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LXR/mTOR/NOX4 SIGNALING AXES: NOVEL THERAPEUTIC TARGETS IN DIABETIC NEPHROPATHY

by

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AN ABSTRACT OF THE THESIS OF

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Background. Podocyte injury has been shown to play a major role in the initiation of Diabetic Nephropathy (DN). The molecular mechanism by which hyperglycemia/diabetes induces podocyte injury is suggested to be multi-factorial and not well defined. Recent research suggests that defective autophagy may play a role in podocyte dysfunction during the onset and development of diabetes. Furthermore, our lab has previously described the importance of Nox4 in the progression of podocyte injury. However, a mechanistic link between NADPH oxidases (Nox)-induced ROS production and the alteration in autophagy has not yet been elucidated. Moreover, the Liver-X-receptor and the mTOR pathways have been recently shown to be associated with autophagic and oxidative stress responses. In this study, we aim to investigate the role of the Nox4/LXR/mTOR axis on autophagy and their possible link to podocyte depletion and injury.

Methods. Both in vitro and in vivo models were used in this study. A conditionally immortalized human podocyte cell line was used for our *in vitro* studies. High-fat diet/low-dose streptozotocin-induced type 2 diabetic mice were used for the *in vivo* studies, as well as high-dose streptozotocin-induced type 1 diabetic mice. Pharmacological means were utilized to alter the expression of NADPH oxidases (GKT), LXR (T0) and the mTOR (Rapamycin) signaling pathways, while podocyte depletion/loss, autophagy alteration and glomerular injury were assessed as the corresponding biological output via Western Blotting, PCR and immunohistological assessments.

Results. Our results reveal that high glucose HG/hyperglycemia induces defective autophagy in both podocytes and isolated glomeruli of type 2 diabetic mice. HG/hyperglycemia reduces LXR mRNA levels and protein expression and activates the mTORC1 pathway. These effects were mediated by an increase in Nox4 protein expression and NADPH oxidase activity, triggering ROS production. Activation of the LXR pathway was shown to decrease diabetes-induced Nox4 expression and activity, and reduce mTORC1 activation, and restore autophagy protein levels. In parallel, inactivation of the mTORC1 pathway using low-dose rapamycin mimicked the effect of LXR activation on ROS production and podocytes injury but did not alter the LXR pathway.

Conclusion. Collectively, our results display the role of Nox4 in autophagy, whereby Nox4 inhibition restored autophagy protein levels and ameliorated podocytes and glomerular injury. These findings highlight a novel signaling axis, the LXR/mTOR axis, which modulates ROS generation and autophagy deregulation in diabetic nephropathy. Thus, LXR activation, mTOR and/or NADPH oxidase inhibition represent a promising therapeutic modality for Diabetic Kidney Disease.

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LIST OF ABBREVIATIONS

4E-BP1:4E-binding protein 1

AngII: Angiotensin II

ATG: Autophagy Related gene

DN: Diabetic nephropathy

GBM: glomerular basement membranes

GFB: glomerular filtration barrier

HG: High Glucose

LXR: Liver- X -receptor

mTOR: mammalian target of Rapamycin

Nox: NADPH oxidase

Rapa: Rapamycin

ROS: Reactive Oxygen Species

SD: slit-diaphragm

T1DM: Type 1 Diabetes Mellitus

T2DM: Type 2 Diabetes Mellitus

TGF-β: Tumor growth factor beta

CHAPTER I

INTRODUCTION

Diabetes Mellitus mostly referred to as Diabetes is a serious and complex metabolic disorder that is clinically manifested by persistent high blood glucose levels. Diabetes is characterized by the inability of the body to produce (Type I) or respond (Type II) to insulin which results in impaired carbohydrate metabolism and hyperglycemia. Hyperglycemia is implicated in increased risk of macrovascular (atherosclerosis) and microvascular complications (retinopathy, nephropathy, and neuropathy), leading to a substantial increase in morbidity and mortality among diabetic patients. Diabetic nephropathy (DN) is a prevalent complication in diabetic patients. It is estimated that approximately 30-40% of diabetic subjects will develop DN through the course of their disease (Saha et al., 2010). DN is currently the primary cause of end-stage renal disease, accounting for approximately 60% of individuals requiring dialysis or renal transplantation in the United States (Foley et al., 2009). Diabetic Nephropathy is multifactorial in nature including both modifiable and nonmodifiable risks. Modifiable risks mainly include hyperglycemia, hypertension, dyslipidemia, and recently smoking (Ausdiab et al., 2004; Scott et al., 2001). On the other hand, non-modifiable risks include but not limited to age, ethnicity, race, and genetic polymorphism (Seaquist et al. 1989).

A. Diabetic Nephropathy Pathogenesis

DN is characterized by structural and functional changes. Structural changes involve glomerular hypertrophy, mesangial matrix expansion, glomerular basement membrane thickening, podocyte loss, tubulointerstitial fibrosis and characteristically, nodular glomerulosclerosis (Kimmelstiel–Wilson nodules) (Mauer, 1994). These structural alterations, in turn, lead to functional changes and vice versa. In early DN there is hyperfiltration and increase albumin excretion, as DN advances, glomerular filtration rate decreases and proteinuria develops.

The kidney glomerulus is composed of three cell types known to be involved in DN: mesangial, endothelial, and podocyte. Early research focused on mesangial cells because of the phenomenon of mesangial cell expansion and it was believed that podocytes tend to be injured in the latest stages. However, in 1997 Pagtalunan *et al*. showed a landmark observation related to the role of podocyte in DN. The study revealed that reduction in podocyte number is strongly associated with albuminuria and loss of GFR in Pima Indians with T2DM (Pagtalunan et al., 1997). Further studies also confirmed the correlation between proteinuria, glomerulosclerosis, and podocyte loss, showing that podocyte loss is possibly a key factor in DN pathogenesis (Lovato et al. 2013).

Podocytes are glomerular epithelial cells that are terminally differentiated consisting of a cell body, primary process, and a branching foot process encircling glomerular capillaries. Podocyte foot processes interdigitate through their basolateral region with adjacent foot processes to form evenly spaced areas covered by slit-diaphragm (SD) proteins (Maezawa et al., 2015). SD is a main component of the glomerular filtration barrier (GFB)

along with fenestrated endothelial cells and the glomerular basement membranes (GBM). GFB is freely permeable to water and selectively permeable to other molecules depending on size and charge. Any change in the milieu, stress or injury can induce rearrangement in the podocyte cytoskeleton manifested by foot-process flattening, widening, and retraction also known as the effacement phenomenon (Mundel et al., 2002). Podocyte injury will, in turn, lead to a disturbance in the GFB, weakening its integrity and causing albuminuria.

B. Hyperglycemia and Podocyte

1. Podocyte Hypertrophy

Several studies have established that the development of DN is associated with glomerular podocyte hypertrophy, however, the pathophysiology of podocyte hypertrophy is still unclear (Herbach et al., 2009; Lin et al., 2016). In order to compensate for the dilation in the GMB, podocyte- unable to divide- should expand to cover the denuded area of the GMB (Gödel et al., 2011). One of the culprits is Angiotensin II (AngII). AngII upregulates the protein expression of the parathyroid hormone-related protein (PTHrP), and cell cycle regulatory protein-p27Kip via TGF-β1, promoting the exacerbation of podocyte hypertrophy under hyperglycemia (Romero et al., 2010). Another way by which hyperglycemia induce podocyte hypertrophy is through activation of mammalian target of rapamycin (mTOR) (Lu et al., 2011). Activation of mTORC1 has shown to increase the expression of CKIs causing podocyte cycle arrest and hypertrophy (N.-H. Kim et al., 2006).

2. Podocyte Epithelial-Mesenchymal Transition

Podocytes have displayed a conversion in phenotype in several nephropathies (Y. Liu, 2004). When Epithelial-Mesenchymal Transition (EMT) occurs the cells lose their original features leading to the disappearance of cell contact, impaired cell polarity, and exhibited characteristics of the mesenchymal markers, such as vimentin, α-smooth muscle actin (α-SMA), and fibroblast-specific protein 1 (FSP1) (Lu et al., 2011). TGF-β activation causes a decrease in epithelial biomarkers such as nephrin, ZO-1, P-cadherin, while upregulation of mesenchymal proteins desmin, matrix metalloproteinase-9, FSP-1 and fibronectin (Li et al., 2008). In another study, it was found that 86% of podocyte found in urine sediments expressed FSP-1, and that level of FSP-1 in kidney specimens was correlated with podocyte loss, proteinuria and severity of DN (Yamaguchi et al., 2009)

3. Podocyte Detachment

Podocytes are attached to the GMB through α3β1 integrin which could be affected by the decrease in either the protein expression of integrin or surface negative charge, cytoskeleton rearrangement, and deglycosylation under Hyperglycemia (Shankland, 2006). Mechanical stress and TGF-β also contribute to the decrease in the expression of α3β1 (Dessapt et al., 2009). A study performed on the urine of 50 patients with type 2 diabetes showed that podocytes were present in the urine of 53% patients with microalbuminuria and 80% with macroalbuminuria, while no podocytes were detected in healthy control, diabetic patients with normoalbuminuric and diabetic patients with chronic renal failure (Nakamura et al., 2000). This suggests that podocyte in urine could be an early marker of DN.

4. Podocyte Apoptosis

In the body, there must be a dynamic equilibrium between proapoptotic and anti-apoptotic signal pathways to maintain the integrity of the internal environment. Podocyte apoptosis contributes to the pathogenesis of DN and is multifactorial (Dai et al., 2017). Concomitantly, Hyperglycemia, AngII, oxidative stress, TGF-β, and AGEs activate apoptotic pathways in podocyte (Dai et al., 2017). Reactive oxygen species (ROS) under hyperglycemia activate both p38MAPK and caspase-3 both *in vivo* and *in vitro* causing proteinuria (Susztak et al., 2006). Other than ROS, AGEs can also activate p38MAPK signal pathway leading to the activation of FOXO4 and apoptosis (Chuang et al., 2007). We have shown before that ROS generation by CYP4A monooxygenases, 20-HETE, and NADPH-oxidases is implicated in podocyte apoptosis both *in vitro* and *in vivo* (A. A. Eid et al., 2009). Recently, Notch1 signal-dependent activation of p53 has shown to be involved in podocyte apoptosis in DN (Niranjan et al., 2008).

C. Oxidative stress and Diabetic Nephropathy

Reactive oxygen species (ROS) are a family of molecules including molecular oxygen and its derivatives, superoxide anion (O_2^-), peroxynitrite ($ONOO^-$), hydroxyl radical (HO^-), hypochlorous acid (HOCl), nitric oxide (NO), hydrogen peroxide (H_2O_2), and lipid radicals. ROS is also referred to as a free radical since many of the ROS have unpaired electrons. ROS is the final common pathway of various signaling pathways involved in the pathogenesis of both macro and micro- complications (Forbes et al., 2008). ROS oxidizes biological molecules like proteins, carbohydrates, lipid, and most importantly DNA in a

phenomenon known as oxidative stress (Brownlee, 2001). As a consequence, ROS generation can eventually lead to endothelial dysfunction, inflammation, fibrosis among others (Jha et al., 2016). Potential sources of ROS include NO synthase and certain other hemoproteins, xanthine oxidase, mitochondrial respiratory chain, and NADH/NADPH oxidases (Forbes et al., 2008). Recently, among all the possible sources of ROS, NADPH oxidases (Noxs) appear to be one of the chief contributors in DN pathogenesis (Jha et al., 2016).

Till today, seven known isoforms of Nox have been identified: Nox1-5 and Double oxidases Duox 1&2. All Noxs have six conserved transmembrane domains, four conserved heme-binding histidine domains along with a NADPH binding site at the end of the C-terminus, and a flavin adenine dinucleotide (FAD) binding region in proximity of the C-terminal transmembrane domain. All Noxs transfer electrons from NADPH to reduce molecular oxygen to superoxide (O_2^-) across the biological membrane (Bedard et al., 2007).

