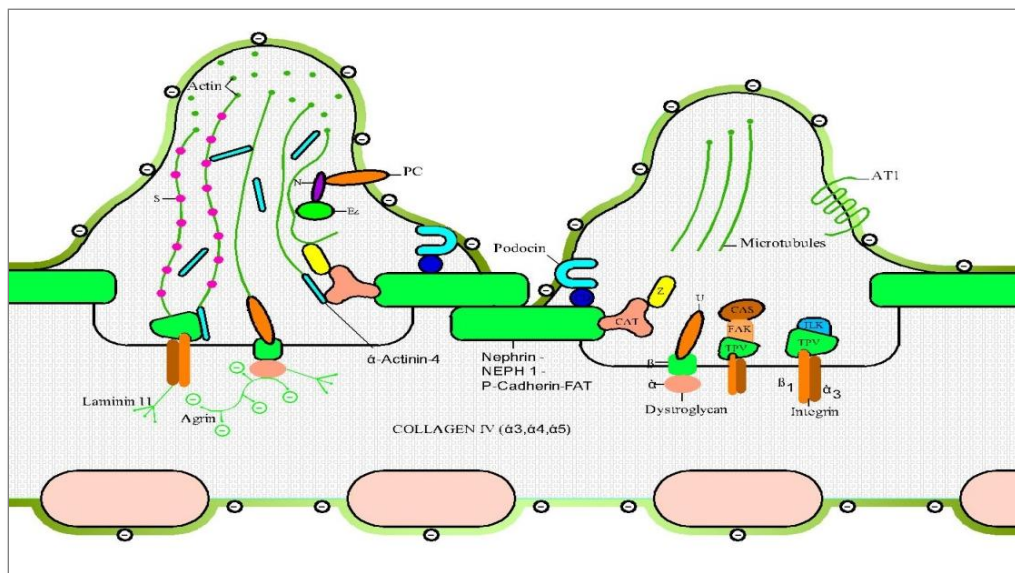


Figure 1: The structural organization of glomerular filtration barrier. The endothelial, the GBM and the most outer side covered with podocytes. Foot processes of neighboring podocytes are interdigitated forming slit diaphragm (the Figure is adapted by author from Pavenstädt H et al., 2003).

B- Diabetes Mellitus

Diabetes Mellitus (DM) is a metabolic disorder of glucose homeostasis clinically characterized by chronic hyperglycemia. The global prevalence of diabetes has dramatically risen over last decades. According to World Health Organization (WHO) estimates, the prevalence of diabetes among adult population (above 18 years) has increased from 4.7% in 1980 to 8.5% in 2014 (WHO, 2016) with 46% of diabetic patients suffer from undiagnosed diabetes (Beagley J et al., 2014). 382 million people, worldwide, are living with diabetes and this number is rapidly increasing with estimation to reach around 600 million by 2035 (Guariguata L et al., 2014). DM is considered the 7th leading cause of mortality and morbidity. It was recently estimated that 8.4% of all-cause deaths were directly related to diabetes, which almost count for 5.1 million deaths (IDF Diabetes Atlas Group et al., 2015).

DM is classified into two major types: Type 1 DM (Insulin-dependent Diabetes Mellitus, IDDM) and Type 2 DM (Non-insulin-dependent Diabetes Mellitus, NIDDM). Type 1 diabetes is associated by insulin deficiency. It occurs as a result of autoimmune disease in which the pancreatic Beta-cells present in the islets of Langerhans that are responsible for insulin secretion are destroyed (Forbes JM et al., 2013). Type 1 diabetes can occur at any age, but tends to develop at early stages of life during childhood. It accounts for 5-10% of diabetic patients. On the other hands, Type 2 diabetes is characterized by peripheral insulin resistance with compensatory hyper-insulin secretion as a response to increased glucose blood level stimulation. Type 2 diabetes accounts for the majority of the diabetic cases and tends to effect adults and overweight people (Forbes JM et al., 2013).

Chronic hyperglycemia is associated with serious organ damage. Increasing rates of death are caused by diabetic complications. These complications begin with acute metabolic complications (Ketoacidosis, hypoglycemia, hyperosmolarity, lactic acidosis) to end up with long-term vascular complications as a result of chronic exposure to hyperglycemia. The long-term vascular complications of DM can be classified into two groups: macro-vascular and micro-vascular diseases. Macro-vascular diseases are caused by the damage of arteries as the accelerated cardiovascular diseases (Forbes JM et al., 2013). While micro-vascular diseases are caused by the damage of small vessels including retinopathy, neuropathy and nephropathy.

Although microvascular complications are associated with morbidity, cardiovascular diseases are the main cause of mortality among diabetic patients (Roper NA et al., 2001). The incidence of microvascular and cardiovascular complications is linked to degree of chronic exposure to hyperglycemia. Interestingly, meta-analysis study performed by Selvin E. and her colleagues has demonstrated that 1% increase in glycosylated hemoglobin results in increased risk of any cardiovascular disease event is by 1.15% and 1.18% in type 1 and type 2 diabetic patients, respectively (Selvin E et al., 2004). Although several attempts were made to study the effect of intensive glucose control on the reduction of diabetic complications, the evidence for its impact on cardiovascular outcomes is still limited.

Multiple clinical trials were conducted to tackle whether intensive glycemic control can prevent diabetes-induced microvascular and cardiovascular events and be able to reduce the risk of mortality. The Diabetes Control and Complications Trial (DCCT) study has shown that intensive insulin therapy delays the development of neuropathy by 60%,

retinopathy by 76% and microalbuminuria by 39% in type 1 diabetes but not the cardiovascular outcomes. In this trial, patients were randomly assigned to either conventional insulin therapy (one or two daily injections achieving a mean of glycated hemoglobin (HbA1c) levels of 9.1%) or intensive insulin therapy (insulin pump therapy or multiple insulin injections achieving a mean of glycated hemoglobin (HbA1c) levels of 7.4%) (DCCT, 1993). Subsequent trials such as EDIC (The Epidemiology of Diabetes Interventions and Complications; DCCT follow-up study) and UKPDS (UK Prospective Diabetes Study) have demonstrated that early intensive therapy can prevent long-term cardiovascular diseases in type 1 and type 2 diabetes (Nathan DM et al., 2005; UKPDS, 1998). Most recently, two interventions (ACCORD and ADVANCE) were performed to investigate if strict glucose control near normoglycemia (HbA1c < 6.5%) can reduce cardiovascular outcomes and mortality in diabetic patients. These clinical trials were terminated due to the severe hypoglycemia and increased rate of death related to cardiovascular diseases in intensive-therapy group (Brown A et al., 2010).

C- Cardiovascular complication of Diabetes

Cardiovascular diseases (CVD) are a major cause of morbidity and mortality in the diabetic population. Diabetic patients are at higher risk of developing CVD than other non-diabetic individuals. The risk of CVD is directly correlated to glucose plasma levels (WHO, 2016). Epidemiological studies performed among U.S. patients have shown that the prevalence rate of CVD and strokes among adult diabetic patients is 68% and 16%, respectively, with increased CVD death rates by 1.8 times (Buse JB et al., 2007). This is mainly due to the occurrence of strokes and myocardial infarction (MI).

The increase of mortality due to cardiovascular complications in diabetic patients is related to several risk factors including hypertension, dyslipidemia, hyperglycemia, obesity and hypercoagulability (Mazzone T et al. 2008).

Hyperglycemia serves as a major risk factor in the development of diabetic cardiomyopathy, comprising functional and structural abnormalities in the heart, including diastolic and/or systolic dysfunction, altered cardiac contractility, cell hypertrophy, apoptosis and interstitial fibrosis (Fonarow GC et al., 2006; Hayat SA et al, 2004). As the disease progresses, there is increased myocyte loss accompanied by myocyte hypertrophy and fibrosis (Mishra TK et al., 2005). These changes are associated with increased oxidative stress that causes further deterioration of the diabetic heart (Asbun J et al., 2006).

D- Microvascular Complications of Diabetes

1- Diabetic Retinopathy

Diabetic retinopathy (DR) affects the peripheral retina, the macula, or both leading to vision loss and irreversible blindness in people with diabetes. It occurs as a result of long term small vessels damage. According to WHO, 2.6% of global blindness is attributed to diabetes (WHO, 2016). The prevalence of DR increases with prolonged exposure to diabetes (Orchard TJ et al., 1990). It has been shown that one third of diabetic people have signs of DR and third of these are suffering from vision-threatening diabetic retinopathy. The severity of DR ranges from mild non-proliferative and moderate non-proliferative to severe proliferative DR. At this advance stage, the secreted growth

factors trigger the abnormal growth of new vessels in the inside surface of the retina. The newly formed blood vessels are fragile allowing fluid leakage to the eye leading to diabetic macular edema (DME) (Harding S et al., 2003).

Epidemiological studies conducted from 1980 to 2008, estimated that global prevalence of DR is much higher in patients with type 1 than those diabetes type 2 diabetes (Lee R et al., 2015). There are several risk factors associated with the development of DR including chronic exposure to hyperglycemia, hypertension, dyslipidemia and obesity. Oxidative stress and metabolic hormones such as leptin and adiponectin appears to have a vital role in the pathogenesis of DR. Vascular endothelial growth factor (VEGF) is another modulator that has a major role in neovascularaization and angiogenesis that is regulated by inflammatory cytokines. VEGF signaling antagonists have been widely used for the treatment of both PDR and DME (Lee R et al., 2015).

2- Diabetic Neuropathy:

Diabetic Neuropathy affects about 10% of patients newly diagnosed with diabetes and more than 50% of patients with longstanding diabetes (Shakeel M. 2014). It results in a number of sensorimotor disorders including pain, loss of proprioception and motor function and infectious ulcers in the feet and legs that often require limb amputation (Edward JL et al., 2008). Additionally, diabetic neuropathy can lead to autonomic dysfunction, which manifests as orthostatic hypotension, fainting, arrhythmias, gastrointestinal dismotility, bloating, diarrhea, etc (Vinik AI et al., 2003).

Diabetic neuropathy can be classified as proximal, focal, autonomic and peripheral, each of which affecting different parts of the body in various ways (Callaghan BC et al, 2012). The most common form of DN is the distal symmetric polyneuropathy, also known as diabetic peripheral neuropathy (DPN). DPN is associated with impaired nerve conduction, abnormal thermal perception, axonal atrophy, demyelination, blunted regenerative potential and loss of nerve fibers, resulting in motor dysfunction (Boulton AJ et al., 2014). Patients diagnosed with diabetic neuropathy also experience an increased sensitivity to pain (hyperalgesia), as well as an increased responsiveness to non-painful stimuli (allodynia) (Dworkin RH et al., 2007a; Dworkin RH et al., 2007 b; Dworkin RH et al., 2005; Jensen MP et al., 2006; Jensen TS et al., 2006). With the progression of the disease, pain is replaced with complete numbness followed by serious foot problems, ultimately resulting in ulcerations and leading to foot amputation (Feldman EL Et al., 1999; Feldman EL et al., 2005; Feldman EL et al., 2002b).

3- Diabetic Nephropathy:

Diabetic Nephropathy (DN) is the leading cause of end-stage renal disease (ESRD) in 20-30% of patients diagnosed with diabetes (Wolf G et al., 2007; Shahbazian H et al., 2013). Knowing the fact that ESRD is a life threatening complication with poor prognosis and high medical cost, several epidemiological studies show that the incidence rate of ESRD due to DN is 3.3% after 30 years of diabetes duration (Narres M et al., 2016) and the risk of mortality associated with chronic kidney diseases tends to increase with time (Sattar A et al., 2012; Groop PH et al., 2009). The prevalence rate of DN in

diabetic patients of Type 2 is much higher than that of Type 1 (Shahbazian H et al., 2013).

Besides the environmental and genetic risk factors that set the stage of the disease, DN occurs as a result of hemodynamic and metabolic alterations. Tremendous alterations in kidney structure and function are associated with DN as early as the first few months after the onset of DM. Hyperfiltration and hyperperfusion are the very early glomerular hemodynamic alterations to occur. Impaired auto-regulation of renal blood flow caused by hyperglycemic conditions causes dilation of afferent arteriole which in turn increases glomerular filtration rate and induces intraglomerular pressure. These alterations contribute to the glomerular injury through mesangial volume and extracellular matrix expansion which eventually leading to glomerulosclerosis (Wolf G et al., 2007; Shahbazian H et al., 2013). Renal hypertrophy is another early histological change of diabetic kidneys. Kidney enlargement in the diabetic course is characterized by hypertrophy and hyperplasia that contribute to the roughly same extent (Rasch R et al., 1983). Glomerular and tubular hypertrophy is also associated with matrix expansion and thickening of basement membranes as a result of progressive accumulation of extracellular matrix protein such as Collagen IV, Laminin, Fibronectin, etc. All these features precede the development of irreversible diabetic renal changes characterized by glomerulosclerosis and tubulointerstitial fibrosis (Wolf G et al., 1999).

Microalbuminuria, which characterized by urinary albumin excretion rate (UAE 30-300mg/day), is considered the earlier clinical manifestation to occur in diabetic patients. It can progress, if left untreated, to develop overt proteinuria (>300mg/day) leading to ESRD where dialysis, renal replacement therapy or transplantation is strongly warranted

(Dirks J et al., 2006). About 25%-30% of Type 1 and 2 diabetic patients develop microalbuminuria within the first 20 years of diabetes (Shahbazian H et al., 2013; Hovind P et al., 2004). Microalbuminuria is associated with early glomerular lesion characterized by thickening of basement membrane, mesangial expansion and accumulation of matrix proteins (Bangstad HJ et al., 1993). It also devastates the metabolic activity of the tubular cells leading to tubulointerstitial fibrosis and subsequent reduction in glomerular filtration rates (Stitt-Cavanagh E et al., 2009).

To date, the most potential therapy used for the treatment of patients with DN is the hypertensive drugs; however these agents delay the progression of the disease but don't prevent the damage. Therefore, comprehensive understanding of the underlying mechanisms in hyperglycemia-induced renal injury is of major importance.

E- Podocytes injury in DN:

A large body of evidence supports the “mesangiocentric” theory as cellular and molecular mechanism for the development of diabetic kidney injury. However, these data couldn't explain the genesis of proteinuria which should be devoted to glomerular filtration barrier for its known function as macromolecular sieve. In support of this crucial role in filtration process, podocytes injury has attracted scientific attention over the last decades and surge of research is now focusing on podocytes loss or progressive dysfunction as an earlier player in the onset of microalbuminuria (Petrakka J et al., 2009). Evidence from both diabetic patients and experimental animal models revealed that albuminuria is associated with podocytes loss. The reduction in podocytes number has been shown to precede and influence microalbuminuria leading to the progression of

renal disease in Pima Indians with Type 2 diabetes (Meyer TW et al., 1999). Additional clinical studies conducted on Type 1 (Steffes MW et al., 2001) and Type 2 diabetic patients demonstrated that the reduction of podocytes number is associated with increased UAE leading to albuminuria (White KE et al., 2002). Progressive podocytes dysfunction and loss is concomitant with altered morphology of podocytes that is characterized by foot processes effacement (Lin JS et al., 2016) and loss of slit diaphragm as a result of altered localization (Rincon-Choles H et al., 2006) or expression of specific slit diaphragm proteins such as nephrin, podocin, ZO-1, etc. (Langham RG et al., 2002; Denhez B et al., 2015).

Although the etiology of podocytes loss is still speculative, there are two mechanisms that describe this loss: apoptosis or detachment from the GBM. Several findings have established that cultured podocytes undergo apoptosis during diabetic course (Meek RL et al., 2013; Ma Y et al., 2016). Data published by our laboratory shows that exposure of mouse podocytes to high glucose resulted in apoptosis through P53-dependent pathway (Eid AA et al., 2009; Eid AA et al., 2010). Podocytes apoptosis was also detected in isolated glomeruli of STZ-induced type1 diabetes (Lee SH et al., 2015). Susztak et al. demonstrated that the increase in podocytes apoptosis coincides with the onset of diabetes and precedes podocytes loss and UAE in Akita and *db/db* mice (Susztak K et al., 2006). Other findings have suggested that the detachment of podocytes from GBM and their loss doesn't exclude podocytes apoptosis, since the detached cells found in urine are viable cells with a limited proliferative capacity followed by dramatic apoptosis (Petermann AT et al., 2003; Mundel P et al., 2003).

In addition to hyperglycemia, several cytokines such as VEGF, TGF-beta and SMAD3 have shown to participate in podocytes injury in diabetic state leading to albuminuria (Ziyadeh FN, 2008; Schiffer M et al., 2001).

F- Oxidative Stress and ROS production:

Oxidative stress is characterized by the increased production of reactive oxygen species (ROS). ROS are free radicals that bind to oxygen molecule and form biologically active substances such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^{\cdot}). ROS play a central role in cellular physiology, and take part in various biological processes such as proliferation, senescence, apoptosis and autophagy (Sedeek M et al., 2013). Under physiological conditions, a subsequent compensatory response of endogenous anti-oxidant network such as superoxide dismutase (SOD), Vitamin E, α -Lipoic acid etc. is activated to detoxify ROS and repair the damage. However, this balance is disrupted in many pathological conditions and the role of antioxidants becomes minimal, so that ROS over production takes place causing oxidative stress and subsequent cellular and tissue damage.

ROS production is enhanced in various tissues through enzymatic and non-enzymatic pathways including glucose autooxidation, the polyol pathway, Advanced Glycation End Products (AGE/RAGE System), metabolites of arachidonic acids, NADPH oxidases, uncoupling of nitric oxide and others (Eid S et al., 2013; Forbes JM et al., 2008; Kuroki T et al., 2003; Brownlee M, 2005). However and according to the unifying hypothesis, the mitochondrial respiratory process is considered as a major source of hyperglycemia-

induced superoxide that initiates the damage and leads to the activation of all the above mentioned sources of ROS (Brownlee M, 2005; Araki E et al., 2010).

G- Oxidative Stress in DN:

Oxidative stress has emerged as critical pathogenic factor associated with early development and progression of diabetic microvascular dysfunction (Araki E et al., 2010; Tilton RG, 2002). In kidneys, hyperglycemia mediates the overproduction of superoxide and H₂O₂ by several cell types including the endothelial, proximal tubular, mesangial, and podocytes (Eid AA et al., 2009; Eid S et al., 2013; Gorin Y et al., 2013). Increased production of ROS has been linked to dysregulation of mesangial contraction, endothelial dysfunction and matrix expansion. Oxidative stress has also been shown to induce tubular injury and apoptosis (Singh DK et al., 2011). Experimental evidence has revealed that glucose-stimulated intracellular ROS production initiates *in-vitro* and *in-vivo* podocytes apoptosis and depletion (Susztak K et al., 2006). It also provokes podocytes hypertrophy. The usage of anti-oxidant enzymes is able to attenuate the damage (Kim NH et al., 2006).

