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MECHANISMS OF PROXIMAL TUBULAR CELL INJURY IN DIABETES: ROLE OF AMPK AND THE CYTOCHROMES P450 ENZYMES

by
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AN ABSTRACT OF THE THESIS OF

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347 million people worldwide suffer from Diabetes, and the prevalence of raised fasting blood glucose levels is increasing vastly by the year, and especially in the Middle East region. Worldwide, more than 3 million people die each year as a consequence of diabetes-related complications. Diabetes is a chronic metabolic disease that involves impaired blood glucose homeostasis. The elevated levels of blood glucose result in several complications among which diabetic nephropathy stands as one of the most serious.

Diabetic nephropathy causes functional and histological changes to renal cells. Studies have shown a relation between certain metabolic pathways and the development of nephropathy. Oxidative stress was also linked and was shown to play a major pathological role in its development, and is manifested by the increased production of reactive oxygen species (ROS).

Studies show that high glucose decreases the phosphorylation of AMP-activated protein kinase (AMPK), which is a serine/threonine kinase that acts as an energy sensor regulated by glucose. The activation of AMPK requires its phosphorylation, and its deactivation was shown to cause a cascade of events that eventually leads to the increase in the production of ROS, mainly the increased expression of NOX4 – a ROS generating NADPH oxidase.

It has also been shown that HG leads to an increase in the expression of Cytochrome P450 (CYP 450), and specifically the 4A subfamily of the enzyme. This up-regulation leads to increased ROS production in the kidneys, mediated by the increased production of 20-HETE.

We hypothesize that the common resulting increased production of ROS due to deactivation of AMPK and increased expression of CYP 450 underlies a relationship between these two enzymes. Our research aims to reveal this cross-talk, and further elucidate the signaling pathway involving them.

In order to achieve this aim, we conducted in-vivo setting experiments, where diabetes was induced in male Sprague-Dawley rats, and different groups were treated with different drugs, among which were AMPK activator and CYP 4A inhibitor.
The expression of proteins was assessed using western blots. Along with that, we also assessed the histological changes in the kidney cortex using different histochemical stains. The ROS production was also measured using Dihydroethidium (DHE) stain and detected via confocal microscopy.

Our project's aims, once achieved, will offer a more profound insight on the signaling pathways controlling the kidney cells' metabolism in diabetes, and eventually leading to diabetic nephropathy. That would further help in advancing the targeted therapy that regresses diabetic nephropathy, and reverses its effects on renal cells and kidney tissues in general.

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LIST OF ABBREVIATIONS

DM:	Diabetes Mellitus
DN:	Diabetic Nephropathy
IDDM:	Insulin-dependent Diabetes Mellitus
NIDDM:	Non-insulin-dependent Diabetes Mellitus
ESRD:	End-stage Renal Disease
ROS:	Reactive Oxygen Species
CYP 450:	Cytochrome P450
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate
AA:	Arachidonic Acid
EET:	Epoxyeicosatrienoic Acid
20-HETE:	20-dihydroxyeicosatetraenoic Acid
AMPK:	Adenosine-Monophosphate Protein Kinase
HET0016:	N′-hydroxyphenylformamidine

INTRODUCTION

A. Diabetes Mellitus

A.1. Definition

Diabetes Mellitus (DM) is a chronic metabolic disease that is characterized by glucose metabolism dysregulation. According to the International Diabetes Atlas, more than 371 million people worldwide suffer from diabetes, and the prevalence of raised fasting blood glucose levels is increasing vastly by the year. Lebanon is one of the world's top ten countries for highest prevalence (%) of diabetes with 16.6% among adults between 20 and 79 years of age. The disease is classified into two major types: Type 1 DM, or insulin-dependent DM (IDDM), and Type 2 DM, or non-insulin dependent DM (NIDDM). IDDM is characterized by the absence of insulin production due to the destruction of the insulin producing β-cells of the pancreas, which are located in the islets of Langerhans. It is commonly referred to as the juvenile type, and has many risk factors including genetic predisposition, diet and environmental factors, and exposure to certain chemicals. Though the risk factors vary, the direct onset of the disease is usually the result of an auto-immune reaction causing the destruction of the β-cells. IDDM is accompanied with high plasma glucose levels. On the other hand, NIDDM is characterized by insulin resistance, or the failure of cells to make use of the insulin in blood. It is usually accompanied by high levels of blood glucose as well as high insulin levels. NIDDM is known as the adult-onset type of DM, and it has several risk factors

similar to type 1. However, the main cause has been shown to be obesity linked with genetic predisposition (Butler *et al.,* 2003). Type 1 and Type 2 diabetes are associated with morbidity and mortality due to the onset and development of complications. In Lebanon, 3222 deaths were attributed to diabetes in the year 2010 alone (Diabetes Atlas).

A.2. Diabetic Complications

Diabetes is accompanied by several serious microvascular and macrovascular complications. Worldwide, more than 3 million people die each year as a consequence of these diabetes-related complications.

A.2.1. Microvascular Complications

The major and most serious of the diabetes-related microvascular complications are retinopathy, neuropathy, and nephropathy (Nathan *et al.,* 1993).

A.2.2. Macrovascular Complications

The main macrovascular complications caused by diabetes are atherosclerosis, cardiovascular, and cerebrovascular diseases (Fowler *et al.,* 2008).

B. Diabetic Nephropathy

Between 20 to 40% of diabetic patients develop Diabetic Nephropathy (DN), that will lead to end-stage renal disease (ESRD) in 40% of the cases (Dronavalli *et al.,* 2008). It is also associated with a high mortality rate where approximately 10% of diabetic patients die of renal failure (van Dieren *et al.,* 2010). DN is initiated by hyperglycemia,

which causes functional and histological changes in the kidneys. Functionally, in the primary stages, glomerular hyperfiltration occurs leading to microalbuminuria. In later stages, it progresses into proteinuria, which ultimately leads to ESRD where the glomerular filtration rate rapidly falls (Nathan *et al.*, 1993). Structural changes, on the other hand, include increase in kidney size (Nathan *et al.*, 1993), podocyte loss, thickening of the glomerular basement membrane, glomerular and tubular epithelial hypertrophy, accumulation of mesangial matrix, tubular atrophy, tubulointerstitial fibrosis, and glomerulosclerosis (Wolf *et al.*, 2004)(Fig. 1).

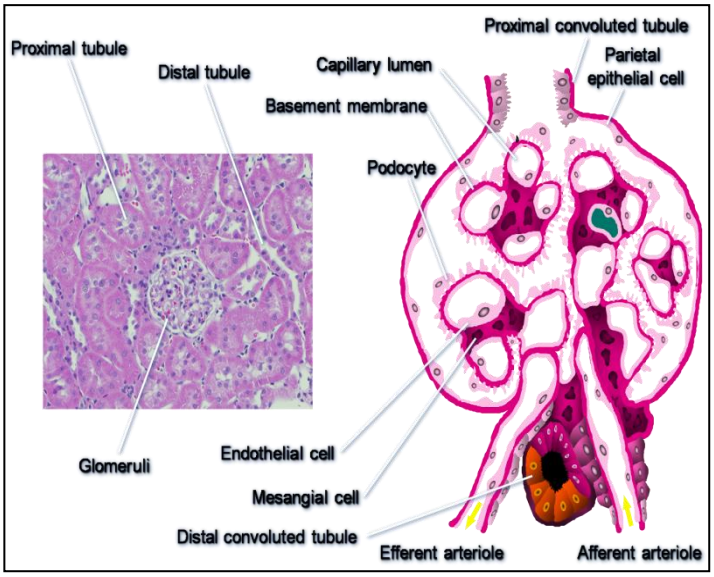

Figure 1. Schematic diagram of the kidney's anatomy and glomerular structure

B.1. Oxidative Stress in Diabetes

DM has been shown to be associated with high oxidative stress (OS) levels (Giuglano *et al.*, 1996) (Li *et al.*, 2003), resulting from the disproportionate production of free radicals and Reactive Oxygen Species (ROS). The increased production is due to biochemical pathways associated with hyperglycemia. The high OS levels play a major role in the pathogenesis of DN (Dronavalli *et al*,. 2008). OS is the state of imbalance between the production of free radicals and ROS on one side, and the body's ability to

rid itself of them by utilizing free radical scavengers or anti-oxidant systems on the other side (Baynes *et al.*, 1991). The sources of OS in diabetes may be amplified by a continuing cycle of metabolic stress, tissue damage, and cell death, leading to further increase in the production of the free radicals. This in turn compromises the efficiency of the free radical inhibitory systems in the body, thus aggravating the OS and accelerating the development of diabetic complications (Baynes *et al.*, 1991).

C. Reactive Oxygen Species

C.1. Role

Reactive Oxygen Species are products of normal cellular metabolism, and have been proven to play vital roles in cell signaling pathways and important physiologic roles in many cellular responses (Droge *et al.*, 2002). However, it has been shown that DM is closely linked to an increase in the production of ROS, and thus leads to the accumulation of ROS in the tissues (Vinod *et al.*, 2012). Increased levels of ROS have toxic effects and they injure cells and tissues, and they have been proven to be the major contributors to the development of the diabetic sequelae, mainly DN (Bonina *et al.*, 2002).

