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ROLE OF CYTOCHROME P450 PRODUCTS OF ARACHIDONIC ACID METABOLISM IN THE RENAL COMPLICATIONS OF DIABETES MELLITUS IN RATS

by
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AN ABSTRACT OF THE THESIS OF

Jessy Joseph Tabetfor Master of Science
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Title: Role of Cytochrome P450 Products of Arachidonic Acid Metabolism in the Renal Complications of Diabetes Mellitus in Rats

Background: Diabetic Nephropathy(DN) is a serious life threatening condition associated with both type I and type II diabetes. It is usually characterized by renal hypertrophy, extracellular matrix accumulation, protenuria which eventually accumulates into renal failure and kidney function loss. Early stages are also often associated with abnormal sodium load handling and consequent hypertension. Such processes are linked by the arachidonicacid(AA) metabolites 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) produced by several cytochrome P450 enzyme isoforms. AA metabolism is altered in rat models of type I diabetes.

Aim: The present study aims at determining the role of 20-HETE and various EETs in renal hypertrophy and extracellular matrix accumulation which contribute to the pathophysiology of diabetic nephropathy. In addition, the role of CYP450 enzymes as promising therapeutic agents in both the development and progression of diabetic nephropathy will be investigated.

Methods: Male Sprague Dawley rats were adopted as animal models for this experiment. Diabetes will be induced by Streptozotocin and renal parameters will be studied at different stages of diabetes. Urine protein excretion will be assessed. Western Blot analysis will be performed to determine levels of CYP4A, Nox4 and fibronectin. Trichrome stains will be used to assess collagen deposition, PAS stains to assess the level of proteoglycan deposition and DHE stain to assess ROS generation. Levels of 20-HETE and EET in urine will be measured at day 28 of the experiment.

Results: Diabetes is associated with renal enlargement, mesangial matrix deposition, collagen deposition, ROS production, fibronectin expression and an increase in the urinary excretion of 20-HETE. Treatment with either HET0016 or BNF reduces these injury indexes.

Conclusion: Our results indicate that hyperglycemia in diabetes has a significant effect on the expression of AA-metabolizing CYPs, manifested by increased AA metabolism, and might thus alter kidney function through alteration of type and amount of AA metabolites; this pathway is through an oxidative stress-dependent mechanism.

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ABBREVIATIONS

DN	Diabetic Nephropathy
20-HETE	20-hydroxyeicosatetraenoic Acid
EET	Epoxyeicosatrienoic Acids
AA	Arachidonic Acid
ROS	Reactive Oxygen Species
CYP450	Cytochrome 450
HET0016	N-hydroxy-N'-(4-butyl-2-methylphenol) Formamidine
BNF	Beta Naphtoflavone

CHAPTER 1
INTRODUCTION

A. Diabetes Mellitus

The world health organization (WHO) defines diabetes mellitus as a metabolic dysfunction of many aspects mainly characterized by chronic hyperglycemia with disturbances in fat, carbohydrate, and protein metabolism due to defects in insulin secretion, action or both (Report of WHO consultation, 1999). Despite the advances made in recent years in the management of diabetes mellitus, diabetes remains a significant threat. According to the most recent statistics, diabetes has been rated as the fifth leading cause of death in 2007 in the US (Heron et al, 2009). This presents a serious challenge to the health care system since people with diabetes have a reduced life expectancy compared with those without diabetes (Morgan et al, 2000).

B. Diabetes Prevalence in Lebanon

A study conducted by World Health Organization (WHO) indicated that the estimated worldwide burden of diabetes will significantly increase in future years, and that deaths from diabetes will double between 2005 and 2030. This is more in developing countries as more than 80% of diabetes deaths occur in low- and middle-income countries (Van Dieren et al, 2010).

Arab populations in the Middle East are characterized by high rates of diabetes. Several studies have been conducted to determine the prevalence of diagnosed and undiagnosed diabetes in Lebanon.

The prevalence of diabetes mellitus in Lebanon is estimated to be approximately 7-8% and that of impaired glucose tolerance 10-11%. Half of the diabetics were previously unknown; this percentage was higher in people aged less than 40 years. It has been noticed that the onset of diabetes in the Lebanese population occurs at a relatively younger age as compared with the western world (Kiers et al, 1992).

In fact, studies conducted recently using the American Diabetes Association criteria for the diagnosis indicate a high prevalence of diabetes in Greater Beirut, which includes Administrative Beirut and its suburban part of Mount Lebanon, as compared to the US population, although larger samples covering more areas in Lebanon need to be estimated in the future (Hirbli et al, 1992; Hirbli et al, 2005).

C. Classification of Diabetes Mellitus

In 1980 the World Health Organization classified DM according to the need of insulin into: (1) insulin dependent diabetes mellitus (IDDM) or type 1 diabetes and (2) non-insulin dependent diabetes mellitus (NIDDM) or type 2 diabetes mellitus. In 1985 type-1 and type-2 terms were omitted. But in the year 1997 the American Diabetes Association (ADA) declared a new classification according to the etiology of the disease: type-1 and type-2 diabetes mellitus. These two terms are still in use till today (Alberti*et al*., 1998; Holt, 2004).

1. Type I Diabetes Mellitus

Type I diabetes is the case where the autoimmune system of the body, for unknown reasons, destroys the insulin-producing pancreatic cells therefore leading to an absolute deficiency in insulin. Patients with this form rely primarily on insulin therapy for survival. It represents 10 to 20 % of all diabetic cases worldwide (Holt, 2004, Seifter et al., 2005) and usually affects children between 4 to 5 years of age and in their adolescence (Holt, 2004).

2. Type II Diabetes Mellitus

Type II Diabetes Mellitus is an epidemic disease that counts for 80-90% of all diabetic cases worldwide (Rhoades and Bell, 2009) usually affecting the adult section of the population.Type 2 diabetes is a form of the disease in which the body produces insulin but either its secretion is not enough and inadequate or the body cannot use it effectively due to a resistance to insulin action (Kuzuya et al, 2001). It is usually characterized by relative insulin deficiency. Patients are usually obese and suffer from insulin resistance;hence, their treatment includes weight loss, diet and exercise.

D. Complications of Diabetes Mellitus

A defect in insulin secretion, insulin action, or both, interrupts carbohydrate, fat and protein metabolism. The first noticeable consequences of diabetes are polydipsia, polyphagia, polyuria, blurred vision, and weight loss. Hence, treatment is of noticeable importance. In addition, Diabetes Mellitus shows two types of

complications namely acute and chronic. Acute complications include hyperglycemia, ketoacidosis and fluid loss. The chronic complications affect the circulatory system thus leading to micro and macrovascular complications. The microvascular complications are characterized by disturbances of blood flow, to the retina causing retinopathy, neuropathy and to the kidney leading to low glomerular filtration rate thus causing nephropathy. Whereas, the macrovascular complications lead to myocardial infarction, coronary vascular disease and stroke (Rhoades and Bell, 2009). All diabetic complications are highly associated with morbidity and mortality (Mahler et al, 1999).

E. Diabetic Nephropathy

Diabetes related kidney disease or diabetic nephropathy is a worldwide critical life threatening complication of diabetes mellitus affecting 25% of patients with type I diabetes and 40% of patients with type II diabetes (Hall, 2006). In addition, diabetic nephropathy is one of the major risk factors for cardiovascular morbidity and mortality (Gilbert and Cooper, 1999). It eventually progresses into renal insufficiency and failure and is one of the leading causes of end stage renal disease. The early phase is clinically silent and later as the lesions accumulate it manifests in the form ofhyperfiltration and increased glomerular filtration rate (GFR) that almost reaches double of the normal value. Kidney size is increased and microalbuminuria develops, which later may progress into frank proteinuria,severe decrease in GFR and the development of hypertension. The mentioned symptoms only manifest themselves after the loss of almost 85% of the nephrons that make up the kidney.

The lesions associated with diabetic nephropathy were originally viewed as glomerulocentric. However, it is now well established that the lesions affect the endothelium, the glomeruli as well as the tubulo-interstitial compartment (Gilbert and Cooper, 1999). An increase in tubular basement membrane mass is one of the earliest renal pathological changes in diabetes accompanied by the development of renal hypertrophy (Jones et al., 1999). Diabetes related kidney disease is usually defined by progressive accumulation of several extracellular matrix proteins including collagen, laminin and fibronectin which are involved in adhesion, migration, survival, differentiation and growth. (Young et al., 1995; Wang et al., 2008). Late stages are mainly characterized by interstitial fibrosis.

Many studies have been conducted to shed a light on the pathogenetic mechanisms that are involved in diabetic nephropathy. Multiple mechanisms have been proposed to be implicated in the changes observed in DN. Hemodynamic, metabolic changes and the complex interaction between the two have all been shown to contribute to diabetes related kidney injury.

The metabolic pathways involved in diabetes are thought to be primarily mediated through the effect of hyperglycemia which is a crucial factor in the development and progression of diabetic nephropathy. Although the etiology of fibrosis observed in overt diabetic nephropathy is not fully established, much of the emphasis has been placed on the role that glucose plays. Much of this evidence comes from in vitro experiments whereby tubular epithelial cells exposed to high glucose concentrations (25mM glucose) were found to develop hypertrophy and an increase in the accumulation of extra cellular matrix. The tubular compartment is well

known to be a major target of hyperglycemia and the exposure to high blood glucose levels is a major contributor to the tubulointerstitial changes seen in overt diabetic nephropathy. Two mechanisms have been postulated to explain how hyperglycemia causes tissuedamage: nonenzymatic glycosylation that generates advanced glycosylation end products and the activation of Protein Kinase C (PKC) (Friedman et al., 1999). Oxidative stress seems tobe a theme common to both pathways (Brownlee et al., 2001).