Nox4 or renal Nox is the most studied Nox in the kidney and it is crucial for the pathogenesis of diabetic kidney disease. Numerous studies have supported the role of Nox-derived ROS in glomerular hypertrophy and mesangial cell expansion in DN (Gorin & Block 2009; Jha, Thallas-Bonke, et al. 2016). In response to high glucose HG, ROS levels were boosted in mesangial cells and were associated with an increase in Nox 4 expression. Eid *et al.* have previously shown that both mRNA and protein expression of Nox1 and Nox4 but not Nox2 are increased upon treating podocytes with HG (A. A. Eid et al., 2013). Moreover, Nox4-derived ROS leads to eNOS dysfunction in diabetes triggering fibrotic injury in mesangial cells. On the other hand, the blocking of NOX4-induced eNOS dysfunction by

the antioxidant sestrin 2-dependent AMPK showed an antifibrotic effect in mesangial cells subjected to HG (A. A Eid et al. 2013). Also, data from our lab described the involvement of the mTOR pathway in the apoptosis of podocytes via upregulation of Nox4 and Nox1 expression and NADPH oxidase activity in DN in Type I model increasing albumin excretion and kidney fibrosis. The same study showed that upon the inhibition of mTORC1 by Rapamycin there is a reverse in the changes observed where glomerular basement membrane thickening (GBM) decreases, foot process effacement is reduced, mesangial expansion is attenuated and albuminuria declines (A. A. Eid et al., 2013). In addition, inhibiting Nox 4 by antisense oligonucleotides to Nox4 (Gorin et al., 2005) or GKT137831 Nox1/4 inhibitor mitigated the diabetes-induced increased mesangial expansion, glomerulosclerosis, and buildup of ECM proteins similar to results obtained in NOX4 knockout type I diabetes model (J. C. Jha et al., 2014).

D. Liver-X-Receptor in Diabetic Nephropathy

The liver X receptors (LXRs) α and β are nuclear hormone receptors that act as a transcription factor and are encoded by *Nr1h3* and *Nr1h2* genes respectively. LXR natural ligands are oxysterols which are derivatives of cholesterol. Thus, LXR is referred to as intracellular 'cholesterol sensors'(Hyvönen et al., 2010). LXR-β is ubiquitously expressed, unlike LXR-α which is expressed mainly in highly metabolic tissues such as liver, macrophages, adipose tissues, intestine, spleen, heart, and kidney. When the ligand binds to LXR, it induces its heterodimerization with Retinoic-X-Receptor (RXR) that will bind to the DR-4 response element with the sequence 5′-GGTTTAAATAAGTTCA-3′ in the promoter of target genes (Hyvönen et al., 2010). LXR target genes include ATP-binding

cassette transporters A1, G1, G5, and G8 (ABCA1, ABCG5, ABCG8) which govern cholesterol efflux, cholesterol ester transport protein (CETP), fatty acid synthase (FAS), apolipoprotein E (Apo E), SREBP-1c, and lipoprotein lipase (LPL) proposing an important role of LXR in lipid and cholesterol metabolism (Steffensen et al., 2004). Interestingly, PPAR response element has been identified in the LXR gene promoter suggesting that PPAR through LXR pathway regulates ABCA1 expression (Ruan et al., 2003).

1.LXR and Renal Lipid Metabolism

Lxrα/β −/− mice showed a pathological renal profile with tenfold higher albumin to creatine ratio compared to Lxrα/β +/+ mice, and an increase in glomerular lipid accumulation and mesangial matrix expansion (Patel et al., 2014). These results correlate with the human pathological changes observed in DN because human biopsies from DN patients showed a significant decrease in the expression of LXRα and LXRβ and lipid accumulation mainly in glomeruli (Lee et al., 1998).

2. LXR role in inflammation and oxidative stress in DN

LXR has shown also to play important role in inflammation and development of DN. LXR activation decreases osteopontin level, expression of several inflammatory genes, such as TGFβ, tumor necrosis factor α (TNFα), monocyte chemoattractant protein-1 (MCP-1), and macrophage infiltration, preventing the development of albuminuria and other pathological aspects of DN (Tachibana et al., 2012). Moreover, recent studies have

implicated a role of LXR in ROS production. LXR alpha expression showed to suppress glycated or acetylated low-density lipoprotein-induced cytokines and ROS in macrophages ameliorating hyperlipidemic-hyperglycemic nephropathy (Kiss et al., 2013). Upon treatment with *N, N*-dimethyl-3beta-hydroxycholenamide (LXR activator) decreased the level of kidney inflammatory and oxidative stress markers were observed (Patel et al., 2014). However, more studies must tackle the interaction between LXR and oxidative stress in DN

E. mTOR signaling in Diabetic Nephropathy

The mammalian target of rapamycin (mTOR) signaling cascade is a preserved pathway found in all eukaryotes that plays a major role in cellular growth, survival, and metabolism. The mTOR catalytic subunit is a serine/threonine kinase that can exist in two forms: mTORC1 and mTORC2, which are generally distinguished by different sets of protein binding partners (Wullschleger et al., 2006). mTORC1, which is rapamycin sensitive along with its main component mTOR, mLST8, and rapamycin-sensitive adaptor protein of mTOR (RPTOR), mediate its effect through p70S6 kinase/S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1), inducing protein synthesis and cell growth (Um et al., 2006). On the other hand, mTORC2 is rapamycin insensitive, its core components are mTOR, mSIN1, mLST8, and the rapamycin-insensitive subunit Rictor mediates its effect through phosphorylation of protein kinase B (PKB/Akt) at Ser473 promoting cell cytoskeletal organization and survival (Sarbassov et al., 2005). Several studies by our group and others described key regulation of mTOR by AMPK (A. A. Eid et al., 2010; Sabatini, 2006)

In DN, studies showed early activation of mTOR-dependent pathways in both diabetic patients and diabetic murine models (Yang et al., 2007; Chen et al., 2009).

Additionally, podocyte-specific activation of mTORC1 demonstrated several features of DN including proteinuria and mesangial expansion (Inoki et al., 2011), while podocyte-specific repression of RPTOR demonstrated beneficial effects on proteinuria (Gödel et al., 2011). Inhibiting mTORC1 by rapamycin has been extensively studied in the kidneys. At low doses, rapamycin beneficially decreased mesangial expansion, tubular epithelial-to-mesenchymal transition (EMT), hypertrophy, macrophage recruitment, GBM thickening, and proteinuria (Chen et al., 2009; A. A. Eid et al., 2013; Y. Yang et al., 2007). *In vitro* studies have displayed that mTORC1 activation affects the expression of podocyte slit diaphragm proteins and cytoskeleton structure (Vollenbroker et al., 2008). Eid *et al.* showed both *in vivo* and *in vitro* that podocyte apoptosis was mediated by activation of the mTORC1 pathway through inactivation of the AMPK/tuberin pathway (A. A. Eid et al., 2013). These data reflect the importance of achieving a level of mTOR signaling that is consistent with energy and cellular stress.

Studies tackling the role of mTORC2 are rare. Yet, its role in DN has been contradictory. While some studies show that mTORC2 knockout unlike mTORC1 knockout in podocytes don't display DN phenotype suggesting that mTORC2 function in podocytes is not crucial as mTORC1 (Gödel et al., 2011), recent studies showed that mTORC2 knockout in podocyte has a protective role against stress-induced damage to the GFB (S. Eid et al., 2016). On the other hand, mTORC2 induces the expression of crucial proteins in DN pathogenesis and progression such as Serum- and glucocorticoid-induced protein kinase 1 (SGK1), protein kinase C (PKC) and the serine/threonine protein kinase Akt/protein kinase

B (Hori et al., 2013). Thus, more studies must be done on mTORC2 to fully understand its role in DN.

F. Autophagy in Diabetic Nephropathy

Autophagy (derived from the Greek word meaning "self-eating") is lysosomal degradation pathway that delivers intracellular constituents like damaged organelles, soluble proteins, protein aggregates, macromolecules to lysosomes in order to maintain intracellular homeostasis and cell integrity especially during stress conditions (Ding et al., 2015). Three types of autophagy have been identified: Macroautophagy referred to as autophagy, Microautophagy, and chaperone-mediated autophagy.

Autophagy is initiated with the formation of the isolation membrane called phagophore around cytoplasmic components. The phagophore matures into double-membraned autophagosome forming at ER-mitochondria contact site in mammalian cells to sequester the cytoplasmic cargo. Then, autophagosome fuses with the lysosome to form autolysosome, and the enclosed contents are degraded and reutilized (Hamasaki et al., 2013).

In order to ensure optimal balance between synthesis and degradation autophagy is tightly controlled. More than 30 autophagy-related (Atg) genes are implicated in autophagosome formation (Mizushima et al., 2011). Autophagy starts by the formation of preautophagosome or pahogophore originated from the Endoplasmic Reticulum. It is a developing membrane that requires Unc-51-like (ULK) 1/2 complex, ULK1/2–ATG13–FIP200–ATG101 to develop these processes is known as initiation (He et al., 2009). Next is the nucleation phase, as the phagophore is developing another complex comprising Beclin-1, VPS34, VPS15 and ATG14L are recruited. Actually, Beclin-1 is phosphorylated by

ULK1/2 and the Beclin complex acts as class III phosphatidylinositol 3 kinases (PI3K) converting PIP2 into PIP3(Liang et al., 2010). Accumulation of PIP3 helps in recruiting WIPI1/2, a scaffold PIP3 protein, and ATG9L, a transmembrane traffic protein (Yang et al., 2017). Two ubiquitin-like conjugation systems, the microtubule-associated protein light chain 3-phosphatidyl ethanolamine (MAPLC3/LC3-PE) and the ATG12–ATG5–ATG16L complex, are involved in autophagosome elongation and closure (Ding et al., 2015). The most indicative step in autophagosome formation is the cleavage of LC3-I into LC3-II mediated by the action of Atg3 and Atg7 (Ravikumar et al., 2010). Once phagosome and mature it fuses with lysosomes involving UVRAG interaction with PI3K complex and subsequent activation of the GTPase RAB7 (Liang et al., 2010).

Accumulating evidence suggest that autophagy plays an important role in many critical aspects of normal and disease states of the kidney (Ding et al., 2015; D. Yang et al., 2017). In DN, the role of autophagy is still controversial. Advocating evidence for the inhibition of autophagy in DN includes but not exclusive to studies showing that diabetic kidneys lack autophagic activity. In kidney cortex tubules of streptozotocin (STZ)-induced early diabetic rats, autophagy was inhibited and was associated with renal hypertrophy (Barbosa et al., 1992) However, there was a regain in autophagy function upon insulin replacement by insulin treatment or islet transplantation (Han et al., 1997). However, in another study done by Inoki et al. Insulin signaling was shown to suppress autophagy via phosphorylation protein kinase B (Akt) activating mTORC1 (Inoki et al., 2003, 2002), which can phosphorylate and inhibit Ulk1, thus inhibiting autophagosome formation (Hosokawa et al., 2009).

Some intracellular stresses that are elevated in DN can also stimulate autophagy. Reactive oxygen species (ROS) can induce autophagy through several mechanisms. For example, exogenous hydrogen peroxide can activate PKR-like kinase (PERK), which will oxidize and activate Atg4 proteases accelerating the proteolytic mature LC3 and inhibition of mTORC1 activity (L. Liu et al., 2008). Accumulation of ROS often involves the activation of mitogen-activated protein kinases, including JNK1 inducing autophagy (Sakon et al., 2003; Webber et al., 2010). Moreover, ROS accumulation in the mitochondria activates mitophagy, the selective autophagic removal of mitochondria, mediated by a mitochondrial kinase, PINK1 accumulation (Vives-Bauza et al., 2010).

Despite the role of autophagy in health and diseases, its role is not yet fully described. Further studies are needed to delineate its role in DN and to identify its potential crosstalk with upstream and downstream signaling pathway.

G. Aim and Hypothesis

Podocyte injury has emerged as a key mediator in the initiation and progression of Diabetic Nephropathy (DN). The molecular mechanism by which hyperglycemia induces podocyte injury is multi-factorial and not well defined. Despite the contradiction of the role played by autophagy in DN, several studies have described the potentiality of defective autophagy mechanisms on the onset and development of diabetes. Furthermore, our lab has described the importance of renal NADPH Oxidases (Noxs) in the progression of podocyte injury. Yet, a mechanistic pathway between Nox-induced ROS production and the alteration in autophagy has yet to be clarified. In parallel, Liver-X-receptor (LXR) and the mTOR pathway have recently been linked to autophagy and oxidative stress. In this study, we aim

to assess the role of Nox/LXR/mTOR signaling axes in autophagy and its possible link to podocyte depletion and injury. Our hypothesis is that hyperglycemia downregulates LXR expression and increases oxidative stress through activating the mTORC/Nox4 pathway leading to impaired autophagy and podocyte depletion and injury. Activating the LXR pathway, inhibiting Nox4 or mTORC1 pathway will reverse the changes observed in DN by restoring the physiological activity of autophagy.

CHAPTER II

MATERIALS AND METHODS

A. Animal Models

All animal procedures were conducted according to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the institutional animal care and use committee at the American University of Beirut. Two animal models for diabetes were used in this study: Streptozotocin (STZ) -induced swiss Webster mice and high-fat diet/low-dose streptozotocin C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) mice.

