AMERICAN UNIVERSITY OF BEIRUT

THE ROLE OF CYTOCHROME P-450 MEDIATED METABOLITES OF ARACHIDONIC ACID IN DIABETIC CARDIOVASCULAR DYSFUNCTION IN RATS

by
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AN ABSTRACT OF THE THESIS OF

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Title: The Role of Cytochrome P-450 Mediated Metabolites of Arachidonic Acid in Diabetic Cardiovascular Dysfunction in Rats

Background: Cardiovascular complications of diabetes mellitus are major causes of the increased mortality and morbidity associated with the disease. Among the manifestations of cardiovascular dysfunction are impaired endothelial mediated vasodilation and vascular responses to vasoactive agents as well as cardiac remodeling with fibrotic changes. Oxidative stress is a major contributor to diabetes induced cardiovascular dysfunction. Recent studies demonstrated significant cardiovascular effects for cytochrome *P*-450 (CYP-450) mediated arachidonic acid (AA) metabolites, 20-HETE (20- hydroxyeicosatetraenoic acid) a potent vasoconstrictor, and EET (epoxyeicosatrienoic acid) a vasodilator and potentially cardioprotective eicosanoid. The role of these products in diabetes induced cardiovascular dysfunction, however, is still controversial.

Aims: The following study aimed to investigate if blocking 20-HETE production through the treatment with 10 mg/kg/day HET0016 (*N*-hydroxy-*N*_-(4-butyl-2 methylphenyl) formamidine), a selective CYP4A isoforms inhibitor, or inducing EETs synthesis by 35 mg/kg/day BNF (β-naphthoflavone), a selective CYP2C11 inducer, in vivo, may decrease diabetes induced cardiovascular dysfunction. CYP-450 has been considered an important source of ROS (reactive oxygen species), so we further investigated the effect of HET0016 and BNF treatment on oxidative stress in diabetes. Our hypothesis was that 20-HETE played a detrimental role while EET's protected against diabetes induced cardiovascular complications.

Methods: Male Sprague- Dawley were divided into four study groups. A dose of 65 mg/kg body weight of streptozotocin (STZ) was used to induce diabetes in three groups. Control (CV) rats were administered the vehicle of STZ (normal saline solution). Two days after STZ, control and diabetic rats (DV) started receiving daily i.p. injections of 5% DMSO in normal saline, while the remaining two groups received daily i.p. injections of either HET0016 (DH group) or BNF (DF group), dissolved in 5% DMSO in normal saline. Blood glucose and body weight were measured, and urine was collected before and on days 16, and 30 after STZ. Urine samples were used to measure the level of 20-HETE and EET. On day 30, rats were sacrificed, and the thoracic aortas were isolated for vascular reactivity studies to phenylephrine, acetylcholine, and SNAP. The abdominal aortas were isolated for protein expression analysis of vascular injury markers: fibronectin, NOX-4,and α-smooth muscle actin (α-SMA). To assess the response to treatment, protein and mRNA expression of CYP2C11 and CYP4A8 were performed. Left ventricular

sections were used to evaluate cardiac collagen deposition, myocytes injury, and ROS generation by the use of Trichrome, PAS staining, and DHE staining respectively.

Results: Urinary excretion of 20-HETE was increased in the DV group and decreased with HET0016 and BNF treatment, while excretion of EETs was only increased in the DF group. Neither diabetes nor treatment with HET0016 or BNF affected the vascular responses to vasoactive agents. NOX-4 protein expression was increased in the DV group relative to CV, and this was decreased with BNF treatment. Trichrome staining showed increased left ventricle collagen deposition in hearts from DV rats that decreased with BNF but not HET0016 treatment. Both HET0016 and BNF treatment decreased the severity of myocyte injury observed in the left ventricle of diabetic treated rats. DHE staining of the left ventricle showed increased ROS in DV heart which was decreased with BNF and HET0016 treatment. There was no difference in the CYP2C11 protein and gene expression among the studied groups. CYP4A8 mRNA gene expression was increased in DH treated rats as compared to DV rats. There was no significant difference in protein expression of fibronectin and α-SMA among the study groups.

Conclusion: Our results show that treatment with BNF, and to a lesser extent with HET0016, decreased the severity of injury induced by diabetes using the cardiovascular parameters measured. These results suggest that 20 HETE may play a deleterious effect in diabetic cardiovascular dysfunction, while EETs provide a protective effect by decreasing collagen deposition, cardiomyocyte injury, and ROS generation in the left ventricle. This study suggests possible therapeutic indications for inhibitors of 20 HETE generation or for EETs or inducers of EET formation. These preliminary results need further confirmation and investigation of mechanisms involved.

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ABBREVIATIONS

AA : Arachidonic acid

Ach : Acetylcholine

BNF : β- naphthoflavone

CYP-450 : Cytochrome P-450

DHET : Dihydroxyeicosatrienoic acids

EET: Epoxyeicosatrienoic acid

HETE : Hydroxyeicosatetraenoic acid

HETE0016: *N*-hydroxy-*N*_-(4-butyl-2 methylphenyl) formamidine

LV : Left ventricle

PAS : Periodic acid Schiff

PE : Phenylephrine

ROS : Reactive oxygen species

sEH : Soluble epoxide hydrolase

SNAP : S-Nitroso-N- acetylpenicillamine

α-SMA : α-Smooth muscle actin

CHAPTER I

INTRODUCTION

A. Diabetes mellitus: an epidemic

The brisk increase in diabetes mellitus (DM) prevalence in the past years made it a growing epidemic with a constellation of health ,social ,and economic burdens (Ding et al.,2005). The number of adults with diabetes is expected to increase from 371 million in 2010 to reach 439 million by the year 2030. Unfortunately, half of the people with diabetes are undiagnosed due to poor screening and disease knowledge.

In Lebanon, the prevalence of diabetes among age groups 20-79 years is estimated to increase from 7.9% in 2010 to reach 9.6% by the year 2030. The Middle East and North Africa regions show a growing trend in diabetes where one in nine adults have diabetes (IDF Diabetes Atlas,2012).

Industrialization along with dietary changes and a sedentary lifestyle have contributed to this increasing trend. This epidemic like nature of Diabetes mellitus resulted in a major search for potential advances in genetic predisposing factors, improved molecular diagnostic methods ,and targeted treatment (Diabetes An old Disease, a New Insight,2012).

B. Overview of diabetes mellitus

The Egyptians were the first to identify diabetes as a disease defined by "the passing of too much urine" . The Greek physician Aretaeus provided the first medical

description of diabetes as "the melting down of flesh and limbs into urine" (Canadian Diabetic Association, history of diabetes). In Greek, diabetes means "to pass through" and mellitus means "honey" (Diabetes An old Disease, a New Insight,2012).

Today diabetes mellitus is classified as a constellation of metabolic disorders characterized by hyperglycemia. The abnormal carbohydrate, fat, and protein metabolism originates from abnormalities in insulin secretion, insulin action, or both (Maraschin et al.,2010). The hallmark of diabetes is hyperglycemia along with polyuria, polydypsia, weight loss, polyphagia, blurred vision, impaired growth, and increased predisposition to infections. Diabetes mellitus is associated with acute life threatening complications due to poor glycemic control as hyperglycemia with ketoacidosis or nonketotic hyperosmolar syndrome. Chronic complications affecting cardiovascular, ocular, renal ,and autonomic nervous systems occur and start to develop even before disease diagnosis is made (Diabetes An old Disease, a New Insight,2012).

C. Classification of diabetes mellitus

The old diabetes classification set by the National Diabetes Data Group (NDDG) in 1979 was based on disease treatment. Diabetes mellitus was classified into insulin dependent diabetes mellitus (IDDM) and noninsulin dependent diabetes mellitus (NIDDM) (Diabetes,1979). With the advances in disease understanding a new classification based on pathogenesis of disease rather than its pharmacology was adopted. The current classification was set by the American Diabetic Association (ADA) in 1997 and updated by the World Health Organization (WHO) in 2006 . Diabetes mellitus was classified into four

categories :type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), other specific types, and gestational diabetes (World Health Organization Report,2006).

1- Type 1 diabetes

Type 1 diabetes mellitus represents 5-10% of the cases of diabetes (Diabetes Care,2010). It is the most prevalent type in children and adolescents, and is characterized by absolute insulin deficiency. T1DM is subdivide into a prevalent type 1a with autoimmune destruction of β-islet cells of pancreas and a minority idiopathic type 1b of unknown causes. The prevalence of T1DM has increased world wide by 2-5% with a prevalence in United States of 1 in 300 by the age of 18 .

This increase has been linked to genetic factors or the interaction between genetic and environmental factors (Maahs et al, 2010).T1DM has shown a strong association with human leukocyte antigen (HLA) with linkage to DQA and DQB genes and influenced by DRB genes. HLA-DR4/DQB1 and HLA-DR3/DQB1,either or both increase diabetes susceptibility and are carried by 90-95% of T1DM children (Mehers and Gillespie, 2008).

The increase in T1DM prevalence in low genetic predisposition indicates the presence of environmental factors such as :viral infections, intake of cow's milk during childhood, and vitamin D deficiency. Viral infections may initiate an autoimmune reaction against the β-islets of the pancreas contributing to the development of T1DM in children. The proteins present in the cow's milk such as bovine insulin, casein and others may trigger an autoimmune attack against the pancreatic β-cells in children (Maahs et al.,2010). Vitamin D deficiency was shown to be associated with T1DM. Vitamin D supplementation in T1DM patients resulted in an improvement of glycemic control, insulin sensitivity, and

β-cell protection (Aljabri et al.,2010). Despite the following associations , the linkage of environmental factors with T1DM etiology is still inconclusive and warrants further interventional investigations (Maahs et al., 2010).

2- Type 2 diabetes

Type 2 diabetes mellitus (T2DM) represents 90-95% of diabetes. T2DM is highly prevalent among adults aged 45-65. It has been reported recently in children and adolescents with the increase in childhood obesity. In the year 2000, 170 million people worldwide had T2DM , and this figure is expected to reach 366 million by the year 2030 (Green et al. ,2004).

This increase in T2DM has been highly linked to increasing obesity. Obesity is a major contributor to T2DM, where it account for 55% of T2DM cases (Abdulfatai et al.,2012). Increased free fatty acid, adipokines, and leptin resistance in obese people contributes to increased insulin resistance (Diabetes An old Disease, a New Insight,2012). T2DM is a complex metabolic disorder characterized by increased hepatic glucose production ,insulin resistance and/or impaired insulin action. T2DM is currently defined as a multi-organ disease resulting from dysfunction at the level of skeletal muscles, adipose tissues, liver, β-cells of pancreas , central nervous system, along with a genetic predisposition.

Insulin resistance is a key player in T2DM pathogenesis. It is defined as an impaired response of target cells to insulin reflecting a defect in insulin-target cell interaction rather than insulin secretion. Insulin resistance at the level of skeletal muscles leads to impaired insulin mediated glucose uptake. Adipokines secreted by adipose tissues

create an inflammatory state that increases insulin resistance and promotes β-cell apoptosis. Obesity in T2DM creates a state of metabolic imbalance where the central nervous system fails to integrate various inputs to adjust body glucose levels. A first degree- positive family history of T2DM is associated with a two-fold increase in the risk of T2DM development (Lin and Sun, 2010).

D. Molecular basis of diabetic complications

Diabetes mellitus is associated with increased mortality and morbidity. According to the WHO, DM will be ranked as the seventh leading cause of death in 2030 (WHO, Diabetes fact sheet 312). Diabetes mellitus causes two types of complications: microvascular and macrovascular. The microvascular complications result from damage to small resistance arteries, arterioles, and capillaries. It manifests as retinopathy, nephropathy, and neuropathy. Macrovascular complications result from damage at the level of large conductance vessels and manifests as coronary artery disease ,cerebrovascular disease ,and peripheral arterial disease (Madonna and De Caterina, 2011).

Diabetes mellitus increases cardiovascular mortality rate by 2-4 fold compared to non-diabetic. Macrovascular complications of myocardial infarctions and stroke are the major causes of mortality among diabetics (Cusick et al., 2005). Microvascular complications contribute to increasing morbidity with 60-70% neuropathy and 60% of non-traumatic lower limb amputations in diabetics. Diabetic nephropathy is the main cause of kidney failure (National Diabetes Fact Sheet,2011)

Diabetes damages cells that are not able to slow down glucose transport when hyperglycemia exists. Compared to normal cells, increased intracellular hyperglycemia was

proven to be the initiator of diabetic damage. Four hypotheses of hyperglycemia induced damage were postulated: (1) increased polyol pathway activity ,(2) intracellular production of advanced glycation end products (AGE) and plasma protein modifications (3) increased hexosamine pathway activity (4) protein kinase C (PKC) activation (Niskikawa et al.,2000).

1- ***Increased polyol pathway activity***

Aldose reductase reduces toxic aldehydes to inactive alcohols. When glucose concentration inside the cell is elevated, aldose reductase reduces glucose to sorbitol consuming NADPH (nicotinamide adenine dinucleotide phosphate-oxidase), a cofactor for glutathione regeneration, hence increasing intracellular oxidative stress (Brownlee ,2005).

2- ***Intracellular production of advanced glycation end products (AGE) and plasma protein modifications***

Auto-oxidation of glucose generates AGE that alter intracellular protein functions, modify extracellular protein in the cell matrix, glycate plasma proteins that bind to AGE receptors and promote an inflammatory response and reactive oxygen species generation (ROS) (Brownlee,2005).

3- ***Increased hexosamine pathway activity***

When glucose level is high inside the cells, it gets metabolized to glucose-6-phosphate then fructose-6-phosaphte (F-6-P). Glutamine fructose-6-phosaphte amidotransferase(GFAT) enzyme converts (F-6-P) to UDP(uridine phosphate)-N-acetyl

glucosamine that binds transcription factors and modifies gene expression. The resultant changes increase vascular damage in diabetes (Brownlee,2005).

4- ***Protein kinase C (PKC) activation***

Hyperglycemia increases diacylglycerol inside the cell, which activates PKC altering the expression of various genes. The alteration results in increased endothelin-1 and decreased endothelial nitric oxide synthase expression ,contributing to increased vascular dysfunction in diabetes (Brownlee,2005).

Clinical trials aimed at blocking one of the mechanisms were disappointing. This indicated that a single upstream event is responsible for the activation of the four damaging pathways. Mitochondrial ROS generation in diabetes is the initiator of the diabetic complications. Superoxide anions generated cause DNA strand breakage thus activating poly(ADP-ribose)polymerase (PARP). PARP is a nuclear repair enzyme normally found in the nucleus in an inactive form and is activated upon DNA damage. Upon activation, PARP makes polymers of ADP-ribose that binds to glyceraldehyde- 3-phosphate dehydrogenase (GAPDH) and other nuclear proteins. GAPDH, an important glycolytic enzyme that shuttles normally in and out of the nucleus plays a role in the DNA repair. Hence, the resulting modifications decrease GAPDH enzymatic activity and switch on the damaging pathways.

The decrease in GAPDH enzymatic activity increases the levels of upstream glycolytic metabolites such as glyceraldehyde- 3-phosphate ,thus activating AGE and PKC pathways. Increased glyceraldehyde- 3-phosphate generates non-enzymatically methylglyoxal the major AGE precursor. Diacylglycerol formed from glyceraldehyde- 3-

phosphate will activate PKC pathway. Moreover, the levels of the glycolytic metabolite fructose-6-phosphate will also increase resulting in the activation of the hexosamine pathway. With the decrease in GAPDH activity the intracellular levels of the first glycolytic metabolite glucose will be increased thus activating the polyol pathway (Brownlee and Giacco, 2010).

Despite the clear elucidation of the diabetic damaging pathways origin, there was a concern regarding to whether both diabetic micro and macrovascular complications would follow the same pathways described above. Prospective studies of HbA1c and diabetic complications, showed a significant 10-fold increase in the risk of microvascular complication when HbA1c increased from 5.5-9.5% , and only two-fold increase of macrovascular complications with the same HbA1c increase (UKPDS,1998). This indicated that hyperglycemia is at the origin of the development of microvascular complications. However, insulin resistance resulting in increased free fatty acid oxidation is at the basis of the development of macrovascular complications (Brownlee and Giacco, 2010).

In brief, both micro and macrovascular complications in diabetes stem from an increased mitochondrial ROS generation due to either hyperglycemia or increased fatty acid oxidation along with insulin resistance, with decreased GAPDH enzymatic activity being the key to the activation of the four damaging pathways. Hence, therapeutic targeting of the ROS generated in diabetes can present a future therapy for the management of diabetic complications.

E. Cardiovascular dysfunction in diabetes

Diabetes is a major risk factor for cardiovascular disease (CVD), affecting both the heart and vasculature. Vascular dysfunction results in retinopathy, nephropathy, peripheral vascular disease (PVD),stroke, and coronary artery disease (CAD). Endothelial dysfunction is at the basis of these complications (Dokken and Triggle,2005). Myocardial damage in diabetes leads to congestive heart failure (CHF), as a result of CVD or diabetic cadiomyopathy (Kawaguchi et al.,1997). CVD increases mortality rate by 3-to 4 times compared to general population (Haffner et al., 1998). Diabetes increases CAD risk by 2- to 4 times and PVD by 10-fold compared to non-diabetic (Laakso ,1999).

1- Vascular endothelium: Role and Mediators

The vascular endothelium is a continuous monolayer of endothelial cells that line the lumen of blood vessels (Verma et al.,2003).It plays an important role in the control of vascular function and structure along with maintaining vascular homeostasis (Furchgott & Vanhoutte, 1989). In addition to maintaining vascular tone, vascular endothelium through the release of various bioactive mediators regulates vascular permeability, clotting and fibrinolytic pathways, smooth muscle growth, inflammatory and oxidative interactions (Aljada and Dandona,2004).

Nitric oxide (NO) released by the endothelium is an important endothelium derived relaxing factor (EDRF) that plays an important role in maintaining vascular tone, opposing endothelium derived contracting factors, inhibiting platelet and leukocytes aggregation, and preventing smooth muscle proliferation (Verma et al.,2003).

The activation of endothelial receptors by acetylcholine, bradykinin, or shear stress results in calcium influx inside the cell activating endothelial nitric oxide synthase enzyme (eNOS). eNOS converts L-arginine to L-citrulline resulting in the synthesis of NO. NO diffuses into vascular smooth muscles and activates guanylate cyclase/cyclic GMP-dependent pathway resulting in vasodilatation (Moncada and Higgs,1993) .Prostacyclin (PGI_2) resulting from cyclo-oxygenase mediated metabolism of endothelial arachidonic acid is another EDRF that leads to vascular smooth muscle relaxation. The endothelium can promote smooth muscle relaxation by hyperpolarizing the membrane through the release of endothelium derived hyperpolarizing factor (EDHF), yet to be elucidated (Vanhoutte et al.,2009). Several molecules have been proposed to act as EDHF such as: potassium ion (K^+), hydrogen peroxide (H_2O_2),and epoxyeicosatrienoic acids (EETs) (Fitzgerald et al.,2005). EDHF contribute to maintaining vascular tone and reactivity in conditions where NO- mediated vascular control is impaired as in diabetes (De Vriese et al.,2000)

2- Vascular dysfunction in diabetes: micro- and macrovascular disease

McVeigh and colleagues were the first to show that endothelial dysfunction in T2DM is associated with impaired vasodilatation in response to endothelial or direct smooth muscle cell stimuli. They postulated that this endothelial dysfunction results from reduced NO release (McVeigh et al., 1992). Endothelial dysfunction is thought to be the key behind development of micro- and macrovascular complications (Schalkwijk and Stehouwer,2005). Endothelial dysfunction is the loss of the endothelium to maintain its

normal functions and regulate the balance between vasodilating and vasocontracting factors, procoagulant and anticoagulant, proliferators and growth inhibitors, with a net outcome of decreased vasodilator, increased inflammatory, and prothrombotic states (Nossaman et al., 2000).