While it is well known than diabetes results in the accumulation of extra cellular matrix proteins, the role by which glucose mediates this effect still needs to be unveiled. Hyperglycemia results in the eventual production of advanced glycosylation end products (AGEs) through the cross linking of glucose with the amino group of matrix proteins, specifically collagen. AGEs accumulate in the serum of patients with renal insufficiency (as these are normally excreted in urine) resulting in the activation of circulating profibrotic cytokines such as TGFß1 (Makita et al., 1991). The activation of the PKC pathway results in the activation of downstream mitogen activated protein kinases (MAPK) and increases the expression of TGFß1 which results in hypertrophy and increased collagen synthesis (Cooper at al., 1998). Upregulation of PKC wasobserved in kidneys of rats with diabetic nephropathy (Koya et al., 1997). It was associated with an upregulation of the protein expression of TGFß1, fibronectin, and collagen type IV. In one study, when STZ induced diabetic rats are treated with the inhibitor of PKC, they show decreased expression of TGFß1, decreased extracellular matrix (ECM) accumulation as well as reduced hyperfiltration and albuminuria (Ishii at al., 1996).

Hemodynamic pathways are primarily involved in the changes seen in the early clinically silent phase of diabetic nephropathy. Early stages start with an impaired autoregulation of the afferent arteriole whereby it is characterized to have a significantly decreased resistance. The decreased resistance facilitates the early processes of hyperperfusion and hyperfiltration. The primary mediators of such changes are nitric oxide (NO), angiotensin II and VEGF (Ziyadeh et al., 2008). These early hemodynamic changes facilitate albumin leakage from the glomerular capillaries and overproduction of mesangial cell matrix, as well as thickening of the glomerular basement membrane and injury to podocytes(Ziyadeh et al., 2008). In addition, increased mechanical strain resulting from these hemodynamic changes can induce localized release of certain cytokines andgrowth factors such as TGFß1 (Wolf et al., 2008). Such cases are traditionally treated with an angiotensin convenrting enzyme blocker. Patients with type I diabetes and nephropathy treated with an ACE inhibitor show markedly improved proteinuria and decreased serum levels of TGFß1 (Sharma et al., 1999).

On an another account, genetic predisposition/susceptibility has been postulated to be involved in diabetic nephropathy. This observation was based on the fact that not all diabetic patients develop diabetes related kidney disease. In patients with type 1 or type II diabetes, the likelihood of developing diabetic nephropathy is markedly increased in those who have a sibling or parent with diabetic nephropathy (Trevisan et al., 1995). One study evaluated PimaIndian families in whom two successive generations had type II diabetes. The likelihood of theoffspring developing overt

proteinuria was 14%if neither parent had proteinuria, 23% if oneparent had proteinuria and 46% if both parents had proteinuria (Pettitt et al., 1990).

F. Arachidonic acid Metabolites in Kidney Function

Arachidonic acid (AA) can be metabolized by one of the following three pathways: the cyclooxygenase, the lipoxygenase or the cytochrome P450 (CYP) monooxygenase pathway. It is by the third pathway that the AA is metabolized by several of the CYP450 isoforms into 20-hydroxyeicosatetraenoic acid (20-HETE) and epoxyeicosatrienoic acids (EETs) among other eicosanoid products (Zeldin, 2001).

The CYP4A and CYP4F isoforms catalyze the formation of 20-HETE. 20-HETE is the main metabolite observed in renal cells and exerts a wide array of regulatory as well as opposing functions depending on the respective location where it is produced, thus manifestingas vasoactive, mitogenic or renal filtration effects (Carroll, 2000). 20-HETE is pro-inflammatory and has an important function in hypertension and in promoting systemic vasoconstriction. It regulates vascular smooth muscle and endothelial cells by influencing their proliferation, migration and survival. In addition, 20-HETE has been shown to induce apoptosis in glomerular epithelial cells, through the upregulation of ROS production (Eid et al., 2009) and to increase endothelial cell proliferation and migration (stimulation of angiogenesis) through the stimulation of superoxide production (Guo et al., 2007).

Also, in the kidney, it blocks re-absorption of sodium by inhibiting the Na^+-K^+-ATPase. Therefore, an aberrant increase in 20-HETE production is expected to have both hemodynamic effects as well as an effect on cellular proliferation and apoptosis, both of which contribute to the resulting tissue hypertrophy.

EETs are produced by the CYP1A and the CYP2C11 isoforms of the cytochrome P450 enzymes. Similarly to 20-HETE, EETs have a multitude of biological roles depending on the site of their production. When compared to 20-HETE, EETs are considered as potent vasodilators (Michaelis et al., 2003; Michaelis et al., 2005; Campbell et al., 2007) and they are thought to be the endothelium derived hyperpolarizing factor (EDHF). The CYP450 metabolites of arachidonic acid contribute to 50% of the total relaxation response in arteries. While the COX pathway represents the remaining 50%, the LOX pathway does not contribute to this response in arteries. Besides their hemodynamic role, EETs are also involved in the regulation of cell proliferation (Harris et al., 1990; Sheu et al., 1995; Chen et al., 1998). Hence, similarly to 20-HETE, anomalous concentrations of EETs are expected to alter cellular function.

G. Oxidative Stress and Diabetic Nephropathy

A high amount of reactive oxygen species is generated in the kidney under normal physiological conditions. However, this high production is counter balanced by effective antioxidant systems of free radical scavengers. Unfortunately,

hyperglycemia/ high glucose tips the balance towards ROS generation through activation of a number of enzymatic and non enzymatic sources in the body.(Nishikawa et al., 2007). Persistent hyperglycemia induced-ROS generation has been shown to play an early role in podocyte damage and apoptosis (Susztack et al., 2006). Evidence suggests that glomerularpodocytes have a key role in early proteinuria, and reducedpodocyte number was reported in patients with diabeticnephropathy (Steffes et al., 1994). Therefore, oxidative stress is speculated to be one of the primary players in diabetic nephropathy. ROS-induced fibrogenic responses include distinct cell functions such as hypertrophy, migration, proliferation, apoptosis, and regulation of extracellular matrix (Grzegorz et al., 2009). The CYP450 enzymes are thought to be one of the major sources of reactive oxygen species in the liver and the kidney cortex (Puntarulo et al, 1998; Fleming et al, 2001; Eid et al, 2009). 20-HETE has been shown by Eid et al (2009) to increase ROS generation in the kidney glomerular epithelia. 20-HETE has also been shown, by Guo et al (2007), to mediate its effects by superoxide generation. One of the most common reactions catalyzed by the CYP450 enzymes is a monooxygenase reaction (the insertion of an oxygen atom, previously activated by the heme group, into an organic substrate).

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$$

This reaction is accompanied by the formation of superoxides/H_2O_2 in the case of uncoupled redox cycling by these enzymes whereby the activated oxygen atom escapes before being incorporated in the substrate. However, the mechanism by

which these ROS producing enzymes in the kidney contribute to diabetic nephropathy remains poorly understood.

H. NADPH in Diabetic Nephropathy

As mentioned above, hyperglycemia facilitates a state of chronic oxidative stress in the renal milieu, the long term consequence of which is the loss of glomerular and tubular cells. In diabetes, this state of oxidative stress is sustained via an over expression of the NADPH oxidase. NADPH oxidase is located in plasma membrane of various renal cell types, including mesangial and proximal tubular cells, vascular smooth muscle cells andendothelial cells (Griendling et al., 1994). The activation of NADPH oxidase results in the generation of superoxides known as reactive free radicals. In the kidney cortex, three different Nox isoforms Nox1, Nox2, and Nox4 have been shown to be expressed (Bedard and Krause, 2007). Nox4 is most abundant and predominantly localized in renal tubular cells, but is also found in glomerular mesangial cells at lower levels. ROS in diabetic nephropathy are thought to be one of the primary mediators of hypertrophy and fibrosis among other processes that eventually lead to renal dysfunction. NADPH oxidase is the predominant enzyme source for ROS generation in fibrotic disease and is now recognized as a key mediator of cell proliferation and matrix accumulation in renal disease (Li et al., 2006). Studies have shown that NADPH-dependent ROS generation is increased in the renal cortex and glomeruli of mice suffering from

diabetes (Gorin et al., 2005; Satoh et al., 2005; Kim et al., 2006). Inhibition of Nox4 using antisense oligonucleotide therapy reduces the diabetes induced ROS generation in the renal cortex and whole kidney as well as glomerular hypertrophy and fibronectin expression in type 1 diabetic rats (Gorin et al., 2005). Thus, NADPHoxidase may be one of potential targets deserving furtherinvestigation in the development of drugs for the treatmentof diabetic nephropathy.

I. 20-HETE in Diabetic Nephropathy

Little is known about the involvement of 20-HETE in physical and physiological changes specifically when it comes to its role in altering organ function in diabetes. The regulation of CYP450 is tissue as well as disease specific. The alteration in expression of CYP450 enzymes and the resulting changes in the levels of their corresponding metabolites have been shown to be implicated in the renal damage observed in diabetes (Enriquez et al., 1999; Dey et al., 2004). 20-HETE has also been shown to play an important role in the regulation of renal as well as cerebral blood flow whereby an increase in the levels of 20-HETE is suspected to play a role in pathogenesis of hypertension and ultimate renal failure (Miyata et al., 2001). Eid et al. (2009) have shown that the levels of 20-HETE are increased compared to those of EETs which are decreased in humans with renovascular disease (high 20-HETE:EET ratio). However, the specific roles of 20-HETE and EETs in diabetic nephropathy are yet to be revealed. Microalbuminuria is one of the earliest detectable abnormalities observed in diabetic nephropathy that leads to abnormal secretion of proteins mainly albumin into the urine (Wolf and Ziyadeh, 2007). The

role of 20-HETE in the process of proteinuria remains to be controversial. In some in vitro experiments where puromycinaminonucleoside (PAN) was used to induce albumin permeability in isolated glomeruli, 20-HETE was shown to protect against proteinuria (McCarthy et al., 2005). On the other hand, a recent study by Eid et al. (2009) demonstrated that the inhibition of 20-HETE production in type 1 diabetic mice results in the reduction of proteinuria. These data suggest the involvement of 20-HETE in states of proteinuria. However, the exact mechanisms and effects of 20-HETE in proteinuria still need to be disclosed.