Swiss-Webster male mice, eight- week old and weighing around 40 g, received a single 100 mg/kg body weight intraperitoneal injection of STZ (Sigma-Aldrich, Steinheim, Germany) dissolved in sodium citrate buffer (0.01 M, pH 4.5). Controls received similar injections of citrate buffer. For the type 2 diabetes-induced mice model, C57BL/6J 6-week old mice were fed High Fat Diet (HFD) for 3 weeks and then 3 consecutive 50mg/kg body weight of STZ over 3 days were injected. Glucose measurement was performed one week after the STZ injection and blood was obtained via tail vein punctures and glucose levels were assessed using a glucometer for both models (Accuchek, Roche). Mice with a fasting blood glucose ≥ 250 mg/dl were considered diabetic. Blood glucose levels were monitored weekly throughout the study.

At the onset of diabetes, animals were divided into the following groups:

C57BL/6J (8- weeks treatment)

- Diabetic animals treated with vehicle
- 40mg/kg body weight of GKT137831, a specific Nox1/ Nox4 inhibitor administered three times a week by oral gavage.
- 5 mg/kg/week of T0901317 (T0), an LXR agonist administered by oral gavage.
- 0.5 mg/kg/body weight of rapamycin (Rapa), a mTORC1 inhibitor administrated three times a week by intraperitoneal injection.

Swiss Webster (5-week treatments)

- Diabetic animals treated with vehicle
- 5 mg/kg/week of T0901317 (T0), an LXR agonist administered by oral gavage.

Glycemia levels (LifeScan One Touch Glucometer; Johnson & Johnson) were monitored 24 h after the treatment and then checked weekly till the mice were sacrificed. All of the mice had unrestricted access to food and water and were maintained in accordance with protocols approved by the Institutional Animal Care and Use Committee. Before sacrifice, mice were placed in metabolic cages for urine collection. Urine albumin was measured using a mouse albumin enzyme-linked immunosorbent assay (ELISA) quantification kit (Bethyl Laboratories) and expressed as micrograms of albumin/24 h. Animals were sacrificed by exsanguination under anesthesia. Both kidneys were removed and weighed. A slice of kidney cortex at the pole was embedded in paraffin or flash-frozen in liquid nitrogen for microscopy and image analyses.

B. Glomeruli isolation

Tissues were obtained from C57BL/6J mice kidneys which weighed 0.2-0.3 g. The cortex was dissected from the kidney and used for the preparation of glomeruli samples. In experiments with tissue slices, the kidney was first carefully divided into cortex, medulla, and papilla, and tissue slices (0.5-mm thick) from these three zones were cut with a Stadie-Riggs tissue slicer. These tissue slices were washed several times in ice-cold KRB. Glomeruli were prepared with a combination of sieving and differential centrifugation. The cortical tissue was minced, strained through a stainless-steel sieve (220-gm opening), and subsequently passed through a hypodermic needle (23-gauge, Sherwood Med. Industries). The resulting suspension of cortical tissue was washed four times in KRB by repeated centrifugation at x200g for 2 mm to eliminate debris of small fragments floating in the supernatant. The resulting pellet was resuspended in about 50 ml of KRB and passed three to five times by x 1g gravity filtration sequentially through each of the three nylon sieves (NITEX) with pore-opening sizes of first 390 µm, then 250 µm, and finally 213 µm. To obtain the glomerular fraction, referred to further as "glomeruli," after filtering through a 213-sm sieve, we passed the resulting suspension through a screen cloth (NITEX #25, about 60-µm pore opening); this step allowed debris and small fragments to pass through, with glomeruli retained on the cloth. The retained glomeruli were recovered by inverting the screen over a glass beaker and by washing the glomeruli out with KRB. glomerular preparations were then pelleted by centrifugation at x 1 500g for 5 mm. Pelleted fractions were gently resuspended in 4 ml of KRB, transferred in pre-weighed polyallomer tubes, and centrifuged at x 1500g for 5 mm; the supernatant was removed by aspiration, and the tubes

were weighed again to determine the approximate wet weight of the preparations. Glomeruli were suspended in fresh KRB so that approximately 4 to 6 mg (wet weight) of glomeruli were contained in 100 µl of KRB suspension. The purity of each glomerular suspension was evaluated by light microscopy and counting of glomeruli. An aliquot of glomerular suspension (50 to 100 µl) was mixed on the microscopic slide (10:1, volume per volume) with 0.1% toluidine blue solution in isotonic saline to visualize cellular elements and was examined under higher magnification (x430) to evaluate first the structure of isolated glomeruli and then at the lower magnification (x 100) to determine the glomerular count of suspension. Isolated glomeruli were without capsules, and the structure was well preserved. The purity of glomeruli was determined by counting at least 200 particles stained with toluidine blue, and only preparations which contained more than 95% of glomeruli were used for subsequent experiments. Glomeruli were also examined microscopically after fixing in 10% formol and staining with hematoxylin-eosin. This examination confirmed the intact structure of glomerular preparations.

C. Cell culture

Human podocytes were obtained from Bristol UK (Moin Slim) as previously described (Saleem et al., 2002) were cultured in media (Sigma-Aldrich, Steinheim, Germany) containing 5 mM glucose normal glucose (NG) or treated with 25 mmol/L glucose (HG) for 48 h in the presence or absence of 20 µM GKT137831 (Nox1/Nox4 inhibitor), in the presence or absence of 5 µM T0901317 (T0) (LXR synthetic ligand) or in the presence or absence of rapamycin (mTORC1 inhibitor) (20 nmol/L). All cultures were maintained at 37°C in a humidified atmosphere of 5% CO2

D. Electron microscopy

For electron microscopy photomicrographs, the kidney cortex was prepared and analyzed as we have previously described (A. A.Eid et al., 2013)In summary, the kidney cortex was cut into 0.5–1-mm3 pieces and fixed overnight in cold 4% formaldehyde and 1% glutaraldehyde in phosphate buffer and then embedded in Epon 812 resin. Plastic sections (0.50 mm) were cut and stained with toluidine blue for identification of representative areas for subsequent sectioning using an ultramicrotome. Ultrathin sections were stained with uranyl acetate and examined and photographed on a JEOL 100CX electron microscope. All electron microscopy photomicrographs were examined in a blind manner. Individual capillary loops were examined and quantified in five glomeruli/group of animals for the degree of foot process effacement as described by Jo et al. (Jo et al., 2007). The procedure adopted for GBM thickening measurement was a modification of the harmonic mean method summarized by Dische (Dische, 1992) and adapted from Jensen et al. (Jensen et al., 1979) and Hirose et al. (Hirose et al., 1982) as described in detail by Carlson et al. (Carlson et al., 1997).

E. NADPH oxidase activity

NADPH oxidase activity was measured in cultured podocytes or in glomeruli isolated from kidney cortex as we have described previously(A. A. Eid et al., 2009). Cultured podocytes were washed five times with ice-cold phosphate-buffered saline and scraped from the plate in the same solution, followed by centrifugation at 800 × *g* for 10 min at 4°C. The cell pellets were resuspended in lysis buffer (20 m*M* KH_2PO_4 [pH 7.0], 1 m*M* EGTA, 1 m*M* phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin). Cell

suspensions or washed glomeruli were homogenized with 100 strokes in a Dounce homogenizer on ice. To start the assay, 20 μg of homogenates were added to 50 m*M* phosphate buffer (pH 7.0) containing 1 m*M*EGTA, 150 m*M* sucrose, 5 μ*M* lucigenin, and 100 μ*M* NADPH. Photon emission expressed as relative light units were measured every 20 or 30 s for 10 min in a luminometer. A buffer blank (<5% of the cell signal) was subtracted from each reading. The lucigenin-enhanced chemiluminescence assay is a reasonably good indicator of Nox4 capacity to generate superoxide indirectly when the probe is used at low concentrations (>20 μ*M*) to avoid artifactual events. Superoxide production was expressed as relative light units/min/mg of protein. Protein content was measured using the Bio-Rad protein assay reagent.

F. Detection of intracellular superoxide in podocytes using HPLC

Cellular superoxide production in podocytes was assessed by HPLC analysis of DHE-derived oxidation products, as described previously (S. Eid et al., 2016). The HPLC-based assay allows separation of superoxide-specific EOH from the nonspecific ethidium, as previously described. Briefly, after exposure of quiescent podocytes grown in 60-mm dishes to the treatment mentioned above for 48 h, cells are washed twice with Hanks' balanced salt solution (HBSS)-diethylenetriaminepentaacetic acid (DTPA) and incubated for 30 min with 50 μ*M* DHE (Sigma-Aldrich) in HBSS–100 μ*M* DTPA. Cells were harvested in acetonitrile and centrifuged (12,000 × *g* for 10 min at 4°C). The homogenate was dried under vacuum and analyzed by HPLC with fluorescence detectors. The isolated glomeruli followed the same protocol. Quantification of DHE, EOH, and ethidium concentrations was performed by comparison of integrated peak areas between the obtained and standard curves

of each product under chromatographic conditions identical to those described above. EOH and ethidium were monitored by fluorescence detection with excitation at 510 nm and emission at 595 nm, whereas DHE was monitored by ultraviolet absorption at 370 nm. The results are expressed as the amount of EOH produced (nmol) normalized for the amount of DHE consumed (*i.e.*, initial minus remaining DHE in the sample; μmol).

G. mRNA analysis

mRNA was analyzed by real-time RT-PCR using the $\Delta\Delta C_t$ method (Eid et al., 2009). Total RNA was isolated from cultured podocyte or mouse isolated glomeruli using an RNeasy Mini kit from Qiagen. mRNA expression was quantified using a Realplex mastercycler (Eppendorf, Westbury, NY) with SYBR green dye and mouse RT^2 qPCR

Primer	Sequence
h-LXR-β	F: TCGTGGACTTCGCTAAGCAA R: TCGAAGATGGGGTTGATGAAC
LXR-β	F: CTTGGTGGTGTCTTCTTGA R: TGTGGTAGGCTGAGGTGTA
Nox4	F: ACTCCCTTCGCCTCTCTTC R: CCTCCCTTGTTCACTCATC
h-Nox4	F: AGCTTGGAATCTGGGTCTT R: GCATCAGTCTTAACCGAACCA
h-podocin	F: ACCGAATGGAAAATGCCTCT R: CATCTTGGGCGATGCTCT
h-Nephrin	F: CTGACAACAAGACGGAGCAG R: GGAGAAGTGAGGAGGACCA

H. Western Blot

Cultured human podocyte cells and mouse kidneys or isolated glomeruli (n=5) were lysed using RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxylate, 150 mM sodium chloride, 100 mM EDTA, 50 mM Tris-hydrochloride, 1% Tergitol (NP40), 1% of the protease and phosphatase inhibitors and 1mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 13,600 rpm for 30 minutes at 4°C. Protein concentration in the supernatants was measured using the Lowry Protein Assay. For immunoblotting, 20-40 μg of proteins were separated on 12-15% polyacrylamide gel Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The blots were blocked with 5% BSA in Tris-buffered saline and then incubated overnight with rabbit polyclonal anti-Nox4 (1:500, Santacruz), rabbit polyclonal anti-p-mTOR ^{ser2448} (1:1000, Cell Signaling), rabbit polyclonal anti-ATG3 (1:500, Cell Signaling),), rabbit polyclonal anti-ATG7 (1:500, Cell Signaling),), rabbit polyclonal anti-Beclin-1(1:500, Cell Signaling),), rabbit polyclonal anti-LXR-α (1:500, Cell Signaling),), rabbit polyclonal anti-podocin (1:500, Abcam), and Goat HSC-70 (1:1000; Santa Cruz Biotechnology) was used as loading control . The primary antibodies were detected using horseradish peroxidase-conjugated IgG (1:1000, Bio-Rad). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software (A. A. Eid et al., 2009).

I. Immunofluorescent staining

3-μm frozen sections of kidney cortex on glass slides were stained with an anti-podocin antibody, followed by Cy3-labeled donkey anti-goat IgG (Chemicon). Sections

were examined by epifluorescence using excitation and band-pass filters optimal for Cy3. Digital images were taken of random glomeruli using a Zeiss Axio fluorescent microscope. Twenty glomeruli were examined per animal for podocin expression. Quantification of podocin immunofluorescence intensity was performed as follows. The obtained colored images were inverted and opened in a gray-scale mode using NIH Image J. Podocin expression was calculated using the formula, [x(density) × positive area (in micrometers squared)]. The positive area of podocin in each glomerulus was estimated as the ratio to the mean area of the glomerulus. The mean area of each glomerular profile was measured manually, tracing the glomerular outline, encircling the area of interest, and calculating the surface area by computerized morphometry using MetaMorph 4.69

J. PAS staining

Sections 4-μm thickness from paraffin-embedded tissues were stained with periodic acid-Schiff. Glomerular areas were quantified using an image analysis system from PAS-stained glomeruli (MetaMorph version 6.1; Universal Imaging).

