

AMERICAN UNIVERSITY OF BEIRUT

CARDIAC FIBROBLAST INJURY IN DIABETES:
NEW INSIGHTS ON THE ROLE OF CYTOCHROMES P450

by
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AN ABSTRACT OF THE THESIS OF

Rafka Bassam Abou Salem for Master of Science
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Title: Cardiac Fibroblast Injury in Diabetes: New Insights On the Role of Cytochromes P450

Background: Diabetic cardiomyopathy (DCM), a serious complication of diabetes, is characterized by systolic and diastolic dysfunction, cardiomyocytes (CM) hypertrophy, and cellular matrix accumulation. Cardiac fibroblasts (CF), which make up around 70% of a rat's heart, play an important role in cardiac remodeling. DCM is associated with altered cytochrome P450 enzyme (CYP) expression and reactive oxygen species (ROS) production. It is known to re-induce the “fetal gene program” and activate autophagy pathway as a cell's way of survival under stressful conditions.

Aim: The present study aims to determine the effect of HET0016 or AUDA treatment on the biological output of the left ventricle (LV) and ROS generation. This work will investigate the mechanism of myocardial cell injury and how the treatments can alter the cross-talk between CYPs and oxidative stress and other possible mechanisms.

Methods: 6 weeks streptozotocin (STZ) - induced diabetic rats received injections of either HET0016 or AUDA for 5 weeks. Afterwards, cardiac function and histological and biochemical parameters were measured. Echocardiography was performed to measure systolic function. Dihydroethidium (DHE) was used to assess intracellular ROS production levels. TUNEL assay was done to detect cellular hypertrophy and apoptotic cells. Western blot was performed to study the protein expression of: α - and β -myosin heavy chain (MHC), CYP2C11 and 4A1/A2/A3, beclin-1 and LC3B, and tau (p-S214). Histological alterations were measured using periodic acid Schiff (PAS) stain.

Results: Treatment with HET0016 or AUDA reversed systolic dysfunction and prevented oxidative stress. AUDA treatment reversed “fetal gene program”. Type 1 diabetes mellitus (T1DM) increased autophagy; beclin-1 protein level was affected by HET0016, while LC3B protein level was affected by AUDA. It also increased tau hyperphosphorylation, while treatment with HET0016 or AUDA reduced tau hyperphosphorylation.

Conclusion: Our results indicate that hyperglycemia leads to ROS generation and systolic dysfunction through altering CYP protein expression. This further leads to “fetal gene program” induction, cardiac cell injury, autophagy and tau hyperphosphorylation.

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LIST OF ABBREVIATIONS

DCM:	Diabetic Cardiomyopathy
T1DM:	Type 1 Diabetes Mellitus
CF:	Cardiac Fibroblast
CM:	Cardiomyocyte
LV:	Left Ventricle
ECM:	Extracellular Matrix
ER:	Endoplasmic Reticulum
DHE:	Dihydroethidium
ROS:	Reactive Oxygen Species
CYP/CYP450:	Cytochrome P450
sEH:	Soluble Epoxide Hydrolase
AA:	Arachidonic Acid
EETs:	Epoxyeicosatrienoic acids
20-HETE:	20-Hydroxyeicosatetraenoic acid
DHETs:	Dihydroxyeicosatrienoic acids
MHC:	Myosin Heavy Chain
DDR2:	Discoidin Domain Receptor 2
STZ:	Streptozotocin

CHAPTER I

INTRODUCTION

A. Diabetes Mellitus and Its Effect on the Myocardium: An Overview

Diabetes is a major public health problem that affects about 20 % of the world population. The incidence of diabetes has increased immensely in the past 10 years. This alone makes it an epidemic disease. Diabetes is a chronic, often debilitating and sometimes fatal disease, in which the body either cannot produce insulin (type 1) or cannot properly use the insulin it produces (type 2). Diabetes is associated with a number of metabolic risk factors that contribute to a high rate of micro- and macrovascular events. In this study we will be focusing on the cardiac complications induced by the onset and progression of type 1 diabetes (1, 2).

T1DM, also known as juvenile diabetes or insulin-dependent diabetes, is a chronic disorder in which the immune cells attack pancreatic beta cells leading to their deficiency. Consequently, very low or no insulin is present in the plasma for uptake of glucose by the cells. Hence, diabetics face the corollary of hyperglycemia on daily basis (3). The onset of T1DM can arise at any age, but most commonly develops between infancy and late 30s. T1DM is rare and comprises 5-10% of all cases related to diabetes (4).

Hyperglycemia serves as a major risk factor in the development of diabetic cardiomyopathy, comprising functional and structural abnormalities in the heart, including diastolic and/or systolic dysfunction, altered cardiac contractility, cell hypertrophy, apoptosis and interstitial fibrosis (5, 6). It has been proposed that oxidative stress can be a possibility for causing deterioration of the diabetic heart (7). As the disease progresses, there is increased myocyte loss accompanied by myocyte hypertrophy and fibrosis (8). These changes in cellular phenotype are accompanied by the reinduction of the fetal gene program (9).