J. EETs in Diabetic Nephropathy

As previously stated for the 20-HETE, the role of EETs in diabetic nephropathy is yet to be elucidated. A group of researchers have recently shown that the overexpression of CYP2J2 epoxygenase with increased EET generation in streptozotocin-induced diabetic mice attenuated microalbuminuria (Chen et al., 2011). On the other hand, another group of researchers have found that increased circulating levels of EETs achieved by the inhibition of the enzyme soluble epoxide hydrolase, responsible for the metabolism of EETs intodihydroxyeicosatrienoic acids (DHETs), contribute to albuminuria in mice models ofdiabetes (Jung et al., 2010). In an another set of experiments, EETs have been shown to reduce hyperglycemia through decreasing ß islet cells apoptosis and therefore enhancing insulin secretion. Similarly to 20-HETE, EETs are one of the major players in proteinuria with an unknown mechanism that needs to be elucidated.

K. Hypothesis of the Study

The levels of cytochrome P450 (CYPs), particularly the ones involved in arachidonic acid metabolism, are altered by hyperglycemia during both the onset as well as the progression of diabetes. This in turn results in the overproduction of various metabolites primarilyepoxyeicosatetraenoic acids (EET), and/or 20-hydroxyeicosatetraenoic acids (20-HETE), which have been shown to have major roles in proliferation and apoptosis. The roles of these metabolites in diabetic nephropathy are controversial, whereby some studies implicated a particular metabolite in the pathogenesis of the disease while others suggested a protective role for it. This study was conducted to further explore and characterize the roles of EET and 20 HETE in diabetic nephropathy, by using drugs that either inhibited the production of 20 HETE (HET0016) or that enhanced the production of EET (BNF). We hypothesized, based on previous studies from Dr. Eid's laboratorythat 20 HETE production in diabetes contributed to the deleterious effects on kidney structure and function whereas EET production served to protect the kidney against such complications. The role of CYP enzymes in contributing to production of reactive oxygen species was also addressed.

L. Aims of the Study

The study has the following specific aims:

Using control rats and rats with streptozotocin induced diabetes mellitus we sought to examine the effect of treatment with HET0016 or BNF on:

a. Development of kidney hypertrophy

b. Induction of fibrosis in the kidney by
    i. Histological examination using specific stains for collagen and matrix deposition
    ii. Expression of fibronectin in the renal cortex
c. Oxidative stresss by
    i. Production of reactive oxygen species in the renal cortex
    ii. Expression of NADPH Oxidase in renal cortex
d. CYP 4F3 expression in renal cortex
e. Levels of 20 HETE and EET in urine

CHAPTER 2

MATERIALS AND METHODS

A. Animal Models

Male Sprague-Dawley rats weighing between 200 and 300 g were used. Rats were purchased from and housed at the American University of Beirut Animal Care Facility. During the period of the study, animals had access to water and standard rat chow (Purina Pellets) and they were placed under a cycle of 12 hours light-12 hours dark in an ambient temperature room. All experiments were conducted with the approval of the Institutional Review Board/Animal House Committee of the American University of Beirut. Rats were subsequently divided into four groups of six animals each.Group 1 consisted of rats injected with sodium citrate buffer (the vehicle for streptozotocin, which was used in other groups to induce diabetes) intravenously through the tail vein, and were also treated with daily i.p injections of 0.2 ml 95% normal saline with 5% DMSO (the vehicle for drugs used to inhibit or induce CYP activity) for a period of 4 weeks. This group will be referred to as the control vehicle (CV) throughout the study.The remaining rats were injected once intravenously through the tail vein with 65 mg/kg body weight streptozotocin (STZ) (Sigma Chemical Company, St. Louis, MI, USA) dissolved in sodium citrate buffer (0.01 M, pH 4.5), under ether anesthesia,in order to induce diabetes and were further subdivided into the remaining three groups. Rats of Group 2 were treated with the same vehicle as the control vehicle group, starting two days after induction

with STZ and for the duration of the study and will be referred to as diabetic vehicle (DV). Rats of group 3 were treated with 10 mg/kg/day of HET0016intraperitoneally (AUB, Chemistry Department), a potent and selective inhibitor of 20-HETE production and rats of group 4 were treated with 35mg/kg/day of Beta-Naphthoflavoneintraperitoneally (BNF Sigma Aldrich), a potent inducer of EET production. Groups 3 and 4 are respectively referred to as DH and DF.

Blood glucose level was checked by Accu-Chek glucometer (Accu-Chek instant test, Roche Diagnostics GmbH, Mannheim, Germany) two days after STZ injection. A non-fasting blood glucose level of above 250 mg/dl was indicative of a diabetic state in rats.Three randomly selected rats of each of the four groups were placed in metabolic cages three days prior to the collection of their urine output on days 0, 14 and 28 of the study. In all groups body weights and blood glucose levels weremonitored on weekly basis throughout for the duration of the study.

B. Western Blot analysis for Fibronectin, NADPH oxidase and CYP protein expression

Animals were sacrificed on day 30 after STZ or vehicle injection by decapitation. Both kidneys were removed and weighed. A piece of the kidney cortex at the pole was embedded in paraffin or flash frozen in liquid nitrogen for microscopy or image analyses. The tissues were later lysed using 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. Theobtainedlysates were then centrifuged at 13200 rpm for 30 minutes at + 4°C. Protein in the supernatants was measured using the Lowry Protein Assay. For immunoblotting, 80µg 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-Nox 4 (1:500, Santa Cruz Biotechnology), rabbit polyclonal anti-CYP4F3(1:1000, Novus), and rabbit polyclonal anti-fibronectin antibody(1:7000, sigma-aldrich). The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:7000, Bio-Rad). Bands were visualized by enhanced chemiluminescence (ECL). Band thickness was determined by a semi-quantitative densitometric analysis (arbitrary unit) using a specific computer software (Image J), and expressed as mean of thickness ± SE.

C. Collagen Deposition Using Masson'sTrichrome Staining

Kidney cortex sections were embedded in paraffin, cut into a 4µm thick section and then mounted on glass slides. The slide was then dried and immersed twice into xylol for 5 minutes for deparaffinization. It was consequently hydrated by the immersion in subsequently decreasing concentrations of alcohol solutions for 3 minutes each (100-95-75% alcohol). The hydration step was followed by rinsing with distilled water for 3 minutes. The slide was then dipped in Bouin solution at 56ºC for 30 minutes. It was then washed with tap water followed by distilled water. The slide was then dipped in Hematoxylin for 3 minutes after which it was washed

with running tap water and distilled water. It was later stained with Biebrich scarlet for 1 minute and rinsed with distilled water. It was then dipped in 5% phospholungstic acid for 10 minutes and washed with distilled water. The slide was subsequently counter stained with light green for 8 minutes followed by a 2 minute tap water wash. The slide was then dehydrated by dipping in alcohol solutions of increasing concentrations (95-95-100% alcohol) for 3 minutes each. The slide was then dipped twice 3 minutes in xylol for clearing. Images were quantified using image J software.

D. Mesangial Matrix Deposition Using Periodic Acid Schiff Staining (PAS)

Kidney cortex sections were embedded in paraffin, cut into a 4μm thick section and then mounted on glass slides. The slide was then dried and immersed twice into xylol for 5 minutes for deparaffinization. It was consequently hydrated by the immersion in subsequently decreasing concentrations of alcohol solutions for 5 minutes each (100-95-75% alcohol). The hydration step was followed by rinsing with distilled water for 3 minutes. The slide was then dipped in 1% periodic acid for 10 minutes. It was then washed with distilled water. The slide was then dipped in Schiff reagent for 10 minutes after which it was washed with running tap water. The slide was subsequently counter stained with Hematoxylin for 3 minutes followed by a 2 minute tap water wash. The slide was then dehydrated by dipping in alcohol solutions of increasing concentrations (95-95-100% alcohol) for 3 minutes each. The slide was then dipped twice 3 minutes in xylol for clearing.

E. Reactive Oxygen Species Detection Using Dihydroethidium Staining (DHE)

The DHE (Calbiochem, Darmstadt, Germany) staining for superoxide was carried out as follows: unfixed kindey cortex samples were cut into 10μm thick sections and mounted on glass slides. DHE (10μml/L) was applied to each tissue section and the slides were incubated in a light-protected humidified chamber at 37ºC for 15 mimutes, Fluorescent images of ethidium-stained tissue were obtained with laser scanning confocal microscope (Zeiss 510 NLO). Ethidium bromide was excited at 488nm and fluorescence was detected at 560 nm long pass filter. Mean fluorescence intensity of the digitized image was measured with Image J software for quantification. Generation of superoxide was demonstrated by red fluorescent labeling. Non stained kidney cortex sections were used as background control. The average of three sections stained with DHE was taken as the value for each animal.

F. Detection of Renal Hypertrophy

Renal Hypertrophy was assessed macroscopically. Wet kidney weight was recorded for all groups. Kidney weight (K.W) to body weight (B.W) ratio [K.W/B.W] was determined for every rat and averaged for every group and served as an index for comparison among different groups.

G. Protein Level in Urine

Urine collected from metabolic cages was used for the measurement of protein levels. Protein levels were measured using the Lowry technique.

H. Assay of AA Metabolites

The levels of 20-HETE and EET were measured in urine collected from all rat groups. Levels were assessed using 20-HETE and EET ELISA kits provided by Detroit R&D Inc. procedures were carried out according to the manufacturer's protocol.