HG induced- oxidative stress has been thoroughly implicated in the pathogenesis of DN through its multiple pathways. Glucose is considered as a major fuel to mitochondrial respiratory chain at which it is converted to pyruvate. This process generates large amount of NADH/NAD⁺ as main electron donor. The flux of glucose in diabetic state increases the electron leakage, *per se*, leading to increased ROS generation (Forbes J et al., 2008). Moreover, it can also activate aldose reductase and stimulate the production of

sorbitol. During this reaction, NADPH generated from the pentose phosphate pathway (PPP) is consumed as a co-factor for the reaction and serves as a source of reductive stress causing cell damage. It also inhibits the replenishment of reduced glutathione, a major substrate for the anti-oxidant glutathione-peroxidase (Singh DK et al., 2011).

On the other side, advanced glycation end products (AGEs) that are formed by non-enzymatic covalent binding of sugar moieties to free amino groups on proteins, lipids and nucleic acid are hastened by hyperglycemia (Singh VP et al., 2014) and oxidative stress. AGE products bind to their receptors (RAGEs) that are usually localized to mesangial cells, proximal tubular cells and podocytes (Forbes et al., 2008; Gu L et al., 2006). RAGEs expression is increased in diabetic state. AGE/RAGE activation increases ROS production and contributes to pathogenesis of tubular cell and podocyte injury in early diabetic nephropathy (Chuang PY et al., 2007). They are also implicated in DN through the alteration of extracellular matrix architecture (Tanji N et al., 2000). Diabetes stimulated- ROS production is also enhanced via the uncoupling of endothelial nitric oxide synthase or eNOS. Normally, NO is released in the vascular lumen and is implicated in the regulation of vascular tone. However in diabetes, the reduction in eNOS activity and the induction of oxidative stress reduces the synthesis and bioavailability of NO, respectively. The presence of excess superoxide anions reacts with NO[•] to form a highly reactive nitrogen intermediate peroxynitrite (ONOO⁻) (Tufro A et al., 2012). Low NO bioavailability and the additional augmentation of ROS production result in vascular endothelial dysfunction and alteration in the intraglomerular hemodynamics (Singh DK et al., 2011, Balakumar P et al., 2009).

We and others have shed light on the important of cytochrome P450 derived-eicosanoids as another potent source of oxidative stress involved in diabetic kidney damage (Eid AA 2009; Eid S et al., 2013). Several studies have reported the contribution of arachidonic acid metabolites 20-HETE and EET in the renal microvascular function (Imig JD. et al., 2013). Data published by our laboratory have indicated the involvement of 20-HETE and EETs in the early stage of DN and their direct effect on proximal tubular cells and podocytes injury. Moreover, 20-HETE appears to be a major inducer of ROS in cultured endothelial cells. It contributes to endothelial dysfunction by uncoupling of endothelial nitric oxide synthase (eNOS) (Cheng J et al., 2008). Furthermore, oxidative stress are shown to modulate the activity of several stress-signaling kinases including mitogen-activated protein kinases (MAPKs), JAK and STAT as well as nuclear factor- κ B (NF- κ B) that further worsen renal injury (Singh DK et al., 2011).

H- Anti-Oxidants as a potential therapy for DN:

Although our understanding of how hyperglycemia-induced oxidative stress enhances the progression of microvascular diseases has advanced over the recent years, the development of effective therapeutic strategies to treat DN in diabetic patients is still limited. Experimental studies on diabetic animal models have revealed the potential effect of various exogenous anti-oxidant enzymes such as Superoxide dismutase (SOD), glutathione reductase and catalases to prevent the progression of DN. However, the application of these drugs in clinical trials has generated disappointing results (Celik T et al., 2010; De Zeeuw et al., 2013). For instance, diabetic patients assigned for chronic oral administration of the antioxidant alpha-tocopherol (Vitamin E), have increased risk of

developing heart failure (Celik T et al., 2010). Similarly for bardoxlone methyl, an antioxidant NRF2 pathway activator was orally given to type 2 diabetic patients in randomized clinical trial to evaluate its effect on reducing the risk of ESRD. This trial was terminated because it was associated with high risk of cardiovascular diseases among bardoxlone methyl-treated group. (De Zeeuw et al., 2013)

Besides the standard therapeutic approach manifested by tight glycemic control, ACE inhibitors are the current therapy used for the treatment of overt nephropathy (HOPE and MICROHOPE substudy, 2000). These inhibitors have also been shown to ameliorate oxidative stress. Therefore, comprehensive understanding of the various sources of ROS generation in diabetes is of great concern, in order to develop a new, more targeted, plausibly designed antioxidant approach. Most recently, we and others have highlight the importance of phagocyte-like NADPH oxidases as cornerstone source of ROS generation in kidney cortex (Block K et al., 2009; Eid AA et al., 2009), in which its activation has been significantly linked to different pathogenesis including DN.

I- ROS – Generating NADPH Oxidases:

NADPH Oxidases (NOXs) are electron transporting membrane enzymes which are solely dedicated to ROS production. NOXs generate superoxide anions ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) by electron transfer from NADH or NADPH to oxygen molecule. The well characterized phagocytic NOX consists of two membrane-bound subunits: gp^{91phox}/NOX2 (the catalytic subunit) and p22^{phox} and cytosolic subunits: p47^{phox}, p40^{phox}, p67^{phox}, and small GTPase/Rac (Bedard K et al., 2007). The stimulation of enzyme activation occurs through assembly of the cytosolic factors with the

membrane-associated subunits (Clark RA, 1999; Leusen JH et al., 1996). NOX-derived ROS contributes to different physiological functions of the vasculature including cell signaling, differentiation, gene expression, oxygen sensing and apoptosis (Sedeek, M et al., 2013a). Studies over the last decade have documented significant NOX-dependent ROS generation in non-phagocytic cells (Bedard K et al., 2007; Clark RA, 1999; Leusen JH et al., 1996). In kidneys, NOX-derived ROS plays a pivotal role in regulation of gene expression, renal flow and alteration of cell fate (Shiose A et al., 2001). It also regulates glucose production and transport (Sedeek, M et al., 2013). NOXs are shown to modulate several mechanistic signaling pathways under pathologic conditions including p38 MAPK and AKT pathway (Gorin Y et al., 2005).

Seven members of NOX family have been identified (Nox1 to Nox5, Duox1, and Duox2). Among these isoforms, at least NOX-1, 2 and 4 are expressed in the renal cortex. NOX4, originally termed Renox, is the most abundant isoform predominantly localized in the proximal tubular (Geiszt M et al., 2000) as well as mesangial cells (Gorin Y et al., 2003) and podocytes (Eid AA et al., 2009). NOX4 is a 578 amino acid protein that exhibits 39% identity to gp91^{phox}/Nox2. It is a constitutively active enzyme that doesn't require the other cytosolic subunits to function (Bedard K et al., 2007). In renal cortex, NOX4 has been found to be sub-localized and regulated in the mitochondria and to lesser extent on plasma membrane (Block K et al., 2009).

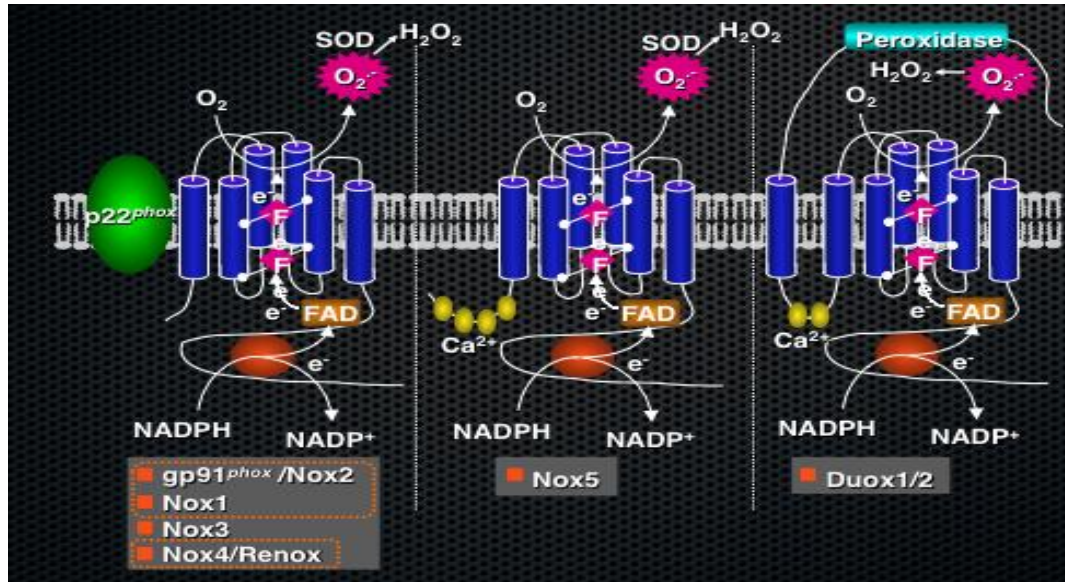


Figure 2: The molecular and structural organization of NADPH oxidases. NOXs are electron-transporting transmembrane enzyme. Its catalytic subunit contains 1) C-terminal dehydrogenase domain featuring a binding site for NADPH and FAD. 2) N-terminal domain consisting of six transmembrane helices that binds to two heme groups. The cytosolic NADPH transfer electrons to FAD, which in turn passes the electrons subsequently to the heme group. The electrons ultimately bind to the oxygen molecule to for superoxide anion (the Figure is adapted by author from Nassif J et al., 2016).

J- NADPH Oxidases (NOXs) in DN:

There is ample evidence that proves the contribution of glucose induced-NOXs in the pathogenesis of DN. We and others have obtained increased NOXs expression, mainly NOX4, in proximal tubular cells (Sedeek M et al., 2010), mesangial cells and podocytes exposed to high glucose. This increase has been associated with mesangial hypertrophy and fibronectin deposition (Sedeek M et al., 2010; Eid S et al., 2013; Gorin Y et al., 2005). Our laboratory has shown that NOX4 mediates the effect of hyperglycemia in inducing podocytes apoptosis. Inhibiting NOX4 activity using an

adenovirus vector has significantly reduced oxidative stress and attenuated the apoptotic effect of HG on mouse podocytes (Eid AA et al., 2010). Similar data were obtained in human podocytes treated with HG in the presence of NOX1/4 inhibitor (Jha JC et al., 2014). Moreover, genetic deletion and pharmacological inhibition of NADPH oxidases results in glomerular injury protection associated with reduced albuminuria and extracellular matrix accumulation in type 1 and type 2 diabetic animal models (Jha JC et al., 2014; Gorin Y et al., 2015; Sedeek M et al., 2013b). Similar data was obtained in cultured human podocytes (Sedeek M et al., 2013b). The enhancement of NOX4 activity was also evident through other oxidant pathways. ANGII and TGF-B are shown to activate PKC that in turns potentiates NOX4 profibrotic activity in early DN (Gorin Y et al., 2003). Similarly, AGE/RAGEs also are known to activate NOXs in tubular cell injury.

Altogether, these results prove that NOX4 is a potent source of oxidative stress responsible for renal injury in diabetes.

K- VEGF and its receptors in Kidneys:

Vascular Endothelial Growth Factor (VEGF) is a homodimeric glycosylated protein and a potent survival factor belongs to PDGF family. In 1986, Senger et al. described VEGF, which originally named vascular permeability factor (VPF), as a highly conserved molecule that enhances vascular permeability in tumors (Senger DR et al., 1986). It was also shown to act as angiogenic, vasculogenic and endothelial mitogenic and migration factor (Kliche S et al., 2001; Klagsbrun M et al., 1996). VEGF family

consists of VEGF-A, B, C, D, E and placental growth factor (Ferrara N et al., 2003). The expression of VEGF-A mRNA and protein has been reported in several cell types within the human and rodent kidney (Simon M et al., 1995; Monacci WT et al., 1993). Podocytes are the major source of VEGF-A in kidney (Brown LF et al., 1992). VEGF-A expression was also obtained in mesangial cells, collecting ducts as well as tubular cells (Simon M et al., 1995; Brown LF et al., 1992; Takahashi T et al., 1995; Feliers D et al., 2010). Podocytes constitutively produce three major VEGF-A isoforms (VEGF 121, 165, and 189) that are formed by alternative messenger RNA splicing (Tufro A et al., 2012). VEGF-A isoforms differ in their secretion and expression properties. Among others, VEGF-A 165 is the most abundant isoform which can be secreted or remain cell-associated or bound to extracellular matrix through its heparin-binding affinity (Ferrara N et al., 2003). This heparin-binding domain appears to be involved in VEGF-A paracrine mitogenic potency (Keyt BA et al., 1996) suggesting the optimal characteristics of VEGF-A 165 bioavailability and bioactivity.

VEGF exerts its effect through its direct binding to functionally distinct tyrosine kinase receptors. VEGF receptors include VEGFR 1 (Flt-1), VEGFR2 (KDR/Flk-1), VEGFR3 and two potential non-signaling co-receptors that also known to amplify VEGFR signaling (neuropilin-1 (Np-1) and neuropilin-2 (Np-2)). In addition to sFlt-1, the soluble form of VEGFR1 that is known for its inhibitory effect (Ferrara N et al., 2003).

VEGF receptors expression in renal glomerulus appears to be heterogeneous. VEGF-A binds with high affinity to VEGFR1 and VEGFR2 that are predominantly expressed on endothelial cells of fetal and adult human kidneys (Klagsbrun M, et al., 1996; Simon M et al., 1995). Endothelium expressed-VEGFR2 is constitutively phosphorylated to maintain

normal fenestration (Maharaj AS et al., 2006). VEGFR1 and VEGFR2 are also detected in mesangial cells (Thomas S et al., 2000; Takahashi T et al., 1995) as well as tubular epithelial cells (Kanellis J et al., 2000). In contrast, the detection of VEGFRs on podocytes is still not fully characterized. Several findings have shown that VEGFR1, 3 and neuropilin-1 have been identified in human and cultured mouse podocytes, but not VEGFR2 (Foster RR et al., 2003; Chen S et al., 2004). On the other hands, some studies observed the expression of VEGFR2 in podocytes foot processes and cell bodies and describe its involvement in podocytes survival (Guan F et al., 2006; Veron D et al., 2010).

L- VEGF-A in Physiology and Pathophysiology

There is increasing evidence about the role of VEGF signaling during kidney organogenesis. VEGF-A is expressed by podocytes precursors during the comma-shaped stage, at which VEGF-VEGFR2 axis becomes active and recruits endothelial cells found in the cleft and adjacent mesenchyme towards the developing glomerular capillaries (Pavenstädt H et al., 2003). Experimental studies using immunological and genetic strategies to suppress VEGF expression have confirmed that VEGF indispensable role in normal development of renal vasculature and fenestrated phenotype acquirement (Tufro A et al., 2012; Pavenstädt H et al., 2003).

Interestingly, podocytes and tubular cells persist to express VEGF-A during the adult stage, proposing its critical role in maintaining a normal glomerular hemodynamics (Wakelin SJ et al., 2004). VEGF-A acts as paracrine survival factor on the endothelial and mesangial cells (Thomas S et al., 2000). It has profound role on glomerular

microvascular permeability through increasing and maintaining endothelial fenestrations (Esser S et al., 1998). As potent vasodilator, VEGF-A is also implicated in the regulation of renal blood flow and glomerular filtration rate through activation of Nitric oxide (Murohara T et al., 1998). VEGF-A stimulates the production of alpha3 chain of collagen IV, a major GBM component (Chen S et al., 2004). Alternatively, VEGF-A functions in an autocrine fashion on podocytes themselves to stimulate their protection from apoptosis, regulate slit diaphragm proteins (Guan F et al., 2006) and alter calcium homeostasis (Foster RR et al., 2003; Wakelin SJ et al., 2004). It was also found that podocytes VEGFR2 is bound to nephrin in multiprotein complex. This complex is activated upon VEGF-A stimulation to provoke significant change in podocytes shape and size (Tufro A et al., 2012; Bertuccio C et al., 2011).

Several data have obtained that the alteration in podocytes VEGF-A expression is associated with various glomerular disease at the both fetal and adult stages. VEGF genetic polymorphisms may contribute to the risk of DN in diabetic patients (Sun L et al., 2014). In addition, the increase in glomerular VEGF expression and activity was observed in patients with glomerulonephritis (Hohenstein B et al., 2010). Eremina V et al., have shown that homozygote podocytes- specific deletion of VEGF-A causes perinatal lethality after 18 hours of birth and the heterozygote deletion causes renal injury concomitant with proteinuria and endotheliosis. Similarly, the overexpression of VEGF-A 164 isoform causes collapsing glomerulopathy (Eremina V et al., 2003). Likewise, Veron D. et al. have demonstrated that podocytes-specific overexpression of VEGF-A in adult mice induces proteinuria and causes structural and functional abnormalities similar to those seen in renal diabetic complications including podocytopathy, glomerular

basement membrane thickening and mesangial expansion (Veron D et al., 2010). It was also shown that VEGF pharmacological deletion in patients treated with neutralizing anti-VEGF (bevacizumab) led to thrombotic microangiopathy (Eremina V et al., 2008). The usage of anti-VEGF in newborn mice leads to disruption of vessels formation which is associated with a decreased in the number of developed nephrons (Kitamoto Y et al., 1997). Therefore, VEGF-A is essential factor in maintaining well-formed podocytes cytoskeleton and glomerular integrity, and a tight regulation of VEGF-A expression is highly demanded to establish normal podocytes phenotype.

M- VEGF-A in DN:

The association of increased VEGF-A production with diabetic complications such in the case of diabetic retinopathy, neuropathy and nephropathy has been well-deliberated (Bolinger MT et al., 2016; Sasso FC et al., 2003; Peng L et al., 2015). Several studies have witnessed the up-regulation of glomerular-VEGFA expression and its receptors in rodent and human diabetic kidneys (Sung SH et al., 2006; Cooper ME et al., 1999; Iglesias-de la Cruz MC et al., 2002; Hovind P et al., 2000). The level of VEGF-A 165 expression appears to vary with the progression of DN. Many reports indicated that elevated podocytes VEGF-A expression was observed during early course of the disease (Hovind P et al., 2000) followed by remarkable drop in patients with heavy proteinuria and severe glomerulosclerosis (Bortoloso E et al., 2004). Several cytokines and growth factors also have the potential to up-regulate VEGF-A expression during diabetic course. Angiotensin II (Feliars D et al., 2010) and TGF-B induced by high glucose ambience stimulates podocytes to produce α 3 Collagen IV through VEGF-A signaling pathway

(Chen S et al., 2004; Iglesias-de la Cruz MC et al., 2002). VEGF-A production is also stimulated by hypoxia and the activation of stress-activated kinases such as NF- κ B, P38MAPK and JNK in diabetes (Evans JL et al., 2002; Pagès G et al., 2000).