C.2. Sources and Effects

ROS are produced by several mechanisms in the cell (Fig.2), but among them, the two main enzymatic

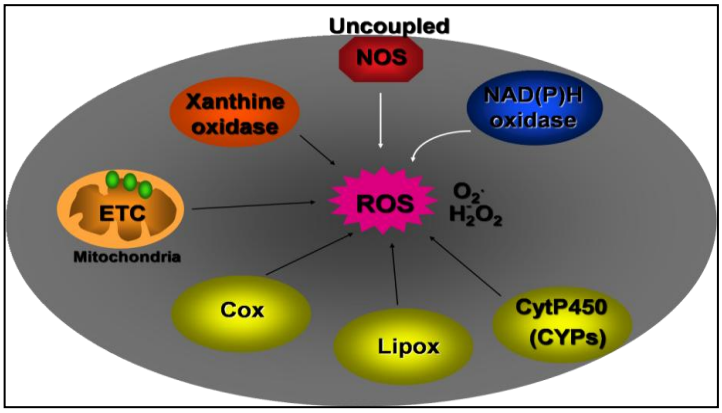

Figure 1. Sources of ROS

reactions that are of importance to this study are NADPH oxidase, and Cytochrome P450 (Puddu *et al.,* 2008). Aside from mitochondrial respiratory chain derived ROS, these two reactions vastly add to the ROS produced, especially in the presence of hyperglycemia. Studies show that antioxidants prevent glomerular and renal hypertrophy, as well as proteinuria, in diabetes suggesting a pathogenic role for ROS in the progression of the disease (Brownlee *et al.,* 2001). CYP 450s are found to be significant sources of ROS in many tissues (Puntarulo *et al.,* 1998). Studies done in our laboratory have shown that high glucose induces ROS production through the upregulation of CYP4A and NADPH oxidase (Eid *et al.,* 2009). The catalytic mechanism for CYPs involves reductive activation of molecular oxygen. The generation of radicals within the active site of CYPs is responsible for their unusual catalytic properties, and small amounts of $O_2^{\bullet -}$ and H_2O_2 are by-products of the normal P450 catalytic cycle. To be catalytically active, microsomal CYPs require a supply of electrons, which are provided by NADPH-cytochrome P450 oxidoreductase (Puntarulo *et al.,* 1998).

D. NADPH in Diabetic Nephropathy

Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidases (NOX) are superoxide anion radical generating enzymes belonging to a family of heme-containing transmembrane electron transport proteins (Fig. 3). Their biological function is electron transportation across biological membranes, and ROS generation. The members of this

family of enzymes that are known till today are seven, and each has a tissue specific distribution (Maghzal *et al.*, 2010). NOX 1, NOX2, and NOX 4 are found in kidney tissues, but NOX 4 is the most abundant (Bedard *et al.*, 2007). NOX-derived ROS' function in the kidneys can be classified into three major categories: regulation of renal blood flow, alteration of cell fate - namely inducing mesangial cell apoptosis and promoting cellular hypertrophy, and regulation of gene expression (Bedard *et al.*, 2007). Gorin et al. show that the expression of NADPH oxidase is increased in the diabetic kidney. The study also shows that the overproduction of ROS as a result of NADPH oxidase play a key role in renal hypertrophy (Gorin *et al.*,2005). This increased oxidative stress contributes to the development of diabetic nephropathy (Balakumar *et al.*, 2009). Other studies have shown a role for NOX-derived ROS in diabetic nephropathy, where NADPH oxidase inhibitors prevent high-glucose induced ROS generation (Asaba *et al.*, 2005). Moreover, different studies confirmed elevated levels of NOX 4 mRNA and protein in DN (Etoh *et al.*, 2003); additionally, treating diabetic animals with NOX 4 antisense mRNA decreases kidney pathology (Gorin *et al.*, 2005). Many studies suggest that

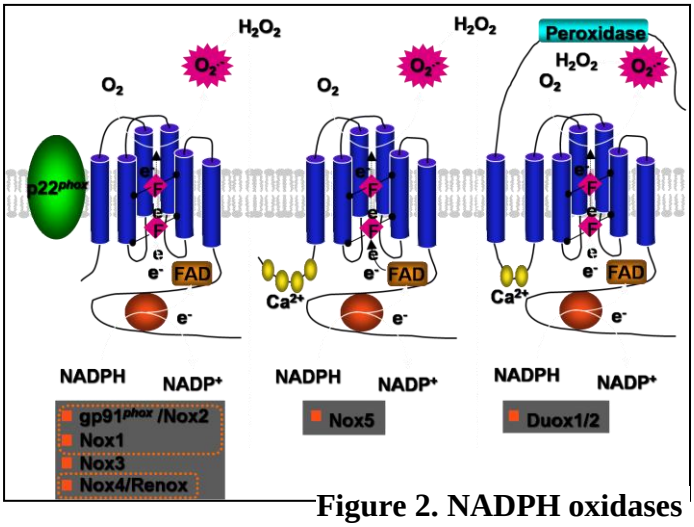

Figure 2. NADPH oxidases

NOX4 act synergistically with other NOX isoforms to generate ROS-dependent damage in diabetic nephropathy.

E. Cytochrome P450 in Diabetic Nephropathy

The Cytochrome P450 (CYP) is a large and diverse family of hemoproteins (Fig. 4). They are enzymes that catalyze the oxidation of various organic substances, and their expression is tissue specific. CYP4A mRNA and protein are expressed in various renal regions (Natarajan *et al.*, 2003). The action of phospholipases in renal cells, leads to the release of the lipid arachidonic acid (AA), which can be further metabolized by three major pathways: the cyclooxygenase pathway, the lipoxygenase pathway, and the cytochrome P450 (CYP) pathway.

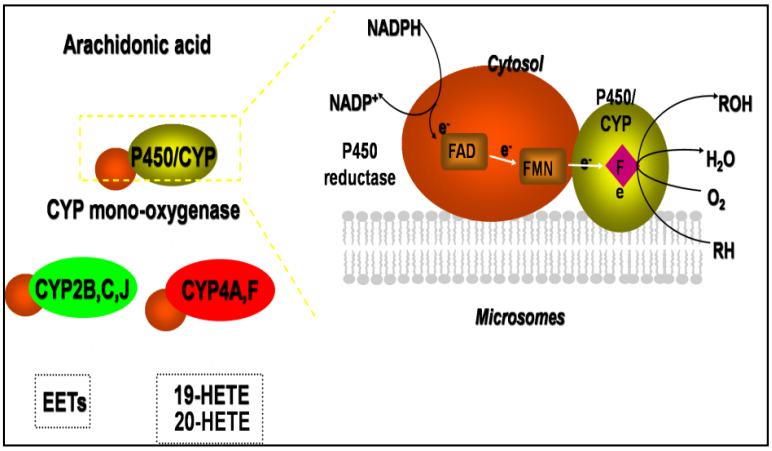

Figure 3. Cytochrome P450 Enzyme

According to the CYP pathway, metabolism of AA leads to the production of several isoforms of epoxyeicosatrienoic acids (EETs), dihydroxyeicosatetraenoic acids, and 19- and 20-HETEs (Natarajan *et al.*, 2003). These metabolites have important physiological, cellular, as well as key pathological functions. 20- HETE, is one of the major CYP eicosanoids produced in the kidney cortex (Eid *et al.*, 2009). 20-HETE,

among several locations, is produced in the thick ascending loop of Henle. It regulates sodium-potassium-chloride transport in this nephron segment (Maier *et al.,* 2001). The predominant CYP450 in the kidney cortex that synthesizes 20-HETE is cytochrome P450 of the 4A family (CYP4A) and CYP 2C11 (Eid *et al.,* 2009-2013). Formation of 20-HETE and EETs is altered in several disease models such as diabetes (Hoagland *et al.,* 2001). Eid et al. show that high glucose, i.e. hyperglycemia, induces an increase in the production of CYP 4A. The increase of CYP4A expression is accompanied by an increase in 20-HETE formation (Eid *et al.,* 2013). Another study also shows that 20-HETE is a mediator in the production ROS (Eid *et al.,* 2009). To further study the specific effect of CYP4A, HET0016 - a potent inhibitor of CYP 4A - was used. HG induced CYP4A protein expression and 20-HETE formation was attenuated by HET0016 treatment (Eid *et al.,* 2013). In parallel, HET0016 reverses the effect of HG-induced fibronectin and collagen IV protein expression and inhibits hypertrophy (Eid *et al.,* 2013).

F. Signaling Pathways in Diabetic Nephropathy

Diabetic nephropathy (DN) is characterized by a plethora of signaling abnormalities that together result in the pathologic hallmarks of DN. High extracellular glucose increases glucose uptake via the facilitative glucose transporter, GLUT1, which leads to the activation of a number of metabolic pathways that result in increased AGE and ROS

generation. In turn, these activate a number of signaling pathways that lead to enhanced ECM production (Brosius *et al.*, 2010).

Reduced bradykinin signaling

The onset and progression of Type 1 DN in humans causes a substantial decrease in bradykinin, which is associated with enhanced renal expression of several genes involved in progressive glomerulosclerosis, including TGF-β1, CTGF, a TGF-βeffector, and p53. In other studies, bradykinin signaling has been found to inhibit IGF-1 activation of Erk 1 and 2 in cultured mesangial cells (Brosius *et al.*, 2010).

Reduced nitric oxide signaling

Nitric oxide (NO) is reduced in diabetic glomeruli in humans with DN. NO suppresses expression of a profibrotic factor known as secreted modular calcium binding protein 1 (SMOC-1), which was shown to stimulate TGF-β and CTGF expression in mesangial cells (Brosius *et al.*, 2010).

JAK/STAT pathway activation

It was shown that high glucose levels lead to tyrosine phosphorylation of Janus kinase (JAK2), which, in turn, phosphorylates and activates the transcription factors, STAT1 and STAT3. JAK2 activation was shown to mediate collagen IV and fibronectin production, TGF-βactivation, and cell growth (Brosius *et al.*, 2010).

mTOR activation

Exposure to high glucose induces mTOR activity in the diabetic kidney. This may be due to reduction in AMP-activated protein kinase signaling .This leads to enhanced cellular metabolism and growth in part via phosphorylation and activation of two major downstream substrates, S6 kinase (S6K)1 and eukaryotic initiation factor 4E binding protein (4EBP)1 (Brosius *et al.,* 2010).

Plasminogen activator inhibitor-1 activation

Multiple studies have found reductions in plasmin activity, due in part to inhibition of tissue plasminogen activator by enhanced levels of plasminogen activator inhibitor (PAI)-1 in DN. Decreased protease activity of plasmin leads to enhanced accumulation of ECM proteins, such as fibronectin (Brosius *et al.,* 2010).

Increased TGF- βsignaling

Subjected to high glucose in diabetes, the glomerular epithelial cell responds by increasing the expression of TGF-β. Podocyte TGF-β may participate in GBM thickening. In a high glucose environment, podocytes increase their deposition of collagen IV (Brosius *et al.,* 2010).