I. Statistical analysis

All data are expressed as mean values obtained for all the parameters mentioned previously, per animal and per group. To assess significance among the different experimental groups, the student t-test was exploited and the analysis of variance for multiple factors (ANOVA) using the Bonferroni penalty method was employed. Values were expressed as arithmetic mean ± standard error of the mean (X ± SE) and P values detected significant differences wherever they existed. Significanceis determined as probability (P) <0.05.

CHAPTER 3

RESULTS

A. Reversal of Diabetes Induced Renal Hypertrophy by HET 0016 and BNF

The mean body weight and plasma glucose levels of all the different rat groups are shown in Table 1. There was a significantly lower body weight in all the diabetic groups compared with CV rats at the end of the study, However, the diabetic group which received BNF showed a significantly higher body weight when compared with the DV group. Similarly, all diabetic groups had a significantly higher plasma glucose level compared with CV group

Renal hypertrophy was assessed grossly by averaging the weights of both kidneys and normalizing to body weight. Renal hypertrophy is represented in figure 1. Diabetic rats showed significantlyhigher kidney weight to body weights when compared with the CV group. Rats treated with HET0016, the potent inhibitor of 20-HETE synthesis, showed significantly lower kidney to body weight than the DV group. Similarly, the group treated with BNF, the potent inducer of EET synthesis, has a significantly lower kidney to body weight ratio and therefore a lower hypertrophy index.

Table 1. Mean glucose levels, body weights, kidney weight and hypertrophy index for all rat groups at day 28

Rat Group	Glucose ± SE(mg/dl)	Body Weight ± SE(g)	Kidney Weight ± SE (g)	K.W/B.W ± SE (Hypertrophy Index)
Control	169.2 ± 11	394.3 ± 16	1.56± 0.02	0.004 ± 0.0002
Diabetic	564.33 ± 21*	305 ± 16*	1.82± 0.05	0.006 ± 0.0003*
Diabetic + HET0016	508.2 ± 39*	311.33 ± 20*	1.71± 0.3	0.005± 0.0006#
Diabetic + BNF	448.33 ± 88*	354.67 ± 5*#	1.68± 0.2	0.004 ± 0.0005#

*P<0.05 vs Control
#P<0.05 vs Diabetic

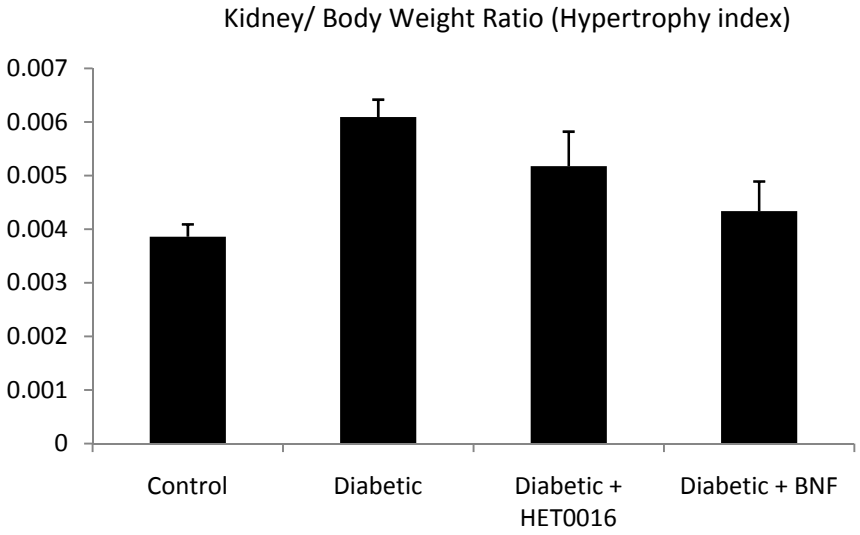


Figure 1. Diabetes induces Renal Enlargement
Hypertrophy index was assessed by taking average kidney weights isolated from type I diabetic rats after 4 weeks of treatment and normalizing to body weight (n=6). Each histogram represents the mean hypertrophy index. Values are the means ± SE. *P<0.05, vs. CV; #P<0.05, vs DV

B. Urinary Protein Excretion

Diabetes is well known toinduce increased protein excretion in the urine. Protein levels in urine samples collected from metabolic cages for all groups, were assessed using the Lowry Protein Assay. The values represented reflect the total protein levels found in the sample. Rats from both CV and DV groups had an increase in urine protein excretion over time with no significant differences between them. The diabetic group receiving HET0016, showed a consistent and non-significant increase in the levels of urine protein, similar to that observed in the previous two groups. The diabetic group receiving BNF, however, had a significantly different patten of urinary protein excretion over time, with essentially no change over the 4 weeks, and this was significantlylower from other groups on day 28.

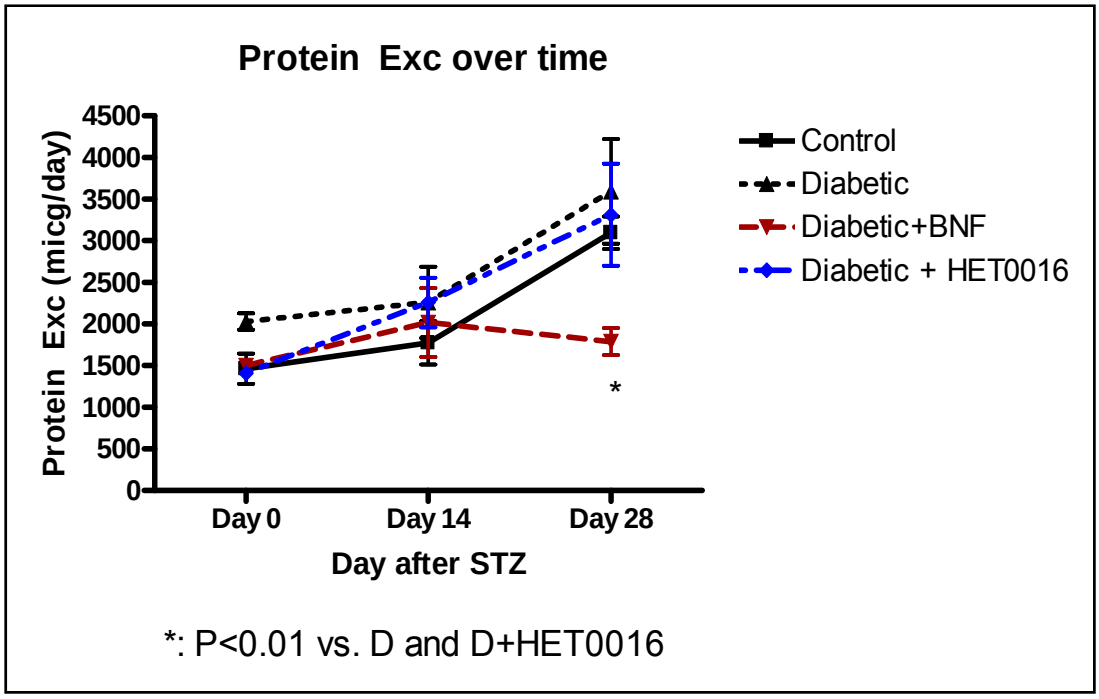


Figure 2. Diabetic Protein Excretion Over Time

Urinary protein excretion in control rats (symbol) in diabetic rats in diabetic rats which received HET0016 and in diabetic rats that received BNF before and on days 14 and 28 after administration of STZ or vehicle. Values are the means ± SE. *P<0.05 vs DV and DH

C. Urinary excretion of 20 HETE and EET

As expected, the urinary excretion of 20-HETE was significantly increased in the diabetic group. This increase was significantly reduced in treatment groups receiving either HET0016 or BNF. The levels of EET did not change in the diabetic group and the group receiving HET0016. However, it was significantly increased in the group receiving BNF.

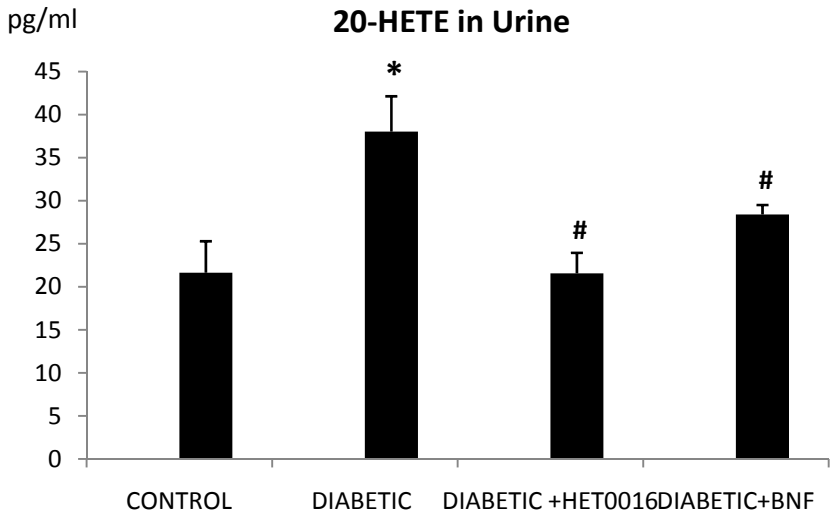


Figure 3.20-HETE production is increased in urine collected from Type I diabetic rats.
Each histogram represents the amount to 20-HETE in the urine sample (n=6). Values are the means ± SE. *P<0.05 C; #P<0.05 vs D