How does increased VEGF-A expression contribute to the onset and progression of renal dysfunction in diabetes and what is the pathophysiological role that VEGF-A exerts are still greatly controversial concerns.

The concomitance of VEGF-A overproduction with proteinuria has been uncovered by a number of studies (Sung SH et al., 2006; Bai X et al., 2014). These studies reveal the deleterious role of VEGF-A in inducing renal microvascular injury. It has been demonstrated that VEGF-A overproduction exacerbates the early progression of glomerular injury through inducing glomerular hypertrophy, hyperfiltration, mesangium expansion, thickening of GBM, podocytes apoptosis and altering slit pore density and nephrin expression (Sung SH et al., 2006; Bai X et al., 2014; Veron D et al., 2011; de Vriese AS et al., 2001), all of which ultimately causes proteinuria. In contrast, Sivaskandarajah G et al., described a new genetic approach that permits specific deletion of VEGF-A 164 from podocytes of STZ-induced diabetic mice. This study suggested the renoprotective role of elevated-VEGF A on microvasculature and its knockout from the glomerulus is deleterious and significantly associated with the genesis of proteinuria and podocytes apoptosis (Sivaskandarajah GA et al., 2012). Therefore, Further investigations are still greatly warranted to precise to role to VEGF-A in the development of DN.

N- VEGF-A and oxidative stress in DN

The well-established detrimental role of oxidative stress on vasculature in diabetic diseases suggests a crosstalk relationship between VEGF-A and ROS production. Superoxides are shown to stimulate VEGF-A expression in diabetic nephropathy (Tilton RG, 2002; Evans JL et al., 2002). VEGF-A activation is regulated by several ROS-dependent pathways including Hypoxia, PKC, AGE/RAGE system, NOXs, nitric oxides (NO), sorbitol pathway. AGEs and its receptors (RAGEs) are been shown to contribute to VEGF-A expression in diabetic glomeruli (Wendt TM et al., 2003). Polyol pathway is also associated in increase of renal and retinal VEGF-A expression in diabetic ambience (Sung JK et al., 2010). Similarly, PKC inhibition was shown to regulate VEGF-A expression in cultured podocytes (Lee EY et al., 2006) and ameliorate albuminuria in diabetic animals (Menne J et al., 2004). Several studies also correlated the HG-stimulated NOXs activation to altered VEGF-A expression. It was demonstrated that NOXs inhibition using apocynin was shown to reduce kidney cortical membranous VEGF-A in type 1 diabetic rat model (Thallas-Bonke V et al., 2008).

Taken Together, studies from the literature have described a contradictory role of VEGF-A in diabetic nephropathy. Therefore, the exact VEGF-A mechanism of action and its crosstalk with the NADPH oxidases-inducing ROS generation and podocyte injury is still unknown.

O- TP53 Induced Glycolysis and Apoptosis Regulator (TIGAR)

Tp53 induced glycolysis and apoptosis regulator (TIGAR) is a recently discovered enzyme which primarily functions as glycolysis regulator in cells. TIGAR, is a highly conserved protein that acts in a manner similar to fructose-2,6-bisphosphatase (Li H et al., 2009). It degrades fructose-2,6-bisphosphate and shifts glycolysis pathway into PPP. Once PPP is activated, TIGAR increases NADPH production that allows the scavenging of ROS by reduced glutathione. This supports its antioxidant role (Bensaad K et al., 2006).

TIGAR is P53 target gene. Microarray analysis reveals that TIGAR exhibits two possible p53 binding sites, one is located upstream of the first exon (BS1) and the other within the first intron (BS2) with higher affinity to p53 (Bensaad K et al., 2006). Therefore, its expression regulates p53-induced apoptosis. However, the significance of TIGAR expression on ROS regulation and apoptosis seems to be cell type and tissue context-dependent (Bensaad K et al., 2006).

TIGAR was shown to be involved in several diseases such as myocardial, renal and brain ischemia (Li M et al., 2014; Kimata M et al., 2010; Kim J et al., 2015) and malignancies (Dodson M et al., 2013). However and to our knowledge, there is still no evidence that observes its expression in the diabetic course. Thus, further investigations are still needed to study TIGAR expression and its effect on apoptosis in diabetic kidney diseases.

CHAPTER II

HYPOTHESIS

Several clinical trials have been established to evaluate the magnitude of strict glucose control on the onset and progression of microvascular complications in patients with type 1 and type 2 diabetes. However, none of these trials gave an exact answer to the major hypothesis set by each study. The DCCT and UKPDS trials showed that intensive glycemic control potentially prevent the development of diabetic microvascular complications and long-term cardiovascular diseases. However, the ACCORD and ADVANCE trials showed that intensive glucose control to near normoglycemia have detrimental effects on cardiovascular outcomes associated with severe hypoglycemia (Brown A et al., 2010). Therefore and despite the importance of glycemic control that is often hard to achieve, gaining additional insights into the mechanistic effect of hyperglycemia on endogenous factor will help design adjunct therapy to prevent or reverse DN.

VEGF-A has emerged as a potent microvascular permeability factor that contributes by virtue to the genesis of proteinuria. In diabetes, VEGF-A expression is increased; however the critical role of this increase is still controversial. Several findings prove that glucose toxicity is partially enhanced by oxidative stress. To our knowledge, there is little evidence that addresses the direct crosstalk between VEGF-A and oxidative stress in DN.

Thus, understanding how altered VEGF-A expression is implicated in kidney injury and how is correlated to oxidative stress is of our great interest.

Our central hypothesis is that high glucose/hyperglycemia increases VEGF-A expression in podocytes. This increase activates NOX4 dependent -ROS generation leading to podocytes injury and albuminuria.

High Glucose/Hyperglycemia

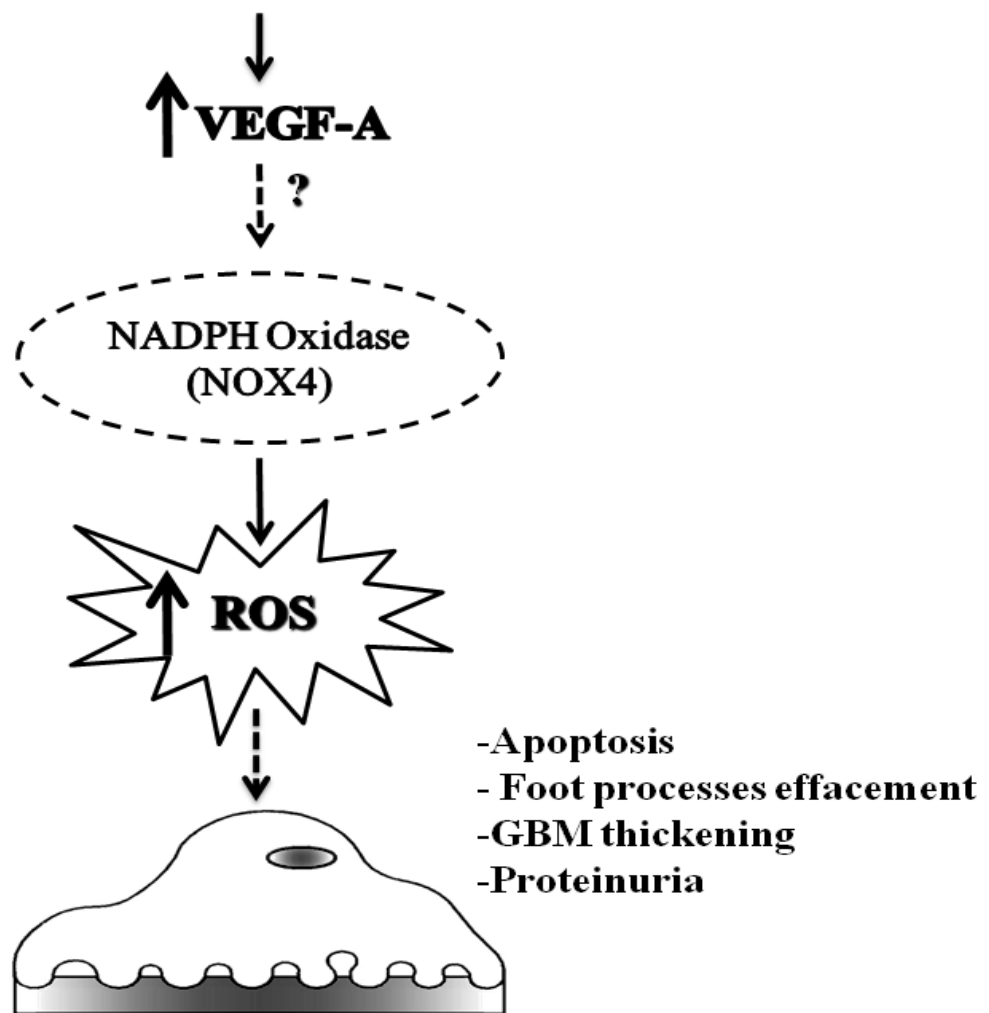


Figure 3: Proposed model for podocytes injury in diabetes

CHAPTER III

MATERIALS AND METHODS

A. Podocytes Culture and siVEGF-A Transfection

Conditionally immortalized rat podocytes were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) containing 1000 mg/L glucose (low concentration of glucose), 10% fetal bovine serum, and 100 unit/ml of penicillin/streptomycin. Podocytes were double transfected with si-RNA against VEGF-A and then incubated in low glucose DMEM serum-deprived medium containing 1% FBS and 100 unit/ml of penicillin/streptomycin for 24hr. Podocytes were treated with either 25mmol/l D-glucose or 30ng/ml rat recombinant VEGF-A 164 (R&D system) for 72hr. 25 mmol/l Mannitol is used as osmotic control.

For siRNA transfection experiments, siGENOME siRNA specific for rat VEGF-A was obtained from Thermoscientific Dharmacon. Si-VEGF-A (100nM) was introduced into the cells by double transfection using X-treme Gene DNA Transfection Reagent (Roche Life Science). 100nM Scrambled siRNAs (non targeting siRNAs) were used as controls to validate the specificity of transfection.

B. Animal model:

Male Sprague-Dawley rats weighing between 200 and 225 g were divided into 4 groups of five animals each. Rats in **group 1** were injected with sodium citrate buffer (0.01 M, pH 4.5) alone and considered as control. **Group 2** rats were injected once intravenously via the tail vein with 55 mg/kg body with streptozotocin (STZ) (Sigma-Aldrich) prepared in sodium citrate buffer to induce type 1 diabetes. After 2 days of STZ injection, rats with glucose blood levels above 250mg/dl were considered diabetic. Following 4 weeks of diabetes, part of the diabetic rats were further subdivided into 2 groups (Group 3 and Group 4), rats in **group 3** were intraperitoneally injected with 3mg/kg SU-5416 (small molecular inhibitors interfere with the ATP-binding site of the receptor kinase and prevents its autophosphorylation) (Aldrich-Sigma) and **Group 4** rats were intraperitoneally injected with 5mg/kg humanized monoclonal anti-VEGF-A neutralizing antibody (bevacizumab or Avastin ®). Control group were injected with equivalent amount of sodium citrate buffer alone. After 2 months of twice a week treatment, all rats were euthanized, and both kidneys were removed and weighed.

A slice of kidney cortex at the pole was fixed with 4% formalin for immunohistochemical analysis or flash-frozen in liquid nitrogen and stored at -80°C for biochemical and image analysis.

All rats were kept in a temperature controlled room and on a 12/12 dark/light cycle and had free food and water access. All protocols were approved by the Institutional Animal Care and Use Committee of the American University of Beirut. The body weight and the

blood glucose levels were monitored every 48h periodically. Blood samples were taken from the tail vein and glucose levels were determined with the glucometer system Accu-Chek. Urine samples were collected using metabolic cages and albumin concentration was measured as micrograms per 24hr.

C- Apoptosis Assays:

1- Cellular DNA fragmentation

Cellular DNA fragmentation ELISA (Roche Applied Science) was used for the detection of BrdU-labeled DNA fragments in culture supernatants and cell lysates. The experimental procedures were handled according to the manufacturer's protocol. Briefly, cells were plated in 12 well-plates. After reaching certain confluency, double-transfected podocytes were treated for 72hr according to the experimental conditions. 5 hrs prior stopping the treatment, 15 μ M BrdU-labeling solution was added to the cells. For ELISA procedures, an anti-DNA coating solution was fixed in the wells of a microplate. Then, BrdU-labeled DNA extracted for cell lysates and culture supernatant were added to the immobilized anti-DNA antibody. The immunocomplexed BrdU-labeled DNA fragments were denatured and fixed on the surface of the microplate by microwave irradiation, in order to improve the accessibility of the antigen BrdU for detection by the antibody. Anti-BrdU-peroxidase conjugate which reacted with the BrdU incorporated into the DNA is then added. The amount of peroxidase bound in the immune complex was photometrically determined, after the addition of the substrate solution. Absorbance was measured at 450 nm against a reference wavelength of 650 nm using a microplate reader (Multiskan Ex).

2- Annexin V-FITC Staining

Annexin V-FITC apoptosis detection kit (Abcam) was used for annexin V and propidium iodide (PI) staining according to the manufacturer's protocol. siVEGF-A transfected podocytes were plated in 6-well plate and treated according to the experimental conditions. After 72hr, cells were detached, washed with ice-cold 1X PBS buffer and centrifuged at 12000 rpm for 5 mins. The pellets was further resuspended with 1X binding buffer and incubated with Annexin V and Propidium Iodide for 5 minutes at room temperature in dark. The number of Annexin V-FITC binding cells that corresponds to the early apoptotic cells, together with the number of necrotic cells labeled with PI was measured by using Fluorescence-Activated Cell Sorter (FACS) at excitation and emission wavelengths of 488 nm and 530 nm, respectively. The percentage of cell death was further assessed.

3- Hoechst Staining

Si-VEGF-A transfected podocytes were plated on cover slips in 6-well plate. Apoptotic nuclei were detected using Hoechst 333425 (Molecular probe). After treatment, cells were washed with ice-cold 1X PBS and fixed with 4% paraformaldehyde. 1 µg/ml of Hoechst solution diluted in distilled water was added to the cells for 30 min at room temperature in dark. The percentage of apoptotic cells were analyzed via fluorescence microscopy at 350nm excitation and 460nm emission.

D. ROS Detection

ROS Generation was assessed using oxidant-sensitive fluorogenic probe Dihydroethidium (DHE) (Invitrogen) reacts with superoxide anions to form the red fluorescent product (2-hydroxyethidium) (Zhao H et al., 2005). Briefly, cortical frozen samples were cut into 4 μm thick sections and placed on glass slides. DHE (20 $\mu\text{mol/l}$) was applied to each tissue section and the slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Fluorescence was detected at excitation and emission wavelengths of 488 and 520 nm, respectively, using laser-scanning confocal microscope. Images were taken at 20x magnification lens from different fields. The average of five sections stained with DHE was taken as the value for each animal. Quantification was done using Zen light Software.

E. Immunohistochemical Analysis

Kidney cortex tissues from each group were fixed in a 4% formalin solution and embedded in paraffin block. Samples were cut into 5 μm thick sections and placed on glass slides. The kidney sections were stained with periodic acid Schiff (PAS) reagent to assess mesangial accumulation, masson trichrome staining to evaluate the collagen IV fibers deposition and hematoxylin and eosin stain (H&E) to review the morphologic changes in the kidney. A quantitative measurement for 15 randomly sampled glomeruli was performed on each group by a blinded observer using Image J software.

F. Real-time RT-PCR

Gene expression in renal cortex was analyzed by real-time RT-PCR using the $\Delta\Delta C_t$ method. Total RNA was extracted from the renal cortex lysate using TRIZOL reagent (Sigma-Aldrich) and converted into cDNA using the Revert First Strand cDNA Synthesis Kit (Qiagen) according to the manufacturer protocol. cDNA was measured by RT² qPCR Biorad CFX96 using SYBR green dye and rat RT² qPCR Primers for NOX4: Forward: 5'- TTC GGG TGG CTT GTT GAA GT -3' and Reverse: R: 5'-TGG GGT CCG GTT AAG ACT GA -3', for TIGAR: Forward: 5'- ATC CGC CAG GCA AGG AGT AG -3' and Reverse: 5'- CCT TCT GCA ACC CCG TAC AT -3', for Collagen IV 5'- CGT GGA TGG TGT ACC TGG TC and Reverse: 5'- ACC TCG TGA GCC ATT GTA GC -3'. GAPDH Forward: 5'- GGG GCT CTC TGC TCC TCC CTG -3' and Reverse: 5'- CGG CCA AAT CCG TTC ACA CCG -3' was used as internal reference gene.

G. Western Blot Analysis

Cuts from the renal cortex tissues were prepared in 500 μ L of lysis buffer containing 0.1% sodium dodecyl sulfate (SDS) ,0.5% sodium deoxycholate, 150 mM sodium chloride, 50 mM Tris-hydrochloride, 100 mM EDTA, 1% Tergitol (NP40), 100 mM PMSF, 1X of protease inhibitor cocktail containing aprotinin and leupeptin and phosphatase inhibitors cocktail (Bio-world). Samples were homogenized using a dounce homogenizer.

Rat podocytes were plated and double transfected with si-RNA against VEGF-A (Dharmacon). After 72hr of treatment with 25 mmol/l Glucose and 30ng/ml VEGF-A, cells were washed twice with 1X cold-PBS (Sigma-Aldrich) and scraped with same lysis Buffer. Homogenates were incubated for 2h at 4 °C and centrifuged at 13,600 rpm for 30 minutes at 4 °C. Proteins in the supernatants were measured using the lowry quantification method (Bio-rad Laboratory). For immunoblotting, proteins (40-80µg) were separated on 12.5% Polyacrylamide SDS- gel Electrophoresis and transferred to nitrocellulose membranes. Blots were incubated with rabbit anti-Nox4 (1:250; Santa Cruz Biotechnology), rabbit anti-VEGF-A (1:500; Abcam), rabbit anti-collagen IV (1:1000; Abcam), and rabbit anti-TIGAR (1:250; Abcam). Goat HSC-70 (1:1000; Santa cruz Biotechnology) was used as loading control. The primary antibodies were detected using horseradish peroxidase–conjugated IgG. Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software.

