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EFFECT OF TREATMENT WITH CAPTOPRIL, HET0016 AND THEIR COMBINATION ON RENAL COMPLICATIONS OF DIABETES IN SPRAGUE DAWLEY RATS

by:

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A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Pharmacology And Toxicology
of the Faculty of Medicine
at the American University of Beirut

Beirut, Lebanon
June 2015

AMERICAN UNIVERSITY OF BEIRUT

Effect of Treatment with Captopril, an Angiotensin Converting Enzyme Inhibitor, and HET0016, a 20-HETE Inhibitor, and their Combination on Renal Complications of Diabetes in Sprague Dawley Rats

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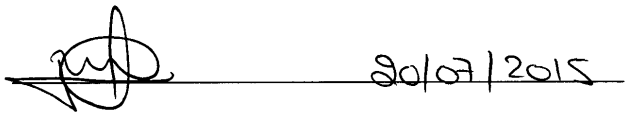

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ACKNOWLEDGMENTS

I would like to thank Dr. Ramzi Sabra for all the support he has provided me from the moment I started this program. You were always here to listen, understand and advise me through the roller coaster ride that these past three years were. Thank you from the bottom of my heart.

I would also like to deeply thank Dr. Assaad Eid for all the times he was there for me. Your technical advice always got me out of dead ends. Your moral support and contagious liveliness lifted my spirits whenever I came nagging, so thanks a million.

My gratitude also goes to the members of the committee: thank you Dr.Zgheib, Dr.Semaan and Dr.Zeidan for your advice and comments when reviewing my work.

My recognition goes to Dr.Eid's laboratory staff for the technical support and advice that laid the ground work for this project.

However, my deepest and most sincere thank you goes to the family that is the 2-12 lab. Rana, Ruweida and Nahed, not only did you help me with my project but you were supportive, understanding and made me feel like I was surrounded by my family. Thank you so much for the love and care that you gave me. George thank you for the support and the advice whenever I needed it. Moustafa thank you for all the technical advice that you provided me with and Lamis for all the help that you gave me in a heartbeat. Both of you made this difficult time easier with all the laughter and good mood that you always have. Thank you to everyone who is part of this beautiful family, you made the journey smoother.

Many many thanks go to Maysaa. My friend, my sister, my partner in crime, the Heckle to my Jeckle. What a journey we went through. Laughs, tears, emotional breakdowns, we've seen it all. Thank you for tolerating my insanity, supporting me when I was feeling down, and always being there for me. You are the reason I didn't give up.

I would also like to thank all my friends who stood by me: Jerome, Murielle, Rita and Georgio. You mean the world to me.

Last but not least, thank you Mom, Dad and my brother Camille. I couldn't have done it without you. You supported me with this knowing it meant that I would be away for three more years. The thought of seeing you once I was done was my light at the end of the tunnel. Thank you for the sacrifices you make for me every day so that I could be here.

AN ABSTRACT OF THE THESIS

Rita Joseph Bassil for Masters of Science
Major: Pharmacology and Therapeutics

Title: Effect of treatment with captopril, an angiotensin converting enzyme inhibitor, and HET0016, a 20-HETE inhibitor, and their combination on renal complications of diabetes in Sprague Dawley rats.

Background: Diabetic nephropathy is one of the major life threatening complications of diabetes mellitus. It is characterized by renal hypertrophy, increased extracellular matrix deposition, increased albumin excretion which will all eventually lead to kidney failure. Administering captopril, an angiotensin converting enzyme inhibitor, will slow down all these processes and delay the onset of nephropathy. Recent studies have shown that 20-HETE, an arachidonic acid metabolite of cytochrome P450 of the 4A family, plays a role in the pathology of diabetic nephropathy and does so not only through its vasoconstrictor properties but through the production of reactive oxygen species (ROS) which mediate the renal damage. *N*-hydroxy-*N*-(4-butyl-2 methylphenyl) formamidine (HET0016) is a potent inhibitor of 20-HETE synthesis and is thought to decrease the production of ROS, leading to a renoprotective effect.

Aim: The present study aims to determine the role of 20-HETE in renal hypertrophy, extracellular matrix deposition and ROS production in a model of diabetes. In addition, the effect of combined administration of captopril and HET0016 on inhibiting the production of 20-HETE and the related outcomes of diabetes, such as ROS production and indexes of kidney injury, will be studied in order to determine if the combined treatment has a positive therapeutic outcome on renal dysfunction.

Methods: Male Sprague-Dawley rats were used in this experiment as animal model. Diabetes was induced using streptozotocin in order to mimic type I diabetes. Rats were then divided into eight groups according to the different treatment they received. Control and diabetic vehicle rats were treated with either normal saline, captopril, HET016 or with a combination of captopril and HET0016. Body weight, blood glucose levels, urine volume, were taken on day 0, day 14 and day 28. On day 28, rats were sacrificed and the kidneys were harvested. Plasma collected on day 28 was used to assess 20-HETE levels in the circulation. Kidney cortex were isolated for protein and mRNA expression of collagen IV, laminin, CYP4A, NOX 4. Kidney cortexes sections were used to evaluate collagen deposition and ROS production by Masson trichrome and DHE staining respectively.

Results: Indexes of kidney injury were present in non-treated diabetic rats, with increased collagen and laminin deposition, increased total urine protein excretion and increased ROS production. Treatment with captopril ameliorated kidney injury by decreasing collagen and laminin expression, decreasing urine protein excretion, ROS production and NOX 4 activation. HET0016 also decreased kidney injury by ameliorating protein excretion in the urine, decreasing collagen IV deposition and expression, laminin expression, NOX 4 expression and ROS production. The combined treatment had the same trend as captopril and HET0016, however protein excretion and NOX 4 expression were lower than each treatment alone. CYP4A expression and 20-HETE plasma levels were both increased in the diabetic group, which can be injurious to the kidney, whereas the levels in the groups treated with captopril, HET0016 and the combined treatment groups were decreased.

Conclusion: Our results show that treatment with captopril and HET0016 and the combination of both ameliorated kidney injury by decreasing ROS production and 20-HETE levels. Our observation suggest that captopril and HET0016 might have a synergistic effect, which can be used as a possible new therapeutic treatment.

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ABBREVIATIONS

Advanced glycation end products……..…..AGEs

Angiotensin II……..…….Ang II

Angiotensin converting enzyme……..….ACE

Angiotensin converting enzyme inhibitor……..…..ACEi

Arachidonic acid……..…….AA

Cardiovascular disease…….……CVD

Cytochrome P450.....CYP

Diabetic nephropathy……..…….DN

Diacyl glycerol…….DAG

End stage renal disease…….....ESRD

Epoxyeicosatrienoic acids……...EETs

Extracellular matrix…….ECM

Fructose-6-phosphate……...F-6-P

Glomerular basement membrane…….GBM

Glomerular filtration rate…….GFR

Hydroxyeicosatetraenoic acid……..HETE

N-hydroxy-N'-(4-butyl-2-methylfenyl) formamide…….HET0016

Insulin dependent diabetes mellitus……..IDDM

Middle East and North Africa……...MENA

Myocardial infarction…….....MI

Nicotinamide adenine dinucleotide phosphate…….NAD(P)H

Non-insulin dependent diabetes mellitus……..NIDDM

Protein kinase C…….PKC

Reactive oxygen species…….....ROS

Renin angiotensin system……..RAS

Thick ascending loop of Henle……..TALH

Transforming growth factor ß-1……. ...TGF-ß1

Type 1 diabetes mellitus…….T1DM

Type 2 diabetes mellitus…….T2DM

Urine albumin excretion…….UAE

World Health Organization…….WHO

CHAPTER I
INTRODUCTION

A. Defining Diabetes Mellitus

The American Diabetes Association defines diabetes mellitus as a metabolic disease caused by defects in insulin secretion, insulin action or both, which results in a state of hyperglycemia. If left uncontrolled, chronic hyperglycemia will eventually lead to the dysfunction and damage of several organs such as the eyes, nerves, blood vessels, heart and kidneys (American Diabetes Association, 2015). Symptoms of hyperglycemia consist of polydipsia, polyuria, blurred vision, weight loss and sometimes polyphagia. Acute consequences of elevated blood sugar include life-threatening ketoacidosis or a non-ketotic hyperosmolar state that could lead to stupor, coma and even death in extreme cases. Hyperglycemia may be present for a long time before a proper diagnosis is made (Alberti et al., 1998).

B. Prevalence of diabetes mellitus

According to the International Diabetes Federation, developing countries hold the majority of people with diabetes. The prevalence in the Middle East and North Africa (MENA) region is the highest in the world at 10.9% of the regional population in 2013. This number is projected to increase by 2035 to reach a whopping 11.3% of the MENA

population ("Internation Diabetes Federation," 2013). The increased occurrence of obesity and physical inactivity as well as population growth and longevity are behind these staggering numbers (Wild et al., 2004).

C. Classifying diabetes mellitus

In 1980 the World Health Organization (WHO) proposed a classification for the different types of diabetes mellitus and named them Insulin Dependent Diabetes Mellitus (IDDM) or Type 1 and Non-Insulin Dependent Diabetes Mellitus (NIDDM) or Type 2. A revision of the classification in 1985 dropped the names Type 1 and Type 2 and added Impaired Glucose Tolerance as well as Gestational Diabetes Mellitus to the classification. With time the terms IDDM and NIDDM were removed and Type I and Type II were retained in order to base the classification on the etiology of the disease rather than the pharmacological course of treatment (Alberti & Zimmet, 1998).

1- Type I diabetes

Type I Diabetes Mellitus (T1DM), also known as IDDM, juvenile onset diabetes or immune-mediated diabetes is characterized by the autoimmune destruction of the pancreatic beta-cells. It usually results in complete insulin deficiency. Several autoantibodies such as islet-cell autoantibodies, insulin autoantibodies, GAD65 autoantibodies and autoantibodies to the tyrosine phosphatases IA-2 and IA-2Beta are markers of immune destruction. 85-90% of patients have one or more of these autoantibodies upon diagnosis of fasting hyperglycemia. The rate of beta cell destruction is

not constant and varies from an individual to another. The peak incidence of T1DM occurs in childhood and early adolescence however it may set off at any age. There is a genetic predisposition to autoimmune destruction of the beta cells as well as several environmental factors, however the latter are not very well defined (American Diabetes Association, 2015).

T1DM forms with no known etiology are classified as idiopathic diabetes. The patient presents with chronic insulinopenia, without proof of autoimmune destruction of pancreatic beta cells, and is prone to episodes of ketoacidosis. A minority of patients fall into this category of Type 1 diabetes mellitus (Alberti & Zimmet, 1998).

2- Type 2 Diabetes

Type 2 diabetes mellitus (T2DM), also known as adult-onset diabetes and NIDDM, is considered an epidemic that affects more than 170 million people in the world. 90-95% of diabetics have T2DM which is characterized by an insulin insensitivity with a gradually decreasing beta-cell function (Stumvoll et al., 2005). The severity of the insulinopathy ranges from insulin resistance with relative insulin deficiency all the way to an insulin secretion defect with insulin resistance. Hyperglycemia develops slowly and in the early stages of the disease the symptoms are not as marked as in T1DM. Hence the patient may not notice the symptoms and the disease can go on undiagnosed for many years (American Diabetes Association, 2015).

The majority of patients with T2DM are obese or have an increased percentage of fat in the abdominal area. It is a well-established fact that obesity creates some sort of insulin resistance which is a stepping stone towards diabetes. Even though an inactive

lifestyle and diet increase the probability of triggering T2DM, an individual's genetic make-up plays a certain role as well. A positive family history of T2DM increases the risk by 2 to 4 folds compared to a negative family history (Stumvoll et al., 2005).

Weight reduction, a more active lifestyle and pharmacological intervention might improve the insulin resistance, however normoglycemia won't be fully restored (American Diabetes Association, 2015).

D. Complications of diabetes

The morbidity and mortality of diabetes is associated with the long term microvascular and macrovascular complications caused by hyperglycemia and reduced insulin sensitivity (Daneman, 2005).