Increased MCP-1/CCR2 signaling

Monocyte chemoattractant protein-1 - a potent chemoattractant of monocytes and macrophages levels are upregulated in glomeruli and tubules of diabetic rodents. Podocyte MCP-1 production increases in response to metabolic mediators, such as AGEs and, especially, TGF-β1 (Brosius *et al.*, 2010).

Increased Wnt/ β-catenin signaling

The Wnt/β-catenin system activity is increased in DN. Overexpression of Wnt-1 exacerbates podocyte foot processes effacement and albuminuria. Similarly, activation of β-catenin results in significant albuminuria. The Wnt/β-catenin pathway downregulates nephrin or redistributes it. β-catenin signaling is actively modulated by integrin-linked kinase (ILK). With ILK significantly upregulated in the diabetic podocyte, the ILK/β-catenin pathway may constitute a mechanism by which podocytes sense GBM dysregulation and react by changing their foot process morphology and slit diaphragm composition (Brosius *et al.*, 2010).

Increased VEGF signaling

Podocytes are the major source of renal VEGF. In most animal models of DN, VEGF levels are elevated. In humans, VEGF levels and downstream signaling are increased in early stages of DN but then appear to be reduced below normal with progressive DN. This early increase in VEGF levels may be due directly to increases in glucose or to

increased signaling via TGF-β, angiotensin II, and hypoxia-inducible factor. Secreted VEGF activates podocytes and induces collagen IV synthesis via PI3K signaling. While VEGF plays a role in podocyte signaling abnormalities that promote changes of early DN, it also has podocyte protective effects, by promoting cell healing in the face of increased stress in diabetes. VEGF has also been strongly implicated in endothelial alterations in DN. As VEGF levels fall in later DN, endothelial growth is halted and apoptosis may occur, leading to capillary loss and perhaps contributing to glomerulosclerosis (Brosius *et al.*, 2010).

Reduction in eNOS

Vascular eNOS activity is altered in diabetes, leads to lower production of NO, and is associated with the development of advanced nephropathy in patients with diabetes. Most reports suggest higher levels of NO production early in diabetes but reduced levels in progressive DN, analogous to the pattern with VEGF. VEGF stimulates eNOS activity in the glomerulus and may exert some of its protective effects through this mechanism. Hyperglycemia reduces eNOS activity in cultured endothelial cells in part by enhancing mitochondrial production of superoxide (Brosius *et al.*, 2010).

Reduced levels of thrombomodulin & activated protein C

Protein C is activated by binding of thrombin to its receptor, thrombomodulin. Activated protein C has been shown to have anti-inflammatory, anti-apoptotic, anti-

thrombotic and fibrinolytic effects. In diabetic patients, function of the endothelial thrombomodulin-protein C system is impaired, therefore, protein C activation is reduced. Mesangial ECM expansion is enhanced in the diabetic mice with impaired protein C activation (Brosius *et al.*, 2010).

Enhanced inflammation & NF- kB activation

Activation of NF-kB, a key inflammatory signaling molecule, appears to be an important part of tubulointerstitial fibrosis and progressive decline in renal function in progressive DN (Brosius *et al.*, 2010).

F.1. Adenosine-Monophosphate Protein Kinase (AMPK)

Studies show that abnormalities in the AMPK signaling pathway also play a major role in DN.

F.1.1. Overview

Adenosine-Monophosphate Protein Kinase (AMPK) belongs to the family of energy sensing enzymes (Misra *et al.*, 2007). It is a downstream constituent of a pathway composed of protein kinases, and is activated by cellular stresses that deplete ATP. AMPK plays a crucial role in protecting cellular functions under energy-restricted conditions, and inhibits mitochondrial ROS production (Viollet *et al.*, 2012). An increase in the AMP/ATP ratio activates AMPK through a mechanism requiring phosphorylation of the α subunit by upstream kinases (AMPKKs) namely LKB1. Upon

activation, AMPK restores cellular ATP by inhibiting ATP consuming cellular processes, and promoting ATP producing processes. AMPK activation occurs by phosphorylation of the catalytic domain at the Thr^{172} site (Misra *et al.*, 2007). AMPK, a serine/threonine kinase, is a heterotrimeric protein consisting of 3 subunits, namely α (isoforms α1 and α2) catalytic subunit, β (β1 and β2) and γ (γ1, γ2, and γ3) regulatory subunits (Eid *et al.*, 2010). The regulatory subunits play a role in maintaining the stability of the complex.

F.1.2. Role of AMPK in Diabetes

Studies show that high glucose - hyperglycemic milieu - inhibits AMPK activation, and increases NOX 1, NOX 4, and NADPH oxidase activity (Eid *et al.*, 2013). Moreover, diabetes-induced renal hypertrophy correlates with diminished AMPK activity and, at the same time, with increased protein synthesis under high glucose conditions (Hallows *et al.*, 2010). It can be concluded from these studies that in DM, there is inhibition of AMPK phosphorylation, thus increasing hypertrophy and ROS production, which in turn exacerbates renal injury, aiding in the progression of DN.

G. Overview of the Anti-Diabetic Drug: Metformin

Metformin is a member of the biguanide family of drugs. It is the primary drug of choice for the treatment of T2D. It doesn't cause hypoglycemia, it is rather an antihyperglycemic agent. It is also an insulin sensitizer, it affects insulin resistance only

without affecting its secretion. It also reduces liver glucose output, probably by inhibition of gluconeogenesis (Stumvoll *et al.,* 1995). It works via several mechanisms, among which is peroxisome proliferator-activated receptor (PPAR)-α. Its target molecule was not known initially, however, it has been discovered that the drug targets AMP-activated protein kinase. Interestingly, Metformin does not activate AMPK by direct phosphorylation via LKB1 – an upstream kinase; instead, its influence on the mitochondria activates AMPK as a secondary effect (Viollet *et al,.* 2012).

H. Hypothesis and Aims of the study

We have shown in our laboratory that diabetes causes the upregulation of CYP 4A in the kidneys. This increase causes the accumulation of ROS in the kidney cortex. On the other hand, the inactivation of AMPK due to diabetes also results in ROS formation and accumulation. In this study, we hypothesize that the upregulation of CYP 4A is a direct factor inducing the inactivation of AMPK. We also propose that the two factors belong to a common signaling pathway, where CYP 4A is upstream of AMPK, whereby diabetes induces this pathway leading to the formation of ROS, thereby increasing the OS in the kidney cortex (Fig. 5). In order to test this hypothesis, this study aims to:

- Identify the alteration in the levels of CYP 4A and pAMPK in the kidney cortex *in-vivo* in the presence of diabetes.
- Investigate the correlation between the variation of their levels and the amount of ROS formation.

- Examine the correspondence between these variations, and establish their link, thus elucidating the components and order of the signaling pathway involved.

In short, in this project we intend to identify the crosstalk betweek AMPK, CYPS, and ROS production that are shown to play a major role in DN. This will, in turn, set the stage to identify and develop new therapeutic measures targeting any of these molecules, in order to treat diabetic patients or in the least alleviate the sequelae of the disease.

Working Hypothesis

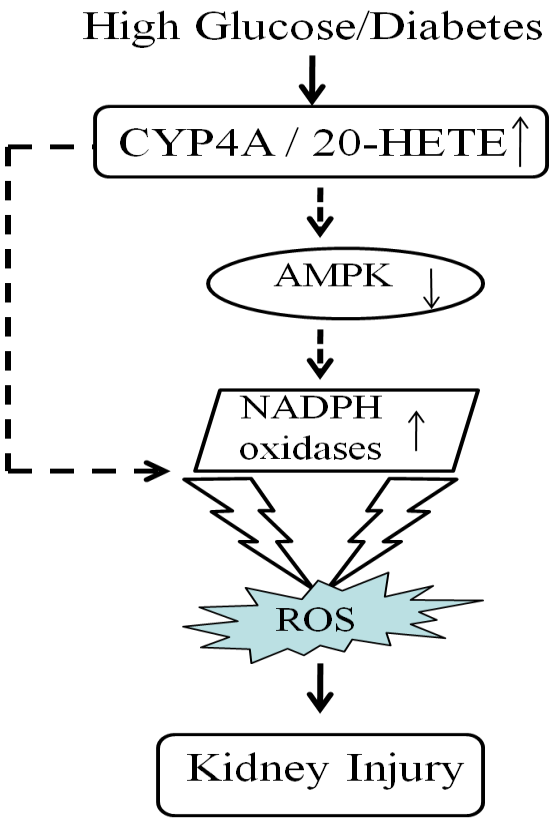

Figure 4. Proposed mechanism of diabetes-induced kidney injury.

Materials and Methods

A. Animal Models

Male Sprague-Dawley rats weighing between 150-200 grams were used in the study. Rats were kept in a temperature conditioned room at 37˚C on a 12/12 dark/light cycle. Food and water were available ad libitum.

In order to induce type 1 diabetes, the rats were injected intravenously through the tail vein with Streptozocin (Sigma-Aldrich, Steinheim, Germany – cat#S0130-1G) at a concentration of 55 mg/kg dissolved in 0.01 M sodium citrate buffer (pH 4.5). Two days after streptozocin (STZ) injection, blood glucose levels were checked using the glucometer (Accu-Check). Animals with blood glucose level more than 250 mg/dl were considered diabetic, and were included in the study. For the purpose of this study, 2 sets of rats were used.

In the first set, the rats were divided into 3 groups of 5 animals each. Rats in group 1 were control rats injected intraperitoneally with sodium citrate buffer. Rats in group 2 were rendered diabetic by STZ injections. Rats in group 3 were rendered diabetic and treated with Metformin (Glucophage – Merck)(100mg/kg intraperitoneally qd). The treatments were initiated 6 weeks after the STZ injections, and continued for 6 weeks, after which they were euthanized and sacrificed.