Microcirculation is controlled by central and local regulators. Central regulation occurs via the autonomic sympathetic and parasympathetic nerves system that act on vascular smooth muscles. Local regulation is mediated by the endothelium and local products of metabolism. Both regulatory mechanisms respond to alterations in blood flow and maintain vascular tone (Lewis,1998). Diabetic autonomic neuropathy (DAN) along with endothelial dysfunction result in microvascular disease (Ewing et al.,1980). Diabetic autonomic neuropathy affects neurotransmitter release and neuronal response. Diabetes is associated with low plasma noradrenalin levels leading to decreased vessel constriction and impaired capillary circulation (Watkins et al, 1998). Decreased response to vasoactive agents contributes to the circulatory abnormality seen in diabetics. Decreased response to noradrenalin was recorded in diabetic rats after 2 to 6 weeks of streptozotocin injection (Lucas P.D., 1985). Diabetes causes impaired endothelium-dependant vasodilatation in response to acetylcholine (Nitenberg et al.,1993). Diabetes decreases NO bioavailability due to insulin resistance or deficiency thus contributing to the development of endothelial dysfunction (Brownlee,2001).

Microvascular disease results not only from impaired vascular homeostasis leading to endothelial dysfunction, but also from morphological changes of the blood vessels altering their function. Hyperglycemia in diabetes leads to capillary basement membrane thickening and protein modification. The capillary basement membrane is composed

primarily of collagen IV, fibronectin ,laminin and heparin proteoglycans. The alteration of the expression and structure of these proteins in diabetes results in the modification of capillary membrane permeability. These capillary morphological changes contribute to the development of the microalbuminuria seen in diabetic nephropathy, retinopathy and vasculopathy (Dokken, 2008).

Atherosclerosis is the major cause of macrovascular disease in diabetes (Dokken,2008). Endothelial dysfunction along with dyslipidemia contribute to the development of atherosclerosis in diabetes. Diabetes is associated with decreased HDL, increased triglyceride, and abundant small dense LDL (Rosenson,2004).

3- Cardiac dysfunction in diabetes:

Diabetes is associated with increased risk of heart failure development (Dokken,2008). The U.K. Prospective study showed that with every 1% increase in HbA1c a 12% increase in heart failure risk resulted (Stratton et al.,2000). Heat failure in diabetes can result from macro- and microvascular disease. Macrovascular disease as hypertension and CAD lead to ischemic and thrombotic events contributing to the development of heart failure. Heart failure due to microvascular disease leads to myocardial injury, fibrosis, and hypertrophy resulting in diabetic cardiomyopathy (Kawaguchi et al.,1997). Diabetic cardiomyopathy is a result of diabetic autonomic neuropathy along with endothelial dysfunction (Dokken,2008). Diabetic autonomic neuropathy impairs coronary flow reserve resulting in diastolic dysfunction. Myocardial injury, fibrosis, and hypertrophy in diastolic dysfunction result from increased free fatty acid utilization, ROS generation, decreased NO levels, and release of inflammatory cytokines (Dokken,2008).

4-Molecular basis of diabetic vascular dysfunction:

Diabetic endothelial dysfunction is associated with a decrease in NO bioavailability. The NO decrease in diabetes is attributed primarily to increased ROS generation. The increase in ROS alters signaling pathways implicated in eNOS activation. Oxygen free radicals lead to eNOS uncoupling, where eNOS shifts from NO to superoxide producing enzyme. Electrons will be diverted from L-arginine to molecular oxygen resulting in superoxide instead of NO synthesis. Endothelial nitric oxide synthase (eNOS) uncoupling results from the oxidation of zinc thiolate cluster of eNOS and its cofactor tetrahydro-L-biopterin along with decreased L-arginine in diabetes. The uncoupling of eNOS will alter eNOS enzymatic activity, increase ROS production and decrease NO synthesis. Nitrosative stress encompassing increased inducible NOS (iNOS) activation and peroxynitrite generation contribute to endothelial and cardiac dysfunction in DM.

Unlike constitutive eNOS, iNOS is increased in vascular tissues under the effect of inflammatory mediators that are increased in DM. Inducible nitric oxide synthase (iNOS) increases NO production in the presence of oxygen free radicals, that react to form peroxynitrite ($ONOO^-$). Peroxynitrite is a strong oxidant that damages the endothelium, VSM, and myocardium by reacting with cellular proteins, damaging DNA and inducing apoptosis (Contemporary Endocrinology: Cardiovascular Endocrinology: Shared Pathways and Clinical Crossroads, Nossaman et al.).

Cytochrome P-450 (CYP-450) mediated arachidonic acid metabolites epoxyeicosatrienoic acids (EETs) and hydroxyeicosatetraenoic acids (20-HETE) affect the eNOS/NO pathway, and thus the development of endothelial dysfunction. EETs increase

eNOS expression and NO synthesis (Deng et al.,2010).Wang et al., showed that cultured bovine aortic endothelial cells incubated with EETs or transfected with CYP-450 - epoxygenases resulted in increased eNOS mRNA and protein expression. The addition of 17-ODYA,CYP-450 inhibitor, to the cells decreased the up regulation in eNOS protein expression mediated by CYP-450 epoxygenases transfection. Cells transfected with CYP-450 epoxygenases showed a three-fold increase in eNOS activity compared to vehicle vector (Wang et al.,2003). Human platelet cells incubated with 11,12-EETs showed an increase in NOS activity and NO production in vitro (Zhang et al.,2008). Mouse aortic endothelial cells in presence of 8,9-EET and 14,15-DHET showed an increase in NO production that was inhibited by L-NAME (Hercule et al.,2009).Thus, EETs by increasing eNOS expression and NO production may play a protective role in the vasculature. On the other hand, 20-HETE added to bovine aortic endothelial cells lead to superoxide generation resulting from eNOS uncoupling. 20-HETE by inhibiting NO synthesis and promoting ROS generation through eNOS uncoupling, augments endothelial dysfunction (Cheng et al.,2008).

Inflammatory processes play a role in the development of diabetic vascular dysfunction. Human aortic endothelial cells exposed to high glucose result in nuclear factor kappa b (NFκB) gene modifications and increase expression of several inflammatory cytokines :monocyte chemoattractant protein-1(MCP-1), *vascular cell adhesion mole*cule-1(VCAM-1), *intracellular adhesion mole*cule-1(ICAM-1),and interleukin-6 (IL-6) that augment cardiovascular disease in DM (Basha et al.,2012).

Cytokine activated human endothelial cells treated with EETs showed an attenuation of inflammatory responses via inhibition of NF-κB–mediated VCAM-1

expression (Node et al.,1999). Peroxisome proliferator-activated receptor (PPAR) transcription factors found in vessels and myocardium possess an anti-inflammatory effect. EETs increase PPAR activation resulting in anti-inflammatory effects (Liu et al.,2005). Apo-lipoprotein E deficient mice infused with angiotensin (II) showed upon treatment with soluble epoxide hydrolase (sEH) inhibitor that inhibits EETs degradation a down regulation of inflammatory mediators, lipid lowering effects, and attenuation of atherosclerosis. sEH through EETs augmentation lead to vasculoprotective effects (Zhang et al., 2009).

NO through its vasodilatory and anti-inflammatory effects protects the vasculature. 20-HETE was shown to oppose these effects through its vasoconstricting and pro-inflammatory properties. In addition to eNOS uncoupling, 20-HETE leads to endothelial dysfunction by stimulating endothelial activation through NF-κB mediated increase of inflammatory cytokines and adhesion molecules (Cheng et al.,2010).

Both EETs augmentation and 20-HETE inhibition may play an essential role in diabetic endothelial dysfunction by influencing eNOS/NO and inflammatory pathways. Based on the above reported studies, CYP-450 arachidonic acid metabolites may influence the development of vascular dysfunction in diabetes by affecting both the synthesis and activity of the eNOS/NO and inflammatory pathways responsible for the vascular dysfunction. This may imply the involvement of CYP-450 arachidonic acid metabolites in the molecular basis of diabetic vascular dysfunction. Thus, Pharmacological therapy aimed at increasing EETs levels or blocking 20-HETE may possess a therapeutic potential in the management of vascular dysfunction in diabetes.

F- Cytochrome P-450 mediated arachidonic acid metabolites and diabetes

1-Cytochrome P-450(CYP-450) metabolites of arachidonic acid

Arachidonic acid (AA) was long known to be metabolized by cyclooxygenase (COX) and lipooxygenase (LOX) enzymes into 5-,12- and 15-hyroxyeicosatetraenoic acid (HETE),prostaglandins, prostacyclins, thromboxanes and leuokotrienes. These lipid mediators play an important role in the regulation of vascular tone,renal and pulmonary functions along with inflammatory reactions (Roman, 2002). In 1981, Capdevila et al. identified a third pathway of AA metabolism via cyp450 (Capdevila et al.,1981). Subsequent studies identified that CYP-450 enzymes in the hepatic and extrahepatic tissues metabolize AA into: epoxyeicosatrienoic acids (EET) and hydroxyeicosatetraenoic acids (HETEs) (Roman,2002). Studies performed on rabbit medullary thick ascending limb (mTAL) pointed to the presence of two CYP-450 derived AA metabolites. One of these metabolites bocks Na^+-K^+-ATPase activity, while the second relaxes blood vessels (Schwartzman et al.,1985).The two CYP-450 metabolites then were identified :20-HETE and 11,12-EET (Schwartzman et al.,1986).

Despite these findings the CYP-450 mediated AA pathway was neglected due to the short half life of metabolites and the lack of CYP-450 inhibitors (Roman,2002). It was not until a decade ago when three remarkable observations on the role of CYP-450 metabolites of AA were made which shed light on the importance of these metabolites in renal and vascular control (Roman,2002). First, CYP-450 gene expression was found to be altered with certain diseases. For instance, CYP4A2 gene was found to be overexpressed in the kidneys of spontaneously hypertensive rats (SHR) and that it was controlled by salt

intake (Iwai and Ingami,1991).Second,it was observed that 20-HETE levels were increased in the kidneys of SHR (Sacerdoti et al.,1988). Finally, it was reported that by selectively depleting renal CYP-450 through the treatment with stannous chloride ($SnCl_2$), blood pressure was restored to normal in SHR. Treatment with $SnCl_2$ leads to the depletion of 20-HETE that is the major renal CYP-450 AA metabolite. This showed that 20-HETE is involved in the regulation of renal vascular function and blood pressure (Escalante et al.,1991).These results demanded further investigations to clearly elucidate the role of CYP-450 metabolites of AA in the control of renal and vascular functions in relevance to various disease conditions.

AA was shown to be metabolized by CYP-450 *w-hydroxylases* into 20-HETE and by *epoxygenases* into EET and dihydroxyeicosatrienoic acids (DHETs) in the brain, heart, blood vessels, lungs, liver, pancreas and intestines. These lipid mediators act as second messengers, paracrine ,and autocrine factors in the control of various physiological processes (Roman,2002).

2- 20-Hydroxyeicosatetraenoic acid

HETEs are produced by AA CYP-450 mediated w-hydroxylation. CYP4F2 is the enzyme that primarily produces 20-HETE in human kidney's (Powell et al.,1998). In rat tissues, isoforms of CYP4A and CYP4F family have been identified in producing 20-HETE. Of the CYP4A enzyme family, CYP4A2 and CYP4A3 are present in the vasculature, where RT-PCR and western blot expression indicated that CYP4A2 is constitutively expressed in liver, kidney and vasculature of rats (Ito et al.,1998). 20-HETE is produced in the vascular smooth muscles of renal, cerebral, pulmonary and skeletal

muscle arterioles (Roman,2002). 20-HETE is a potent vasoconstrictor of small arteries and arterioles with little or no effect on large arteries or aorta. This warrants further investigation, but it can be attributed to the difference in K^+ channel expression, the site of 20-HETE activity (Muthalif et al.,1998).

20-HETE plays an important role in the regulation of vascular tone (Roman,2002). Membrane stretch, angiotensin II(AgII) and norepinephrine (NE) activate phospholipase C (PLC) that synthesizes inositol triphosphate (IP3) and diacylglycerol (DAG).IP3 stimulates the release of Ca^{2+} from endoplasmic reticulum. Elevation in intracellular Ca^{2+} ,activates Ca^{2+} sensitive phospholipase A2 (PLA) and DAG lipase to release AA and produce 20-HETE. 20-HETE blocks the calcium-activated potassium (K_{Ca}) channel depolarizing the membrane and enhancing the Ca^{2+} influx through the L-type Ca^{2+} channel. A net outcome of vascular smooth muscle contraction results (Gebremedhin et al., 1998).

20-HETE ,a potent vasoconstrictor, was shown to induce hypertension in both normotensive and SHR through the inhibition of calcium-activated potassium (K_{Ca}) channels (Stec et al.,1997). The main site of 20-HETE production is the rat kidney through CYP4A2 (Schwartzman et al.,1996).Bovine aortic endothelial cells incubated with 20-HETE showed a decrease in NO release and increased generation of superoxide anions as a result of 20-HETE induced eNOS uncoupling. Thus, 20-HETE has been linked to endothelial dysfunction and its development (Cheng et al.,2009). Rats transfected with CYP4A2 adenovirus had higher levels of 20-HETE and lower levels of NO compared to control rats. The increased vascular synthesis of 20-HETE contributed to endothelial dysfunction development in these rats, manifested as decreased acetylcholine mediated relaxation, increased oxidative stress and hypertension development (Wang et al.,2006).

Androgen induced hypertension and endothelial dysfunction were reversed upon the use of HET0016 (*N*-hydroxy-*N*_-(4-butyl-2 methylphenyl) formamidine) , a 20-HETE synthesis inhibitor, in vivo (Singh et al., 2007). In addition to impairment in NO activity, 20-HETE causes endothelial dysfunction by promoting endothelial activation and the release of proinflammatory cytokines and adhesion molecules (Ishizuka et al.,2008).

It has been noted that along with 20-HETE level elevation in some hypertensive animal model, an associated hyperglycemia or hyperinsulinemia coexisted (Lai et al.,2012). Stroke prone hypertensive rats showed elevated 20-HETE levels along with hyperglycemia (Williams et al.,2010). SHR showed elevated 20-HETE along with hyperinsulinemia (Potenza et al.,2005). CYP4A, the major CYP-450 isoform implicated in 20-HETE production, expression is increased with diabetes. Streptozotocin (STZ) diabetic rats showed an increase in CYP4A expression and 20-HETE production in renal and cardiac microsomes (Yusif et al.,2009). Transgenic CYP4F2 mice showed an increase in hepatic 20-HETE levels along with the development of hypertension and hyperglycemia compared to wild type. These alterations were reversed with the use of 20-HETE synthesis inhibitor HET0016 (Lai et al.,2012). In a human study with metabolic syndrome, a significant elevation in 20-HETE plasma and urinary levels was detected (Tsai et al.,2009).

The increased expression of CYP4A mediated 20-HETE production in STZ treated rats with its vasoconstricting properties may play an impotent role in affecting the development of diabetes induced vascular dysfunction. Moreover, 20-HETE increased levels have been associated with hyperglycemia and insulin resistance. Thus, blocking 20-HETE may possess a new therapeutic approach in the treatment of DM and its associated vascular complication.

3- Epoxyeicosatrienoic acid

AA is metabolized by CYP-450 epoxygenases to yield EETs and DHETs. Unlike 20-HETE, several CYP-450 enzymes in different tissues are implicated in EETs production (Roman,2002).CYP2C8 is the major hepatic epoxygenase in humans (Zeldin et al.,1996). CYP2C8 and CYP2C9 are the major isoforms forming EETs in human endothelial cells. Cyp2J2 is the primary enzyme that catalyzes EET production in the extrahepatic tissues in human heart, pancreas, lungs and kidney (Roman,2002). In the liver of male rats CYP2C11 and CYP2J3 are the major epoxygenases. CYP2J3 contributes to EET production in the heart, pancreas, liver, lung and kidney of rats. CYP-450 epoxygenase isoforms show different region and stereospecificty with respect to the EET formed; similarly the response to EETs in the vasculature is regio- and stereospecific (Roman,2002).

EETs are short lived metabolites and are rapidly degraded to DHETs by the enzyme soluble epoxide hydrolase (sEH). The physiological role of these DHETs is still controversial. DHETs were thought to be the inactive form of EETs, but recently 11,12-DHET was found to produce coronary vasodilatation (Spector ,2009). EETs are produced primarily by the endothelial cells , where as the vascular smooth muscles are the major source of 20-HETE (Campbell and Fleming,2010). sEH inhibitors are being used in various studies to augment EETs activity, and have been found to decrease blood pressure, prevent cardiac hypertrophy, prevent vascular smooth muscle proliferation, improve renal hemodynamic and protect against hypertension induced renal damage (Spector,2009).

EETs exert their effects through different signal transduction pathways. EETs are considered as endothelium derived hyperpolarizing factors (EDHF) and posses

vasodilatory, anti-inflammatory, antiapoptotic ,angiogenic and cardioprotective effects (Specor,2009).EETs exert their effects through autocrine and paracrine pathways.

Autocrine effects are mediated by the activity of EETs on the endothelium. Paracrine effects result from EETs released into the extracellular fluid space between the endothelium and vascular smooth muscle (Campbell and Fleming, 2010). EETs hyperpolarize or relax vascular smooth muscle by activating Gαs protein coupled receptors or transient receptor potential channel (TRP) vanilloid-type 4. EETs released by endothelium bind Gαs coupled receptors activating adenylyl cyclase and cAMP production. cAMP activates protein kinase A (PKA) that phosphorylates ATP sensitive K^+ channel resulting in K^+ efflux and membrane hyperpolarization. TRP4V channels on smooth muscles activated by endothelial EET allow Ca^{2+} influx. Increased intracellular Ca^{2+} promotes Ca^{2+} release form endoplasmic reticulum and activation of Ca^{2+} activated K^+ channel (BKca). K^+ efflux through the channel leads to membrane hyperpolarization and relaxation (Campbell and Fleming,2010).