H. Statistical analysis

Results are expressed as means ± standard errors (SE). Statistical significance was assessed by one-way ANOVA (ANOVA), followed by Tukey's posttest when more than two variables were analyzed. Two group comparisons were performed by Student's t-test. Statistical significance was determined as a probability (P value) of less than 0.05. All statistical analysis was performed with Prism 6 software (GraphPad Software).

CHAPTER III

RESULTS

A. HG induces podocyte injury in Culture

Podocyte injury starts from slit diaphragm protein alteration, podocyte depletion, and foot process effacement, leading to albuminuria. Nephrin and podocin, two slit-diaphragm associated proteins, are localized between the podocyte foot processes. Mutation of either protein can result in foot process effacement and massive proteinuria (Boute et al., 2000; Kestilä et al., 1998). In our study human podocytes were cultured in the presence of normal glucose (NG: 5 mM), high glucose (HG: 25 mM) alone or in combination with 5 μM T0901317(T0), 20 nmol/L Rapamycin, or 20 μM GKT137831 for 48 hrs. Our results showed that treatment with HG downregulates significantly nephrin and podocin **(figure 1 A-B)** mRNA levels, implicating podocyte injury which is reversed significantly upon activating LXR, inhibiting mTORC1 or Nox4.

A B

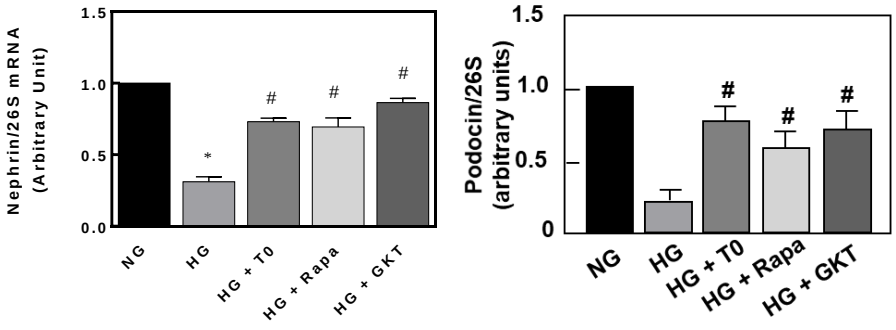


Figure1. Nephrin and Podocin mRNA levels. Human Podocytes were cultured in the presence of normal glucose (5mM), high glucose (HG: 25 mM) alone or in combination with T0901317(T0) an LXR synthetic ligand (5 µM), Rapamycin mTORC1 inhibitor (20 nmol/L), or GKT137831 (Nox1/Nox4 inhibitor) (20 µM) for 48hrs. mRNA levels of Nephrin (A) or podocin (B) decreased upon treatment with HG indicating podocyte loss, however, upon treatment nephrin levels (A) and podocin (B) significantly increased compared to the HG. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus HG.

B. High Glucose activates NADPH oxidases 4-derived ROS in cultured human podocytes.

From our work as well as others, ROS is suggested to be the final common denominator of the pathways implicated in diabetic complications. More importantly, the NADPH oxidase family is considered to be major sources of ROS in cells and tissues (Gorin et al., 2005; N.-H. Kim et al., 2006). For that, human podocytes were cultured in the presence of normal glucose (NG: 5 mM), high glucose (HG: 25 mM) alone or in combination with 5 µM T0901317(T0), 20 nmol/L Rapamycin, or 20 µM GKT137831 for 48 hrs.

Our results show an increase in Superoxide generation evaluated in HG-treated cells when compared to normal glucose. Interestingly, ROS levels significantly decreased upon treatment with T0, Rapamycin, or GKT **(Figure 2A)**. To assess the role of NADPH oxidases

in superoxide generation, NADPH Oxidase Activity Assay was performed. As expected, NADPH enzymatic activity increased upon treatment with HG compared to normal glucose and decreased upon the treatment with T0, Rapa, or GKT **(Figure 2B).** Consistent with our results, mRNA expression level of Nox4 was upregulated in HG and then downregulated upon treatment T0, Rapa, or GKT **(Figure 2C)** of interest GKT treatment.

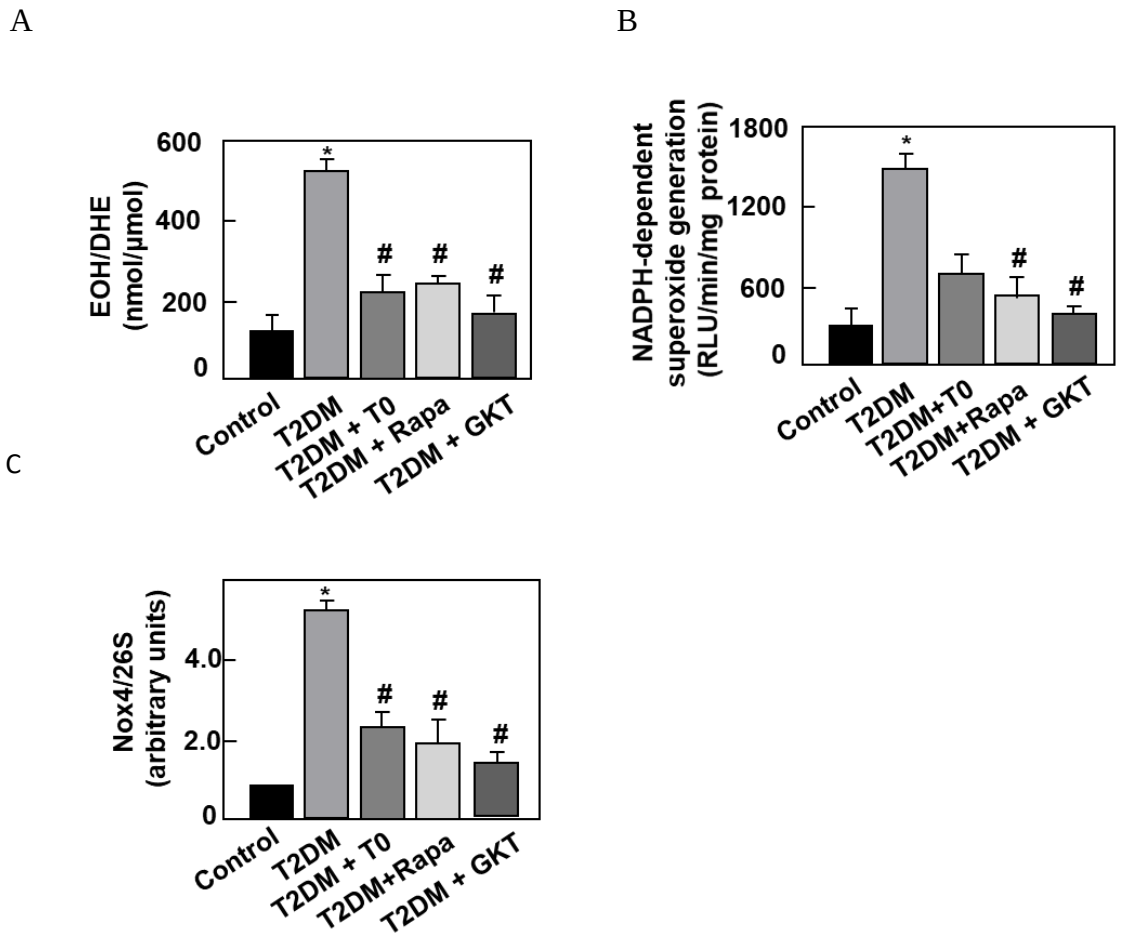


Figure 2. Assessment of ROS production in cultured human podocyte in the Diabetic mimicking milieu. Human Podocytes were cultured in the presence of normal glucose (5mM), high glucose (HG: 25 mM) alone or in combination with T0901317(T0) an LXR synthetic ligand (5 µM), Rapamycin mTORC1 inhibitor (20 nmol/L), or GKT137831 (Nox1/Nox4 inhibitor) (20 µM) for 48hrs. (A) Superoxide generation increased upon

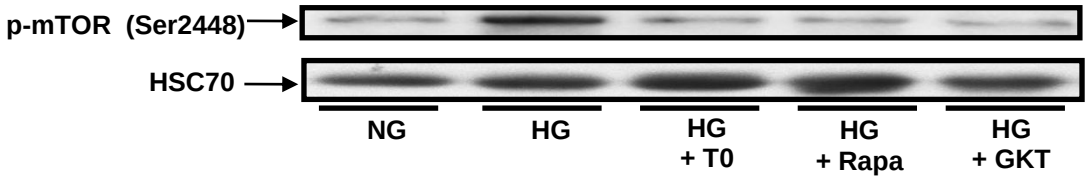
treatment with HG which was reversed upon treatment with T0, Rapa, or GKT measured by HPLC. (B) to determine the role of Noxes in ROS production, NADPH Oxidase Activity Assay was performed. Superoxide generation activity was heightened upon treatment with HG which was decreased upon treatment with T0, Rapa, or GKT. (C) these results were paralleled by an increase in Nox4 mRNA levels in the diabetic mimicking milieu which was reduced upon treatment. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus HG.

C. LXR-mTOR-Nox4 Signaling Axes

In order to assess the efficiency of our treatment in reversing HG-induced mTORC1 activation, LXR inhibition, and Nox4 upregulation, human podocytes were cultured in the presence of normal glucose (NG:5mM), high glucose (HG: 25 mM) alone or in combination with 5 μM T0901317(T0), 20 nmol/L Rapamycin, or 20 μM GKT137831 for 48 hrs. Our results show that HG activates mTORC1 pathway (increase in the p-mTOR Ser2448 expression **(Figure 3A)** inactivates LXR (decrease in LXR mRNA **(Figure 3B)**) and activates NADPH oxidase Nox4-induced ROS production as assessed by an increase in ROS production and Nox mRNA levels **(Figure 2C).** Treatment with T0 inhibited mTORC1 pathway as measured by a decrease in p-mTOR at Ser 2448 **(Figure 3A)**, activates LXR (increase in LXR protein levels **(Figure 3B)**) and decreases NADPH oxidase Nox4-induced ROS production as assessed by a decrease in ROS production and Nox mRNA levels **(Figure 2C).** Treatment with Rapa inactivates the mTORC1 pathway as measured by a decrease in p-mTOR at Ser 2448 **(Figure 3A)**, doesn't significantly affect LXR **(Figure 3B)**, and decreases NADPH oxidase Nox4-induced ROS production as assessed by a decrease in ROS production and Nox mRNA levels **(Figure 2C).** Treatment with GKT inhibits mTORC1 pathway as measured by a decrease in p-mTOR at Ser 2448 **(Figure 3A)**, activates LXR (increase in LXR protein levels **(Figure 3B)**) and decreased NADPH oxidase Nox4-

induced ROS production as assessed by a decrease in ROS production and Nox mRNA levels **(Figure 2C).**

Together, these results highlight that LXR is upstream of mTORC1, and that Nox4 can be activated by LXR inhibition, which is, in turn, activates mTORC1 that by another positive feedback activates further Nox4 Signaling pathway leading to increased ROS production, thus, further injury.