In the second set, the different groups of rats were injected with STZ followed by a daily injection with 5 units of NPH insulin supplemented with regular insulin (Novo

Nordisk Pharmaceuticals Inc., Princeton, NJ) subcutaneously or were treated with a specific inhibitor of CYP4A, the *N*-hydroxy-*N*-(4-butyl-2 methylphenol) formamidine; HET0016 (2.5 mg/kg/day; subcutaneously). Het0016 is a N′-hydroxyphenylformamidine derivative which was proven to be a potent and highly selective 20-HETE synthase inhibitor (Sato *et al.,* 2001). Drugs were administered 72 h after STZ injection for one month, after which they were euthanized and sacrificed.

All rats were maintained in accordance with protocols approved by the Institutional Animal Care and Use Committee of the American University of Beirut.

When the duration of the treatments ended, both kidneys were removed and weighed. A slice of the kidney cortex at the pole was embedded in paraffin or flash-frozen in liquid nitrogen for histological studies, western blotting, and image analyses. Tubules were isolated by different sieving techniques (Eid *et al.*, 2009; 2010) and used for microsome isolation, NAD(P)H oxidase activity measurements and western blotting.

B. Protein Lysis

Cuts of kidney cortex were dounced with RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS) ,0.5% sodium deoxylate, 150mM sodium chloride, 50mMTris-hydrochloride, 100 mM EDTA, 1% Tergitol (NP40), and 1% of the protease and phosphatase inhibitors. The lysates were then kept on a rotator for 2

hours at 4°C. Afterwards, they were centrifuged at13,600 rpm for 30 minutes at 4°C as well. The supernatant was then taken discarding the cell debris.

C. Isolation of microsomes

Tubules isolated from kidney cortex were homogenized in a 10 mmol/l potassium phosphate buffer, pH 7.7, containing 250 mmol/l sucrose, 1 mmol/l EDTA, 10 mmol/l magnesium chloride, 2 μmol/l leupeptin, 1 μmol/l pepstatin, 2 μg/ml aprotinin, and 0.1 μmol/l PMSF. Microsomes were prepared by differential centrifugation as described (Eid *et al.*, 2009)(Wolf *et al.*, 1999) and used for CYP 4A protein detection by Western blotting and 20-HETE measurement by high-performance liquid chromatography (HPLC).

D. Western Blot Analysis

Proteins in the supertant were quantified using DC Protein Assay (Bio-Rad laboratory, Ca, USA). For immunoblotting, 40μg of proteins were separated on 10% Polyacrylamide gel Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The membranes were blocked with 5% low fat milk in Tris-buffered saline and then incubated with rabbit polyclonal anti-Nox1(1:1000; Santa Cruz Biotechnology), rabbit polyclonal anti-Nox 4 (1:500, Santa Cruz Biotechnology), rabbit polyclonal phospho-AMPK α antibody (1:500, R&D), rabbit polyclonal AMPK α antibody (1:500, abcam), rabbit polyclonal anti-CYP4A12 (1:1000, abcam), rabbit polyclonal anti-collagen IV

antibody(1:100, abcam) and rabbit polyclonal anti-fibronectin antibody(1:7000, sigma-aldrich). The primary antibodies were detected using the appropriate horseradish peroxidase–conjugated IgG (1:1000, Bio-Rad). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software.

E. Detection of Intracellular ROS

DHE (Dihydroethidium) was used to measure intracellular ROS in kidney cortex. DHE or hydroethidine is a cell-permeable compound that, upon entering the cells, interacts with $O_2^{\bullet-}$ to form 2-hydroxyethidium, which in turn interacts with nucleic acids to emit a bright red color detectable qualitatively by fluorescent microscopy. Studies have proven that it detects essentially superoxide radicals, is retained well by cells, and may even tolerate mild fixation (Owusu-Ansah *et al*., 2008). This assay has been adapted for specific and quantitative detection of $O_2^{\bullet-}$ from biological samples, making it the best compound to use in detecting intracellular ROS (Cai *et al.*, 2007).

Parrafin embedded tissues were cut using a microtome at a 4 micron thickness. DHE solution with a concentration of 5μM was put to cover the tissues, and incubated for 30 minutes, at 37˚C. ROS was then detected at 2 time points (t=0 and t=30 mins). DHE was measured in cortical tissues as described (Maalouf *et al.*, 2012). Fluorescence was measured at a wavelength of 560nm using a Zeiss LSM710 Laser

Confocal Microscope. Images were taken at 40X magnification lens from different fields. Quantification was done using the Zen light Software.

F. NADPH Oxidase Assay

NADPH oxidase activity was measured by the lucigenin-enhanced chemiluminescence method as described (Eid *et al.*, 2009; 2010). Superoxide production was expressed as relative chemiluminescence (light) units (RLU)/mg protein. Protein content was measured using the Bio-Rad protein assay reagent.

G. Histological Examination

The kidney cortices were processed and embedded in paraffin. Sections were cut using a microtome at 4 micron thickness. The slides were then stained with Periodic Acid Schiff stain and Masson trichrome stain for histological observations, and analysis of kidney damage. The PAS method works by exposing the tissue to periodic acid. This acts as an oxidizing agent which oxidizes glycol linkage of carbohydrates. This oxidation creates dialdehydes, that when exposed to Schiff's reagent, create an insoluble magenta compound which is similar to the basic fuchsin dye within the Schiff's reagent. The method is useful as a reaction to detect accumulation of carbohydrates in tissues like glycogen, mucin, and basement membrane. (McManos *et al.,* 1948). Masson Trichrome stain is another histochemical stain that aims to detect the accumulation of collagen fibers. It is

useful to study areas of renal interstitial and glomerular fibrosis, and matrix expansion.

H. 20-HETE production

Levels of 20-HETE were measured in isolated microsomes by HPLC as described (Eid *et al.,* 2009). In short, [1-14C]-labeled arachidonic acid (50–100 μmol/l) was dried down and resuspended in the reaction mix containing 50 μg microsomes, 30 mmol/l isocitrate, and 0.2 unit isocitrate dehydrogenase in reaction buffer (100 mmol/l potassium phosphate, pH 7.4, 5 mmol/l magnesium chloride, and 1 mmol/l EDTA). After incubation at 37°C for 5 min, the reaction was initiated by the addition of NADPH to a final concentration of 1 mmol/l. Aliquots were removed at 30, 60, and 90 min, and the reaction was stopped by the addition of 100% methanol. The precipitated proteins were then pelleted by centrifugation (using a microcentrifuge), and the samples were stored at -20°C until analyzed. The metabolites were separated via HPLC on a C-18 column using an acetonitrile/H20 gradient and identified by coelution with labeled standards.

I. Immunohistochemistry

Localization of cellular fibronectin and CYP4A were assessed by immunoperoxidase histochemistry using mono- or polyclonal antibodies. Frozen cortical sections (6 μm thick) were fixed and permeabilized in acetone for 10 min and then rehydrated in PBS-0.1% BSA for 15 min. Sections were incubated with

0.6% hydrogen peroxide in methanol to block nonspecific peroxidase activity and 0.01% avidin, 0.001% biotin to block localization of endogenous activity before the addition of the appropriate blocking immunoglobulin for 15 min. Sections were incubated with primary antibodies for 30 min in a humidified chamber at room temperature. They were then washed three times in PBS-0.1% BSA and then incubated with biotinylated secondary anti-mouse IgG for 30 min at room temperature. Bound antibody was identified by immunoperoxidase ABC staining following the manufacturer's instructions (Vector Laboratories, Burlingame, CA). The sections were then dehydrated and mounted with Permount (Sigma) and viewed by bright-field microscopy.

J. Statistical Analysis

The data were analyzed and expressed as mean ± S.D (n = 3). The statistical difference between the normal, diabetic, and treated was analyzed by paired t-test. The results were considered statistically significant, if $p\text{-}value < 0.05$.

Results

A. Hyperglycemia induces kidney hypertrophy

Blood glucose levels, body and kidney weights were studied in the different group of rats after 1 month of diabetes onset. Untreated diabetic rats, diabetic rats treated with HET0016, and diabetic rats treated with metformin had equivalently elevated blood glucose concentration at the end of the study period compared with the control rats, while rats treated with insulin showed a significant decrease in hyperglycemia. Body weights at the end of the study were similarly lower in the diabetic rats, in rats treated with HET0016, and in rats treated with metformin compared with control rats, while rats treated with insulin had their body weights similar to control rats. Total kidney weight and kidney hypertrophy assessed by kidney weight to body weight ratio were significantly increased in diabetic rats compared to non-diabetic control animals. Diabetic rats treated with insulin or with HET0016 showed a significant decrease in kidney weight and kidney weight to body weight ratio when compared to diabetic rats. However, diabetic rats treated with metformin, showed an increase in kidney weights and kidney weight to body weight ratio when compared to control rats (table 1,2).

Table 1. Glucose level, body weight, kidney weight, kidney weight to body weight ratio after 1 month of diabetes. Values are the means ±S.E. from 10 animals (n =10) for each group.					
Group	**n**	**Blood Glucose (mg/dl)**	**Body weight (g)**	**Kidney Weight (g)**	**Kidney weight/body weight (g/kg)**
Control	10	190 ± 17.6	310 ± 5.0	1.047 ± 0.05	3.342 ± 0.09
Diabetes	10	402 ± 25.4*	242 ± 9.6*	1.298 ± 0.05*	5.412 ± 0.19*
Diabetes + HET0016	10	398 ± 21.3*	264 ± 11.3*	1.098 ± 0.05#	4.186 ± 0.17#
Diabetes + Insulin	10	210 ± 18.5#	295 ± 14.3	1.056 ± 0.04#	3.765 ± 0.20#
*$p \leq 0.05$ *versus* control rats. #$p \leq 0.05$ *versus* diabetic rats.					

Table 1: Glucose level, body weight, kidney weight, kidney weight to body weight ratio of control rats, STZ-induced type 1 diabetic rats, type 1 diabetic rats treated with insulin and type 1 diabetic rats treated with HET0016. Values are the means ± S.E. from 10 animals for each group. *P<0.05 vs. control; #P<0.05 vs. type 1 diabetic rats.