CYP-450 epoxygenase pathway through EETs generation plays an important role in blood pressure regulation. sEH gene deletion (Ephx2-/-) in DOCA-salt (deoxycorticosterone acetate plus high salt mice models) hypertensive mice resulted in lower blood pressure and attenuated renal inflammation and injury compared to wild type DOCA-salt mice (Manhiani rt al.,2009). sEH cortical protein expression was up regulated in rats with angiotensin (II) induced hypertension compared with normotensive rats. The administration of intraperitoneal sEH inhibitor lowered blood pressure in hypertensive rats (Imig et al.,2002). sEH gene (Ephx2) was identified as a susceptibility gene for hypertension associated heart failure in rats (Monti et al.,2008). Genetic deletion of sEH

and pharmacological treatment with t-AUBC (selective inhibitor of sEH [*trans*-4-[4-(3-adamantan-1-ylureido) cyclohexyloxy]-benzoic acid (*t*-AUCB)] prevented hyperglycemia in STZ treated mice. sEH treated diabetic mice showed an increase in insulin secretion and decrease β-cell apoptosis (Luo et al.,2010).

CYP-450 epoxygenase are altered with disease. A decrease in CYP2C isoforms and increase in CYP4A isoforms been documented in obesity and diabetes (Luo and Wang,2011). Hyperglycemic mice treated with sEH inhibitor had improved insulin signaling and sensitivity (Luria et al.,2011). Being involved in the regulation of blood pressure ,inflammatory cascades and glucose homeostasis ,EETs appear to play an important role in protecting the CV system in DM (Guglielmino et al.,2012).

The ability of EETs to increase insulin secretion and decrease β-cell apoptosis, along with the their anti-inflammatory and vascular protective effects makes them an interesting target in the pharmacotherapy of DM and its associated cardiovascular complications. Hence, the increase in EETs level and induction of synthesis may play an important role in ameliorating diabetes induced cardiovascular dysfunction.

G- 20-HETE and EET in diabetic cardiovascular dysfunction

20-HETE increased vascular reactivity in diabetes, thus contributing to diabetes induced vascular dysfunction. The vascular dysfunction was manifested as augmented response to vasoconstrictors and impaired response to vasodilators. Renal artery and mesenteric vascular beds of STZ treated male Wistar rats showed an augmented vasoconstriction response to noradrenalin (NA), enodothelin-1(ET-1), and angiotensin(II) (AgII). Treatment with HET0016 or ABT (1-aminobenzotriazole) a 20-HETE and EET

inhibitor , reduced these enhanced vascular responses in the perfused vascular mesenteric beds, and were able to correct the impairment in vasodilator response to carbachol ,histamine, and SNP (S-Nitroso-N- acetylpenicillamine) (Yousif et al.,2009).

An important finding by Yousif et al. showed that 20-HETE mediated vascular hyper reactivity may not be related to increased CYP4A protein expression in the renal vasculature of diabetic rats as it was in the kidneys (Yousif et al.,2009). Instead, 20-HETE mediated increase in vascular reactivity is due to alterations at the level of signal transduction pathways promoting an increase in 20-HETE level or enhanced vascular effects (Muthalif et al.,1998; Muthalif et al.,2000;Yousif et al.,2009). This implies that blocking the production of 20-HETE may not be sufficient to ameliorate the diabetes related vascular dysfunction, since various signal transduction pathways tend to influence 20-HETE mediated vascular effects.

Endothelial dysfunction in diabetes is associated with increased 20-HETE productions impairing NO mediated vasodilation through superoxide generation (Wang et al.,2006). HET0016 through inhibiting 20-HETE synthesis restores normal NO dependent vasodilation (Yousif et al.,2009). Hence, HET0016 by blocking 20-HETE synthesis, tends to reduce ROS induced vascular dysfunction in diabetes.

Microsomes from hearts of diabetic rats showed a two-fold increase in 20-HETE production compared to control (Yousif et al.,2009). 20-HETE elevation in diabetic animals contributes to ischemic injury and cardiac dysfunction (Han eta l.,2007;Shimajo et al.,1993). Thus, blocking 20-HETE production may possess a cardioprotective effect in diabetes through preventing diabetes induced cardiac fibrosis and remolding. Inhibition of sEH, thus increasing bioavailability of EETs, improved myocardial perfusion in

cardiovascular dysfunction linked to increased oxidative stress as in diabetes and hypertension (Davis et al.,2002;Schmelzer et al.,2005). Thus, EETs may decrease oxidative stress induces cardiac dysfunction. Cardiac function studies on diabetic hearts following ischemic/reperfusion studies were impaired compared to control. Pretreatment of diabetic hearts with CDU (sEH inhibitor) or HET0016 enhanced cardiac recovery after ischemic/reperfusion injury (Yousif et al.,2009).

Mesenteric vascular beds and carotid artery of SHR and Wistar-Kyo (WKY) rats treated with L-NAME to induce endothelial dysfunction were used for vascular studies in presence of HET0016 and CDU. HET0016 and not CDU restored vasoconstrictor response to PE in both vessels. While ,CDU and not HET0016 was able to restore vasodilator response to carbachol in carotid artery only. Benter and Yousif explained that CYP-450 metabolites tend to act differently in different vascular beds. Endothelial dysfunction associated with impaired NO activity increases 20-HETE formation, since NO inhibits 20-HETE synthesis in VSMC. Thus, HET0016 and not CDU, by blocking 20-HETE production was able to correct abnormal vascular response to PE in conditions of low NO. Vasodilator response restored with CDU indicates that sEH by increasing EETs can restore vasodilator response in conditions of impaired NO production (Benter and Yousif,2010).

Glucotoxicity in diabetes contributes to cardiovascular dysfunction and remodeling. EETs being implicated in the regulation of blood pressure, inflammation, and glucose homeostasis were suggested to play a role in protecting the heart against structural and functional alterations of hyperglycemia. Cardiac myocytes of left ventricle from control, untreated and APAU (1-(1-acetypiperidin-4-yl)-3-adamantanylurea, sEH inhibitor) treated diabetic UCD-T2DM rats (obtained by breeding Sprague-Dawley and Zucker

diabetic rats) were evaluated for structural remodeling, hypertrophic protein expression and intracellular [Ca^{2+}] reflective of cardiac contractility. APAU treatment ameliorated structural remodeling, decreased protein expression of hypertrophic and remodeling markers (brain natriuretic peptide (BNP) and sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA)), restored normal Ca^{2+} store in sarcoplasmic reticulum and Ca^{2+} transient amplitude. EETs were shown to possess an important role in protecting the heart against structural remodeling and hypertrophic changes associated with diabetes (Guglielmino et al.,2012).

H- Epoxyeicosatrienoic acids and endothelium derived hyperpolarizing factors

Vessels treated with NO and COX inhibitors, vasodilate in presence of Ach, bradykinin and AA. Thus, the endothelium releases mediators other than NO and prostacyclin that lead to vasodilatation. It was determined that the substance released induce vasodilation by hyperpolarizing vascular smooth muscles (VSM).The use of K^+ channel blockers or the elevation of extracellular K^+ abolished the hyperpolarization and vasodilation response. The substance was referred to as EDHF. EETs are produced by the endothelium and result in vasodilation through the activation of KCa channel and membrane hyperpolarization. This raised the question whether EETs are EDHF (Roman, 2002) .

Methacholine induced vasodilation of precontracted bovine coronary arteries with intact endothelium. The relaxation was blocked by the use of miconazole or SKF 525A (CYP-450 inhibitors), TEA (an inhibitor of KCa), or by increasing extracellular K^+ level. These findings indicated that methacholine produces endothelium dependent relaxation via

hyperpolarization that is CYP-450 mediated. Bovine coronary arteries perfusate treated with labeled AA in presence of methacholine showed an increase in EETs when measured using HPLC (high performance liquid chromatography). Thus, CYP-450 metabolites of AA, EETs, act as EDHF in response to methacholine in small coronary arteries (Campbell et al.,1996). The use of β- naphthoflavone, CYP1A and CYP2C inducers, increased EETs production and enhanced EDHF responses (Popp et al.,1996). Campbell et al., showed also that the 14,15-EET is the major regioisomer released in the vasculature. The addition of indomethacin ,nitro-L- arginine (L-NA), and bradykinin to bovine coronary arteries resulted in endothelium dependent relaxation that is inhibited by CYP-450 inhibitors (Campbell et al.,2001).

However, despite the various studies regarding the potential role of EETs as EDHF, the results are still inconclusive. Moreover, the chemical and functional identity of EDHF is highly dependent on vessel type, size, and species studied. EETs as EDHF have a greater vasodilating potential in small arteries and arterioles than in large arteries. A number of substances are suggested to be EDHF candidates : potassium ion, EETs, C-type natriuretic peptide, hydrogen peroxide, and anandamide (Campbell and Falck,2007).

A debate was raised regarding the site of action of EETs as EDHF and their mechanism of action. Do EETs act as autocrine factors on the endothelium or as transferable factors on VSM ? The discovery of EET selective antagonist 14,15-EEZE (14,15-epoxyeicosa-5(Z)-monoenoic acid) helped in solving the debate. Using a dual-cannulated, tandem perfused bovine coronary artery bioassay , Gauthier et al., were able to show that EETs act as endothelium derived transferable relaxing factors, stimulated by bradykinin, inhibited by 14,15-EEZE ,and dilate VSM.

The bioassay consisted of donor arteries with intact endothelium connected to detector arteries without endothelium. Bradykinin,14,15-EET and 14,15-EEZE were added to vessels. Detector arteries were preconstricted with U46619 ,a vasoconstrictor. Bradykinin added to donor arteries resulted in vasodilation of the detector arteries. Removing the endothelium of the donor arteries in presence of bradykinin abolished the vasodilation. Thus, bradykinin stimulated the release of an endothelium derived transferable factor.

The addition of 14,15- EET to preconstricted detector arteries resulted in a dose dependent vasodilation, while 14,15-EEZE blocked vasodilation in detector arteries without affecting vasodilation in donor arteries. This indicated that a transferable relaxing factor works at the level of VSM to promote vasodilation. The inhibition of vasodilation with 14,15-EEZE implies that EDHF is 14,15-EET.

Perfusate obtained from donor arteries with bradykinin showed upon mass spectrometry analysis the presence of 14,15 and 11,12-EETs. The fact that donor arteries did not vasodilate in presence of 14,15-EET and the relaxation was not blocked by 14,15-EEZE, yet 14,15-EET existed in the coronary perfusate implies the presence of a signaling pathway ought to be clarified or an unknown candidate promoting EETs release to VSM (Gauthier et al.,2001).

Campbell and Fleming elaborated that EETs act as EDHF via two proposed mechanisms: "transfer of factor " and "transfer of hyperpolarization ". Arteries in different organs and sites, may utilize either or both of these mechanisms.

The traditional transfer of factor mechanism is characterized by EETs released from endothelium as transferable factors that hyperpolarize VSM by acting on BKCa

channel. The transfer of hyperpolarization occurs when EETs act in an autocrine way on EC (Campbell and Fleming, 2010) . EETs lead to Ca^{2+} influx thro TRPV4 channels found on blood vessels and activated by AA CYP-450 mediated metabolites (Watanabe et al.,2003). Ca^{2+} influx in response to 5,6 and 8,9-EET is abolished in EC of TRPV4 null mice (Vriens et al.,2005). Hence, endothelial CYP-450 metabolite through TRPV4 channel activation result in vasodilation (Earley et al.,2005).The increase in intracellular Ca^{2+} release leads to microsome Ca^{2+} depletion and activation of CYP-450 mediated metabolism of AA in EC and further release of EETs (Quilley and McGiff et al.,2000). The net increase in intracellular Ca^{2+} activates small conductance (SK) and intermediate conductance (IK) K_{Ca} channels leading to hyperpolarization and K^+ efflux into subendothelail space. Potassium ion activates sodium-potassium- ATPase or inward rectifying K+ channel on VSM that mediate K^+ ion efflux. Gap junctions between EC and VSM contribute to transfer of hyperpolarization (Campbell and Fleming, 2010).

EDHF may play a compensatory role in diabetic endothelial dysfunction by regulating blood flow along with vascular tone and reactivity as a result of decreased NO availability. Results regarding the role of EDHF in T1DM are conflicting. EDHF mediated responses are decreased in mesenteric, carotid artery and renal circulation of STZ diabetic rats. Opposing these findings is an increase in EDHF vasodilation in femoral and mesenteric arteries of STZ treated animals. Type 2 diabetes mellitus is associated with unchanged or augmented EDHF mediated relaxation in response to impaired NO and PGI2 vasodilation. Differences in diabetes models used, disease stage, vessel type and size, and study conditions all affect the differences in EDHF relaxation results.

The results regarding EDHF contribution to diabetes are still inconclusive. Because of the unknown nature of EDHF, studies aimed at augmenting EDHF pathways to asses therapeutic outcome in diabetic vascular dysfunction are very few. With further investigations, EDHF can become a therapeutic target for the treatment of diabetic vascular dysfunction and its associated complications (Gao et al.,2011).

I- Diabetic cardiovascular dysfunction and oxidative stress

Oxidative stress is an increase in ROS generation and/or a decrease in the cellular antioxidant defense mechanisms. Oxidative stress is a key element in the development of cardiovascular disease in diabetes. CYP-450, NADPH oxidases, and uncoupled eNOS are among the enzymes involved in ROS generation in diabetes (Ding and Triggle,2005).

Endothelial cells in presence of hyperglycemia showed an increase in mitochondrial ROS generation (Camici et al.,2007). STZ induced diabetic mice showed higher plasma super oxide levels compared to control mice (Matsumoto et al.,2003). Diabetic patients plasma showed low levels of glutathione, glutathione peroxidase and catalase activity that are responsible for decreasing oxidative stress (Dave and Kalia,2007). The administration of super oxide dismutase normalized the increase in ROS production in diabetic animals (Reis et al.,2008). Oxidative stress contributes to the development of cardiac structural and functional changes termed as diabetic cardiomyopathy (Khular et al.,2010). ROS overproduction in the heart of ob/ob and db/db mice resulted in increased apoptosis (Barouch et al.,2003). Hence, treatment targeting the increased oxidative stress in diabetes is integral to the vascular and cardiac protection in diabetes.

NADPH oxidase (NOX) enzyme system is a major source of ROS generation. NOX enzymatic activity was shown to be increased in vascular tissues of diabetic patients (Guzik et al.,2002). NOX is a transmembrane enzyme found inside organelles and generates superoxides. It has several isoforms NOX(1-5). NOX activation decreases NO synthesis, and through ROS generation increases eNOS uncoupling (Shen, 2010). Accumulated evidence show that NADPH oxidases of the NOX family especially NOX4 are a major source of oxidative stress in cardiovascular disease (San Martin et al.,2007; Thandavarayan et al.,2009).

NOX4 expressed in heart and cardiomyocytes show an increased activity in diabetes (Gao and Mann,2009). The left ventricle (LV) of diabetic rats showed an increase in NOX4 protein expression compared to controls. NOX4 protein expression decrease with anti-sense oligonucleotide (AS) for NOX4 in diabetic treated group. Dihydroethidium (DHE) staining for ROS in LV of diabetic rats was increased compared to control and reduced with AS NOX4 treatment. Hence, NOX4 was shown to be the major source of ROS in the LV of diabetic rats generated in the early stages of T1DM (Eid et al.,2012).

Myocardial injury in response to NOX4 induced ROS generation leads to the re-expression of embryonic markers of cardiac development and cardiac remodeling through extracellular matrix deposition. α-Smooth muscle actin (α-SMA) protein expression occurs during cardiac development and upon cardiomyocytes differentiation, α-SMA will be gradually replaced by α-skeletal actin and α-cardiac actin. The protein expression of α-SMA were increased in diabetic rats and treatment with AS NOX4 reduced the protein expression. Extracellular matrix remodeling in diabetic cardiomyopathy is represented by

increased fibronectin and collagen deposition in the heart. Fibronectin protein expression was increased in diabetic group and reduced with AS NOX4 treatment (Eid et al.,2012).

Cardiac fibrosis was assessed using Masson Trichrome staining. Interstitial fibrosis was higher in LV of diabetic rats compared to control, and AS NOX4 treatment ameliorated this increase (Eid et al.,2012).

NOX-4 induced cardiac ROS generation in diabetes resulted in cardiac fibrotic changes and remodeling detected by DHE and Trichrome staining. In addition to that, NOX-4 induced increase in ROS was associated with the increased expression of cardiac injury markers as α-SMA and fibronectin. The decrease in NOX-4 expression tended to correct the following abnormalities. With 20-HETE being involved in the increase generation of ROS through NO uncoupling, blocking 20-HETE production may be a promising therapeutic target in the correction of diabetes related oxidative stress. Moreover, EETs have shown to possess a cardioprotective effect and may also be an attractive target in the amelioration of oxidative stress induced diabetic cardiovascular dysfunction.

Although CYP-450 epoxygenases have been shown to act as EDHF in the vasculature, CYP2C9 in coronary endothelial cells increased ROS generation. Sulphafenazole, a selective CYP2C9 inhibitor , inhibited CYP-450 epoxygenase mediated ROS generation (Fleming at al.,2001).Sulphafenazole was able to correct the impairment in acetylcholine mediated vasodilation in patients with coronary artery disease by inhibiting CYP2C9 mediated ROS generation (Fichtlscherer et al.,2004). CYP2C9 ROS synthesis increases endothelial dysfunction through decreasing NO bioavailability. Sulphafenazole was able to correct cardiac function, decrease infarct size ,and ROS production in an ischemic/reperfusion model of rats' heart (Khan et al.,2007).

These data suggest that CYP2C9 can simultaneously act as a source of EDHF and ROS. It is the imbalance in the generation of ROS depending on the disease condition that will influence the net outcome (Ding and Triggle, 2005). 20-HETE is an important source of ROS, also 20-HETE added to rat cardiomyocytes increased NADPH oxidase activity and ROS generation (Zeng at al.,2010). The use of 20-HETE inhibitors may decrease NADPH oxidase activity and ROS generation. Despite the promising role of EETs in the treatment of diabetes induced cardiovascular dysfunction, the ability of CYP-450 mono-oxygenases to generate ROS may confound their protective role. Thus, further investigations are warranted in this field.

CHAPTER II

AIMS OF THE STUDY

Previous studies showed that CYP-450 metabolites of AA are altered in various disease conditions as in diabetes. 20-HETE and EETs, were shown to influence hyperglycemia and β-cell function in diabetes. Their effects on the vasculature and the heart have been documented, and there is some evidence that 20-HETE levels are elevated in diabetes as is the expression of CYP4A and may contribute to the development of the vascular dysfunction seen in diabetes. In contrast, EETs seem to play a protective role in DM ,where the increase in EET levels improved insulin sensitivity ,may play a cardioprotective role through preserving myocytes structure, and may act as EDHF to ameliorate vascular dysfunction in diabetes.

In this study, we hypothesize that diabetes associated cardiovascular dysfunction may be ameliorated or reversed through enhancing EETs activity or blocking 20-HETE activity in vivo. Moreover, with the CYP-450 being a source of ROS generation and with oxidative stress being the key to diabetic complication s ;we assume that by inducing EETs or inhibiting 20-HETE we may influence ROS induced cardiovascular damage in DM. We will use two drugs to test our hypothesis: HET0016, a selective blocker of CYP4A that will inhibit HETE production, and BNF a CYP1A and CYP2C11 inducer that will induce EET production. To test the following hypothesis, we will study the following:

1- Effect of treatment with HET0016 and BNF on the protein and mRNA expression of CYP4A and CYP1A or CYP2C11. We will also measure the levels of 20-HETE and

EET in urine collected on day 28 of the experiment for the control rats treated with vehicle (CV), diabetic rats treated with vehicle (DV), diabetic rats treated with HET0016 (DH), and diabetic rats treated with BNF (DF).