H. Measurement of NADPH Oxidase enzymatic activity

NADPH oxidase activity was measured in transfected rat podocytes grown in serum-free medium and treated with D-glucose and exogenous VEGF-A for 72 hrs or isolated kidney cortex tissues. Briefly, podocytes were washed twice in ice-cold PBS and proteins were extracted using Lysis Buffer that contains 20 mmol/l KH_2PO_4 , pH 7.0, 1 mmol/l EGTA, 1 mmol/l phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin, and 0.5 µg/ml leupeptin. Similarly, cuts of kidney cortex tissues were homogenized with dounce homogenizer on ice. Samples were left to rotate in the lysis buffer for 30mins at 4°C.

Total protein concentration was determined using Bio-rad protein assay reagents. 25 µg of lysate were added to an Assay Buffer containing 50 mmol/l phosphate buffers, pH 7.0, containing 1 mmol/l EGTA and 150 mmol/l sucrose. 5 µmol/l lucigenin, and 100 µmol/l NADPH were further added. Photon emission expressed as relative light units was measured every 30 s for 5 min in a luminometer. Superoxide production was expressed as relative light units (RLU) per milligrams (mg) of protein.

I. Podocyte Enumeration

Dual-label immunohistochemistry was used to identify and count podocytes relative to GMB. 5-µm paraffin embedded sections of kidney cortex on glass slides were hydrated and stained with rabbit anti-NPH2 antibody (abcam) and mouse anti-Collagen IV (Millipore), followed by staining with secondary antibodies Alexo-Fluor 568 goat anti-rabbit IgG and Alexa Fluor 488 goat anti-mouse IgG (Molecular probe), respectively. After several washes, kidney cortex sections were preserved on cover slips by anti-fade mounting medium with DAPI (Abcam) for fluorescence detection of nuclei. Sections were examined by confocal microscopy using excitation and band-pass filters optimal for FITC, Cy3, and DAPI. Digital images representing each fluorochrome were taken of random glomeruli. 15–20 glomerular cross-sections per animal were photographed in each color channel. The images were merged and color-balanced using Zen Light software, and the number of podocytes was counted in projected images in a blind fashion. Podocin-positive podocytes were counted as well.

J. TUNEL Assay

TUNEL staining using TAC TdT DAB (Diaminobenzidine) Kit (Trevigen) was performed to assess podocytes apoptosis according to the manufacturer's instructions. Briefly, paraffin-embedded kidney cortex tissues were deparaffinized and incubated with proteinase K solution to permeabilize the membrane. Tissues were pre-treated with hydrogen peroxide to reduce the non-specific background staining. After immersing the slides in 1X TdT Labeling Buffer, tissues were incubated with Labeling Reaction Mix containing TdT DNTP Mix, TdT Enzyme, Mn^{2+} cation and 1X TdT Labeling Buffer for 1h at 37°C. The reaction was stopped by immersing the tissues in 1XTdT stop buffer for 5mins and then covered with streptavidin-horseradish peroxidase (Strep-HRP) solution until brown color is developed. Tissues were further incubated with DAB solution and counterstained using methyl green. The apoptotic cells exhibit dark brown staining that is typically associated with cell condensation. The number of TUNEL-positive cells was counted in 15 randomly selected glomeruli (magnification $\times 40$) from each animal by blinded observer and the percentage of apoptotic cells per glomerular section was calculated.

K. Statistical analysis

Results are expressed as means \pm SE. Statistical significance was assessed by student's unpaired *t* test. Significance was determined as probability (*p*) less than 0.05.

CHAPTER IV

RESULTS

A. HG induced-VEGF-A expression and activity in cultured podocytes

Alteration of VEGF-A protein expression was measured in rat podocyte exposed to 5mmol/l D-glucose (NG) or 25mmol/l D-glucose (HG) for 6h, 24h and 72h. Our results show that HG-induced early increases in VEGF-A expression at 6h and persistent up to 72h (**Figure 4A**). To assess the biological output of VEGF-A on podocytes, different concentrations of exogenous recombinant rat VEGF-164 (rVEGF-A) was used on cultured podocytes for 72h. Our results show that HG-induced podocyte injury as assessed by the Annexin V staining assay, was mimicked by the use of at least 30ng/ml of VEGF-A (**Figure 4B**), while lower doses of VEGF had no effect on apoptosis. To confirm the effect of VEGF-A on apoptosis, the cellular DNA fragmentation assay was used and rat podocyte were treated with 30ng/ml of rVEGF-A for 24h, 48h and 72h (**Figure 4C**). Our data show that at 72h, VEGF-A becomes detrimental to the cells as compared to the control non treated podocytes. In all of these experiments 25mmol/l of

mannitol was used as an osmotic control and our results show that it had no effect on the podocyte phenotypic changes.

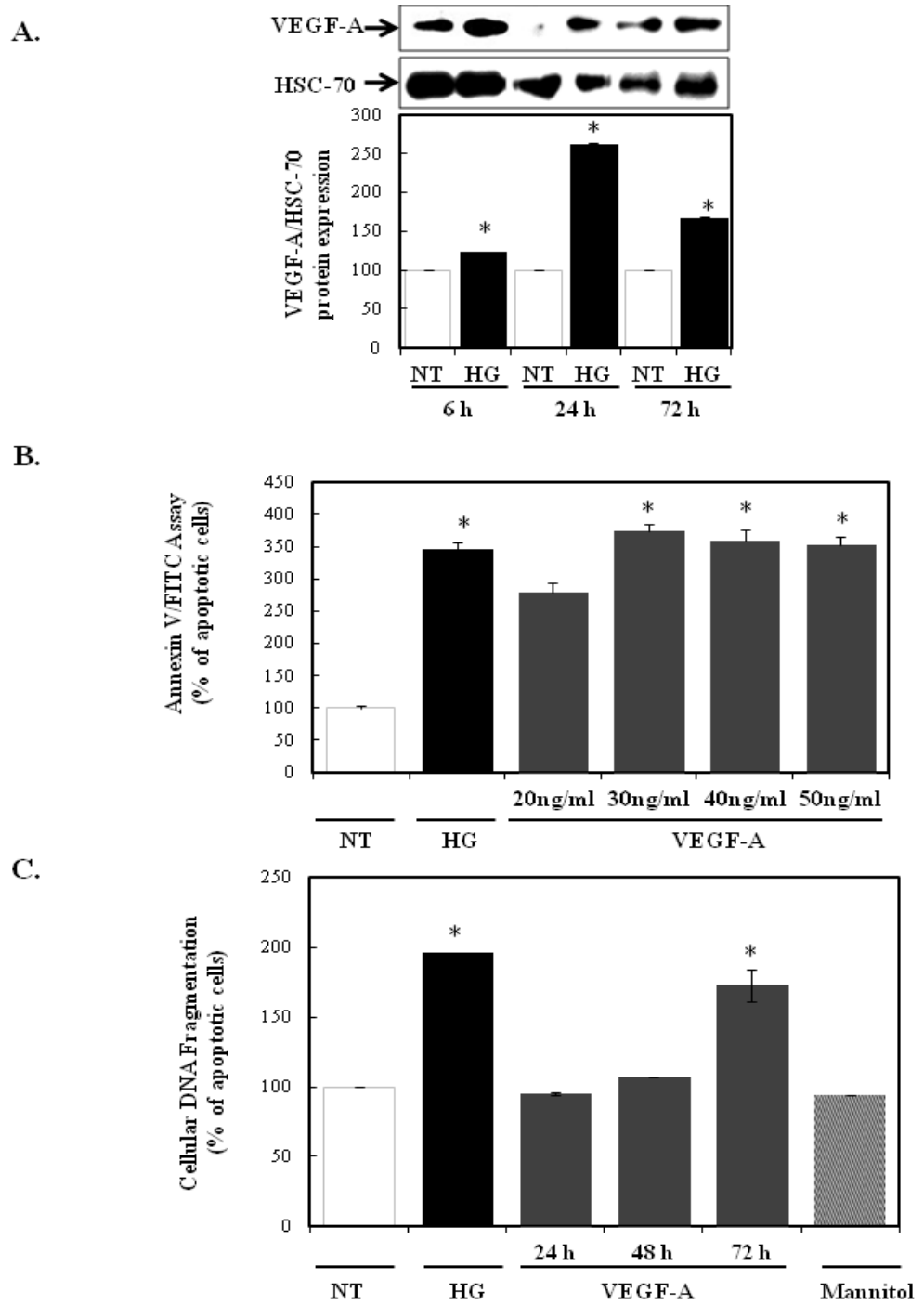


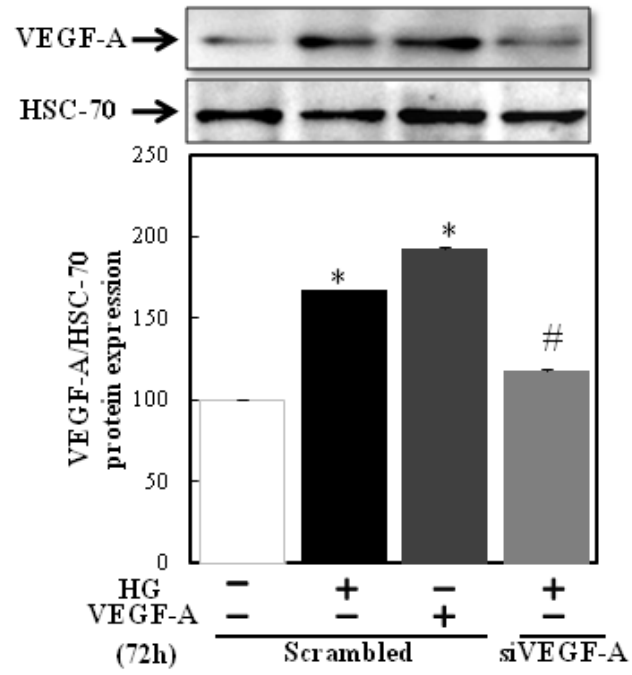
Figure 4: HG-induced VEGF-A expression and activity in cultured podocytes. Rat podocytes were serum starved, then treated with 25mmol/l glucose (HG) for 6h, 24h and 72h. Western blot analysis was performed to assess VEGF-A expression in HG-treated cells compared to non-treated cells (NT). HSC-70 was included as a loading control and the corresponding histogram represents the intensity band quantification as measured by image J software of VEGF-A three independent experiments for each time points (A). Annexin V/FITC staining was done to examine the dose effect of recombinant rat VEGF-A (20ng/ml, 30ng/ml, 40ng/ml and 50ng/ml) on podocytes apoptosis. The percentage of apoptotic cells was calculated (n=3) (B). Cellular DNA Fragmentation was used to measure the time course of rVEGF-A activity on rat podocytes. Cells were treated with either HG or 30ng/ml rVEGF-A for 24h, 48h, 72h. Mannitol was used as osmotic control (C). All values are the mean \pm SE. * $P < 0.05$ vs. NT; # $P < 0.05$ vs. HG

B. VEGF-A regulates HG-induced podocytes Apoptosis

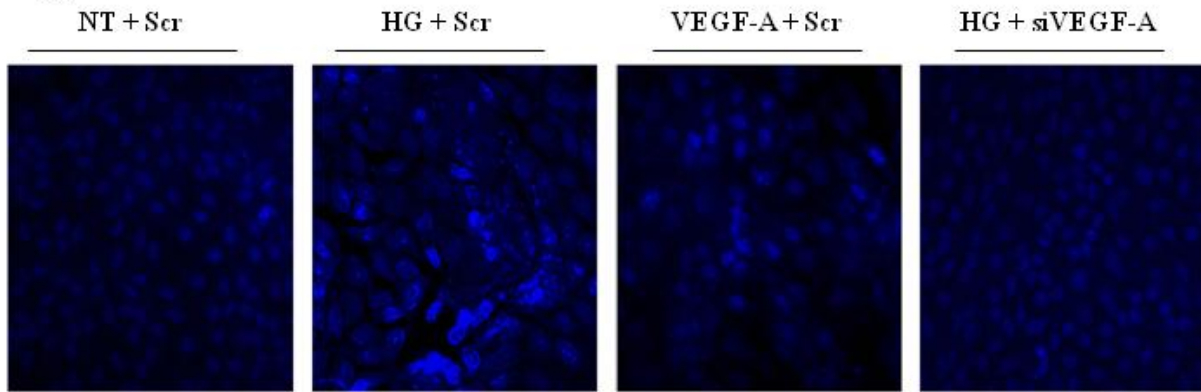
To further assess the role of VEGF-A production on diabetes-induced podocyte apoptosis, VEGF-A expression was inhibited by using small interfering RNA (siVEGF-A). First, the efficiency of the transfection was assessed, and the results show that siVEGF-A significantly decreases its expression in the diabetic milieu, while the use of scrambled siRNA showed no effect (**Figure 5A**).

More importantly, our results confirmed our previous observation that exposure of rat podocytes to HG for 72hr significantly increases apoptosis as measured by Hoechst staining (**Figure 5B and C**), and cellular DNA fragmentation (**Figure 5D**), an effect that is significantly reversed by the blockade of VEGF-A using siVEGF-A and mimicked by the treatment with 30ng/ml of rVEGF-A 164. Taken together, these results demonstrate that increased VEGF-A expression mediates the effect of HG in inducing podocytes apoptosis and could provide an insight into podocytes VEGF-A autocrine role.

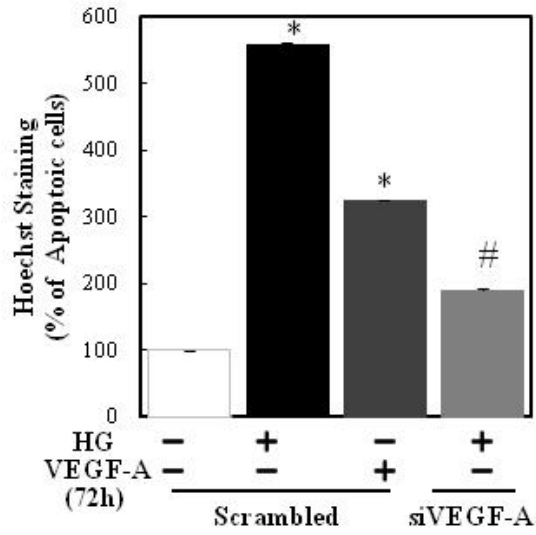
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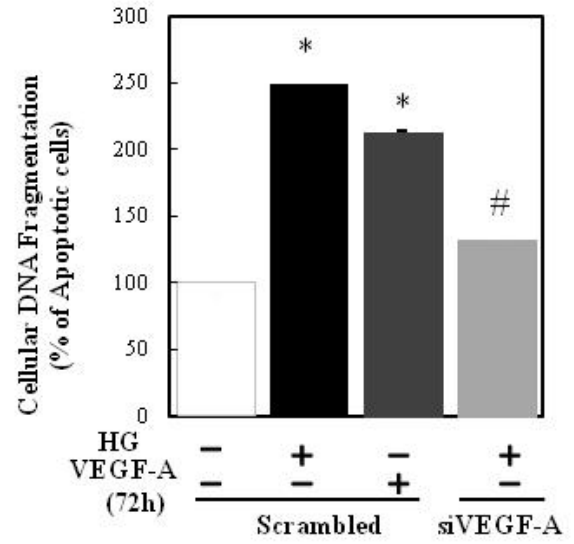


Figure 5: VEGF-A regulates HG-induced podocytes Apoptosis. Rat podocytes were double transfected with siVEGF-A, serum deprived and treated either with 25mM glucose or 30ng/ml VEGF-A for 72hr. VEGF-A protein expression was measured by Western blot analysis to assess the efficiency of siVEGF-A transfection. Non-treated and HG-treated cells were also double transfected with non-targeting scrambled siRNA (A). Apoptosis was measured using Hoechst staining (B), ELISA for cellular DNA fragmentation (D) and Annexin V/PI staining. Quantification of Hoechst-positive cells (% of apoptosis) from three different experiments (C). All values are the mean \pm SE from four independent experiments. * $P < 0.05$ vs. NT; # $P < 0.05$ vs. HG.

C. VEGF-A induces podocytes apoptosis through regulating NOX4 expression and activity

We have previously showed that HG-induced podocyte apoptosis was mediated by the increase in Nox4-stimulated ROS production (Eid AA et al., 2010). However the exact mechanism by which Nox4 exerts its deleterious effect is still not known. Therefore, we next examined if VEGF-A induces podocytes apoptosis through ROS-dependent pathway in which NADPH oxidase Nox4 is involved. As expected, an increase in superoxide anions was observed upon treating cells with either 25mM glucose or 30ng/ml exogenous VEGF-A as measured by the NADPH oxidases activity (or superoxide anions generation) (**Figure 6A**). This effect was reversed upon treating the cells with siVEGF-A. These results were paralleled by an increase in Nox4 protein expression in the presence of HG or 30ng/ml exogenous VEGF-A and reversed by siVEGF-A (**Figure 6C**). Taking altogether, our results indicate that VEGF-A enhances ROS generation and induces oxidative stress in cultured podocytes. This effect occurs through the involvement of NOX4.