The chronic hyperglycemia is at the root of microvascular complications that include diabetic retinopathy, nephropathy and neuropathy, as well as accelerated macrovascular atherosclerosis disease which affects arteries that supply vital organs such as the heart, the brain in addition to the lower extremities. The latter puts the patient at an increased risk of myocardial infarctions, strokes and limb amputations while microvascular complications are the leading causes of blindness, end stage renal disease (ESRD) and several different neuropathies (Brownlee, 2001).

1- Molecular mechanisms of hyperglycemia induced damage

Four different hypotheses have risen in order to explain the microvascular and macrovasuclar complications caused by the hyperglycemia: increased flux through the

polyol pathway, increased formation of advanced glycation end products (AGEs), activation of protein kinase C (PKC) and increased flux through the hexosamine pathway.

In normal glucose concentration, a small percentage of glucose goes through the polyol pathway. However in cases of hyperglycemia, the concentration of intracellular glucose is increased which causes an over-activation of the polyol pathway. Aldose reductase, the first enzyme in the pathway, catalyzes the reduction of glucose with the consumption of nicotinamide adenine dinucleotide phosphate (NAD(P)H). Glucose is excessively converted to sorbitol while consuming large amounts of NAD(P)H. The resultant decrease in intracellular NAD(P)H causes a decrease in reduced glutathione regeneration which could increase intracellular oxidative stress (Forbes et al., 2013).

Increased intracellular glucose is the primary causative factor in the formation of intracellular and extracellular AGEs. Intracellular auto-oxidation of glucose yields several different intracellular carbonyls. These carbonyls react with the amino group of intracellular and extracellular proteins which will result in the production of AGEs. Intracellular AGEs precursors damage cells by three different mechanisms. First, the intracellular modification of proteins including transcription factors. Second, AGEs that diffuse out of the cell can modify extracellular matrix components which affects cell signaling and cause cellular dysfunction. Lastly, extracellular AGE precursors can interact with plasma proteins and modify them to cause the production of inflammatory cytokines and reactive oxygen species (ROS)(Brownlee, 2005).

Intracellular hyperglycemia also leads to activation of PKC due to overproduction of diacyl glycerol (DAG). However DAG is not the only activator or PKC; the increased

activity of the polyol pathway and AGEs production also increase PKC activation. The activation also causes the upregulation of several downstream pathological mechanisms such as PKC-induced activation of transforming growth factor-ß1 (TGF-ß1) and NAD(P)H-oxidase (Kanwar et al., 2011).

Usually, when glucose goes through glycolysis, it is transformed into glucose-6-phosphate then fructose-6-phosphate (F-6-P) and then onto the glycolytic pathway. Some of that F-6-P gets shunted into the hexosamine pathway in which an enzyme labeled as GFAT converts F-6-P into UDP-N-acetylglucosamine. When the latter is integrated in serine and threonine residues of transcription factors, we end up with pathological changes in gene expression such as overexpression of TGF-ß1 (Brownlee, 2005).

2- Macrovascular complications of diabetes mellitus

Macrovascular complications of diabetes are due to arterial damage. Major complications include cardiovascular disease (CVD) manifested as myocardial infarctions (MI), cerebrovascular disease exhibiting as strokes, myocardial dysfunction and peripheral vascular disease. (Forbes & Cooper, 2013). These clinical end results are preceded by pathological processes that affect the structure and function of arteries and are measured by arterial compliance, intima-media thickness and distensibility. One of these processes is atherosclerotic vascular disease (Daneman, 2005). Atherosclerosis is an intricate pathological process that involves several cell types and numerous cell-to-cell interactions. It starts out with a fatty streak and progresses to the formation of more a complex atherosclerotic plaque. The atherosclerotic plaque might occlude the blood vessel at the site

of formation or might become unstable and rupture, embolizing distal vessels which will lead to MI, unstable angina or even cerebral stroke (Forbes & Cooper, 2013).

3- Microvascular complications of diabetic mellitus

Microvascular disease affects the small blood vessels in several different organs. The most encountered microvascular complications are diabetic retinopathy, neuropathy and nephropathy.

The most prevalent microvascular complication is diabetic retinopathy. It involves the perturbation of all the retinal tissue components. The first sign of retinal damage is microaneurysms. The hypoperfusion of regions of the retina will progressively cause leakage of fluid into the eye, inflammation and swelling of the macula, labeled diabetic macular edema. A process called neovascularization comes next. These new vessels grow in an unchaperoned manner which will lead to hemorrhages and persistent blindness (Eisma et al., 2015).

Neuropathy is defined as damage to the peripheral nervous system. It is a debilitating disorder and diabetes is the most common causative agent. Patients diagnosed with neuropathy present with one or more of the following symptoms: numbness, tingling, pain and/or weakness. The symptoms are symmetrical with a more sensory involvement than motor. The loss of sensation in severe cases of diabetic neuropathy puts the patients at risk of developing ulcers which could lead to amputations of the lower extremities. Diabetic neuropathy can be life altering specially the lives of the patient with neuropathic pain which is very difficult to treat (Callaghan et al., 2012). Diabetic neuropathy not only affects the somatic division of the peripheral nervous system but also the autonomic division.

Orthostatic hypotension can develop as well as gastrointestinal symptoms which can affect the efficiency of oral medications.

E. Diabetic Nephropathy

Diabetic Nephropathy (DN) is one of the microvascular complications of diabetes. It is the leading causative agent of ESRD in adults. There is a high rate of mortality in patients with DN. The increased risk of macrovascular complications, such as MI and strokes, caused by DN account for more than half of the excess deaths (Caramori et al., 2003). Clinically, DN is characterized by the progressive development of proteinuria along with a decline in the glomerular filtration rate (GFR). The decline stretches over 10-20 years and if left untreated could lead to fatal uremia (Forbes & Cooper, 2013). Protein leakage is measured using albumin as a marker because of its size and charge which makes it impossible for it to leave a properly functioning glomerulus as well as it's abundance in the serum. In albuminuria there is a several fold increase in the urine albumin excretion (UAE) compared to the usually extremely low levels. Albuminuria in DN is divided into microalbuminuria and macroalbuminuria (Powell et al., 2013). Microalbuminuria develops as the first clinical symptom of DN. It may or may not progress to macroalbuminuria, also known as overt proteinuria, and ultimately ESRD if left untreated (Wolf et al., 2007).

1- Morphological Changes

The diabetic kidney is usually enlarged due to both hypertrophy and hyperplasia (Forbes & Cooper, 2013). Glomerular hypertrophy encompasses mesangial cell

proliferation, accumulation of extracellular matrix (ECM) proteins and thickening of the glomerular basement membrane (GBM). The increase in GBM width will ultimately lead to nodular glomerulosclerosis, also known as Kimmelstiel-Wilson lesions (Kanwar et al., 2011). The expansion of the mesangial matrix is caused by the excessive deposition of fibronectin and collagen IV, more specifically collagen α1 (IV) and α2 (IV) chains in the mesangial area, and collagen α3(IV) and α4(IV) in the GBM (Wolf & Ziyadeh, 2007). Morphological changes also occur in the tuberointerstitium. Tubular hypertrophy followed by the thickening of the tubular basement membrane as well as interstitial fibrosis are among the changes. The fibrosis is due to a transformation of tubular cells into interstitial cells, a process called epithelial-to-mesenchymal transformation which causes ECM to build up in the renal interstitium (Kanwar et al., 2011).

In the early phases of DN, the GFR is increased due to glomerular hyperthrophy that increases the filtration area, to tubular expansion, as well as hemodynamic factors such as tubular-glomerular feedback and angiotensin II affecting the tone of the afferent and efferent arterioles. As the tubule expands, more of the urinary filtrate is reabsorbed which increases the GFR through a positive feedback mechanism. This hyperfiltration causes the kidneys to filter excess amounts of glucose, proteins, growth factors and cytokines, which can trigger pathological pathways such as redox abnormalities, fibrosis and inflammation (Forbes & Cooper, 2013; Wolf & Ziyadeh, 2007; Yacoub et al., 2015). This hyperfiltration of temporarily “enhanced” kidney function in the early stages is accompanied by normal blood pressure (BP) and microalbuminuria that is not always evident. The GFR will eventually drop back to normal, the BP will rise and the microalbuminuria becomes

obvious which is explained by the mesangial expansion. When macroalbuminuria occurs it is followed by a decline in GFR with an increase in BP. Histologically this stage is explained by the appearance of Kimmelstiel-Wilson nodules and glomerulosclerosis (Appel, 2013).

2- Reactive oxygen species

ROS are oxygen radicals formed as byproducts of several different biological reactions. The ROS that induce renal damage include the superoxide anion ($\bullet O_2^-$), the non-radical hydrogen peroxide (H_2O_2), hydroxyl free radical ($\bullet OH$) and peroxynitrite ($ONOO^-$). The transformation of oxygen (O_2) into the free radical $\bullet O_2$- is the initial step in the formation of all ROS. $\bullet O_2^-$ is very unstable in aqueous solution and catalyzed into H_2O_2 by superoxide dismutase within seconds. H_2O_2 is then transformed into the highly reactive hydroxyl radical or detoxified into water by catalase and glutathione dismutase (Forbes & Cooper, 2013; Gill et al., 2006).

Under homeostatic conditions, production of ROS is neutralized by antioxidants. However when the generation of ROS becomes too excessive, due to hyperglycemia for instance, antioxidant consumption is overwhelmed and a state of oxidative stress occurs. Oxidative stress has negative effects on various tissues and may cause injury. It is a well-established fact that it contributes in a major way to the development of diabetic complications, including diabetic nephropathy, by activating the four major biochemical pathways mentioned previously: increased flux in the polyol pathway, AGEs formation, hexosamine pathway flux and activation of PKC (Ha et al., 2008; Noh et al., 2011). Reactive oxygen species can act as a signal amplifier. PKC, TGF-ß1 and Angiotensin II

(Ang II), to name a few, are all activated by ROS. Once activated, their signaling cascade ends up producing even more ROS, therefore creating a sort of positive feedback loop (Ha et al., 2008; Lee et al., 2003).

The generation of ROS occurs via two systems, primarily through the oxidative phosphorylation in mitochondria, the major source of superoxide (Nishikawa et al., 2000) and through NAD(P)H-oxidase system in much smaller amounts. NAD(P)H oxidase was first discovered in phagocytic cells as a complex producing superoxide anion as a line of defense against pathogens. The phagocytic oxidase, termed phox, is a membrane bound cytochrome made up of two subunits: $gp91^{PHOX}$ and $p22^{PHOX}$. With time, several $gp91^{PHOX}$ homologs were identified in a variety of tissues and named NOX 1 through NOX7 (Geiszt et al., 2004). In the kidney, NOX 2 is expressed in podocytes, mesangial cells and in the endothelium. NOX 3 is found in the fetal kidney and in the distal convoluted tubules (Gill & C.S., 2006). The most abundant NAD(P)H oxidase in the kidney is NOX 4, previously termed Renox (Geiszt et al., 2000). Block et al. located NOX4 in membranes and mitochondria in the kidney (Block et al., 2009). High glucose levels increase the expression of NOX which leads to an overproduction of ROS, resulting in a state of oxidative stress, causing an expansion of the mesangium and increasing collagen and fibronectin deposition (Asaba et al., 2005; Lee et al., 2003).

F. The renin-angiotensin system in diabetic nephropathy

The renin-angiotensin system (RAS) has been strongly linked to the complications of diabetes, leading to DN (Giacchetti et al., 2005). When the body is challenged by fluid loss or sodium restriction, the RAS comes into play to adjust the situation. Renin is secreted in the kidney by the juxtaglomerular apparatus, triggered by a decrease in the levels of sodium chloride delivered to the macula densa cells. Renin converts angiotensinogen into angiotensin I (Ang I). Ang I is then cleaved by Ang I converting enzyme (ACE) into Ang II, which is the active form. Ang II interacts with its receptors Ang II type 1 (AT 1) and Ang II type 2 (AT 2) (Luther et al., 2011).

