



AMERICAN UNIVERSITY OF BEIRUT

EFFECT OF HET0016, CAPTOPRIL, AND THEIR  
COMBINATION ON THE CARDIOVASCULAR  
COMPLICATIONS OF DIABETES IN SPRAGUE DAWLEY  
RATS

by  
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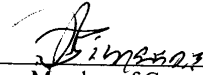
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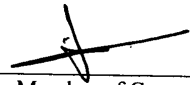
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## AN ABSTRACT OF THE THESIS OF

Maysaa Ahmad Hamza for Master of Science  
Major: Pharmacology and Therapeutics

Title: Effect of HET0016, Captopril, and their combination on the cardiovascular complications of diabetes in Sprague Dawley rats.

**Background:** Cardiovascular complications associated with diabetes mellitus are one of the leading causes of death. Oxidative stress can contribute to these complications that include cardiomyopathy and vascular dysfunction. Angiotensin has been known to be a mediator of these complications, however recent studies have demonstrated a role for CYP450 mediated arachidonic acid eicosanoids. These include 20-HETE a potent vasoconstrictor associated with diabetic injuries, and EET a potent vasodilator thought to be cardioprotective. Furthermore an interaction between angiotensin and 20-HETE has been implied in several studies.

**Aims:** To study the effect of inhibiting 20-HETE formation, by HET0016, on the cardiovascular system in streptozotocin (STZ) induced diabetic rats. In addition, this study aimed to investigate if captopril, an ACE inhibitor, would have an effect on 20-HETE formation. Moreover, we aimed to study if there would be a beneficial outcome from a combined treatment of HET0016 and captopril.

**Methods:** Male Sprague Dawley rats were divided into 8 groups for this study. Diabetes was induced in 4 groups by STZ injection, the other 4 groups were controls and injected normal saline instead (STZ vehicle). Two days later glucose levels were measured to prove the induction of diabetes. Then 4 treatments were given for 28 days: Vehicle (normal saline), Captopril (ACE inhibitor), HET0016 (20-HETE formation inhibitor), and a combination of captopril + HET0016. At day 28, rats were sacrificed, plasma samples were taken and used to measure the level of 20-HETE. The thoracic aorta was excised and used for vascular reactivity study in response to phenylephrine, acetylcholine, KCl, and SNAP. The abdominal aorta and left ventricle were used to evaluate collagen deposition by Trichrome stain. Both these tissues were also used to determine protein and mRNA expression of CYP4A1 and CYP2C11, as well as the expression of several markers of injury including  $\alpha$ -SMA,  $\alpha$ -MHC,  $\beta$ -MHC, NOX-1, and NOX-4. Left ventricular sections were used to assess ROS production by DHE staining.

**Results:** 20-HETE levels in plasma were increased in diabetic vehicle group (DV), in contrast no increase was observed in diabetic rats that received any of the 3 treatment regimens. Vascular responses showed no changes except for the response to SNAP (NO donor), where DV had an impaired dilatory response; this was corrected by all

treatments. Trichrome stain displayed increased collagen deposition in DV which was prevented by treatment with captopril and the combination of captopril + HET0016; however treatment with HET0016 prevented collagen deposition in left ventricle only. CYP4A1 expression was increased in DV compared to control, and was prevented by HET0016 and the combination of treatments. CYP2C11 expression was decreased in DV but was not corrected by any treatment.  $\alpha$ -SMA expression was increased in DV on an mRNA level, this was only decreased in the group treated with captopril. An isoform switch from  $\alpha$ -MHC to  $\beta$ -MHC was observed in DV, but not in diabetic rats treated with HET0016 or the combination treatment. NOX-4 levels were unchanged among the different groups, NOX-1 levels were increased on an mRNA level in DV; this increase was not observed in any of the treated groups. DHE stain showed an increase in ROS production which was decreased by all the different treatments.

**Conclusion:** These results show a role for 20-HETE in diabetic cardiovascular disease since HET0016 treatment significantly decreased markers of injury in the cardiovascular system. An interaction between angiotensin and 20-HETE pathways is possible since captopril treatment was able to decrease the levels of 20-HETE in plasma. As for the results of combining both treatments, it was not conclusive since in some parameters there was an additional benefit from combining the drugs, and in other parameters there was not. Further investigation is required to validate these findings, and to better understand the mechanism of action behind these mediators of cardiovascular injury.

## CONTENTS

ACKNOWLEDGEMENT.....	V
ABSTRACT.....	VI
LIST OF ILLUSTRATIONS.....	XI
LIST OF TABLES.....	XIII
LIST OF ABBREVIATIONS.....	XIV

### Chapters

I-INTRODUCTION.....	1
A- Diabetes Mellitus: General Overview.....	1
1. Prevalence of Diabetes.....	1
2. Definition of Diabetes Mellitus .....	2
3. Classification.....	2
i- Type 1 Diabetes Mellitus.....	2
ii- Type 2 Diabetes Mellitus.....	3
iii- Other Specific Types.....	4
iv- Gestational Diabetes.....	4
B- Mechanism of Diabetic Complications.....	4
1. Increased Polyol Pathway Flux.....	5



2.	Increased Intracellular Formation of Advanced Glycation End-products.....	5
3.	Activation of Protein Kinase C.....	6
4.	Increased Flux through the Hexosamine Pathway.....	6
C-	ROS production and NADPH oxidases.....	7
D-	Cardiovascular Complications in Diabetic Patients.....	9
1.	Cardiomyopathy.....	9
2.	Vascular Dysfunction.....	11
E-	Arachidonic Acid Metabolites of Cytochrome P450.....	13
1.	20-HETE and EET Formation.....	14
2.	20-HETE and EET Function in Vasculature.....	14
3.	20-HETE and EET Function in the Heart.....	16
F-	RAS Pathway and Diabetic Complications.....	18
1.	Angiotensin II in Diabetic Cardiovascular Disease.....	19
2.	Angiotensin Converting Enzyme Inhibitor vs. Angiotensin Receptor Blocker.....	20
G-	Association Between Angiotensin and Arachidonic Acid Metabolites.....	21
II-	AIMS OF THE STUDY.....	22
III-	MATERIALS AND METHODS.....	24
A-	Animal Model.....	24
B-	Vascular Reactivity Study.....	26
C-	Western Blot Analysis.....	27

D- Polymerase Chain Reaction (q-PCR).....	29
E- Histological Staining.....	30
F- Enzyme Linked Immunosorbent Assay (ELISA).....	31
G- Immunohistochemistry DHE stain for ROS production.....	32
H- Statistical Analysis.....	32
<b>IV- RESULTS.....</b>	<b>33</b>
A- Blood Glucose Levels.....	33
B- Heart and Body Weight Changes.....	34
C- Collagen Deposition in Extracellular Matrix.....	35
D- Levels of CYP4A1/A2/A3 and CYP2C11 in Abdominal Aorta and Left Ventricle.....	40
E- Levels of 20-HETE in the Plasma.....	47
F- Markers of Heart Injury.....	48
G- Assessment of ROS Production.....	54
H- Functional Activity of Thoracic Aorta.....	62
<b>V- DISCUSSION.....</b>	<b>68</b>
<b>REFERENCES.....</b>	<b>75</b>

## ILLUSTRATIONS

Figure	Page
Figure (1): Blood glucose levels at d0, d2, d14, and d28.....	34
Figure (2a): Quantification of Masson's Trichrome stain in abdominal aorta.....	36
Figure (2b): Histological staining using Masson's Trichrome method on Abdominal Aorta and visualized using a light microscope.....	37
Figure (3a): Quantification of Masson's Trichrome stain in Left Ventricle.....	38
Figure (3b): Histological staining using Masson's Trichrome method on Left Ventricle and visualized using a light microscope.....	39
Figure (4a): Western Blot analysis of CYP4A1/A2/A3 in Left Ventricle.....	41
Figure (4b): q-PCR results of CYP4A1/A2/A3 in Left Ventricle.....	42
Figure (5a): Western Blot analysis for CYP4A1/A2/A3 in Abdominal Aorta.....	43
Figure (5b): q-PCR results of CYP4A1/A2/A3 in Abdominal Aorta.....	44
Figure (6a): Western Blot Analysis of CYP2C11 in Left Ventricle.....	45
Figure (6b): q-PCR results of CYP2C11 in Left Ventricle.....	46
Figure (7): q-PCR results of CYP2C11 in Abdominal Aorta.....	46
Figure (8): Quantification of ROS production by DHE immunostain.....	47
Figure (9a): Western Blot analysis for $\alpha$ -MHC in Left Ventricle.....	49
Figure (9b): q-PCR results for $\alpha$ -MHC in Left Ventricle.....	50
Figure (10a): Western Blot Analysis of $\beta$ -MHC in Left Ventricle.....	51
Figure (10b): q-PCR results of $\beta$ -MHC in Left Ventricle.....	52
Figure (11a): Western Blot analysis of $\alpha$ -SMA in Left Ventricle.....	53
Figure (11b): q-PCR results of $\alpha$ -SMA in Left Ventricle.....	54

Figure (12a): Western Blot analysis of NOX-1 in Left Ventricle.....	55
Figure (12b): q-PCR results of NOX-1 in Left Ventricle.....	56
Figure (13a): Western Blot analysis of NOX-4 in Left Ventricle.....	57
Figure (13b): q-PCR results of NOX-4 in Left Ventricle.....	58
Figure (14): q-PCR results of NOX-1 in Abdominal Aorta.....	58
Figure (15a): Western Blot analysis of NOX-4 in Abdominal Aorta.....	59
Figure (15b): q-PCR results of NOX-4 in Abdominal Aorta.....	60
Figure (16a): DHE quantification for ROS production in Left Ventricle.....	60
Figure (16b): DHE stain of Left Ventricle visualized by confocal microscope.....	61
Figure (17a): Response curve of Thoracic Aorta to vasoconstrictive effect of PE with intact endothelium.....	63
Figure (17b): Response curve of Thoracic Aorta to vasoconstriction of PE in absence of endothelium.....	64
Figure (18): Response curve of Thoracic Aorta to vasoconstriction of KCl.....	65
Figure (19): Response curve of thoracic aorta to vasorelaxation of Ach.....	66
Figure (20): Response curve of Thoracic Aorta to vasodilation of SNAP in absence of endothelium.....	67

## TABLES

Table	Page
Table (1): Differentiation of treatment between different groups.....	26
Table (2): Forward and reverse primers used with annealing temperatures.....	30
Table (3): Comparison between heart weight, body weight, and a ratio between the two at day 28.....	35

## ABBREVIATIONS

Diabetes Mellitus = DM  
NADPH oxidase = NOX  
Advanced Glycation End-products = AGE  
Receptor for Advanced Glycation End-products = RAGE  
Reactive Oxygen Species = ROS  
Nuclear Factor k Beta = NFkB  
Mitogen Activated Protein Kinase = MAPK  
Fibronectin = FN  
Collagen = CGN  
Alpha Smooth Muscle Actin =  $\alpha$ -SMA  
Alpha Myosin Heavy Chain =  $\alpha$ -MHC  
Beta Myosin Heavy Chain =  $\beta$ -MHC  
Endothelial Nitric Oxide Synthase = eNOS  
Nitric Oxide = NO  
Arachidonic Acid = AA  
Cytochrome P450 = CYP  
Phospholipase A2 = PLA2  
Diacylglycerol = DAG  
Hydroxyeicosatetraenoic acid = HETE  
Epoxyeicosatrienoic acid = EET  
Dihydroxyeicosatrienoic acid = DiHETE  
Soluble Epoxide Hydrolase = sEH  
N-Hydroxy-N'-(4-butyl-2-methylphenyl)formamidine = HET0016  
Vascular Smooth Muscle Cells = VSMC  
Protein Kinase C = PKC  
Renin Angiotensin System = RAS  
Angiotensin II = AngII  
Angiotensin Type 1 Receptor = AT1  
Angiotensin Converting Enzyme inhibitor = ACEi  
Angiotensin Receptor Blocker = ARB  
Streptozotocin = STZ  
Polymerase Chain Reaction = PCR  
Phenylephrine = PE  
Acetylcholine = Ach  
S-Nitroso-N-acetylpenicillamine = SNAP  
Dihydroethidium = DHE

# CHAPTER I

## INTRODUCTION

### **A- Diabetes Mellitus: General Overview**

Diabetes Mellitus (DM) is a very serious health disorder that affects several organs in the body (Neville and Sidawy 2012). The effects of diabetes are so diverse that one would argue the necessity of several doctors from different fields to ensure proper treatment (Kaul, et al. 2013). Patients with diabetes are at risk of many pathologies including renal disease, blindness, neuropathy, and cardiovascular disease (Harcourt, et al. 2013). This is not a curable disorder, but rather requires a lifetime treatment (Kaul, et al. 2013). That is why it is considered to be one of the expensive medical conditions with expenses spent on direct medical costs that reached 176 billion in 2012 (National Diabetes Clearinghouse 2012).

#### ***1- Prevalence of Diabetes***

The number of people diagnosed with diabetes mellitus is rising worldwide over the course of years. In the year 2013, the number of adults with diabetes was estimated to be 381.8 million and it is predicted to increase to up to 591.9 million by 2035 (Guariguata, et al. 2014).

In the Middle-East and North Africa region (MENA), the prevalence of diabetes has increased due to many factors mainly urbanization and lifestyle changes. In 2013 countries in the MENA region were among the areas with the highest rates of diabetes worldwide, where 9.2% of the adult population were diagnosed which is equivalent to 34.6 million people. This number is expected to increase to almost 67.9 million in 2035.

Lebanon ranks the 7<sup>th</sup> in the top 10 countries for prevalence in the MENA region with 14.99% of diabetic cases (Majeeda, et al. 2014). In a population-based study in Lebanon, diabetes predominantly type 2 was found to be a leading cause of mortality, where people diagnosed with it has reached 8.5% of the population (Costanian, et al. 2014).

## **2- Definition of Diabetes Mellitus**

Diabetes is a group of metabolic disorders, mainly affecting glucose metabolism, which manifests by a characteristic high blood glucose level (Harcourt, et al. 2013). In addition to carbohydrate disturbances, there is disturbance in fat and protein metabolism which lead to long term damage and failure of many organs (Alberti and Zimmet 1998) especially the kidneys, eyes, nerves, heart, and blood vessels (American Diabetes 2004). This mainly occurs due to either defective insulin production or defective insulin response or both (Alberti and Zimmet 1998).

## **3- Classification**

Patients can be categorized according to four etiological classifications of diabetes mellitus which are: Type 1 DM, Type 2 DM, other specific types, and gestational diabetes with the main types being Type 1 and Type 2 DM (Maraschin, et al. 2010).

### **i. Type 1 Diabetes Mellitus**

This type is also referred to as Insulin Dependent Diabetes Mellitus (IDDM) or as juvenile-onset diabetes mellitus (American Diabetes 2004). It accounts for about 10% of patients with diabetes and is most commonly seen in children and teenagers (Harcourt, et al. 2013) even though it can occur in adults and is then referred to as Latent Autoimmune Diabetes in Adults (LADA) (Kerner and Bruckel 2014). It is defined as an acute autoimmune disorder that leads to the destruction of  $\beta$ -cells of the



islets of Langerhans in the pancreas which causes lack of insulin production and consequently hyperglycemia (Harcourt, et al. 2013). In normal conditions high blood glucose suppresses glucagon release, but in case of T1DM  $\alpha$ -islets become defective and release even more glucagon, thus worsening the hyperglycemic state (Ozougwu 2013). Patients with this type can have different autoantibodies against:  $\beta$ -islet cells, insulin, glutamic acid decarboxylase ( $GAD_{65}$ ), tyrosine phosphatases IA-2 and IA-2 $\beta$ , and zinc transporter 8 (ZnT8) (American Diabetes 2015). Treatment of T1DM necessitates insulin for the survival of patients (Richesson, et al. 2013).

ii. Type 2 Diabetes Mellitus

It is also referred to as Non-Insulin Dependent Diabetes Mellitus (NIDDM) or as adult-onset diabetes mellitus (American Diabetes 2004). It accounts for about 85% of the cases worldwide (Harcourt, et al. 2013) and most commonly seen in adults over the age of 40 (Ozougwu 2013). T2DM is characterized by insulin resistance where cells no longer respond to the stimulus once insulin is released (Harcourt, et al. 2013). It is also associated with insulin deficiency or defective insulin production (Kerner and Bruckel 2014); so even though insulin might be present, glucose does not enter cells and remains in the blood stream causing hyperglycemia (Harcourt, et al. 2013). The danger of this type is that it can go undiagnosed for years since it needs gradual development for symptoms to start showing (American Diabetes 2004). A combination of genetic predisposition and environmental factors such as obesity, high fat diet, lack of exercise, stress, smoking, and age can affect insulin resistance, adding to the pathogenesis of T2DM (Ozougwu 2013). That is why treatment includes a healthy diet and an active lifestyle in addition to pharmacotherapy (Richesson, et al. 2013).

iii. Other Specific Types

This group of diabetes include: exocrine pancreas disease (ex. pancreatitis), viral infections, endocrinopathies (ex. Cushing's Syndrome), drug induced, certain genetic defects, and insulin receptor antibodies (Maraschin, et al. 2010)

iv. Gestational Diabetes

This type is referred to as the pregnancy associated form of glucose dysfunction. Its prevalence ranges from 1% to 14% according to the country being studied (American Diabetes 2004) since certain ethnicities have a higher risk (Alberti and Zimmet 1998).

## **B- Mechanism of Diabetic Complications**

Diabetes mellitus is a disorder that is currently being referred to as the epidemic of the century since patients diagnosed with it have increased by 50% in the last decade (Gray and Jandeleit-Dahm 2014). These patients will suffer particular symptoms including hyperglycemia, polyuria, polydipsia, and susceptibility to infections (American Diabetes 2004). On the long term they are at risk of developing vascular complications tenfold more than non-diabetic individuals, these complications can be divided into microvascular and macrovascular (Gray and Jandeleit-Dahm 2014).

Microvascular complications affects small blood vessels that eventually lead to nerve, retinal, and kidney dysfunction. On the other hand, macrovascular complications affect large arteries leading to myocardial infarctions, stroke, and amputations (Gray and Jandeleit-Dahm 2014).

Under a hyperglycemic state, cells generally decrease glucose transport to maintain constant intracellular concentrations. However, cells that are unable to reduce glucose influx will end up with high intracellular glucose concentrations. Cells having high intracellular hyperglycemia are those that will suffer from diabetic complications (Heilig, et al. 1995). Four main hypotheses were considered as explanation for hyperglycemic induced diabetic.

### ***1- Increased Polyol Pathway Flux***

Aldose reductase is an enzyme that reduces toxic aldehydes into inactive alcohols. In normal conditions it metabolizes a small percentage of glucose, however when intracellular glucose becomes high it reduces high amounts of glucose to sorbitol that is later oxidized to become fructose. When changing glucose to sorbitol, the polyol pathway will consume NADPH which is essential for the cell to recycle the anti-oxidant glutathione from the oxidized to the reduced form. The polyol pathway leads to decreased NADPH which leads to a decrease in active glutathione and thus an increase in oxidative stress (Vedantham, et al. 2011).

### ***2- Increased Intracellular Formation of Advanced Glycation End-products (AGEs)***

Intracellular and extracellular formation of AGE precursors occur from the auto-oxidation of glucose into glyoxal, glyceraldehyde-3-phosphate to methylglyoxal, and amadori products to 3-deoxyglucosone (Madonna and Caterina 2011). These reactive dicarbonyls will interact with amino groups of proteins and form AGEs (Brownlee 2001). These precursors will cause cellular damage by different mechanisms. The first mechanism is by binding to intracellular proteins and altering their structure and function. This becomes even more important when it affects proteins responsible for regulating gene transcription. The second mechanism is by altering extracellular matrix

molecules affecting the signaling cascade between the matrix and the cell eventually leading to cellular dysfunction. Finally the third mechanism is when AGE precursors bind to circulating proteins and modify them, these modified proteins will then bind to RAGE (AGE receptors) and in turn, activated RAGEs will stimulate ROS production (Brownlee 2005) as well as changing gene expression to induce vascular inflammation and endothelial dysfunction (Madonna and Caterina 2011).

### ***3- Activation of Protein Kinase C***

Diacylglycerol is produced intracellularly when the levels of glucose increases, it is a cofactor for activating Protein Kinase-C (PKC) particularly the  $\beta$  and  $\delta$  isoforms (Koya, et al. 1997). The  $\beta$  isoform of PKC is particularly activated in the aorta and the heart (Madonna and Caterina 2011). Upon activating PKC, many changes occur on the level of gene expression in a way that decreases what is essential for normal function and maintenance of homeostasis, and at the same time increases what is responsible for pathological conditions (Brownlee 2005).

For example, production of endothelial nitric oxide synthase responsible for vasodilation is decreased while production of endothelin-1 that causes vasoconstriction is increased, resulting in blood flow abnormalities. Active PKC is also responsible for activating membrane NADPH oxidases leading to an increase in ROS formation. PKC is also responsible for the activation of nuclear factor- $\kappa$ B (NF $\kappa$ B) which activates various pro-inflammatory genes particularly in the vascular system (Kuboki, et al. 2000).

### ***4- Increased Flux through the Hexosamine Pathway***

Glucose is mainly metabolized by glycolysis; fructose-6-phosphate is an intermediate in this glycolytic pathway. Once glucose concentrations are high inside the cell, the formation of fructose-6-phosphate is increased and some of it gets diverted into

another pathway where glutamine fructose-6 phosphate amidotransferase converts it to glucosamine-6-phosphate and then to UDP-N-acetyl glucosamine. This glucosamine is a substrate for O-linked acetylglucosamine (O-GlcNAc) transferases that cause posttranslational modification of serine and threonine residues of cytoplasmic and nuclear proteins (Du, et al. 2000).

All of these different mechanism that lead to diabetic complications can be linked through the overproduction of ROS. In diabetes, the increase of glucose levels causes an increase in the amount of ROS being produced. This increase activates poly(ADP-ribose)polymerase which reduces the activity of GAPDH. And since GAPDH is necessary for glycolysis, reducing its activity leads to an accumulation of upstream glycolytic intermediates (Du, et al. 2003).

An accumulation of glyceraldehyde-3-phosphate can produce methylglyoxal thus activating the AGE pathway, it can also form diacylglycerol (DAG) which leads to PKC pathway activation. Fructose-6-phosphate is another intermediate whose levels are increased when GAPDH activity is decreased, this will activate the hexosamine pathway. Finally we get an accumulation of the first substrate which is glucose, it is responsible for activating the polyol pathway (Brownlee 2005).

### **C- ROS Production and NADPH oxidases**

Diabetic patients have a high amount of ROS in their system as well as a reduced amount of anti-oxidants (Jay, et al. 2006). ROS are highly reactive signaling molecules whose production is noted to be increased particularly in the cells that are

affected and damaged in the case of diabetes mellitus (Brownlee 2005; Harcourt, et al. 2013).

There are many sources of ROS including xanthine oxidase, cyclooxygenase, mitochondrial respiratory chain, cytochrome P450 enzymes, and nitric oxide synthase (Jiang, et al. 2011). However the main source is from enzyme complexes such as NADPH oxidase (NOX) (Gray and Jandeleit-Dahm 2014). All sources produce ROS as a secondary byproduct, but in the case of NOX its main function is producing these reactive species (Jiang, et al. 2011).