Table 2. Glucose level, body weight, kidney weight, kidney weight to body weight ratio after 1 month of diabetes. Values are the means ±S.E. from 5 animals (n =5) for each group.					
Group	**n**	**Blood Glucose (mg/dl)**	**Body weight (g)**	**Kidney Weight (g)**	**Kidney weight/body weight (g/kg)**
Control	5	106.6 ± 4.9	344 ± 10.1	1.669 ± 0.04	4.861 ± 0.07
Diabetes	5	558 ± 82.9*	272 ± 12.2*	1.775 ± 0.15	6.477 ± 0.34*
Diabetes + Metformin	5	542.4 ±63.5*	284.8 ±20.7*	1.813 ± 0.09	6.493 ± 0.51*
*$p \leq 0.05$ *versus* control rats.					

Table 2: Glucose level, body weight, kidney weight, kidney weight to body weight ratio of control rats, STZ-induced type 1 diabetic rats, and type 1 diabetic rats treated with metformin. Values are the means ± S.E. from 5 animals for each group. *P<0.05 vs. control

B. Hyperglycemia induces CYP4A protein expression and 20-HETE formation.

The levels of CYP4A and 20-HETE formations were assessed in the different groups of rats. There was a significantly higher expression of CYP4A in proximal tubular microsomes, isolated from diabetic animals compared to those in controls. As expected, the induction of CYP4A expression was greatly reduced in diabetic rats treated with HET0016 (***Fig. 6A, 6B***), a well-known potent inhibitor of CYP4A, and thus 20-HETE formation. Also, levels of CYP4A protein expression were significantly lower in diabetic rats treated with insulin when compared to diabetic rats, and were similar to those observed in control rats (***Fig. 6A, 6B***). Consistent with the induction of AA-metabolizing CYPs, metabolism of AA was increased in the diabetic animals, but fell back to control levels in the diabetic animals treated with insulin or with HET0016. The levels of 20-HETE followed the same pattern (***Fig. 6C***, ***6D)*** as shown by a chromatogram of HPLC separation of AA and the various metabolites formed in the reaction.

Figure 6:

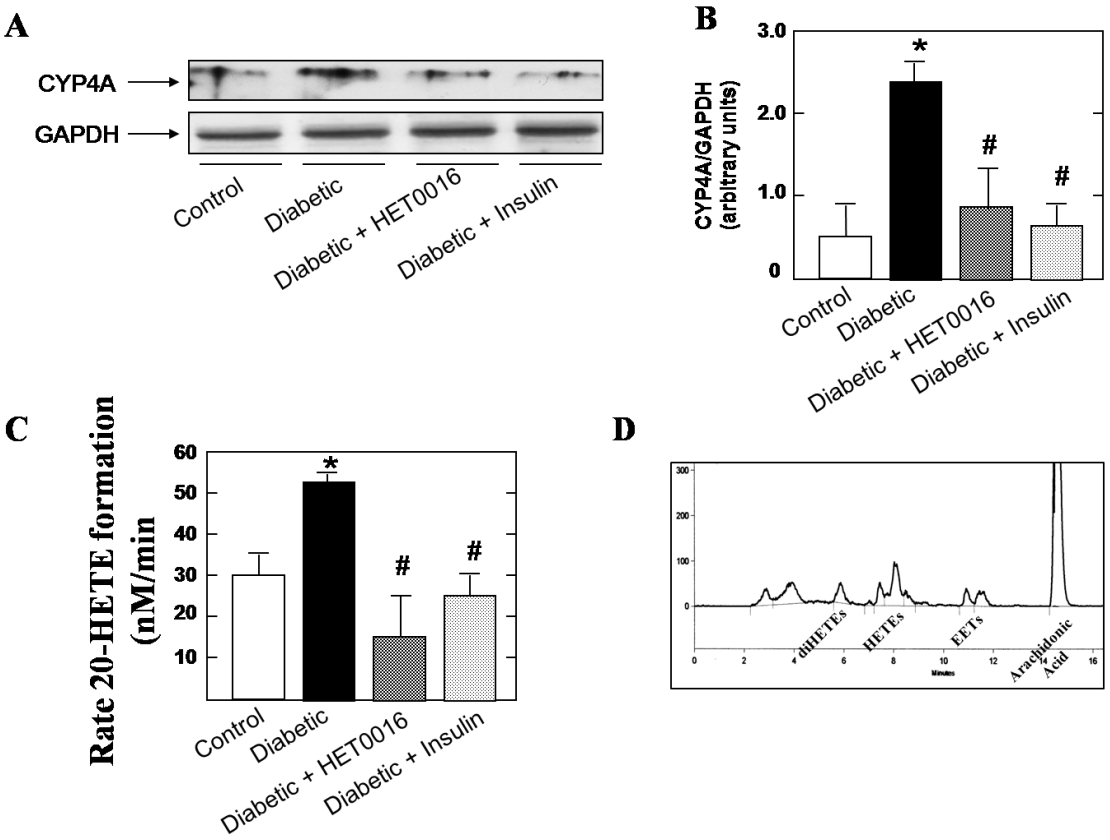

Figure 5. Hyperglycemia induces CYP4A protein expression and 20-HETE formations. *A and B,* expression of CYP4A protein was determined by direct immunoblotting of homogenized microsomes isolated from proximal tubules isolated from kidney cortex. GAPDH was included as a control for loading and the specificity of change in protein expression. Representative results of Western blot analysis were obtained from 5 independent samples from each group. *C,* Microsomes (0.25 mg of protein) prepared from isolated proximal tubules from kidney cortex were incubated with 10 μmol/l arachidonic acid in the presence of NADPH. 20-HETE was separated by reverse-phase HPLC. Values of five independent experiments (n = 5) are the means ± SE. *P<0.05 vs. control; $^{\#}$P<0.05 vs. type 1 diabetic rats. *D,* Typical HPLC chromatogram showing arachidonic acid metabolites produced by rat kidney microsomes.

C. CYP4A and 20-HETE mediate diabetes-induced kidney hypertrophy and tubulointerstitial changes in type 1 diabetic rats.

Renal hypertrophy, as measured by the increase in kidney weight/body weight ratio (***Table 1***), and tubulointerstitial changes, assessed by fibronectin expression (***Fig. 7A-7D***), was induced in diabetic rats when compared to control rats, concomitant with an increase in kidney CYP4A expression and 20-HETE formations (***Fig.6A-6C***). Hyperglycemia-induced hypertrophy (***Table 1***) and increased fibronectin expression (***Fig. 7A-7D***) were abrogated in the kidneys of diabetic rats treated with HET0016 or treated with insulin. Our results indicate that diabetes has a significant effect on the expression levels of AA-metabolizing CYP4A in the rat kidney, which is manifested as increased AA metabolism, and might thus be expected to alter kidney function due to alteration of 20-HETE produced.

Figure 7:

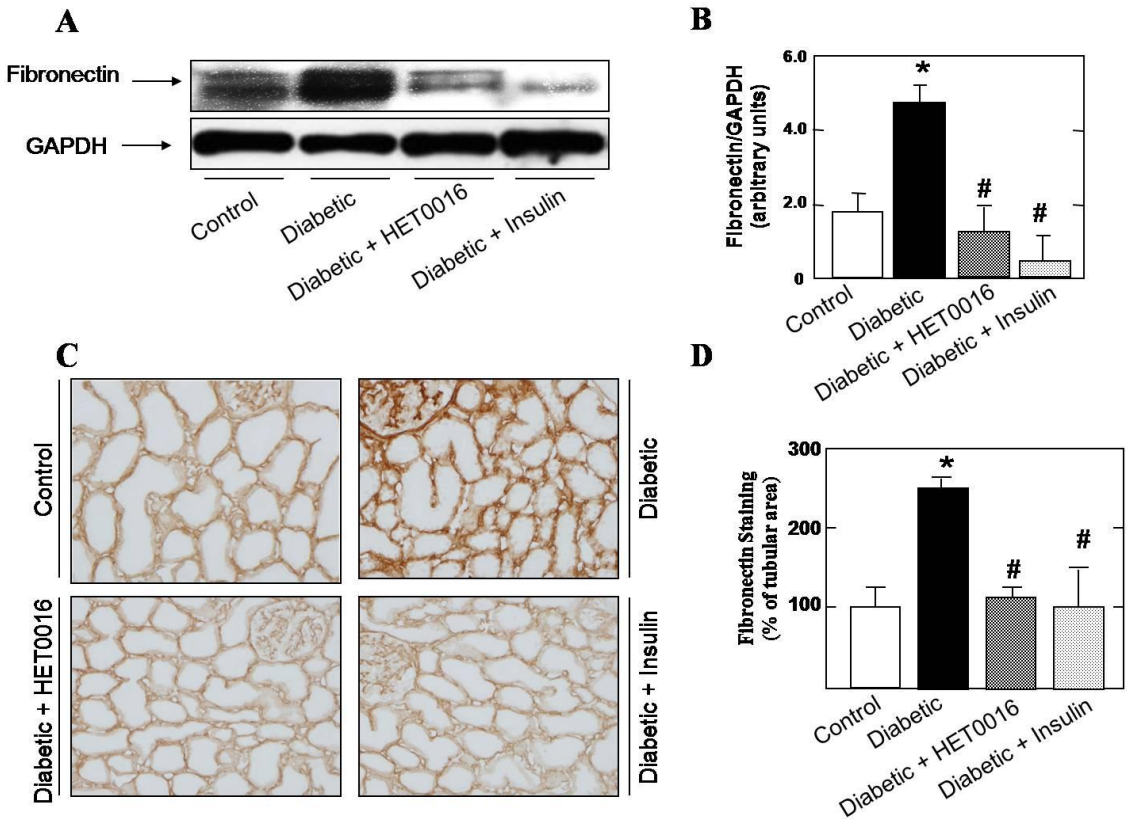

Figure 6. CYP4A and 20-HETE mediate diabetes-induced fibronectin expression in type 1 diabetic rats. *A and B,* expression of fibronectin protein was determined by direct immunoblotting of homogenized tubules isolated from kidney cortex. GAPDH was included as a control for loading and the specificity of change in protein expression. Representative results of Western blot analysis were obtained from 5 independent samples from each group. Values are the means ± SE. *P<0.05 vs. control; $^{\#}$P<0.05 vs. type 1 diabetic rats. *C,* fibronectin localization were detected by anti-fibronectin using immunoperoxidase staining of kidney sections from control, diabetic rats, and diabetic rats treated with either HET0016 or insulin. D, quantitation of fibronectin expression in kidney cortical tubules using the Image-Pro Plus 4.5 software. The histograms represent means ± S.E. of 25 individual sections from six individual rats in each group. *P<0.05 vs. control; $^{\#}$P<0.05 vs. type 1 diabetic rats.