Early studies on the RAS in DN focused on the systemic levels of RAS. Whereas the circulating levels of renin are normal or even low in diabetic states, an intrarenal RAS appears to be activated (Price et al., 1999). Ballerman et al. (1984) first reported that angiotensin II receptor density in the kidneys of 3 weeks diabetic rats decreased, which suggests an increase in Ang II generation (Ballermann et al., 1984). It was shown that Ang II concentration in several different renal compartments is higher that the systemic concentration. This suggest that intrarenal RAS is regulated independently of systemic RAS, which can explain the difference between the low plasma renin and high Ang II levels in diabetes(Seikaly et al., 1990).

Over activity of the RAS has detrimental effects on the kidney and contributes to the progression of DN to ESRD by altering renal function. Ang II has many physiological and biochemical effects that could contribute to DN. Hemodynamic effects include systemic hypertension, vasoconstriction of the afferent and efferent arterioles in the renal

circulation leading to increased glomerular pressure causing proteinuria and mesangial cell contraction which reduces the filtration surface area (Leehey et al., 2000; Roscioni et al., 2014). Non-hemodynamic effects of Ang II on the kidney are similar to the effects of high glucose. Glucose and Ang II both activate PKC, the previous through de novo synthesis of DAG by the polyol pathway and the latter through AT 1 receptors (Leehey et al., 2000). Additive effects of high glucose and Ang II can also increase PKC activation, which will then increase the production of TGF-ß1. AngII, through TGF-ß1, stimulates ECM synthesis and deposition in glomerular mesangial and proximal tubules, increases transcription and synthesis of collagen IV and is associated with cellular hypertrophy (Kagami et al., 1994; Wolf et al., 1997). Ang II is also a source of superoxide because it activates NOX 4 in the kidney. The NOX activation stimulates protein synthesis in mesangial and tubular cell which leads to renal hypertrophy. Some of the extracellular components regulated by ROS in the kidney include fibronectin, collagen and TGF-ß1 (Garrido et al., 2009).

ACE inhibitors (ACEi) are drugs that inhibit the angiotensin converting enzyme in the RAS system in order to reduce Ang II levels (Schrijvers et al., 2004). They have been studied extensively over the years for their renoprotective effects in diabetes. In a study conducted in by Lewis et al. (1993) it was established that captopril, an ACEi, protects the kidney and slows down the progression of DN. It was also concluded that captopril's protective mechanism of action is not purely antihypertensive (Lewis et al., 1993). Not only does it reduce interglomerular capillary pressure, several studies showed that in type 1 diabetic patients ACEi had reduced interstitial fibrosis, prevented glomerular hypertrophy and decreased urinary albumin levels in microalbuminuric patients (Nankervis et al., 1998;

Osterby et al., 2000; Ravid et al., 1998; Rudberg et al., 1999). These observations lead us to the conclusion that ACE inhibition influences structural changes in the kidney. The effect of ACEi on TGF-ß1 in the kidney has also been examined. Sharma et al. (1999) observed that, upon administration of captopril for 6 months to diabetic patients, plasma TGF-ß1 levels dropped, and this paralleled the prevention of progression of DN (Sharma et al., 1999). Interrupting the RAS decreases TGF-ß1 levels and is associated with reduced albuminuria and hypertrophy (Erman et al., 2004). As previously mentioned, Ang II activates NAD(P)H oxidase in the kidney; so inhibiting ACE will decrease the amount of ROS produced. The indirect inhibition of NAD(P)H oxidase, by blocking the formation of Ang II, reduces mesangial expansion and albuminuria in the kidney and is beneficial in delaying diabetic nephropathy (Onozato et al., 2002).

G. Cytochrome P450 metabolites of arachidonic acid

Arachidonic acid (AA), released by the action of phospholipases, are metabolized by three different pathways in the kidney: the lipoxygenase pathway which forms leukotrienes and 5-, 12- and 15-hydroxyeicosatetraenoic acids (HETEs); the cyclooxygenase pathway which leads to the formation of prostaglandins, prostacyclins and thromboxane; and the cytochrome P450 (CYP) pathway which metabolizes AA into several HETEs including 20-HETE and epoxyeicosatrienoic acids (EETs) (Natarajan, 2003).

1- Cytochrome P450 in the kidney.

In the kidney, more precisely in the glomerulus, proximal tubules and thick ascending loop of Henle (TALH), AA is mainly metabolized by CYP450 enzymes of 4A and 2C families into 20-HETE and EETs respectively (Maier et al., 2001).

Four isoforms of the CYP4A family have been identified in the rat: CYP4A1, 4A2, 4A3 and 4A8; all four are found in the kidney. Enzymes from the CYP4B and CYP4F also participate in the formation of 20-HETE in the kidney but to lesser extents (Roman, 2002).CYP4A mRNA has been identified along the rat nephron and renal vasculature. CYP4A2, CYP4A3 and CYP4A8 are found in the glomerulus, proximal tubules, cortical collecting ducts, and TALH (Ito et al., 1998). CYP4A1 and 4A3 are highly expressed in the kidney of neonatal rats, however their levels decrease in adulthood. CYP4A2 is not expressed in neonates, but its expression increases with age until they become the main isoform in the kidney (Omata et al., 1992). Nguyen et al. proved that these isoforms possess different catalytic efficiencies for the production of 20-HETE. Even though CYP4A2 is the most abundant in the kidney, it appears that CYP4A1's catalytic activity is 10 times greater than both CYP4A2 and A3 (Nguyen et al., 1999). In the human kidney, CYP4A11 and CYP4F2 are highly expressed in proximal tubules. Both enzymes contribute to the formation of 20-HETE (Lasker et al., 2000).

EETs are produced by a handful of CYP enzymes: 1A, 2B, 2C, 2D, 2E, 2J and 4A families, present in various tissues. CYPs of the 2C and 2J subfamilies are highly expressed in the proximal tubules of the kidney. CYP2C are the main enzymes responsible for the generation of EETs in epithelial and endothelial cells of the kidney (Imaoka et al., 2005;

Imig, 2005; Roman, 2002). CYP2J2 has emerged as the primary enzyme involved in the formation of EET in human; it is widely spread in extrahepatic tissues including the kidneys where CYP2J2 is expressed in the proximal tubule and medullary collecting duct (Ma et al., 1999; Roman, 2002).

2- 20-HETE and EETs in diabetic nephropathy.

In diabetes, the expression of CYP4A and CYP2C11 is altered. CYP4A expression and function is enhanced and 20-HETE production is increased while CYP2C11 is downregulated and EET production is decreased (Shimojo et al., 1993). These alterations are implicated in the renal damages of diabetic nephropathy (S. Eid, Maalouf, et al., 2013). 20-HETE overproduction bears hemodynamic and pathologic consequences on the kidney. From a hemodynamic aspect, 20-HETE is a known potent vasoconstrictor widely present in the renal microvessels that plays a vital role in regulating vascular tone (Alonso-Galicia et al., 2002). Ang II increases the expression of CYP4A and the production of 20-HETE while decreasing the levels of EETs by increasing their degradation (Croft et al., 2000; Zhao et al., 2003). Further analysis on the interaction between Ang II and 20-HETE production came to the conclusion that 20-HETE increased ACE expression. This suggests a positive feedback interaction between 20-HETE and the RAS (Imig, 2010).

Alteration in CYP4A and CYP2C and their respective metabolites 20-HETE and EETs contribute to the complications of diabetic nephropathy. Hyperglycemia upregulates the expression of CYP4A which increases the production of 20-HETE in the kidney. 20-HETE stimulates the production of ROS by activating the NAD(P)H oxidases NOX 1 and NOX 4. The production of superoxide anion by 20-HETE causes deposition of ECM

components such as fibronectin and collagen IV and leads to kidney hypertrophy. Eid et al (2013) found that with the increase in CYP4A expression and 20-HETE formation causing a state of oxidative stress, the expression of TGF-ß1 was also increased. As mentioned previously, TGF-ß1 contributes to the progression of diabetic nephropathy by promoting kidney hypertrophy and deposition of collagen IV and fibronectin (A. A. Eid et al., 2009; S. Eid, Abou-Kheir, et al., 2013; S. Eid, Maalouf, et al., 2013) . However it is important to note that Luo et al. (2009) found that in STZ induced diabetic rats CYP4A expression decreased and consequently 20-HETE levels were also decreased (Luo et al., 2009). These opposed findings show that the role of CYP4A and 20-HETE in diabetes is still controversial.

Hyperglycemia causes a decrease in EETs in the kidney. The inhibition of EETs induces production of ROS in the kidney and mediates the effects of high glucose on proximal tubular cells by increasing the deposition of collagen IV and fibronectin and causing hypertrophy, which suggests a renoprotective effect of EETs in diabetic nephropathy. It appears to be that overproduction of 20-HETE and decreased levels of EET cause renal injury in diabetes (S. Eid, Maalouf, et al., 2013).

N-hydroxy-N'-(4-butyl-2-methylfenyl) formamide (HET0016) is the first reported selective and most potent inhibitor of 20-HETE synthesis (Sato et al., 2001). It is highly selective for the CYP4A isoforms that produce 20-HETE. Miyata et al (2001) reported that HET0016 selectively inhibited the production of 20-HETE but not EETs in rats and humans microsomes (Miyata et al., 2001). Studies have shown that the administration of HET0016 in STZ induced diabetic rats or in cells exposed to high glucose decreased ROS

production, NOX 4 expression, deposition of ECM and renal hypertrophy (S.Eid et al. 2013; A.A. Eid et al., 2009). These observations suggest that HET0016 might have a positive therapeutic outcome in delaying the onset of diabetic nephropathy.

CHAPTER II
RATIONALE AND AIMS

The renin-angiotensin system in diabetes is unregulated. The overproduction of angiotensin II paves the way to diabetic nephropathy by increasing intrarenal pressure, activating cytokines such as TGF-ß1 which lead to extracellular matrix deposition, and creating a state of oxidative stress which causes the deposition of extracellular matrix and cellular hypertrophy. ACE inhibitors, such as captopril, delay the onset of diabetic nephropathy. By blocking the production of angiotensin II, the cascade of events that follow, such as the activation of TGF- ß1 or the production of reactive oxygen species, is inhibited.

Previous studies examined the role of 20-HETE, a CYP450 metabolite of arachidonic acid, and its involvement in diabetes mellitus. Its effect on renal hemodynamics and physiology makes it a contender in the progression of diabetic nephropathy. 20-HETE is a potent vasoconstrictor and will affect kidney hemodynamics by constricting kidney microvessels. It will also lead to the production of reactive oxygen species which affect kidney function due to the deposition of extracellular matrix components. It is important to note that angiotensin II activates CYP4A and causes overproduction of 20-HETE, which will then activate the production of angiotensin II in a positive feedback manner. Inhibiting the production of 20-HETE by administering HET0016, was shown to slow down the complications that are associated with its over production.

In this study, we hypothesized that diabetic nephropathy may be ameliorated or reversed by giving a combination of an ACE inhibitor, captopril, and a 20-HETE inhibitor HET0016. Since angiotensin II plays a role in the activation of 20-HETE and is itself involved in the progression of diabetic nephropathy through the renin angiotensin system, we assume that inhibiting its production and the production of 20-HETE by combining the treatments will yield a better outcome. This will be accomplished through examining:

1- Effect of captopril and HET0016 on kidney hypertrophy by calculating kidney weight to body weight ratio and histological examination of mesangial expansion.
2- Effect of captopril and HET0016 on kidney fibrosis by measuring collagen deposition in histological section, mRNA and protein levels of extracellular matrix components (laminin and collagen IV).
3- Effect of captopril and HET0016 on generation of reactive oxygen species by measuring mRNA and protein expression of the NAD(P)H oxidase NOX 4 and using DHE stain on renal tissue.
4- Effect of captopril and HET0016 on the expression of CYP4A1/A2/A3 in the kidney.
5- Effect of captopril and HET0016 on total urine protein excretion.
6- Effect of captopril and HET0016 on 20-HETE plasma levels.