Hypertrophy is the compensatory response of the myocardium to increased work, most often resulting from elevated pressures or increases in blood volume. Dilatation with stretching of the myocytes, while initially improving contractility becomes detrimental. The defining features of hypertrophy are enhanced protein synthesis, an increase in cardiomyocyte size and an increase and higher organization of the sarcomere, together with fetal gene activation (10). In addition to the changes in the myocytes, hypertrophy also includes changes in the extracellular matrix and the microvasculature. Various hypertrophic signaling pathways have been associated with protection of the heart from apoptosis (11, 12), as well as a predisposition of cardiac myocytes to apoptosis (13, 14). However, the mechanisms by which high glucose exerts its deleterious effects on the other types of cells in the myocardium, particularly on cardiac fibroblasts, remain poorly understood.

B. Cardiac Fibroblasts and Diabetic Cardiomyopathy

DCM is a major cause of morbidity and mortality in the diabetic population. DCM leads to myocardial dysfunction, even in the absence of discernible coronary artery, valvular, or hypertensive disease, and predisposes diabetic subjects to heart failure and increased mortality. Hyperglycemia plays a significant role in myocardial cell injury. Hyperglycemia and the resulting oxidative stress lead to abnormal gene expression and activate signal transduction pathways involved in the development and progression of cardiac hypertrophy, apoptosis and fibrosis (15).

A human's myocardium, which is very similar in composition to that of rats, is composed mainly of cardiac fibroblasts and cardiomyocytes, which comprise around 60-70% and 30-40%, respectively. This was also assessed to be likewise in the left ventricle (16, 17).

In particular, CF play a role in cardiac remodeling through synthesis and breakdown of ECM during pathological states, but also perform other functions under normal conditions (18). They are characterized by “low turnover unless stimulated by physiological or pathophysiological signals” (19). They communicate with other cells, including cardiomyocytes, endothelial cells and other fibroblasts. Such cell-to-cell communication affects the electrophysiological properties of the heart (18); it can influence it passively by hindering the electrical transmission from cell to cell, or actively (if coupled to CM) by synchronizing the electrical activity in the distant CM (19). In addition, cardiac fibroblasts can alter extracellular matrix (ECM) composition by generation and/or degradation, and can differentiate into myofibroblasts in response to a stimulus (18). Due to CF's wide variety of functions, it can contribute to myocardial hypertrophy, cell proliferation, and even apoptosis (20).

LV mechanical abnormalities, a diastolic dysfunction in particular, have been described in 40-50% of diabetic patients diagnosed with unknown cardiac complaint (21). Echocardiography revealed LV hypertrophy due to pathological changes in the cardiac tissue. This has been linked to ROS production and excessive ECM generation by CF inducing ventricular remodeling (7, 22). Accordingly, a possible correlation between CF morphological/functional changes and the development of DCM might exist, since the literature on the effect of hyperglycemia on CF is very limited (23).

C. Reactive Oxygen Species and Diabetic Cardiomyopathy

ROS production can be altered in a cell and tissue specific manner. There exists a correlation between diabetic cardiomyopathy and oxidative stress, in which excessive ROS production plays a major role in inducing ventricular remodeling that is characterized with hypertrophy, fibrosis and cardiac dysfunction (22). Diabetes and hyperglycemia lead to the overproduction of ROS in cardiac cells. This results in cardiac cell death, myocardial fibrosis and hypertrophy, and ultimately causing remodeling and dysfunction to the heart (24, 25). Mitochondria and NADPH oxidase are a major source for production of ROS.

We and others showed that CYP450 isoforms also can produce ROS in different organs of the body (26, 27). CYPs are enzymes that metabolize arachidonic acid into metabolites such as epoxyeicosatrienoic acids (EETs) and 20-hydroxyeicosatetraenoic acid (20-HETE) (28, 29). They are highly expressed in the myocardium (30). In a rat heart, CYP4A is the major enzyme that catalyzes hydroxylation of AA forming 20-HETE, and CYP2C11 catalyzes the epoxidation of

AA to EETs. Furthermore, CYP4A11 and CYP4F2 are responsible for 20-HETE formation in humans, while CYP2J2 synthesizes EETs (31).

CYP2C is one isoform that not only produces EETs but also is said to produce ROS. This initial burst of ROS is suggested to mediate EET-cardioprotective role (32). Also, studies conducted on humans propose that 20-HETE may be important in the development of cardiovascular disease in the metabolic syndrome (33). Another study showed that a reduction in alcohol intake was correlated with a significant decrease in blood pressure and urinary 20-HETE excretion, as well as decreased ROS production. This can lead to the conclusion that there might be a possible link between increased 20-HETE production and oxidative stress (34).

ROS scavengers are a potential therapeutic target to at least preserve myocardial function under hyperglycemic-established oxidative stress (35). However, the involvement of CYPs family in the heart to produce significant ROS, leading to DCM remains poorly understood.