NOX are protein complexes, they have a catalytic trans-membrane subunit with regulatory proteins in the membrane and the cytosol. There are different subunits each form is different and has its own ROS product causing different dysfunctions. For example NOX 1 produces mainly superoxide and is responsible for endothelial dysfunction. NOX 4, which is the most distributed form, produces mainly hydrogen peroxide and is responsible for pressure overload to the heart, heart failure, and angiogenesis (Altenhöfer, et al. 2012).

NADPH oxidase can be activated by cellular stress including chemical factors, physical factors, changes in intracellular environment, inflammatory factors, and membrane lipid metabolites (Jiang, et al. 2011). Increased level of glucose and free fatty acids can activate NADPH oxidase complexes and this is shown to be mediated by PKC activation as well as AGEs. Another strong activator of the NOX family is Angiotensin II particularly in vascular cells associating it with hypertension and diabetic complications (Gray and Jandeleit-Dahm 2014; Rosen, et al. 2001).

NOX derived ROS are associated with vascular diabetic complications through nitric oxide inactivation, kinase activation, growth and cell adhesion, endothelial cell

proliferation and migration, activation of transcription factors, and neointimal formation. Angiotensin II activated NOX in the heart is associated with cardiac hypertrophy, fibrosis, and heart failure through Akt and MAPK activation (Bedard and Krause 2007).

## **D- Cardiovascular Complications in Diabetic Patients**

It is established that diabetes is one of the leading causes of death, this occurs mainly from cardiovascular diseases associated with diabetes (Schmidt 2014). Diabetic patients can suffer from many different cardiovascular complications including cardiomyopathy and vascular dysfunction (Shamhart, et al. 2009).

### ***1- Cardiomyopathy***

Cardiomyopathy is a very common early marker of diabetic complications (Miki, et al. 2013). It is characterized by morphological changes in the ventricular muscle as well as a decrease in the activity of the myocardium eventually leading to ventricular contractile dysfunction and diastolic dysfunction (Cagalinec, et al. 2013; Taye, et al. 2013).

Morphological changes affect the left ventricular myocytes as early as one week causing a significant decrease in their length and width observed using 3D confocal imaging (Cagalinec, et al. 2013); this is indicative of misarranged heart structure (Dyntar, et al. 2006). In addition to morphological changes, the sensitivity of cardiac myocytes to calcium was shown to be decreased as evidenced by a prolonged action potential duration and a slow decay of calcium transient; both these factors add up causing impairment in heart contractility (Pereira, et al. 2006).

Hypertrophy is another marker of cardiomyopathy and is generally accompanied by metabolic remodeling (Kolwicz, et al. 2013). A decrease in phosphocreatinine, the heart's energy reserve, is observed as well as a decrease in the ATP production from oxidative metabolism driving the heart to get its energy from fatty acid oxidation thus accumulating lipids in the heart (Liao R, et al. 1996). This metabolic profile causes energy deprivation, reduced contractility, and reduced efficiency ending in heart failure (Kolwicz, et al. 2013).

Myocardial fibrosis can also affect a diabetic heart and impair its performance by causing stiffness to the ventricle. This is accompanied by collagen deposition and accumulation in the extracellular matrix exacerbating the myocardial stiffness (Zhao, et al. 2014). Fibroblasts are a type of cells found in the heart that provide structural support. In pathologies such as diabetes, fibroblasts are differentiated into myofibroblasts (Kim, et al. 2014); these myofibroblasts can cause remodeling by forming fibronectin (FN) and increasing collagen (CGN), which is necessary for healing the diabetically-induced stressed heart (Bruder, et al. 2010). Myofibroblasts should undergo apoptosis after completing their function, however due to high glucose in diabetes this does not occur, instead there is a persistent fibrotic inflammatory state causing scar tissue (Fowlkes, et al. 2013). Protein markers for the differentiation from fibroblast into the stressed myofibroblast include alpha-smooth muscle actin ( $\alpha$ -SMA) that are indicative of an active myofibroblast. Levels of  $\alpha$ -SMA, FN, and CGN have been shown to be increased in animal models with diabetes by collagen gel test (Fowlkes, et al. 2013), gel electrophoresis of PCR products (Kim, et al. 2014), and Western blots (Mohamed, et al. 2013).



Diastolic dysfunction is the earliest known sign for cardiomyopathy (Schannwell, et al. 2002), where streptozotocin induced diabetic rats exhibited diastolic dysfunction after 4 weeks (Litwin, et al. 1990). This occurs when the myosin heavy chain (MHC), a contractile part of the heart muscle sarcomere, switches isoforms from the adult  $\alpha$ -MHC to the fetal  $\beta$ -MHC form. MHC determines the speed of cardiac contractions since it determines the rate of cross-bridging, after switching isoforms  $\beta$ -MHC form displays slow cross bridge kinetics and prolong the cross bridge attachment time contributing to diastolic dysfunction (Chung, et al. 2014; Rundell, et al. 2004). This isoform switch has been documented by many studies which performed Western blot analyses as well as PCR analyses showing a significant decrease in  $\alpha$ -MHC and an increase in  $\beta$ -MHC (Giger, et al. 2007; Maalouf, et al. 2012).

## ***2- Vascular dysfunction***

Vascular calcification and collagen deposition comprise part of the vascular dysfunction since they lead to impaired vascular compliance. Diabetes is associated with an increase in ROS production, and in turn it activates the hexosamine pathway that is responsible for O-GlcNAcylation of AKT at the phosphorylation site promoting vascular smooth muscle cell calcification. Echocardiography analysis showed an increase in pulse wave activity indicating aortic stiffness at 16 weeks after streptozotocin injection (Heath, et al. 2014). ROS can also activate the AGE/RAGE mechanism and AGEs can interact with collagen making it resist turnover causing stiffness to the vascular system (Miki, et al. 2013). RAGEs are located in the vasculature next to areas that produce AGE and when they bind to their receptor they can cause an engorged endothelium, disarranged elastic fibers, and disorganized smooth muscle cells that can be visualized by H&E histological stain (Xiang, et al. 2012).

Hyperglycemia can impair endothelium dependent relaxation, and this is hypothesized to be mainly mediated by uncoupling of endothelial nitric oxide synthase (eNOS) decreasing the availability of nitric oxide (NO) (Dikalova, et al. 2010). eNOS in normal conditions produces NO which is responsible for endothelium-dependent vasodilation, and has a role in vascular smooth muscle cell differentiation and platelet aggregation (Griendling and FitzGerald 2003). Substances that induce vasodilation will interact with endothelial receptors causing intracellular calcium influx which activates eNOS causing it to convert L-arginine to L-citrulline thus forming NO. NO will then diffuse into the vascular smooth muscle cells activate cyclic GMP and cause vasodilation (Moncada and Higgs 1993). The superoxide form of ROS can interact with NO forming peroxynitrate, and the latter oxidizes tetrahydrobiopterin (BH<sub>4</sub>) reducing its availability. BH<sub>4</sub> is essential for the activity of eNOS and without it eNOS becomes uncoupled; thus, instead of forming NO it transfers electrons to molecular oxygen forming more superoxide (Guzik, et al. 2002). The loss of NO contributes to the development of atherosclerosis since the beneficial functions of NO such as inhibiting leukocyte adhesion, inhibiting platelet aggregation, anti-atherogenic properties, and the inhibition of vascular smooth muscle cell proliferation are lost (Williams, et al. 1998). ROS dependent endothelial vasodilation impairment was validated when rats overexpressing NOX had an impaired response to acetylcholine induced relaxation. Also by Western blot, eNOS dimers were shown to be reduced indicating possible uncoupling (Dikalova, et al. 2010).

## **E- Arachidonic Acid Metabolites of Cytochrome P450**

Arachidonic Acid (AA) is known to be metabolized by cyclooxygenases and lipoxygenases into prostaglandins, thromboxanes, and leukotrienes; all of which have essential roles in regulating the vascular tone, inflammatory response, as well as renal and pulmonary function (Funk 2001; Roman 2002). However, a third metabolic pathway for AA was first described in 1981 by Capdevilla who reported that AA can be metabolized by cytochrome P450 enzymes (CYP) in the liver and kidney forming hydroxyeicosatetraenoic acids (HETEs) and epoxyeicosatrienoic acids (EETs) (J. Capdevilla, et al. 1982; J. Capdevilla, et al. 1981); the latter can be further metabolized into dihydroxyeicosatrienoic acids (DiHETEs) (Roman 2002). CYP enzymes can metabolize AA into different eicosanoids according to the type of tissue, CYP isozyme, and species (Kroetz and Xu 2005). For example in the cardiovascular system, CYP1A1 can be found in the heart, endothelial cells, and vascular smooth muscle cells (VSMC) and can synthesize HETE; while CYP2B1 is found only in endothelial cells and forms EET (Elbekai and El-Kadi 2006).

The expression and activity of CYP enzymes can be altered in pathophysiological conditions such as diabetes, and an increase in total CYP was measured spectrally and by Western blot analysis in hepatic microsomes of diabetic rats (John B. Schenkman, et al. 1989). AA metabolized by CYP enzymes will mainly form EETs, DiHETEs, and 20-HETEs in kidneys, blood vessels, and the heart (Roman 2002). These products have been implicated as key players in hypertension since they play an important role in modulating the vascular tone with other functions including the control of cellular proliferation and inflammation (Kroetz and Zeldin 2002), however their role in diabetes is not fully studied in all tissues.

### ***1- 20-HETE and EET Formation***

CYP4A is the major enzyme that catalyzes hydroxylation of AA forming 20-HETE in rats, in humans CYP4A11 and CYP4F2 are responsible for 20-HETE formation. As for EETs, they can be formed by many CYPs that can cause epoxidation of AA, but the major enzyme in rats is CYP2C11 and in humans is CYP2J2 (Roman 2002). Spectral assessment and immunoblotting showed an increase in the formation of CYP4A1/A2/A3 in diabetic rats from 57.7 pmol/mg to 128.2 pmol/mg and a decrease in CYP2C11 isoform from 404 pmol/mg to 157 pmol/mg after 3 weeks of diabetic induction (Barnett, et al. 1990; Shimojo, et al. 1993). EETs can be metabolized by soluble epoxide hydrolase (sEH) into DiHETE which have similar EET vasodilating activity, but the binding affinity in tissues is 20-fold greater for EETS than for DiHETE. All these eicosanoids undergo final metabolism by conjugation with glutathione s-transferase, oxidation by cytochrome P450, cyclooxygenases, and by esterification into glycerophospholipids (Kroetz and Zeldin 2002).

### ***2- 20-HETE and EET Function in Vasculature***

Diabetes mellitus is associated with an impaired vascular reactivity, where there is an enhanced vascular response to vasoconstriction and a decreased vascular response to vasodilation. Cytochrome P450 metabolites of AA have been implicated to mediate this dysfunction. EETs and 20-HETEs have opposing effects on the vasculature where EET is a potent vasodilator and 20-HETE is a potent vasoconstrictor (Kroetz and Zeldin 2002).

There are studies that show that EET is an endothelial derived hyperpolarizing factor (EDHF) that is responsible for relaxing vascular smooth muscle cells (VSMC), they can be produced in response to certain agonists such as bradykinin or acetylcholine

(Campbell and Fleming 2010). EETs open large conductance calcium-activated potassium channels thus hyperpolarizing the membrane and causing vasodilation (Pratt, et al. 2001). CYP2C11 inhibitors attenuated the EDHF effect of EETs thus further confirming its role as a vasodilator (Fleming 2000). In addition, EETs have an anti-inflammatory role since they inhibit cytokine induced endothelial cell adhesion molecule expansion and prevents leukocyte adhesion to the vascular wall (Campbell 2000).

In Sprague-Dawley rats that develop vascular dysfunction in pathologies such as hypertension and diabetes, Wang et al showed an upregulation of CYP4A isoform, that is responsible for 20-HETE production, on both mRNA and protein levels in their arteries (Wang, et al. 2005). Studies performed on rat preglomerular microvessels indicated that 20-HETE mediates angiotensin II and endothelin stimulated vasoconstriction. 20-HETE inhibit the opening of calcium activated potassium channel leading to membrane depolarization, it also enhances calcium entry through voltage-sensitive calcium channels causing vasoconstriction (Fan, et al. 2013). 20-HETE mediated vasoconstriction is implicated in hypertension since studies in which CYP4A1 anti-sense oligonucleotide was administered to spontaneously hypertensive rats showed a decrease in 20-HETE amount with a decrease in mean arterial pressure as well (Wang, et al. 2001). Another mechanism of action for 20-HETE mediated constriction is thought to be through activating PKC because experiments performed on cat cerebral VSMC and rat renal VSMC prove that inhibition of PKC will block the vasoconstrictive properties of 20-HETE (Lange, et al. 1997; Sun, et al. 1993).

Another interaction that occurs in vasculature is between 20-HETE and nitric oxide (NO). In physiological conditions NO can bind to heme containing enzyme, so it

was hypothesized that NO can bind to the heme group of CYP4A and inhibit 20-HETE formation, which will then help NO activate calcium-dependent potassium channels leading to vasodilation. This was tested by incubating AA with NO donors and upon increasing the concentration of NO donor the levels of 20-HETE production was decreased (Magdalena Alonso-Galicia, et al. 1997). In certain pathologies such as hypertension or diabetes, levels of CYP4A are increased above normal causing an abnormal increase in 20-HETE formation. This will cause eNOS uncoupling, which was measured by Western blot analysis, and decreased NO availability, measured by a quantification kit (Cheng, et al. 2010). Inhibition of 20-HETE resulted in an enhanced NO mediated vasodilation.

### ***3- 20-HETE and EET Function in the Heart***

Diabetic cardiomyopathy is one of the complications that can be fatal for diabetic patients, however its underlying mechanisms are not fully understood (Ma, et al. 2013). Gene expression in cultured myocytes from rat heart indicated an expression of CYP2C11, which produces EETs, as well as CYP4A1 and CYP4A3, which produce 20-HETE (Thum and Borlak 2000). Yousif et al studied the effect of CYP mediated AA metabolites on cardiac dysfunction in diabetic rats after ischemia/reperfusion. In their study the levels of 20-HETE were increased after ischemia/reperfusion. Administering a 20-HETE inhibitor (HET0016) or a sEH inhibitor (CDU), which would inhibit the metabolism of EETs, proved to be cardioprotective through decreasing the size of the infarct, a decrease in DNA fragmentation, and reduced TUNEL-positive cells which indicates less apoptosis (Yousif, et al. 2009).

Cardiac hypertrophy is a sign of cardiomyopathy that can lead to heart failure. It has been reported that the levels of 20-HETE were increased while EET levels were

decreased in diabetes mediated cardiac hypertrophy, and that inhibiting 20-HETE formation or inhibiting EET metabolism would be protective (Alsaad, et al. 2013). These findings were consistent with those of Zordoky et al who indicated a decrease in CYP2C11 accompanied by an increase in CYP4A1 and CYP4A3 in the heart of rats with cardiac hypertrophy induced by isoprotrenol (Zordoky, et al. 2008). Nuclear Factor kappa B (NF<sub>κ</sub>B) can cause hypertrophy and an increase in the size of cardiomyocytes when activated. It is also responsible for the activation of proinflammatory cytokines (Alsaad, et al. 2013). 20-HETE activates NF<sub>κ</sub>B in endothelial cells and cardiac myocytes, while EETs inhibit it (Ishizuka, et al. 2008; Node, et al. 1999). Mitogen activated protein kinase (MAPK) pathway can cause cell proliferation and apoptosis, and can change cellular functions of cardiomyocytes (Vatner, et al. 2000). It can also be associated with contractile dysfunction, ventricular dilation, and cardiac remodeling (See, et al. 2004). 20-HETE has been shown to activate this pathway and as such was implicated in its detrimental effects on the heart (Akbulut, et al. 2009). 20-HETE is also responsible for activating metalloproteinases which are enzymes responsible for matrix remodeling (Wei Yu, et al. 2011); on the other hand these enzymes are inhibited by EETs (Moshal, et al. 2008).

Cardiac contractility can also be affected in diabetes, where too much calcium can accumulate intracellularly impairing the heart's ability to relax. An sEH inhibitor was given to type 2 diabetes rat model, and by Western blot analysis the downregulation of the sarcoplasmic reticulum Ca<sup>2+</sup> ATPase was attenuated when compared to untreated diabetic rats. This shows better Ca<sup>2+</sup> reuptake when the levels of EET are maintained in cardiac tissue (Guglielmino, et al. 2012).

All of these data indicate a protective role for EETs in the heart and a harmful one for 20-HETE. That is why sEH inhibitors to maintain EET levels and 20-HETE inhibitors to decrease its levels, are of therapeutic interest. One of the 20-HETE production inhibitors, N-Hydroxy-N'-(4-butyl-2-methylphenyl)formamidine, or HET0016, has gained popularity since it is a selective, non-competitive and irreversible inhibitor of CYP4A isoform (Seki, et al. 2005).

#### **F- RAS Pathway and Diabetic Complications**

The renin-angiotensin system (RAS) is a very complicated system, and it is thought to play a role in mediating diabetes induced cardiovascular complications (Gray and Jandeleit-Dahm 2014). It was initially associated with systemic blood pressure regulation and hypertension since it increases sodium and fluid retention, as well as causing vasoconstriction (Luther and Brown 2011). This system is activated when renin is produced by the kidney, it is triggered by low sodium delivery to the macula densa cells, low blood pressure, as well as an activated sympathetic nervous system. Renin then cleaves angiotensinogen, secreted by the liver, into angiotensin I which is the inactive form. Angiotensin I is subsequently cleaved by angiotensin converting enzyme (ACE) into angiotensin II (AngII), the main effector molecule of the RAS system. There are at least two specific AngII receptors, angiotensin type 1 (AT1) and angiotensin type II (AT2), which seem to display opposite activities with AT1 causing vasoconstriction and AT2 causing vasodilation. AT1 receptors generally modulate the effect of AngII in pathological conditions (Forbes and Cooper 2013).

The described system is termed “Classical RAS” and is responsible for systemic circulating AngII levels which is associated mainly in developing the pathological state



of hypertension. A more current view distinguishes between systemic and local RAS, the latter produces AngII intracellularly independent from circulating levels (Kumar, et al. 2012). The differentiation between the two systems is important in case of diabetic complications since studies have shown that under high glucose, intracellular levels of AngII were significantly increased while circulating AngII levels were either reduced or remained normal (Giacchetti, et al. 2005; Singh, et al. 2007). The AT1 receptor was also found to be up regulated in diabetic hearts (Miki, et al. 2013).

### ***1- Angiotensin II in Diabetic Cardiovascular Disease***

AngII is responsible for activating several mechanisms that can cause diabetic cardiovascular complications (Kumar, et al. 2012). Through AT1, AngII will activate NADPH oxidases increasing cellular ROS production (Griendling, et al. 2000). In vasculature, ROS production in vascular smooth muscle cells is mediated by NOX1 (Lassègue, et al. 2001); while in the heart, it is through NOX2 activation (Hingtgen, et al. 2006). AngII mediated ROS production can cause the uncoupling of eNOS, reducing NO bioavailability, and contributing to endothelial dysfunction (Rajagopalan, et al. 1996). This was shown by Dikalova et al, since acetylcholine-induced endothelium dependent vasodilation, was impaired by an AngII infusion, and this was prevented by using a NOX inhibitor (Dikalova, et al. 2010).

AngII activates phospholipase C producing inositol triphosphate and diacylglycerol which increases intracellular calcium, leading to the contraction of vascular smooth muscle cells as well as cardiac myocytes that will eventually lead to diastolic dysfunction (Masuda, et al. 2012; Yan, et al. 2002). Cardiac hypertrophy, fibroblast proliferation, and collagen deposition can be induced by AngII through

activating MAPK (Izumiya, et al. 2003) as well as by increasing mRNA expression of NF<sub>κ</sub>B by 2-folds (Tadevosyan, et al. 2010).

## ***2- Angiotensin-Converting Enzyme Inhibitor vs. Angiotensin Receptor Blocker***

Inhibiting the RAS pathway at any point can have a helpful effect against AngII mediated dysfunctions. Angiotensin-converting enzyme inhibitors (ACEi) are a group of drugs that inhibit the conversion of angiotensin I, the inactive form, to angiotensin II, the active form. Captopril is the first FDA approved ACEi developed in 1977 for treating essential hypertension (Vukelic and Griendling 2014). Another group of drugs that can inhibit RAS pathway are angiotensin receptor blockers (ARBs) that block AT1 receptor and thus inhibiting the AngII downstream signaling cascade (Zhang, et al. 2008). In treating hypertension ACEi or ARB can be used without any significant difference between the result of either treatment (Kikta and Fregly 1982; Zhang, et al. 2008).

In Diabetes, oxidative stress is increased, administering captopril, an ACEi, to diabetic rats was shown to act as an anti-oxidant by decreasing ROS formation (Araujo, et al. 2011). In addition to that effect, ACEi have been shown to reduce cardiovascular complications by improving endothelial function and reducing cardiac remodeling (Nevelsteen, et al. 2013). ACEi was shown to improve cardiac contractility as well by preventing calcium disorders that can occur (Zoja, et al. 2011)

Both ACEi and ARBs have been used in the treatment of cardiovascular and renal damage in diabetes (Luther and Brown 2011). But when differentiating between local and systemic AngII concentrations and effects, ACEi were shown to have a more beneficial effect when compared to ARBs (Kumar, et al. 2012). A meta-analysis comparing both group of drugs also showed a significant reduction in major

cardiovascular events such as myocardial infarction and heart failure in patients treated with ACEi with no significant changes for ARB treatment (Cheng, et al. 2014).

### **G- Association of Angiotensin and Arachidonic Acid Metabolites**

Similarities in the mode of action of AngII and 20-HETE, a metabolite of CYP4A mediated AA metabolism, led to a hypothesis that these two might be related. Evidence suggests that the activation of AT1 receptors by AngII will phosphorylate and activate phospholipase A<sub>2</sub> which in turn produces AA and its metabolites including 20-HETE and EET. 20-HETE are thought to enhance AngII mediated vasoconstriction by enhancing calcium entry (Griendling, et al. 2000; Sarkis, et al. 2004). At the same time adding 20-HETE to cultured endothelial cells resulted in an upregulation of a couple of genes including ACE whose expression increased 5.76 fold (Imig 2010; Sodhi, et al. 2010). These studies indicated the presence of a positive feedback system between AngII and 20-HETE. Further testing demonstrated a reduced AngII mediated vasoconstriction in response to an inhibitor of 20-HETE formation, validating an integrated role between them (Chu, et al. 2000).

EETs on the other hand are cardioprotective, and in order to prove an association between EET and AngII, a study was performed using EETs as a treatment for AngII mediated cardiac remodeling. Normal mice and transgenic mice expressing CYP2J2, an isoform that produces EETs, were given AngII infusion, Those expressing CYP2J2 had significantly lower cardiac hypertrophy, cardiac dysfunction, and reduced collagen deposition proving an antagonistic link where EETs inhibit the effects of AngII in pathology (He, et al. 2015).