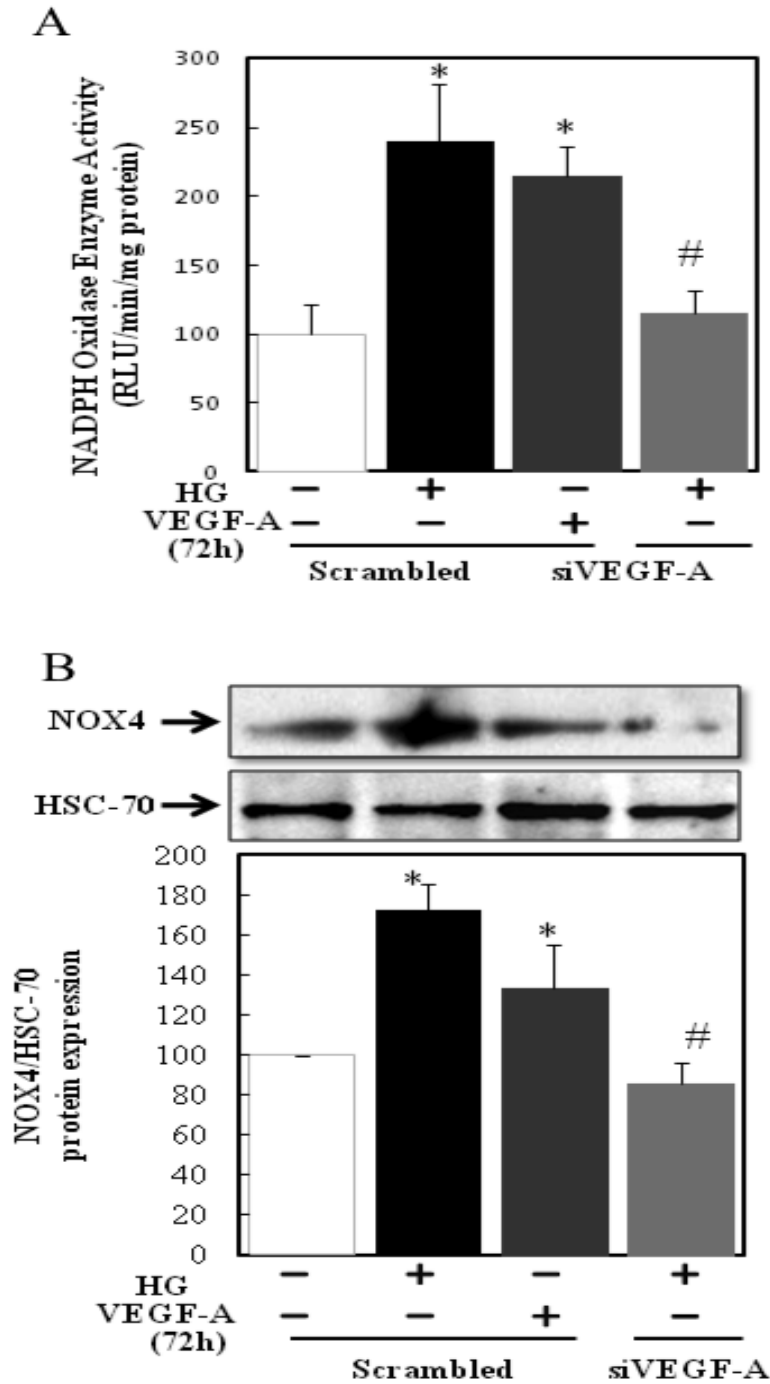


Figure 6: VEGF-A induces podocytes apoptosis through regulating NOX4 expression and activity. Transfected rat podocytes were treated with either high glucose (25 mM) or rVEGF-A (30 ng/ml) for 72hr. NADPH-dependent superoxide generation was assessed by lucigenin-enhanced chemiluminescence. Superoxide anion was expressed as relative chemiluminescent light units (RLU) per milligram of protein per minute ($n = 3$) (A).

Protein expression of Nox4 was determined by Western blotting analysis. HSC-70 was included as a loading control and the corresponding histogram represents the intensity band quantification as measured by image J software for four independent experiments (B). * $P < 0.05$ vs. NT; # $P < 0.05$ vs. HG.

D. VEGF-A regulates TIGAR expression in cultured podocytes

To our knowledge, there is no study that addresses the regulation of TIGAR expression in diabetic context. In this study, TIGAR protein expression was upregulated in podocytes exposed to either 25mM glucose or 30ng/ml VEGF-A for 72hr. This increase was significantly attenuated in cells transfected with siVEGF-A (**Figure 7**). These results suggest that the anti-oxidative role of TIGAR is launched, however, it couldn't reverse the increased in ROS production in the presence of HG. Further experiments are still needed to identify the exact role of TIGAR in diabetic nephropathy.

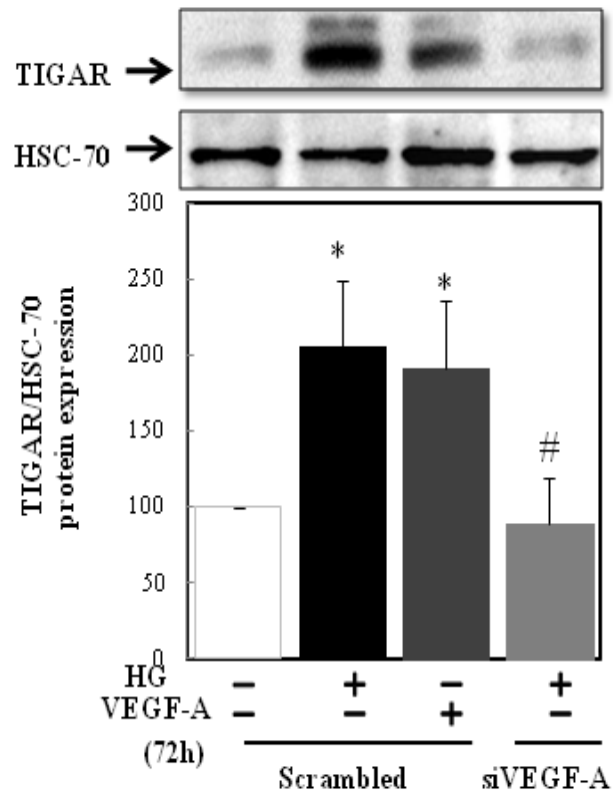


Figure 7: VEGF-A regulates TIGAR expression in cultured podocytes. Protein expression of TIGAR was determined by Western blotting analysis. HSC-70 was included as a loading control and the corresponding histogram represents the intensity band quantification as measured by image J software for three independent experiments. * $P < 0.05$ vs. NT; # $P < 0.05$ vs. HG.

E. Involvement of VEGF-A expression in kidney injury of Type 1 diabetic animal model

To determine the *in-vivo* relevance of our finding in cultured podocytes, STZ induced type 1 diabetic rat model was used. The importance of VEGF-A signaling in diabetes was investigated by using two different pharmacological approaches: SU-5416,

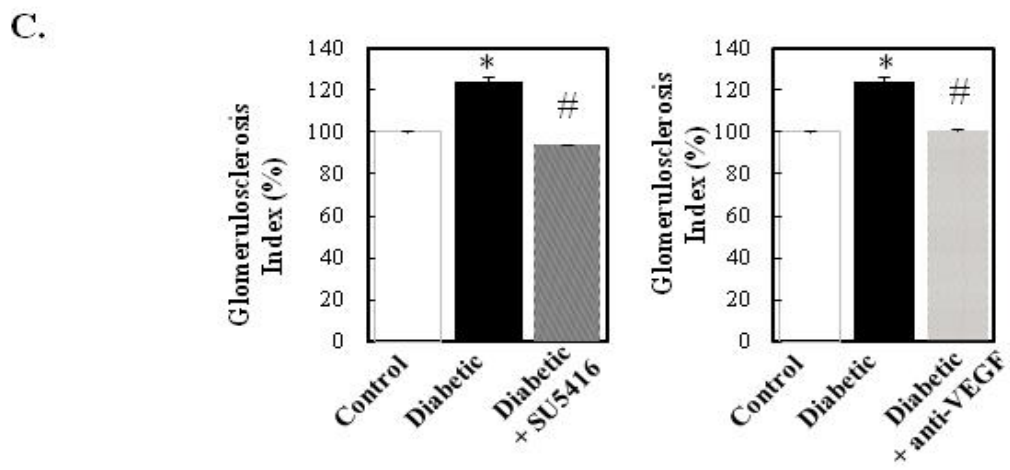
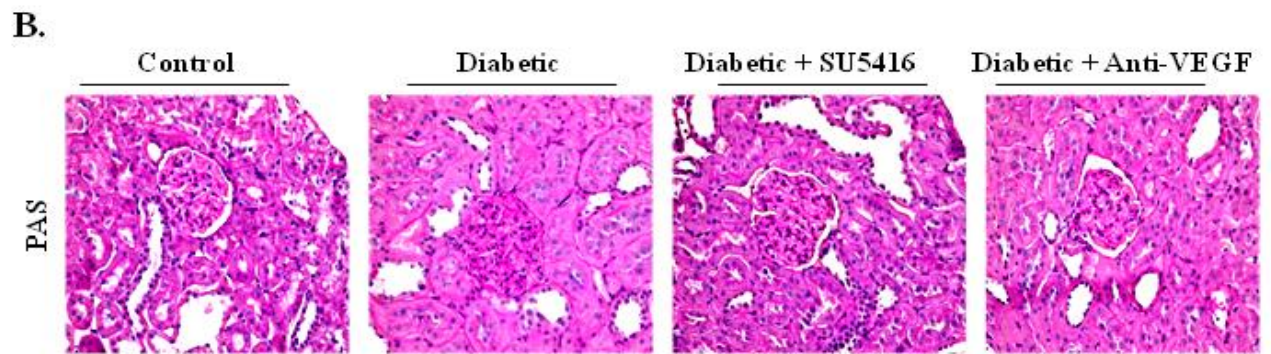
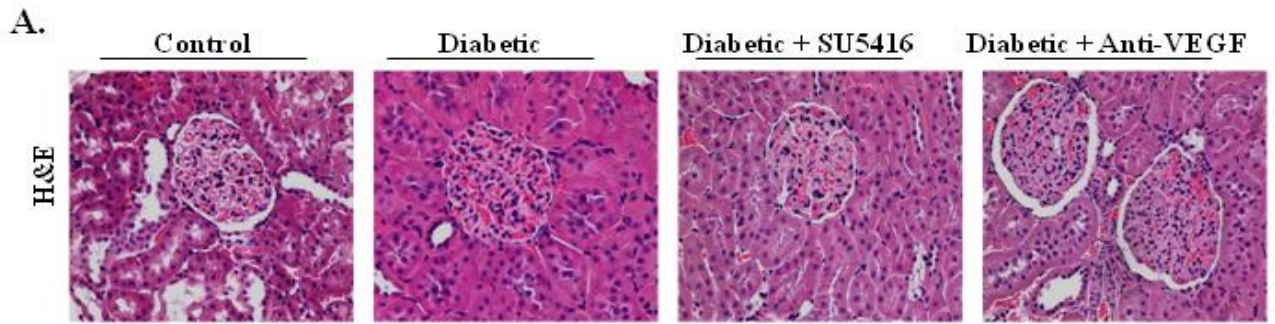
a selective tyrosine kinase inhibitor that blocks VEGF receptor autophosphorylation and humanized monoclonal VEGF antibody (becacuzimab or Avastin ®). Male Sprague-Dawley rats were injected with a single dose of 55mg/kg streptozotocin (STZ) and kept diabetic for 4 weeks after which they were divided into 3 groups: 1) diabetic rats treated with the drugs vehicle, 2) diabetic rats treated with either 3mg/kg of the tyrosine kinase inhibitor (SU-5416) or 3) 5mg/kg anti-VEGF. After 4 weeks of treatment rats were euthanized and kidney cortices were isolated for further analysis. The results obtained from these groups were compared to a control non-diabetic group. The functional parameters of the kidneys were assessed and are presented in **Table 1**. In brief, blood glucose levels and urine excretion rate (ml/24hr) were significantly elevated in diabetic rats as compared to controls. Total kidney to body weight ratio, an index of renal hypertrophy, was also elevated in diabetic rats. Blockade of VEGF signaling did not show any significant alteration in blood glucose levels nor on urine output. Renal hypertrophy was decreased with the use of SU5416 when compared to untreated diabetic rats, while Avastin, did not show any reduction in the hypertrophy index.

	Blood glucose Average	Kidney weight (grams)	Body weight(grams)	Total kidney/body weight	Urine Excretion (ml/day)
Control	118.4 ± 3.3	4.2 ± 0.2	610.7 ± 23.2	0.007 ± 0.0002	23.7 ± 1.9
Diabetic	441.7 ± 28.4*	4.9 ± 0.2*	395.5 ± 18.7*	0.013 ± 0.0009*	155.2 ± 23.6*
Diabetic + SU5416	486.7 ± 17.5	4.2 ± 0.3 ^{0.06}	306.2 ± 18.9 [#]	0.014 ± 0.0007	112.7 ± 25.2
Diabetic + Anti-VEGF	473 ± 23.3	4.9 ± 0.5	335.7 ± 37.2	0.015 ± 0.0013	96.5 ± 12.1

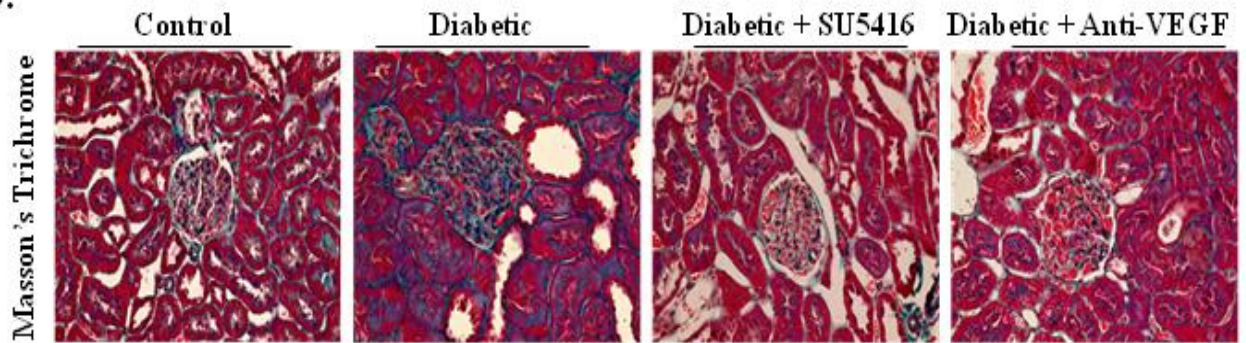
Table 1: Blood glucose levels, kidney weight, body weight, kidney to body weight ratio and urinary excretion (ml/24hr) after 8 weeks of treatment. Value are the mean \pm S.E. n=4 for each group.*p<0.05 vs. Control rats # p<0.05 vs. untreated diabetic rats

F. VEGF blockade attenuates renal injury in STZ –induced diabetic rats

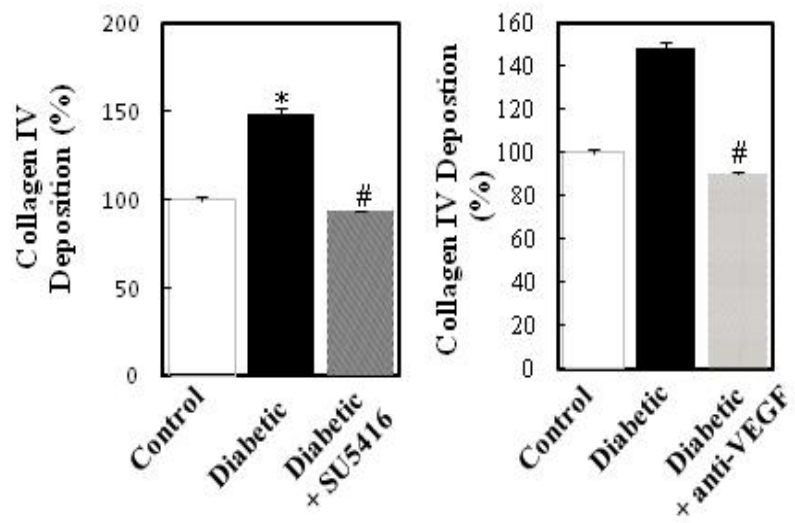
Immunohistochemical studies were performed to determine the degree of renal cellular damage induced by diabetes. Glomerulosclerosis and matrix protein accumulation were assessed in glomerular area of renal cortex of the four groups of rats: the control group, the diabetic group and the diabetic rats treated either with SU5416 or anti-VEGF. H&E staining showed an increase in the glomerular size in diabetic rats as compared to the control. Treatment with VEGF inhibitors restored the glomerular size (**Figure 8A**). Histological examination using PAS staining showed was an increase in the ECM protein accumulation in the glomerulus of diabetic rats compared with the control. The blockade of VEGF was able to reverse tissue injury (**Figure 8B and C**). Similarly, Masson Trichrome staining showed a significant increase in the collagen IV fibers expression in the glomerulus of diabetic kidney compared to that of the controls. Inhibition of VEGF signaling significantly decreased the collagen IV deposition (**Figure 8D and E**). These results were further confirmed by RT-PCR and western blot analysis, in which collagen IV gene and protein expression was increased in kidney cortical tissues of diabetic rats and this increase was significantly lowered upon inhibiting VEGF signaling (**Figure 8F and G**, respectively). These results provide evidence that blockade of VEGF signaling ameliorate glomerular injury observed in diabetes.



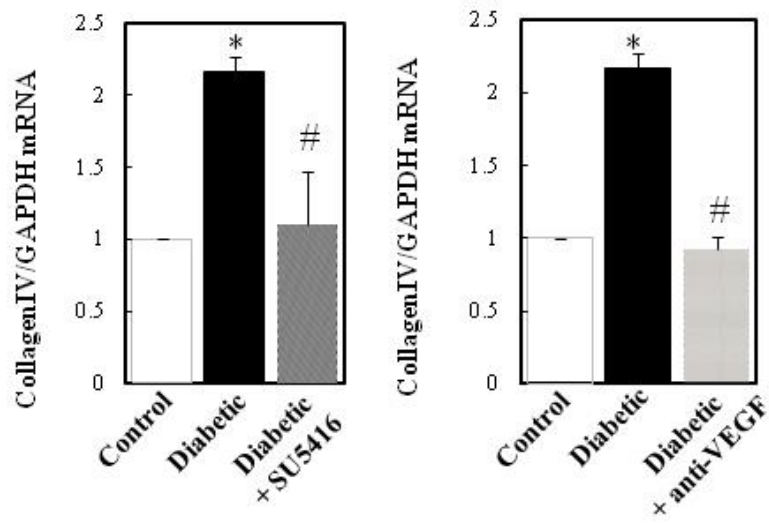
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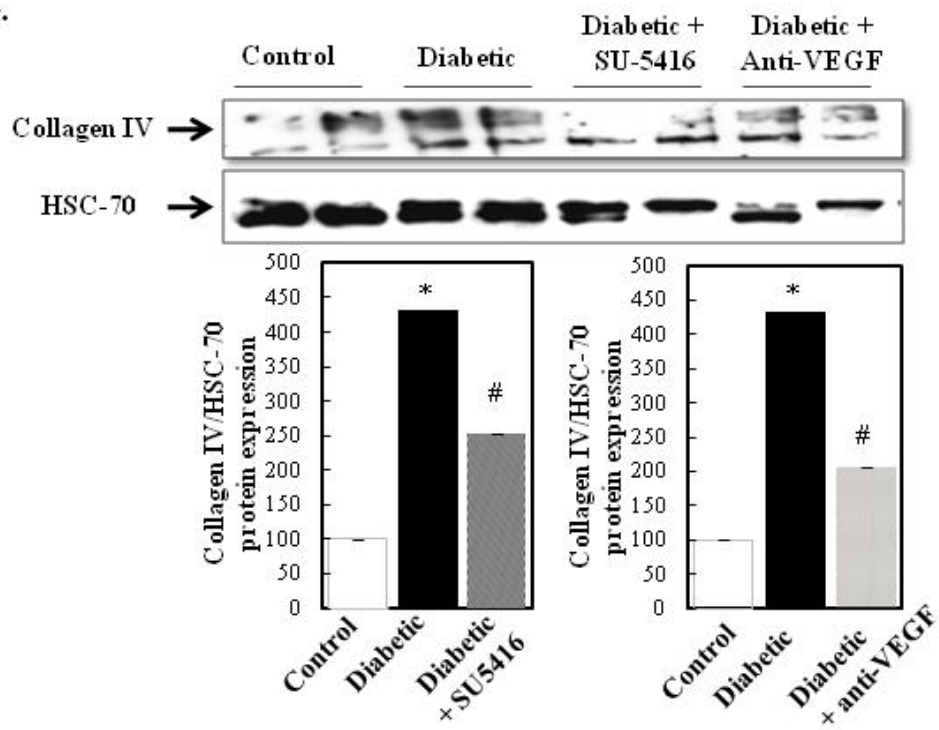


Figure 8: VEGF blockade attenuates renal injury in STZ –induced diabetic rat. The representative photomicrographs represent the immunohistochemical demonstration of morphological changes (A), matrix expansion (B) and collagen IV deposition (D) that were assessed using Hematoxylin and eosin stain (H&E), periodic acid Schiff (PAS) reagent and Masson's trichrome staining, respectively. The corresponding histograms represent the intensity quantification per glomerular area using blinded observer evaluation of 5 randomly chosen glomeruli from 3 rat kidney cortices from each group (C and E). QT-RT PCR was used to assess the gene expression of Collage IV in kidney cortex (F) Collagen IV expression was measured using Western blot in kidney cortex (D).The corresponding histograms represent the intensity quantification measured by imageJ software (n=4 per group). The values are the means \pm SE. *P<0.05 vs controls and #P<0.05 vs untreated diabetic rats.