2- Effects of HET0016 and BNF treatment on the vascular responses to Ach, phenylephrine (PE), and SNAP (S-Nitroso-N- acetylpenicillamine) in CV,DV,DH and DF groups.

3- Effects of HET0016 and BNF treatment on markers of vasculopathy in diabetes by investigating the protein expression of fibronectin,NOX-4,and α-SMA in CV,DV,DH and DF groups.

4- Effects of HTE0016 and BNF treatment on the development of oxidative stress in DM by evaluating NOX-4 protein along with Dihydroehtidium (DHE) staining.

5- Effects of HET0016 and BNF treatment on the cardiovascular remodeling in DM by evaluating left ventricle collagen deposition and cardiomyocytes injury in CV,DV,DH, and DF groups.

The fact that CYP-450 mediated metabolites of AA play a role in the development or regression of diabetes induced cardiovascular dysfunction makes them a suitable therapeutic target for the management of diabetes associated cardiovascular complications. Hence, pharmacological treatment targeting the induction of EETs production and the inhibition of 20-HETE synthesis may provide a new add on treatment to DM pharmacotherapy

CHAPTER III

MATERIALS AND METHODS

A. Animal models

Male Sprague-Dawley rats, weighing 200-300g, were housed in cages and placed in a room at a constant temperature of 23°C in a 12:12 light-dark cycle. All animal procedures were maintained in accordance with the ethical requirements set by the Institutional Animal Care and Use committee of the American University of Beirut.

Diabetes was induced by the administration of a single dose intravenously via the tail vein of 65mg kg^{-1} body weight of streptozotocin (STZ) in sodium citrate buffer (0.01M, pH 4.5). Control rats were injected intravenously via tail vein with sodium citrate buffer alone. Forty-eight-hour after the injection, the onset of diabetes was verified by measuring blood glucose levels using (Accu-Chek® Performa Glucometer, Roche).

Rats were divided according to body weight compatibility into four treatment groups each. Group(1): (CV) control non-diabetic rats injected with 5 % dimethyl sulfoxide in normal saline (DMSO) *intraperitonealy (i.p.), daily* (*n=10*). Group(2) : (DV) diabetic rats treated i.p., daily with 5 % DMSO (*n=10*). Group(3): (DH) diabetic rats that were injected i.p. , daily with 10 mg kg−1 of N-hydroxy-N′-(4-butyl-2-methylphenyl) formamidine (HET0016) , a selective inhibitor of CYP4A isoforms (4A1,A42,4A3, and 4A8) (*n=10*). Group(4): (DF) diabetic rats injected (i.p.), daily with 35 mg kg−1 of beta-naphthoflavone (*BNF), an inducer of CYP1A or CYP2C11(n=10).*

A subset of rats were placed in metabolic cages three days prior to urine collection (*n=24*). Food and water intake were also measured. All biochemical parameters were taken on days 0,14, and 28 the day of the sacrifice.

B. Enzyme-linked immunosorbent assay (*ELISA*): 20-HETE & EET measurement in urine using ELISA

Urine samples collected on day 28 of the experiment for the subset of rats placed in metabolic cages were used for the measurement of 20-HETE and EET levels. After being processed, the urine samples were used to determine 20-HETE and EET levels using a competitive ELISA kit (Detroit R&D,Inc.). The samples were loaded into a 96-well plate coated with anti-20-HETE or anti-14,15-DHET. The 20-HETE or 14,15-DHET in the samples competed with the 20-HETE-HRP conjugate or 14,15-DHET-HRP conjugate that were loaded into the wells. The amount of HRP-conjugate bound to the coated antibody inversely reflected the levels of 20-HETE or EET. The amount of conjugate bound to each well was detected by the addition of tetra methyl benzadine (TMB) that upon binding to HRP conjugate resulted in color changes. The colorimetric changes were read on a spectrophotometer at 450nm.

C. Vascular reactivity in isolated perfused aortic rings

After 28 days of treatment rats were decapitated under carbon dioxide narcosis. The thoracic aorta from rats was obtained and placed immediately into a Petri dish having Kreb's Henseleit solution containing: NaCl (118.3mM), KCl (4.7mM), CaCl2 (2.5mM), MgSO4 (1.2mM), NaHCO3 (25mM), KH2PO4 (1.2mM) and glucose (11.2mM),

equilibrated with 95% O2 and 5% CO2.The isolated aorta was cut into rings of 4-5 mm long. In some rings the endothelium was removed by rubbing the inner surface with a forceps inserted into the lumen.

Aortic rings were placed horizontally into a stainless wire and connected to a force displacement transducer for recording isometric tension. The rings were immersed in a water-jacket chamber filled with 25ml of Kreb's Henseleit solution kept at 37 °C and aerated constantly with 95% O2 and 5% CO2 gas. After an equilibration time of 45 min, endothelial integrity was assessed by contracting the aortic rings with 1μM of phenylephrine (PE),then recording the relaxation response to 1μM of acetylcholine (Ach). The percentage of intact endothelium was calculated based on an endothelium test formula. Aortic rings with a percentage relaxation $\geq$ 80% were considered as having intact endothelium, and those with a percentage relaxation <5% were considered endothelium denuded aortic rings. After washing and equilibration in Kreb's buffer solution to achieve equal tension baselines, the endothelium intact rings were subjected to one of the following:

- Responses to increasing cumulative concentrations of acetylcholine (Ach) 0.001μM, 0.01μM,0.1μM,1μM,and10μM) after precontraction with phenylephrine (PE) (1μM)
- Responses to increasing cumulative concentrations of phenylephrine (PE). (0.01μM,0.1μM,1μM, and 10μM).

The endothelium denuded rings were exposed to increasing cumulative doses of SNAP (NO donor) (0.1 μM,1 μM,10 μM, and 100μM) after precontraction with PE (1μM).

D. Protein Expression Analysis: Western Blot

The abdominal aorta was isolated for protein expression analysis. Mortar and pestle previously immersed in liquid nitrogen were used to homogenize frozen abdominal aortic tissues. The homogenized tissues were transferred to eppendorfs containing RIPA lysis buffer (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) . After two hour incubation with RIPA buffer at 4°C, the tissue lysates were centrifuged at a speed of 13,600 rpm for 30 minutes at 4°C.

Proteins in the supernatant were quantified based on 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-Nox4 (1:500, Santa Cruz Biotechnology), rabbit polyclonal anti-CYP4A(1:1000, abcam), rabbit polyclonal anti-CYP2C11(1:1000, abcam), rabbit polyclonal anti-alpha smooth muscle actin antibody (1:500, abcam) and rabbit polyclonal anti-fibronectin antibody(1:9000, sigma-aldrich). The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:7000, Bio-Rad). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software.

E. mRNA analysis : Real time RT- PCR

Real-time RT-PCR was used to determine specific gene expression. Data obtained was analyzed using 2(-Delta Delta C(T)) method (Livak *et al*., 2001). Tissue abdominal

aortic RNA was isolated using TRIzol reagent (Sigma Aldrich, Steinheim, Germany). mRNA was quantified using Icycler with SYBR green dye and rat RT2qPCR Primers (Bio-Rad Laboratory, CA, USA) for CYP4A8 and CYP2C11.GAPDH was used as an internal reference gene.

Primer	Sequence	Annealing Temperature(**ºC)**
CYP4A8	F:ATCCAGAGGTGTTTGACCCTTAT R:AATGAGATGTGAGCAGATGGAGT	55.8 **ºC**
CYP2C11	F: ACAAGGACAATCCTCGGGAC R:AAGGGTAAACTCAGACTGCGG	56.9 **ºC**

Table 1. Oligonucleotide primer sequences and conditions employed for real-time PCR

F. Immunohistostaining assays : Masson's trichrome & Periodic acid-Schiff (PAS)

Left ventricle(LV) heart tissues were stained with Masson's trichrome and Periodic acid-Schiff (PAS) . Masson's trichrome is used to stain collagen deposits green, and Periodic acid-Schiff (PAS) is used to stain cardiomyocytes nuclei deep blue showing any changes at the level of cardiomyocytes arrangements and cardiac muscle injury. These stains are used to assess fibrotic changes reflecting histologically cardiac dysfunction in

diabetes (Yan *et al*.,2004). Frozen LV heart tissues were cut into 3mm thick cuts, incubated for 48 hours in formaldehyde, and then embedded in paraffin.

Paraffin embedded tissues were cut into 4-5 μm thickness and mounted on glass slides. Tissue sections were then stained by trichrome and PAS and examined under light microscope. LV heart tissues were divided into six sections and three images were taken per section.

Trichrome stains were quantified using Image J software. PAS stains were quantified using a semi-quantitative scale of 1-4,where 1, normal histology; 2, cell swelling and nuclear condensation with loss of up to one-third of the nuclei; 3, same as for score 2, but greater than one-third and less than two-thirds of the cells profile showing nuclear loss, and 4, greater than two-thirds of the cells profile showing nuclear loss.

G. DHE staining and ROS measurement

Frozen left ventricle samples that were obtained on day 28 of the experiment from CV,DV,DH, and DF rats were cut into sections of 10μm thickness and mounted on glass slides. Dihydroethidium (DHE) staining applied to each slide was used for the measurement of superoxide anions. DHE is an oxidative fluorescent dye that interacts with the superoxide anions to form the DNA-binding fluorophore ethidium bromide. DHE (10μmol/l) was applied to the LV section, after which the slides were incubated at 37 °C for 15 minutes in a light-protected humidified chamber. The fluorescence of the ethidium stained tissues was detected using a laser scanning confocal microscope with ethidium bromide fluorescence being detected at excitation and emission wavelength of 488 and

560nm respectively. Superoxide anions detected in the LV tissues appeared as red fluorescent labeling that were compared with unstained LV sections kept as a background control. The mean fluorescence intensity of the images was measured using Image J software for quantification.

H. Statistical analysis

Results are represented as mean ± SEM. Statistical significance is determined using student's unpaired t-test. For the concentration response curves, results were compared using 2-way Analysis of Variance (ANOVA) with one factor being "Group" and the other "Concentration". P-value <0.05 is considered as statistically significant.

CHAPTER IV

RESULTS

A. Body weight, blood glucose, heart weight and cardiac hypertrophy index

Body weight was significantly decreased in the DV group compared to CV group on days 14 and 28 of the study ($P<0.05$).DH group showed a significantly higher baseline body weight compared to DV group on day 0 of the study ($P=0.024$). Although DF group showed a significantly lower baseline body weight compared to control, BNF treatment decreased body weight on day 14 and 28 of the study compared to DV group ($P<0.05$). Blood glucose levels among CV, DV, DH, and DF groups showed no significant difference at baseline level (D=0) ($P>0.05$). However, after STZ injection (D=14 and 28), the blood glucose level was significantly higher in the DV group than that with CV group ($P<0.05$). The glucose level in the HET0016 or BNF treated groups was similar to that in the DV group ($P >0.05$) (Table 2A).

Heart weight was significantly lower in the DV group as compared to CV group ($P=0.012$). However, the cardiac hypertrophy index was increased in the DV group compared to CV group ($P=0.017$). BNF treated rats showed a significantly higher heart weight when compared to DV group. However, there was no difference with respect to the cardiac hypertrophy index between the DV and DF group. HET0016 treatment showed no difference in heart weight and cardiac hypertrophy index when compared to DV group ($P<0.05$) (Table 2B).

B. 20-HETE and EETs measurement in urine: Increased 20-HETE level with diabetes and decreased with HET0016 and BNF treatment. EETs level increased with BNF treatment only

Urine samples collected on day 28 of the experiment were used to measure the levels of 20-HETE and EET using ELISA kit. 20-HETE levels in urine were significantly elevated in DV group as compared to CV group ($P<0.05$). Moreover, treatment with HTE0016 and BNF resulted in a significant decrease in 20-HETE levels in both DH and DF group as compared to DV group ($P<0.05$) (Figure 1A).

EETs level was not altered in diabetes. There was no significant difference in EETs level among DV and CV group ($P>0.05$). Treatment with HET0016 had no influence on EET level in urine ,and thus there was no significant difference among DV and DH treated groups ($p>0.05$) . BNF treatment increased EET levels significantly compared to DV group ($p>0.05$) (Figure 1B).

C. Vascular function studies among control, diabetic, diabetic+HET0016 and diabetic+ BNF treatment groups

1-Evaluation of response to phenylephrine in aortic rings with intact endothelium

There were no significant difference in the response of aortic rings to PE among the four groups, although it tended to be lower in DV vs. CV group and lowest in the group treated with BNF (35mg/kg/day) (Figure 2).

2-*Evaluation of response to acetylcholine in precontracted aortic rings with intact endothelium*

The response to Ach were not significantly different among the four group of rats (P>0.05) (Figure 3).

3- Evaluation of response to SNAP in aortic rings with denuded endothelium

Although not significant the response to SNAP tended to be greater in DV vs. CV aortic rings (P>0.05). The response of aortic rings from rats treated with BNF (35mg/kg/day) and HET0016 (10mg/kg/day) were closer to those observed in CV group, although they were not significantly different from DV group (P>0.05) (Figure 4).

D. CYP-450 mediated AA metabolites and protein expression of vascular injury markers

1-HET0016 and BNF treatment did not decrease fibronectin protein expression

Fibronectin expression was elevated in the CV compared to DV group. Fibronectin was slightly higher in the DV compared to DH and DF groups . However, both differences in fibronectin protein expression among CV and DV groups (P=0.06) , and between DV compared to DH and DF groups were statistically insignificant (P=0.24 and 0.08).Thus treatment with BNF or HET0016 did not decrease fibronectin protein expression in diabetic rats abdominal aorta (Figure 5 A-B).

2-NOX-4 protein expression increased in DV rats and was lowered with BNF treatment

NOX-4 protein expression was significantly increased in DV compared to CV group (P=0.018). NOX-4 protein expression was also increased in DV compared to DH and DF groups. Treatment with HET0016 and BNF decreased NOX-4 protein expression

compared to DV group. BNF treatment resulted in a significant decrease in NOX-4 protein expression levels in DV compared to DH group . BNF restored NOX-4 protein expression to control levels (Figure 6 A-B).

3- α-SMA protein expression was not altered among CV,DV,DH, and DF groups

α-SMA was slightly decreased in diabetic vehicle compared to control (P=0.049). DH and DF groups showed increased α-SMA protein expression compared to DV that was insignificant (P=0.05). Overall, α-SMA protein expression was not altered significantly among the study groups, and the treatment with BNF or HET0016 did not influence α-SMA protein expression in the abdominal aorta of diabetic rats (Figure 7 A-B).

4-BNF (35mg/kg/day) did not increase CYP2C11 protein and mRNA expression in DF group.HET0016(10mg/kg/day) did not increase CYP4A8 mRNA expression in DH group.

CYP2C11 protein expression was comparable among the study groups with minor decrease in the DV group. BNF i.p. treatment did not result in increase CYP2C11 protein expression in DF group. There was no significant difference among CYP2C11 protein expression between the study groups (P>0.05) (Figure 8 A-B). CYP2C11 mRNA expression was increased in the DH group, and no increase was shown in the DF group compared to DV group (Figure 8C). CYP4A8 mRNA was assessed to evaluate 20-HETE cardiovascular effects and effectiveness of HET0016 i.p. treatment, since protein expression analysis of CYP4A was difficult to detect. CYP4A8 mRNA expression was

increased in the DH and DF treated groups compared to DV, and no decrease in CYP4A8 mRNA expression was seen with HET0016 treated rats.(Figure 8D).

E. Masson's trichrome and Periodic acid-Schiff staining of left ventricle in control, diabetic vehicle, diabetic+HET0016 ,and diabetic +BNF

Masson's trichrome used to stain collagen deposits green was applied to left ventricle sections of (A) CV, (B) DV, (C) DH, and (D) DF rats respectively (Figure 9). The green color intensity was increased in the LV sections of DV compared to CV rats, hence reflecting increased collagen deposition in the heart of diabetic rats (Figure 9A,B, and E) ($P<0.05$). BNF (35mg/kg/day) treatment was able to decrease collagen deposition in the left ventricle of DF as compared to DV rats ($P<0.05$) (Figure 9B,D, and E). Treatment with HET0016 (10mg/kg/day) did not ameliorate collagen deposition compared to DV group ($P>0.05$) (Figure 9B,C, and E).

Periodic acid-Schiff staining (PAS) was applied to left ventricle sections of (A) CV, (B) DV, (C) DH, and (D) DF rats respectively (Figure 10). PAS was used to assess fibrotic changes in the left ventricle through the staining of proteoglycans in the extracellular matrix, along with cell injury and nuclear loss. Diabetic rats (DV) showed a score of 2.8 that showed significant cell swelling and nuclear condensation, with loss of up to one-third of the nuclei (Figure 10 A and B) (Table3). DH and DF LV stains showed a significant decrease in blue color stain with a score of 1.7 and 1.4 ,respectively. Treatment with BNF and HET0016 significantly decreased cardiomyocytes injury compared to diabetic ($P<0.05$) (Figure 10C and D)(Table3).

F. DHE staining of LV section and ROS generation in control, diabetic, diabetic +HET0016,and diabetic +BNF .

DHE staining of the LV sections of diabetic rats treated with vehicle showed an increase intensity of red fluorescence as compared to control rats indicative of increased ROS generation in the LV tissue of diabetic rats (Figure 11A and B). Treatment with HET0016 and BNF resulted in a decrease of ROS generation as evident by the decreased intensity of red fluorescence (Figure 11C and D). Moreover, quantification of the red fluorescence showed a significant increase in the ROS generation in the DV rats compared to CV rats ($P<0.05$) (Figure 11E).DH and DF groups showed a significant decrease in diabetes mediated ROS generation when compared to DV LV tissue (Figure 11E).

CHAPTER V

DISCUSSION

This study was conducted to examine the roles of 20 HETE and EET's in the vascular pathology that complicates diabetes mellitus. In the rat model we used, in which diabetes was induced by streptozotocin injection and in which studies were conducted one month later, we were able to document the following abnormalities associated with diabetes: cardiac hypertrophy, evidenced by an increase in heart weight to body weight ratio, an increase in NOX4 protein expression in the abdominal aorta, an increase in ROS generation in the left ventricle , increased deposition of collagen and cardiomyocytes injury in the left ventricle (as evidenced by Masson trichrome and PAS staining), associated with an increased urinary excretion of 20 HETE but not of EET's. Other markers of vascular pathology were unchanged, notably fibronectin expression (indicative of collagen deposition) and alpha SMA expression. Importantly, vascular reactivity to vasoactive agents including acetylcholine (endothelium dependent vasodilator), SNAP (endothelium independent vasodilator) and phenylephrine (vasoconstrictor) were not changed by diabetes.

NOX-4 protein expression indicative of increased ROS generation was significantly increased in the DV rats compared to CV rats. This finding coincides with the literature regarding the role of oxidative stress and ROS generation in the development of diabetic cardiovascular disorders (Shen,2010).