CHAPTER III
MATERIALS AND METHODS

A. Animal Models

Male Sprague-Dawley rats (220-300 grams) were made diabetic by a single intravenal injection of streptozotocin (65 mg/kg/0.2 mL) dissolved in citrate buffer through the tail vein. Control rats were injected with and equal volume of the vehicle also through the tail vein. Induction of diabetes was confirmed 48 hours by measuring blood glucose level using an Accucheck glucometer (Roche, Germany),

Rats were subsequently divided into eight groups according to their treatment. Group (1) control non-diabetic rats given normal saline (C.V); group (2) diabetic rats given normal saline (D.V); group (3) control non-diabetic rats treated with 50 mg/kg/day of captopril, an angiotensin converting enzyme inhibitor, dissolved in normal saline (C.Cap); group (4) diabetic rats treated with captopril (D.Cap); group (5) control non-diabetic rats treated with N-hydroxy-N'-(4-butyl-2-methylphenyl) also known as HET0016, a potent inhibitor of 20-HETE, dissolved in 5% dimethylsulfoxide at a dose of 2.5 mg/kg/day (C.Het); group (6) diabetic rats treated with HET0016 (D.Het); group (7) control non-diabetic rats treated with a combination of captopril and HET0016 (C.Comb); group (8) diabetic rats treated with a combination of captopril and HET0016 (D.Comb). Normal saline and captopril were administered through an Alzet osmotic pump inserted subcutaneously, dorsally at the scapular region. The pump releases its content at a rate of

2.5 uL/hour for a period of 28 days.HET0016 was administered subcutaneously on a daily basis.

Group	Treatment	Method
Control Vehicle (C.V)	Normal Saline	Osmotic pump
Diabetic Vehicle (D.V)	Normal Saline	Osmotic pump
Control Captopril (C.Cap)	Captopril	Osmotic pump
Diabetic Captopril (D.Cap)	Captopril	Osmotic pump
Control HET	HET0016	Subcutaneous injection
Diabetic HET	HET0016	Subcutaneous injection
Control combination	Captopril	Osmotic pump
	HET0016	Subcutaneous injection
Diabetic Combination	Captopril	Osmotic pump
	HET0016	Subcutaneous injection

Table 1: Type and method of treatment per group.

Rats were housed in the Animal Care facility in the American University of Beirut in individual cages. The room temperature was at a constant 23 degrees with a light-dark cycle of 12 hours each. On days 0, 13 and 27, rats were put in metabolic cages for a period of 24 hours where food and water intakes were monitored, weight and blood glucose level were measured and urine and plasma were collected. Blood glucose levels were also measured once a week for control rats and twice a week for diabetic rats.

On day 28, rats were sacrificed by decapitation and several organs were harvested: left ventricle, both left and right kidney cortexes, thoracic and abdominal aortas and both

sciatic nerves. Kidney weights were measured before flash freezing them with liquid nitrogen and stored at -80 degrees. Part of the left ventricle, abdominal aorta and left kidney cortex were fixated in 4% formaldehyde for histological studies.

All experiments were conducted with the approval of the Institutional Review Board/Animal House Committee of the American University of Beirut. Rats had access to unlimited water and food pellets throughout the experiment except when they were placed in metabolic cages where food and water intake was monitored.

B. Protein expression analysis by western blot

Kidney tissues were grinded using a mortar and pestle immersed in liquid nitrogen to maximize the protein extraction. The homogenized tissue was lysed using RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate, 150 nM NaCl, 100 nM EDTA, 50 mM Tris-HCl pH 8, 1% tegitol (NP-40) and 1% protease and phosphatase inhibitors). After placing the eppendorfs on a rotor in a cold room for two hours, they were centrifuged for 30 minutes at 13,600 rpm at 4°C. The supernatant was collected and stored at -20°C.

Proteins in the supernatant were quantified using Lowry Protein Assay. For immunoblotting, 30 μg of proteins were separated on either 8% or 10% Polyacrylamide gel Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred on nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). Membranes were blocked in 5% low-fat milk dissolved in Tris-buffered saline and successively incubated with rabbit polyclonal anti-laminin (1:1000, Abcam), rabbit polyclonal anti-collagen 4 (1:1000, Abcam), rabbit

polyclonal anti-CYP4A1/A2/A3 (1:2000, Abcam) and goat polyclonal anti-Nox4 (1:500, Santacruz Biotechnology). The house keeping protein used was a goat polyclonal anti-HSC-70 (1:1000, Santacruz Biotechnology). All primary antibodies were visualized using anti-rabbit polyclonal IgG (1:20000) except for anti-Nox4 where goat anti-mouse IgG was used (1:7000, Santacruz Biotechnology). Bands were visualized using enhanced chemiluminescence (Biorad Laboratories, CA, USA) and quantified using Image J.

C. mRNA quantitation using RT-qPCR

In order to extract the RNA, kidney cortex samples were dounced in Trizol (Sigma-Aldrich, Steinheim, Germany). After centrifugation at 1200 rpm at 4°C, chloroform was added and the samples were centrifuged again at 12000 x g at 4°C for 30 minutes. The aqueous phase was collected, mixed with isopropyl alcohol and centrifuged again at 12000 x g at 4°C for 30 minutes. A white pellet, containing the RNA appears at the bottom. After discarding the supernatant, ethanol was added for washing. Samples were centrifuged at 7500 x g for 5 minutes at 4°C. After removing the ethanol, the samples were left to air dry for 30 minutes. Once dried, 15 μL of RNAase free water was added to the sample. The sample's concentration and purity were measured using Nanodrop ND-1000 spectrophotometer.

The RNA was then transformed into cDNA using the RevertAid first strand cDNA synthesis kit (Thermo Fischer Scientific, USA) according to the manufacturer's protocol. Briefly, RNA samples were mixed with oligo$(dT)_{18}$ primer and nuclease free water and

incubated at 65°C for 5 minutes. After placing the samples on ice, the following reagents were added in the described order: 5X reaction buffer, Ribolock RNAase inhibitor, 10 nM dNTP mix and finally RevertAid reverse transcriptase. Samples were then incubated for 60 minutes at 42°C followed by 5 minutes at 70°C for 5 minutes.

mRNA was quantified using CFX Connect (BioRad, CA, USA) with SYBR green dye and rat forward and reverse primers for CYP4A1/A2/A3, fibronectin, collagen IV, laminin and Nox 4. GAPDH was used as an internal control. The primer sequences are as follows:

Primer	Sequence	Annealing Temperature
CYP4A1/A2/A3	F: GGCCACTTCGACCCTTTCAA	60.44
	R: CTGCCAAAGCATATGGCACAG	60.2
Collagen α1 (IV)	F: CGTGGATGGTGTACCTGGTC	59.6
	R: ACCTCGTGAGCCATTGTAGC	59.8
Laminin	F: AGAAAAGGGTCAACAGGGCAA	60.1
	R: GACAGTGCCCAGGAAGAAGT	60.2
Nox 4	F: TTCGGGTGGCTTGTTGAAGT	60.11
	R: TGGGGTCCGGTTAAGACTGA	60.18
GAPDH	F: GGGGCTCTCTGCTCCTCCCTG	60.4
	R: CGGCCAAATCCGTTCACACCG	60.2

Table 2: Oligonucleotide primer sequences and conditions employed for RT-qPCR

Results were then analyzed using the ΔΔCT method.

D. 20-HETE levels in the plasma by enzyme-linked immunosorbent assay (*ELISA*)

Plasma samples collected on the day of sacrifice (day 28) were used for the measurement of 20-HETE levels using a competitive ELISA kit (Detroit R&D Inc.). Sample and assay preparation protocols were provided by the manufacturer. After unthawing the plasma samples, they were acidified using acetic acid. Ethyl acetate was then added at a 1:1 ratio. Samples were then centrifuged for 10 minutes at 2000 rpm. The top organic phase was collected and the interphase discarded. Ethyl acetate was added to the aqueous phase and the extraction was repeated twice more. Once all the organic phase was collected, it was lyophilized in order to get the extracted sediments.

The sediments were then saponified using 20% KOH and incubated for an hour at 50°C. They were diluted in distilled water and their pH adjusted using 20% formic acid. Extraction with ethyl acetate was repeated and the organic phase obtained was lyophilized.

After lyophilization, dimethylformamide (DMF), sample dilution buffer (SDB) and distilled water were added to the samples. Standards were prepared by serial dilution. The pre-coated ELISA plate was provided by the manufacturer. Blanks, standards and samples were loaded onto the plate followed by the HRP conjugate in every well except the blanks. The plate was incubated at room temperature for two hours after which it was washed and TMB was added to every plate. After an incubation of 30 minutes, the reaction was stopped by adding 2N sulfuric acid. The place was read SAME AS LOWRY at 450 nm.

E. Collagen deposition by Masson Trichome Staining

Fresh kidney tissue that was fixated in 4% formaldehyde was embedded in paraffin. 4 μm sections were put on a slide and left to dry. The slide was then dewaxed using xylol, rehydrated with decreasing concentrations of ethanol and washed in distilled water. The slide was then dipped in Bouin's solution, staining it yellow, for 30 minutes at 50°C. After again and stained with scarlet red. It was then washed, put in 5% phosphotungistic acid, washed again and finally stained with light green. A last wash was performed, the slide was dehydrated with increasing concentrations of ethanol and cleared with xylol. Collagen deposition was quantified using ImageJ.

F. Mesangial matrix deposition with Periodic Acid Schiff

Fresh kidney tissue that was fixated in 4% formaldehyde was embedded in paraffin. 4 μm sections were put on a slide and left to dry. The slide was then dewaxed using xylol, rehydrated with decreasing concentrations of ethanol and washed in distilled water. It was then dipped in 0.5% periodic acid, which was freshly prepared, washed in distilled water and dipped in Schiff reagent. The slide was subsequently washed with distilled water, dehydrated with increasing concentrations of ethanol and cleared with xylol. Mesangial expansion was quantified using ImageJ.

G. Reactive oxygen species detection by DHE staining

Frozen sections of unfixed kidney cortex samples were cut into 6μm thick sections and mounted on glass slides. 10μml/L of DHE (Calbiochem, Darmstadt, Germany) was applied to

each tissue section and the slides were incubated in the dark at room temperature for 30 minutes. Fluorescent images of ethidium-stained tissue were obtained with laser scanning confocal microscope (Zeiss 510 NLO). Ethidium bromide was excited at 570 nm and fluorescence was detected at 590 nm long pass filter. Generation of superoxide was demonstrated by red fluorescent labeling. Mean fluorescence intensity of the digitized image was measured with Image J software for quantification.

H. Total urine protein quantification

Urine collected on day 28 from rats in metabolic cages was used for the protein quantification. Total urine protein was quantified using the Lowry technique.

I. Statistical analysis

Results are represented as mean ± SEM. Statistical significance is determined using student's unpaired t-test. P-value <0.05 is considered as statistically significant.

CHAPTER IV
RESULTS

A. Blood glucose, body weight, kidney weight and renal hypertrophy index

Blood glucose level, prior to injection showed no significant difference. However when the diabetic groups D.V, D.Cap, D.Het and D.Comb, were injected with STZ for induction of diabetes (day 0) blood sugar levels increased significantly compared to their respective control groups (day 2) ($P<0.005$). On day 28, there was no significant difference among the four different diabetic groups. When comparing glucose values from day 0 and day 28 of the control rats given the same treatment, there was a significant increase in the blood glucose levels (table 3).

Body weight was significantly decreased in the D.V group compared to the C.V group ($P<0.005$). Diabetic rats treated with captopril, D.Cap, had a significantly higher body weight compared to D.V ($P<0.05$), however it was still significantly lower than its control ($P<0.005$). Weight in the D.Comb was significantly higher than D.V ($P<0.005$) and almost equal in value to its control C.Comb ($P>0.05$). D.Het weight is not significantly higher than D.V but it has a tendency to increase even if it's still significantly lower than its control C.Het ($P<0.005$) (table 4).