## CHAPTER II

### AIMS OF THE STUDY

From the various studies and literature, we know that in diabetes there are a lot of complications that target various organs. One of the serious complications is its effect on the cardiovascular system, with a very complex mechanism of dysfunction. Cytochrome P450 enzymes are associated with diabetic disorders since different isoforms have altered expression. For instance CYP4A expression is enhanced in diabetes, it is responsible for producing 20-HETE. This eicosanoid has been shown to mediate part of the diabetic pathology. On the other hand the expression of CYP2C, that forms EETs, is decreased. Studies have shown a helpful role for EET in reversing diabetic induced complications. Local tissue angiotensin II was also proved to be increased in diabetes, elucidating a role for it in causing diabetic dysfunctions. Recent studies formed a link between AngII and 20-HETE, which suggested a positive feedback loop between the two.

In this study we examined whether administering HET0016, an inhibitor of CYP4A which inhibits the formation of 20-HETE, will reverse diabetic damage to the cardiovascular system. We also examined whether the beneficial effect of Captopril, an ACE inhibitor, in diabetes may be mediated in part by inhibiting AngII mediated 20-HETE production. So our hypothesis was that by decreasing the levels of angiotensin, captopril would decrease the levels of 20-HETE thus reducing cardiovascular dysfunction in DM. Thus, giving a combined treatment of HET0016 and Captopril may

show better results than either drug on its own. To test the hypothesis, the following aims were put:

- To study the effect of HET0016, Captopril, or both on the expression of CYP4A and CYP2C which directly correlates to the levels of 20-HETE and EET respectively; Western blot and q-PCR were performed to check their protein and mRNA expression.
- To determine the level of 20-HETE present in the plasma of diabetic vs control animals following treatments with HET0016, captopril, and their combination. ELISA test was performed on plasma samples collected at day 28.
- To assess the level of cardiovascular injury; Western blot and q-PCR analysis was done on markers of injury such as  $\alpha$ -SMA,  $\alpha$ -MHC,  $\beta$ -MHC, NOX-1, and NOX-4 in control vs diabetic rats subjected to similar treatments.
- To check the functional vascular response to vasodilating or vasoconstricting stimuli, vascular reactivity study was performed checking the response to phenylephrine, potassium chloride, acetylcholine, and SNAP (NO donor) on the thoracic aorta of each group.
- To test the amount of ROS production in the form of superoxide anion; dihydroethidium stain (DHE) was used on left ventricular tissue sections from different treated groups.
- To visualize cardiovascular remodeling, Masson's Trichrome Stain was done to assess the amount of collagen deposition in the abdominal aorta and the left ventricle.

# CHAPTER III

## MATERIALS AND METHODS

### **A- Animal Model**

We used male Sprague-Dawley rats weighing between 220 and 325 g; and they were divided into 8 groups each group containing 8 rats (n=8). Diabetes was induced in 4 groups by administering a 65mg/Kg intravenous dose, through the tail vein, of Streptozotocin (STZ) dissolved in sodium citrate buffer (0.01M, pH4.5). Control rats were given an equal volume of STZ vehicle which is sodium citrate buffer. Two days later blood glucose levels were measured (AccuCheck Performa, Roche) to verify the induction of diabetes and we started the different treatments.

Group 1 included control non-diabetic rats treated with normal saline as a vehicle (CV). Group 2 was diabetic rats treated with normal saline as vehicle (DV). Group 3 was control non-diabetic rats treated with captopril (CCap), while group 4 was diabetic rats treated with captopril (DCap). Groups 3 and 4 were treated with 50mg/Kg of Captopril by implanting a pump (Alzet osmotic pump model 2ML4) subcutaneously through an incision dorsal to the scapular region. The pump releases a dose of 2.5 $\mu$ L/hr for 28 days. 2 hours before implantation, rats were given an intra-peritoneal injection of 1mg/100g Gentamicin, and they were anaesthetized with a mixture of 75mg/Kg Ketamine and 7.5mg/Kg Xylazine also administered intra-peritoneally. Group 5 included control non-diabetic rats treated with HET0016 (CHet), and group 6 included diabetic rats treated with HET0016 (DHet). Groups 5 and 6 were treated with a daily subcutaneous injection of HET0016 (N-hydroxy-N-(4-butyl-2- methylphenol)

formamidine) in a dose of 2.5mg/kg/day dissolved in 0.1mL 100% DMSO. HET0016 is a specific inhibitor of 20-HETE formation by selectively inhibiting the CYP4A isoforms. Group 7 was control non-diabetic rats treated with both captopril and HET0016 (CComb), while group 8 was diabetic rats treated with both captopril and HET0016 (DComb). Groups 7 and 8 were treated with a combination of both Captopril as a subcutaneous pump, and HET0016 as a daily subcutaneous injection. The different groups and treatments are summarized in Table 1.

The duration of the treatment was 28 days during which the rats had unrestricted access to food and water, were put in a 12 hour light to 12 hour dark cycle, and were maintained in accordance with the recommendations of the Institutional Animal Care and Use Committee of the American University of Beirut. During this time rats were placed in individual metabolic cages for urine collection as well as food and water intake measurements on days 0, 14, and 28. Also, blood glucose levels were measured once per week for control rats and every 3 days for diabetic rats (AccuCheck Performa, Roche).

After 28 days all rats were euthanized and sacrificed, and the heart and both thoracic and abdominal aorta were excised. The heart weight was measured and then part of it and part of the abdominal aorta were kept in 4% formalin for histological analysis. The thoracic aortas were immediately used for vascular reactivity studies. The remaining parts were frozen in liquid nitrogen and stored at -80°C.

<b>Abbreviation</b>	<b>Groups</b>	<b>Treatment</b>	<b>Method</b>
CV	Control	Saline	Pump
DV	Diabetic	Saline	Pump
Ccap	Control	Captopril	Pump
Dcap	Diabetic	Captopril	Pump
Chet	Control	HET0016	SC injection
Dhet	Diabetic	HET0016	SC injection
Ccomb	Control	Captopril + HET0016	Pump + SC injection
Dcomb	Diabetic	Captopril + HET0016	Pump + SC injection

Table. 1 Differentiation of treatment between different groups.

## **B- Vascular Reactivity Study**

On day 28 of the experiment, rats were sacrificed by decapitation and the thoracic aorta was obtained and immediately put in Krebs Henseleit solution (118mM NaCl, 4.7mM KCl, 2.5mM CaCl<sub>2</sub>, 1.2mM MgSO<sub>4</sub>, 25mM NaHCO<sub>3</sub>, 1.2mM KH<sub>2</sub>PO<sub>4</sub>, 11.2mM glucose).

The aorta was cut into five rings each piece is 4-5 mm in length. Using a forceps the inner lining of two of the rings was gently rubbed in order to remove the endothelium; for the remaining three rings the endothelium was maintained.

The aortic rings were placed onto a wire connected to a force displacement transducer to record their tension. They were placed in individual chambers containing Krebs solution, gas equilibrated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>, and maintained at 37°C. These preparations were left to equilibrate then the endothelial integrity was tested by the ability of 10µM acetylcholine to relax the aortic rings after they have been contracted with 1µM phenylephrine. Aortic rings exhibiting  $\geq 70\%$  relaxation were



considered to have an intact endothelium while those exhibiting  $\leq 5\%$  relaxation were considered rings with denuded endothelium.

After washing, the aortic ring were equilibrated in Krebs solution to have equal tension baselines (2g) and then subjected to one of the following:

- Response to increasing concentration of phenylephrine (PE) (0.01 $\mu$ M, 0.05 $\mu$ M, 0.1 $\mu$ M, 0.3 $\mu$ M, 0.5 $\mu$ M, 1 $\mu$ M, 5 $\mu$ M) with endothelium.
- Response to increasing concentration of PE (0.01 $\mu$ M, 0.05 $\mu$ M, 0.1 $\mu$ M, 0.3 $\mu$ M, 0.5 $\mu$ M, 1 $\mu$ M, 5 $\mu$ M) without endothelium.
- Response to increasing concentration of KCl (20mM, 40mM, 60mM, 80mM, 100mM) with endothelium.
- Response to increasing concentration of acetylcholine (Ach) (0.001 $\mu$ M, 0.01 $\mu$ M, 0.05 $\mu$ M, 0.1 $\mu$ M, 0.5 $\mu$ M, 1 $\mu$ M, 5 $\mu$ M, 10 $\mu$ M) after pre-contracting with PE (1 $\mu$ M) with endothelium.
- Response to increasing concentration of SNAP (S-Nitroso-N-acetylpenicillamine) which is a Nitric Oxide donor (0.05 $\mu$ M, 0.1 $\mu$ M, 0.3 $\mu$ M, 0.5 $\mu$ M, 1 $\mu$ M, 5 $\mu$ M, 10 $\mu$ M) after pre-contracting with PE (1 $\mu$ M) without endothelium.

### **C- Western Blot Analysis**

At the day of sacrifice, the heart was collected and then the left ventricle was cut out in addition to the abdominal aorta, flash frozen in liquid nitrogen, and stored at -80°C. Later, part of the tissues was homogenized in liquid nitrogen using a mortar and pestle. The now powdered tissue was added to an eppendorf tube containing RIPA lysis buffer (0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxylate, 300mM sodium

chloride, 100 mM EDTA, 100mM Tris-HCl pH 8, 1% Tergitol (NP-40), 1mM phenylmethanesulfonylfluoride (PMSF), protease inhibitor cocktail, and phosphatase inhibitor cocktail). They were incubated for 2 hours at 4°C and then centrifuged at 14,000 rpm for 30 minutes at 4°C. The supernatant containing the proteins was collected and stored at -20°C. Protein concentration was measured using Lowry Protein Assay for colorimetric quantification using different BSA concentrations for the standard curve.

For immunoblotting, 40µg of proteins for left ventricular tissue and 60 µg of proteins for aortic tissue, were separated on 10% polyacrylamide gel electrophoresis (BioRad Laboratory, CA, USA) at 80 volts for 2 hours. The gel was then transferred to a nitrocellulose membrane (BioRad Laboratory, CA, USA) and blocked with 5% low fat milk dissolved in Tris-Buffered Saline for 1 hours. After that membranes were incubated with one of the following overnight:

- Goat polyclonal anti- $\alpha$ MHC (1:500, Santa Cruz Biotechnology)
- Mouse monoclonal anti- $\beta$ MHC (1:200, Santa Cruz Biotechnology)
- Rabbit polyclonal anti- $\alpha$ SMA (1:1000, Abcam)
- Rabbit polyclonal anti-CYP4A1+A2+A3 (1:2000, Abcam)
- Rabbit polyclonal anti-CYP2C11 (1:2000, Abcam)
- Rabbit polyclonal anti-NOX1 (1:500, Santa Cruz Biotechnology)
- Rabbit polyclonal anti-NOX4 (1:500, Santa Cruz Biotechnology)

The primary antibodies were detected using the appropriate horseradish peroxidase conjugated IgG (1:20,000 for rabbit, 1:10,000 for goat and mouse).

Bands were visualized by enhanced chemi-luminescence ECL (BioRad), and then semi-quantified by densitometric analysis using Image J software.

#### **D- Polymerase Chain Reaction (q-PCR)**

Real time PCR was performed on left ventricle and aortic samples that have been frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ . mRNA was isolated using Trizol reagent (Sigma Aldrich, Steinheim, Germany) then chloroform was added to separate the solution into three phases where the upper aqueous phase contains the RNA. The upper phase is collected and isopropyl alcohol is added, then this mixture is centrifuged at 14,000 rpm for 20 minutes at  $4^{\circ}\text{C}$ . When the centrifugation is done, there will be a white pellet containing the RNA which is then reconstituted in nuclease free water and stored at  $-80^{\circ}\text{C}$ .

The concentration, integrity, and purity of the RNA was checked by nanodrop (ND-1000 spectrophotometer). The RNA was then changed to cDNA using RevertAid First Strand cDNA Synthesis kit. Samples were amplified using Thermal cycler (CFX-384 Touch Real Time PCR detection system, BioRad) with SYBR green and rat primers for  $\alpha$ -MHC,  $\beta$ -MHC,  $\alpha$ -SMA, CYP4A1, CYP2C11, NOX-1, and NOX-4. GAPDH was used as internal reference gene. Results were analyzed using  $\Delta\Delta\text{C}(t)$  method. (Table 2)

Primer	Sequence	Annealing Temperature (°C)
$\alpha$ -MHC	F: AGTGACAGGATGACGGATGC R: TAGGCGCTCCTTCTCTGACT	59.82 60.03
$\beta$ -MHC	F: CCAAGGGCCTGAATGAAGAGT R: CAGGTCTCAGGGCTTCACAG	59.99 60.04
$\alpha$ -SMA	F: GCCATCAGGAACCTCGAGAAG R: GAGCCATTGTCACACACCAG	60.47 59.12
CYP2C11	F: ACAAGGACAATCCTCGGGAC R: AAGGGTAAACTCAGACTGCGG	59.39 60.00
CYP4A1	F: GGCCACTTCGACCCTTTCAA R: CTGCCAAAGCATATGGCACAG	60.54 60.20
NOX-1	F: GCTCCAGACCTCCATTTGACA R: TG TTCAGCCCCAACCAAGAAA	60.00 60.34
NOX-4	F: TTCGGGTGGCTTGTTGAAGT R: TGGGGTCCGGTTAAGACTGA	60.11 60.18

Table 2. Forward and reverse primers used with annealing temperatures

### E- Histological Staining

Upon sacrifice, a piece of the left ventricle and a piece of the abdominal aorta were cut and put in 4% formaldehyde for 48 hours. After that, the tissues were embedded in paraffin blocks and cut into pieces that are 4-5 $\mu$ m in thickness and mounted on glass slides.

Tissues were then stained with Masson's Trichrome stain to visualize any cardiac dysfunction, it is used to assess the amount of collagen deposition in tissues by staining them green.

Tissues were examined under a light microscope, each slide was divided into four sections and five pictures were taken per section. Analysis was performed using Image J software.

#### **F- Enzyme Linked Immunosorbent Assay (ELISA)**

In order to measure the levels of 20-HETE, plasma samples were collected at day 28 and analyzed using a competitive ELISA kit (Detroit R&D, Inc.). Samples are prepared by adding equal part ethyl acetate and collecting the upper organic phase, then the collected liquid is turned into sediment by lyophilization for 3 hours per 1 mL. This is followed by saponification with 20% KOH solution and incubation for 1 hour at 50°C. Extraction with ethyl acetate is performed again collecting the upper phase containing saponified lipids and drying them for the second time with lyophilization.

The acquired sediment is stored at -20°C, it is later dissolved in N,N-dimethyl formamide prior to loading the ELISA plate. We add the plasma samples to a 96 well plate coated with anti-20-HETE followed by 20-HETE-HRP conjugate. 20-HETE present in the plasma will compete with the HRP-conjugates to bind to the antibodies. Upon adding tetra methyl benzidine it bind to the HRP conjugates bound to the wells and results in a change of color read by a spectrophotometer at 450nm, this determines the level of HRP conjugate. The levels of 20-HETE found in the plasma is inversely proportional to the binding of the conjugates. From the standards provided with the kit, a standard curve is drawn from which the concentration values can be extrapolated.

### **G- Immunohistochemistry DHE stain for ROS production**

Left Ventricle tissue, which was frozen directly after sacrifice on day 28, was cut into pieces 4-5µm in thickness and mounted on glass slides. DHE stain (dihydroethidium) is used to determine the amount of Reactive Oxygen Species (ROS) present in the tissues since it interacts with superoxide anions to form a fluophore.

DHE was diluted with PBS 1x to reach a concentration of 10µmol/L of which 40µL were added to one section on the slide and incubated in the dark at room temperature for 30 minutes. Using a confocal microscope, the ethidium bromide fluorescence can be detected at excitation wavelength of 488nm and an emission wavelength of 560nm. The superoxide anions will be visualized as red fluorescence and quantification of the fluorescence is done using Image J software.

### **H- Statistical Analysis**

Results are represented as Mean ± Standard Error Mean. Statistical significance is determined using student's unpaired t-test. P-value <0.05 is considered as statistically significant. When comparing control groups vs diabetic groups of any treatment (\*) is used when p<0.05. And when comparing DV to diabetic treated groups (#) is used when p<0.05.

## CHAPTER IV

### RESULTS

#### **A- Blood Glucose Levels**

On day 0 there was no difference in blood glucose levels between the different groups. Two days after STZ / normal saline injections, glucose levels were measured to prove rats injected with STZ developed hyperglycemia. All diabetic rats injected with STZ showed a significant increase in glucose levels on day 2 compared to day 0 and compared to control groups injected with saline that showed no change in glucose. This hyperglycemia was maintained in diabetic rats of all groups throughout the study, while control rats showed no significant changes. (Figure 1)

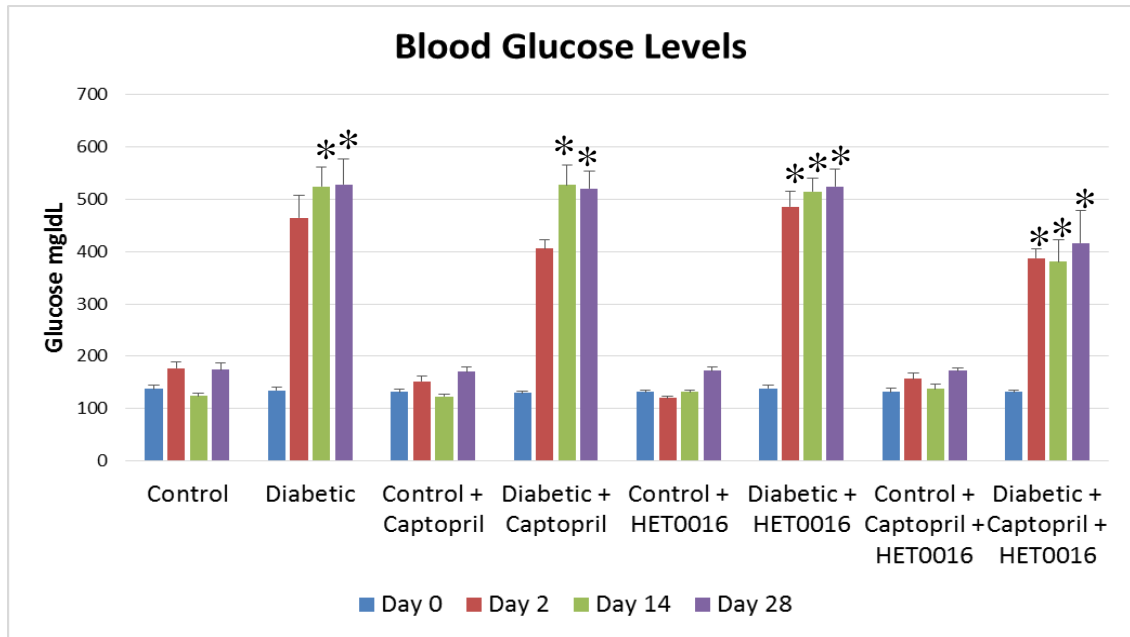


Figure. 1 Blood glucose levels at d0, d2, d14, and d28 after administration of streptozotocin or its vehicle in 8 groups of rats treated: HET0016, Captopril, or their combination.

\* is used for  $p < 0.05$  when comparing Control vs Diabetic groups receiving the same treatment.

## B- Heart and Body Weight Changes

When comparing the four control non-diabetic groups (CV, Ccap, Chet, Ccomb), there was no significant difference in their body weight. Body weight of diabetic rats were significantly lower when compared to the controls receiving similar treatment except for treatment with combination that did not exhibit any change when comparing Ccomb to Dcomb. However there was a significant increase in body weight when comparing diabetic untreated rats with diabetic treated with either captopril or the combination. Diabetic rats treated with HET0016 did show a different weight change compared to diabetic untreated.

As for heart weight, it was significantly lower in DV at day 28 when compared to CV. In the diabetic groups treated with HET0016, captopril, and the combination,



heart weight was significantly higher than that in DV and was almost similar among groups. There was no change in heart weight when comparing the control groups. Heart weight to body weight ratio is an index of cardiac hypertrophy; no significance was observed among the groups however there is a general tendency for the ratio to increase with diabetes when compared to controls (Table 3).

Groups at day 28	Heart weight (g)	Body weight (g)	H.W/B.W (mg/g)
Control Vehicle	1.33 ± 0.05	432.63 ± 14.85	3.07 ± 0.05
Diabetic Vehicle	0.88 ± 0.04 *	281 ± 12.4 *	3.14 ± 0.05
Control + Captopril	1.2 ± 0.01	410.5 ± 6.62	2.92 ± 0.06
Diabetic + Captopril	1 ± 0.04 #	318.44 ± 11.15 #*	3.13 ± 0.05
Control + HET0016	1.2 ± 0.04	390.56 ± 13.98	3.08 ± 0.03
Diabetic + HET0016	1.05 ± 0.07 #	299.2 ± 16.55 *	3.42 ± 0.14
Control + Captopril + HET0016	1.1 ± 0.06	388.75 ± 15.61	2.78 ± 0.07
Diabetic + Captopril + HET0016	1.08 ± 0.02 #	358.8 ± 10.38 #^	3.03 ± 0.05

Table 3. Comparison between heart weight, body weight, and the ratio of heart weight to body weight which is a marker for cardiac hypertrophy at day 28. (n=8)

\* is used if  $p < 0.05$  when comparing controls and diabetic receiving same treatment.

# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated.

^ is used if  $p < 0.05$  when comparing diabetic treated with both captopril and HET0016 to diabetics receiving only one of the treatments

### C- Collagen deposition in the Extracellular Matrix

Masson's Trichrome is a stain that determines collagen deposition in tissues by staining them green. In both tissues, the abdominal aorta and the left ventricle, collagen deposition was significantly increased in DV when compared to CV. This increase was prevented in diabetic groups treated with captopril or treated with a combination of captopril and HET0016 in both tissues. However diabetic rats treated with HET0016 (Dhet) by itself showed no significant change in collagen deposition in the aorta when compared to DV, at the same time there was no significant increase in collagen when

compared to Chet. But in the left ventricle tissue Dhet rats showed a decreased level of collagen compared to DV just as much as was observed with the other treatments.

(Figure 2a,b) (Figure 3a,b)

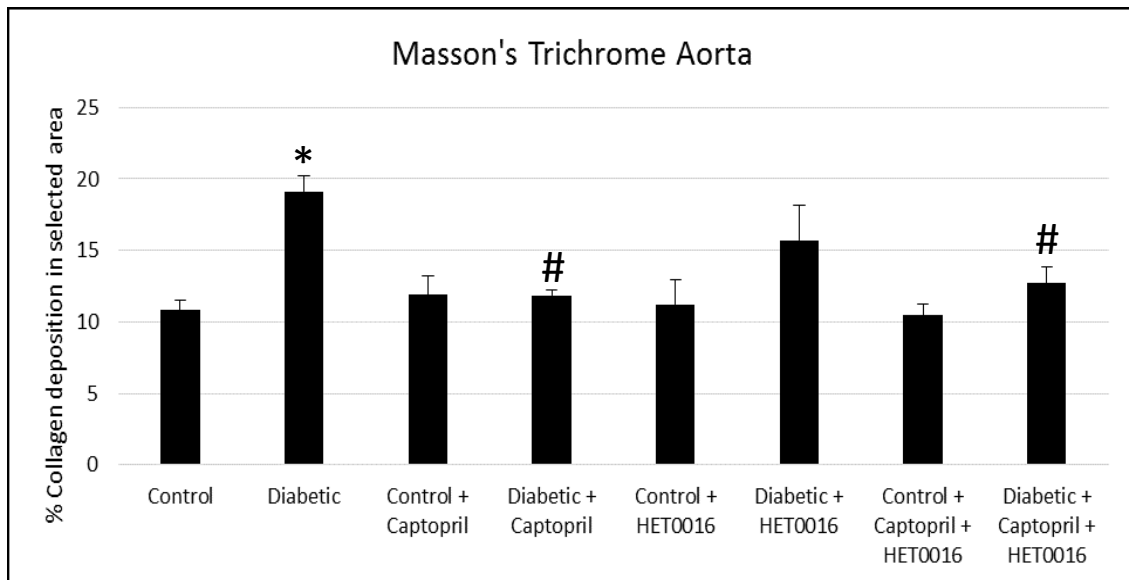


Figure 2a. Quantification of Masson's Trichrome stain in Abdominal Aorta after 28 days of treatment with Captopril, HET0016, and a combination of both drugs. (n=8)  
\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.  
# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated.