The lack of change in fibronectin and alpha SMA is different from what was obtained by Maalouf et al., where it has been reported that after 2 weeks of diabetes in rats

injected with 55mg/kg body weight of STZ, the protein expression of fibronectin and α-SMA in the LV were significantly increased as compared to control rats (Maalouf et al.,2012). A longer duration of diabetes may be needed in our study module to induce physiologically significant increase in vascular pathology markers. The following study was intended to be conducted for one, three, and six months, but because of the time constrains we have conducted the one month study only. Another possible explanation to this discrepancy, is the greater protein content in the LV as compared to the abdominal aorta, where protein quantification and detection was difficult. The vascular reactivity studies to PE were similar to previous results from our laboratory; however we had previously shown a significant though slight decrease in response to Ach and a definite significant increased response to SNAP in diabetic rats relative to control rats. Both these observations were not reproduced here, although the trends observed were in the same directions. Therefore, an increase in the number of animals may be warranted here to render these differences significant.

As for the effects of treatment with HET0016 and BNF, we found that:

1) both BNF and HET0016 treatment decreased 20-HETE levels in urine collected on day 28 of the experiment

2) treatment with BNF (35mg/kg/day) and HET0016 (10mg/kg/day) did not alter vascular reactivity to the vasoactive agents used in DH and DF rats when compared to DV rats.

3) the protein expression of the abdominal aorta injury markers as fibronectin and α-SMA were not altered in the DH and DF treated groups,

4) the increase in NOX-4 protein expression observed in the diabetic group was decreased with BNF but not HET0016 treatment ,

5) the increased ROS staining in the LV of diabetic rats was decreased with both BNF and HET0016 treatment

6) BNF, but not HET0016, treatment decreased collagen deposition and cardiomyocytes injury in the LV of DF rats when compared with DV rats.

These results suggest that that the increase in EETs levels induced by BNF treatment, which was documented as increased urinary excretion of EET in DF relative to other groups, may be proposed as a pharmacotherapeutic intervention that needs to be further investigated in the management of oxidative stress related diabetic cardiovascular dysfunction and cardiac fibrosis seen in diabetes and may have potential for clinical application. Moreover, BNF treatment contributed to a decrease in the injurious marker 20-HETE as evident with 20-HETE measurements in urine. This may be attributed to BNF induced EET beneficial effects on decreasing disease progression and hence the levels of the injurious 20-HETE.

Although there were significant changes induced by diabetes in NOX4 protein expression in the aorta no vascular functional abnormalities of note could be observed. This, as mentioned earlier, may be a result of the small number of animals used, or alternatively, may mean that more time is needed for the injury to translate into functional impairment, and that 4 weeks of diabetes may not be the ideal time to study these interventions for their functional significance.

The increase in EETs through the use of BNF or the inhibition of 20-HETE production by HET0016 had no effect on the vascular responses . Hence, our findings were

similar to those reported by Yousif et al., where the treatment i.p with HET0016 (2.5mg/kg/day) was unable to correct the vascular impairment to NE, Ag(II), and ET-1 nor restore the vasodilatory response to carbachol, histamine, and SNP in the renal artery rings of STZ treated rats . HET0016 treatment was able to correct the vascular impairment in the perfused mesenteric vascular beds Moreover, the response to carbachol in the isolated renal aortic rings was not different in diabetic compared to control, that was similar to our results with the response of thoracic aortic rings in the DV and CV group. Most of the vascular studies utilizing HET0016 to assess the role of CYP-450 AA mediated metabolites in the amelioration of vascular dysfunction were performed in vivo on renal artery and mesenteric vessels or in vitro on carotid aortic rings and bovine aortic endothelial cells .Our study may be the first to investigate in vivo through vascular studies the role of CYP-450 AA mediated metabolites in the amelioration of vascular dysfunction in thoracic aortic rings.

The lack of effect of HET0016 in our study despite the decreased ROS generation evident with the DHE staining, may be explained by the following: 1) HET0016 is highly lipid soluble and tends to accumulate in the brain and kidney at concentrations 4-10 times that in the plasma, thus it may have not reached concentrations sufficient to block vascular 20-HETE (Yousif et al., 2009), 2) the expression of CYP4A tends to be greater in micro-vessels <100 μm in diameter such as the mesenteric vessels that seem to respond better to HET0016 as compared to large conduit vessels like the thoracic aorta (Roman,2002), 3) Different vessels may respond differently as with the difference in the vascular responses of HET0016 treatment in the isolated renal rings and the perfused mesenteric vascular beds (Yousif et al.,2009).Thoracic aortic rings may seem to possess a comparable vascular

response to that of the renal aortic rings. Thus, the evaluation of the dose inhibitory effect of HET0016 on the CYP4A expression and 20-HETE level in the thoracic aorta is needed. Further in vivo studies regarding the role of EETs and 20-HETE in the vasculature are warranted.

Treatment with BNF was able to decrease NOX-4 mediated ROS generation as evident by the DHE staining of ROS and through the down regulation of NOX-4 protein expression. This can be explained by the induction of EETs synthesis through the use of BNF. While CYP2C9 epoxygenase has shown to be a source of ROS ,CYP2J2 epoxygenase have shown to decrease ROS generation (Deng et al.,2010). Thus EETs have been shown to possess anti-oxidant property, but further in vivo studies regarding the role of EETs in the amelioration of diabetes induced oxidative stress is needed.

We were not able to evaluate the inhibitory effect of HET0016 treatment on the protein expression of CYP4A, and the mRNA gene expression of CYP4A8 increased in the DH treated group compared to the rest, probably suggestive of a positive feedback due to decreased 20-HETE level with HET0016. This can be attributed to :1) we used whole abdominal aorta to evaluate CYP4A protein expression rather than the isolated microsomes which would require the pooling of more rats. CYP-450 are abundant in the microsomes of the cell, and their isolation concentrates CYP-450 for protein expression analysis, 2) HET0016 may act by modulating CYP4A enzymatic activity rather than expression, and 3) it may be attributed to technical errors such as the lack of primer specificity ,thus requiring the development of new primers to be used upon study continuation. CYP2C11 protein expression was also not altered with BNF treatment, although BNF treatment was able to decrease NOX-4 protein expression and increase urinary EETs level in the DF group. This

may be attributed also to:1) the fact that isolated microsomes were not used, 2) BNF may act by alternative mechanism affecting activity rather than induction or 3) BNF may have decreased ROS by an alternative mechanism that is EETs independent and is ought to be investigated.

A role for EETs in the protection against cardiac fibrosis and remodeling in diabetes has been postulated (Guglielmino et al.,2012). In our study the DF group showed a decrease in LV fibrosis and collagen deposition as compared to DV group. Trichrome staining of the LV showed a decrease in the collagen deposition with the BNF treated group compared to DV group. PAS staining of extracellular matrix expansion reflective of cellular fibrotic changes was decreased with BNF treatment. These results correlate with BNF mediated decrease in NOX-4 protein expression and ROS staining. Thus, the BNF associated cardiac protection may be attributed to the decrease in ROS generation and oxidative stress mediated cardiac injury.

As a conclusion, we provide preliminary evidence that treatment with BNF (35mg/kg/day) decreases diabetes induced ROS generation in the aorta by decreasing NOX-4 protein expression, and this is associated with protection against cardiac hypertrophy, remodeling and fibrosis. Our results are still preliminary regarding the role of EETs and 20-HETE in the complications and potential management of diabetes induced cardiovascular dysfunction in vivo. Further in vivo studies are needed to further elucidate this role and the mechanisms involved.

Table (2).

(A) Blood glucose and body weight (B) Heart weight and Heart weight/ body weight (cardiac hypertrophy index) in control, diabetic vehicle , diabetic +HET0016, and diabetic +BNF rats on days 0,14,and 28 of the experiment.

A.

	Blood Glucose (mg/dl)			Body weight (g)		
Time (days)	0	14	28	0	14	28
Control Vehicle	132.2±2.4	129.5±3.7	169.2± 11	239.5±3.4	317.5±6	394.33±16.2
Diabetic Vehicle	129.8±3.3	*406.3±30.2	*564.3±21.2	265.7±2	*265.8±9	*305±16.5
Diabetic + HET0016 (10mg/kg)	127.3±4.8	284±64.1	489.8±38.6	# 247.5±7.2	270.7±14.7	311.3±20.1
Diabetic + BNF (35mg/kg)	129.3±2.5	345.3±61.2	448.3±88.1	# 252.83±2.3	# 292.2±5.6	# 354.7±5.4

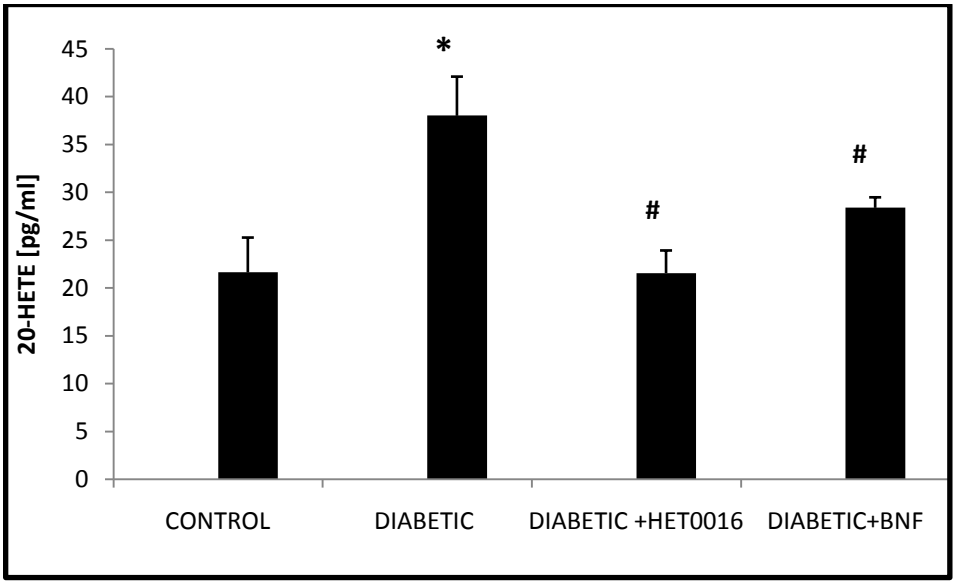
B.

	Heart weight (g)	Heart weight/ body weight (mg/g)
Time (days)	28	28
Control Vehicle	1.31±0.05	3.24±0.06
Diabetic Vehicle	*1.1±0.06	*3.7±0.15
Diabetic + HET0016 (10mg/kg)	1.21±0.07	3.65±0.11
Diabetic + BNF (35mg/kg)	# 1.45±0.06	3.72±0.03

Values are represented as Mean ± SE of the mean.
*P<0.05 comparing control vs. diabetic vehicle; # P<0.05 compared with diabetic vehicle

Figure 1.

A.


20-HETE [pg/ml]
45
40
35
30
25
20
15
10
5
0
*
#
#
CONTROL
DIABETIC
DIABETIC +HET0016
DIABETIC+BNF

B.

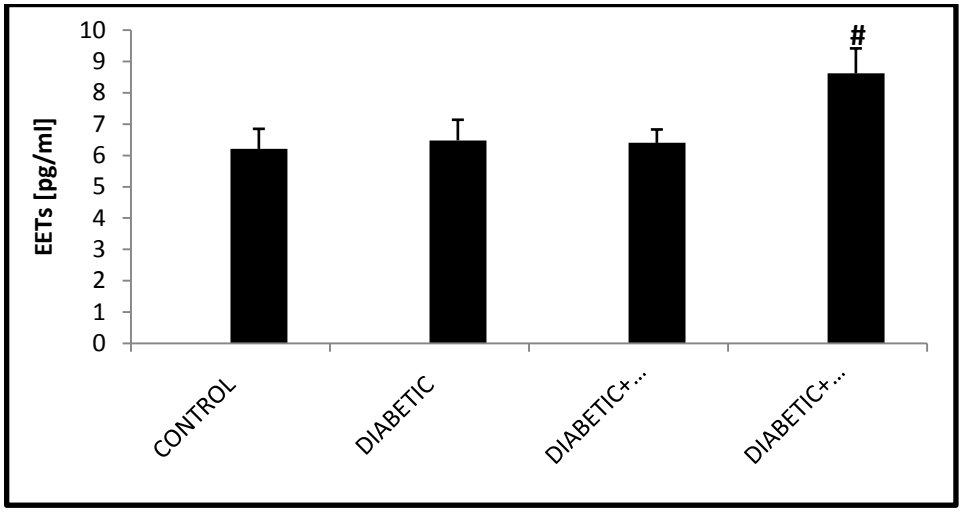

EETs [pg/ml]
10
9
8
7
6
5
4
3
2
1
0
#
CONTROL
DIABETIC
DIABETIC+...
DIABETIC+...

Figure 1. 20-HETE levels increased with diabetes and decreased with HET0016 (10mg/kg/day) and BNF (35mg/kg/day) treatment. EETs level increased with BNF treatment in DF rats.

Histograms representing (A) 20-HETE and (B) EETs levels in urine collected on day 28 of the study, quantified using EETs and 20-HETE ELISA kit. Values are represented as Mean ± SE of the mean; results from 24 rats. *P<0.05, control vs. diabetic; #P<0.05, diabetic vs. diabetic +HET0016 or diabetic +BNF

Figure 2.

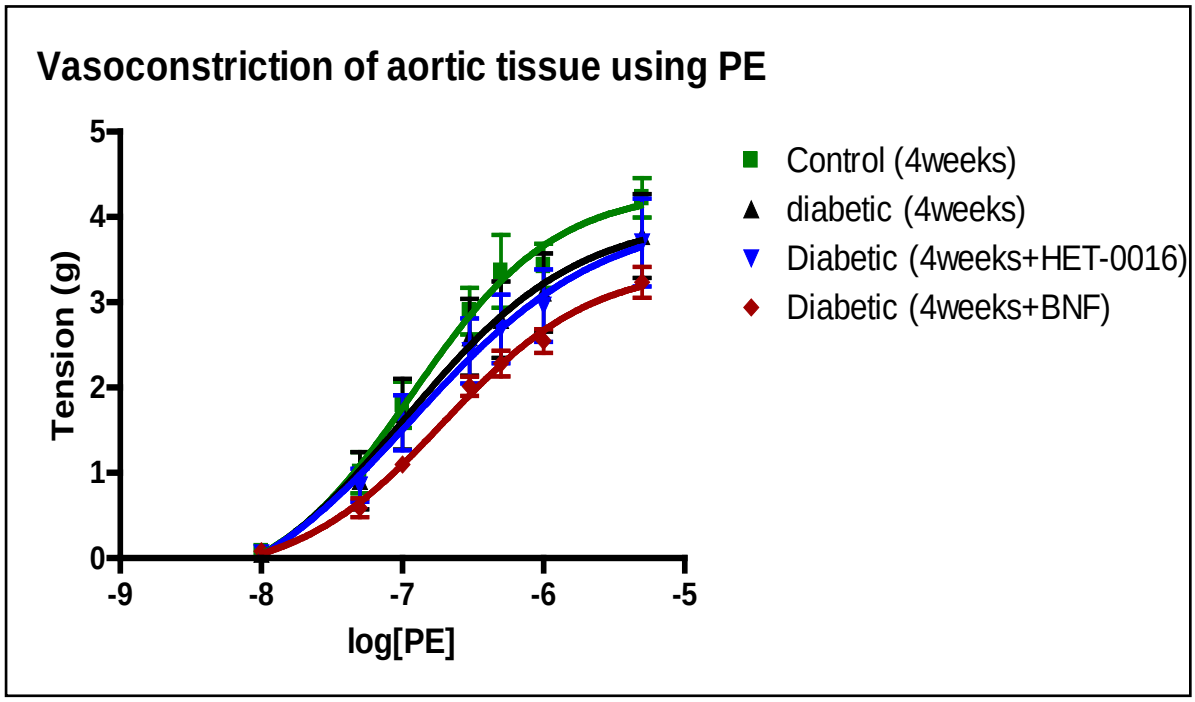


Figure 2. Response to increasing concentrations of Phenylephrine (PE) in endothelium intact aortic rings after 28 days in control,diabetic,diabetic+HET0016,and diabetic +BNF treated groups. Values are represented as mean± SE of the mean.

No significant difference was observed among the groups, ANOVA "Group" effect $^{\#}P>0.05$ (P=0.5906). No significant difference was observed between diabetic vs. diabetic+HET0016 or vs. diabetic+ BNF (*P>0.05)

Figure 3.

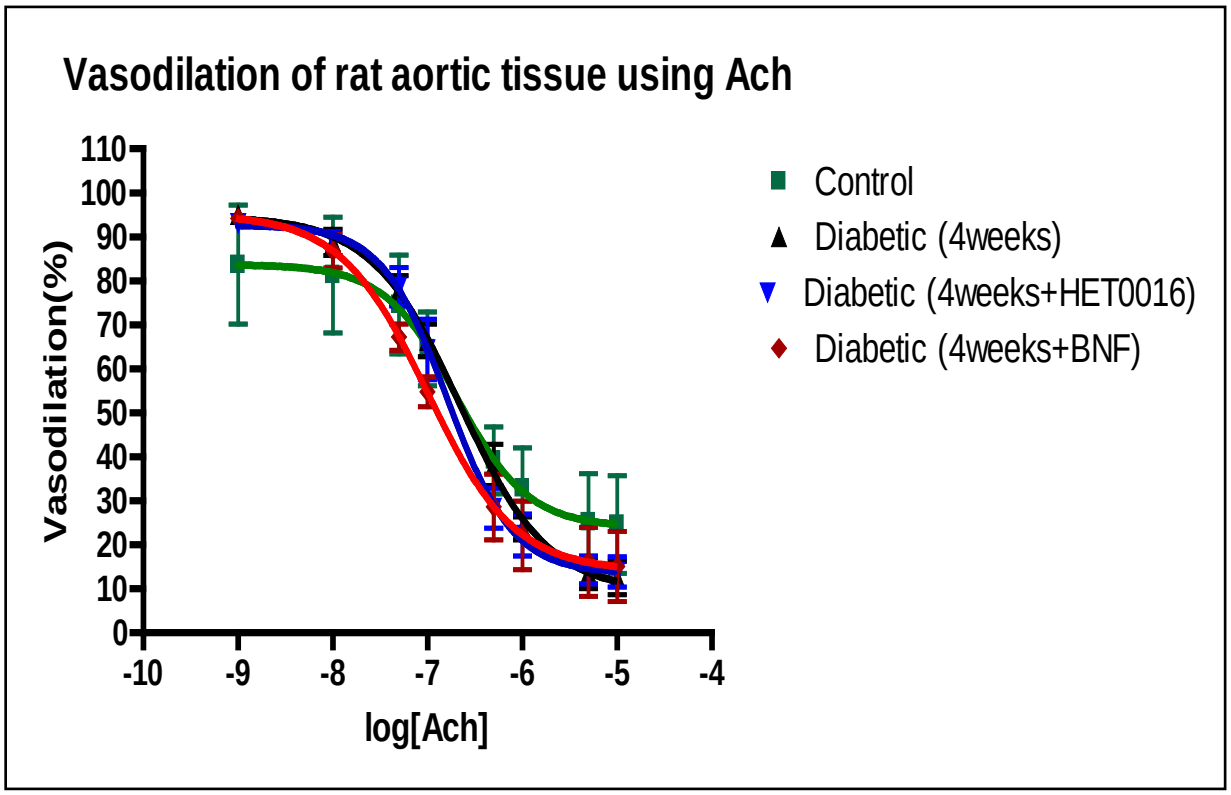


Figure 3. Response to increasing concentrations of Acetylcholine (Ach) in endothelium intact precontracted aortic rings after 28 days in control,diabetic,diabetic+HET0016,and diabetic +BNF treated groups. Values are represented mean± SE of the mean.