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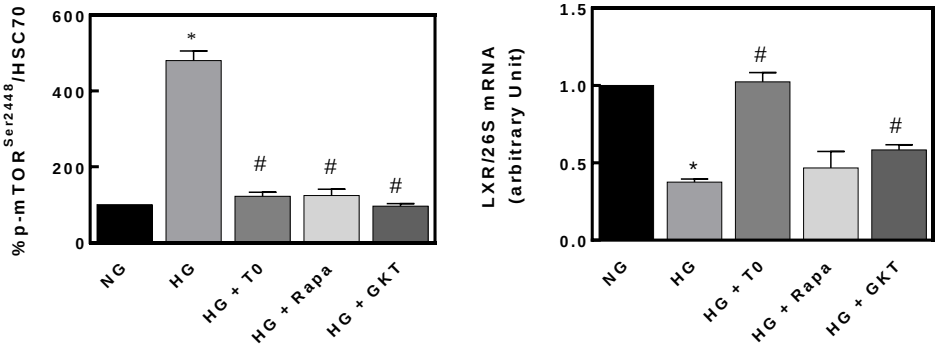


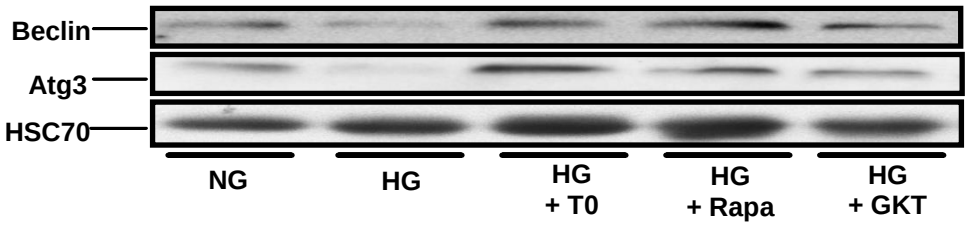
B

Figure 3. LXR-mTOR-Nox4 interaction. Human Podocytes were cultured in the presence of normal glucose (5mM), high glucose (HG: 25 mM) alone or in combination with T0901317(T0) an LXR synthetic ligand (5 µM), Rapamycin mTORC1 inhibitor (20 nmol/L), or GKT137831 (Nox1/Nox4 inhibitor) (20 µM) for 48hrs. To check the effects of the treatment on mTORC1, LXR, and NADPH oxidase signaling pathway, western blot for p-mTOR was performed (A) p-mTOR levels increased under HG and decreased upon the three-treatment indicating that mTOR might be downstream both LXR and Nox4. (B) mRNA levels for LXR were assessed as shown above. LXR mRNA levels decreased upon treatment with HG and increased upon treatment with T0 and GKT only indicating that

mTOR is below LXR. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Diabetic.

D. HG induce podocyte loss and attenuate autophagy through LXR-mTOR-Nox4 signaling axes.

Podocyte injury is a strong predictor of DN onset and progression, yet, the mechanism behind podocyte loss is poorly identified. In order to study the role of autophagy in the podocyte injury, human podocytes were cultured in the presence of normal glucose (NG:5mM), high glucose (HG: 25 mM) alone or in combination with 5 µM T0901317(T0), 20 nmol/L Rapamycin, or 20 µM GKT137831 for 48 hrs. Results were compared to cultured human podocyte exposed to 5mM of glucose (NG). Our results show that HG inactivates autophagy process as assessed by Beclin and ATG3 protein expression. This effect was reversed by T0, Rapa, and GKT treatment that was able to restore the physiological levels of autophagy **(figure 4A-B)**.



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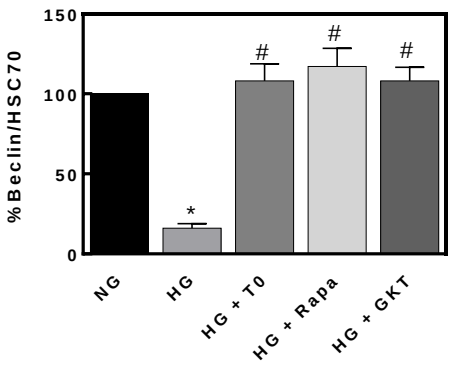

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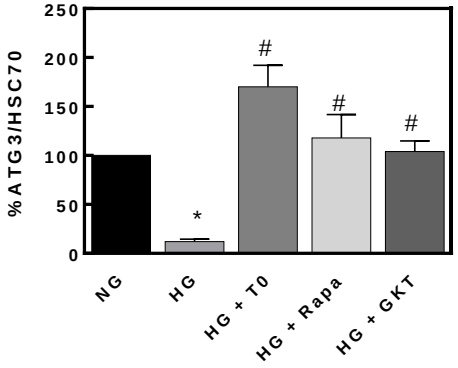


Figure 4. Assessment of autophagy expression. Human Podocytes were cultured in the presence of normal glucose (5mM), high glucose (HG: 25 mM) alone or in combination with T0901317(T0) an LXR synthetic ligand (5 μM), Rapamycin mTORC1 inhibitor (20 nmol/L), or GKT137831 (Nox1/Nox4 inhibitor) (20 μM) for 48hrs. Autophagy proteins Beclin-1(A) and ATG3 (B) were downregulated upon treatment with HG and upregulated upon the administration of the three treatments T0, Rapa, or GKT. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus HG.

E. Hyperglycemia activates NADPH oxidase-derived ROS in glomeruli of type 2 diabetic mice.

In order to assess if our *in vitro* observations correlate with the changes seen in the kidney of type 2 mouse model, mice were divided into 5 groups each with n=10 labeled CTR, T2DM, T2DM +T0, T2DM +GKT, and T2DM +Rapa as described in the section of material and methods. The metabolic characteristics of these mice are presented in **(Table 1)**.

Our data show that in isolated glomeruli, superoxide generation increased in diabetic mice compared with their control littermates (**Figure 5A**). Concurrently, diabetes-induced ROS generation was accompanied by an increase in NADPH oxidase activity and Nox4 mRNA levels (**Figure 5B-C**). Interestingly, the use of either T0, Rapa,
or GKT decreased NADPH oxidase activity (**Figure 5B)** and Nox4 mRNA levels (**Figure 5C**) and reduced ROS overproduction (**Figure 5A**).

These results were paralleled by upregulation of protein levels of p-mTOR at its activating site Ser2448 in the diabetic mice which were downregulated upon treatment with rapamycin, T0, or GKT. These results confirm our cell culture results and suggest that LXR activation downregulate mTORC which is downstream LXR confirmed by LXR mRNA levels **(figure 6B),** while rapamycin didn't affect LXR gene expression Furthermore, downregulation of Nox 4 by GKT leads to inactivation of mTORC1 **(figure 6A)** Also, rapamycin treatment decreased Nox4 mRNA levels **(figure 5C)**, suggesting that Nox4 is in a positive feedback loop that activates mTORC1 and this will lead to more activation of the superoxide generation by further activating Nox4.

Table1: Metabolic characteristic of the mice. Values are the means ± S.E. from 10 animals for each group. *P<0.05 or 0,001 *vs.* control mice; #P<0.05 or 0,001, *vs.* diabetic mice.

	Control	**Diabetic**	**Diabetic + T0**	**Diabetic + GkT**	**Diabetic + rapamycin**
Glucose level (mM)	140 ± 10	490 ± 10*	485 ± 16*	485 ± 18*	485 ± 18*
Body weight (g)	28 ± 1.5	34.5 ± 1.9*	33.5 ± 1.8*	34.1 ± 1.7*	33.2 ± 1.9*
Kidney weight (g)	0.23 ± 0.03	0.41 ± 0.04*	0.29 ± 0.02*	0.27 ± 0.07#	0.27 ± 0.07#
Kidney weight/body weight (g/kg)	8.19 ± 0.6	12.1 ± 0.9*	8.9 ± 0.9*	8.7 ± 0.4#	8.3 ± 0.4#
Urine flow rate (ml/24h)	0.75 ± 0.035	8.5 ± 0.7*	8.0 ± 0.5*	8.5 ± 0.5*	8.5 ± 0.5*

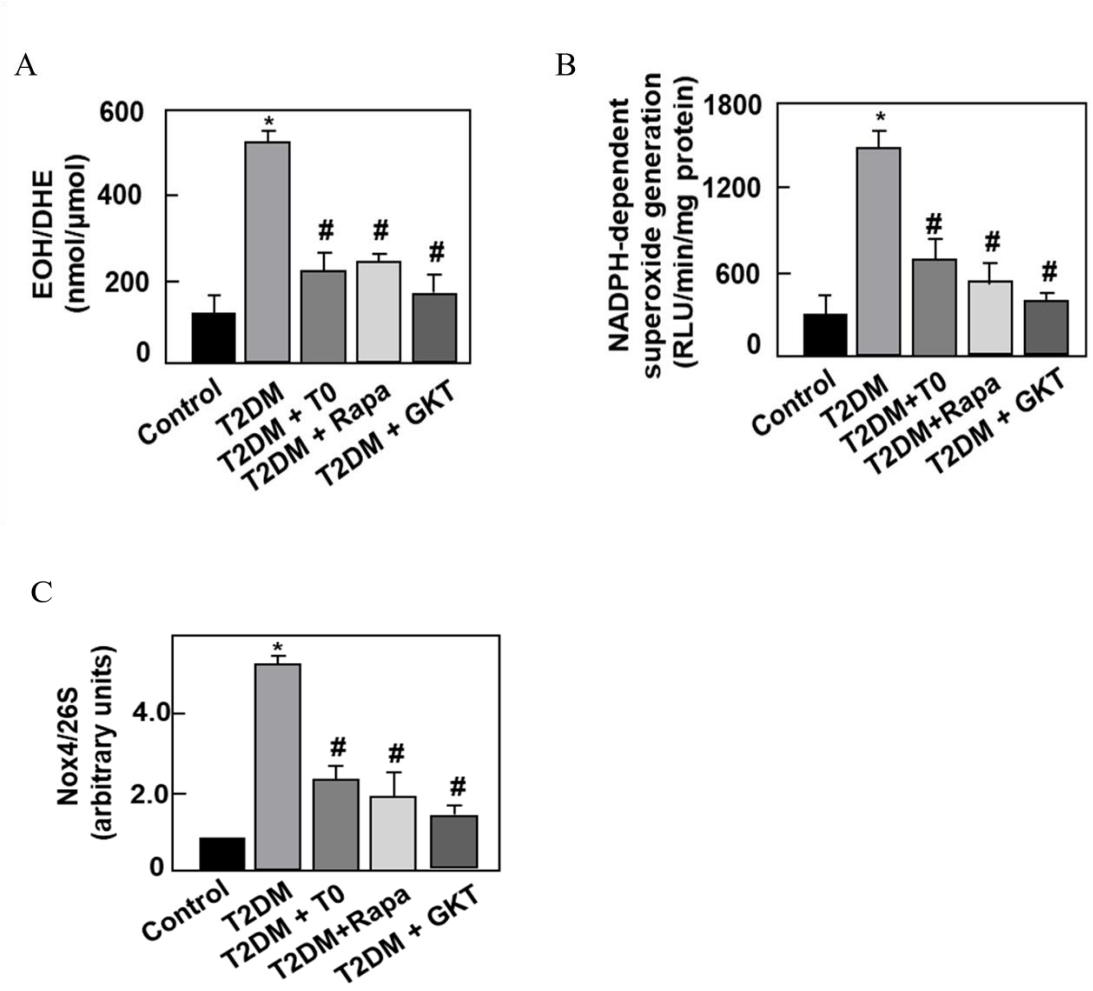


Figure 5. Assessment of ROS production in glomeruli isolated from type 2 diabetic mice with or without treatment. Glomeruli were isolated for the 5 groups of mice. Group one Control, Group 2 T2DM, Group 3 T2DM+T0, Group 4 T2DM+ Rapa, and Group 5 T2DM+GKT. (A) Superoxide generation evaluated using HPLC Showing high levels of ROS generation in diabetic mice compared to the controls, which was reversed upon treatment with T0, Rapa, or GKT. (B) NADPH-dependent superoxide generation was assessed by lucigenin-enhanced chemiluminescence. Consistently, the Noxs' activity increased in the diabetic state compared to the control and decreased again upon treatment. (C) Histogram showing relative mRNA of NOX4. As expected the mRNA levels of Nox4 is higher in the diabetic state than the treated ones. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Diabetic