D. AMPK activation by metformin reduces hyperglycemia-induced matrix protein accumulation, glomerular hypertrophy, and collagen IV deposition.

The levels of collagen IV and fibronectin were evaluated in the different groups of rats. There was a significant increase in the expression of both type IV collagen and fibronectin in the kidney cortices taken from diabetic rats compared to that in controls. Predictably, the activation of AMPK greatly reduced these levels in diabetic rats treated with metformin (***Fig. 10A-10D***). Histological examination showed that there were high levels of matrix protein accumulation and collagen deposition in the diabetic cortex. PAS stains showed higher levels of mesangial matrix deposition, and an increased glomerular size. Meanwhile, slides stained with Masson's trichrome stain showed increased collagen deposition in the kidney cortex. AMPK activation, i.e. treatment with metformin, showed a decrease both in matrix accumulation and collagen depositions when compared to that of diabetic rats, as well as a decrease in the glomerular volume (***Figs. 8A-8B, 9***). These results provide evidence that hyperglycemia induces hypertrophy by inactivating AMPK.

Figure 8:

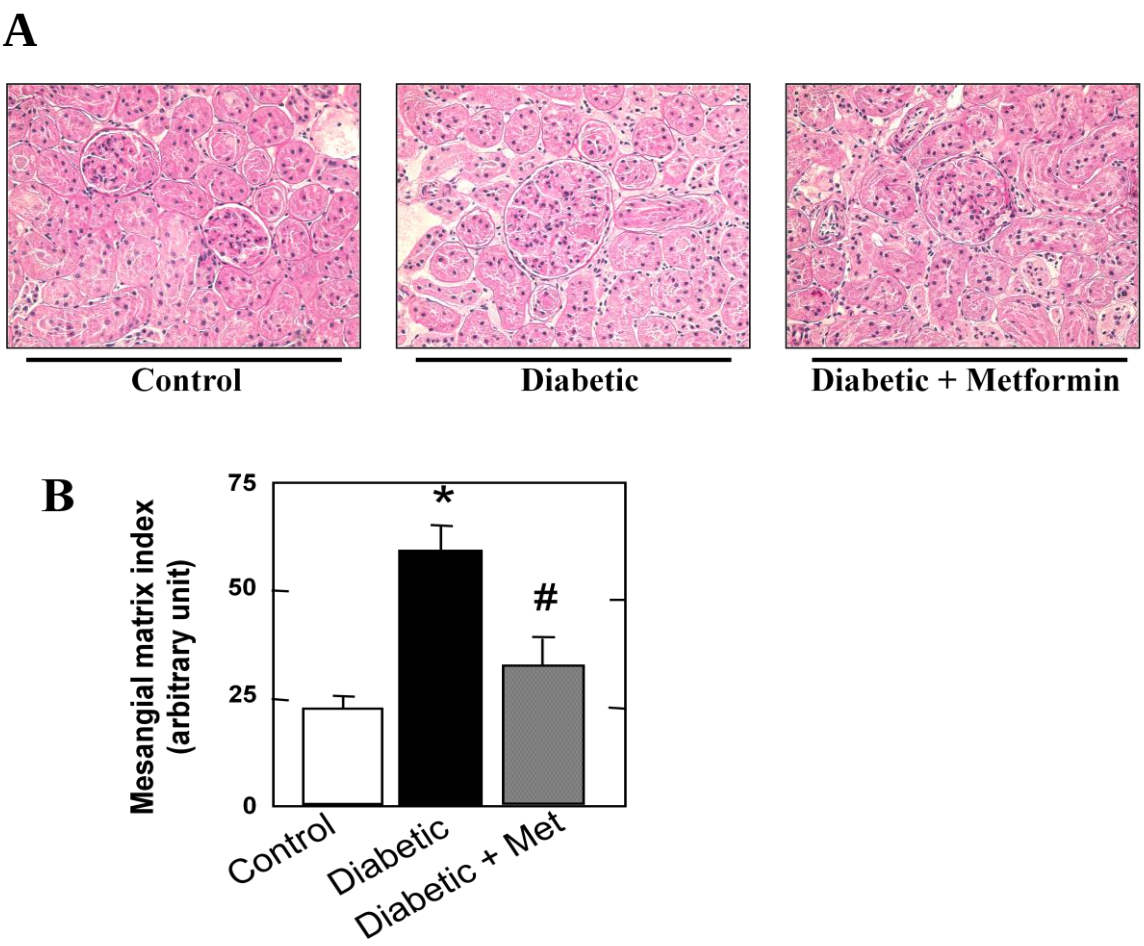

Figure 8. AMPK activation by metformin reduces hyperglycemia-induced matrix protein accumulation and glomerular hypertrophy. Cortical kidney sections stained with periodic acid-Schiff (PAS) and the increase in mesangial matrix was measured in different Group of rats. (A)The pictures display representative glomeruli and Proximal tubules of PAS-stained sections in the different group of rats. (B) The mesangial matrix index represented the ratio of mesangial matrix area divided by tuft area. All data are means ± SD from 6 different rats. $^{*}P < 0.05$ vs. diabetic, $^{\#}P < 0.05$ vs. diabetic + metformin**.**

Figure 9:

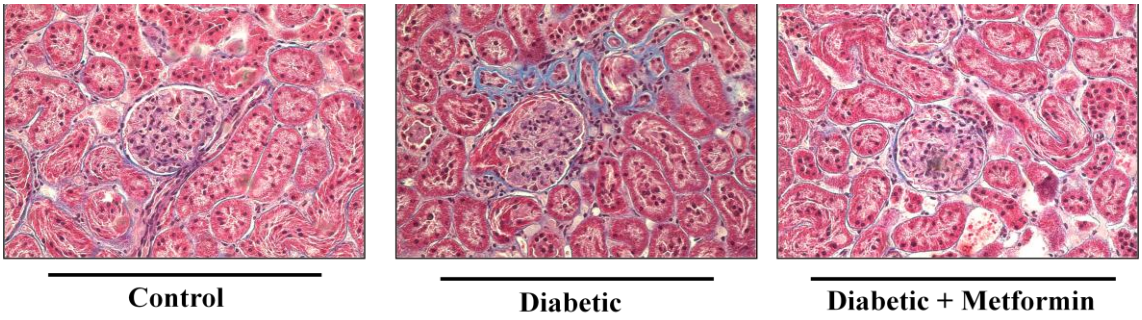

Figure 9. AMPK activation by metformin reduces collagen deposition. Cortical kidney sections stained with Masson's trichrome stain.The pictures display representative glomeruli and Proximal tubules of trichrome-stained sections in the different group of rats.

Figure 10:

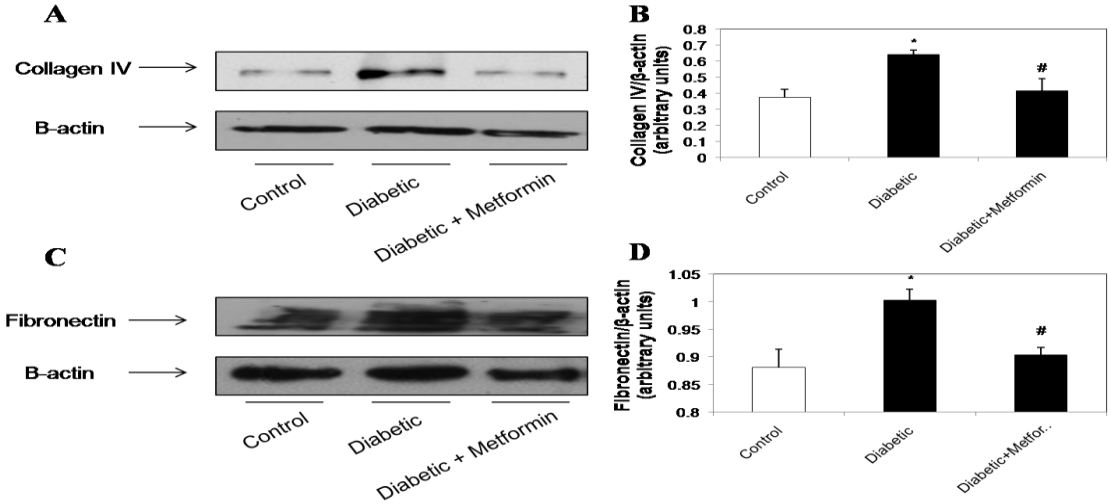

Figure10. **AMPK activation by metformin reduces hyperglycemia-induced matrix protein accumulation, and collagen deposition.** *A and B,*expression of collagen IV protein was determined by direct immunoblotting of homogenized kidney cortex. B-actin was included as a control for loading and the specificity of change in protein expression. Representative results of Western blot analysis were obtained from 3 independent samples from each group. Values are the means ± SE. *P<0.05 vs. control; #P<0.05 vs. type 1 diabetic rats. *C and D,* expression of fibronectin protein was determined by direct immunoblotting of homogenized kidney cortex. B-actin was included as a control for loading and the specificity of change in protein expression. Representative results of Western blot analysis were obtained from 3 independent samples from each group. Values are the means ± SE. *P<0.05 vs. control; #P<0.05 vs. type 1 diabetic rats.