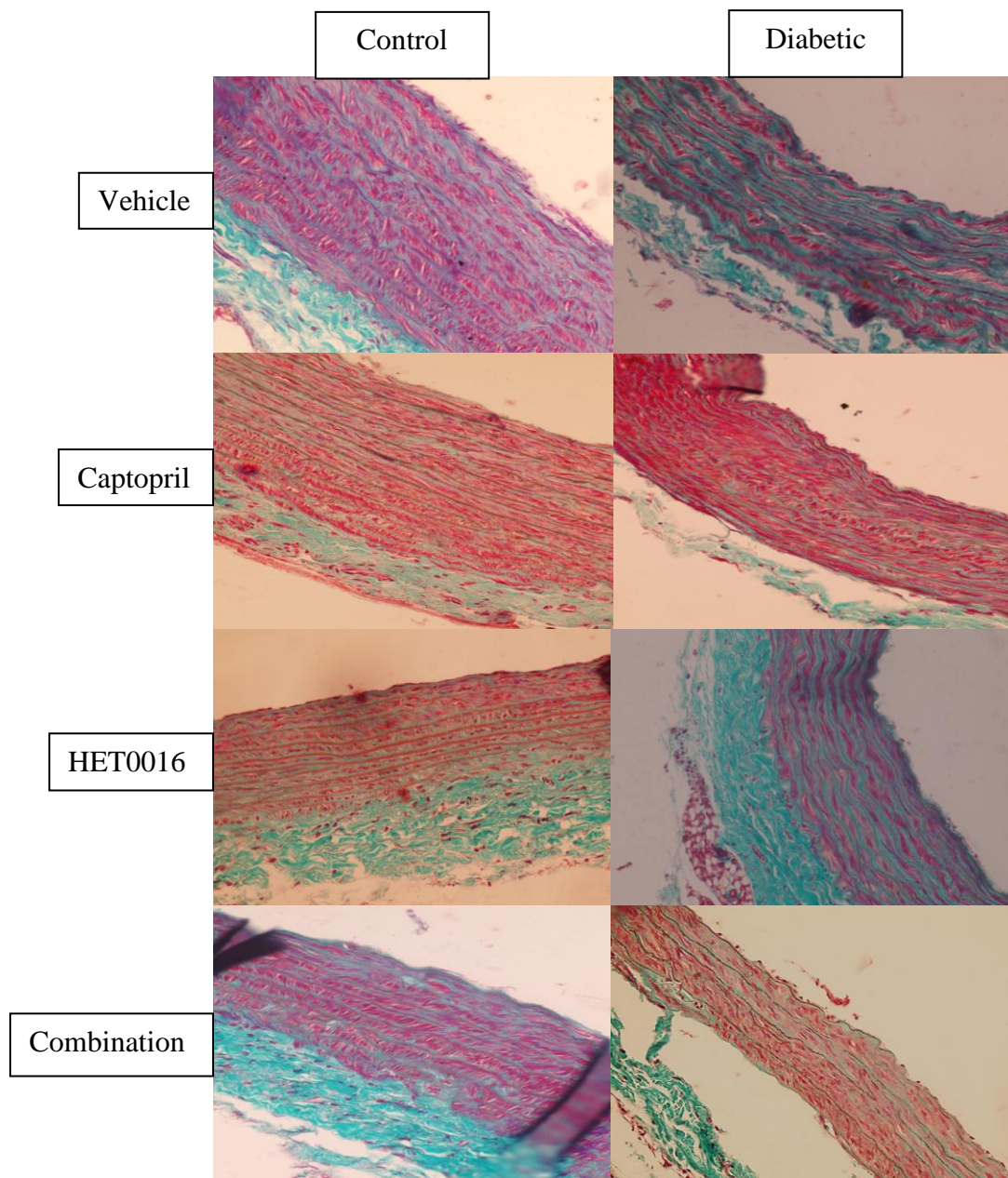


Figure 2b. Histological staining using Masson's Trichrome method on Abdominal Aorta and visualized using a light microscope. Green color depicts collagen deposition. (n=8)

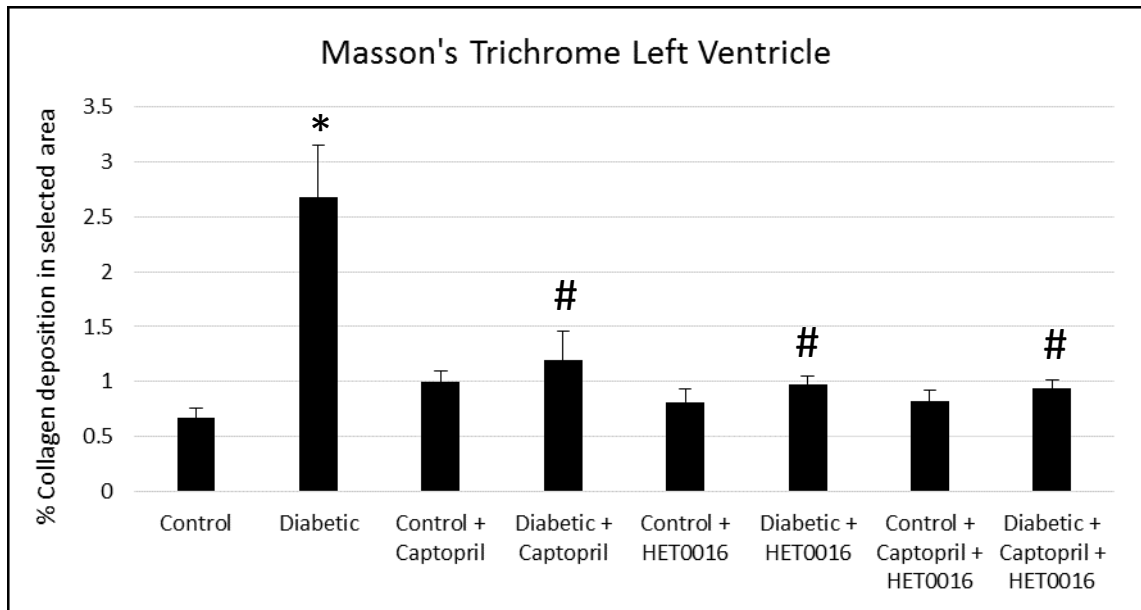


Figure 3a. Quantification of Masson's Trichrome stain in Left Ventricle after 28 days of treatment with Captopril, HET0016, and a combination of both drugs. (n=8)

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated.

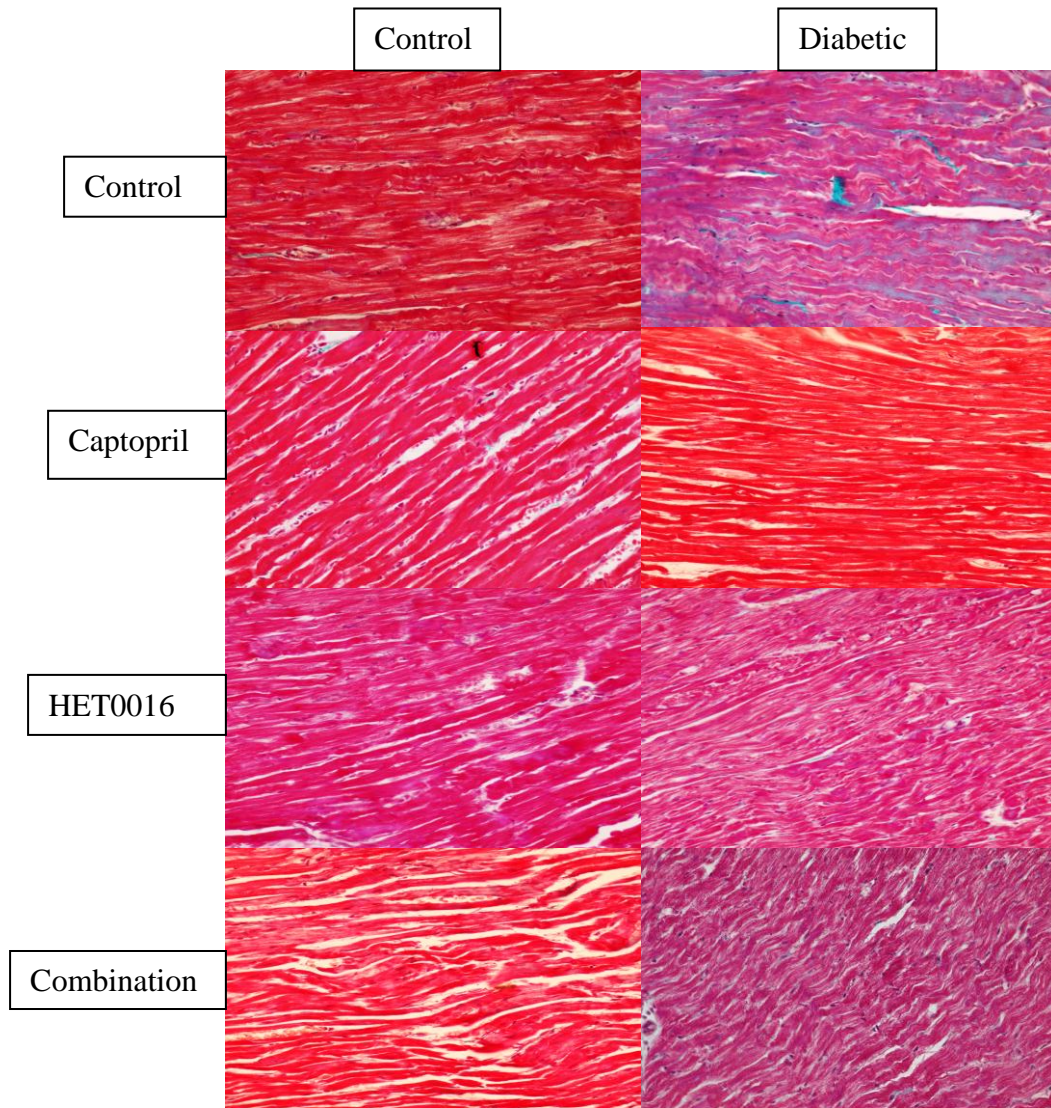


Figure 3b. Histological staining using Masson's Trichrome method on Left Ventricle and visualized using a light microscope. Green color depicts collagen deposition. (n=8)

#### **D- Levels of CYP4A1/A2/A3 and CYP2C11 in Abdominal Aorta and Left Ventricle**

Expression of CYP4A1/A2/A3 that produces 20-HETE as well as expression of CYP2C11 that produces EET was studied at a protein level by Western blot and at an mRNA level by q-PCR.

CYP4A1/A2/A3 levels were significantly elevated in DV when compared to CV in the aorta and left ventricle on both protein and mRNA level. Both tissues also showed that the increase in CYP4A1/A2/A3 was significantly less in Dhet and Dcomb groups when compared to DV on protein and mRNA levels.

However diabetic rats treated with captopril (Dcap) displayed discrepancies among the results. In the aorta, on a protein level Dcap did decrease CYP4A1/A2/A3 expression but not to a significant level. For mRNA expression in aortic tissue, Dcap decreased CYP4A1 significantly but not as effectively as other treatments since it was still significantly higher than Ccap. In the left ventricle, Western blot results showed that Dcap did not decrease CYP4A1/A2/A3 expression, and q-PCR results showed that Dcap slightly reduced CYP4A1 expression but not significantly.

No change was observed in either protein or mRNA expression for CYP4A1/A2/A3 in aorta or ventricle for control groups. (Figure 4a,b)(Figure 5a,b)

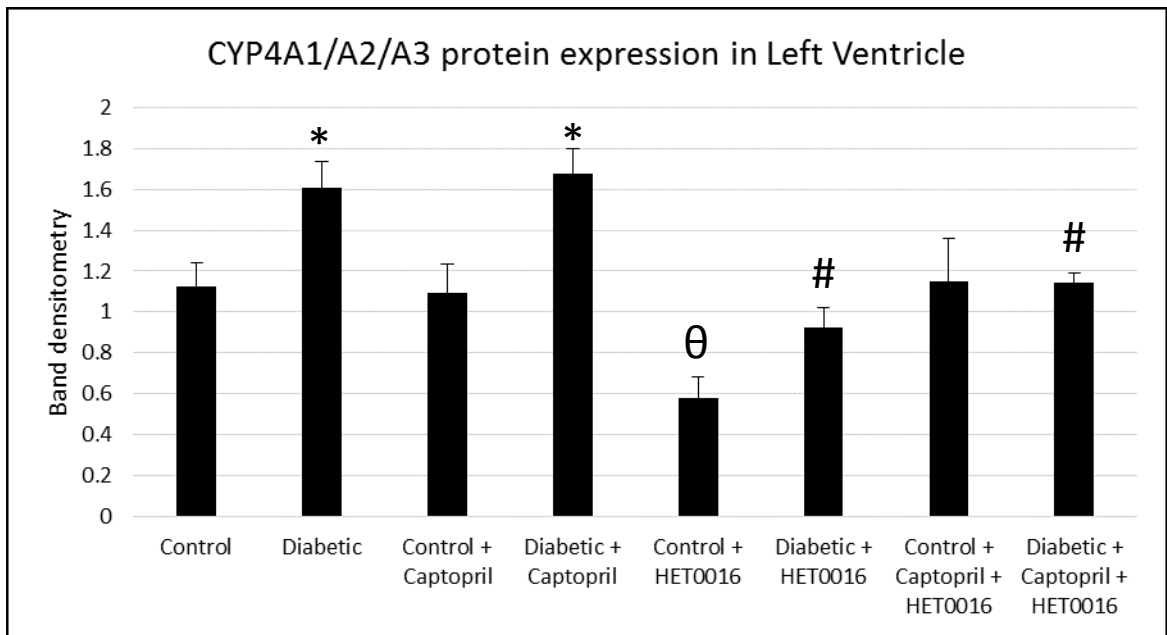
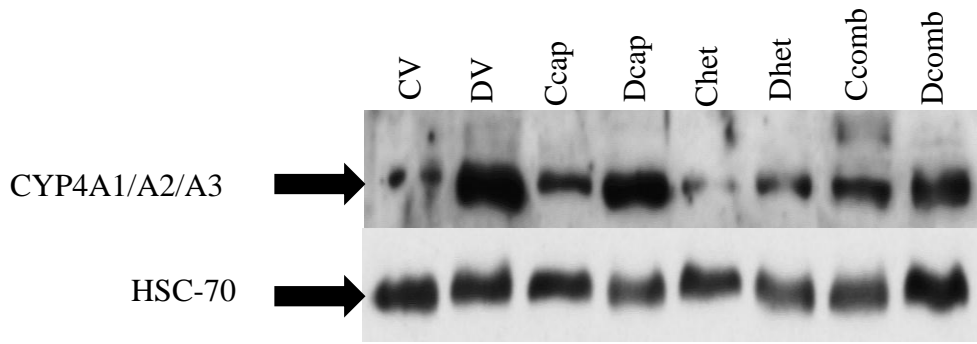


Figure 4a. Western blot analysis for protein expression of CYP4A1/A2/A3 in Left Ventricle at day 28. (n=8)

Treatments: Vehicle (V), Captopril (cap), HET0016 (het), and combination of drugs (comb).

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment

# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated

Θ is used if  $p < 0.05$  when comparing control non-treated to controls treated

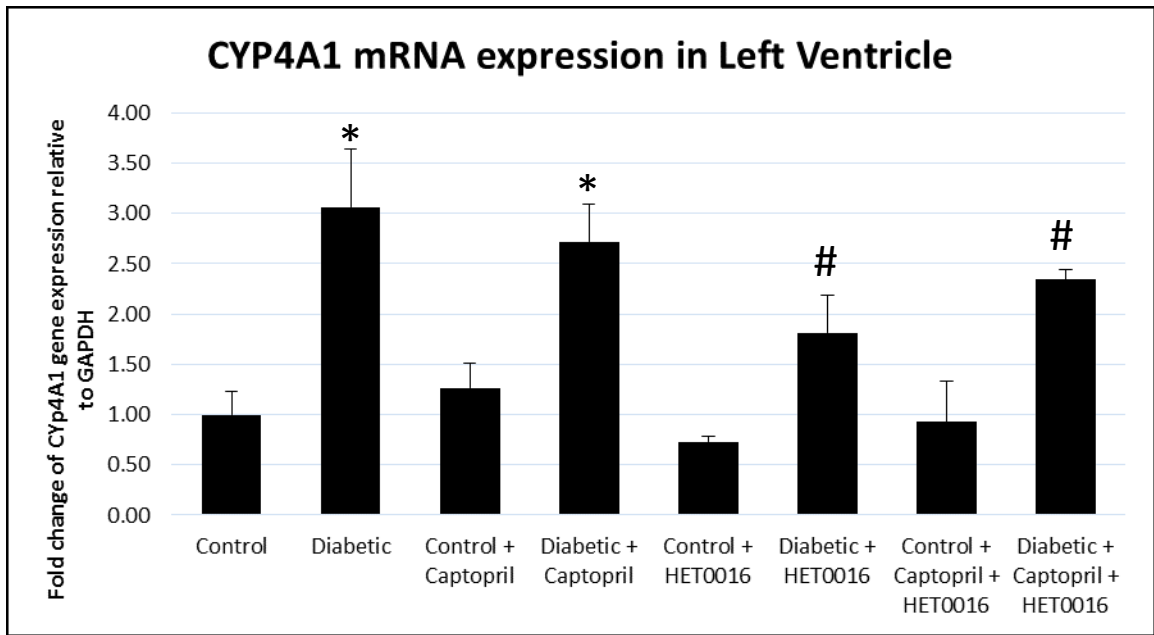


Figure 4b. q-PCR results for mRNA expression of CYP4A1 in Left Ventricle at day 28 of the treatments with Captopril, HET0016, and a combination of both drugs. (n=6)  
 \* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment  
 # is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated.



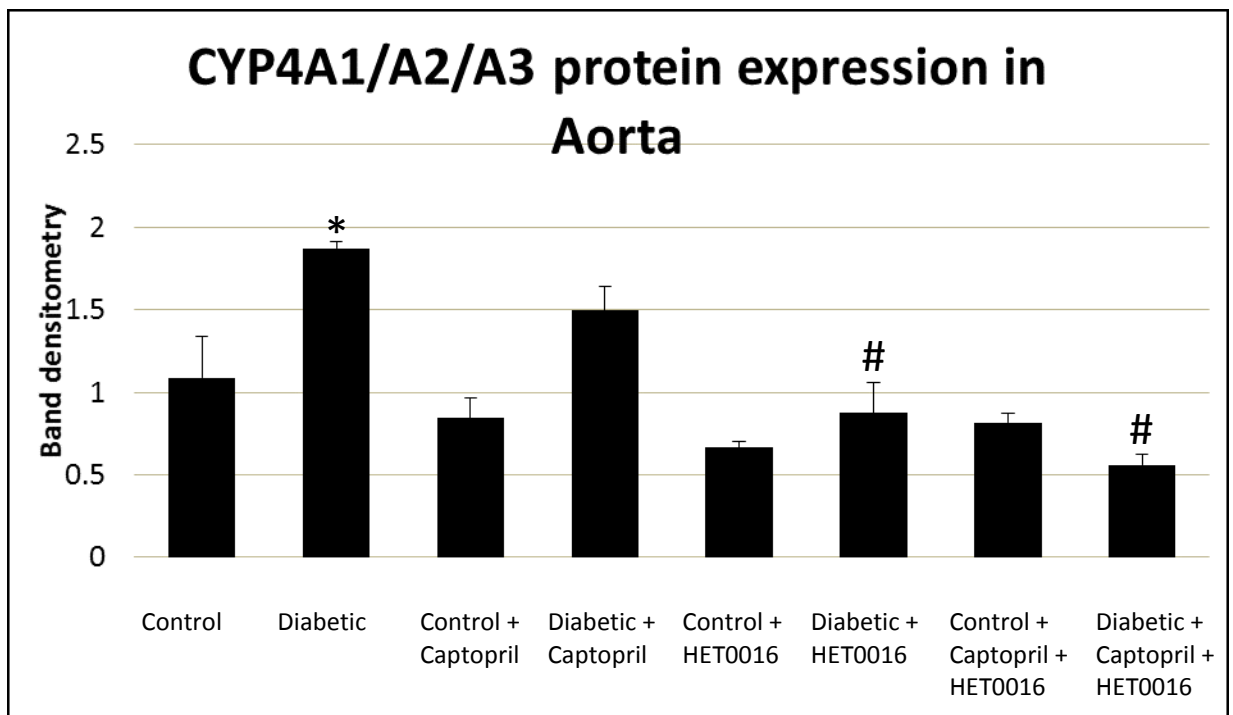
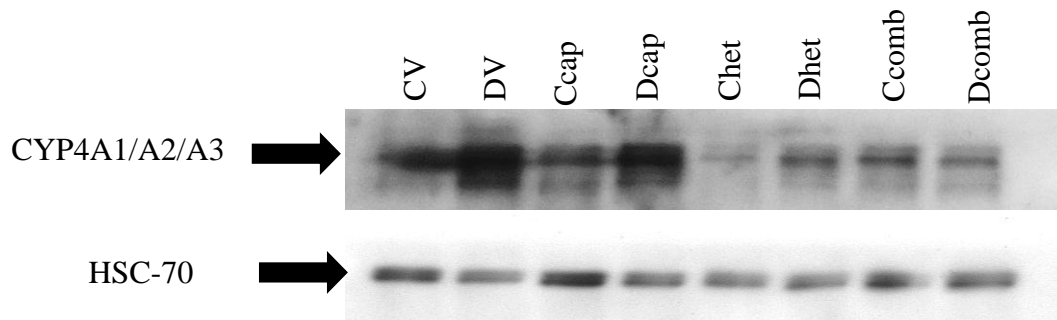


Figure 5a. Western blot analysis for protein expression of CYP4A1/A2/A3 in Abdominal Aorta at day 28. (n=8)

Treatments: Vehicle (V), Captopril (cap), HET0016 (het), and combination of drugs (comb).

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment

# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated.