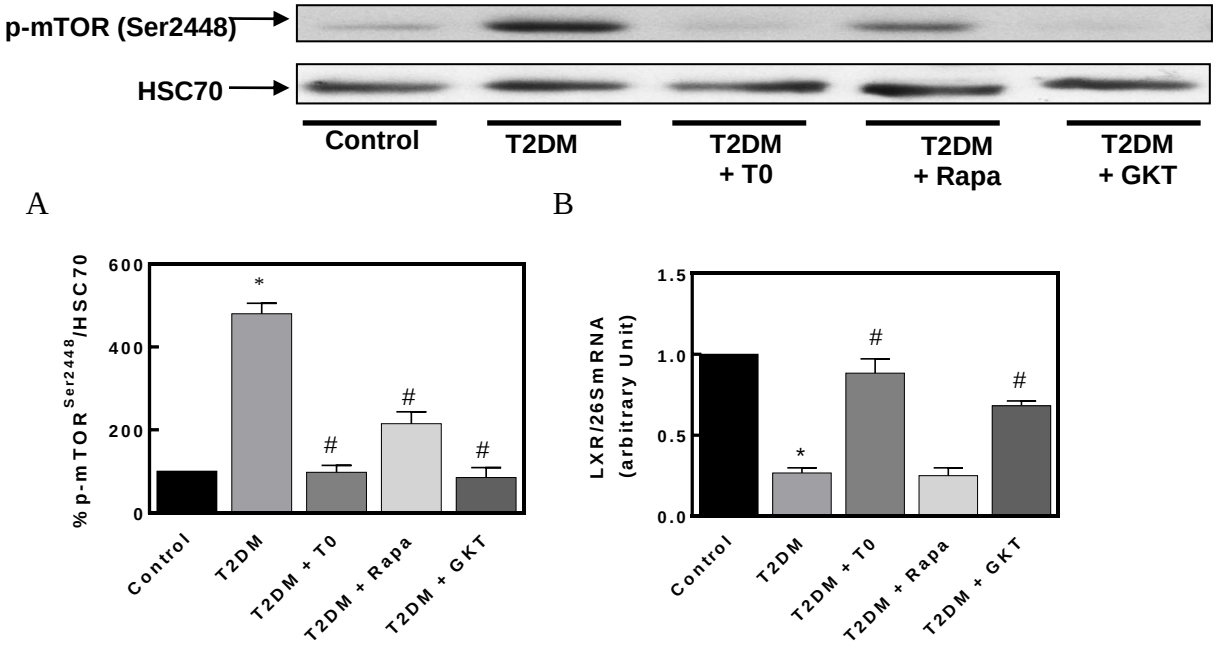
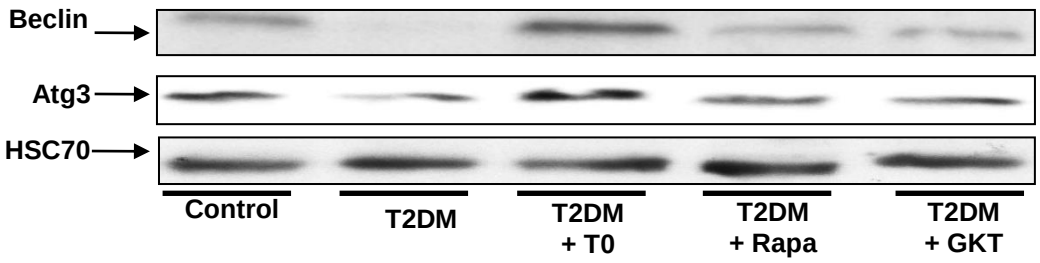


Figure 6. LXR-mTOR-Nox4 interaction. Isolated glomeruli from the 5 groups of mice. Group one Control, Group 2 T2DM, Group 3 T2DM+T0, Group 4 T2DM+ Rapa, and Group 5 T2DM+GKT were homogenized and used for Western Blot and PCR(A) p-mTOR levels increased under hyperglycemia and decreased upon the three-treatment indicating that mTOR might be downstream both LXR and Nox4. (B) LXR mRNA levels decreased in diabetic state and increased upon treatment with T0 or GKT. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Diabetic.

F. Activation of the Liver X Receptor (LXR), inhibition of mTORC, or downregulation of Nox4 and lead to the upregulation of autophagy in type 2 diabetic mice.

Results show that Hyperglycemia induces an alteration in the autophagy process as assessed by Beclin-1 and ATG3 protein expression (figure 6A-B). Treatment with T0, Rapa, or GKT reverses hyperglycemia-induced autophagy process elevates and restores protein levels of ATG3 and Beclin.



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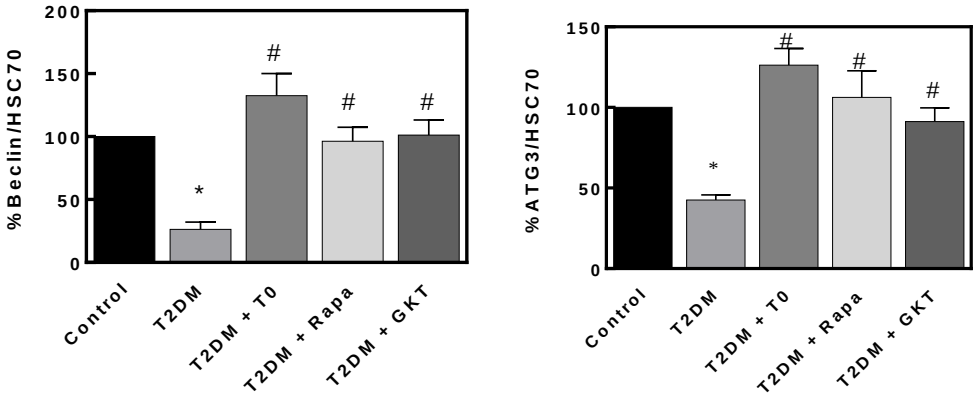


Figure 7. Autophagy levels in Glomeruli. Isolated glomeruli from the 5 groups of mice. Group one Control, Group 2 T2DM, Group 3 T2DM+T0, Group 4 T2DM+ Rapa, and Group 5 T2DM+GKT were homogenized and western blot was performed. (A) Western Blot quantification of Beclin. Beclin protein levels decreased under hyperglycemia and increased upon treatment with T0, Rapa, or GKT (B) Western Blot quantification of ATG3 protein. ATG3 protein levels decreased in diabetic state and increased upon treatment with T0 and GKT and Rapa. This shows that autophagy is attenuated in the diabetic state, and the activation of LXR or inhibition of the mTORC1 or Nox4 restore it. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Diabetic.

G. LXR/mTOR/Nox4 axes regulate GBM thickening, foot process effacement, podocyte depletion, and albuminuria.

In order to correlate the biochemical and physiological changes observed in type 2 diabetic mice with a glomerular injury. Histological studies were done using Transmission Electron Microscope (TEM) on kidney cortex slices of the 5 group of mice **(Figure 9A)**. Electron microscopic analysis revealed a significant increase in GBM thickening in the diabetic mice compared to the control **(Figure 8B),** paralleled by a marked foot processes effacement **(Figure 8C).** Remarkably, activating LXR with T0, or inhibiting mTORC1 by rapamycin, or inhibiting Nox4 by GKT lead to a significant reversal of these glomerular changes **(figure 8B-C)**. Furthermore, slit diaphragm protein alteration assessed by podocin expression is observed in the diabetic mice and reversed by the different treatment T0, Rapa, or GKT. **(Figure 9A-B)**. In parallel, mice were set in metabolic cages for 24hs and urine was then collected to measure albumin levels. Albumin levels increased significantly in diabetic mice compared to the control which then decreased upon treatment with T0, Rapa, or GKT **(Figure9C)**

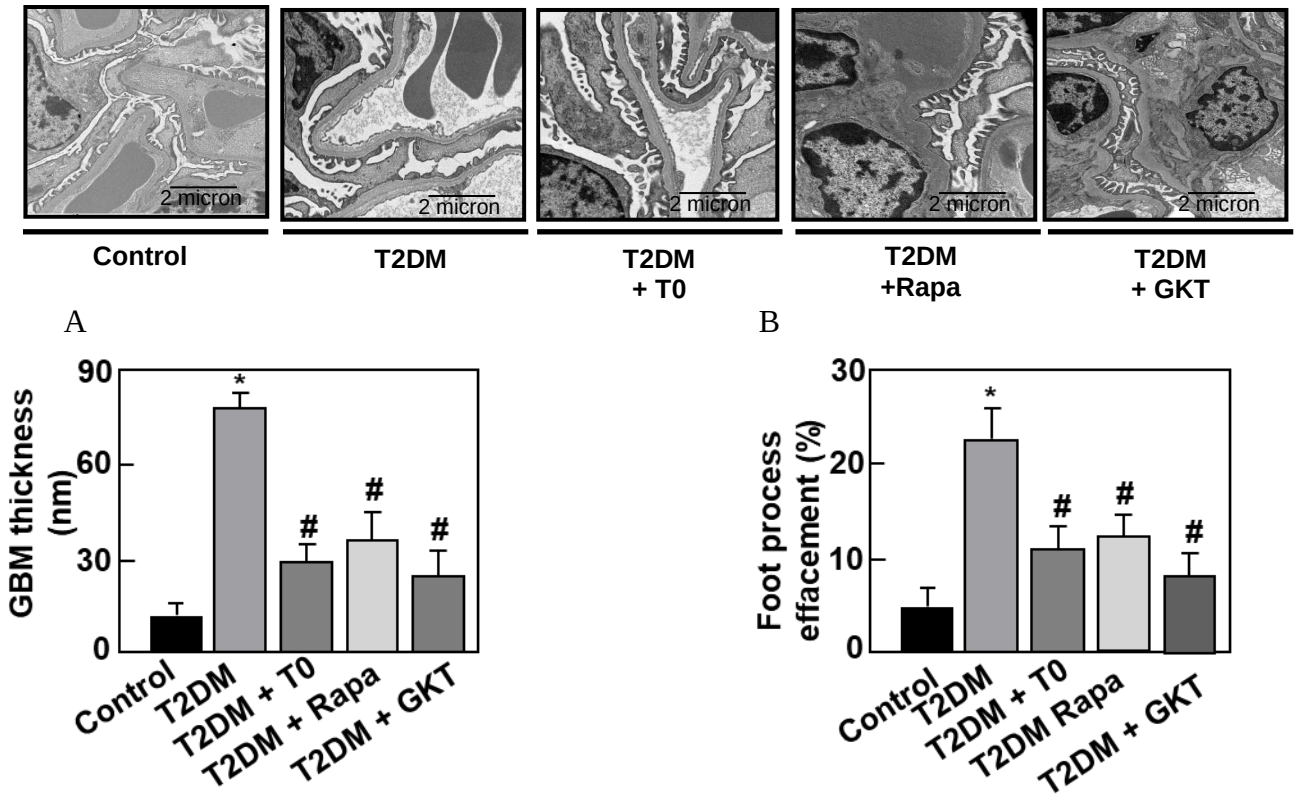


Figure 8. **LXR/mTOR/Nox4 axes regulate GBM thickening and foot process effacement.** Kidney cortex isolated for the 5 groups of mice. Group one Ctr, Group 2 T2DM, Group 3 T2DM+T0, Group 4 T2DM+ Rapa, and Group 5 T2DM+GKT were used for TEM observation. (A) Representative transmission electron photomicrographs of the glomerular cross-section. (B) Histograms representing the thickness of the GBM measured in nm and (C) semiquantitative analysis of foot process effacement of glomeruli from each group of animals. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Diabetic.