G. Modulation of VEGF-A protein expression by VEGF inhibitory drugs

VEGF-A expression was measured in the renal cortex of control, untreated diabetic and diabetic rats treated with either SU-5416 or VEGF neutralizing Ab. In agreement with the previous studies, VEGF-A immunostaining was significantly increased in diabetic rats (**Figure 9**). Interestingly and upon treatment with SU-5416, VEGF-A expression was doubled ($p=0.06$). These obtained results suggest that VEGF-A expression was stimulated as a compensatory effect to overcome the inhibition of VEGF signaling. On the other hand, VEGF-A protein expression was significantly reduced in diabetic animals treated with anti-VEGF.

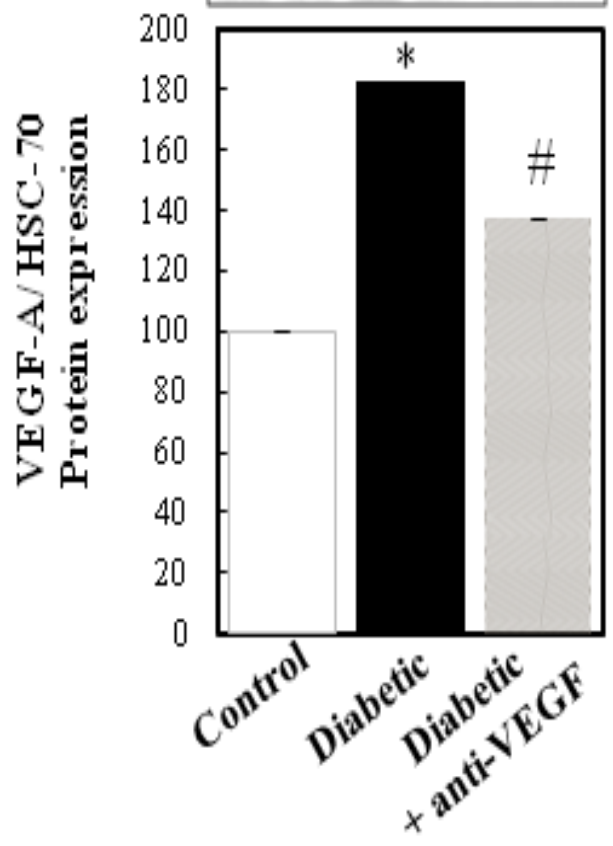
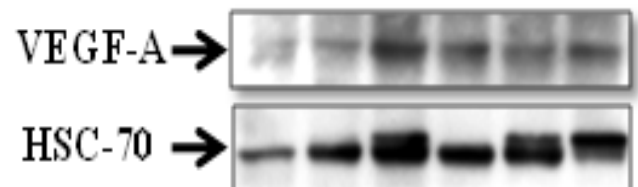
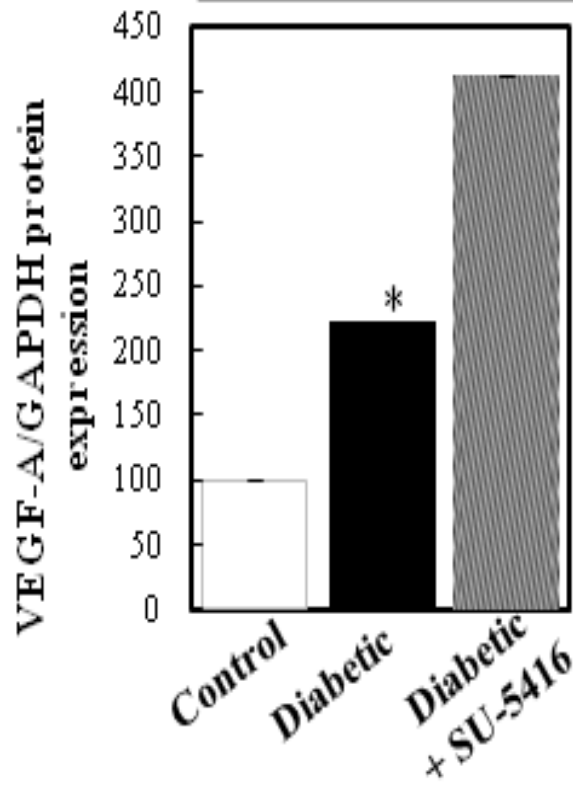
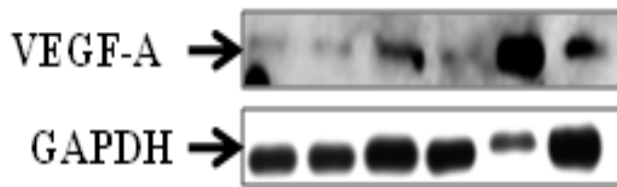
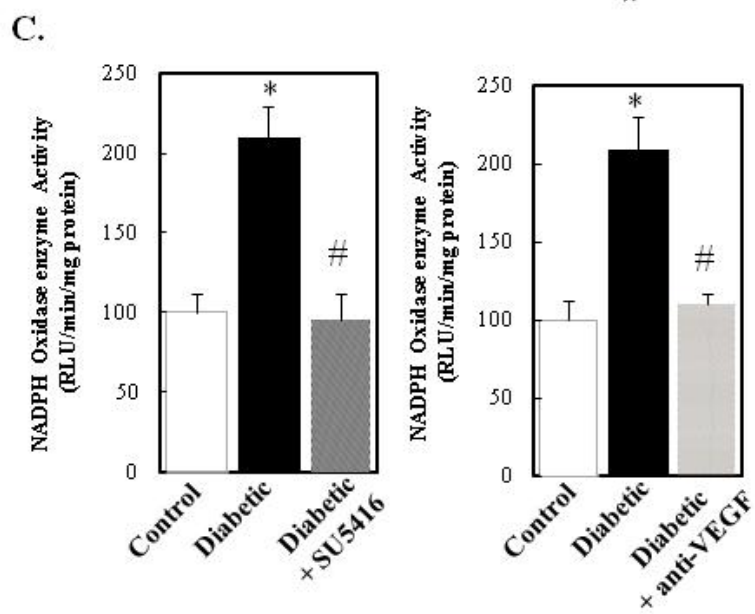
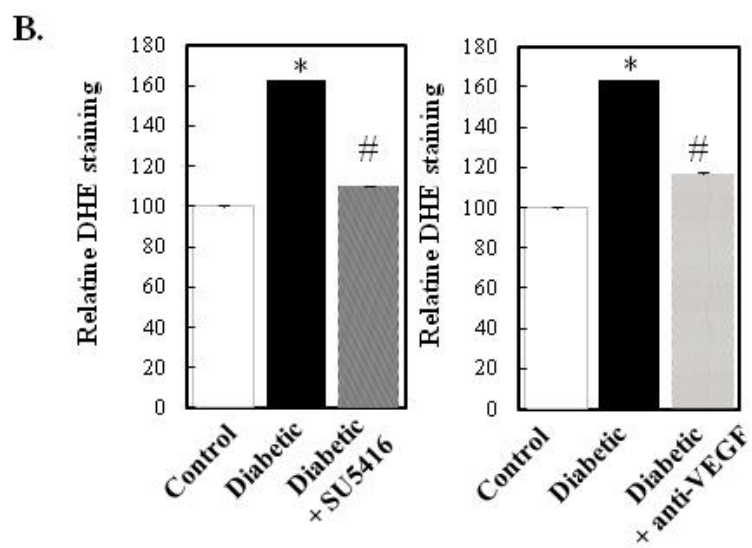
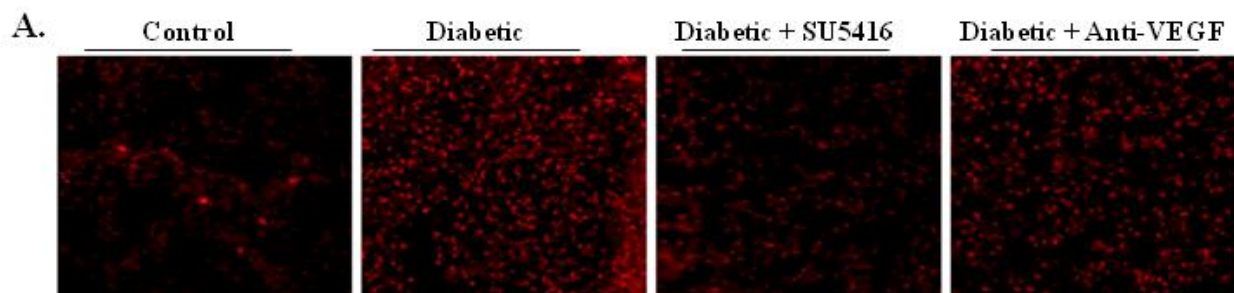


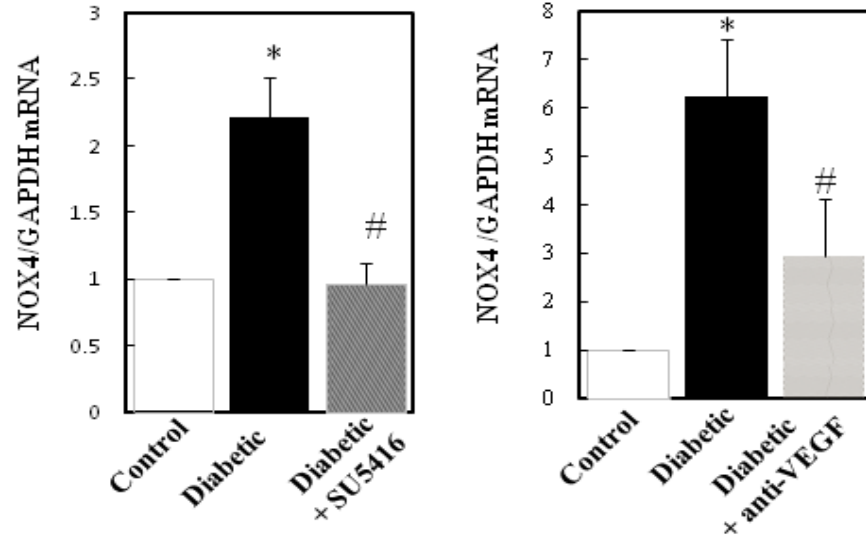
Figure 9: Modulation of VEGF-A protein expression by VEGF inhibitory drugs. VEGF-A protein expression was measured in the kidney cortex of control, untreated diabetic and diabetic rats treated with either 3mg/ml of SU-5416 or 5mg/ml neutralizing anti-VEGF. The corresponding histogram represents the intensity band quantification measured by imageJ software (B). The values are the means \pm SE. *P<0.05 vs. controls and #P<0.05 vs. untreated diabetic rats (n=4 per group).

H. HG-induced VEGF-A enhances ROS generation and up regulates NOX4 expression and activity in Type 1 diabetes

To further investigate if VEGF mediates the effect of hyperglycemia in inducing oxidative stress *in-vivo*. ROS generation was detected using (DHE) stain (**Figure 10A and B**). The obtained results show a significant increase in ROS production in diabetic rats. Pharmacological blockade of VEGF signaling was able to reverse this effect up to normal levels. Similar to our previous findings, NADPH oxidase enzyme activity was enhanced in Type 1 diabetic animals (**Figure 10C**) compared to controls. The enzymatic activity of NOXs was reduced upon VEGF inhibition. VEGF was also shown to regulate NOX4 gene and protein expression (**Figure 10D and E**, respectively). NOX4 expression was elevated in diabetic rats; this increase was reversed in diabetic rats treated with either SU5416 or anti-VEGF as compared to untreated diabetic rats.



D.



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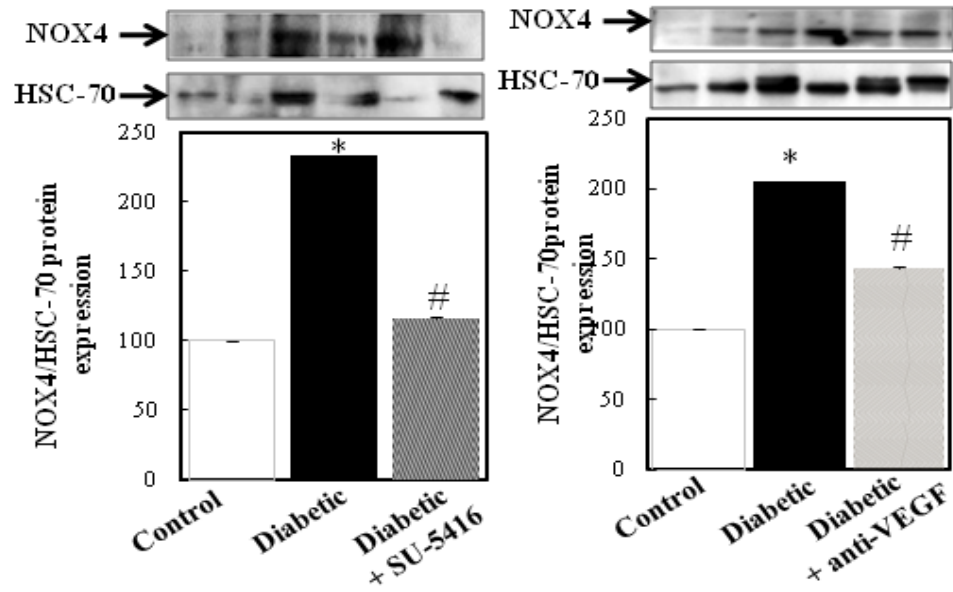
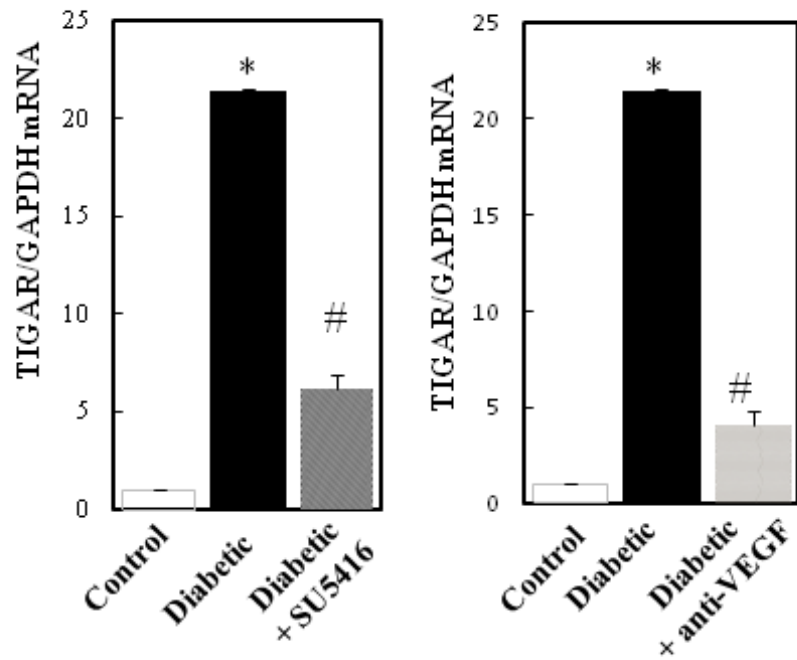


Figure 10: HG-induced VEGF-A enhances ROS generation and upregulates NOX4 expression and activity in Type 1 diabetes. ROS generation was detected using oxidant-sensitive fluorogenic probe dihydroethidium (DHE) stain. Fluorescence was measured at a wavelength of 560nm and the images were taken at 20x magnification lens from different fields. The figure represents fluorescent images of kidney cortex (A) and the corresponding histograms analysis represents the fluorescence intensity quantification of ROS (B). NADPH-dependent ROS generation in kidney cortex tissues was measured by lucigenin-enhanced chemiluminescence. Superoxide anion is expressed as relative chemiluminescent light units (RLU) per milligram of protein per minute (C). Nox4 mRNA levels were also assessed by real-time RT-PCR. The values represent the relative induction referred to GAPDH mRNA levels (D). NOX4 protein expression was measured using western blot and the corresponding histograms represent the intensity band quantification of the 4 groups normalized to HSC-70 expression (E). All values are the means \pm SE. *P<0.05 vs control; #P<0.05 vs. untreated diabetic rats.

I. VEGF-A regulates TIGAR expression in Type 1 diabetes

TIGAR expression was also assessed in STZ-induced Type 1 diabetic rats. TIGAR gene and protein expression was significantly increased in STZ-induced diabetic rats. These results are consistent with the obtained *in-vitro* findings (**Figure 11A and B**). TIGAR expression was reversed in diabetic rats treated with VEGF inhibitors suggesting that VEGF-A can regulate TIGAR expression in Type 1 diabetes.