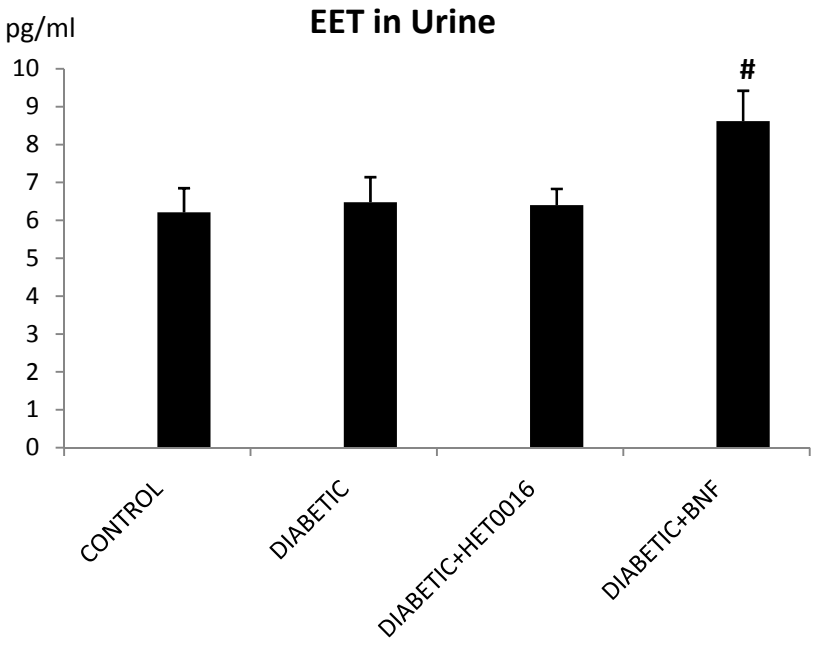


Figure 4.EETs production is increased in urine collected from Type I diabetic rats treated with BNF. Each histogram represents the amount to 20-HETE in the urine sample (n=6). Values are the means ± SE.#P<0.05 vs D

D. Collagen and Matrix Deposition

In order to determine the contribution of 20-HETE and EETs to kidney injury in a hyperglycemic milieu in terms of matrix protein accumulation and the resulting fibrosis, we assessed the levels of fibronectin protein expression as well as collagen deposition.Fibronectin levels were assessed through immunoblotting. Fibrosis, assessed by fibronectin protein expression, tended to be higher in the kidney cortex isolated from diabetic rats when compared to levels in kidney cortex isolated from control non diabetic rats; however this difference did not reach statistical significance. Diabetic rats treated with either HET0016 or BNF showed lower levels of fibronectin protein expression in the kidney cortex compared with those of DV rats (figure 5). As for collagen deposition, which was assessed through the Masson Trichrome stain,it was significantly greater in the diabetic group when compared with the control non diabetic group. Both treatment groups show a significantly lower collagen accumulation more remarkable in the DF group (figures 4 and 5)

The PAS stain was used to stain and detect polysaccharides such as glycogen and other glycolipids and glycoproteins.

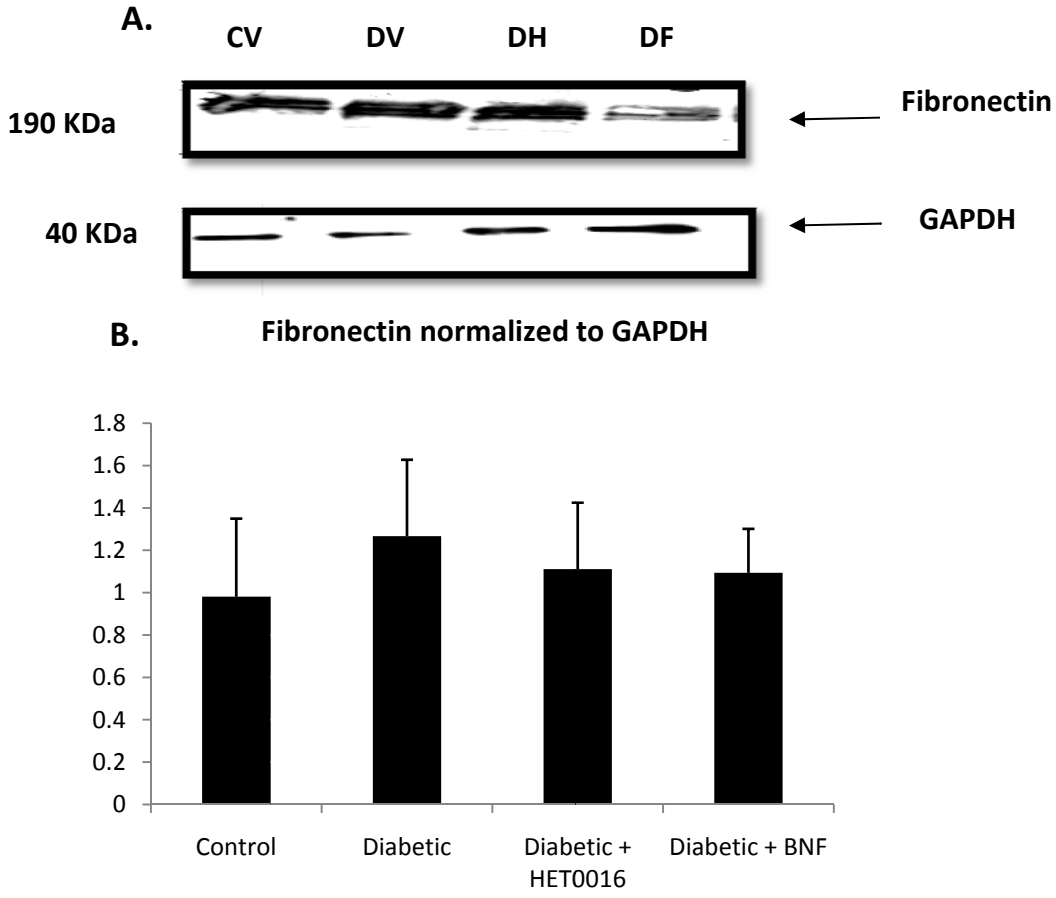
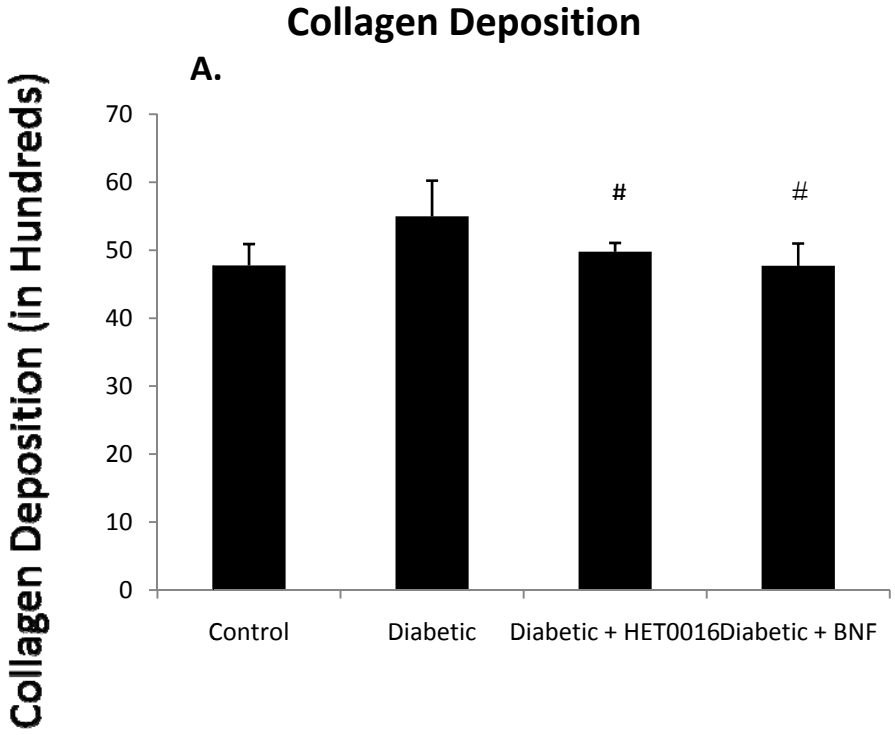


Figure 5.Fibronectin protein expression in control rats and in diabetic rats that received HET0016 or BNF.
Western blot analysis of renal cortex derived from type I diabetic rats after 4 weeks of STZ administration and treatment with HET0016 and BNF revealing the expression of Fibronectin (A) along with their semi-quantifications (B). The Western blots are quantified by densitometry. Each histogram represents the ratio of the intensity of the Fibronectinband factored by the GAPDH band, used as a loading control (n=6). Values are the means ± SE.


Collagen Deposition
A.
Collagen Deposition (in Hundreds)
70
60
50
40
30
20
10
0
#
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Control
Diabetic
Diabetic + HET0016
Diabetic + BNF