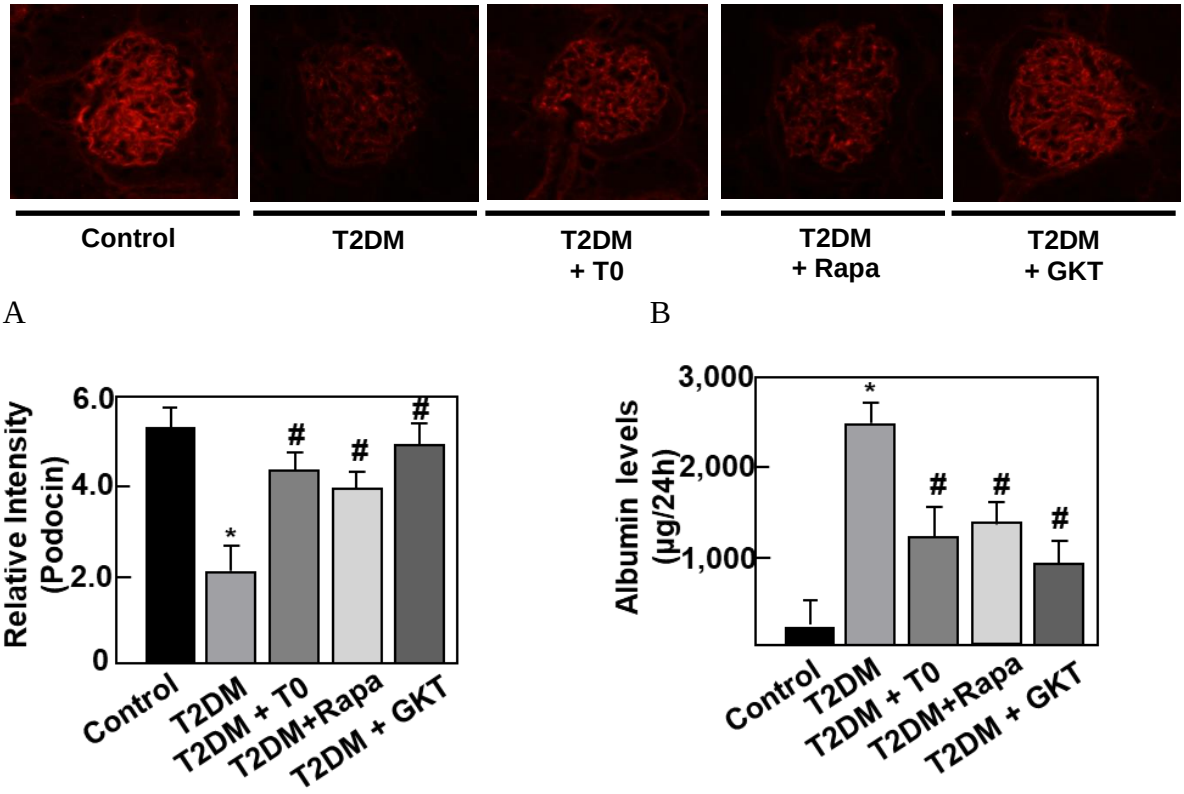


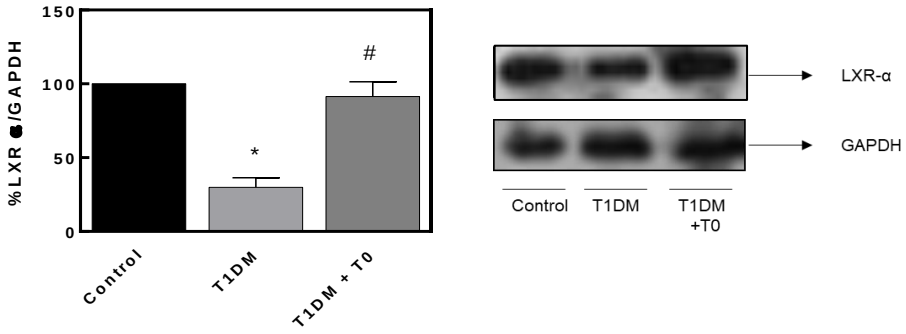
Figure 9. LXR/mTOR/Nox4 axes regulate podocyte loss and albuminuria.
Kidney cortex isolated for the 5 groups of mice. Group one Ctr, Group 2 T2DM, Group 3 T2DM+T0, Group 4 T2DM+ Rapa, and Group 5T2DM+GKT were stained with an antibody against podocin. (A) Representative immunofluorescence images of glomeruli stained with podocin (Red). (B) Histogram showing the relative intensity of podocin. In parallel (C) mice were placed in metabolic cages, urine was collected and albumin levels were measured and expressed as micrograms of albumin per 24 h. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Diabetic.

H. Hyperglycemia is responsible for Nox4-derived ROS generation and altered autophagy.

Kidney cortices were removed from the animals and our results show that HG induces LXR inactivation and Nox4 activation **(Figure 10A-B)**. These were paralleled by an alteration in the autophagy process as assessed by Beclin-1 and ATG7 downregulation **(Figure 11A-B)**. Autophagy alteration correlated with podocyte injury as assessed by

podocin downregulation **(Figure 12)** and an increase in glomerular fibrosis (PAS staining) **(figure 13)**. These results suggest that hyperglycemia is a major contributor to autophagy physiological alteration leading to glomerular injury. Also, in order to assess the role of LXR in the process, mice treated with T0 show a reversal in the observed biochemical and pathophysiological changes. More studies should be performed to assess the role of the Nox/mTOR axes in this process.

A



B

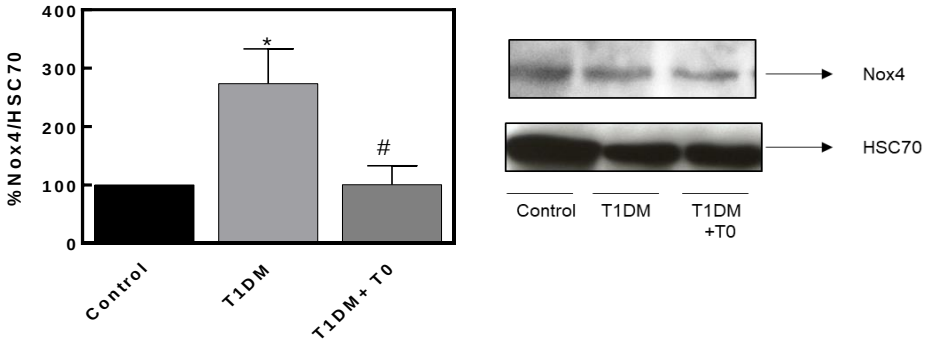


Figure10. Hyperglycemia induces ROS and inactivate LXR leading to podocyte loss in Type I diabetic model. Kidney cortex sections were taken from the three animals' group: Control, T1DM, and T1DM+T0 homogenized to perform western blot. (A) LXR levels in the diabetic state decreased compared to the Control. Upon treatment with TO LXR levels increased compared to the diabetic state. (B) Nox4 levels in the diabetic state increased compared to the Control. upon treatment with T0 Nox4 levels decreased compared to the diabetic state. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Diabetic

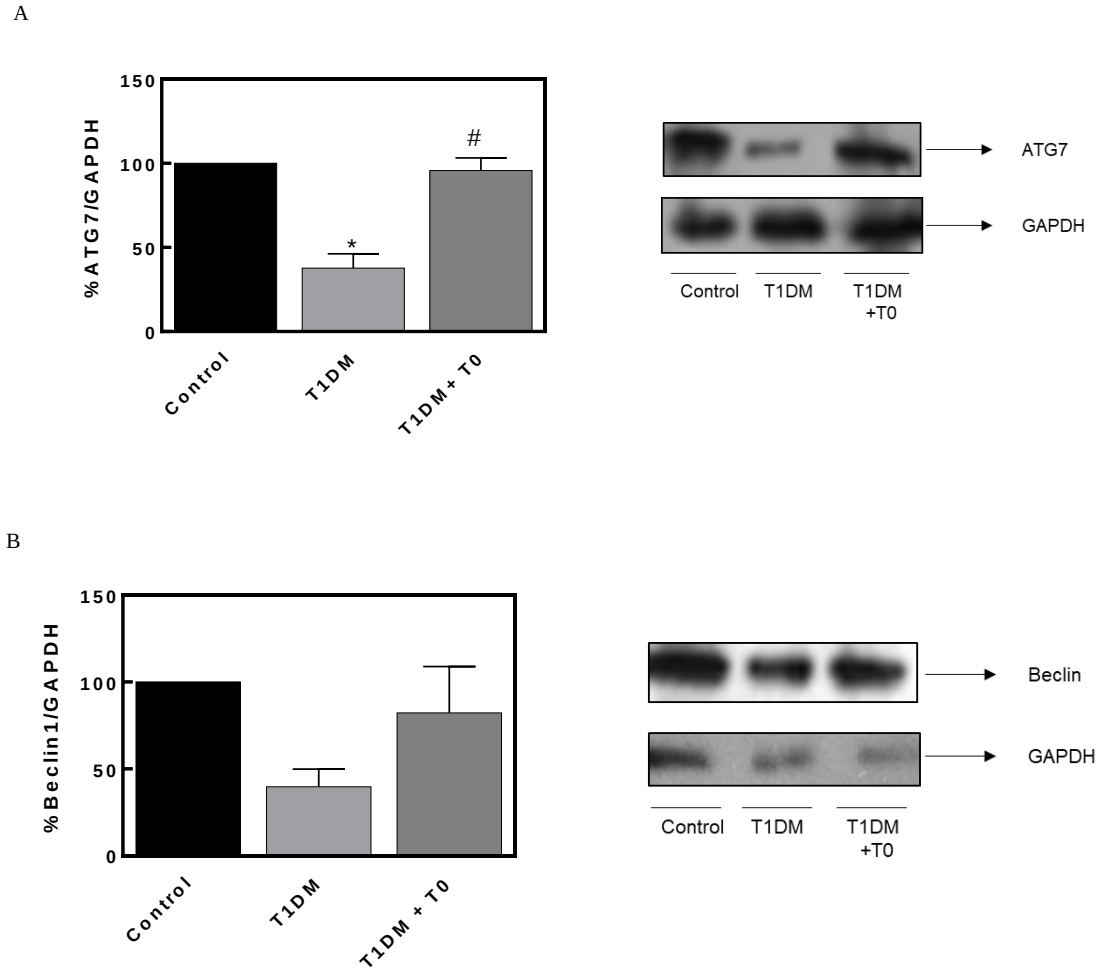


Figure 11. Autophagy levels in diabetic and treated state. Kidney cortex sections were taken from the three animals' group: Control, T1DM, and T1DM+T0 homogenized to perform western blot. Both ATG7 (A) and Beclin (B) levels decreased in the diabetic state compared to the control and then increased upon treatment with T0. This indicates that Hyperglycemia and no other factors alter autophagy in diabetic state and that LXR is important in autophagy. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Diabetic.

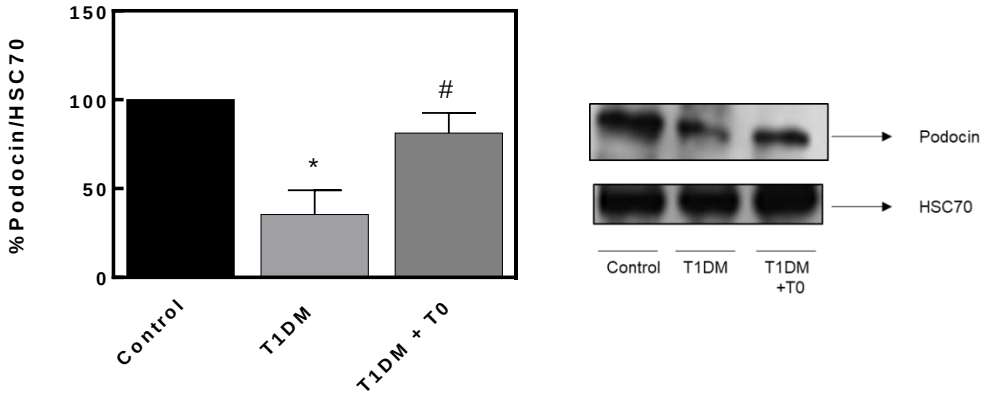


Figure 12. Podocyte Injury. Kidney cortex sections were taken from the three animals' group: Control, T1DM, and T1DM+T0 homogenized to perform western blot. Podocin levels significantly decreased in diabetes compared to the controls, while upon treatment with T0 podocin levels increased compared to the diabetic. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Diabetic

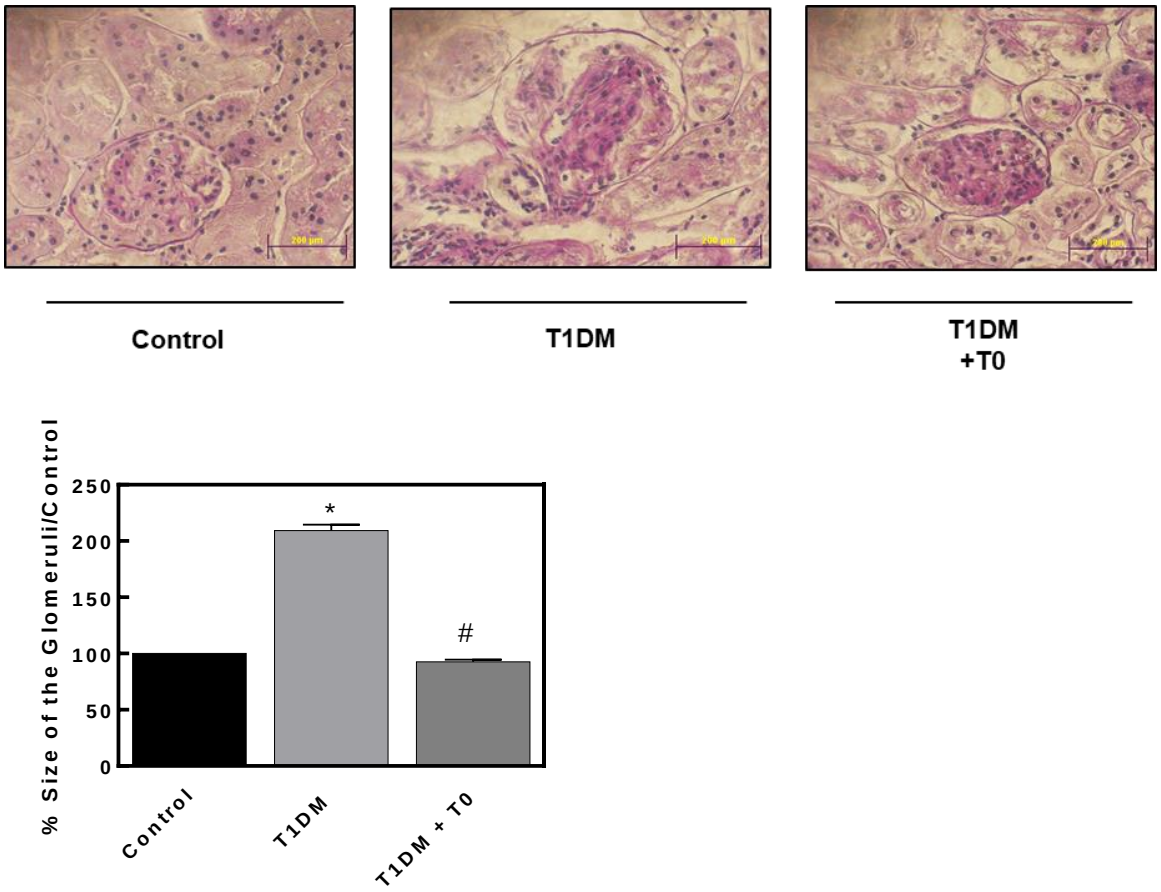


Figure 13. Kidney Histopathology. Kidney cortex sections were taken from the three different groups: Control, T1DM, and T1DM+T0 cut into thin slices and stained with PAS. The size of the glomeruli significantly increased in the diabetic state, while upon treatment with T0 the glomeruli retained a normal size. Values are the means ± SE from three independent experiments. *, $P < 0.05$ versus control; #, $P < 0.05$ versus Diabetic