E. 20-HETE generated by CYP4A mediates the effect of diabetes on ROS generation and NADPH oxidase activity.

Diabetes and hyperglycemia are accompanied by increased generation of reactive oxygen species (ROS). In our study, ROS production, assessed by superoxide measurements was performed in the different groups of rats. As expected, hyperglycemia induced ROS production in diabetic rats (***Figs. 11A-11B***). Hyperglycemia-induced ROS production was inhibited in diabetic rats treated with either HET0016 or insulin (***Figs. 11A-11B***). Our results also showed that NADPH dependent superoxide generation was increased in diabetic rats when compared to control rats and this increase was inhibited by both HET0016 and insulin (***Fig. 11C***). These results suggest a crosstalk between CYPs alteration and NADPH oxidase activation known to play a major role in diabetic nephropathy.

Figure 11:

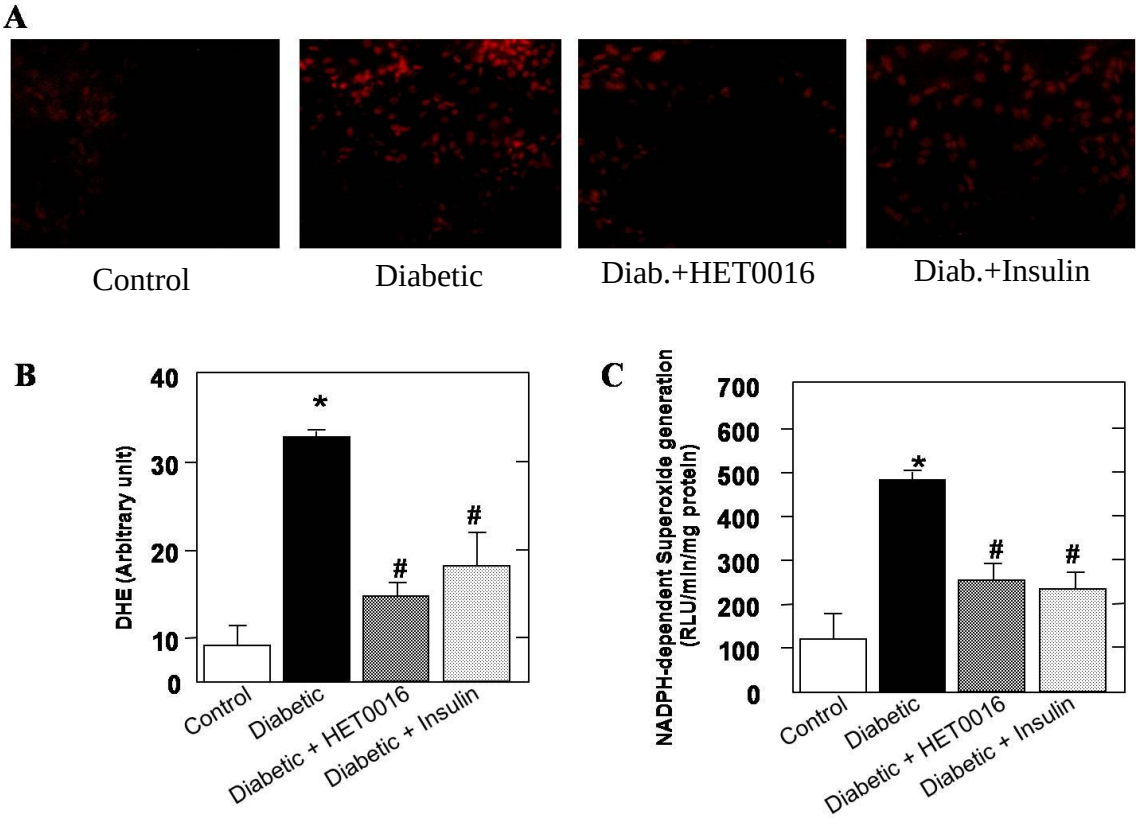

Figure 11. **20-HETE generated by CYP4A mediates the effect of high glucose on ROS generation and NADPH oxidase activity.** *A,* Superoxide production assessed by DHE staining. *B,* Quantitation of ROS production in kidney cortex using the Image-Pro Plus 4.5 software. The histograms represent means ± S.E. of 25 individual cortical areas from six individual rats in each group. *P<0.05 vs. control; #P<0.05 vs. type 1 diabetic rats. *C,* NADPH oxidase activity in cortical homogenates. NADPH-driven superoxide production was expressed as RLU/min/mg protein. Values are the means ± S.E. of the activities from the kidney cortex of six animals for each group. *P<0.05 vs. control; #P<0.05 vs. type 1 diabetic rats.

F. AMPK activation by metformin treatment reduces NADPH oxidases NOX1 and NOX4 protein expression in diabetic rats.

The levels of expression of the NADPH oxidases NOX1 and NOX4 in the kidney cortices were assessed. NOX1 and NOX4 levels were notably higher in diabetic animals compared to those of controls. Consistent with our results, the activation of AMPK by metformin significantly decreased the expression levels of NOX1 and NOX4 in diabetic rats treated with metformin (***Fig. 12A-12D***). These results suggest a crosstalk between AMPK inactivation and the increase in NADPH oxidases protein expression, both occurring in diabetes.

Figure 12:

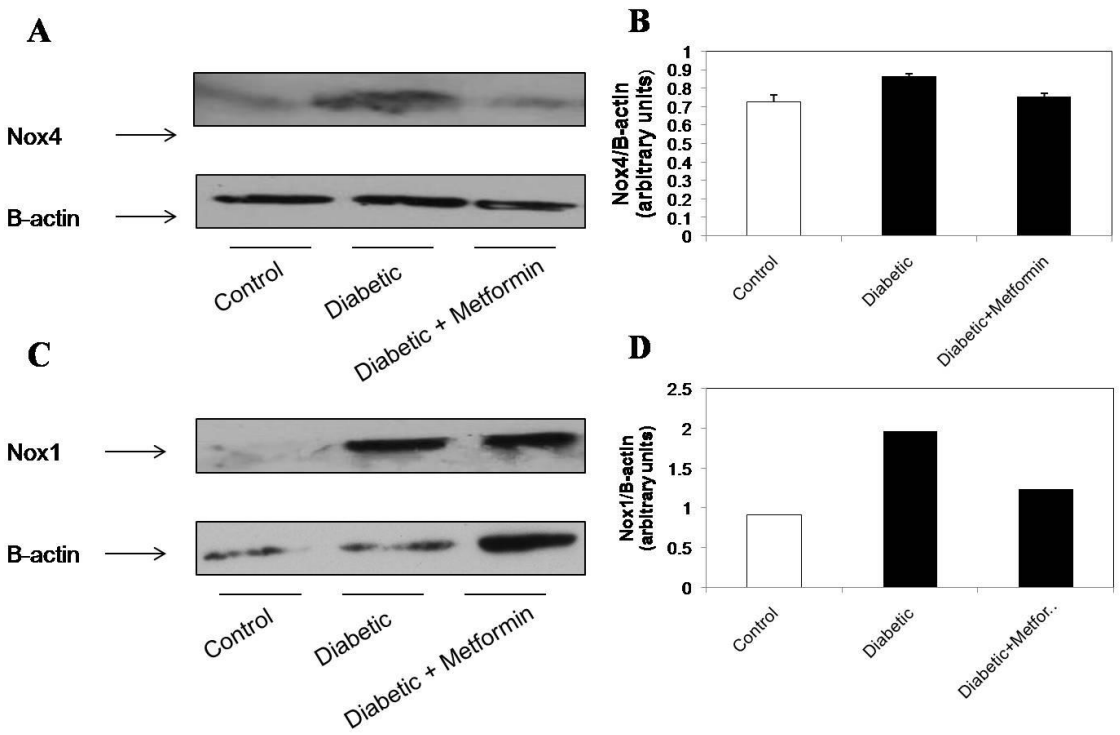

Figure 7. AMPK activation by metformin treatment reduces NADPH oxidases NOX1 and NOX4 protein expression in diabetic rats. *A and B*, expression of NOX4 protein was determined by direct immunoblotting of kidney cortex. B-actin was included as a control for loading and the specificity of change in protein expression. Representative results of Western blot analysis were obtained from 1 sample from each group. *C and D*, expression of NOX1 protein was determined by direct immunoblotting of kidney cortex. B-actin was included as a control for loading and the specificity of change in protein expression. Representative results of Western blot analysis were obtained from 1 sample from each group.

G. CYP4A and 20-HETE mediate hyperglycemia-induced AMPK inactivation.

In order to further dissect the mechanisms leading to DN, we performed experiments to study if there is a correlation between CYP4A/20-HETE alteration and AMPK inactivation. The assessment was done by comparing the level of p-AMPK expression relative to AMPK expression. As predicted, the level of p-AMPK expression was markedly decreased in diabetic rats compared to control rats, and thus AMPK activation was decreased. However, levels of p-AMPK increased significantly in rats treated with HET0016. Also, rats treated with insulin brought back AMPK phosphorylation close to control levels. This result suggests a crosstalk between CYP 4A production and AMPK activation, hinting that both are involved in the same mechanistic signaling pathway. (***Fig.13A-13B***)

Figure 13:

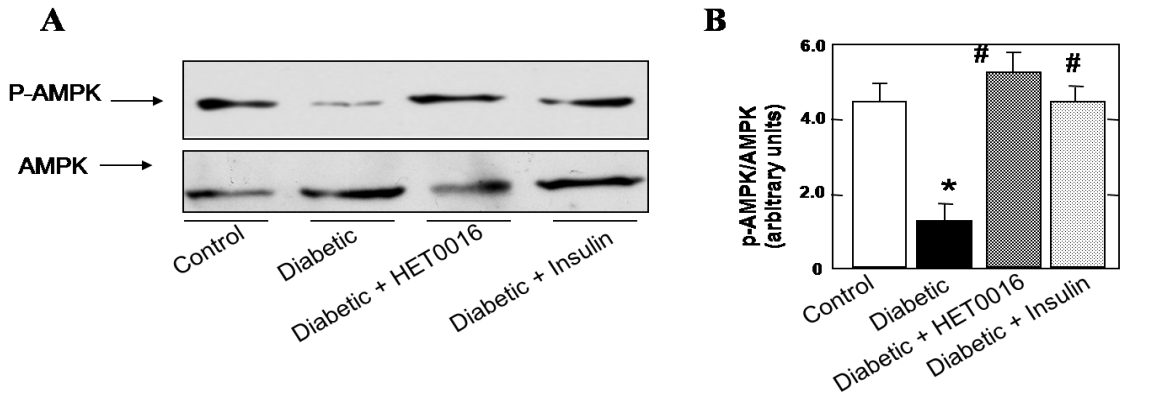

Figure 8. CYP4A and 20-HETE mediates hyperglycemia-induced AMPK inactivation. *A and B*, expression of p-AMPK protein was determined by direct immunoblotting of kidney cortex. AMPK was included as a control for the specificity of change in protein expression. Representative results of Western blot analysis were obtained from 5 independent samples from each group. Values are the means ± SE. *P<0.05 vs. control; $^{\#}$P<0.05 vs. type 1 diabetic rats.