The weights of the right and left kidneys were measured and their average was used to calculate the kidney weight to body weight ratio in order to assess for renal hypertrophy. The diabetic groups all have an increased ratio compared to their control. There was no

difference between D.V, D.Cap and D.Het; their values are almost equal. However the ratio for D.Comb is lower even if not significantly (table 4).

Group	Blood glucose ± SE Day 0 (mg/dL)	Blood glucose ± SE Day 2 (mg/dL)	Blood glucose ± SE Day 28 (mg/dL)
Control + Vehicle	137.75 ± 5.80	178 ± 10.59	174 ± 12.72 θ
Diabetic + Vehicle	134.37 ± 6.43	381 ± 61.33 *	528 ± 45.83 *
Control + Captopril	132.12 ± 5.24	152 ± 10.11	170 ± 8.98 θ
Diabetic + Captopril	130.33 ± 2.42	406 ± 16.50 *	522 ± 31.91 *
Control + Het0016	131.62 ± 3.35	120 ± 3.77	172 ± 7.35 θ
Diabetic + Het0016	138.3 ± 6.21	454 ± 40.52 *	524 ± 31.67 *
Control + Combination	131 ± 6.27	157 ± 10.37	173 ± 5.02 θ
Diabetic + Combination	131.6 ± 2.94	352 ± 29.62 *	416.6 ± 65.22 *

Table 3: Average blood glucose levels on day 0, day 2 and day 28

*P<0.05 control vs diabetic from same treatment
#P<0.05 D.V vs diabetic treated;
θP<0.05 control d0 vs control d28 from same treatment

(N=8)

Group	Body Weight ± SE (g)	Kidney Weight ± SE (g)	K.W/B.W Ratio ± SE
Control + Vehicle	421.75 ± 23.76	1.70 ± 0.13	0.00404 ± 0.00017
Diabetic + Vehicle	281.00 ± 11.61 *	1.65 ± 0.14	000614 ± 0.00022 *
Control + Captopril	410.50 ± 6.19	1.76 ±0.08	0.00429 ± 0.00025
Diabetic + Captopril	318.44 ± 10.51 * #	2.01 ± 0.12 #	0.00631 ± 0.00021 *
Control + Het0016	390.50 ± 13.06	1.54 ± 0.09	0.00381 ± 0.00014
Diabetic + Het0016	299.20 ± 15.70 *	1.95 ± 0.16	0.00622 ± 0.00032 *
Control + Combination	388.75 ± 14.60	1.56 ± 0.08	0.00400 ± 0.00011
Diabetic + Combination	358.80 ± 9.84 *	1.92 ± 0.07 * #	0.00548 ± 0.00029 *

Table 4: Body weight, kidney weight and kidney weight to body weight ratio

*P<0.05 control vs diabetic from same treatment
#P<0.05 D.V vs diabetic treated;
(N=8)

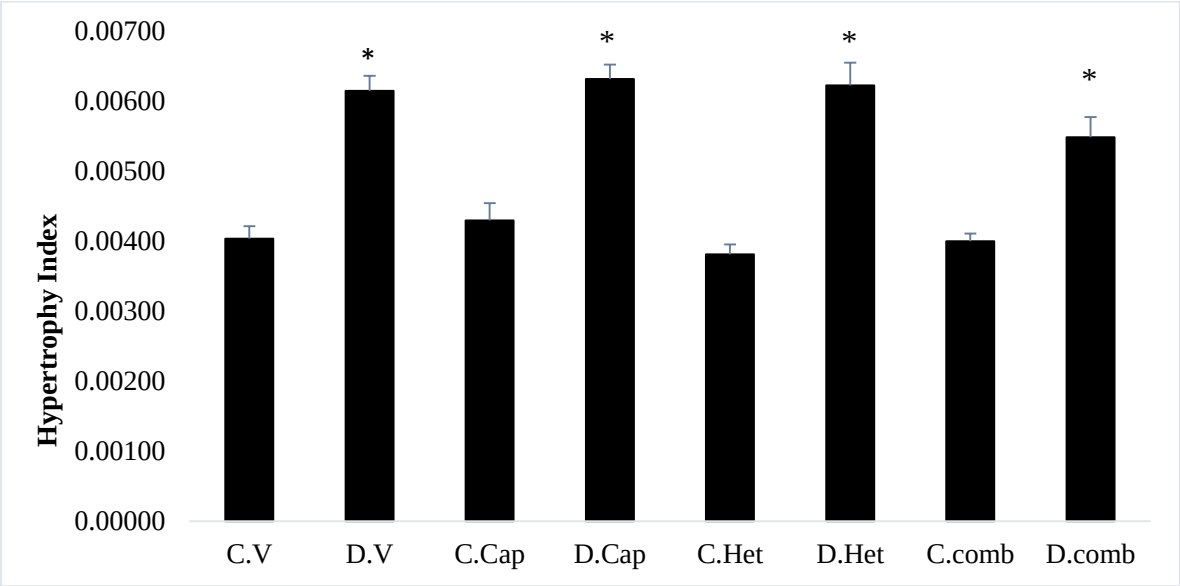


Figure 1: Kidney hypertrophy index

*P<0.05 control vs diabetic from the same treatment
#P<0.05 D.V vs diabetic treated
(N=8)

B. Urinary protein excretion

It is well established that diabetes induces increased urine protein excretion. Urine protein levels on day 28 differed between groups. D.V had a significantly higher protein concentration compared to C.V. All the control groups had similar protein concentrations whereas all the diabetic groups had higher concentration than their corresponding control. However, D.cap, D.Het and D.comb were significantly lower than their vehicle counter group (Figure 2).

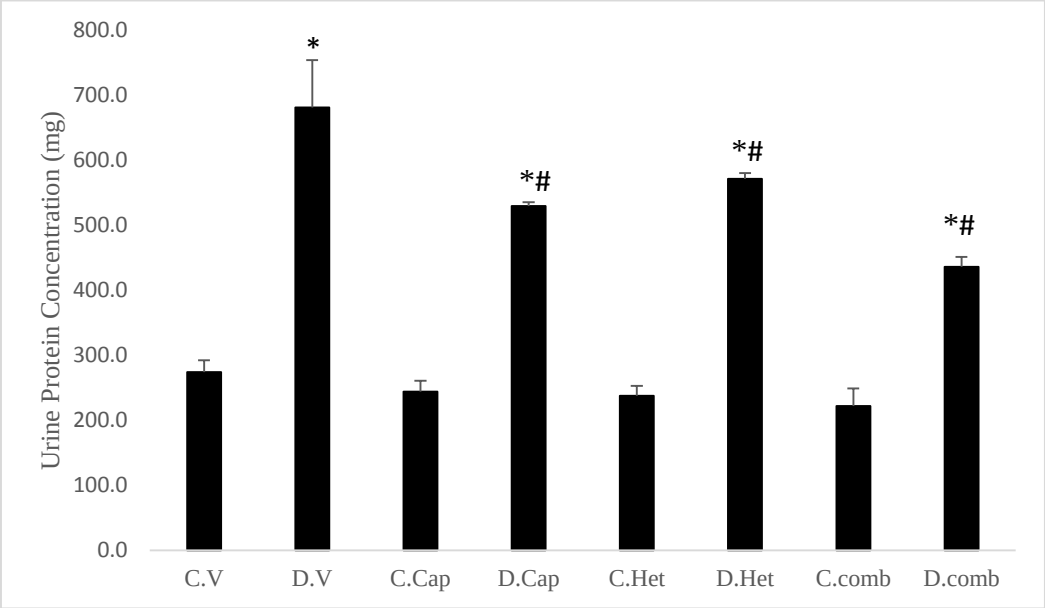


Figure 2: Total protein concentration in day 28 urine samples

*P<0.05 control vs diabetic from the same treatment
#P<0.05 D.V vs diabetic treated
(N=8)

C. Collagen deposition in the kidney

Collagen deposition was assessed using histological kidney section stained with Masson's trichome. D.V collagen deposition is significantly higher than C.V whereas all the diabetic groups, D.Cap, D.Het and D.Comb, were significantly lower than D.V and went back to their baseline value (Figure 3).

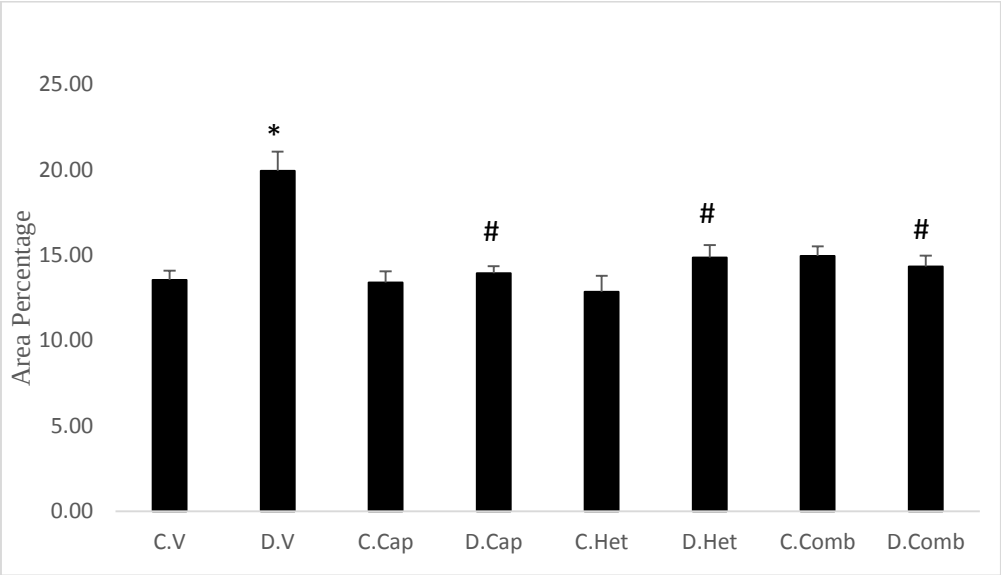


Figure 3: Percentage of collagen deposition in kidney tissue

*P<0.05 control vs diabetic from the same treatment
#P<0.05 D.V vs diabetic treated

(N=8)