CHAPTER IV

DISCUSSION

Altered autophagy has been identified as a key player in the pathogenesis of a variety of diseases including diabetes (D. Yang et al., 2017). Building evidence have implicated a critical role for autophagy in regulating many crucial aspects of normal and disease conditions in the kidney (Ding et al., 2015). Recent studies have linked defective autophagy to podocyte loss and dysfunction (Yi et al., 2017). Podocytes are known to be long-lived, highly differentiated cells that rely on autophagy for their survival. Evidence demonstrate that podocytes have a high level of basal autophagy for the purpose of cellular homeostasis (Fang et al., 2013; Hartleben et al., 2010). Podocyte-specific deletion of the *ATG5* gene induced podocyte loss, increased proteinuria and glomerulosclerosis because of the accumulation of oxidized and ubiquitinated protein along with ER stress. Moreover, the induction of proteinuria by puromycin aminonucleoside or Adriamycin in the *ATG5*-podocyte-specific deletion mice led to excessive podocyte loss, glomerulosclerosis and albuminuria compared to the control (Hartleben et al., 2010). Hence, autophagy protects podocytes. Several hypotheses can be addressed on how autophagy protects the podocytes. Autophagy removes damaged or unwanted organelles in the podocyte and prevents their accumulation which might help the podocyte to cope with stress. Another possibility is that autophagy activation decreases apoptosis. Autophagy degrades protein aggregates and misfolded proteins decreasing ER stress and thus, attenuating apoptosis (Cheng et al., 2015). Moreover, autophagy and apoptosis share some critical proteins, therefore, the activation of

autophagy leads to the depletion of these proteins required for apoptosis. For example, Beclin 1, which is important in the assembly of the autophagic protein complex, and at the same time, regulates apoptosis by antagonizing Bcl-2 (Wei et al., 2008). Further studies are required to delineate the mechanisms of how autophagy protects podocytes under pathological conditions especially in diabetic nephropathy.

Therefore, these studies emphasize the magnitude of both constitutive and stimulated autophagy as chief protecting mechanisms against aging and podocyte loss and injury. In diabetes molecular mechanism by which hyperglycemia induces defective autophagy in podocytes is still unclear. Here we want to show that high glucose (HG) induces defective autophagy in human podocyte measured by both ATG3 and Beclin levels that were significantly attenuated in cultured human podocyte. To confirm the role of hyperglycemia in attenuating autophagy we used type 1 and type 2 diabetic mice models. In both models, autophagy protein levels were decreased emphasizing more on the role of hyperglycemia in negatively affecting autophagy.

To investigate the mechanism by which hyperglycemia downregulated autophagy we first studied the mTOR pathway since it is known to induce podocyte injury and to play a crucial role in autophagy homeostasis. mTOR, a serine/threonine kinase is described in the presence of an alteration in cellular energy or increase in growth factors. mTOR activation, in turn, controls cell growth and proliferation. Our group and others have shown the negative effect of the upregulation of the mTORC1 pathway in the glomeruli of diabetic animals contributing to matrix accumulation, GBM thickening, foot process effacement, and

podocyte loss/apoptosis, which is further confirmed in this study (A. A. Eid et al., 2013; S. Eid et al., 2016; Inoki et al., 2011; Vollenbroker et al., 2008). Hereby, we described the role of mTOR in regulating autophagy in the diabetic milieu. mTORC1 is shown to inhibit autophagy through phosphorylation of eukaryotic translation initiation factor 4E (eIF-4E) binding protein 4EBP1 (Wullschleger et al., 2006). mTORC1 also prevents the autophagy-initiating UNC-5 like autophagy activating kinase (ULK) complex assembly by phosphorylating complex components such as autophagy-related gene 13 (ATG13) and ULK1/2 (Kim et al. 2011). Another mechanism by which mTORC1 mediates autophagy inhibition is through phosphorylation of ATG14L in the VPS34 complex (Yuan et al. 2013). In this study, we show that inhibition of mTORC1 using Rapamycin restores autophagy protein levels ATG3 and Beclin in both Type II diabetic mice and cultured human podocyte. Thus, the mechanism by which mTORC1 activation induces podocyte loss in DN might be through negatively controlling autophagy. In fact, Rapamycin is a well-known immunosuppressive drug that is also a negative regulator of the mTORC1 pathway. Rapamycin is shown to have positive effects on the progression of DN in both type 1 and type 2 diabetic models one way by reducing transforming growth factor-b1 and hypertrophy (Inoki et al., 2011; Lloberas et al., 2006; Sakaguchi et al., 2006; Sataranatarajan et al., 2007).

Alteration in the intracellular metabolism involving an increase in reactive oxygen species (ROS) production has been also implicated in diabetic nephropathy pathogenesis (Bedard et al., 2007; A. A. Eid et al., 2013; Forbes et al., 2008; Jay C. Jha, Banal, et al., 2016). ROS produced by NADPH-oxidases, particularly Nox4, have been shown to reduces HG-induced podocyte apoptosis through a p53-dependent pathway (A. A. Eid et al., 2010).

Moreover, we have previously demonstrated that activation of the AMPK pathway in podocyte reduces podocyte injury via downregulation of Nox4 expression and inhibition of ROS production in DN (A. A. Eid et al., 2010). Interestingly, we found that Nox4 is positioned downstream mTORC (Eid et al. 2013).

However, the link between ROS production and autophagy is still under massive investigation. In recent years, scientists started to recognize that oxidative stress and autophagy are intricately connected. Accumulating evidence suggest that ROS act as upstream modulators of autophagy induction (Scherz-Shouval et al., 2007). It is suggested that ROS production activates PKR-like kinase (PERK), which then oxidizes Atg4 proteases via eIF2a phosphorylation. Oxidization of ATG4 will lead eventually to increase in the level of mature LC3 (L. Liu et al., 2008). A recent study done by Ma et al. showed that treating podocyte cells with high glucose for 24 hours induced the generation of mitochondrial ROS along with an increase in autophagy (Ma et al., 2013). In our study, we show that treatment with HG induces ROS production measured by HPLC mainly due to Noxes activity in particular Nox4. However, autophagy level as suggested by the decrease in both Beclin and ATG3 is attenuated after treating human cultured podocyte with HG for 48hrs. One possibility for the discrepancy in the results is the duration of treatment. Upon treatment with HG, the excessive ROS generation would damage the mitochondrial membrane causing the leakage of ROS into the cytoplasm (Ravikumar et al., 2010). Autophagy, as mentioned above, is responsible for the degradation of affected protein and organelles, thus, it is expected to be activated. However, upon chronic exposure of the cells to HG autophagy will no longer able to match the overwhelming ROS effect leading to podocyte injury. This will

eventually lead to a number of pathological changes like GM thickening, foot process effacement, and albuminuria. Interestingly the effect was reversed upon treatment with either GKT, Rapamycin or LXR activator T0. Treatment with GKT a Nox1/4 inhibitor decreased ROS production as expected and increased autophagy in cultured human podocyte and in the type2 diabetic model. Interestingly inhibiting Nox4 decreased the phosphorylation of mTORC suggesting a loop interaction between Nox4 and mTORC1 giving the possibility that Nox4 downregulate autophagy through activation of mTORC1.

Liver X receptor is a nuclear hormone receptor that acts as a transcription factor. It is activated by oxysterols endogenously or synthetically by T0901317 (T09) and GW3965. Two different, yet highly homologous, isoforms of LXR have been described, LRX-α and LXR-β (Steffensen et al., 2004). While LXR-β is ubiquitously found in all the tissues LRX-α is found in highly metabolic tissues such as liver, intestine, adipose, macrophages, and kidneys. The major role of the LXR is regulating the genes involved in lipid metabolism like SREBP-1c, ATP-binding cassette transporters A1, G5, and G8, ApoE among others (Wu et al., 2004). In the kidney, unlike liver, LXR stimulate the efflux of lipids and cholesterol in mesangial cells (Wu et al., 2004). Crosstalk between LXR and renin-angiotensin-aldosterone system (RAAS) has well been studied. LXRs have been shown to regulate renin the rate-limiting enzyme in the RAAS *in vivo* (Kuipers et al., 2010). In diabetic nephropathy Patel et al. showed that *Lxrα/β* $^{-/-}$ mice exhibited a tenfold increase in the albumin: creatinine ratio and a 40-fold increase in glomerular lipid accumulation compared with *Lxrα/β* $^{+/+}$ mice. When diabetes was induced in *Lxrα/β* $^{-/-}$ accelerated mesangial matrix expansion and glomerular lipid accumulation was observed as well as an increase in both inflammatory and

oxidative stress markers (Patel et al., 2014). To our knowledge, we are the first group to report the interaction between LXR/mTOR/ Nox4 axis in diabetic nephropathy. Our results showed that administration of T0 reversed the biological changes induced by HG in cultured cells or hyperglycemia in both animal models. The mechanism by which LXR ameliorate diabetic nephropathy is still unclear. Here we tried to find a correlation between LXR and two known pathways involved in DN. T0 administration induced a decrease in phosphorylation of mTOR at Ser 2448 while administration of Rapa didn't affect LXR level suggesting that LXR is upstream mTOR and exerts an inhibitory effect on mTOR. LXR activation leads to a decrease in ROS production as measured by HPLC and a decrease in NADPH-oxidase activity proposing a role of LXR in attenuating oxidative stress. To understand how LXR decreases ROS production we checked for Nox4 levels upon treatment with T0. In both diabetic models and in cultured podocyte Nox4 mRNA or protein levels were decreased implying an inhibitory effect of LXR on Nox4. However, upon treatment with GKT LXR levels increased hinting at a negative feedback or loop between LXR and Nox4. LXR has been shown to induce autophagy in the myocardium. Here we showed that LXR activation increased autophagy protein levels both *in vitro* and *in vivo* giving rise to another possibility of how LXR ameliorate diabetic nephropathy.

In summary, our data provide evidence for a novel function of the LXR/mTOR /Nox4 signaling axes in HG/Hyperglycemia- derived reactive oxygen species generation and podocyte autophagy deregulation that contributes to glomerular/podocyte injury in diabetes. Thus, LXR activation, mTOR and/or Nox4 inhibition may represent a therapeutic modality of diabetic kidney disease.

One limitation of the study is the number of mice. It is important to replicate our work on a large cohort of mice. It is also interesting to use different knockout mice for more specificity. Another limitation of the study is relying only on checking for autophagy proteins as an indicator of autophagy. Autophagy kit or confocal microscopy could be a solution. T0 has some undesirable side effects, so finding a better LXR activator with fewer side effects is crucial. GKT is a dual Nox1/4 inhibitor, thus, finding a specific Nox4 inhibitor will give more specific results.

Future Perspectives:

In order to strengthen our study further work should be accomplished *in vitro* and *in vivo:*

- autophagy must be assessed at different time points and different treatment duration.
- Activator/inhibitor for autophagy must be used to study the effect of autophagy alone or how autophagy affects the LXR/mTOR/ Nox4 pathway in the kidney.
- the mTORC2 pathway must be studied in these models since we have reported a role of mTORC2 in DN (S. Eid et al., 2016).
- Usage of LXR alpha or beta knockout mice and activating autophagy would be an investigation worth pursuing to fully grasp how autophagy works through LXR.
- Specific inhibitor for Nox4 other than GKT which is a dual inhibitor for Nox1/4 can be used.
- Specific KO Nox4 mice can be used
- Specific mTORC1 KO mice can be used

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