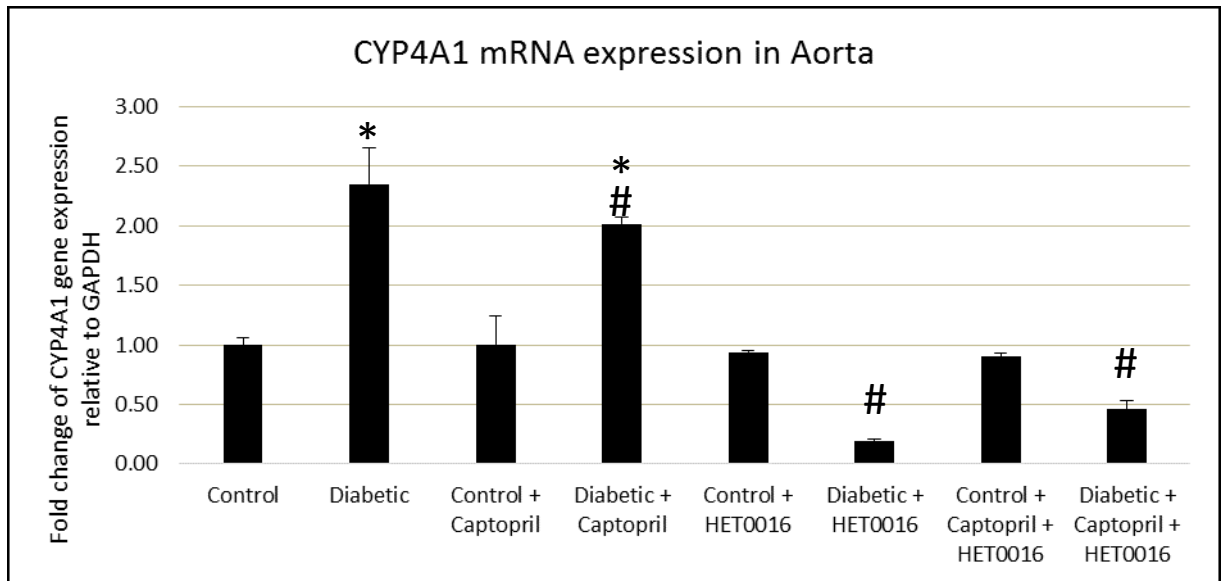


Figure 5b. q-PCR results for mRNA expression of CYP4A1 in Abdominal Aorta at day 28 of the treatments with Captopril, HET0016, and a combination of both drugs. (n=6)  
 \* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatments.  
 # is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated

CYP2C11 expression on the other hand was shown to be decreased in DV compared to CV on a protein and mRNA expression in the aorta and left ventricle. This decrease was not corrected by any of the treatments used in this study. (Figure 6a,b) (Figure 7)

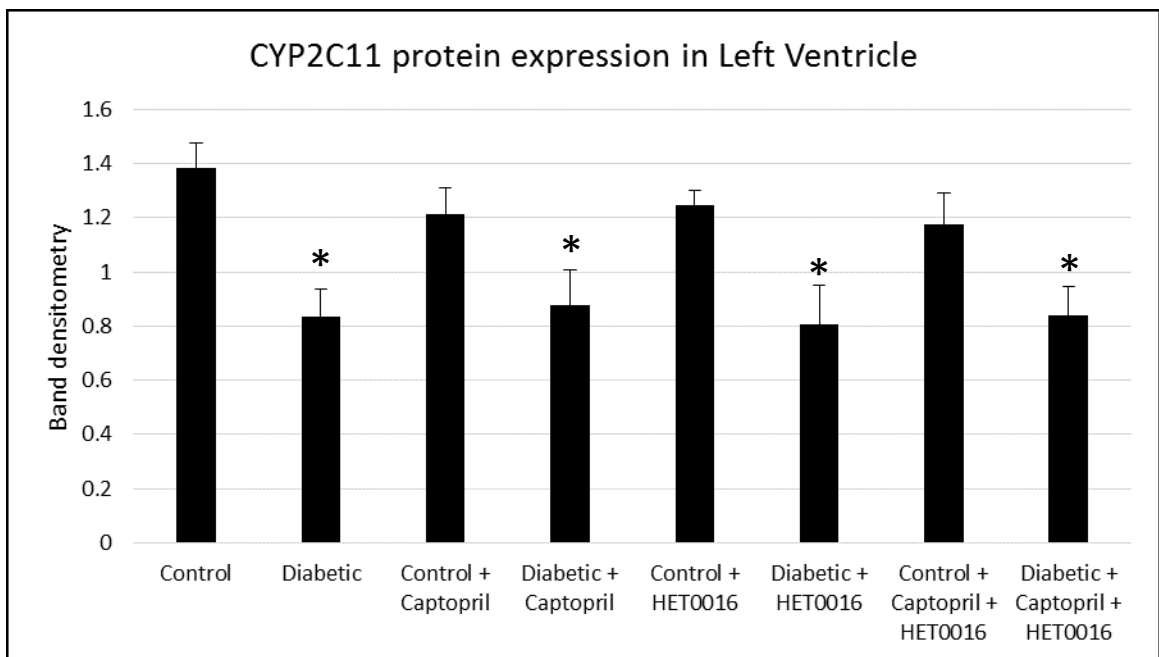
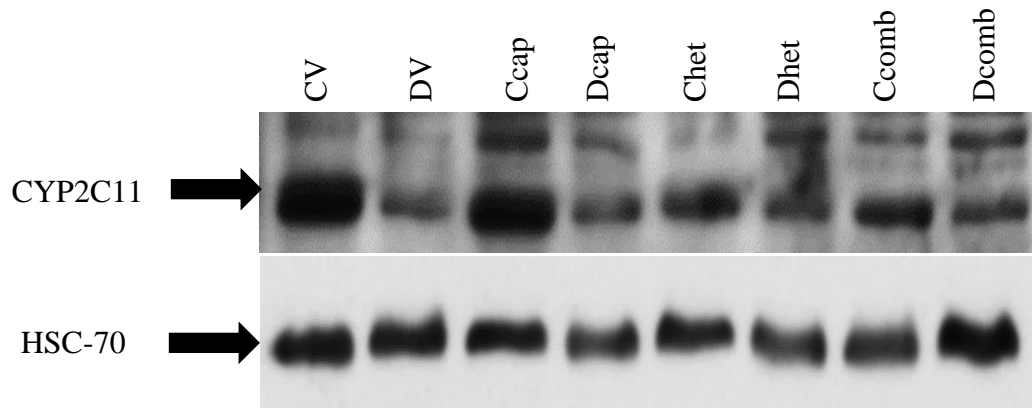


Figure 6a. Western blot Analysis for protein expression of CYP2C11 in Left Ventricle at day 28 of the treatment. (n=8)

Treatments: Vehicle (V), Captopril (cap), HET0016 (het), and combination of drugs (comb).

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

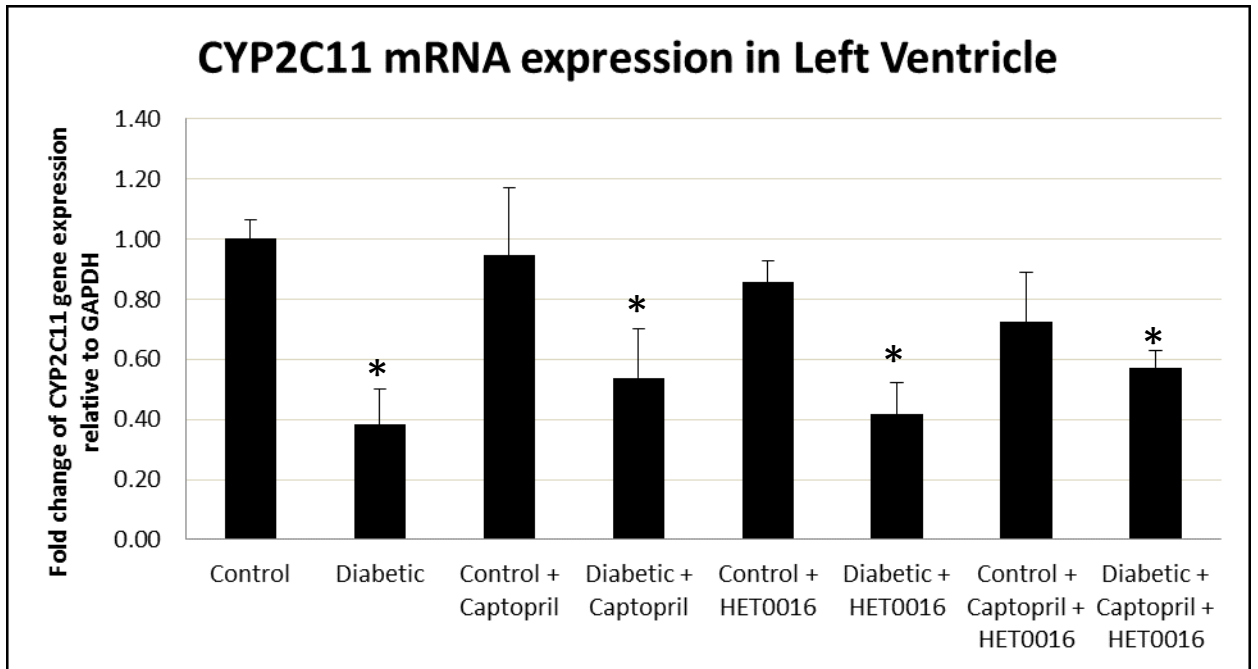


Figure 6b. q-PCR results for mRNA expression of CYP2C11 in Left Ventricle at day 28 of treatment with Captopril, HET0016, and a combination of both drugs. (n=6)  
\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

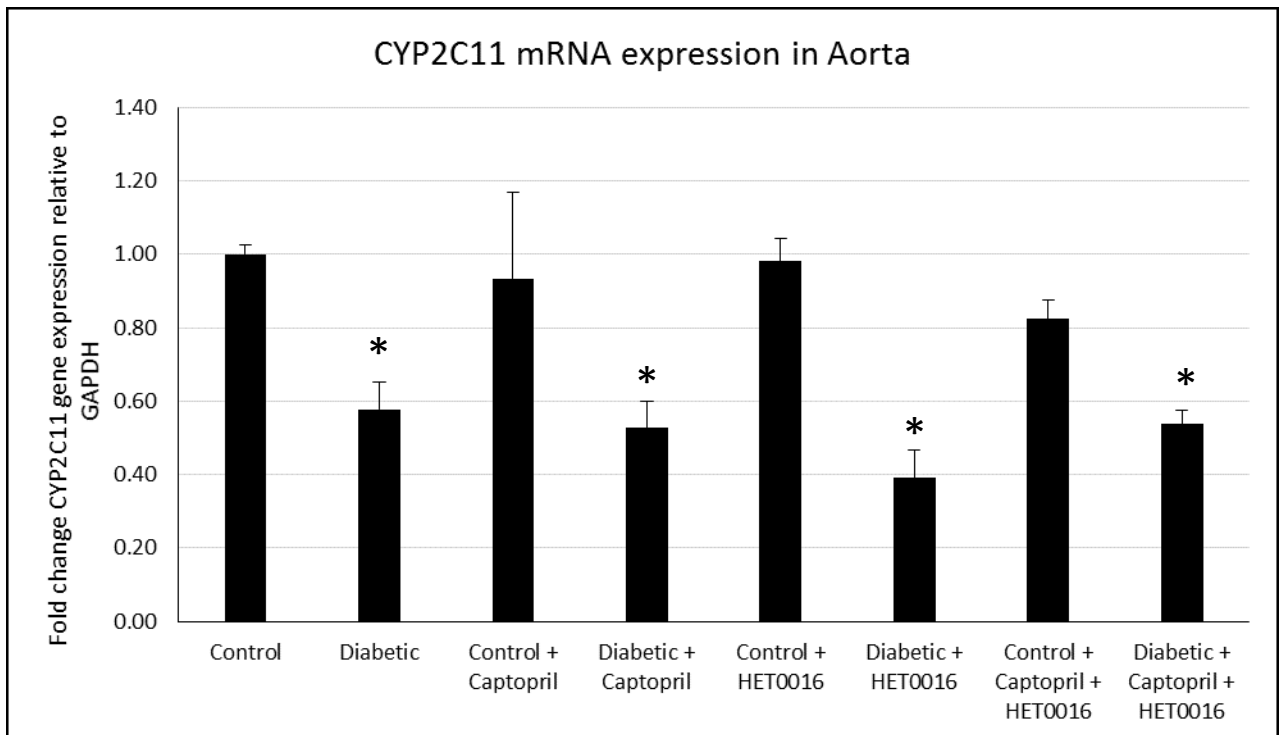


Figure 7. q-PCR results for mRNA expression of CYP2C11 in Abdominal Aorta at day 28 of treatment with Captopril, HET0016, and a combination of both drugs. (n=6)  
\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

### E- Levels of 20-HETE in the plasma

Levels of 20-HETE in plasma collected at day 28 was assessed by an ELISA kit. It displayed a significant increase in 20-HETE levels in diabetic rats when compared to control. Diabetic rats treated with either HET0016 or a combination of HET0016 and captopril had a significantly lower 20-HETE levels compared to DV, almost back to normal levels when compared to their control group. Treatment with captopril did lower 20-HETE levels in plasma but not significantly when compared against DV. (Figure 8)

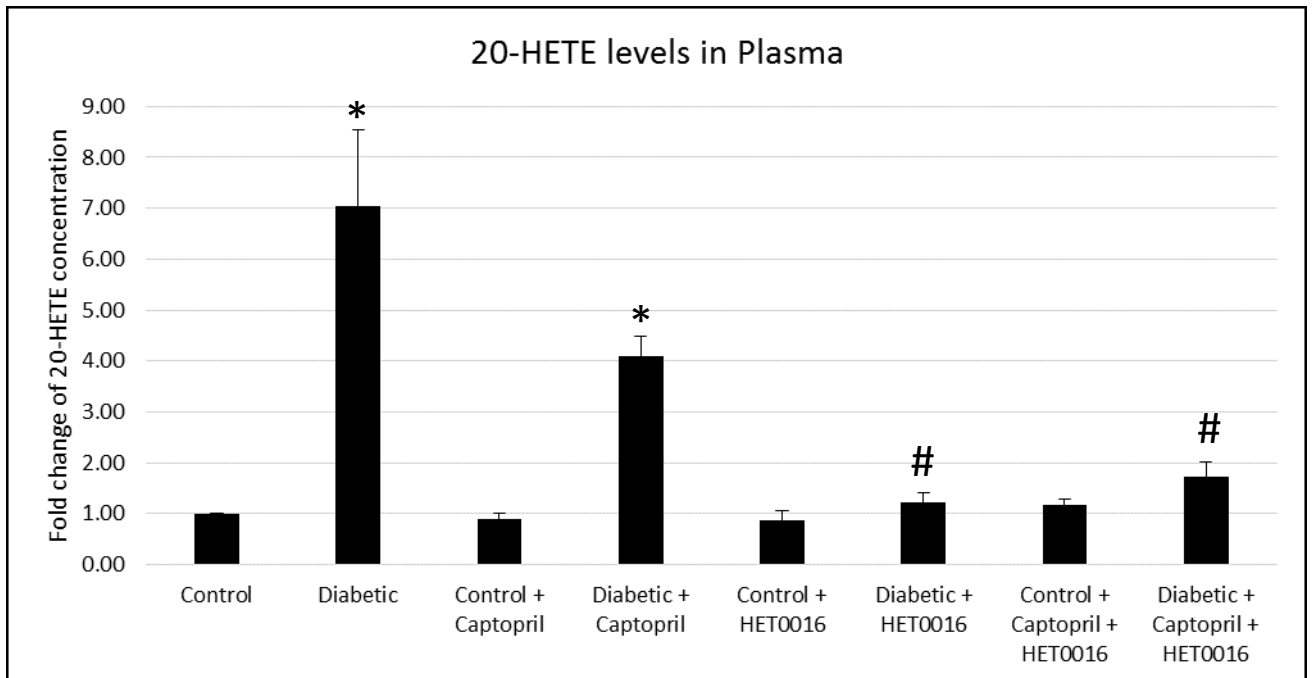


Figure 8. Quantification of 20-HETE production in plasma collected at day 28 by ELISA method for rats (n=4) receiving treatments with Captopril, HET0016, and a combination of both drugs

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated.

## **F- Markers of Heart Injury**

Levels of  $\alpha$ -MHC,  $\beta$ -MHC, and  $\alpha$ -SMA were studied by Western blot and q-PCR in left ventricular tissue.  $\alpha$ -MHC levels were significantly decreased in DV when compared to CV in protein and mRNA levels. Due to the shift in isoforms, this was accompanied with a significant increase in  $\beta$ -MHC protein and mRNA expression in diabetic untreated rats. On a protein level the decrease in  $\alpha$ -MHC was significantly reversed by giving a combined treatment of HET0016 and captopril but not with either by itself. On an mRNA level  $\alpha$ -MHC was increased by all treatments but displayed a significant increase for Dhet and Dcomb only.  $\beta$ -MHC levels showed the same response in treatment for protein and mRNA expressions where all treatments reversed the increase shown in diabetes, but only Dhet and Dcomb displayed a significant decrease when compared to DV (Figure 9a,b) (Figure 10a,b). As for  $\alpha$ -SMA, protein expression showed no significant change between all groups, but mRNA expression showed a significant increase in DV compared to CV. Dcap was the only group that showed a significant decrease in  $\alpha$ -SMA compared to DV, but it was still significantly higher than Ccap (Figure 11a,b).

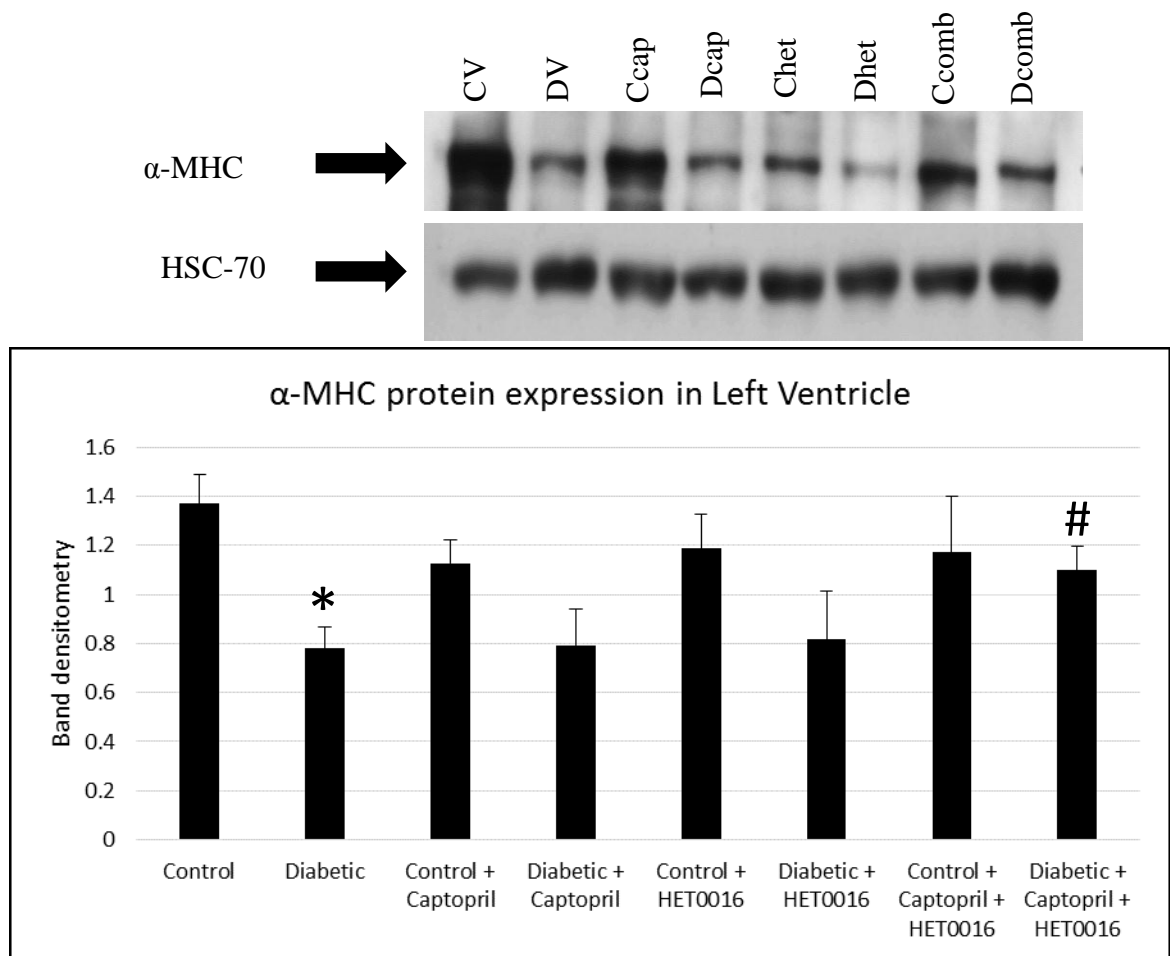


Figure 9a. Western blot analysis for protein expression of  $\alpha$ -MHC in Left Ventricle at day 28 after treatments. (n=8)

Treatments: Vehicle (V), Captopril (cap), HET0016 (het), and combination of drugs (comb).

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated.