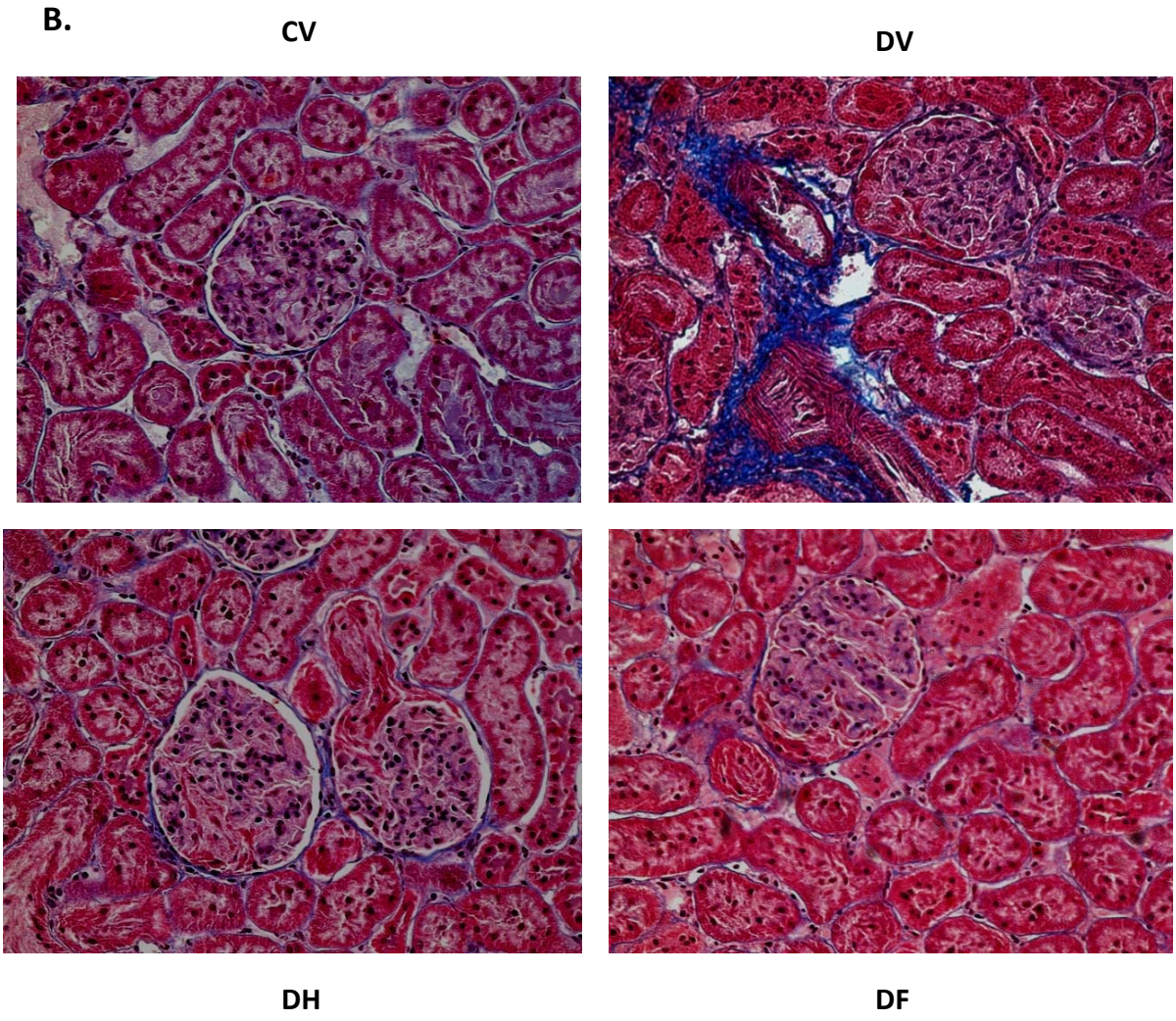


Figure 6. Diabetes induces Collagen deposition Assessment of extracellular matrix (ECM) deposition by Masson's trichrome staining. Quantification of kidney cortex positive for ECM deposition area(A) Representative micrographs of Masson's trichrome–stained kidney sections (B). Each histogram represents amount of collagen (n=6). Values are the means ± SE., #P<0.05 compared with DV

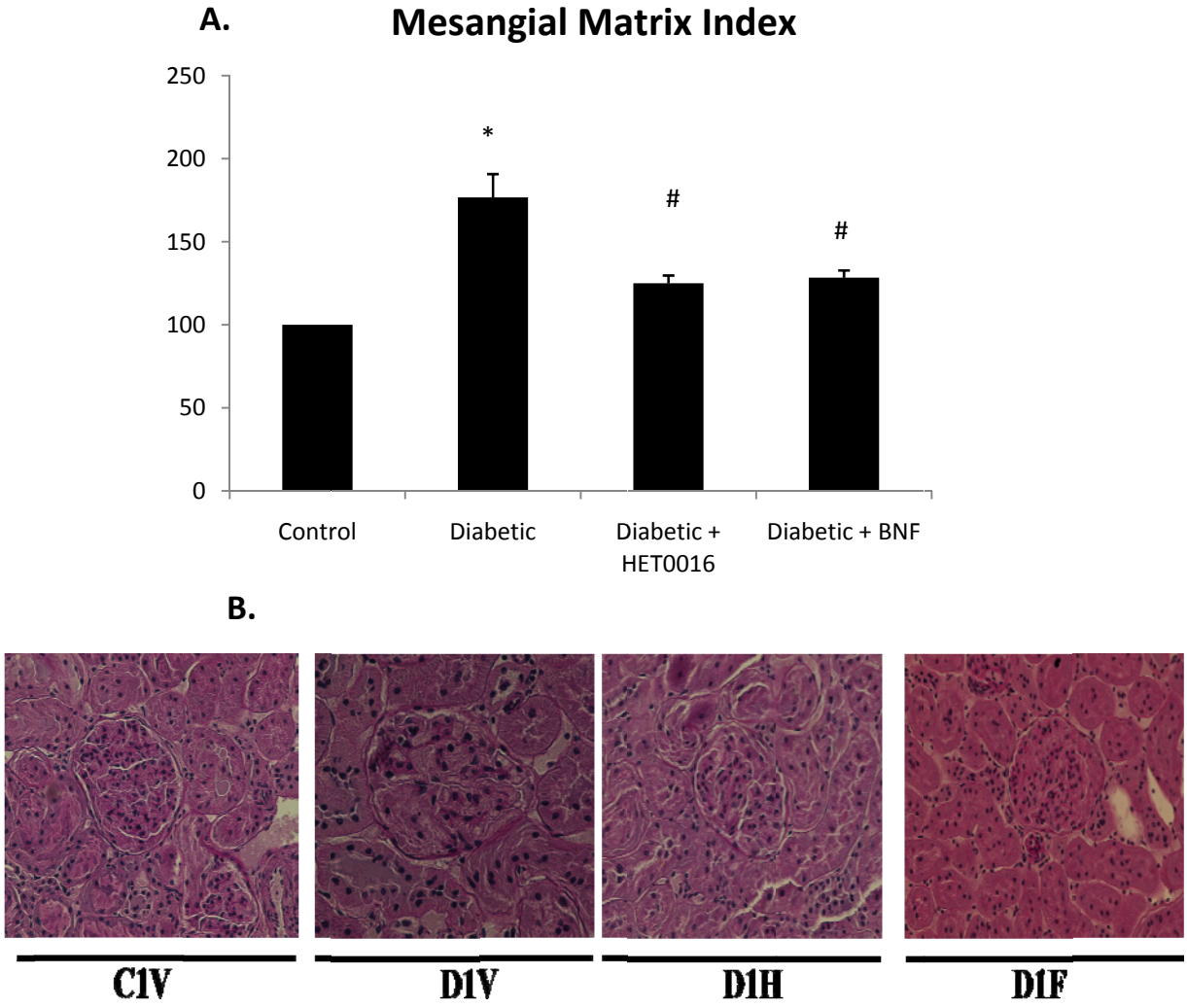
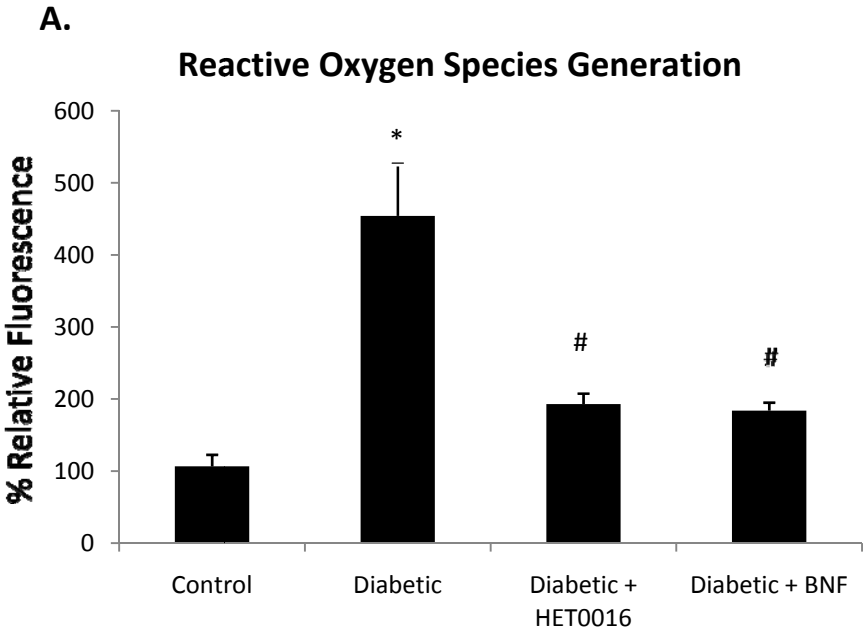


Figure 5. Diabetes induces ECM deposition.
Cortical kidney sections stained with periodic acid-Schiff (PAS) and the increase in mesangial matrix was measured in different Group of rats. (A)The mesangial matrix index represented the ratio of mesangial matrix area divided by tuft area. (B)The pictures display representative glomeruli and Proximal tubules of PAS-stained sections in the different group of rats . All data are means ± SE (n=6). $^{*}P < 0.05$ vs. CV, $^{\#}P < 0.05$ vs.DV.

E. Renal production of reactive oxygen species



B.