No significant difference was observed among the groups, ANOVA "Group" effect $^{\#}P>0.05$ (P=0.7914). No significant difference was observed between diabetic vs. diabetic+HET0016 or vs. diabetic+ BNF (*P>0.05)

Figure 4.

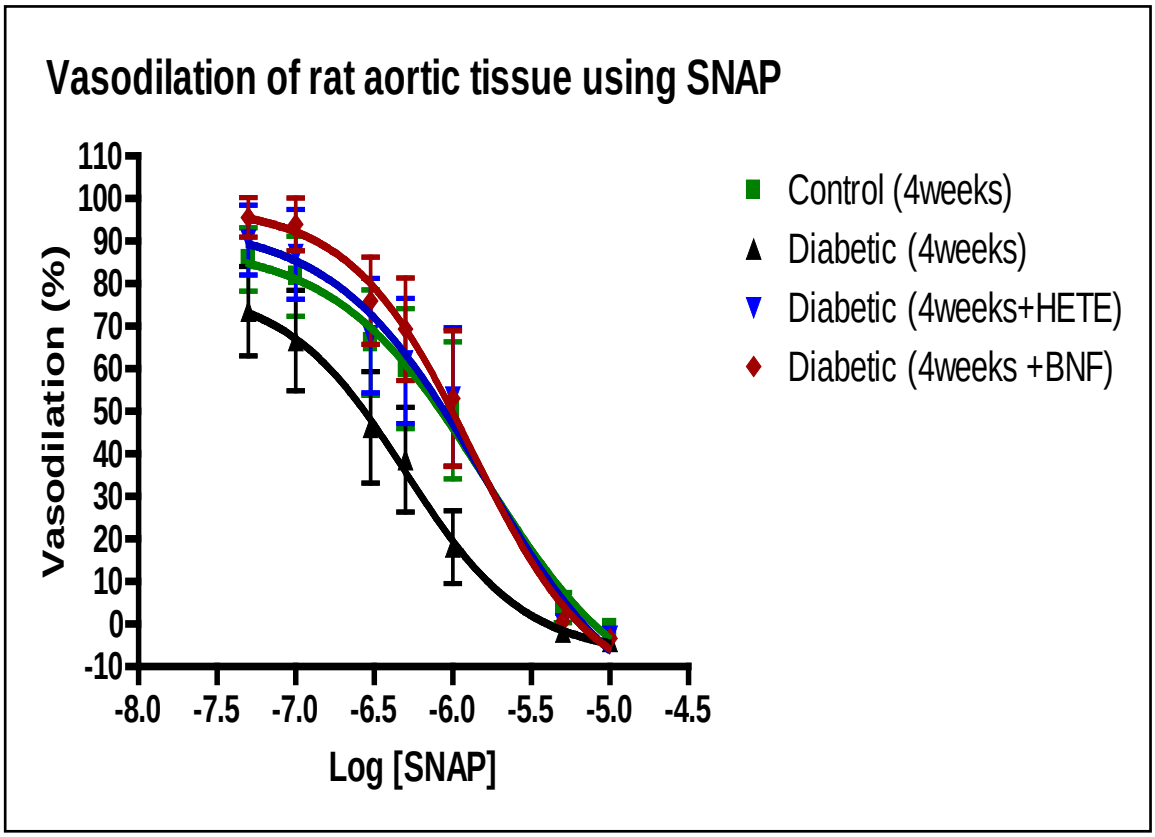
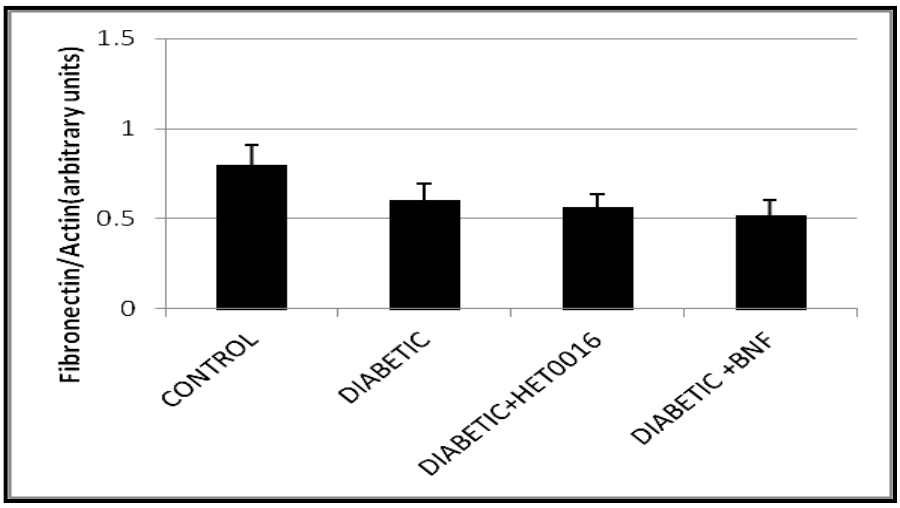


Figure 4. Response to increasing concentrations of SNAP (S-Nitroso-N-acetylpenicillamine) in endothelium denuded aortic rings after 28 days in control,diabetic,diabetic+HET0016,and diabetic +BNF treated groups. Values are represented as mean± SE of the mean.

No significant difference was observed among the groups, ANOVA "Group" effect $^{\#}P>0.05$ (P=0.7914). No significant difference was observed between diabetic vs. diabetic+HET0016 or vs. diabetic+ BNF (*P>0.05)

Figure 5.

A.



B.

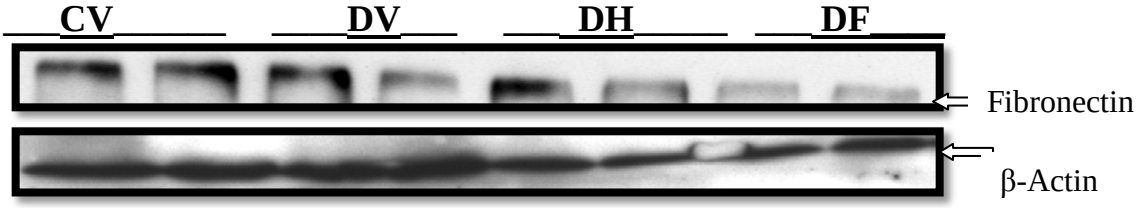
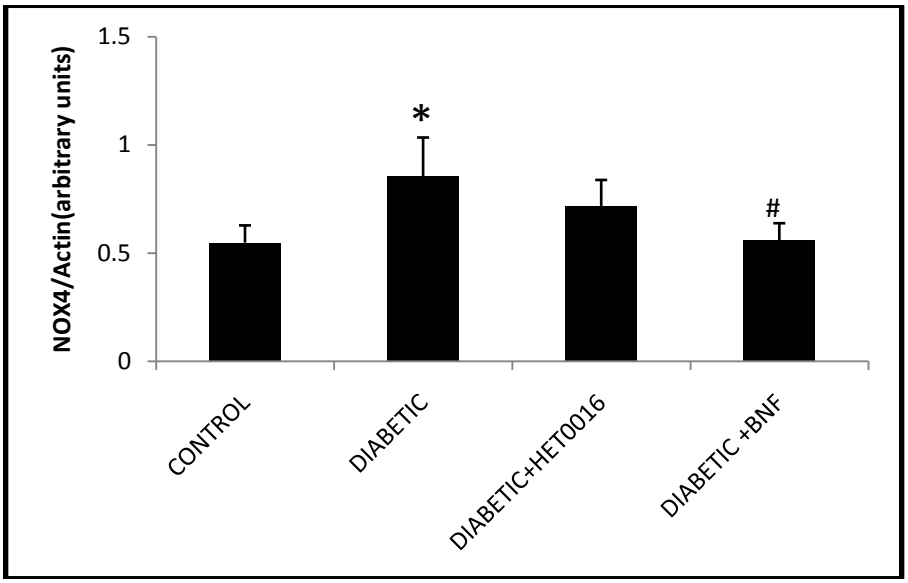


Figure 5. Treatment with HET0016 (10mg/kg/d) or BNF (35mg/kg/d) did not affect fibronectin protein expression in diabetes.

Representative western blot of fibronectin protein expression in abdominal aortic tissues of control, diabetic , diabetic + HET0016 or BNF treated male Sprague Dawley rats after 28 days (B). Western blots are quantified by densitometry and are representative of six independent experiments (n=24). Each histogram represents the ratio of the intensity of the fibronectin band factored by β-actin band used as a loading control (A). Values are represented as mean ± SE of the mean. *$P<0.05$, control vs. diabetic vehicle, and $^{\#}P<0.05$ diabetic vehicle vs. diabetic+HET0016 and diabetic +BNF.

Figure 6.

A.



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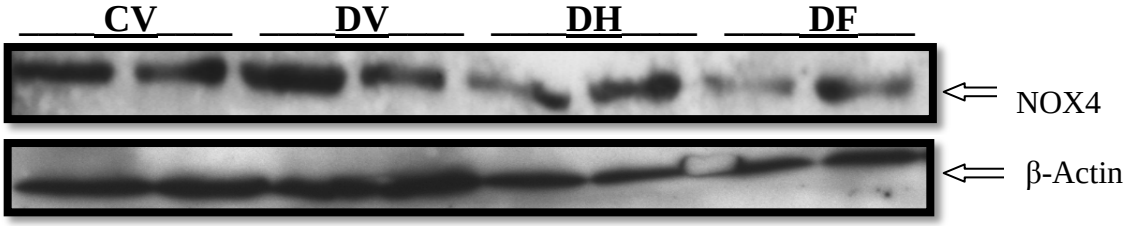
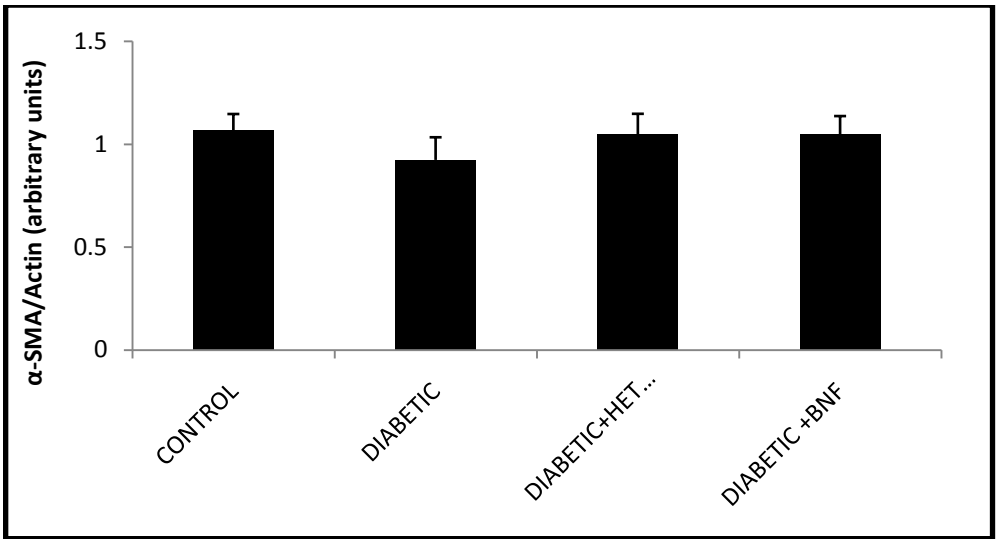


Figure 6. NOX4 protein expression increased in diabetes contributing to cardiovascular dysfunction. BNF (35mg/kg/d) treatment down regulates NOX4 protein expression compared to diabetes. Representative western blot of NOX4 protein expression in abdominal aortic tissues of control, diabetic , diabetic + HET0016 or BNF treated male Sprague Dawley rats after 28 days (B). Western blots are quantified by densitometry and are representative of six independent experiments (n=24). Each histogram represents the ratio of the intensity of the NOX4 band factored by β-actin band used as a loading control (A). Values are represented as mean ± SEM. *P<0.05, control vs. diabetic, and $^{\#}$P<0.05 diabetic vs. diabetic+HET0016 and diabetic +BNF

Figure 7.

A.



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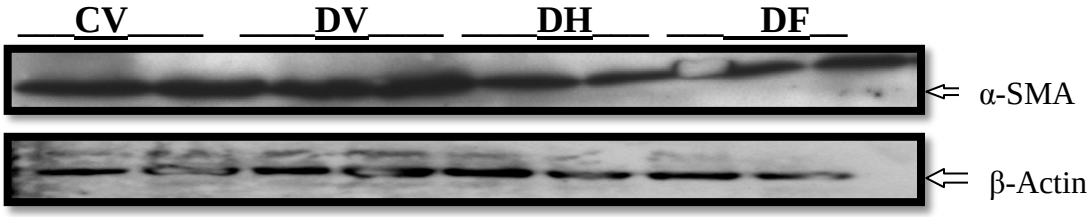
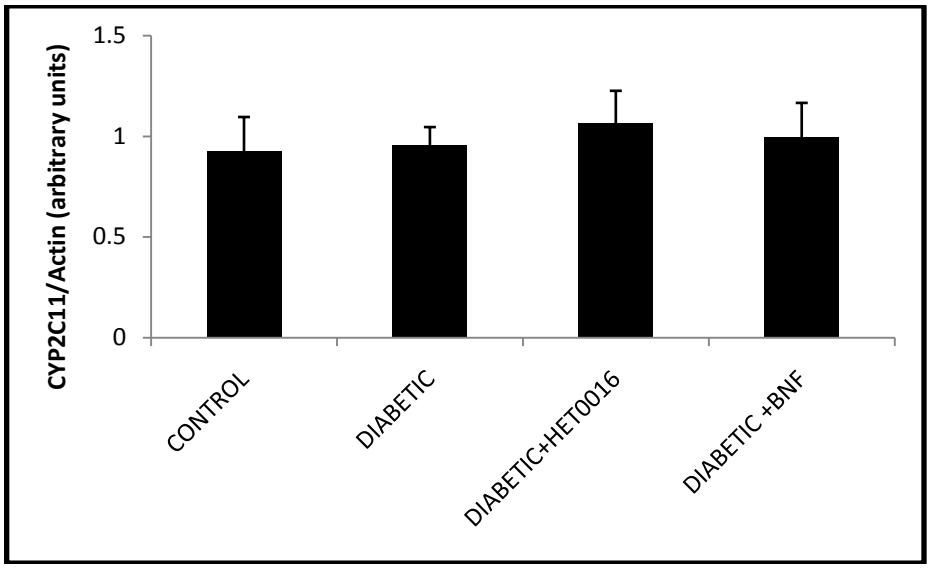


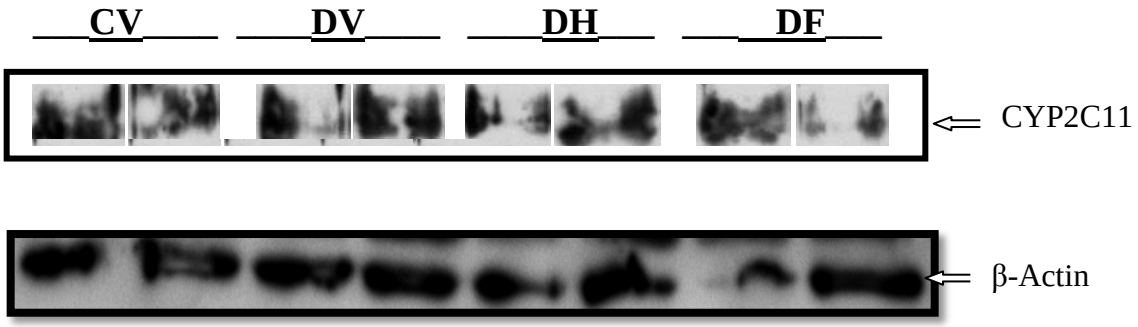
Figure 7. Treatment with HET0016 (10mg/kg/d) or BNF (35mg/kg/d) did not affect α-SMA protein expression in diabetes. Representative western blot of α-SMA protein expression in abdominal aortic tissues of control, diabetic , diabetic + HET0016 or BNF treated male Sprague Dawley rats after 28 days (B). Western blots are quantified by densitometry and are representative of six independent experiments (n=24). Each histogram represents the ratio of the intensity of the α-SMA band factored by actin band used as a loading control (A). Values are represented as mean ± SE of the mean. *P<0.05, control vs. diabetic vehicle, and $^{\#}$P <0.05 diabetic vehicle vs. diabetic +HET0016 and diabetic + BNF.

Figure 8.

A.


CYP2C11/Actin (arbitrary units)
1.5
1
0.5
0
CONTROL
DIABETIC
DIABETIC+HET0016
DIABETIC +BNF

B.


CV
DV
DH
DF
CYP2C11
β-Actin

C. D.

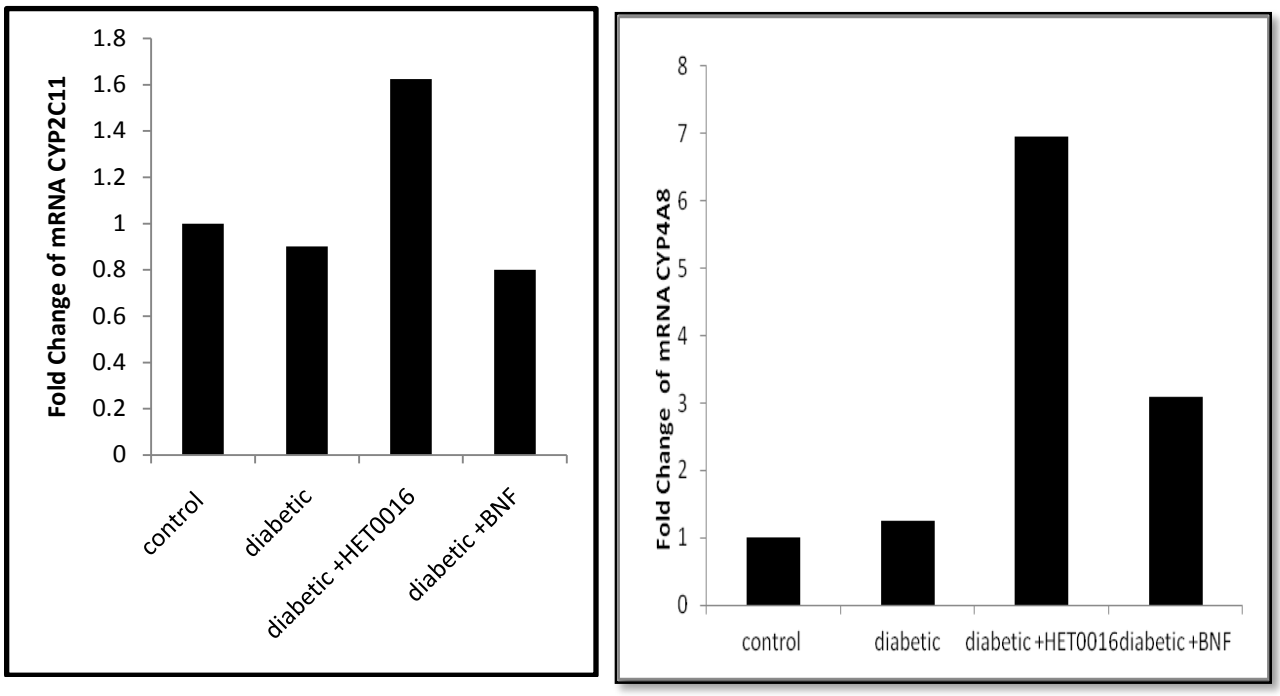


Figure 8. Treatment with BNF (35mg/kg/d) did not increase CYP2C11 protein and mRNA expression in diabetic +BNF group. HET0016 treatment (10mg/kg/d) decreased CYP4A8 mRNA expression in HET0016+diabetic vs. diabetic vehicle.