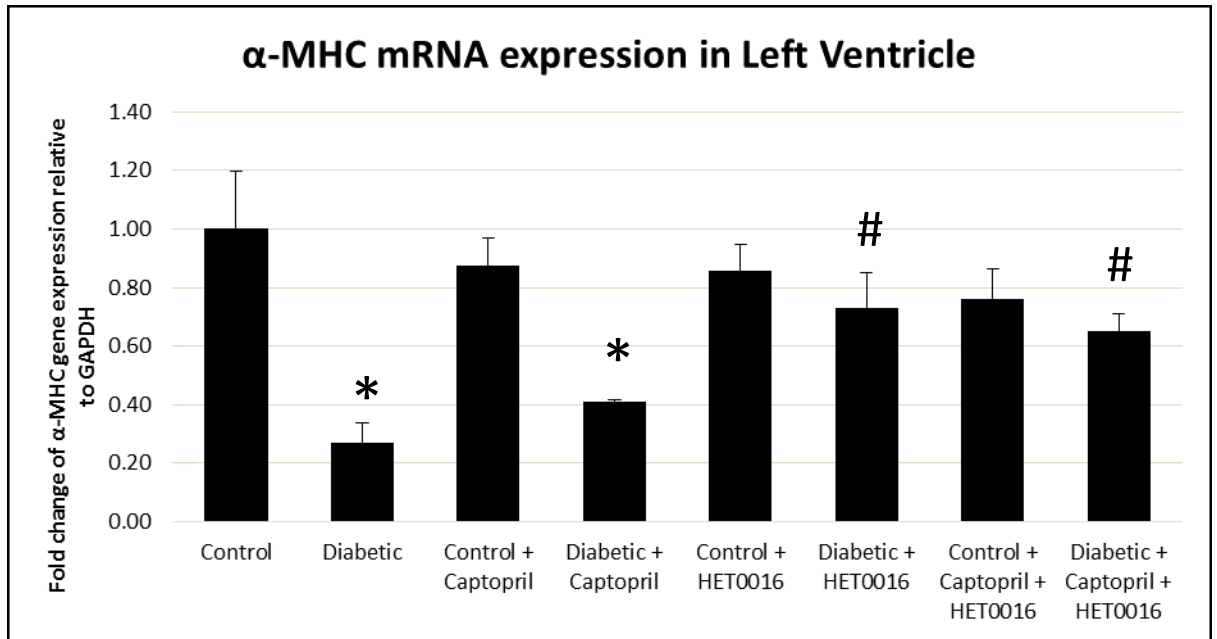


Figure 9b. q-PCR results for mRNA expression of  $\alpha$ -MHC in Left Ventricle at day 28 of treatments with Captopril, HET0016, and a combination of both drugs. (n=6)

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

# is used if  $p < 0.05$  when comparing diabetic untreated and diabetic treated.



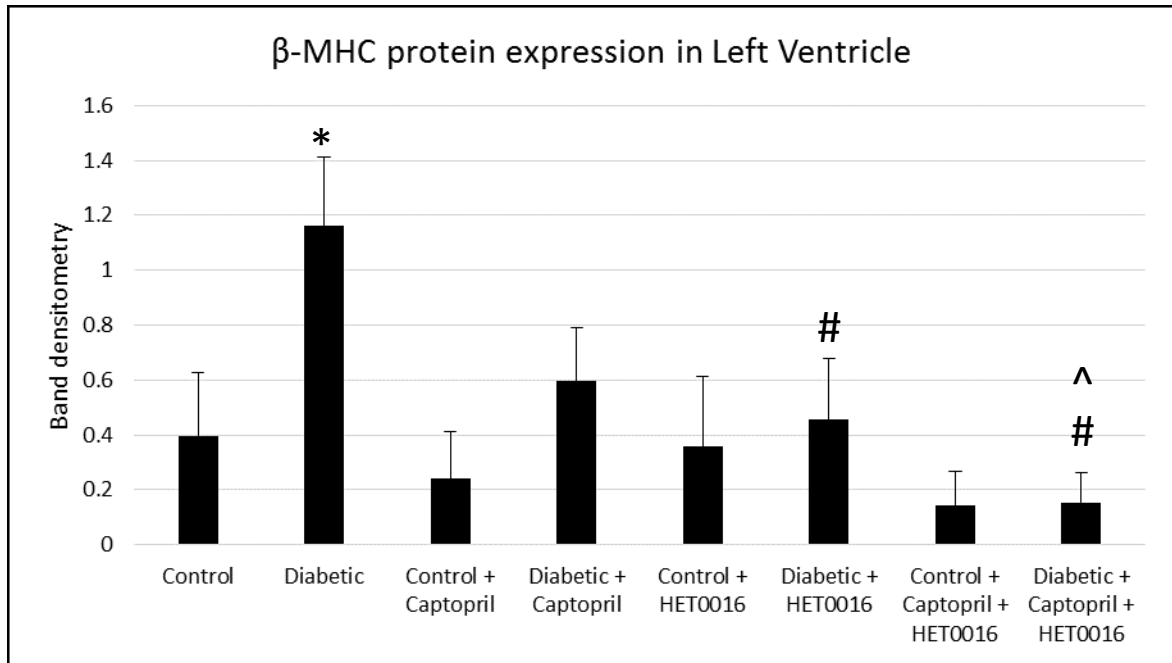
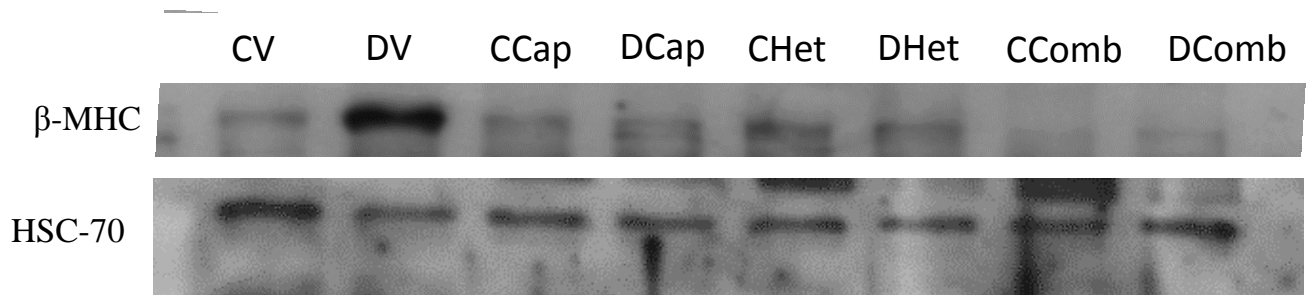


Figure 10a. Western blot Analysis for expression of  $\beta$ -MHC in Left Ventricle at day 28 of different treatments. (n=8)

Treatments: Vehicle (V), Captopril (cap), HET0016 (het), and combination of drugs (comb).

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated.

^ is used if  $p < 0.05$  when comparing diabetic treated with both captopril and HET0016 to diabetics receiving only one of the treatments

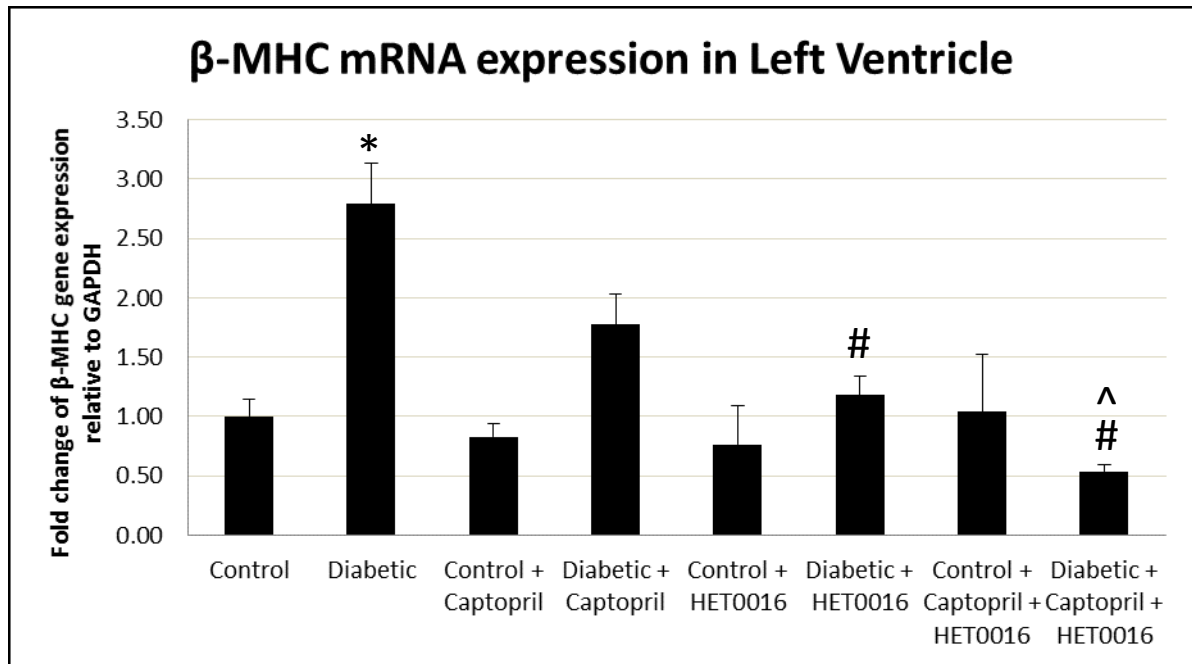


Figure 10b. q-PCR results for mRNA expression of β-MHC in Left Ventricle at day 28 of treatments with Captopril, HET0016, and a combination of both drugs. (n=6)

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment

# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated.

^ is used if  $p < 0.05$  when comparing diabetic treated with both captopril and HET0016 to diabetics receiving only one of the treatments

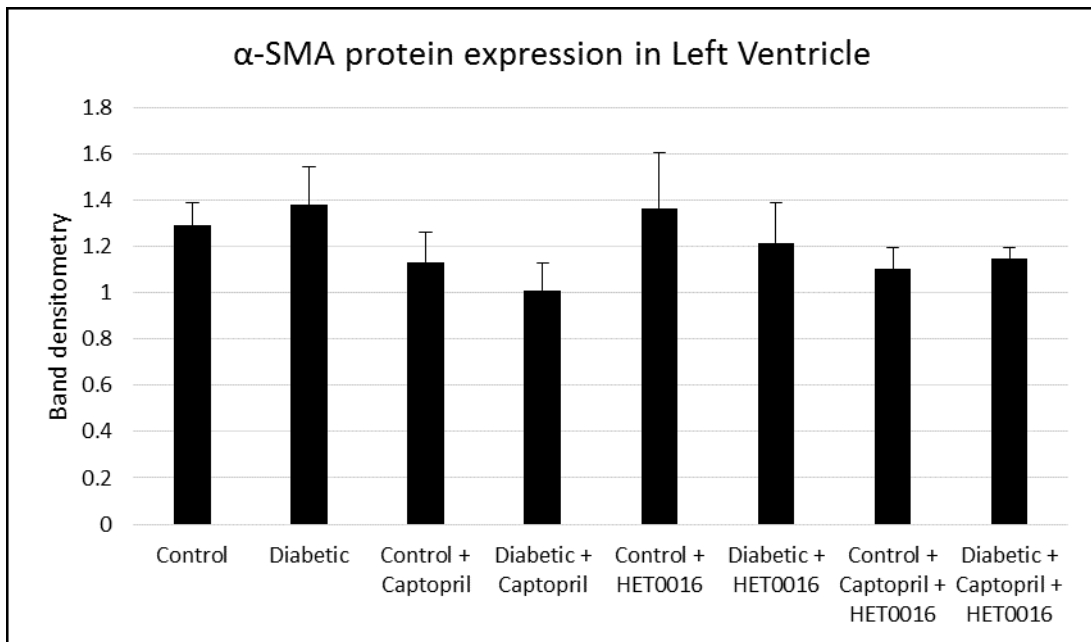
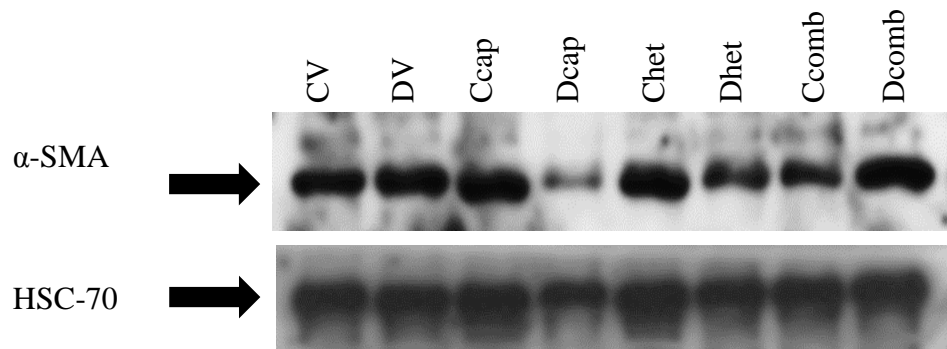


Figure 11a. Western blot analysis for protein expression of  $\alpha$ -SMA in Left Ventricle at day 28 of treatments. (n=8)  
Treatments: Vehicle (V), Captopril (cap), HET0016 (het), and combination of drugs (comb).

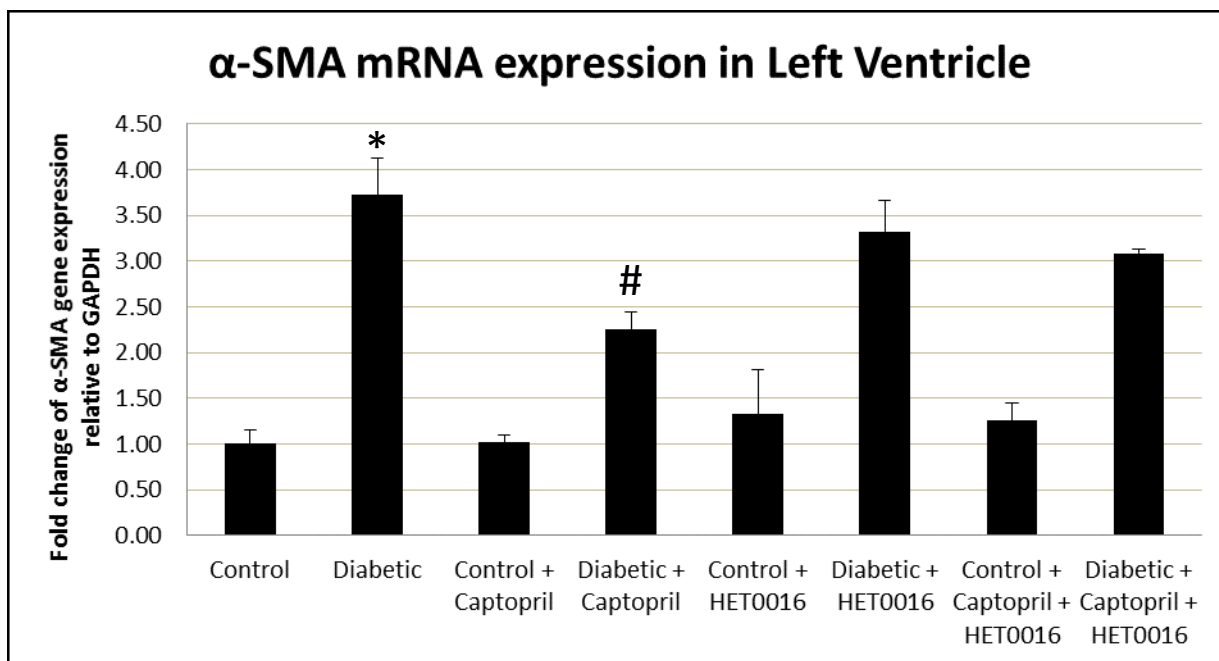


Figure 11b. q-PCR results for mRNA expression of  $\alpha$ -SMA in Left Ventricle at day 28 of treatments with Captopril, HET0016, and a combination of both drugs. (n=6)

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated.

### G- Assessment of ROS production

In order to determine the amount of ROS being produced, NADPH oxidase (NOX) expression was studied by Western blot and q-PCR. Immunostaining for ROS was also performed using DHE. NOX-1 and NOX-4 showed no significant changes among all groups at both a protein and mRNA level. However in the left ventricle, PCR analysis showed a significant increase for NOX-1 to in DV group compared to CV, this increase was not seen in diabetic treated groups (Figure 12a,b) (Figure 13a,b) (Figure 14) (Figure 15a,b). As for DHE stain, immunofluorescence indicating ROS was significantly increased in DV compared to CV. All treatments significantly decreased ROS production when compared to DV (Figure 16a,b).

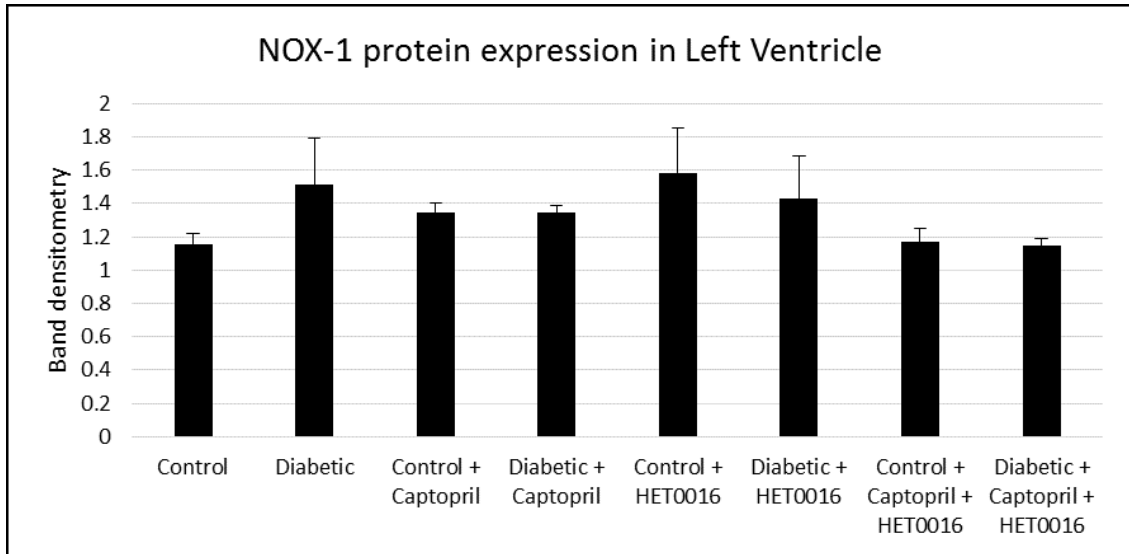
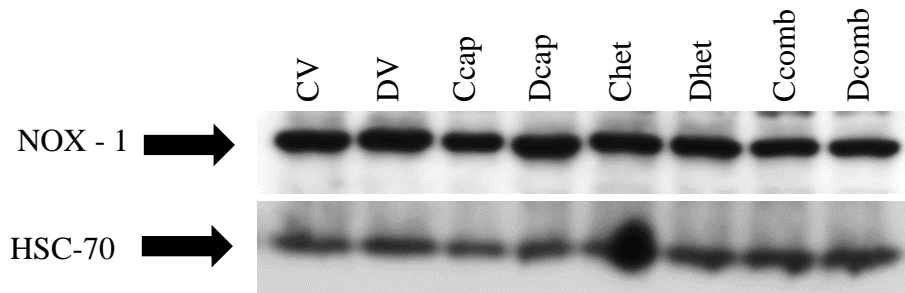


Figure 12a. Western blot analysis for protein expression of NOX-1 in Left Ventricle at day 28 of treatments. (n=8)  
Treatments: Vehicle (V), Captopril (cap), HET0016 (het), and combination of drugs (comb).

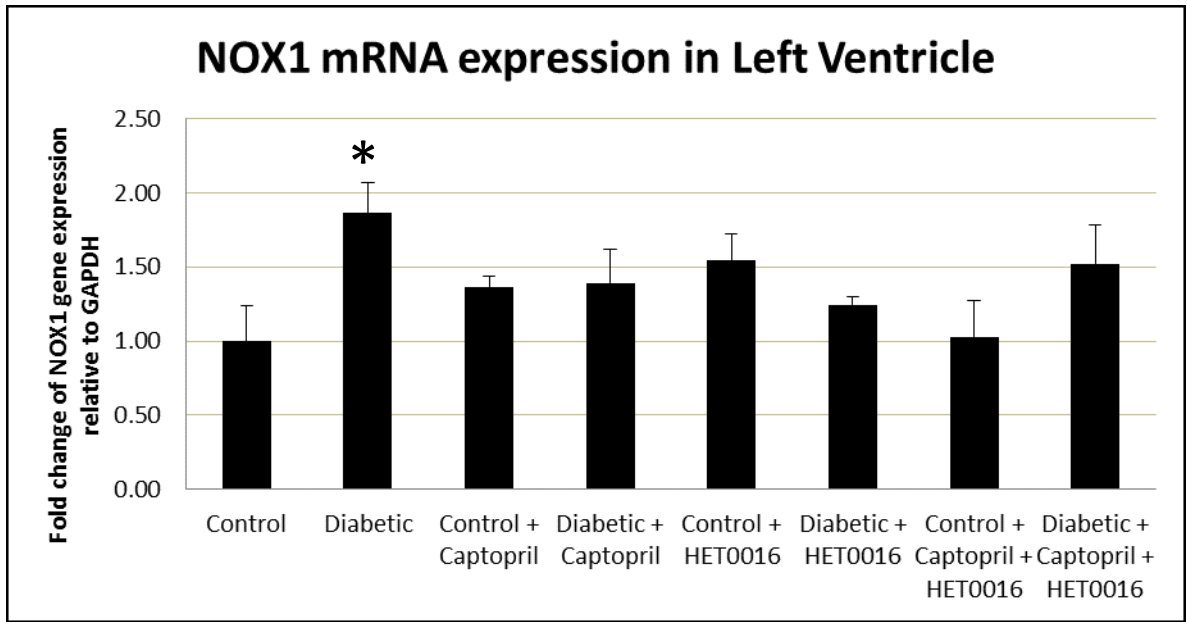


Figure 12b. q-PCR results for mRNA expression of NOX-1 in Left Ventricle at day 28 of treatments with Captopril, HET0016, and a combination of both drugs. (n=6)

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment

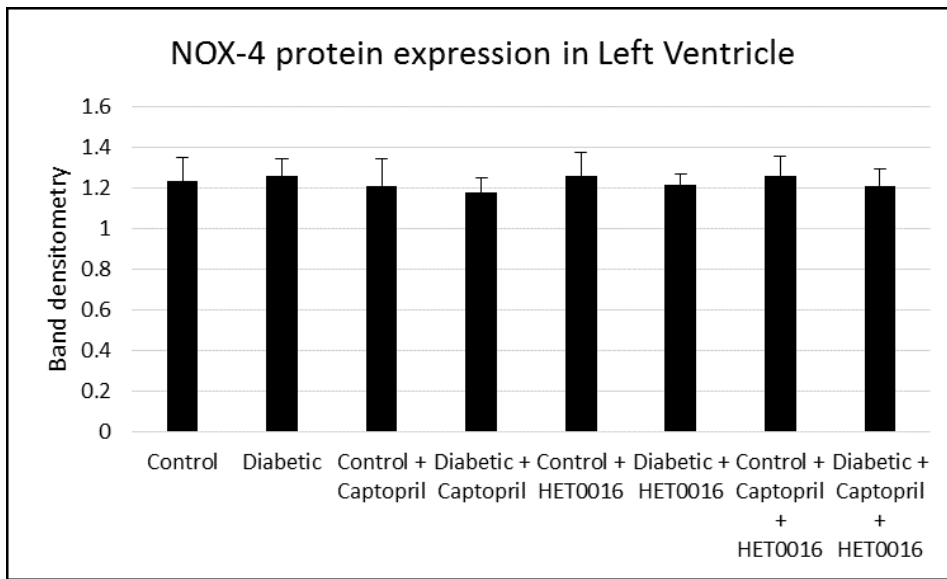
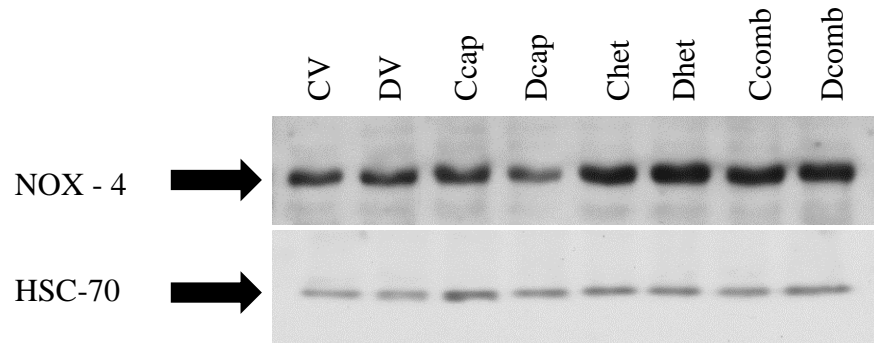


Figure 13a. Western blot analysis for protein expression of NOX-4 in Left Ventricle at day 28 of treatments. (n=8)  
Treatments: Vehicle (V), Captopril (cap), HET0016 (het), and combination of drugs (comb).