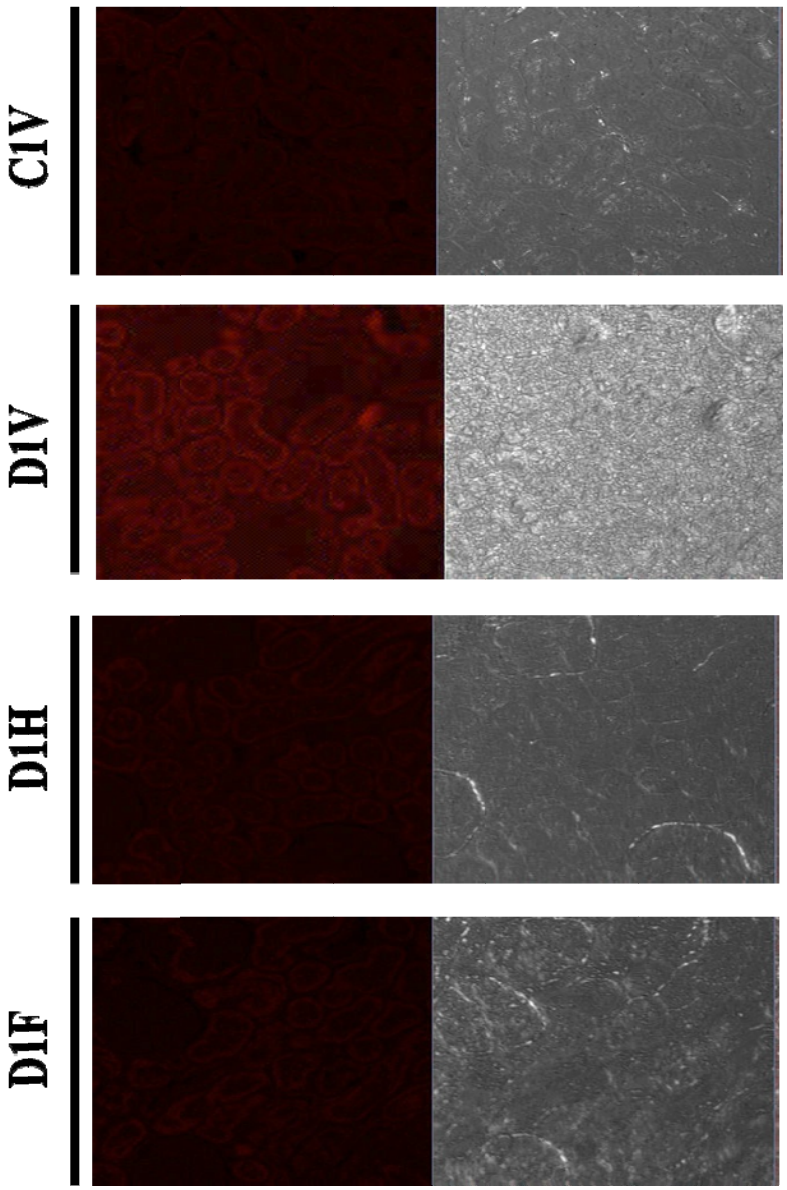


Figure 8. Diabetes induces ROS generation.
Cortical kidney sections stained with Dihydroethidium (DHE) and the increase in ROS generation was measured in different group of rats. (A) Each histogram respresents mean fluorescence intensity of the digitized image. (B)The pictures display representative glomeruli and Proximal tubules of DHE-stained sections. All data are means ± SE (n=6). $^{*}P < 0.05$ vs. CV, $^{\#}P < 0.05$ vs. DV.

F. Renal CYP4F3 expression

Diabetes did not induce any significant change in the expression of CYP4F3 in renal cortex. Similarly, neither treatment with HET0016 nor with BNF had any effect on CYP4A expression (figure 7)

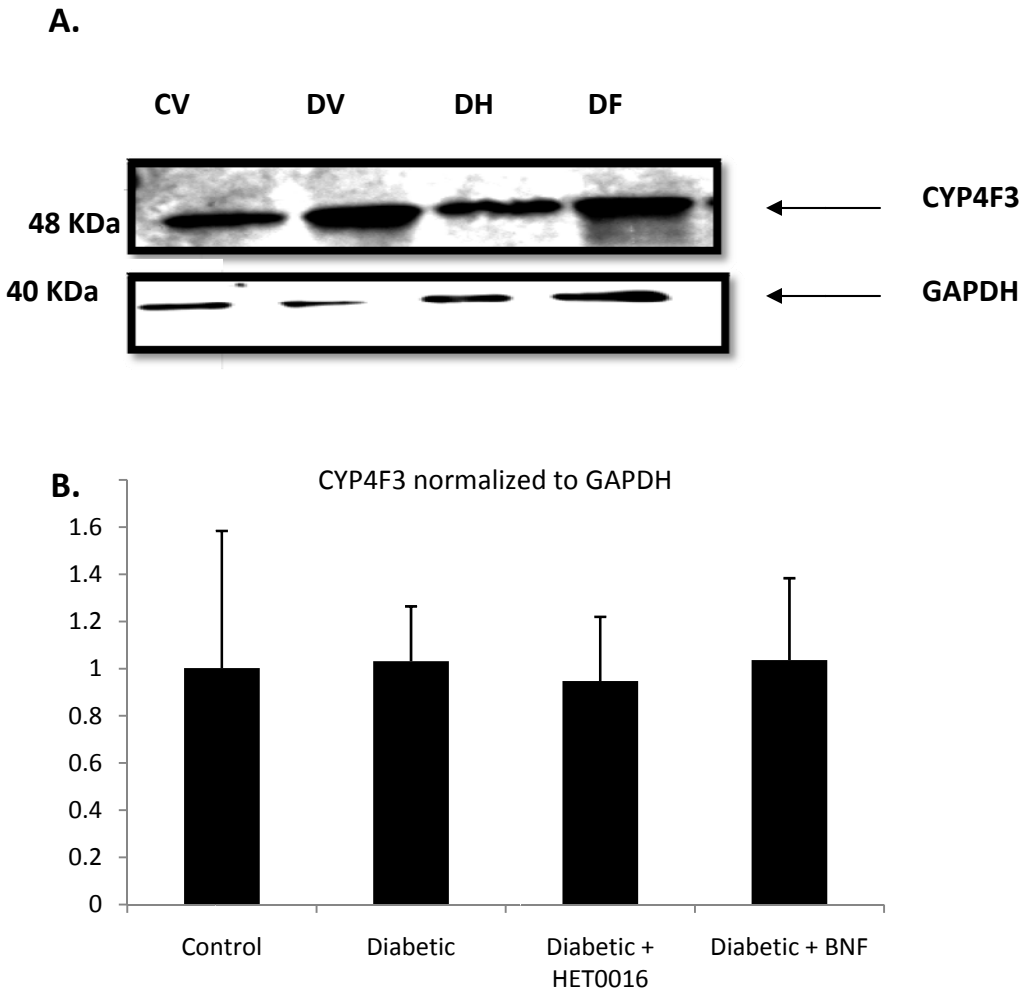


Figure 9.CYP4F3 protein expression in control rats and in diabetic rats that received HET0016 or BNF. Western blot analysis of renal cortex derived from type I diabetic rats after 4 weeks of STZ administration and treatment with HET0016 revealing the expression of CYP4F3(A) along with their semi-quantifications(B). The Western blots are quantified by densitometry. Each histogram represents the ratio of the intensity of the CYP4F3 band factored by the GAPDH band, used as a loading control (n=6). Values are the means ± SE.

G. Renal NADPH oxidase expression

The Noxfamily of NADPH oxidases are enzymes that can produce superoxide and other downstream ROS in cells and tissues (Bedard and Heinz Krause, 2007). Measurement of Nox 4 expression in the kidney of animals from the 4 groups of rats did not reveal any significant differences among them (figure 8).

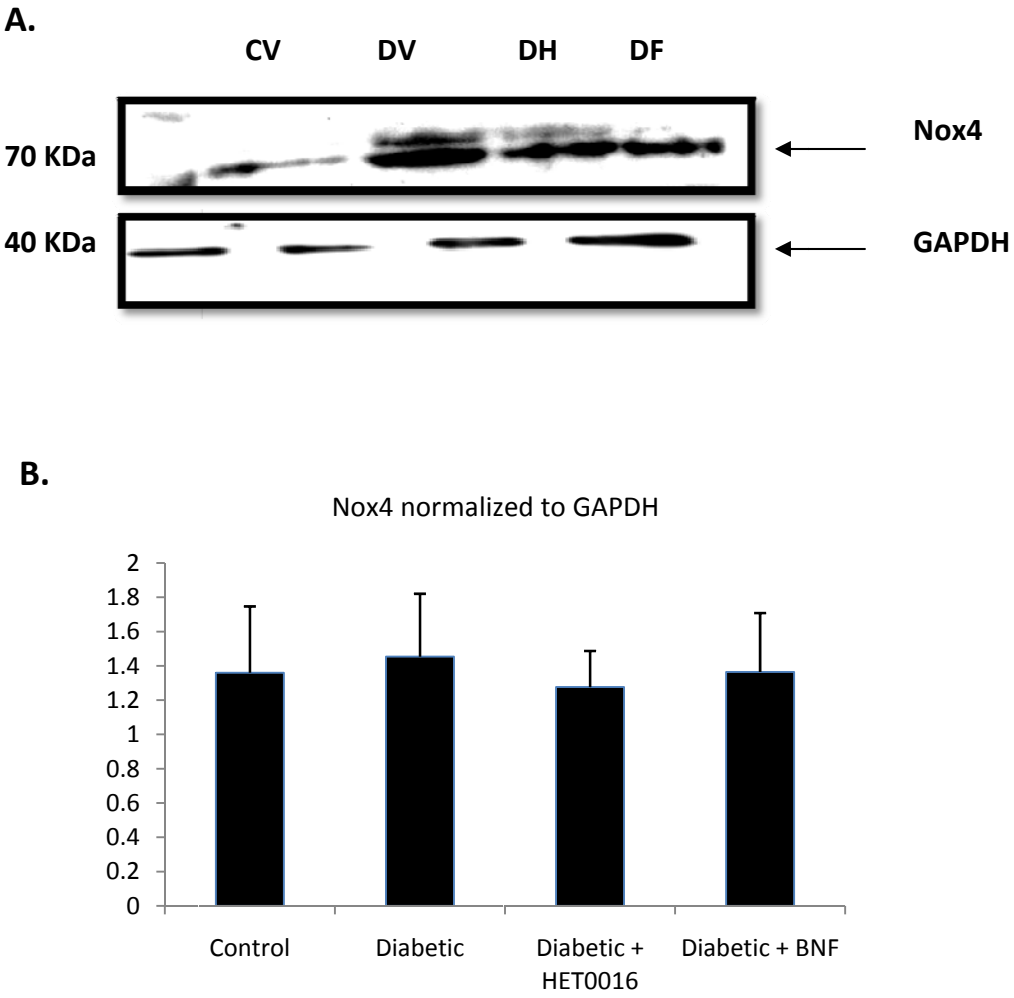


Figure 10.Nox4 protein expression in control rats and diabetic rats that received HET0016 or BNF.
Western blot analysis of renal cortex derived from type I diabetic rats after 4 weeks of STZ administration and treatment with HET0016 and BNF revealing the expression of Nox4(A) along with their semi-quantifications(B). The Western blots are quantified by densitometry. Each histogram represents the ratio of the intensity of the Nox4 band factored by the GAPDH band, used as a loading control (n=6). Values are the means ± SE.