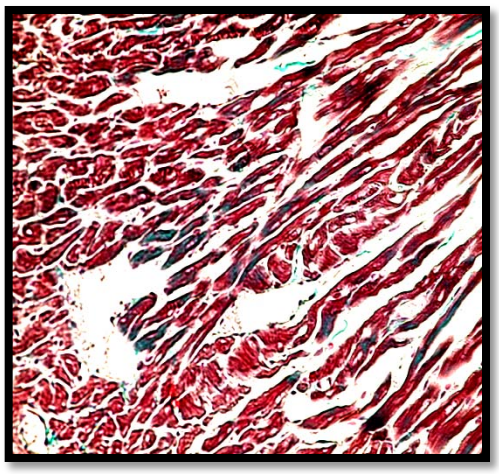
Representative western blot of CYP2C11 protein expression in abdominal aortic tissues of control, diabetic , diabetic + HET0016 or BNF treated male Sprague Dawley rats after 28 days (B). Western blots are quantified by densitometry and are representative of six independent experiments (n=24). Each histogram represents the ratio of the intensity of the CYP2C11 band factored by actin band used as a loading control (B). The values represent the relative induction of CYP2C11 measured by RT-PCR relative to GAPDH mRNA levels (n=16) (C). The values represent the relative induction of CYP4A8 measured by RT-PCR relative to GAPDH mRNA levels (n=16) (D). Values are represented as mean ± SEM. *$P<0.05$, control vs. diabetic vehicle, and $^{\#}P<0.05$ diabetic vehicle vs. diabetic + HET0016 and diabetic +BNF

Figure 9.

CV

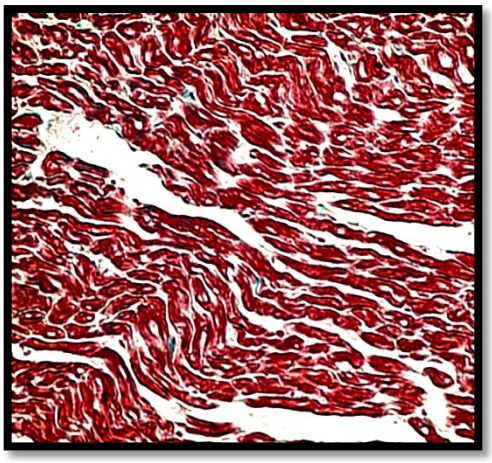
(A)

DV



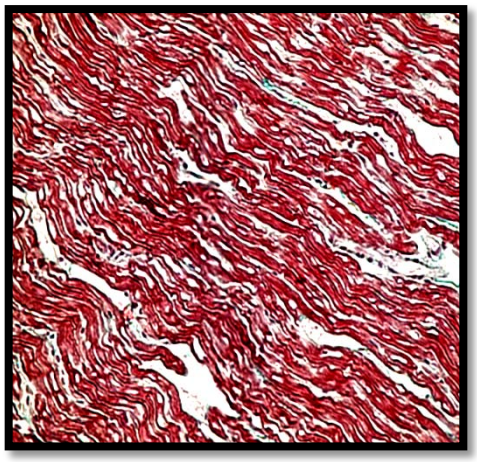
(B)

DH



(C)

DF



(D)

E.

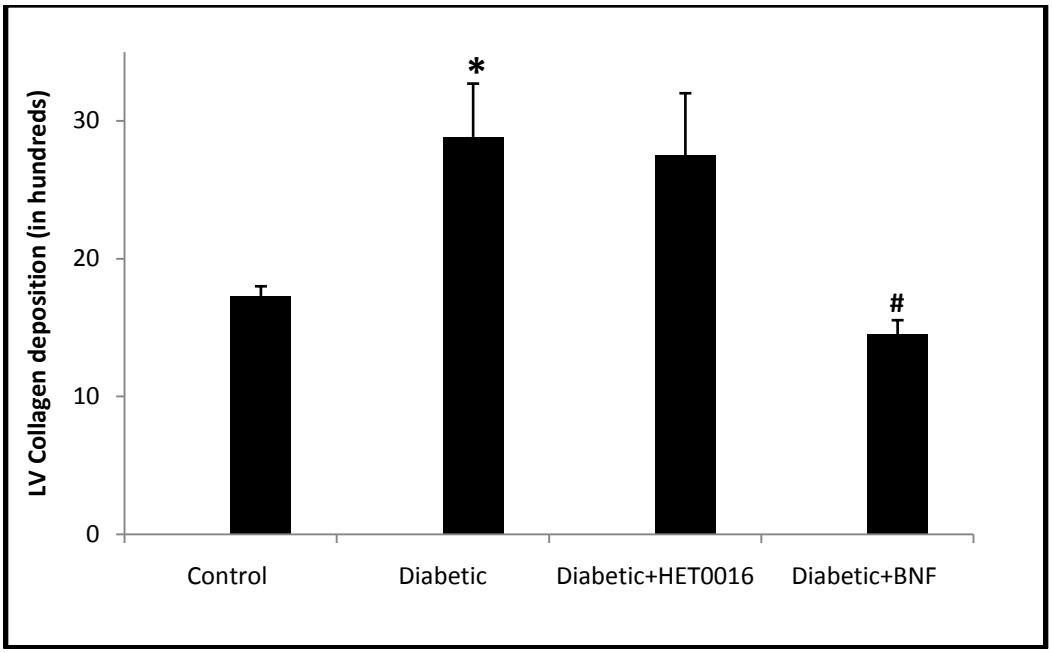


Figure 9. Histopathological analysis of heart left ventricle sections with Masson's Trichrome staining (fibrosis: green color) form (A) control, (B) diabetic vehicle,(C) diabetic +HET0016,(D) diabetic+ BNF rats. Histogram representing the quantification of trichrome staining; results from 20 rats (E). *P<0.05, control vs. diabetic; #P<0.05, diabetic vs. diabetic +HET0016 or diabetic +BNF

Figure 10.

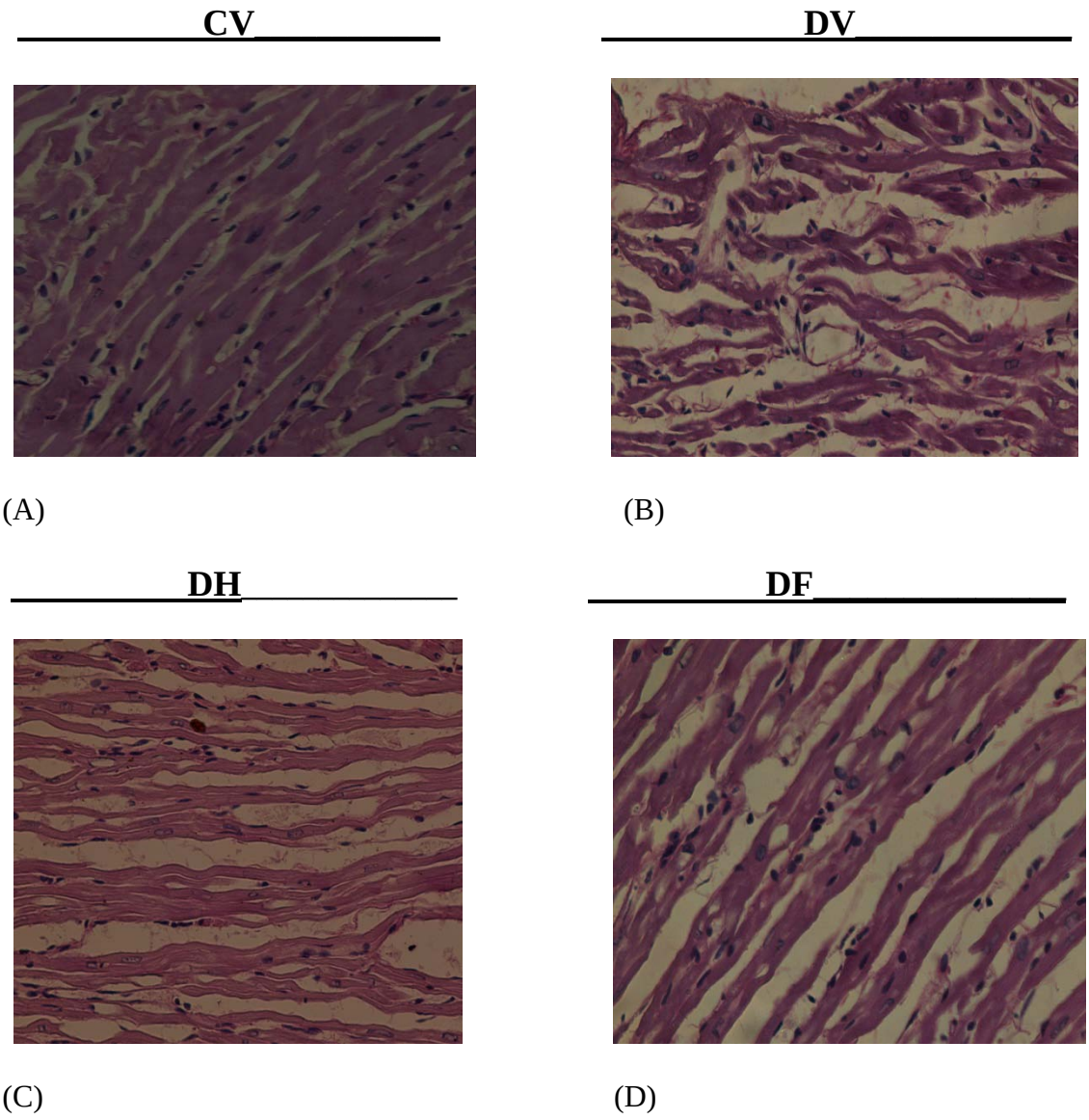


Figure 10. Histopathological analysis of heart left ventricle sections with Periodic acid-Schiff staining (fibrosis: blue color) form (A) Control,(B) Diabetic+ vehicle,(C) Diabetic +HET0016,(D) Diabetic+ BNF rat

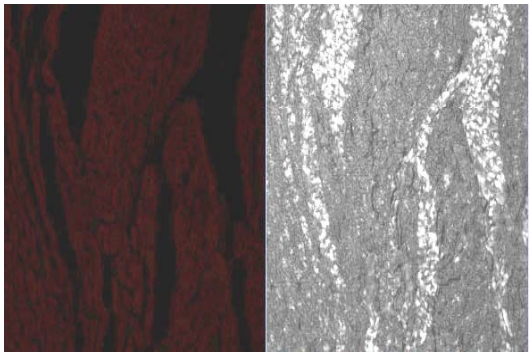
Table 3.

RAT	PSA Scoring
CONTROL	1
DIABETIC	* 2.8 ± 0.6
DIABERIC+HET0016	#1.7 ± 0.3
DIABTIC+BNF	#1.4 ± 0.4

Semi-quantification of Periodic acid-Schiff staining using a score: 1-4. 1, normal histology; 2, cell swelling and nuclear condensation, with loss of up to one-third of the nuclei; 3, same as for score 2, but greater than one-third and less than two-thirds of the cells profile showing nuclear loss, and 4, greater than two-thirds of the cells profile showing nuclear loss .
The total score per left ventricle was calculated by addition of all the calculated scores of each section/rat *;* results from 8 rats. *P<0.05, control vs. diabetic; #P<0.05, diabetic vs. diabetic +HET0016 or diabetic +BNF

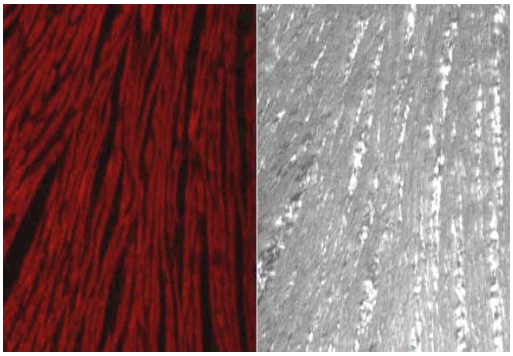
Figure 11.

C1V



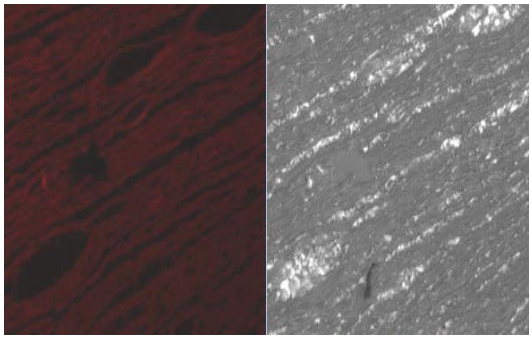
(A)

D1V



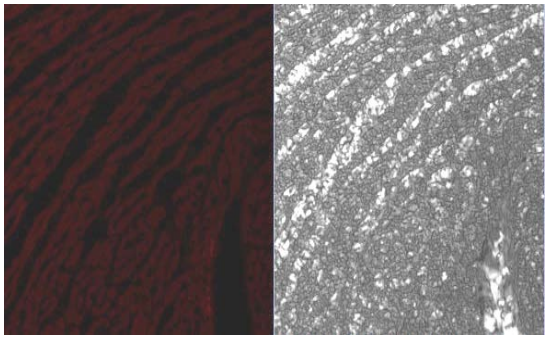
(B)

D1H



(C)

D1F



(D)

E.

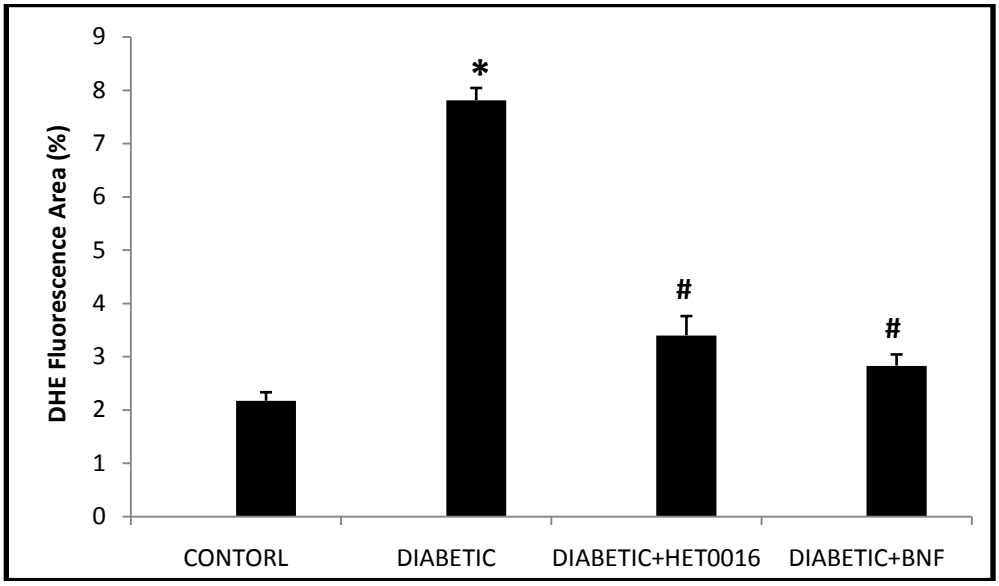


Figure 11. Increased ROS generation in the LV sections of DV compared to CV rats. Treatment with HET0016 (10mg/kg/day) and BNF (35mg/kg/day) decreased ROS generation.

Histopathological analysis of heart left ventricle sections with Dihydroethidium bromide staining for ROS detection (ROS: red color) of (A) CV, (B) DV, (C) DH, and (D) DF groups. Histogram representing the quantification of ROS (E) ; results from 24 rats. Values are represented as Mean ± SE of the mean. *P<0.05, control vs. diabetic; #P<0.05, diabetic vs. diabetic +HET0016 or diabetic +BNF

REFERNCES

Abboud and Bassila et al.,2009.Temporal changes in vascular reactivity in early diabetes mellitus in rats: role of changes in endothelial factors and in phosphodiesterase activity .*Am J Physiol Heart Circ Physiol* ,297, H836–H845

Abdulfatai B. Olokoba, Olusegun A. Obateru, Lateefat B. Olokoba,2012. Type 2 Diabetes Mellitus: A Review of Current Trends. *Oman Medical Journal*,27(4),269-273 acid: a new target for the treatment of hypertension. *J Cardiovasc Pharmacol* ,56,336–344

Aljabri et al.,2010. Glycemic changes after vitamin D supplementation in patients with type 1 diabetes mellitus and vitamin D deficiency .*Ann Saudi Med*. , 30(6),454–458.

American Diabetic Association (ADA),2011.Data from the 2011 National Diabetes Fact Sheet and risk of complications in patients with type 2 diabetes (UKPDS 33),1998. *Lancet*, 352:837– 853

Basha et al.,2012. Endothelial Dysfunction in Diabetes Mellitus: Possible Involvement of Endoplasmic Reticulum Stress? .*Experimental Diabetes Research*, 14

Betsy B. Dokken, 2008. The Pathophysiology of Cardiovascular Disease and Diabetes: Beyond Blood Pressure and Lipids. *Diabetes Spectrum* , 21, 3.

Camici GG et al.,2007.Genetic deletion of p66(Shc) adaptor protein prevents hyperglycemia-induced endothelial dysfunction and oxidative stress. *Proc Natl Acad Sci U S A.* , 104(12),5217-22.

Capdevila J et al.,1981.The oxidative metabolism of arachidonic acid by purified *P*-450. *Biochem Biophys Res Commun* ,101, 1357–1363

Campbell et al.,1996. Identification of Epoxyeicosatrienoic Acids as Endothelium Derived Hyperpolarizing Factors .*Circulation Research*, 78,415-423

Campbell WB et al.,2001. Role of epoxyeicosatrienoic acids as endothelium-derived hyperpolarizing factor in bovine coronary arteries. *Med Sci Monit*. ,7, 578–584.