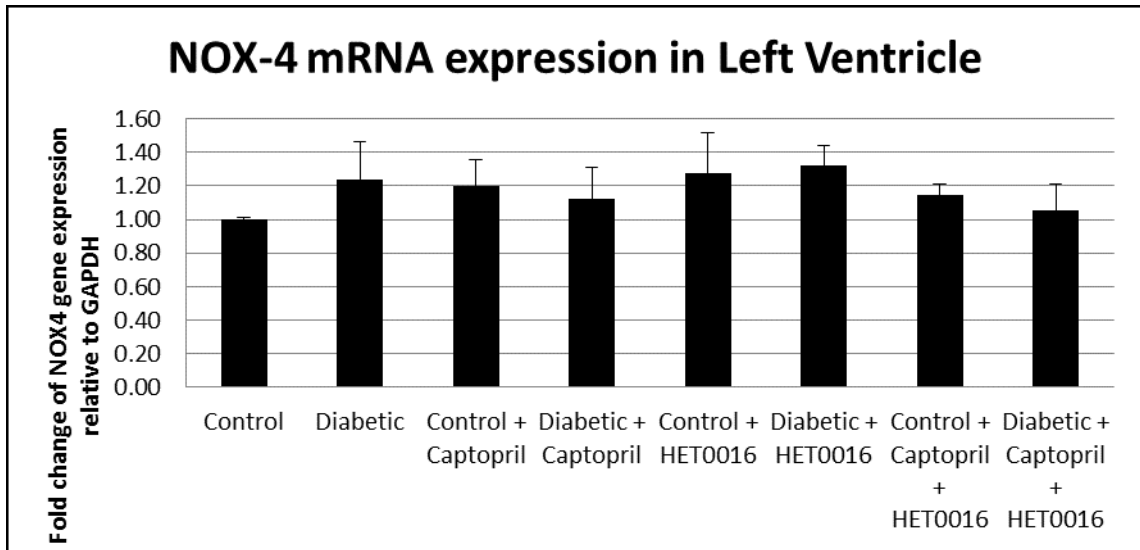


Figure 13b. q-PCR results for mRNA expression of NOX-4 in Left Ventricle on day 28 of treatments with Captopril, HET0016, and a combination of both drugs. (n=6)

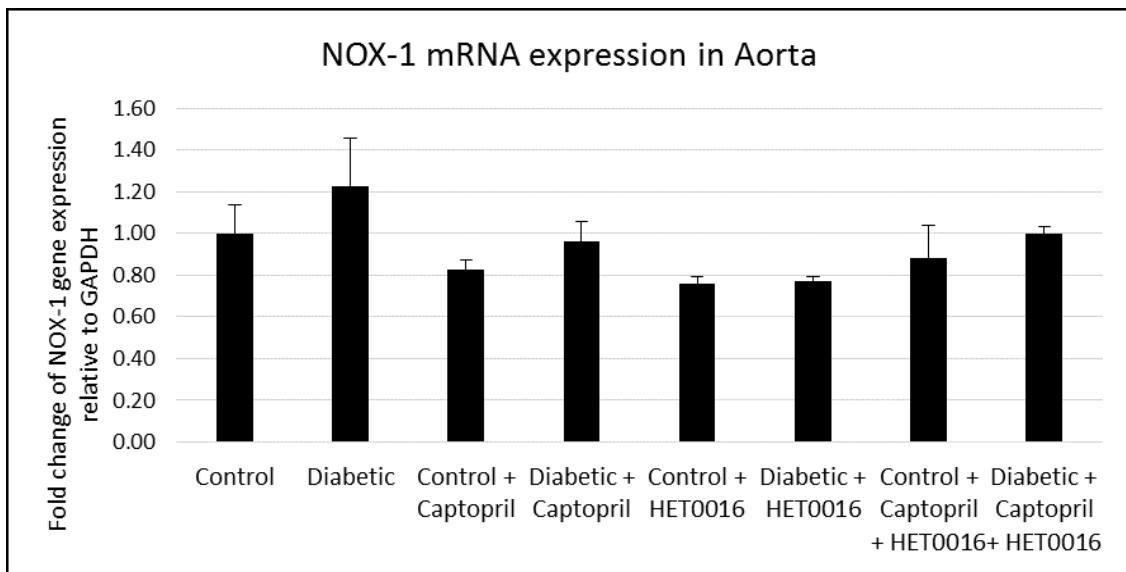


Figure 14. q-PCR results for mRNA expression of NOX-1 in Abdominal Aorta on day 28 of treatments with Captopril, HET0016, and a combination of both drugs. (n=6)



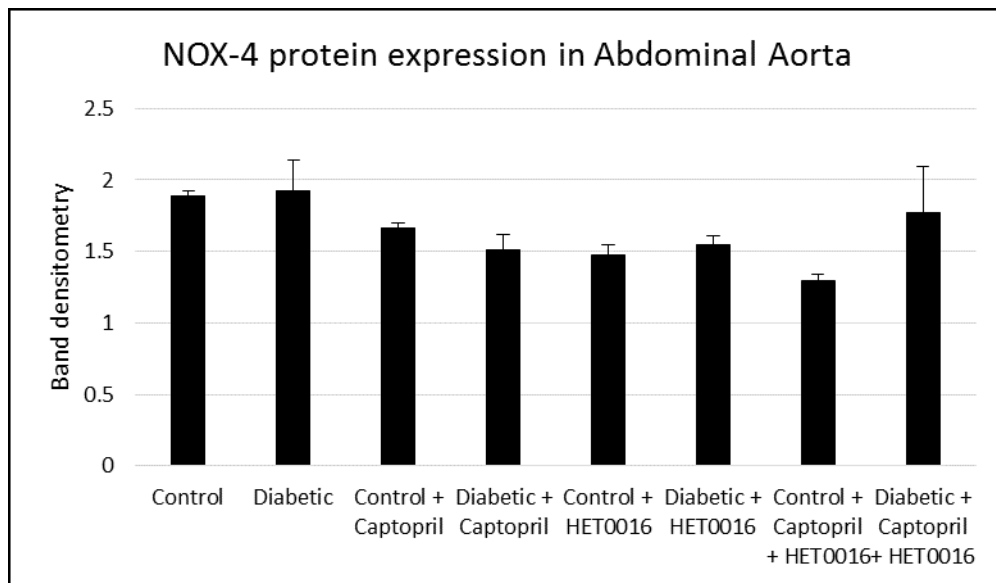
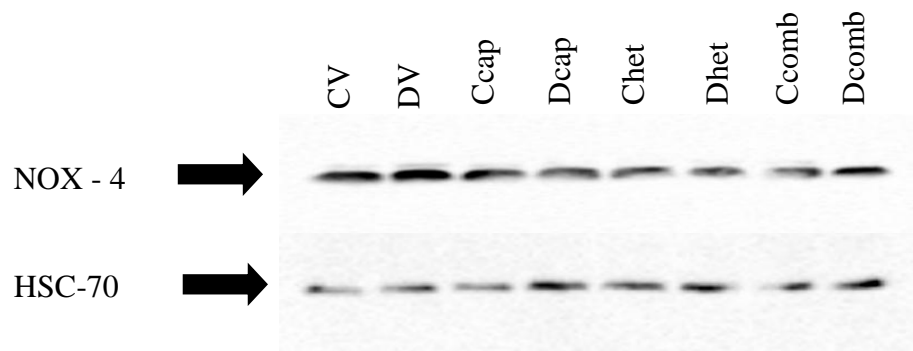


Figure 15a. Western blot analysis for protein expression of NOX-4 in Abdominal Aorta at day 28 of treatments. (n=6)  
Treatments: Vehicle (V), Captopril (cap), HET0016 (het), and combination of drugs (comb).

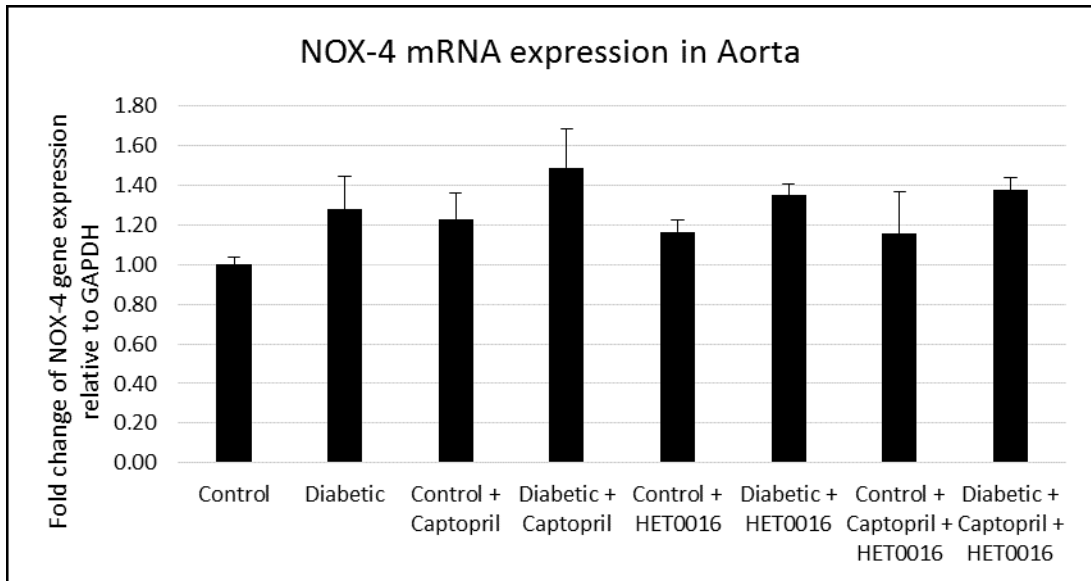


Figure 15b. q-PCR results for mRNA expression of NOX-4 in Abdominal Aorta at day 28 of treatments with Captopril, HET0016, and a combination of both drugs. (n=6)

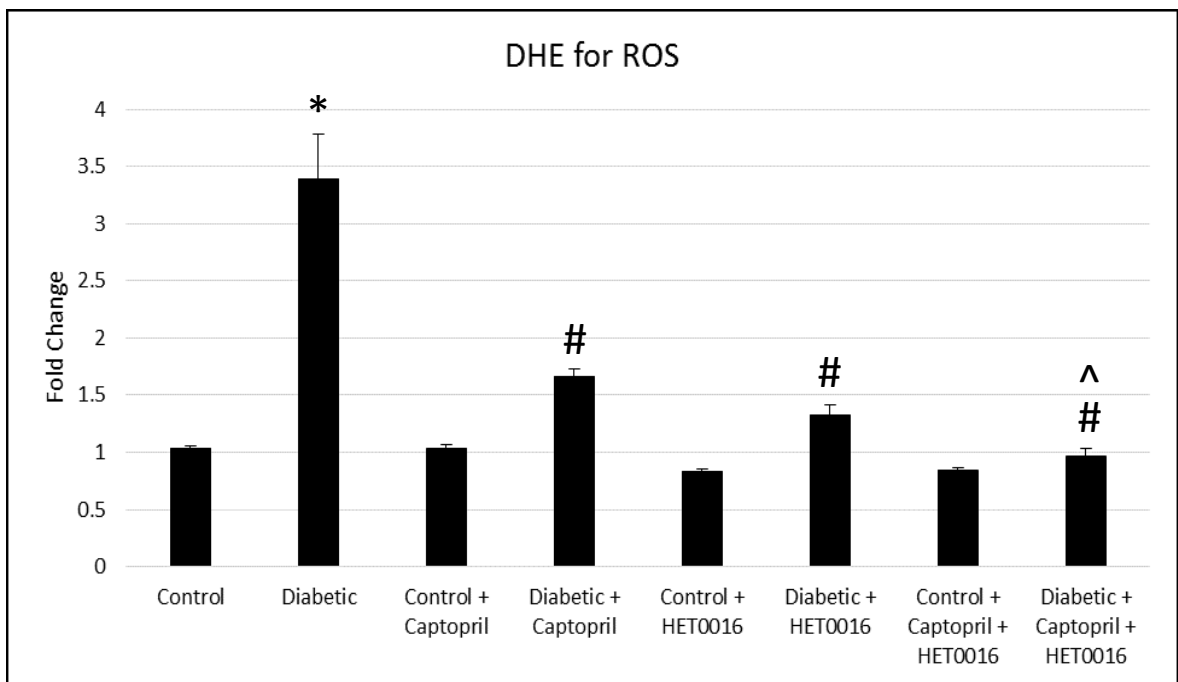


Figure 16a. DHE quantification for ROS production in Left Ventricle at day 28 of treatments with Captopril, HET0016, and a combination of both drugs. (n=3)

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment

# is used if  $p < 0.05$  when comparing diabetic untreated to diabetic treated

^ is used if  $p < 0.05$  when comparing diabetic treated with both captopril and HET0016 to diabetics receiving only one of the treatments

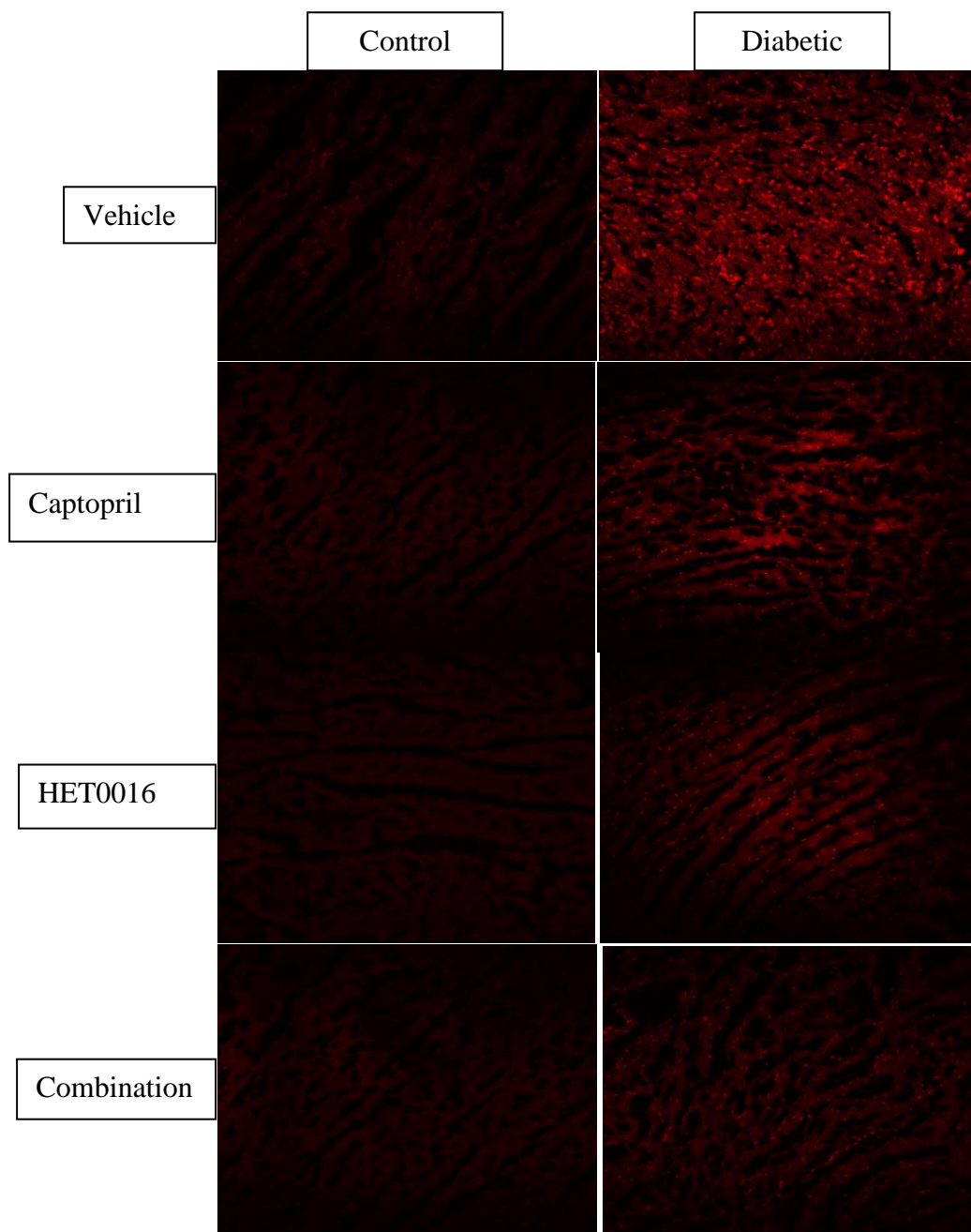


Figure 16b. DHE stain of Left Ventricle visualized by confocal microscope. Red fluorescence depicts amount of ROS production. (n=3)

## **H- Functional activity of Thoracic Aorta**

Vascular reactivity study was performed on the thoracic aorta to study the vasoconstriction and vasodilation response to increasing concentrations of different stimuli. No significant changes were observed between diabetic untreated and control untreated in response to phenylephrine (Figure 17a,b), KCl (Figure 18). Treatment with captopril in diabetic rats was the only treatment to show a significant decrease in vasoconstriction when compared to controls treated with captopril in response to the vasoconstrictive phenylephrine and KCl. No changes were observed in response to acetylcholine for any of the treated groups (Figure 19). Vascular reactivity to SNAP, a NO donor, displayed a significant decrease in the vasodilation response of the DV group compared to CV (Figure 20). This was prevented by all treatments.

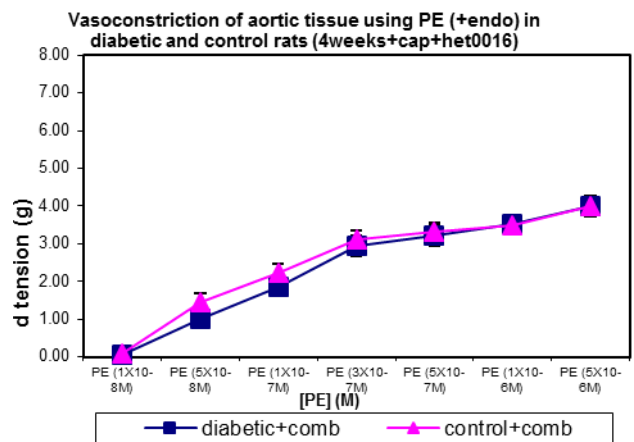
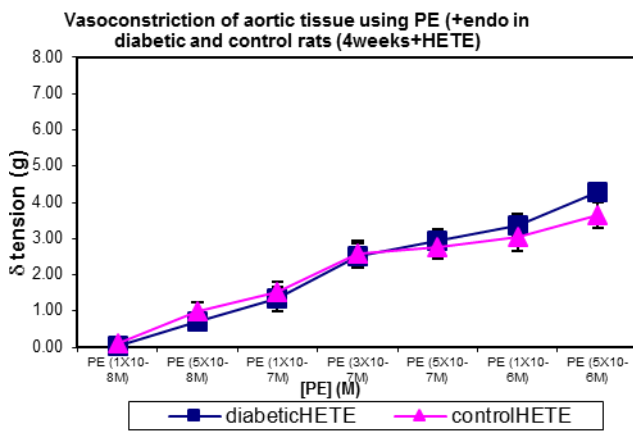
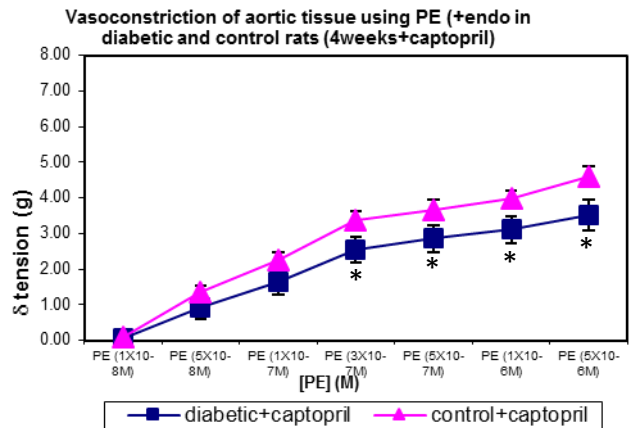
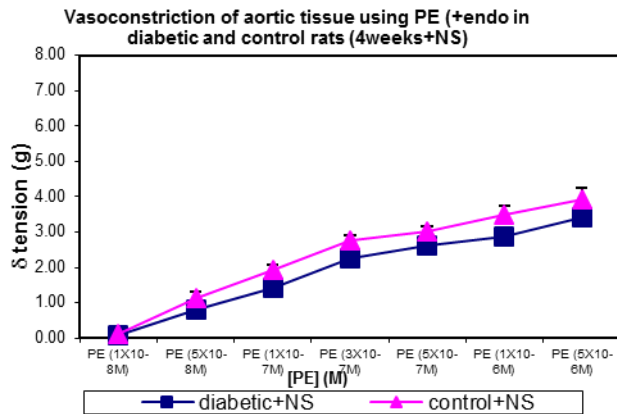


Figure 17a. Response curve of Thoracic Aorta to vasoconstrictive effect of PE with intact endothelium on day 28 of treatments with normal saline (NS), Captopril (cap), HET0016 (HETE), and a combination of both drugs (comb). (n=8)

\* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

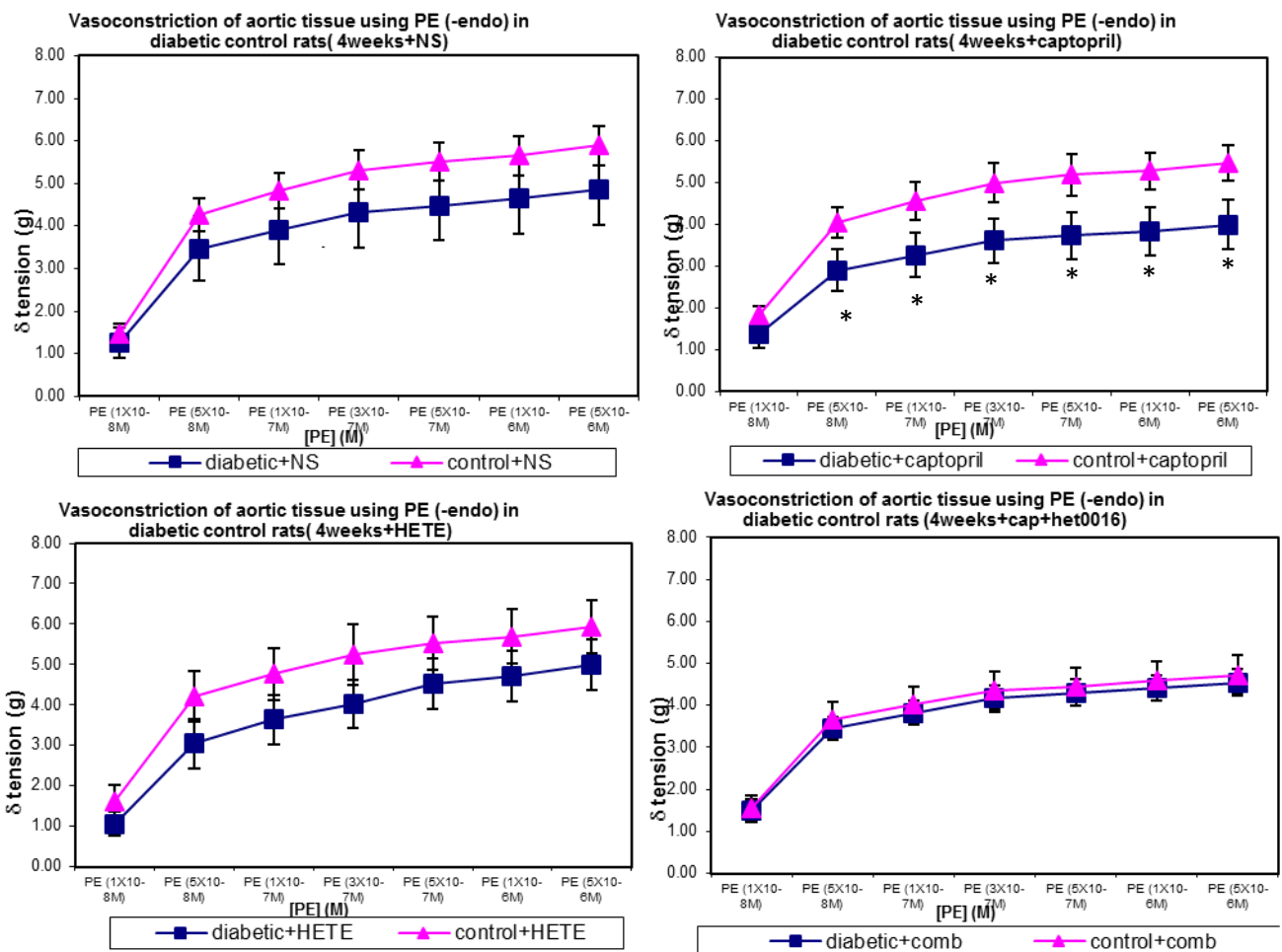


Figure 17b. Response curve of Thoracic Aorta to vasoconstriction of PE in absence of endothelium on day 28 of treatments with normal saline (NS), Captopril (cap), HET0016 (HETE), and a combination of both drugs (comb). (n=8)  
 \* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