DISCUSSION

This study was conducted to evaluate the role of EETs and 20 HETE in diabetic nephropathy using drugs that either induce EET production (BNF) or that inhibit 20-HETE synthesis (HET0016). Several approaches and markers were used to assess the development of renal injury and the effects of the drugs. Thus, it was clear that diabetic rats had the following abonormalities: there was evidence of renal hypertrophy (increased kidney weight and kidney weight to body weight ratio), increased deposition of matrix proteins in the glomeruli (PAS staining), increased generation of ROS (DHE staining), and increased production of 20 HETE as evidenced by increased urinary excretion rates. Collagen deposition in the renal cortex (Masson Trichroome staining) and fibronectin expression (Western blotting) tended to increase in the diabetic rats, but this was not statistically significant. Finally we were unable to detect an increased expression of NADPH oxidase or of CYP4F3 expression as we had anticipated based on published results. Surprisingly, urinary protein excretion rates were increased over time in both control and diabetic animals, a result that suggested that control rats themselves were subjected to some form of stress or injury.

The reasons for the discrepancy between our results and reported results in the literature in similar models of diabetes may relate to the small number of animals used. The results show a clear tendency for increased fibronectin expression and collagen deposition in the diabetic grouop relative to controls, but with considerable

variability within the same group; a higher number of experimental animals may render this change significant. On the other hand, the results with NADPH oxidase expression are not readily explained considering the strong evidence in the literature supporting its increased expression at similar stages of diabetes in this model. As for CYP450 expression, the fact that we used whole tissues rather than microsomal preparations for measuring its expression may explain the lack of effect. All previous studies which demonstrated an increased expression of CYP4A were done on microsomal preparations; this is the first study that attempted to do so in whole tissue extracts.

Turning to the effect of drug treatment on the above changes induced by diabetes, it was clear that treatment with both HET0016 and BNF significantly reduced the kidney weight and kidney weight / body weight ratio. The significant ECM accumulation observed in the DV group was significantly reduced in both HET0016 and BNF treated groups. Similarly, the tendency for increased fibronectin expression and collagen deposition described in the DV group tended to be reversed in both treatment groups as well, with a significantly lower collagen accumulation seen in the BNF treated group compared with the DV group.Since we speculated that ROS are primary players in diabetic nephropathy, we then looked into ROS generation (DHE stain). The significant increase in generation of ROS species seen in the diabetic group was reversed by treatment with either HET0016 or BNF. We did not observe any effect of treatment with either HET0016 or BNF on NOX4 or CYP4F3 expression. As for urinary excretion rate, a noteworthy observation was

the major and significant reduction observed in the BNF treated group relative to all the other groups on days 28 of the study.

Proximal tubular epithelial cell apoptosis is an early feature of diabetic kidneys detected at the onset of diabetes (Ortiz et al., 1997). Oxidative stress plays a key role in the development and progression of diabetic complications, It has been well documented that hyperglycemia leads to tubulointerstitial injury (Park et al., 2001; Hall, 2006), and that diabetes induces apoptosis in proximal tubular epithelial cells via ROS generation (Allen et al., 2003; Liu et al., 2008). Brezniceanu et al. (2007) found that limiting ROS production in the proximal tubules by overexpressing catalase, an enzyme that inactivates H_2O_2, reduces many of the diabetic complications including apoptosis. However, the molecular mechanisms of action of oxidative stress as well as the precise sources of ROS have not been fully characterized. NADPH oxidases are expressed in the kidney cortex (Bedard and Krause, 2007), with a prominent expression of Nox1 and Nox4 in the proximal tubules (Gill and Wilcox, 2006). When exposed to high glucose, different cell types rapidly generate ROS (Inoguchi et al., 2000; Eid et al., 2009). Mitochondrial electron transport chain, NADPH oxidases, and CYP450 are considered as potential sources of ROS in cells and tissues (Shah and Channon, 2004). However, CYP4A-dependent 20-HETE is highly produced in proximal tubules (Zhou et al., 2008) and exerts a wide range of regulatory and opposing functions depending on the location of its production (Natarajan et al., 2003). In this study, we showed that diabetes was associated with significant ROS generation which was reversed by HET0016

treatment. However, we were not able to categorically identify the sources of these ROS as the changes in the protein expressions of CYPs and NAPDH oxidases, the postulated diabetic sources of ROS, were not evident. On the other hand, we were able to show that diabetes was associated with a significant increase in the production of 20-HETE which is well known to be produced by CYP4A/F with an ability to induce ROS production, upregulate Nox4 and increase extracellular matrix protein expression. Inhibition of 20-HETE synthesis by the use of HET0016 significantly reduced oxidative stress and mesangial matrix accumulation. These results, therefore, suggest that in diabetes mellitus, increased production of 20 HETE may contribute to renal injury and that treatment with a CYP4A seletive inhibitor may have a beneficial effect in maintining renal structural integrity and function.Contradictory data have been reported regarding the exact role of 20-HETE. Hoff et al. (2011) showed that 20-HETE was overexpressed in ischemic kidney tissue and increases tubular epithelial cell apoptosis. Moreover, Zeng et al. (2010) demonstrated that 20-HETE increases NADPH-derived ROS production in cardiac myocytes. CYP4A-dependent 20-HETE production has also been associated with the tubular cell injury found in renal ischemia/reperfusion injury and that CYP4A-dependent ROS generation mediates tubular cell apoptosis (Nilakantan et al., 2008). On the other hand, some studies provide opposite data to the above, whereby 20-HETE was shown to mediate epithelial cell proliferation in cystic renal disease (Park et al., 2008). Similarly, Dhanasekaran et al. (2009) reported that 20-HETE inhibited apoptosis and protected pulmonary artery endothelial cells.

It is known that proximal tubular cells exposed to high glucose undergo increased matrix protein accumulation, mainly collagen IV and fibronectin (Gilbert and Cooper, 1999). Additionally, ROS play an important role in the progression of fibrosis (Barnes and Gorin, 2011). Recent studies demonstrated that the abnormal matrix production, induced by TGF-β1 required ROS generation and subsequent activation of mitogen-activated protein kinase (MAPK) (Rhyu et al., 2012). New et al. have identified insulin-like growth factor I, as a mediator of fibronectin expression through Nox4-dependent ROS generation. However, the precise source of ROS is yet to be determined. In our study, we have provided indirect evidence that CYP4A-dependent 20-HETE generation is a potential major source of ROS in the proximal tubules since diabetes-induced mesangial matrix protein expression was significantly reduced with the administration of HET0016, as were fibronectin expression and collagen IV accumulation, though not significantly. This was also reflected in the effect on kidney hypertrophy. Collectively, these data indicate that 20-HETE formation can induce matrix protein accumulation, and that this effect is likely mediated by enhanced ROS production. The CYP2C family, catalyzing the synthesis of 11, 12-EET is highly expressed in the proximal tubules (Huang et al., 2006). The regulation of EET production is tissue and disease specific (Natarajan et al., 2003). Our data showed that whereas diabetes per se did not induce a change in urinary excretion of EETs, treatment with BNF significantly increased it, thus attesting to the efficacy of this treatment. The results demonstrate that diabetes-induced mesangial matrix accumulation and collagen deposition were significantly reduced with BNF treatment. Similarly

fibronectin protein expression was reduced in the group of rats receiving BNF though not significantly. As mentioned earlier, ROS play an important role in diabetic fibrosis. ROS generation was significantly reduced in the treatment group receiving BNF. Again, the beneficial effect of BNF treatment was reflected in the reduction in kidney hypertrophy tha was observed in the diabetic rats. As for the role of EETs in proteinuria, the administration of BNF significantly decreased urinary protein excretion when compared to both non treated diabetic rats and diabetic rats treated with HET0016. This is in agreement with a recent study which showed that the overexpression of CYP2J2 epoxygenase increased EET generation in streptozotocin-induced diabetic mice and attenuated microalbuminuria (Chen et al., 2011). In contrast, another study showed that the inhibition of the enzyme soluble epoxide hydrolase, which causes an increase in the bioavailability of EETs, contributed to albuminuria in mice models of progressive renal disease (Jung et al., 2010).

In conclusion, our observations provide preliminary evidence that in early diabetes, CYP 450 metabolites of arachidonic acid, specifically 20-HETE and EET, may play a role in the pathogenesis or modulation of diabetes induced renal injury. Our hypothesis that generation of 20 HETE may contribute to renal injury is substantiated by the fact that inhibition of its synthesis by HET0016 had significant salutary effects on several indexes of renal injury. In contrast, EET generation may play a role in protecting the kidney against injury since induction of its synthesis with BNF has similar protective effects on the kidney in this model of diabetes.

Further studies are needed to increase the number of animals, to explore the longer term effects of the drugs in later stages of diabetes, and to examine the mechanisms involved, including the role of reactive oxygen species and Nox4 activation. An investigation into the reasons for increased proteinuria in control rats housed in the Animal Care Facility is warranted as well.

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