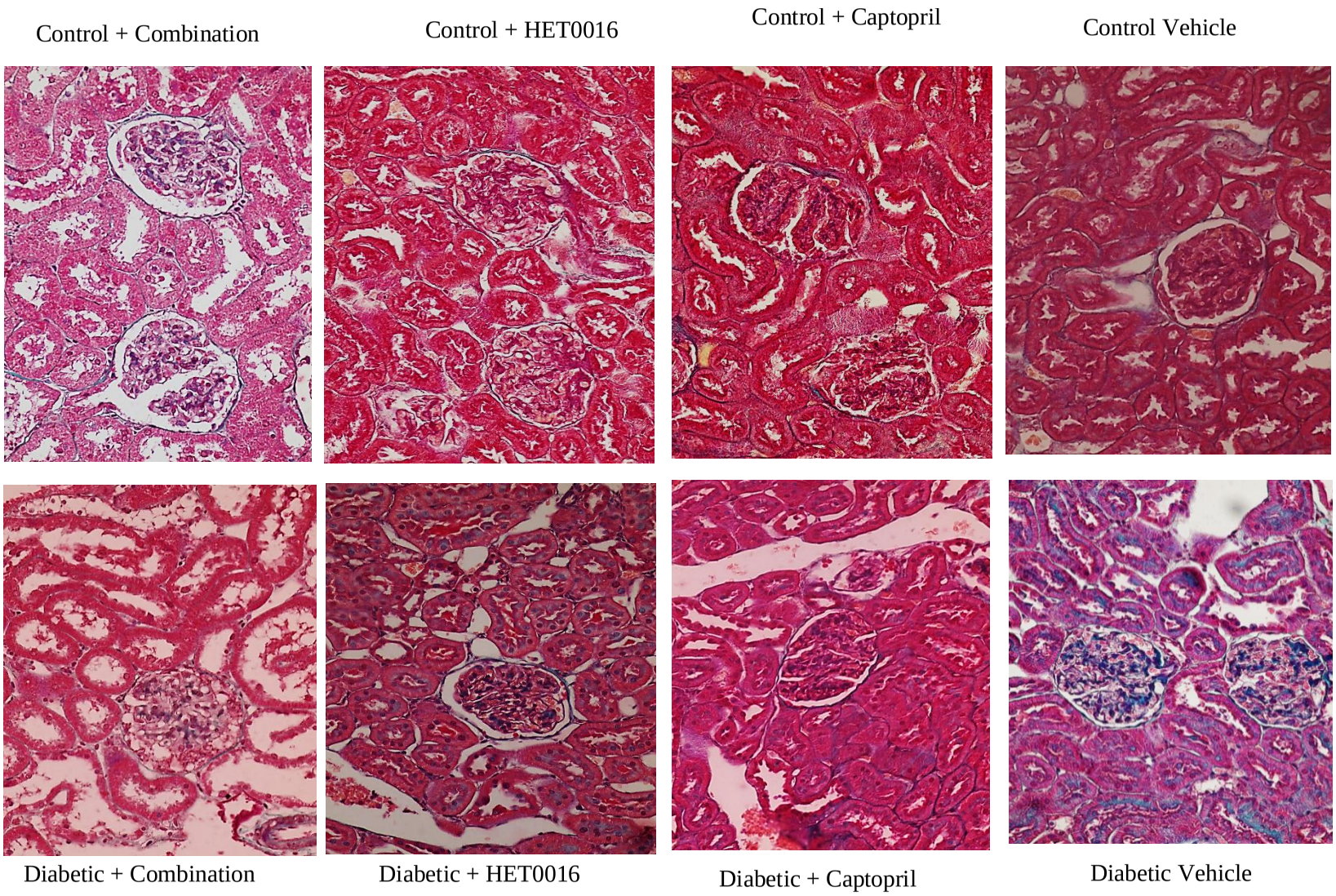
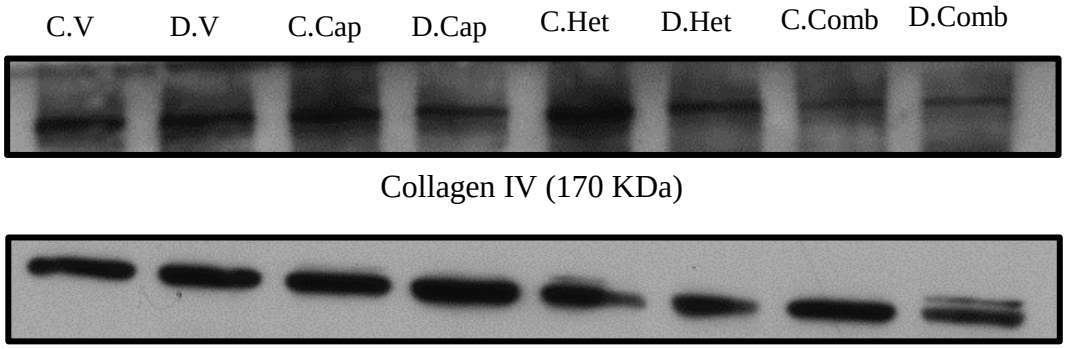

Control + Combination
Control + HET0016
Control + Captopril
Control Vehicle
Diabetic + Combination
Diabetic + HET0016
Diabetic + Captopril
Diabetic Vehicle

Figure 4: Kidney cortex stained with Masson Trichrome to assess collagen deposition.

Collagen IV protein expression was also assessed by immunoblotting. Collagen expression was significantly higher in the diabetic vehicle group. Diabetic groups treated with captopril, HET0016 and the combination of captopril and HET0016 had a significantly lower collagen expression (Figure 5).



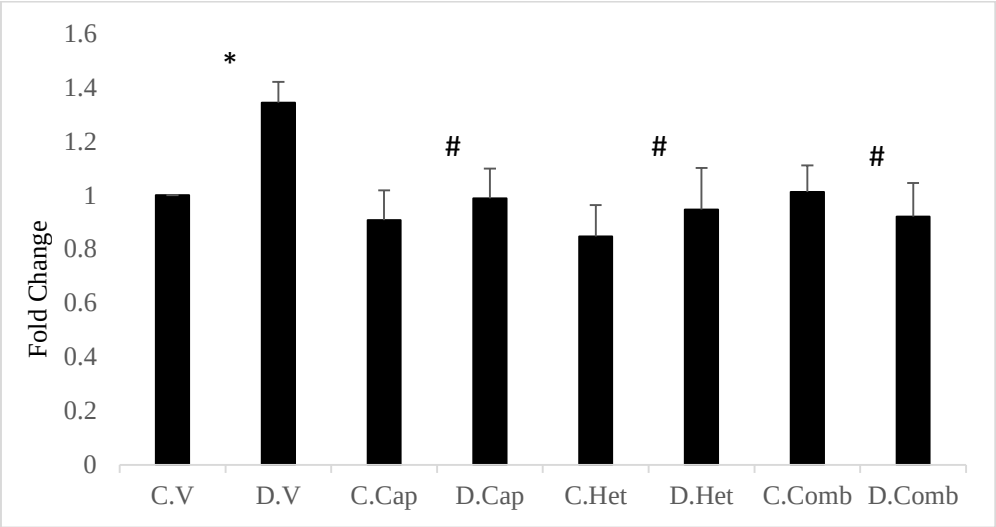
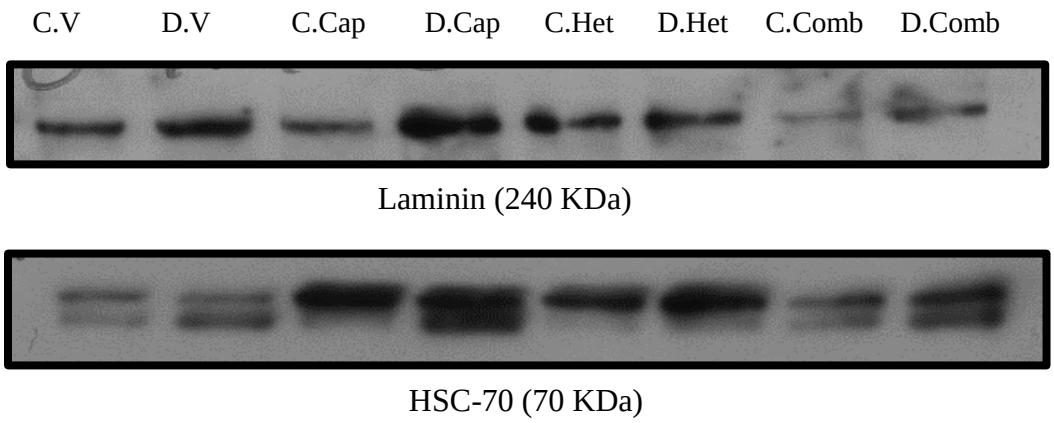


Figure 5: Collagen expression in the kidney cortex

*P<0.05 control vs diabetic from the same treatment
#P<0.05 D.V vs diabetic treated
(N=5)

D. Expression of extracellular matrix components

Protein and mRNA analysis was carried out for laminin, a component of the glomerular basement membrane. Protein expression for laminin was increased in D.V compared to C.V. All three treated diabetic groups had significantly lower protein expression of laminin compared to D.V. There was no significant difference between the control and diabetic group of each treatment (Figure 6).



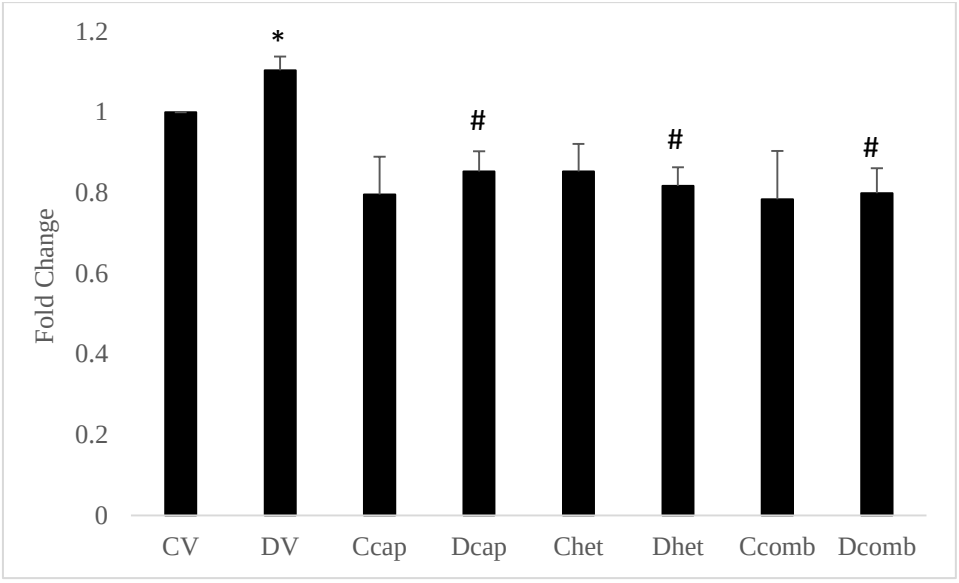


Figure 6: Laminin protein expression in the kidney cortex.

*P<0.05 control vs diabetic from the same treatment
#P<0.05 D.V vs diabetic treated;
(N=5)

The results of mRNA expression of laminin in the kidney cortex, were parallel to the protein expression results (Figure 7)

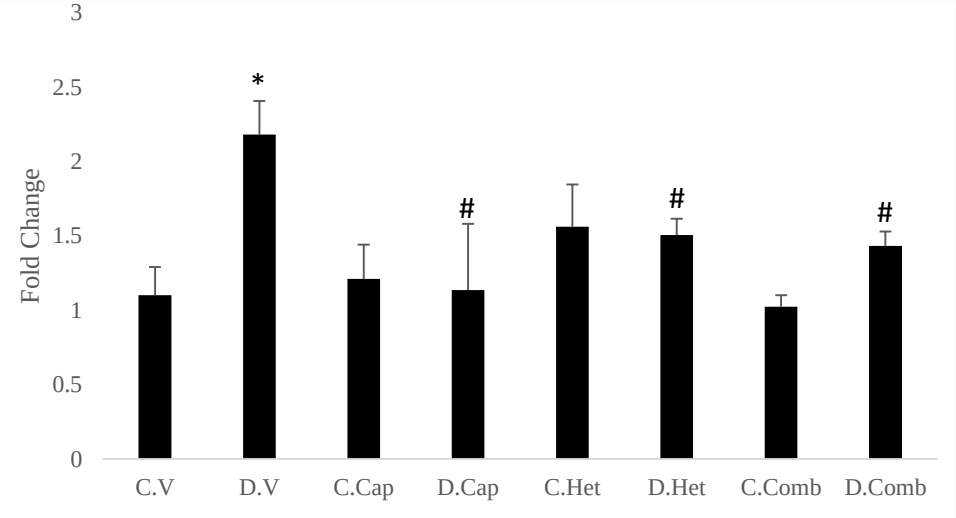


Figure 7: mRNA expression of laminin in the kidney cortex

*P<0.05 control vs diabetic from the same treatment
#P<0.05 D.V vs diabetic treated;
(N=5)

E. 20-HETE measurement in plasma

20-HETE levels were measured using ELISA. Levels were significantly higher in the diabetic non treated group D.V compared with the C.V group. Administration of HET0016 dropped the levels of 20-HETE in D.Het to same level of its control C.Het. We can see the same effect in the combination treatment (D.Comb). In the D.Cap group, levels of 20-HETE decreased but not significantly. They were still higher than their control C.Cap (Figure 8).