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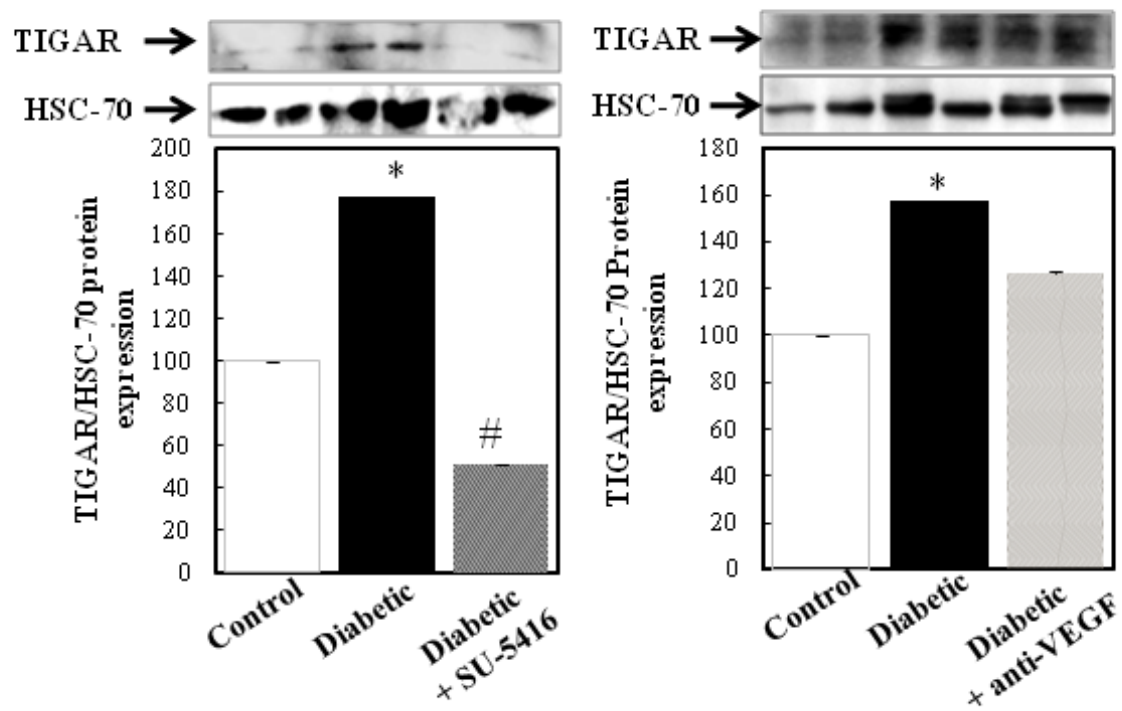
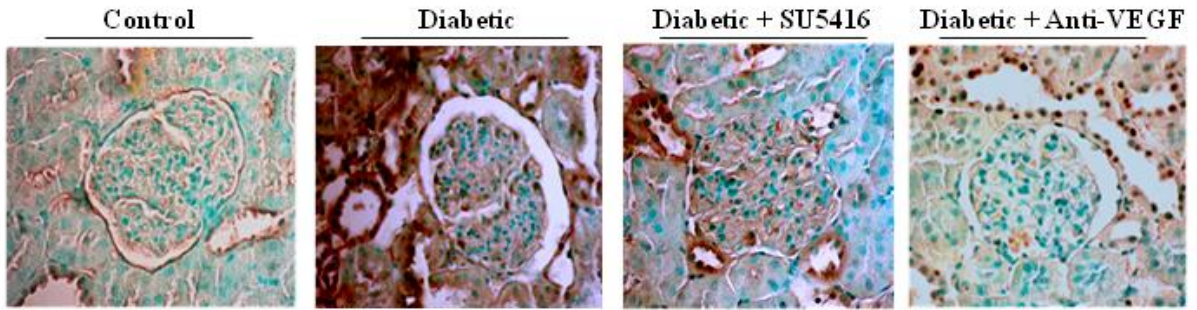


Figure 11: VEGF-A regulates TIGAR expression in Type 1 diabetes. TIGAR gene expression was measured in the kidney cortex of control, untreated diabetic and diabetic rats treated with VEGF inhibitors (n=3 per group) (A). The immunoblots represent TIGAR protein expression in kidney cortex and the corresponding histograms represent the intensity band quantification measured by imageJ software (B). The values are the means \pm SE. *P<0.05 vs. controls and #P<0.05 vs. untreated diabetic rats (n=4 per group).

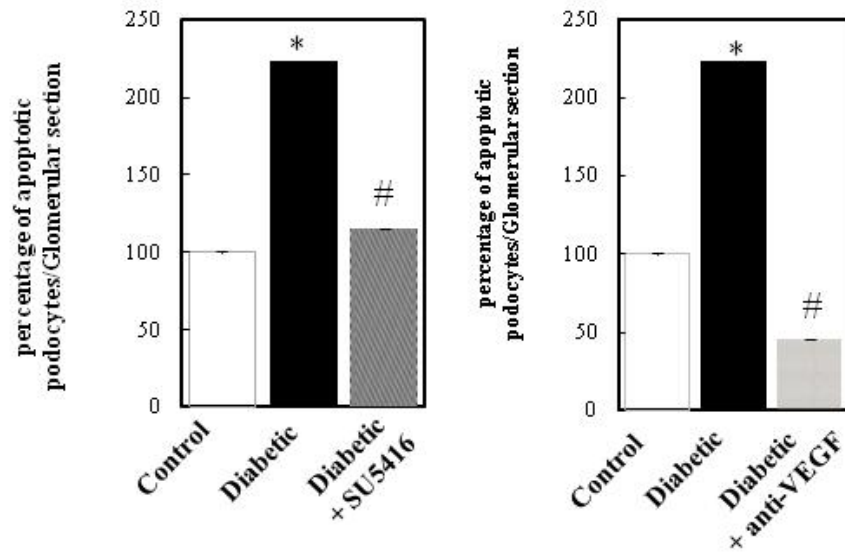
J. VEGF contributes to podocytes apoptosis and reduction in slit diaphragm proteins in Type 1 diabetes

Altered podocytes-derived VEGF-A expression contributes to podocytes apoptosis in diabetes as shown in (**Figure 12A and B**). Apoptotic podocytes number was significantly increased in diabetic animals; this increase was reversed upon blocking VEGF-A signaling. Moreover and as represented by the immunofluorescence photomicrographs, podocin protein expression was reduced in diabetic rats (red label) (**Figure 12C and D**); this reduction was associated with decrease in podocytes number per glomerular section. Blockade of VEGF-A overproduction was able to ameliorate podocin expression and preserve podocytes loss (**Figure 12C and E**).

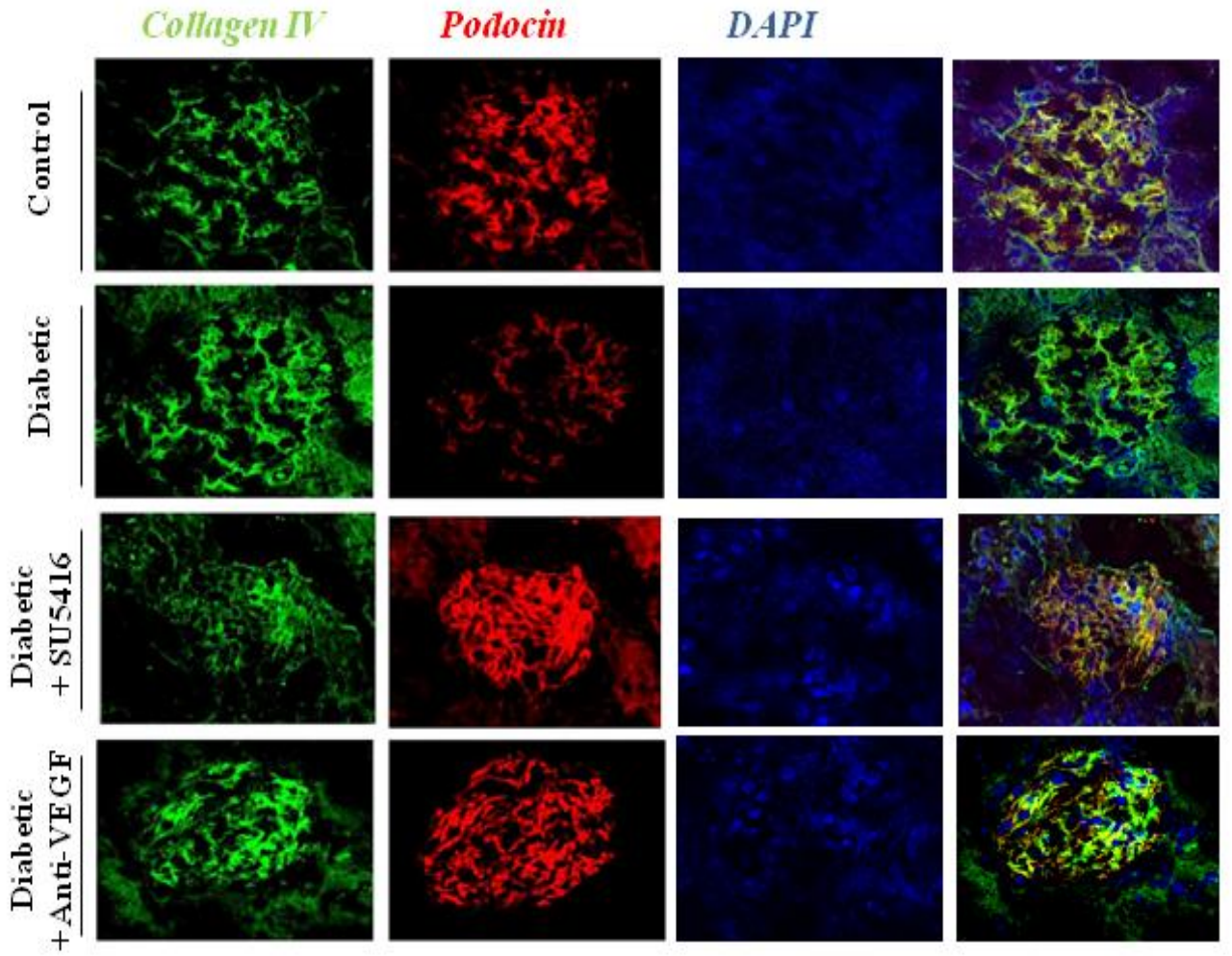
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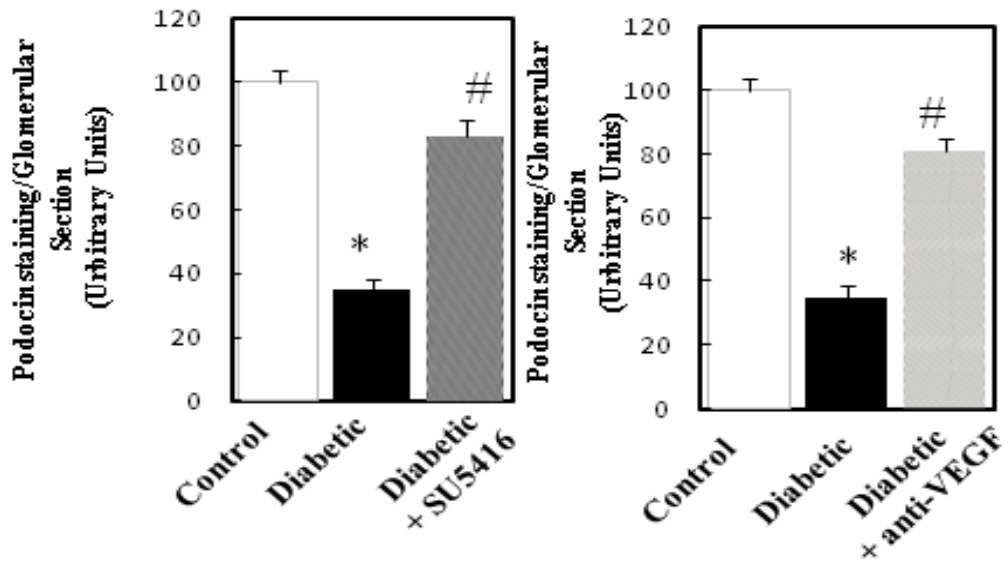
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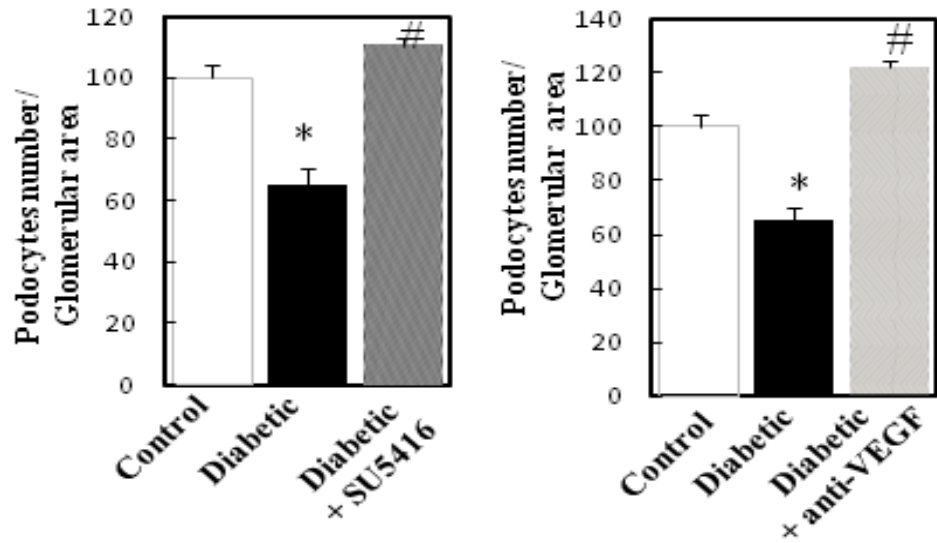


Figure 12: VEGF-A contributes to podocyte apoptosis and reduction in slit diaphragm protein expression in glomeruli of Type 1 diabetic rats. Podocytes apoptosis was measured in the control, untreated diabetic and diabetic rats treated with either SU-5416 or anti-VEGF using TACS TdT DAB assay. The apoptotic cells exhibit dark brown staining that is typically associated with cell condensation (A). The corresponding bar graphs represent the percentage of apoptotic cells per glomerular area using blinded observer evaluation of 12 randomly chosen glomeruli from 3 rat kidney cortices from each group (B). Representative dual-labeled immunofluorescence images of glomeruli stained with collagen IV (green), podocin (red), and 4',6-diamidino-2-phenylindole (blue) (C). podocin protein expression was decreased in diabetic rats and improved upon the treatment with both SU-5416 and anti-VEGF (D). Podocyte number per glomerular section was lower in diabetic rats, with a significant increase in podocyte number in diabetic rats treated with VEGF inhibitors ($n = 3$ per group) (E). Values are the means \pm SE. * $P < 0.05$ vs. control, # $P < 0.05$ vs. Untreated diabetic rats ($n = 3$ per group).

K. Blockade of VEGF-A signaling attenuates diabetes-induced albuminuria

Untreated rats, Diabetic and diabetic rats treated with either SU5416 or anti-VEGF were placed in metabolic cages for 24 h. Urine was collected and albumin levels were measured and expressed as milligrams of albumin per 24 h. As shown by the histogram (**Figure 13**), we have significant increase in a UAE in diabetic rats as compared to control. This increase was significantly attenuated to normal levels after blocking VEGF-A signaling. Thus, blocking VEGF-A attenuates albuminuria in type 1 diabetes.

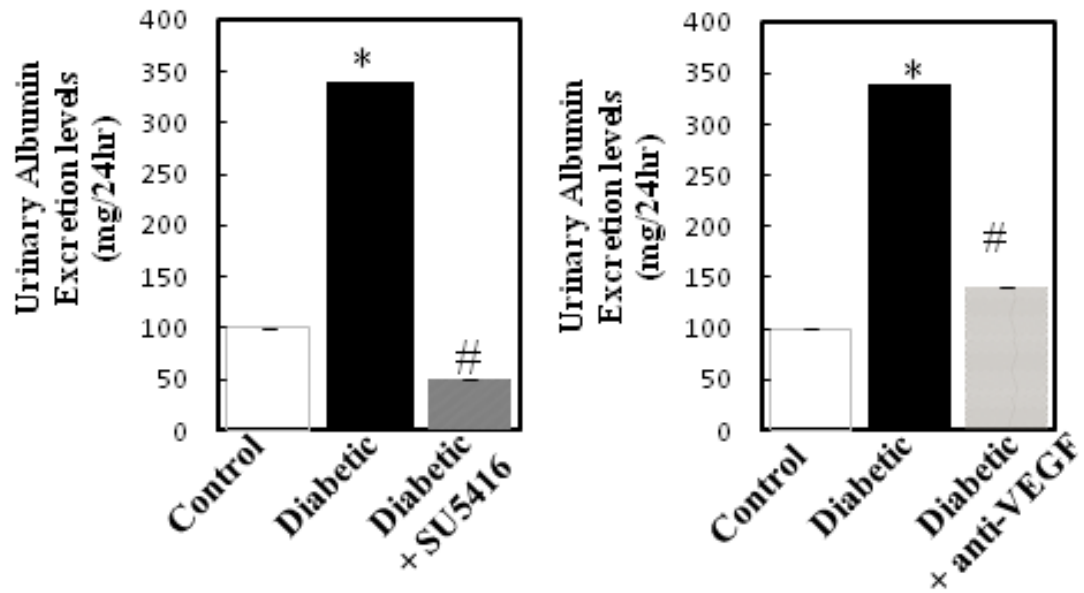


Figure 13: Blockade of VEGF-A signaling attenuates diabetes-induced albuminuria. Untreated rats, Diabetic and diabetic rats treated with SU-5416 or neutralizing anti-VEGF were placed in metabolic cages for 24 h. Urine was collected and albumin levels were measured and expressed as milligrams of albumin per 24 h. Values are the means \pm SE. * $P < 0.05$ vs. controls; # $P < 0.05$ vs. untreated diabetic rats ($n = 3$ per group).