Campbel B. William and Fleming Ingrid,2010. Epoxyeicosatrienoic Acids and Endothelium-Dependent Responses .*Pflugers Arch*. , 459(6), 881–895

Cheng et al., 2008. 20-Hydroxyeicosatetraenoic acid causes endothelial dysfunction via eNOS uncoupling. *Am J Physiol Heart Circ Physiol* ,294,H1018– H1026.

Cheng et al., 2010. 20-Hydroxy-5,8,11,14-eicosatetraenoic Acid Mediates Endothelial Dysfunction via IB Kinase-Dependent Endothelial Nitric-Oxide Synthase Uncoupling. *The journal of pharmacology and experimental therapeutics* , 332, 332,57–65

Chmelzer et al.,2005. Soluble epoxide hydrolase is a therapeutic target for acute inflammation. *Proc. Natl Acad. Sci. U.S.A.*, 102, 9772–9777.

Cusick et al ,*2005.* Associations of Mortality and Diabetes Complications in Patients With Type 1and Type 2 Diabetes Early Treatment Diabetic Retinopathy Study report no. 27.*Diabetes Care* ,28,617–625.

Dandona P, Aljada A.,2004. Advances in diabetes for the millennium: diabetes and the endothelium. *Med Gen Med* ,6,3 ,6.

Dave GS and Kalia K,2007. Hyperglycemia induced oxidative stress in type-1 and type-2 diabetic patients with and without nephropathy. *Cell Mol Biol (Noisy-le-grand),* 53(5),68-78

David M Maahs, Nancy A West, Jean M. Lawrence, MPH, Elizabeth J Mayer-Davis, (2010). Chapter 1: Epidemiology of Type 1 Diabetes. *Endocrinol Metab Clin North Am*,39(3),481–497.

Davis et al.,2002. Inhibitors of soluble epoxide hydrolase attenuate vascular smooth proliferation. *Proc Natl Acad Sci U S A.*, 99,7, 4752.

De Vriese AS, Verbeuren TJ, Van de Voorde J, Lameire NH, Vanhoutte PM,2000. Endothelial dysfunction in diabetes. *Br J Pharmacol,* 130, 963–974.

Deng Yangmei et al,2010.Cytochrome P450 epoxygenases, soluble epoxide hydrolase, and the regulation of cardiovascular inflammation. *Journal of Molecular and Cellular Cardiology,* 48 , 331–341

Earley et al.,2005.TRPV4 forms a novel Ca^{2+} signalling complex with ryanodine receptors and BK channels.*Circ Res.* ,97,1270 –1279.

Escalante B. et al.,1991.Chronic treatment with tin normalizes blood pressure in spontaneously hypertensive rats. *Hypertension* ,17, 776–779

Ewing DJ, Campbell D, Clarke BF,1980.The natural history of diabetic autonomic neuropathy.*Q J Med* ,49, 95–108

Fichtlscherer Stephan et al.,2004. Inhibition of Cytochrome P450 2C9 Improves Endothelium-Dependent, Nitric Oxide–Mediated Vasodilatation in Patients With Coronary Artery Disease. *Circulation*,109,178-183.

Fitzgerald SM, Kemp-Harper BK, Tare M, Parkington HC,2005. Role of endothelium-derived hyperpolarizing factor in endothelial dysfunction during diabetes. *Clin Exp Pharmacol Physiol* ,32, 482–487.

Fleming et al.,2001.Endothelim Derieved Hyperpolarzing Factor Synthase (Cytochrome P450 2C9) Is a Functionally Significant Source of Reactive Oxygen Species in Coronary Arteries.*Circ Res.* , 88,44-51.

Furchgott, R.F. & Vanhoutte, P.M. ,1989. Endothelium derived relaxing and contracting factors. *FASEB J*., 3, 2007 ± 2018.

Gao L and Mann GE,2009. Vascular NAD(P)H oxidase activation in diabetes: a double-edged sword in redox signaling. *Cardiovasc Res,* 82,9–20

Gauthier et al.,2005. 14,15-Epoxyeicosatrienoic Acid Represents a Transferable Endothelium-Dependent Relaxing Factor in Bovine Coronary Arteries. *Hypertension*. ,45,666-671

Gebremedhin D. et al., 1998.Cat cerebral arterial smooth muscle cells express cytochrome *P*-450 4A2 enzyme and produce the vasoconstrictor 20-HETE which enhances L-type Ca current. *J Physiol (Lond)* ,507, 771–781

Giacco Ferdinando and Brownlee Michael,2010. Oxidative Stress and Diabetic Complications . *Circ Res* ,107,1058-1070.

Guglielmino et al.,2012. Pharmacological inhibition of soluble epoxide hydrolase provides cardioprotection in hyperglycemic rats .*Am J Physiol Heart Circ Physiol* ,303, H853–H862,

Guzik TJ et al.,2002. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation.* ,105(14),1656-62

Haffner SM, Lehto S, Ronnemaa T, et al. 1998. Mortality from coronary heart disease in subjects with type 2 diabetes and in nondiabetic subjects with and without prior myocardial infarction. *N Engl J Med*, 339,229–34.

Han et al., 2007. Reciprocal relationships between abnormal metabolic parameters and endothelial dysfunction. *Curr. Opin. Lipidol*., 18, 58–65.

Hanley AJ, Williams K, Stern MP, Haffner SM,2002.: Homeostasis model assessment of insulin resistance in relation to the incidence of cardiovascular disease: the San Antonio Heart Study. *Diabetes Care*, 25,1177–1184.

Hercule HCet al.,2009. Interaction between P450 eicosanoids and nitric oxide in the control of arterial tone in mice. *Arterioscler Thromb Vasc Biol* , 29,54–60.

Hong Ding and Chris R Triggle, (2005). Endothelial Cell Dysfunction and the Vascular Complications Associated with Type 2 Diabetes: Assessing the Health of the Endothelium. *Vasc health risk manag*,1(1),55-71

Imig JD, Zhao X, Capdevila JH, Morisseau C, Hammock BD.,2002. Soluble epoxide hydrolase inhibition lowers arterial blood pressure in angiotensin II hypertension. *Hypertension* ,39,690–4.

Impairment of coronary vascular reserve and ACh-induced coronary vasodilation in diabetic patients with angiographically normal coronary arteries and normal left ventricular systolic function. *Diabetes, 42*(7), 1017-1025.

Ingrid Fleming et al.,2001. Endothelium-Derived Hyperpolarizing Factor Synthase

Ishizuka et al.,2008. 20-Hydroxyeicosatetraenoic acid stimulates nuclear fac-tor-kappaB activation and the production of inflammatory cytokines in human endothelial cells. *J Pharmacol Exp Ther* ,324,103–110.

Ito O. et al.,1998 . Localization of cytochrome *P*-450 4A isoforms along the rat nephron. *Am J Physiol Renal Physiol* ,274, F395–F404

Iwain and Inagami T,1991.Isolation of preferentially expressed genes in the kidneys of hypertensive rats. *Hypertension* ,17, 161–169

Kathryn et al.,2000.Is EDHF an epoxyeicosatrienoic acid? .*Trends Pharmacol Sci.* ,21(4),121-4

Kawaguchi M et al.,1997.A comparison of ultrastructural changes on endomyocardial biopsy specimens obtained from patients with diabetes mellitus with and without hypertension. *Heart Ves* ,1,267–274

Kay L. Mehers and Kathleen M. Gillespie,2008. The genetic basis for type 1 diabetes. *British Medical Bulletin,* 88, 115–129.

Khan Mahmood et al.,2007. Cardioprotection by Sulfaphenazole, a Cytochrome P450 Inhibitor: Mitigation of Ischemia-Reperfusion Injury by Scavenging of Reactive Oxygen Species,*JPET* ,323,813–821

Khullar M et al., 2010. Oxidative stress: a key contributor to diabetic cardiomyopathy. Can J Physiol Pharmacol. ,88(3),233-40

Laakso M. 1999. Hyperglycemia as a risk factor for cardiovascular disease in type 2 diabetes. *Prim Care*, 26,829–39.

Lewis DH,1998.The effect of trauma on the regulation of the microcirculation .*Pathophysiol* ,5 ,1,191

Liu Y. et al.,2005.The antiinflammatory effect of laminar flow: the role of PPARgamma, epoxyeicosatrienoic acids, and soluble epoxide hydrolase. *Proc Natl Acad Sci U S A* , 102,16747–52.

Lucas, P. D. (1985). Effects of streptozotocin-induced diabetes and noradrenaline infusion on cardiac output and its regional distribution in pithed rats. *Diabetologia*, *28*(2), 108-112.

Luo Pengcheng et al.,2010. Inhibition or Deletion of Soluble Epoxide Hydrolase Prevents Hyperglycemia, Promotes Insulin Secretion, and Reduces Islet Apoptosis .*JPET* ,334,430–438

Luo Pengcheng and Wang Mong-Heng ,2011.Eicosanoids, β-Cell Function, and Diabetes. *Prostaglandins Other Lipid Mediat.* , 95(1-4), 1–10.

Luria A. et al.,2011. Soluble epoxide hydrolase deficiency alters pancreatic islet size and improves glucose homeostasis in a model of insulin resistance. *Proc Natl Acad Sci USA*, 108, 9038–9043.

Maalouf M Rita. and Eid A.Assaad et al.,2012. Nox4-derived reactive oxygen species mediate cardiomyocyte injury in early type 1 diabetes. *Am J Physiol Cell Physiol* ,302, C597–C604.

Madonna Rosalinda and De Caterina Raffaele,2011. Cellular and molecular mechanisms of vascular injury in diabetes — Part I: Pathways of vascular disease in diabetes. *Vascular Pharmacology* ,54 , 68–74

Manhiani M, Quigley JE, Knight SF, Tasoobshirazi S, Moore T, Brands MW, et al.,2009. Soluble epoxide hydrolase gene deletion attenuates renal injury and inflammation with DOCA-salt hypertension. *Am J Physiol Renal Physiol* , 297,F740–8.

Maraschin F, Murussi N, Witter V, Silveiro SP, 2010. Diabetes mellitus classification. *Arq Bras Cardiol.*,95(2), e40-6

Matsumoto S et al.,2003. Confirmation of superoxide generation via xanthine oxidase in streptozotocin-induced diabetic mice .*Free Radic Res*. ,37(7),767-72

McVeigh G, Brennan GM, Johnston GD et al., 1992. Impaired endothelium-dependent and independent vasodilation in patients with type 2 (non insuline-dependent) diabetes mellitus. *Diabetologia* ,35,771–776

Moncada S, Higgs A. ,1993.The L-arginine-nitric oxide pathway. *N Engl J Med* ,329(27),2002–12.

Monti et al. ,2008.Soluble epoxide hydrolase is a susceptibility factor for heart failure in a rat model of human muscle cell proliferation. *Proc. Natl Acad. Sci. U.S.A.*,99, 2222–2227.

Muthalif MMet al.,1998. 20-Hydroxyeicosatetraenoic acid mediates CaM kinaseII-induced mitogen-activated protein kinase activation in vascular smooth muscle cells. *Proc Natl Acad Sci USA* ,95,12701–6.

Muthalif MM et al.,2000. Contribution of Ras-GTPase GTPase/MAP kinase and cytochrome P450 metabolites to deoxycorticosterone-salt-induced hypertension.*Hypertension* ,35,457–63.

National Diabetes Data Group (NDDG), 1979. Classification and diagnosis of diabetes mellitus and others categories of glucose intolerance. *Diabetes* , 28,1039-57.

Nishikawa Takeshi, Edelstein Diane, and Brownlee Michael,2000. Indirect effects of high glucose the missing link: A single unifying mechanism for diabetic complications. *Kidney International,58, 77., S-26–S-30*

Nitenberg, A et al., (1993). Impairment of coronary vascular reserve and ACh-induced coronary vasodilation in diabetic patients with angiographically normal coronary arteries and normal left ventricular systolic function. *Diabetes, 42*(7), 1017-1025.

Nossaman et al., From: Contemporary Endocrinology: Cardiovascular Endocrinology: Shared Pathways and Clinical Crossroads Research Support: NIH Grant HL62000, HL77421, ES10018, and RR16456.,

Potenza et al. ,2005. Insulin resistance in spontaneously hypertensive rats is associated with endothelial dysfunction characterized by imbalance between NO and ET-1 production.*Am J Physiol Heart Circ Physiol*, 289,H813–H822

PowelL PK et al.,1998. Metabolism of arachidonic acid to 20-hydroxy-5,8,11,14-eicosatetraenoic acid by *P*-450 enzymes in human liver: involvement of CYP4F2 and CYP4A11. *J Pharmacol Exp Ther* ,285, 1327–1336

Quilley et al.,2003.Anandamide and arachidonic acid use epoxyeicosatrienoic acids to activate TRPV4 channels. *Nature*. ,424,434 –438.

Reis JS et al.,2008. Oxidizing and reducing responses in type 1 diabetic patients determined up to 5 years after the clinical onset of the disease. *Acta Diabetol.* ,45(4),221-4.

Roglic G, Green A et al.,2004.Prevalence of diabetes. Estimates for the year 2000 and projection tor 2030. *Diabetes Care*, 27,1047- 1053.

Rosenson R,2004.Clinical role of LDL and HDL subclasses and apolipoprotein measurement. *ACC Curr J Rev*, 33–37

Sacerdoti D et al.,1988. Renal cytochrome *P*-450-dependent metabolism of arachidonic acid in spontaneously hypertensive rats. *Biochem Pharmacol* ,37, 521–527

San Martin A et al.,2007. Reactive oxygen species-selective regulation of aortic inflammatory gene expression in Type 2 diabetes. *Am J Physiol Heart Circ Physiol,* 292,H2073–H2082

Schalkwijk and.Stehouwer,2005.Vascular complications in diabetes mellitus: the role of endothelial dysfunction. *Clinical Science*109,2, 143–159.

Schwartzman ML et al.,1985.Renal cytochrome *P*-450-related arachidonate metabolite inhibits Na1,K ATPase. *Nature* ,314, 620–622.

Schwartzman ML, Abraham NG, Carroll MA, et al.,1986. Regulation of arachidonic acid metabolism by cytochrome P-450 in rabbit kidney. *Biochem J* ,238,283–90.

Schwartzman ML, da Silva JL, Lin F, Nishimura M and Abraham NG ,1996.Cytochrome P4504A expression and arachidonic acid omega-hydroxylation in the kidney of the spontaneously hypertensive rat. *Nephron* ,73,652–663.

Shen GX,2010. Oxidative stress and diabetic cardiovascular disorders: roles of mitochondria and NADPH oxidase .*Can J Physiol Pharmacol.* ,88(3):241-8..
Shimojo et al., 1993. Changes in amounts of cytochrome P450 isozymes and levels of catalytic activities in hepatic and renal microsomes of rats with streptozotocin-induced diabetes. *Biochem. Pharmacol*., 46, 621– 627.

Singh H, Cheng J, Deng H, Kemp R, Ishizuka T, Nasjletti A, and Schwartzman ML ,2007.Vascular cytochrome P450 4A expression and 20-hydroxyeicosatetraenoic acid synthesis contribute to endothelial dysfunction in androgen-induced hypertension. *Hypertension,* 50,123–129

Spector A.Arthur,2009. Arachidonic acid cytochrome P450 epoxygenase pathway. *J. Lipid Res. ,* 50, S52–S56.

Stec DE, Mattson DL and Roman RJ ,1997. Inhibition of renal outer medullary 20-HETE production produces hypertension in Lewis rats. *Hypertension* ,29, 315–319.

Stratton et al.,2000.Association of glycaemia with macrovascular and microvascular complications of type2 diabetse (UKPDS 35): prospective observational study. *BMJ* ,321,405–412

Subodh Verma, Michael R. Buchanan, Todd J. Anderson,2003.Endothelial Function Testing as a Biomarker of Vascular Disease. *Circulation* ,108,2054-2059

Thandavarayan RA et al.,2009. p38α mitogen-activated protein kinase prevents cardiac apoptosis and remodeling after streptozotocin-induced diabetes mellitus. *Am J Physiol Heart Circ Physiol* ,297,H911–H919

Tsai et al., 2009. 20-HETE and F2-isoprostanes in the metabolic syndrome: the effect of weight reduction. *Free Radic Biol Med* ,46,263–270

UK Prospective Diabetes Study (UKPDS) Group: Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes.*(UKPDS 33*). 352:837– 853.

Vanhoutte et al.,2009. Endothelial dysfunction and vascular disease .*Acta Physiol* , 196, 193–222

Vriens J et al.,2005. Modulation of the Ca2 permeable cation channel TRPV4 by cytochrome P450 epoxygenases in vascular endothelium. *Circ Res*. ,97,908 –915.

Wang et al., 2003.Up-regulation of endothelial nitric-oxide synthase by endothelium-derived hyperpolarizing factor involves mitogen-activated protein kinase and protein kinase C signaling pathways. *J Pharmacol Exp Ther* ,307,753–643

Wang J.S. et al.,2006.Endothelial dysfunction and hypertension in rats transduced with CYP4A2 adenovirus. *Circ. Res*., 98, 962–969.

Watkins, P. J., & Thomas, P. K. (1998). Diabetes mellitus and the nervous system .*Journal of Neurology, Neurosurgery & Psychiatry, 65*(5), 620-632

Williams JM, Murphy S, Burke M, Roman RJ ,2010. 20-Hydroxyeicosatetraeonic acid: a new target for the treatment of hypertension. Cardiovasc Pharmacol 56:336–344.

World Health Organization (WHO), Geneva,2006. Definition and Diagnosis of Diabetes Mellitus and Intermediate Hyperglycemia: Report of a WHO/ID F Consultation.

World Health Organization (WHO),2009. Global prevalence of diabetes: estimates for the year 2000 and projection s for 2030.

Xue Gao et al.,2011. Endothelium-derived hyperpolarizing factor and diabetes. *World J Cardiol* , 26; 3(1), 25-31

Yi Lin and Zhongjie Sun,2010. Current views on type 2 diabetes. *Journal of Endocrinology*,204,1-11

Yousif M. H. M. et al.,2009. Role of 20-hydroxyeicosatetraenoic acid in altering vascular reactivity in diabetes. *Autonomic & Autacoid Pharmacology*, 29, 1–12.

Yousif M. H. M et al.,2009. Cytochrome P450 metabolites of arachidonic acid play a role in the enhanced cardiac dysfunction in diabetic rats following ischaemic reperfusion injury.*Autonomic & Autacoid Pharmacology* , 29, 33–41

Yousif and Benter,2010. Role of 20-Hydroxyeicosatetraenoic and Epoxyeicosatrienoic Acids in the Regulation of Vascular Function in a Model of Hypertension and Endothelial Dysfunction *Pharmacology* ,86:149–156

Zeldin DC et al.,1996. Biochemical characterization of the human liver cytochrome *P*-450 arachidonic acid epoxygenase pathway. *Arch Biochem Biophys* ,330, 87–96

Zhang L et al.,2008. 11,12-Epoxyeicosatrienoic acid activates the l-arginine/nitric oxide pathway in human platelets. *Mol Cell Biochem* ,308,51–6

Zhang LN et al. ,2009.Inhibition of soluble epoxide hydrolase attenuated atherosclerosis, abdominal aortic aneurysm formation, and dyslipidemia. *Arterioscler Thromb Vasc Biol* , 29,1265–70.