Discussion

Hyperglycemia has been shown to be the direct causative of renal hypertrophy in diabetic patients. Renal hypertrophy is manifested macroscopically by the enlargement of kidneys, and microscopically by the increase in cell size and in expression levels of matrix proteins (Bilous *et al.,* 1989). Hypertrophy is one of the major and primary indications for the progression of DN (Wolf *et al.,*1999). Oxidative stress has also emerged as a critical pathogenic factor in the development of DN (Baynes *et al.,*1991). Oxidative stress escalates in diabetes, due to the increase of ROS generation. Many studies show that elevated ROS production accompanies hyperglycemic conditions (Ha *et al.,* 1994)(Onozato *et al.,* 2002). Treatment with antioxidants has been attempted; however, the results were inconclusive (Kuroki *et al.,* 2003). The ROS in the kidneys are mainly, as many studies prove, produced by CYP 4A and multicomponent phagocyte-like NADPH oxidases – namely NOX1 and NOX4, which are major contributors to the development and progression of DN (Geist *et al.*, 2004)(Jones *et al.,* 1995). Being heme-containing monooxygenases, CYPs activate oxygen prior to incorporating it into a substrate; futile or redox cycling of the enzyme, where activated oxygen "escapes" before its incorporation into organic products, leads to the formation of superoxide and H_2O_2 (Puntarulo *et al.,* 1998). Cytochromes P450 have been shown, in certain studies, to be significant sources of oxidative stress in liver and coronary arteries (Fleming *et al.,* 2001). Recently, CYP 4A was shown to be upregulated under hyperglycemic conditions, and leads to the increased production of 20-HETE in the

kidneys (Eid *et al.*, 2013)(Schwartzman *et al.*, 1996). Many studies have shown that 20-HETE itself stimulates superoxide production (Guo *et al.*, 2007). In addition, there is evidence that 20-HETE mediates cytotoxicity and apoptosis in ischemic kidney tubular epithelial cells, and that it exerts its deleterious effect through the generation of ROS (Nilakantan *et al.*, 2008)(Baliga *et al.*, 1996). Therefore, inhibiting 20-HETE - by inhibiting CYP4A - inhibits NADPH oxidases, and thus diminishes the oxidative stress (Eid *et al.*, 2009). On another note, hyperglycemia has been also proven to cause the inactivation of AMPK. The inactivation of AMPK, according to some studies, is one reason for the elevated production of ROS as well (Eid *et al.*, 2010). Put together, the previously mentioned facts lead to the conclusion that diabetes increases the expression of CYP 4A, inactivates AMPK, raises levels of NADPH oxidases, and eventually leads to high levels of ROS in the kidneys. The accumulation of ROS escalates the oxidative stress in the kidney, aggravating DN in diabetic patients. In this study, we hypothesized that there exists a relation between CYP 4A and AMPK, linking the two in a single signaling pathway. For this reason, we studied the effect of diabetes on the: expression of CYP 4A, production of 20-HETE (A.A. metabolite by means of CYP 4A), matrix proteins accumulation, level of AMPK activation, expression of NADPH oxidases, and ROS production. In addition to diabetes alone, we studied the effect of HET0016 - inhibitor of CYP 4A -, Metformin - AMPK activator -, and insulin - an anti-hyperglycemic agent used to mimic low glucose conditions (control)- on diabetes.

Our results clearly show the hyperglycemia led to the development of hypertrophy

in the kidneys. This was proven by assessing kidney weight to body weight ratio, collagen and fibronectin protein expression - identified by western blot -, and accumulation - identified by histochemical stains. The kidney to body weight ratio was significantly increased in diabetic rats compared to control rats. This ratio was brought back to levels close to normal (control) by treatment with HET0016 and insulin. This measurement proved that Het0016 is able to reverse the effect of diabetes, and stop renal hypertrophy. Leading to the conclusion that CYP 4A is a contributor to renal hypertrophy. Fibronectin and collagen IV protein expression was remarkably elevated in diabetic rats; treatment with Het0016, metformin, and insulin decreased this expression to levels close to control levels. In parallel, histochemical staining showed that the accumulation of matrix proteins and collagen deposition was significantly increased in diabetic rats, and as expectedly treatment with Het0016, metformin, and insulin lessened this accumulation. The stains also showed an increased volume of the glomerulus in the diabetic rats, which decreased in the treated rats. The increased volume of the glomerulus in diabetic rats, without an increase in the glomerular number indicates that the individual cell size is increased. This leads to the conclusion that there is hypertrophy in diabetes, and it is partially reversed by the treatments. These two experiments confirm the claim arguing that higher levels of CYP 4A and the inactivation of AMPK are direct contributors for the upregulation and amassing of matrix proteins in the kidney cortex, characteristic of diabetic nephropathy.

Other experiments performed proved that hyperglycemia induces the expression of

CYP 4A, and thus increases the production of 20-HETE. We assessed the expression of CYP 4A and the results showed that it is raised in diabetic rats, and it diminishes significantly in diabetic rats treated with HET0016, as well as rats treated with insulin. Levels of Cyp 4A, being responsible for metabolizing AA, is directly proportional with the levels of 20-HETE, AA metabolite. HPLC chromatography showed an increase in AA metabolism, as well as an increase in 20-HETE production. This result is confirmed by previous studies published by our laboratory that show that high glucose causes the upregulation of CYP 4A, and thus the increased production of 20-HETE (Eid *et al.*, 2013).

In order to evaluate the oxidative stress, and levels of ROS production, a series of experiments were conducted on different groups of rats. The results showed that diabetes caused an elevation in the level of NADPH oxidases expression. NOX 1 and NOX4 levels increased in diabetic rats, and reduced back to levels close to normal in diabetic rats treated with metformin. NADPH oxidase activity was also measured, and was showed to increase in diabetic rats, and were decreased markedly in diabetic rats treated with HET0016, and diabetic rats treated with insulin. ROS generation was measured using fluorescence microscopy and DHE staining in all groups of rats. The results showed that ROS generation is enhanced in diabetic rats significantly compared to control rats. However, in diabetic rats treated with HET0016, metformin, or insulin, ROS generation was found to be drops back to levels close to control rats. NADPH oxidase activity, NOX1 and NOX4 expression, and ROS levels are indicators of

oxidative stress. So this series of experiments proved that diabetes causes an elevation in ROS production and oxidative stress. This is stopped by inhibiting CYP 4A, activating AMPK, or removing the hyperglycemic milieu. This verifies that oxidative stress rises due to the upregulation of CYP 4A on one hand, and due to inactivating AMPK on another hand.

The final experiment intended to show the variation of p-AMPK - the active form of AMPK - in relation with CYP 4A upregulation and inhibition. Using western blot, we showed that p-AMPK levels significantly diminishes in diabetic rats. p-AMPK expression increased notably, however, in rats treated with HET0016, and rats treated with insulin in comparison with the diabetic rats. The level of p-AMPK was compared relative to AMPK expression, in order to evaluate precisely the degree of activation, or inactivation, of AMPK under the different conditions. The results show that the elevation in CYP 4A levels causes the inactivation of AMPK, while the inhibition of CYP 4A activates AMPK in measurable quantities approaching control levels. Treatment with insulin also led to the same result as treatment with HET0016.

Many studies show that AMPK is inactivated by the action of hyperglycemia (Eid *et al.,* 2013) (Hallows *et al.*, 2010). However, little is known about the mechanism by which this inactivation occurs. Meanwhile, a study concerning AMPK activation and ROS production revealed that AMPK activation increases the production of ROS and loss of mitochondria membrane potential induced by high glucose (Kim *et al.*, 2007). For now, our study demonstrates a relation between CYP 4A and AMPK. Our results

suggest that hyperglycemia-induced upregulation of CYP 4A causes an increased production of 20-HETE, leading to the elevated production of ROS, and also contributing to renal hypertrophy. This notion is backed up by several studies showing that CYP 4A, in fact, contributes to renal hypertrophy and ROS accumulation in tissues due to the action of 20-HETE. Nonetheless, our results show that hyperglycemia causes the inactivation of AMPK, also leading to hypertrophy and increased generation of ROS. Subsequently, we can state that CYP 4A might regulate the activation of AMPK under hyperglycemic conditions. Our results substantiate the thought of CYP 4A and AMPK belonging to one signaling pathway, which is activated by hyperglycemia, where the rise in CYP 4A leads to ROS generation as well as AMPK inactivation. This inactivation, in turn, causes further production of ROS in the kidney tissue, hence adding to the oxidative stress in the kidney. Consequently, this signaling pathway leads to kidney injury, aiding in the progress of diabetic nephropathy, and ultimately leading to ESRD.

The main concern of our project was to investigate new pathways that are implicated in the initiation and progression of diabetic nephropathy. Our aims are targeted towards finding new therapeutic approaches to ease the consequences of diabetes and its complications, mainly nephropathy. The assertion of the presence of such a relation between CYP 4A and AMPK, with the proof that CYP 4A is upstream of AMPK opens new horizons for targeted therapy, and for new research to further elucidate this pathway, and other pathways, perhaps also linked to this one, in order to

achieve better therapies and discover new ways to stop the initiation of nephropathy, reverse its effects, or in the least stop its progression. Thus, in addition to the glycemic control, inhibitors of the CYP4A family may be therapeutically useful in the treatment of diabetic nephropathy.

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