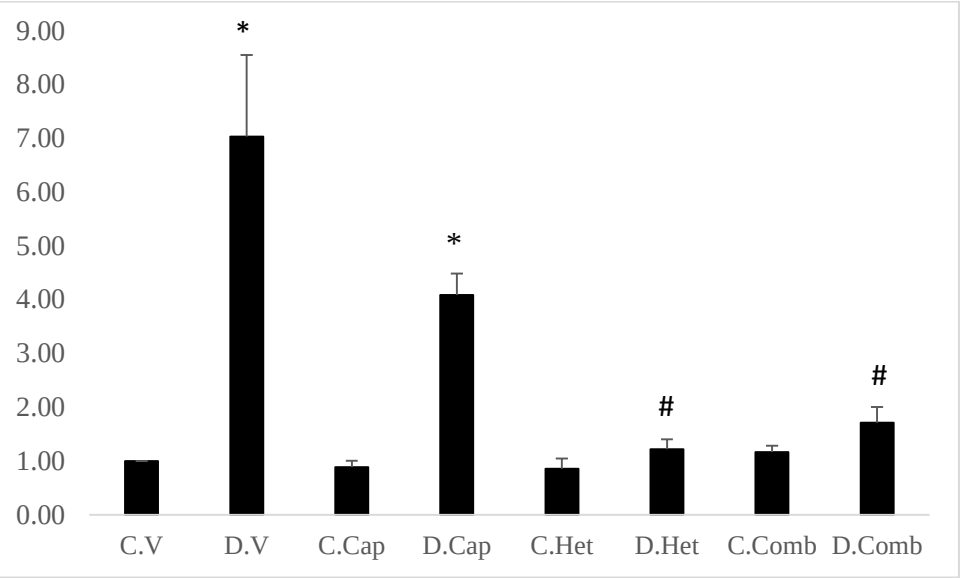
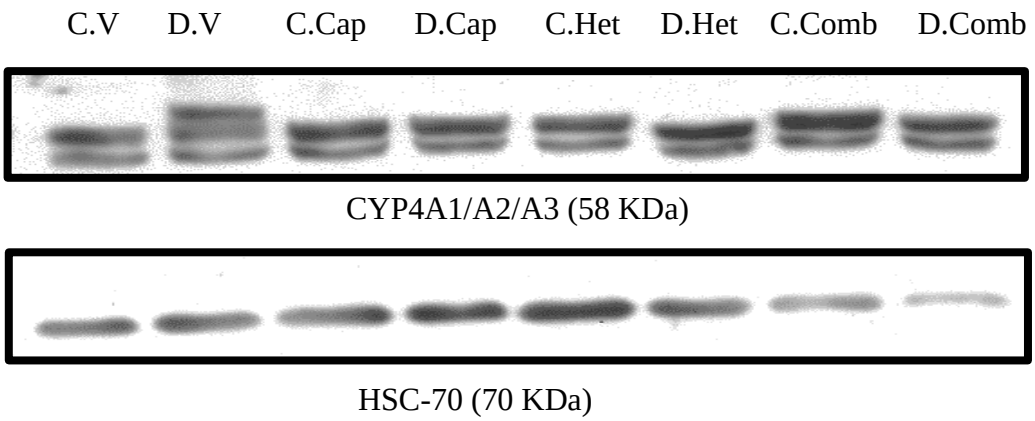


Figure 8: 20-HETE levels in plasma measured by ELISA

*P<0.05 control vs diabetic from the same treatment
#P<0.05 D.V vs diabetic treated
(N=4)

F. CYP4A1/A2/A3 expression in the kidney cortex

Protein expression of the cytochrome responsible for the production of 20-HETE, CYP4A1/A2/A3, was measured by immunoblotting. We observed a significant overexpression of CYP4A in D.V when comparing it to C.V. D.cap has a lower protein expression of CYP4A than D.V but it was not statistically significant. D.Het and D.comb have significantly lower protein expression compared to D.V, and the values were similar to those in their respective control groups (Figure 9).



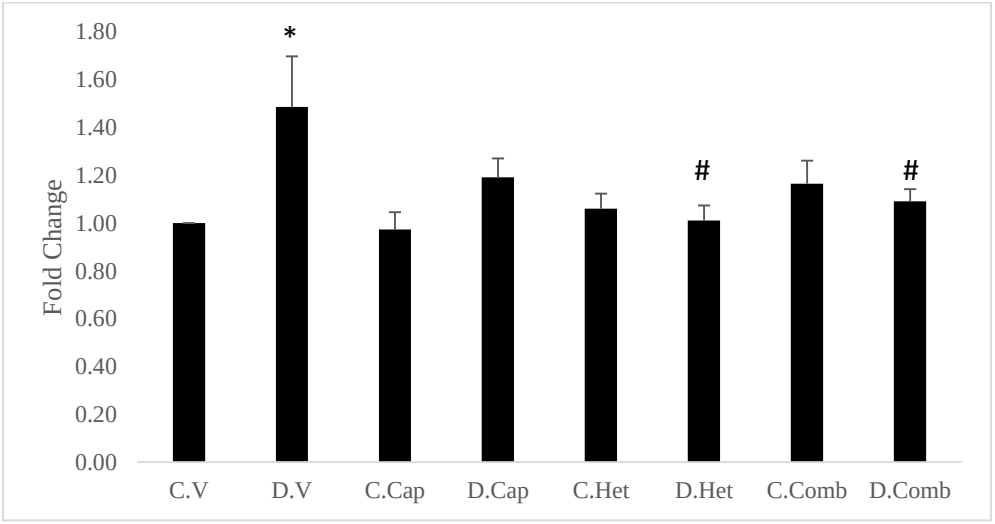


Figure 9: Protein expression of CYP4A1/A2/A2 in the kidney cortex

*P<0.05 control vs diabetic from the same treatment
#P<0.05 D.V vs diabetic treated
(N=5)

The mRNA quantification by RT-qPCR paralleled the results observed in the protein quantification for CYP4A, except that D.Cap expression of CYP4A was similar to the D.V expression (Figure 10)

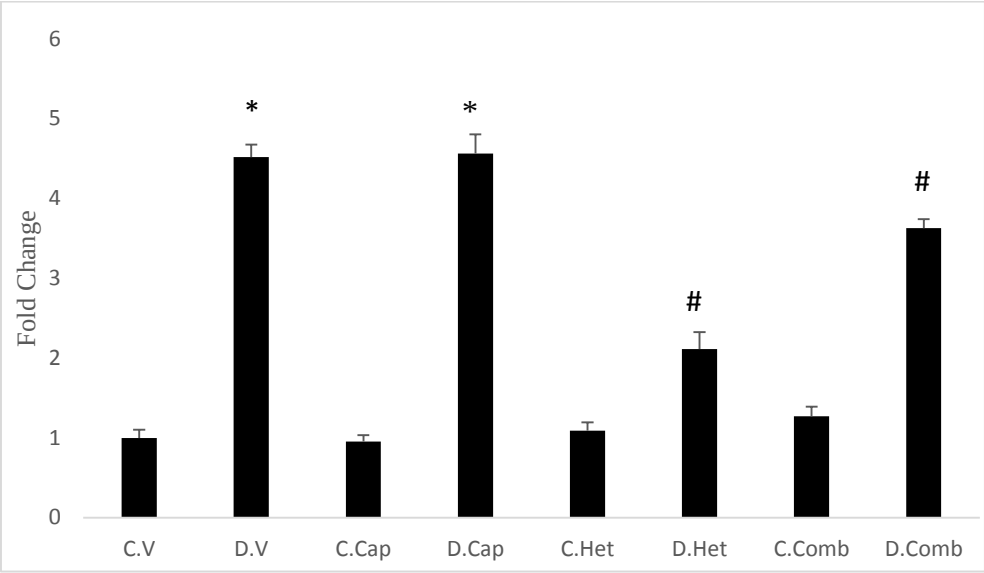


Figure 10: mRNA expression of CYP4A1/A2/A3 in the kidney cortex

*P<0.05 control vs diabetic from the same treatment
#P<0.05 D.V vs diabetic treated
(N=3)

G. Expression of NOX 4 in kidney cortex

The mRNA expression of NOX 4 was quantified by PCR. The expression of NOX 4 was significantly elevated in D.V compared to C.V. It was also significantly elevated in comparison to D.Cap,in which the levels were very different from its respective control group, and D.Comb which had a similar level to its control. Even though we see a decrease in the levels of NOX 4 in the diabetic rats treated with HET0016, the decrease is not statistically significant to D.V and significantly higher than its control (Figure 11).

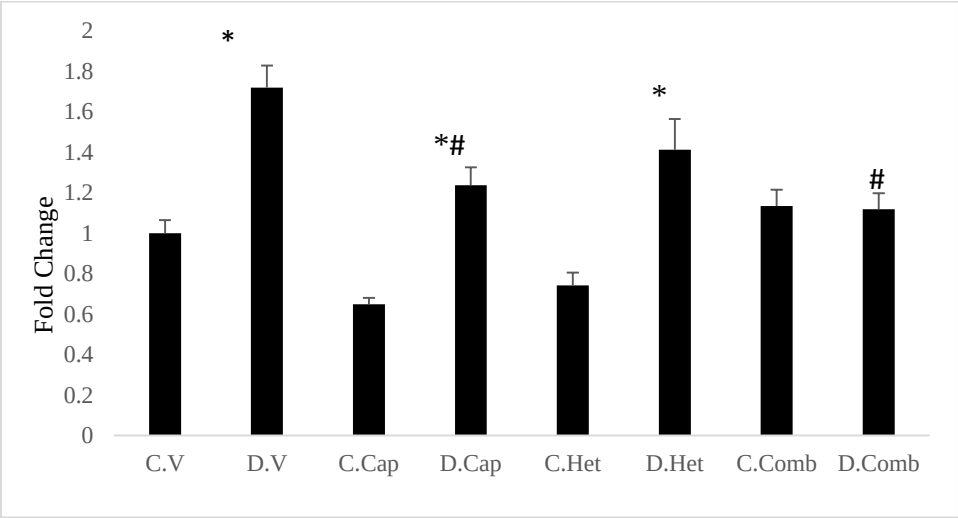
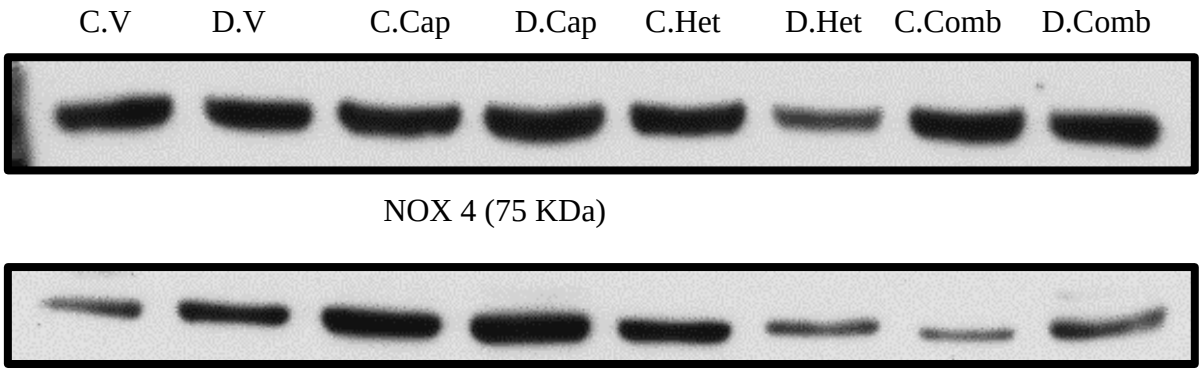


Figure 11: NOX 4 mRNA expression in the kidney cortex

*P<0.05 control vs diabetic from same treatment
#P<0.05 D.V vs treated diabetic
(N=3)

Western blot analysis of NOX 4 in the kidney did not show any significant change in all of the groups, whether control or diabetic (Figure 12)