CHAPTER V

DISCUSSION

Microalbuminuria is the earliest clinical manifestation of Diabetic Nephropathy (DN). We and others focused on podocytes studies, as key mediator in the initiation of diabetic kidney diseases (Wolf G et al., 2007). Podocytes injury is characterized by major phenotypic changes characterized by foot processes effacement, loss of slit diaphragm proteins, and reduction in podocytes number and density (Steffes MW et al., 2001; Pagtalunan ME et al., 1997) thus, leading to microalbuminuria (Ziyadeh FN et al., 2008; White KE et al., 2002). The role of glucose in inducing podocyte depletion has been well established (Eid AA et al., 2009; Eid AA et al., 2010; Bai X et al., 2014; Ma Y et al., 2016), however, the mechanistic pathway, likely suggested to be multi-factorial, remains to be elucidated. In this study, we show that diabetes/HG-induced podocyte phenotypic changes and injury is likely to be mediated through a VEGF-A dependent mechanism. VEGF exerts its effect by enhancing oxidative stress contingent with an increase in Nox4. Inhibition of VEGF prevents podocytes apoptosis *in-vitro* and ameliorates oxidative stress, podocytes loss, reduction of slit diaphragm proteins and ultimate albuminuria in type 1 diabetic rat model. Moreover, to our knowledge, we are the first to set the path of the involvement of TIGAR in DN.

The fact that chronic exposure to HG induces podocytes apoptosis is supported by a large body of evidence (Susztak K et al., 2006). Several studies tried to identify the mechanisms of action by which glucose induces podocyte injury, in which, podocytes

derived-VEGF has emerged as a major mediator in the pathogenesis of DN (Mima A et al., 2012). Experimental and clinical investigations have reported the association of early increase of VEGF-A expression with the onset of renal injury (Kim NH et al., 2000; Han SH et al., 2008; Kim NH et al., 2005), while others described the opposed effect (Sivaskandarajah GA et al., 2012; Sung SH et al., 2006). In this study, we investigate the potential deleterious role of VEGF-A expression in cultured rat podocytes and in animal models of diabetes. Our results show, that in the presence of HG, VEGF-A expression and release increase at 6h and this effect is sustained till 24h. These results are consistent with previous findings that show a rapid increase in VEGF-A protein levels observed at 6h and peaked at 24h, but not at 48h as compared to non-treated mouse podocytes (Han SH et al., 2008). More importantly, our results show that podocytes apoptosis is mediated through a VEGF-dependent mechanism and blockade of the VEGF- signaling using siRNA was able to reverse podocytes apoptosis.

Based on its original role as a potent inducer of vascular permeability (Senger DR et al., 1986), the concomitance of VEGF overproduction with proteinuria has been uncovered by a number of studies (Sung SH et al., 2006; Chen S et al., 2004; Iglesias-de la Cruz MC et al., 2002). There is controversial data reporting the critical role of VEGF in diabetic kidneys (Sivaskandarajah GA et al., 2012; Sung SH et al., 2006). Several genetic and pharmacological approaches of VEGF inhibition or over-expression have been developed to examine its crucial role in the development of diabetic kidney diseases. Veron D et al., have established a type 1 diabetic mice model that over expresses VEGF-A 164 in podocytes upon induction with doxycycline. This model has shown the local effect of VEGF-A signaling at glomerular filtration barrier microenvironment in causing nodular

glomerulosclerosis and massive proteinuria (Veron D et al., 2010). In addition to genetic blockers, the usage of neutralizing antibodies against VEGF has also been shown to ameliorate renal changes, attenuate UAE and hyperfiltration in experimental models of type 1 and type 2 diabetes (Flyvbjerg A et al., 2002; de Vriese AS et al., 2012). Sung SH et al., have demonstrated that the blockade of VEGF signaling using tyrosine kinase inhibitor reduces albuminuria in db/db mouse model (Sung SH et al., 2006). All these findings reveal that the increased VEGF expression exacerbates the early progression of diabetic glomerular injury. In contrast, Sivaskandarajah G et al., described a new genetic approach that permits specific deletion of VEGF-A 164 from podocytes of STZ-induced diabetic mice, suggesting the renoprotective role of elevated-VEGF A on microvasculature and its contribution to proteinuria and podocytes apoptosis (Sivaskandarajah GA et al., 2012).

For this purpose, STZ- induced type 1 diabetic rats were treated with two different pharmacological inhibitors that block VEGF signaling. SU5416 is selective VEGFR inhibitor that has been shown to have good efficacy without measurable toxicity on animal treatment (Fong TA et al., 1999), and anti-VEGF (bevacizumab or Avastin) is neutralizing monoclonal anti-body that is widely used for treatment of diabetic retinopathy and several malignancies. Predictably, our results demonstrate that VEGF expression was enhanced in renal cortices of STZ-induced type 1 diabetic rats after 8 weeks of diabetes. However and unlike previous results that show no significant difference in glomerular VEGF-A expression in SU-5416-treated mice as compared to diabetic rats (Sung SH et al., 2006), VEGF-A immunostaining was doubled upon blockade of VEGF signaling using SU-5416. One possible explanation that VEGF-A

expression can be stimulated as compensatory effect to overcome VEGF-A signaling inhibition especially that its expression is sustained by different stimuli that might be acting in a positive feedback fashion (Iglesias-de la Cruz MC et al., 2002; Chen S et al., 2004). In parallel, the treatment with anti- VEGF was able to sequester VEGF-A and significantly decrease its expression in the renal tissues. In addition, we also show the implication of VEGF-A in diabetic renal injury as measured by the renal structural and functional parameters in STZ-induced diabetic animals. VEGF-A over expression exacerbates matrix expansion and glomerular injury. VEGF-A enhances the expression of Collagen IV expression, a major component of the GBM as compared to diabetic rats. The blockade of VEGF-A was able to reverse this effect. In contrast with the study which reported that VEGF-A acts only in a paracrine loop (Sison K et al., 2010), our data show that podocytes are the key target for VEGF-A function. Consistently with our *invitro* findings, inhibiting VEGF was able to reduce podocytes apoptosis, improve podocin levels, preserve podocytes number and reverse albuminuria in type 1 diabetic animals. These observations prove the deleterious role of VEGF-A and confirm its plausible autocrine mode of action.

Oxidative stress is one of the critical pathogenic factors involved in the development of DN (Forbes JM et al., 2008; Brownlee M. et al., 2005). Several attempts have been made to use antioxidant in clinical trial as potential therapy for the treatment of diabetic patients with DN, however the results were disappointing (de Zeeuw D et al., 2013). We and others have established that ROS produced by NADPH oxidases, mainly NOX4, is involved in podocytes injury in early diabetic stage (Gorin Y et al., 2005). Data published by our laboratory have shown that inhibiting NOX4 activity using an adenovirus vector

encoding a dominant negative form of the enzyme (AddN-NOX4) has significantly reduced the apoptotic effect induced by HG on mouse podocytes (Eid AA et al., 2010). In the present study, we demonstrated that VEGF-A mediates the effect of HG on regulating NOX4 activity and expression. Podocytes exposure to either glucose or exogenous VEGF-A enhanced NADPH oxidases activity and NOX4 expression. Inhibition of VEGF-A using siRNA *in-vitro* significantly attenuates NOX4 activity and expression as well as ROS generation in cultured cells. Similar results were obtained in type 1 diabetic animals where both SU5416 compound and anti-VEGF were able to significantly reduce HG-augmented ROS production and NOX4 expression. Moreover, several studies have documented that oxidative stress regulates VEGF-A expression in renal tissues. Inhibiting NOXs using gene-targeting or pharmacological approaches reduced renal VEGF-A expression, serum and urinary concentration (Thallas-Bonke V et al., 2007; Thallas-Bonke V et al., 2010; Nam SM et al., 2009). Jha JC et al., have shown that blocking NOX4 using GKT137831, a NOX1/4 inhibitor was able to reduce VEGF-A expression in glomeruli of diabetic ApoE^{-/-} mice (Jha JC et al., 2014). The present study provides the first evidence that VEGF-A is acting upstream NOX4 and its inhibition prevents oxidative stress- induced podocytes apoptosis. These results contrast sharply the generally accepted view on VEGF-A role as a downstream mediator rather than being a keystone in the initiation and progression of the disease.

A still-growing surge of research has linked the contribution of TIGAR to several malignancies and diseases (Kim J et al., 2015; Dodson M et al., 2013). TIGAR primarily functions as a regulator of glucose breakdown in the cells. When TIGAR is activated, it acts as fructose-2-6-bisphosphatase to block glycolysis and shift the pathway toward PPP.

This allows the production of NADPH and serves the anti-oxidant function of TIGAR (Bensaad K et al., 2006). Many findings reported the increase of TIGAR expression under several mild and severe stress signals such as ischemia and tumorigenesis (Eleftheriadis T et al., 2016, Hoshino A et al., 2012, Ye L et al., 2013). However and to our knowledge, there is no existing empirical study that addresses TIGAR expression and function in diabetic kidney. In this study, we demonstrated for the first time that the exposure of cultured podocytes and renal cortical tissues to hyperglycemia increases TIGAR expression. TIGAR protein level was double in HG-treated podocytes as compared to untreated cells that also express a basal level of TIGAR. These results are in contrary with a previous study (Kim J et al., 2015) showing that TIGAR is only detected in proximal tubular cells and absent in other renal cells. Moreover, we also showed that VEGF-A mediates the effect of HG in altering TIGAR expression. Genetic and pharmacological blocking of VEGF-A signaling was able to dramatically attenuate TIGAR levels.

Furthermore, P53 is a transcription factor that is known to control different cellular responses including cell cycle arrest, senescence and apoptosis. Our laboratory has previously indicated that HG induces podocytes apoptosis through the activation of pro-apoptotic p53 protein and that p53 acts downstream NOX4 (Eid AA et al., 2010). Although its effect appears to be highly controversial, TIGAR exerts part of its activity through P53-dependent manner (Bensaad K et al., 2006). TIGAR plays a protective role on DNA damage-induced apoptosis in several malignancies such as hepatocellular carcinoma and osteosarcoma cells (L Ye et al., 2013; Bensaad K et al., 2006). Under hypoxic conditions, inhibition of p53-induced TIGAR significantly reduced apoptosis in

ischemic myocardium (Hoshino A et al., 2012; Kimata M et al., 2010). Opposing results were obtained in ischemic kidney and brain (Li M et al., 2014; Kim J et al., 2015). Interestingly, increasing TIGAR expression abrogate ROS production and reduces apoptosis in mild hypoxic renal injury, however and under severe stress, sustained increase in TIGAR expression didn't affect elevated ROS production, and wasn't able to shift the glycolytic pathway to PPP (Kim J et al., 2015). In this report, we show that the increase of TIGAR expression was concomitant with increased ROS generation and podocytes apoptosis. Our data suggests that TIGAR might have renoprotective role as endogenous anti-oxidant enzyme in which its expression is increased to overcome NADPH oxidases-induced oxidative stress, but it couldn't reverse the paralleled increase in ROS generation. However, further investigations are still greatly warranted to precise the role of TIGAR in renal diabetic context.

In conclusion, we have proved that VEGF-A over production in diabetes has a detrimental effect on podocytes. It causes podocytes apoptosis and loss, reduction in slit diaphragm proteins and albuminuria. VEGF-A enhances ROS generation through the activation of NOX4. We also address for the first time the role of VEGF-A in regulating TIGAR expression in cultured podocytes and type 1 diabetic rat model. Our results suggest that VEGF-A is a major pathophysiological factor and its blockade can provide important therapeutic potential for the treatment of DN.

CHAPTER VI

FUTURE DIRECTIONS

This work has set the stage for future work to straighten our findings. The discovery of altered VEGF-A role as a keystone for the induction of oxidative stress in the early development of DN is a crucial step to further progress in this research. Data published by our laboratory have shed light on the importance of Cytochrome P450 derived-eicosinoids as a major source of ROS in kidneys. Altered cytochrome p450 expressions and their metabolites production enhance Noxs expressions and exacerbate renal injury in diabetes. It would be of great interest to investigate the cross talk between VEGF-A and Cytochrome p450 derived- eicosinoids on podocytes injury and albuminuria.

In addition, we are the first to show that TIGAR expression is up regulated in diabetic podocytes through VEGF-A- dependent pathway. Further investigations are still strongly warranted to elucidate its critical function. For the purpose, blocking TIGAR expression using small interfering RNA and evaluating its biological output can help gain new insights into its role on cultured podocytes in hyperglycemic ambience. As an anti-oxidant and p53-inducible gene, this can also provide evidence about the mechanistic pathway by which it exerts its effect and how it is correlated with Noxs alteration.

CHAPTER VII

ANNEX A

mTORC2 Signaling Regulates Nox4-Induced Podocyte Depletion in Diabetes

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Aim: Podocyte apoptosis is a critical mechanism for excessive loss of urinary albumin that eventuates in kidney fibrosis. Oxidative stress plays a critical role in hyperglycemia-induced glomerular injury. We explored the hypothesis that mammalian target of rapamycin complex 2 (mTORC2) mediates podocyte injury in diabetes. **Results:** High glucose (HG)-induced podocyte injury reflected by alterations in the slit diaphragm protein podocin and podocyte depletion/apoptosis. This was paralleled by activation of the Rictor/mTORC2/Akt pathway. HG also increased the levels of Nox4 and NADPH oxidase activity. Inhibition of mTORC2 using small interfering RNA (siRNA)-targeting Rictor *in vitro* decreased HG-induced Nox1 and Nox4, NADPH oxidase activity, restored podocin levels, and reduced podocyte depletion/apoptosis. Inhibition of mTORC2 had no effect on mammalian target of rapamycin complex 1 (mTORC1) activation, described by our group to be increased in diabetes, suggesting that the mTORC2 activation by HG could mediate podocyte injury independently of mTORC1. In isolated glomeruli of OVE26 mice, there was a similar activation of the Rictor/mTORC2/Akt signaling pathway with increase in Nox4 and NADPH oxidase activity. Inhibition of mTORC2 using antisense oligonucleotides targeting Rictor restored podocin levels, reduced podocyte depletion/apoptosis, and attenuated glomerular injury and albuminuria. **Innovation:** Our data provide evidence for a novel function of mTORC2 in NADPH oxidase-derived reactive oxygen species generation and podocyte apoptosis that contributes to urinary albumin excretion in type 1 diabetes. **Conclusion:** mTORC2 and/or NADPH oxidase inhibition may represent a therapeutic modality for diabetic kidney disease. *Antioxid. Redox Signal.* 25, 703–719.

Keywords: diabetic nephropathy, reactive oxygen species, NADPH oxidases, mTORC2

ANNEX B

Mechanism of Diabetic Nephropathy: Role of VEGF-A

Kawthar Braysh, Fuad Ziyadeh, Abdu Jurjus, Assaad A. Eid

Manuscript in preparation

Microalbuminuria is the earliest clinical sign to occur in diabetic nephropathy. Podocytes-derived Vascular Endothelial Growth Factor (VEGF-A) is a potent microvascular permeability factor that has a major role in regulating renal hemodynamics. In diabetes, early increase of glomerular VEGF-A expression is associated with the onset of renal injury, however its critical role in the disease progression is still greatly controversial. In this study, we postulated that VEGF-A mediates HG effect on podocytes injury through the involvement of NADPH oxidases. **Methods:** Conditionally immortalized rat podocytes and STZ-induced type 1 diabetic rat model were used in this study. Si-VEGF-A transfected cells were treated with either 25mM glucose or 30ng/ml exogenous rat VEGF-A 164. Diabetic rats were injected with either SU-5416 or anti-VEGF neutralizing antibody to block VEGF-A signaling. Apoptosis, proteinuria, ROS production, gene and protein expression as well as enzymatic activities were assessed in cultured podocytes and renal cortices. **Results:** Excessive VEGF-A expression and release induce oxidative stress and causes podocytes apoptosis in HG milieu. Blocking VEGF-A expression using siRNA ameliorates ROS production through decreasing NOX 4 expression and activity and prevents podocytes apoptosis. VEGF-A expression was also enhanced in renal cortices of STZ-induced type diabetic animals. This increase was concomitant the up regulation of oxidative stress, loss of podocytes, reduction in slit diaphragm proteins and ultimate microalbuminuria. Inhibiting VEGF-A signaling using two different inhibitory drugs (SU-5416 and anti-VEGF) was able to decrease NOX4 expression and thereby attenuate ROS production. It also reduced podocytes apoptosis, restored podocin levels and improved urinary albumin loss. We also address for the first time that VEGF-A regulates TIGAR expression in cultured podocytes and type 1 diabetic rat model. **Conclusion:** Inhibiting VEGF-A signaling using genetic and pharmacological approaches proves the detrimental role of VEGF-A on glomerular injury and confirm its plausible autocrine mode of action.

Keywords: VEGF-A, reactive oxygen species, NADPH oxidase 4, TIGAR, apoptosis and diabetic nephropathy

ANNEX C

The crosstalk between VEGF-A and Eicosanoids in Diabetic Nephropathy

Kawthar Braysh, Fuad Ziyadeh, Abdu Jurjus, Assaad A. Eid

Manuscript in preparation

Oxidative stress is one of the critical pathogenic factors involved in the development of DN. CYP450s are a significant source of ROS in kidneys. CYP450 metabolizes arachidonic acid (AA) into hydroxyeicosatetraenoic acids (20-HETEs) and epoxyeicosatrienoic acids (EETs). EETs are produced by CYP 2B, 2C and 2J and are known as potent regulators of renal hemodynamics. We have previously shown that the alteration of CYP2C expression in diabetic kidneys is involved in proximal tubular cells injury. **Aim:** In this study, we explored the cross talk between VEGF-A and altered CYP2C expression in diabetic glomerular injury. **Methods:** Streptozotocin (STZ)-induced type 1 diabetic rats were treated with s-EH inhibitor (AUDA) for 5 weeks. Functional, histological and biochemical parameters were measured. Histochemical staining analysis was used to measure morphological and structural alterations. Dihydroethidium (DHE) was used to assess intracellular ROS production levels. Western blot and PCR were performed to study the protein and gene expression, respectively. **Results:** The accumulation of EET by using a selective s-EH inhibitor (AUDA) was shown to have a protective role in diabetic kidneys through the attenuation of glomerular hypertrophy and fibrosis. This reduction was associated with the decrease of ROS generation as well as NADPH oxidases expression and activity. CYP2C was also shown to modulate VEGF-A expression in the renal glomerulus. **Conclusion:** Our results show that increased podocytes-VEGF-A expression is tightly correlated with the alteration of CYP2C expression suggesting that the VEGF-A blockade could have an important therapeutic potential in the treatment of diabetic nephropathy.

Keywords: Diabetic nephropathy, VEGF-A, CYP2C, EETs, reactive oxygen species, NADPH oxidases

CHAPTER VIII

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