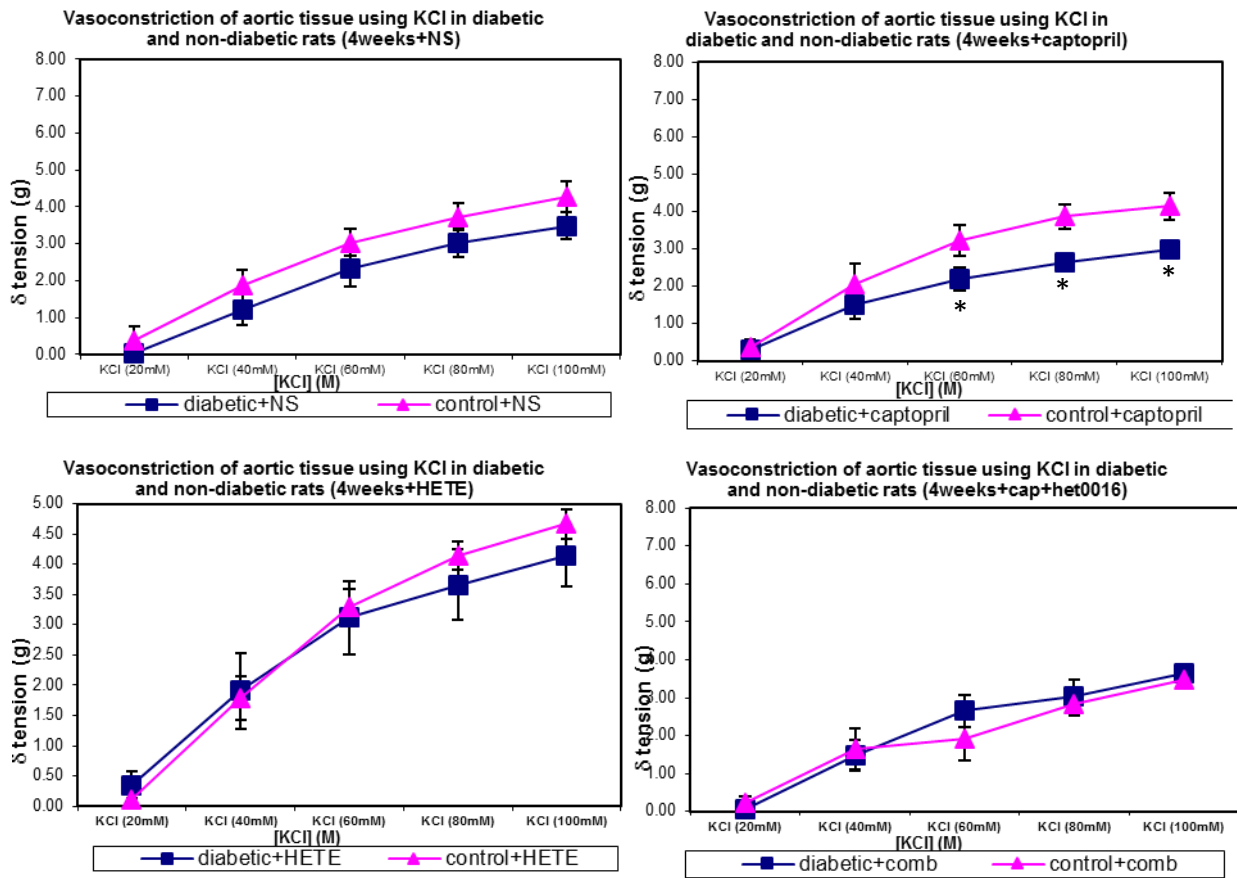


Figure 18. Response curve of Thoracic Aorta to vasoconstriction of KCl in presence of endothelium on day 28 of treatments with normal saline (NS), Captopril (cap), HET0016 (HETE), and a combination of both drugs (comb). (n=8)  
 \* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

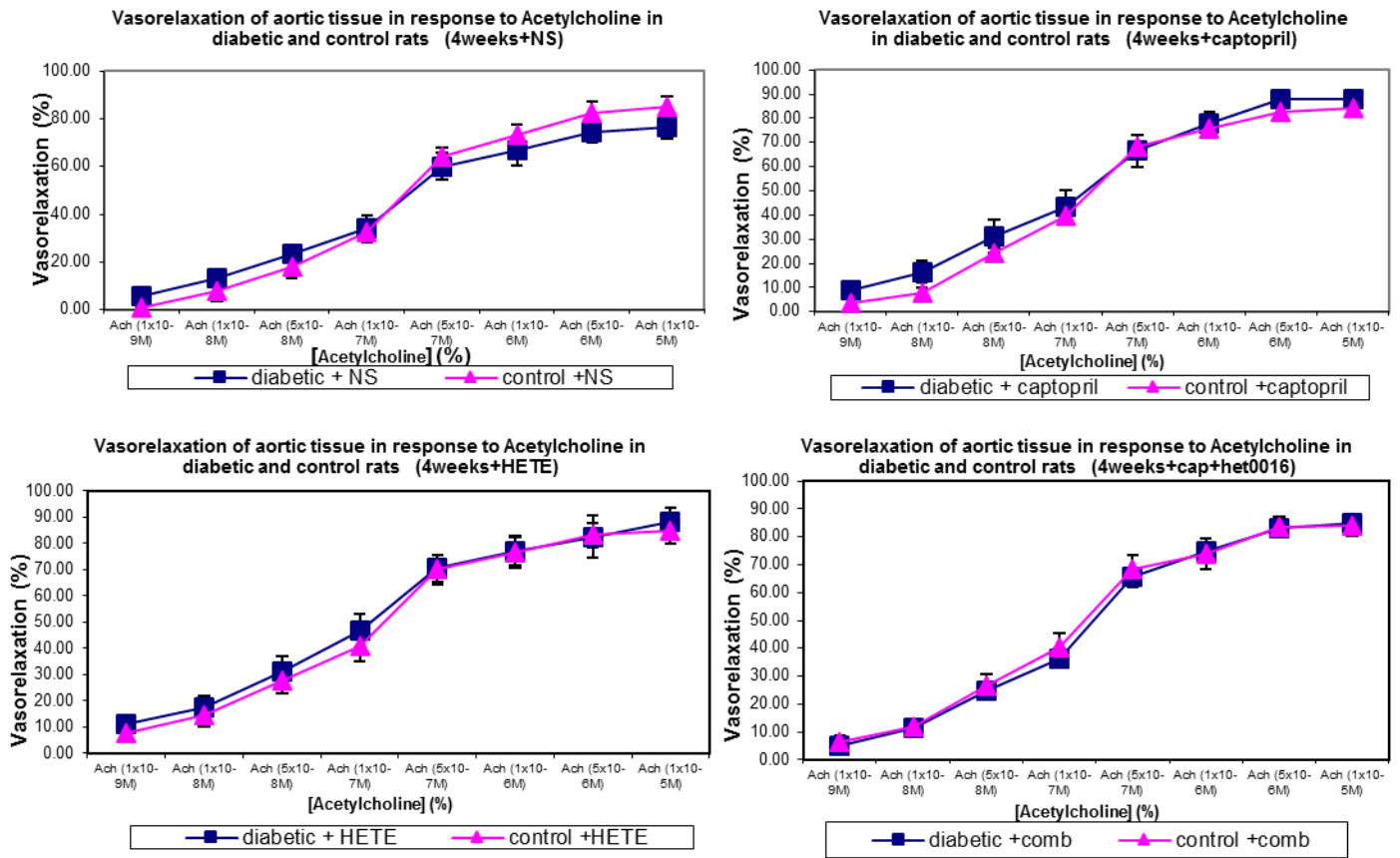


Figure 19. Response curve of thoracic aorta to vasorelaxation of Ach in presence of endothelium on day 28 of treatments with normal saline (NS), Captopril (cap), HET0016 (HETE), and a combination of both drugs (comb). (n=8).



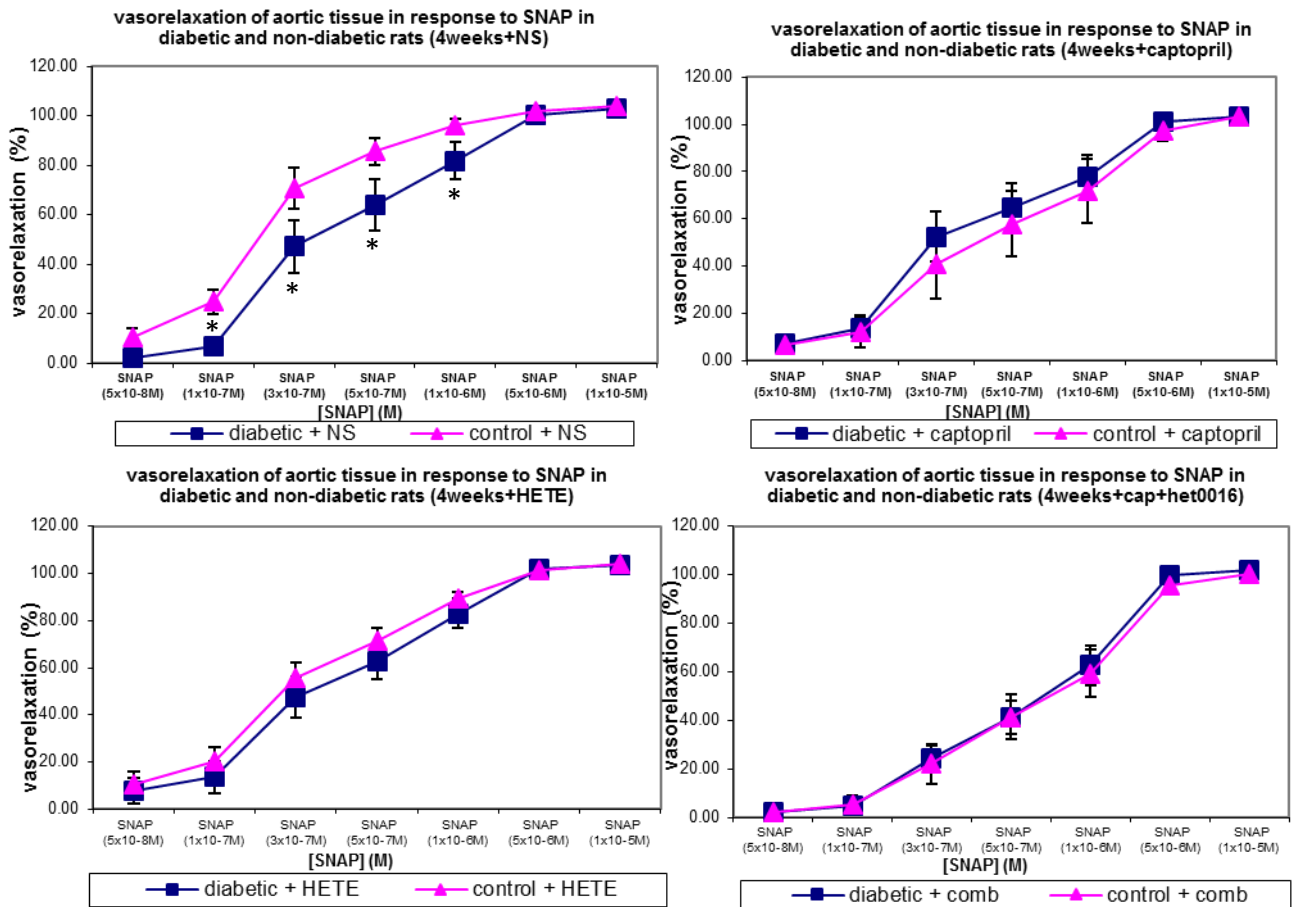


Figure 20. Response curve of Thoracic Aorta to vasodilation of SNAP in absence of endothelium on day 28 of treatments with normal saline (NS), Captopril (cap), HET0016 (HETE), and a combination of both drugs (comb). (n=8)  
 \* is used if  $p < 0.05$  when comparing controls and diabetics receiving same treatment.

## CHAPTER V

### DISCUSSION

Levels of glucose were increased in STZ induced rats at d2 in comparison to d0 indicating that they did develop diabetes. This increase was not prevented by any treatment and the hyperglycemia was maintained for all diabetic rats through the study. This is an indication that any change that occurs in the studied parameters concerning diabetic rats, will be due to the treatment itself and not due to a correction in blood sugar.

We measure heart weight, body weight, and the ratio of heart weight to body weight in order to assess index of hypertrophy. There was no significant changes in the ratio which means there was no observed hypertrophy even though markers of hypertrophy such as  $\alpha$ -MHC and  $\beta$ -MHC were changed. This could mean that a longer period of time was required for cardiac hypertrophy to occur. Body weight and heart weight on the other hand were significantly improved with captopril treatment compared to non-treated diabetics, however the best outcome was seen with a combined treatment of captopril and HET0016 where a general wellbeing was observed despite the presence of hyperglycemia.

CYP2C11 expression was studied in aortic and ventricular tissues on a protein and mRNA levels. Its expression was decreased in diabetic rats when compared to control rats which is also reported in other literature (Barnett, et al. 1990; Shimojo, et al. 1993). None of the treatments were able to elevate the level of CYP2C11 back to normal levels. This was expected for treatment with HET0016 since it is a selective

inhibitor for CYP4A isoform responsible for 20-HETE formation (Seki, et al. 2005). As for AngII, by binding to AT1R it stimulates the release of AA and its metabolism into both eicosanoids 20-HETE and EET, this could explain why using an ACEi will not increase the amount of EET produced (Mehta and Griendling 2007). This indicates that any reversal in diabetic dysfunction observed in this study is not due to increased EET presence but due to a decrease in 20-HETE/AngII levels.

The levels of CYP4A1/A2/A3 were measure in aorta and ventricle. It was shown to be significantly higher in diabetic rats when compared to the control. This is consistent with the literature where studies have shown that CYP4A1/A2/A3 levels increase on a protein and mRNA level in diabetes (Barnett, et al. 1990; Shimojo, et al. 1993), as well as studies showing an increased 20-HETE production in diabetic heart (Yousif, et al. 2009). Treating diabetic rats with HET0016, which is an inhibitor of 20-HETE formation by inhibiting CYP4A isoforms, showed a significant decrease in the expression of CYP4A1/A2/A3 on a protein and mRNA in aorta and left ventricle (Seki, et al. 2005). Treatment with a combination of HET0016 and captopril also displayed similar results to the treatment of HET0016 alone. Treatment with captopril on the other hand showed a tendency to decrease CYP4A1/A2/A3 but not enough to be considered significant. Measurement of 20-HETE in plasma showed a similar trend where 20-HETE was found to be increased in DV compared to CV, this elevation was attenuated by treatment with captopril, HET0016, and a combination of both drugs. This indicates a possible role for AngII in the formation of 20-HETE since inhibiting it with an ACEi lead to the decrease of 20-HETE in plasma.

Collagen deposition was increased in DV in abdominal aorta and left ventricle which is consistent with the literature (Goldin, et al. 2006). In both tissues treatment

with captopril or a combination of treatment significantly reduced this collagen deposition. Different results are seen in the treatment of HET0016 where collagen was not significantly decreased in aorta but was significantly decreased in left ventricle. This is proof for the role of AngII in collagen deposition since inhibiting it with ACEi was able to reduce collagen in tissue. When considering 20-HETE, it could have a role in attenuating collagen deposition since this was observed in left ventricle. But since this was not observed in aorta maybe the left ventricle tissue has an enhanced response to HET0016 compared to the aorta. Also since there was a decrease in collagen in the aortic tissue just not enough to be considered significant, a larger number of experiments might be required to show significance.

Markers of cardiac injury other than collagen deposition were studied including  $\alpha$ -MHC,  $\beta$ -MHC,  $\alpha$ -SMA. In diabetes induced myocardial injury, there seems to be an activation of fetal genes which is shown by downregulating  $\alpha$ -MHC, and upregulating  $\beta$ -MHC and  $\alpha$ -SMA. The  $\beta$  isoform of MHC is expressed in fetal life and is replaced by the  $\alpha$  isoform, the shift back to the  $\beta$  isoform is an indication of early cardiac dysfunction in diabetes.  $\alpha$ -SMA is also found in fetal life where it is responsible for cardiomyocyte differentiation, its presence in diabetic heart is a sign of hypertrophied myocytes and its considered as another early marker of myopathy (Maalouf, et al. 2012). In this study there is a shift in MHC from the  $\alpha$  to the  $\beta$  isoforms which indicates contractile dysfunction in myocytes, this is observed by other studies as well exhibiting the same isoform switch (Chung, et al. 2014; Giger, et al. 2007). There was a tendency with all treatments to increase  $\alpha$ -MHC, but it was only significant in Dcomb when checking protein expression and significant in Dhet and Dcomb when checking mRNA expression. Similarly a tendency to decrease the expression of  $\beta$ -MHC was observed

with all treatments, but it was only significant in Dhet and Dcomb on a protein and mRNA level. In the literature, some studies associate  $\beta$ -MHC expression with hypertrophied cardiac cells, other studies show an increased expression without hypertrophy (Pandya and Smithies 2011). In this study  $\beta$ -MHC was increased without a change in hypertrophy since heart weight to body weight ratios showed no significant changes. These results show a possible role for 20-HETE in shifting MHC isoforms and in the progression of cardiac myopathy even if the duration may not have been enough to show evidence of hypertrophy. As for  $\alpha$ -SMA, Western blot analysis did not show any significant differences between all groups but Dcap did tend to be slightly lower than the other groups. PCR results on the other hand showed a significant increase in  $\alpha$ -SMA mRNA expression in DV group compared to CV, treatment with captopril was the only treatment that showed a significant decrease in  $\alpha$ -SMA. Studies performed on diabetic rats showed an increase in  $\alpha$ -SMA expression in heart (Kim, et al. 2014; Mohamed, et al. 2013). The inconsistent results in this study between mRNA and protein expression of  $\alpha$ -SMA can be explained by a possible protein degradation or by an anomaly in mRNA translation to protein, further studies should be performed. In total the results indicate that treatment with captopril, HET0016, or both can ameliorate certain aspects of cardiac injury.

Functional activity of thoracic aorta was studied by checking its vasodilation and vasoconstriction reactivity to various stimuli. There was no significant alteration in the response to Ach in the study even though an impaired Ach mediated vasodilation in diabetes has been reported, however it was an 8 week study (Bhardwaj, et al. 2014). Response to PE and KCl also did not show any significant changes between control and diabetic rats, treatment with captopril in response to these vasoconstrictors was

decreased in diabetes displaying an active role for captopril in decreasing vasoconstriction when compared to controls. Other studies reported an increased response in diabetic untreated rats to the contractive stimuli of both PE and KCl (El-Bassossy, et al. 2013). There was significant decreased reaction to vasodilation in response to SNAP (NO donor) between DV and CV, this was corrected by all treatments. This could suggest that the aorta still maintained its normal activity however an early form of vascular reactivity dysfunction is occurring suspected by a decreased vasodilator in response to NO at a level beyond NO synthesis. More time might be required to induce a severe vascular dysfunction since we worked a model of early diabetes.

ROS production was studied by Western blot and PCR analysis of NOX expression in the aorta and left ventricle, as well as staining superoxide anion with DHE in ventricle tissues to visualize the extent of ROS found in the tissue. NOX-1 and NOX-4 expression were reported to be distributed in cardiomyocytes and vascular smooth muscle cells (Lassègue, et al. 2012). In this study NOX-4 expression showed no significant changes between all groups in both tissues. However NOX-1 had the tendency to increase in DV compared to its CV on a protein level, and was significantly increased on an mRNA level; this difference between the untreated diabetic and control rats was not seen when comparing any treated diabetics with their control. Studies in the literature show an increase in mRNA and protein expression of NOXs in diabetes (Sedeek, et al. 2010). NOX-1 and NOX-4 protein expression was studied in diabetic hearts after 2 weeks of STZ injection, no changes were observed in NOX-1 expression however levels of NOX-4 were significantly increased in diabetic rats (Maalouf, et al. 2012). NOX-1 was shown to increase in vasculature of diabetic rats causing

atherosclerosis and endothelial dysfunction by uncoupling eNOS (Gray, et al. 2013; Youn, et al. 2012). A change in NOX expression could have required more time (>4weeks) to be significantly altered in this study, although a tendency to increase expression was observed. DHE stain however showed a significant increase in ROS presence when comparing DV to CV. This increase was prevented significantly in all treatments studied. This means that decreasing ROS production is a factor in correcting diabetes induced cardiovascular dysfunctions. Sources of ROS other than NOXs can be implicated since NOX expression was not significantly changed. However it is possible that NOX activity was enhanced even if its expression was not altered; a NADPH oxidase assay should be performed to test for the activity of NOX.

As a conclusion, 20-HETE production can be considered one of the various mediators in diabetic cardiovascular dysfunction. Inhibiting its production by HET0016 was found to prevent the increased expression of several markers of dysfunction. AngII and 20-HETE could have complementary roles in diabetic dysfunction, since some results had a better outcome when both drugs were used such as restoring  $\alpha$ -MHC isoform. Effect of ACEi had a tendency to decrease markers of diabetic dysfunction, however in most cases it was not enough to be considered significant. This could be explained by considering the differential effect of captopril on the systemic and local renin angiotensin system. These results have important pathophysiological and therapeutic implications since they propose an important role for 20-HETE in mediating cardiovascular dysfunction in diabetes which requires further investigation. Also these results suggest that therapeutic interventions targeting HETE may be clinically relevant and effective, this warrants further investigation as well. Future investigations can go

towards understanding the pathophysiology and applications in diseased states after more conclusive studies. (Frustaci, et al. 2000).



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