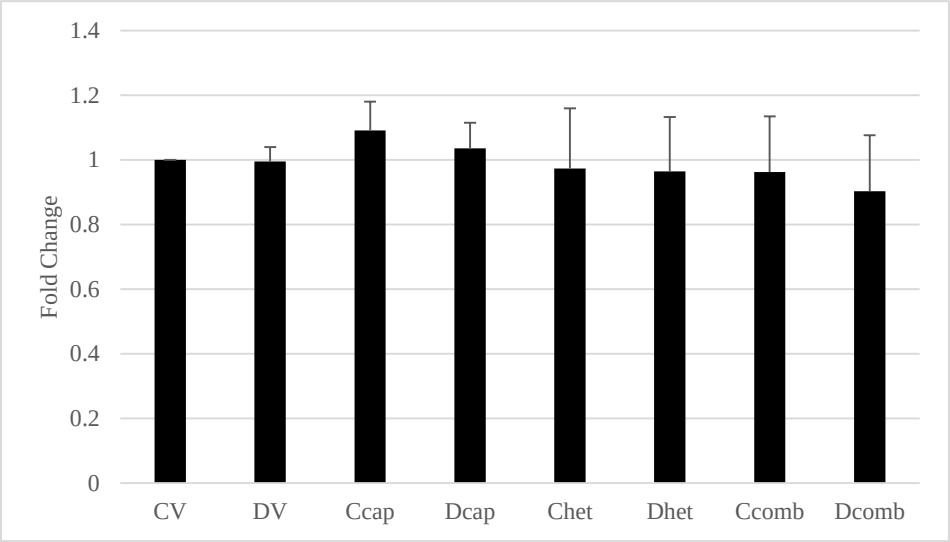


Figure 12: Protein expression of NOX 4 in the kidney cortex

*P<0.05 control vs diabetic from same treatment
#P<0.05 D.V vs treated diabetic
(N=5)

H. Renal ROS production

Reactive oxygen species production by kidney tissue was assessed by staining frozen sections with DHE using a confocal microscope. The amount of ROS detected in the D.V group was significantly higher than C.V. It was also significantly higher than all of the diabetic treated groups, whether captopril, HET0016 or the combination captopril and HET0016 was given. Compared to their control, D.Cap, D.Het and D.Comb were not significantly different (Figure 13)

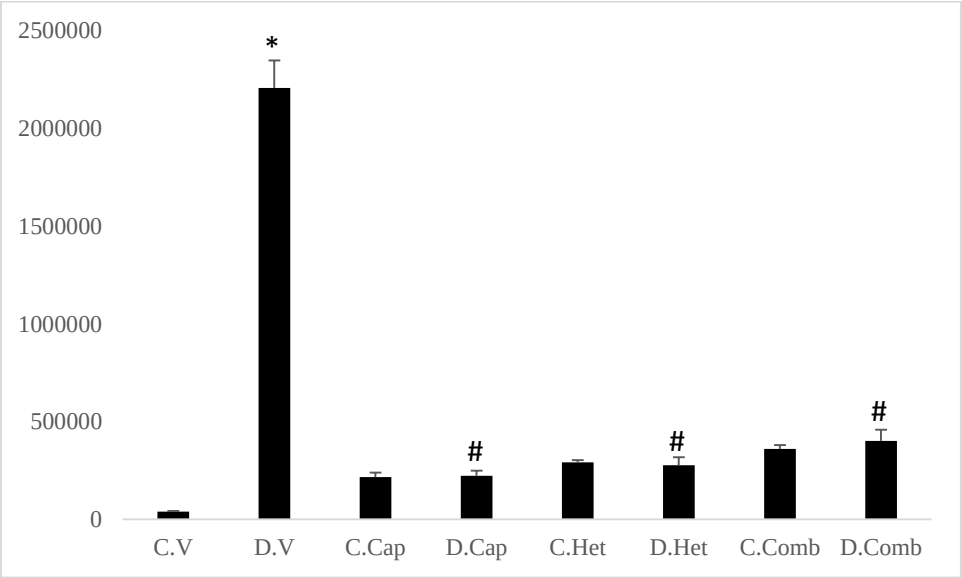


Figure 13: Amount of reactive oxygen species produced in the kidney

*P<0.05 control vs diabetic from same treatment
#P<0.05 D.V vs diabetic treated;
(N=3)

Control Vehicle
Diabetic Vehicle
Control + Captopril
Diabetic + Captopril
Control + HET0016
Diabetic + HET0016
Control + Combination
Diabetic + Combination

Figure 14: Kidney cortex frozen sections stained with DHE to assess ROS production.

CHAPTER V
DISCUSSION

This study was conducted in order to assess the role of 20-HETE, an arachidonic acid metabolite, in diabetic nephropathy as well as the effect of the combined treatment of captopril, an angiotensin converting enzyme inhibitor, and HET0016, a 20-HETE synthesis inhibitor on diabetes induced renal damage.

In order to detect renal injury, several factors have been examined. Kidney weight to body weight ratio in diabetic rats treated with the vehicle was significantly higher than the control rats indicating the kidney hypertrophy may have occurred. Treatment with HET0016, captopril, and their combination did not prevent this change. It should be noted that kidney weight was wet weight rather than dry weight which would have provided a more accurate reflection of hypertrophy. Renal injury was also assessed by studying the deposition of collagen IV and laminin in the kidney cortex. Masson trichrome stain showed an increase in collagen deposition in renal tissue, western blot analysis showed an increase in the expression of both collagen IV and laminin and RT-qPCR analysis showed an increased in laminin mRNA in the diabetic vehicle group, which was expected in the early stages of diabetes consistent with previous findings (Mason, 2003). Urine protein excretion on day 28 was also increased in diabetic non treated rats which is a sign of kidney damage. It is well established that chronic hyperglycemia is a source of ROS (Ha et al., 2008) which was observed in the increased level of ROS in the diabetic vehicle group when we stained kidney tissue with DHE.

A chronic state of hyperglycemia, like the case of the non-treated diabetic rats, induces CYP4A1/A2/A3 and subsequently the overproduction of its metabolite 20-HETE (S. Eid, Maalouf, et al., 2013) as shown here by the increased protein expression and mRNA expression of CYP4A1/A2/A3 as well as the increased levels of 20-HETE in the plasma of diabetic rats, contrary to the observations made by Luo et al (2009) who detected a decreased expression of CYP4A and 20-HETE production in diabetic rats (Luo et al., 2009). Eid et al. (2009) have shown than an increased expression of CYP4A and 20-HETE are a source of ROS in diabetes and that 20-HETE induces NOX 4 activation (A. A. Eid et al., 2009), also a source of ROS (Lee et al., 2003), which coincides with the increased NOX 4 mRNA expression in the diabetic group compared to the control group as well as the increased ROS production observed by DHE staining.

Urinary protein excretion was significantly lower in the diabetic groups treated with captopril, HET0016 and the combination relative to the diabetic non-treated group. All treatments also decreased collagen protein expression and deposition as seen in stained histological sections. Collagen expression in the diabetic treated groups was similar to their control with no significant difference. The same is observed in laminin protein and mRNA expressions, where laminin expression dropped back to baseline level in the diabetic rats treated with captopril, HET0016 and the combined treatment. It is well established that captopril improves protein excretion in diabetes by decreasing extracellular matrix deposition and ameliorating proteinuria (Lewis et al., 1993) as well as decreasing angiotensin II production which reduces intracapillary pressure and improves GFR (Nankervis et al., 1998). However the role of HET0016 in directly ameliorating kidney

function in diabetes has not been fully elucidated. The decreased kidney scarring might be an indirect effect of the inhibition of 20-HETE which will be discussed further on. There was a tendency for combined treatment with captopril and HET0016 to further reduce protein excretion relative to the diabetic group treated with vehicle but this was not significant.

20-HETE plasma levels decreased in diabetic rats treated with captopril but it was not significant. In diabetic rats treated with HET0016, a 20-HETE synthesis inhibitor, plasma 20-HETE levels went back to their control values. The same thing was observed in the combined treatment. The protein expression of CYP4A mirrored the 20-HETE plasma levels results, where the expression in the rats treated with captopril is lower but not significantly whereas the diabetic groups treated with HET0016 and the combination had similar protein expression when compared to their control. HET0016 is a drug that specifically inhibits CYP4A production of 20-HETE (Miyata et al., 2001) so we were expecting an extremely significant decrease in CYP4A protein expression and 20-HETE levels in the groups treated with HET0016 and the combined treatment. Studies have found that angiotensin II increases the expression of CYP4A which increases the production of 20-HETE (Croft et al., 2000). According to Imig (2010) 20-HETE in turn increases the expression of angiotensin converting enzyme in a positive feedback manner. The observed decrease in 20-HETE plasma levels and CYP4A expression in diabetic rats treated with captopril was expected even if it was not significant. mRNA expression of CYP4A in the groups treated with HET0016 and the combination is decreased, however when it came to captopril the expression was as high as the diabetic non treated group which does not

correlate with the observations made in the protein expression and 20-HETE plasma levels. This discrepancy needs to be further studied by increasing the number of samples for mRNA analysis.

Nox 4 mRNA expression was significantly lower in the diabetic group treated with captopril and with the combined treatment. Treatment with HET0016 decreased the mRNA expression as well but not to the point of significance. NOX 4 expression was lower in the combined treatment than both treatment alone, which suggest a synergistic effect when it comes to inhibiting NAD(P)H oxidases. Angiotensin II activates NAD(P)H oxidases (Garrido & Griendling, 2009), so inhibiting its production with captopril decreased its expression in groups treated with captopril and the combination. According to Eid et al. (2009), 20-HETE increases the expression of NOX 4 (A. A. Eid et al., 2009), so administering HET0016 will inhibit its expression as well, which is what we have observed even if the decrease was not significant. The NOX 4 expression did not change on a protein level. This can be explained by the fact that mRNA levels do not always correlate with protein expression due to several factors such as transcription, translation and protein degradation.

ROS production in all treated diabetic groups was significantly decreased compared to the non-treated diabetic group, whether the treatment was captopril, HET0016 or a combination of both. Angiotensin II and 20-HETE are both sources of ROS (Leehey et al., 2005), so the decreased ROS production was expected. 20-HETE induces renal damage by increasing extracellular matrix deposition through ROS production (Guo et al., 2007). The decreased expression of laminin and collagen in diabetic rats treated with HET0016 can be

explained by the decreased ROS production when diabetic rats where treated with HET0016.

The decrease in urine protein excretion observed in the group given the combined treatment of captopril and HET0016 is greater than with each treatment alone. The same trend can be observed in the mRNA expression of NOX 4. These observations suggest the possibility that HET0016 and captopril may have a synergistic effect when administered together. When evaluating collagen deposition by Masson and immunoblotting, the combined treatment brought back collagen expression to the same level as the control just like the individual treatments of captopril and HET0016. The same effect was observed in laminin expression, CYP4A expression, 20-HETE plasma levels and ROS production. The fact that the combined treatment brought everything back to baseline level, just like the individual treatments of captopril and HET0016, did not allow us to identify whether the effect was due to synergism between the two drugs or the effect of one of the drugs only.

In conclusion, inhibiting 20-HETE production by administering HET0016 to diabetic rats lowered kidney injury: urine protein excretion was lower, as were collagen deposition and expression, laminin expression and ROS production compared to the diabetic non-treated group. This indicates that 20-HETE overproduction plays a role in kidney injury in diabetes. The diabetic group treated with captopril had lower 20-HETE levels in the plasma and CYP4A expression tended to decrease which indicates that there is a cross talk between 20-HETE and angiotensin II. The combined treatment of captopril and HET0016 presents a potential for therapeutic intervention and could ameliorate the outcomes of diabetic nephropathy by decreasing ROS, deposition of extracellular matrix

and renal hemodynamics. More studies are needed to confirm and extend these findings. A longer period of diabetes prior to starting the treatment would show us how the combination of HET0016 and captopril affect the damage that already occurred or a longer period of treatment would show the long term effect of the combined treatments on kidney physiology.

CHAPTER VI

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