D. Arachidonic Acid Metabolites and Cardiac Function

Arachidonic acid (AA) can be metabolized along one of three pathways: the cyclooxygenase, the lipoxygenase or the CYP450 monooxygenase pathway. In the third pathway, AA is metabolized by several CYP450 isoforms, which are mainly located in the endoplasmic reticulum, to produce 20-HETE and EETs among other eicosanoid products (36, 37). EETs are cardioprotective, while 20-HETE is injurious to the cardiovascular system (38). Soluble epoxide hydrolase (sEH), which is highly expressed in the myocardium, converts EETs to less metabolically active compounds known as

dihydroxyeicosatrienoic acids (DHETs) (39). A maladaptive change in CYP and sEH expression will affect AA metabolism and bring about negative consequences (38).

20-HETE and EETs are highly lipophilic, and thus, exist at very high concentrations within the tissue. 20-HETE, a potent vasoconstrictor, is primarily produced by CYP4A and CYP4F subfamilies and has been shown to be induced in cardiac hypertrophy and heart failure. On the other hand, EETs, which are vasodilating eicosanoids, are mainly formed by CYP 2C and CYP 2J subfamilies, but can also be metabolized by sEH to a less active form, known as DHETs. If the EETs are not degraded, EETs are rapidly stored into cell membrane phospholipids (38, 40). However, unlike CYP 2J, CYP 2C not only produces EET but also detrimental ROS which may affect cellular homeostasis and target mitochondria (41).

Diabetic cardiomyopathy is a serious complication that can be fatal for diabetic patients; however its underlying mechanisms are not fully understood (42). A correlation exists between CYPs and the development of CH. An inhibition of sEH or an increase in EETs not only reduce CH but can even reverse the case (38). In contrast to EETs, 20-HETE can stimulate NADPH-oxidase to produce ROS, which in turn can lead to cellular apoptosis, hypertrophy, and eventually cardiomyopathy (39, 43, 44). Also, it has been suggested that an initial burst of ROS is vital for EETs to exert their cardioprotective effect (37).

The effect of CYP-mediated AA metabolites on diabetic rats in cardiac dysfunction has been studied post-ischemia/reperfusion. According to this study, the levels of 20-HETE were increased after ischemia/reperfusion. Administering a 20-HETE inhibitor (HET0016) or an sEH inhibitor (CDU) 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 (45). Such data indicate that EETs play a protective role while 20-HETE exerts its detrimental effects on the heart. Hence, 20-HETE inhibitors to reduce its levels and sEH inhibitors to maintain EET levels are of therapeutic interest to treat the diabetic heart and other affected organs.

E. Fetal Gene Program Induction and Diabetic Cardiomyopathy

Left ventricular remodeling is an adaptive mechanism, which is characterized by alterations in the myocardial architecture and increased LV hypertrophy to oppose the elevated wall stress. This is established through rearrangement of extracellular matrix proteins, including collagen, and myocyte hypertrophy to halt any further distortions to the wall. Ventricular compliance is reduced leading to diastolic dysfunction, which will eventually advance to contractile dysfunction.

β -MHC, the fetal isoform, is a marker for pathological hypertrophy. It represents a whole “fetal gene program” that is usually very low or absent in healthy adult heart, but is expressed under pathological conditions, and pathological cardiac hypertrophy in particular. On the other hand, the adult isoform α -MHC is present normally but is reduced as a response to stress, such as diabetes (46, 47). Furthermore, such change is correlated with decreased contractility in view of the fact that α -MHC exhibits higher actin-activated ATPase (48). The effect of hyperglycemia on MHC expression within cardiac cells is still not clearly understood.

Alpha-smooth muscle actin is a marker for cardiac fibroblast differentiation into myofibroblast. This occurs when cardiac injury and accumulation of matrix proteins are evident. The reason behind this is that cardiac fibroblasts are responsible for matrix component synthesis in a healthy heart, and any stress that strikes the myocardium will lead to the switch to myofibroblasts and further detrimental consequences (49, 50). Vimentin and Discoidin Domain Receptor 2 (DDR2) are also accepted as markers for CF (51, 52).

F. Tau Hyperphosphorylation and Diabetic Cardiomyopathy

Tau is a microtubule-stabilizing protein (53). It is functionally modulated by phosphorylation, since the ability of Tau to bind and stabilize microtubules correlates inversely with its phosphorylation which may facilitate its self-assembly. Tau hyperphosphorylation occurs under stressful conditions; they tend to dissociate from cytoskeleton and aggregate. Tau protein is widely expressed in rat tissues and at relatively high levels in the heart (54). However, until now there are no studies that reveal any change that may occur to tau in any cardiac disease, including diabetic cardiomyopathy.

Oxidative stress plays a vital role during diabetes, cardiovascular disorders and neurodegenerative diseases. In the case of Alzheimer's disease, increased tau phosphorylation is thought to be linked to increased ROS production, leading to neuronal dysfunction and ultimately neuron death. It has been suggested that phosphorylation of tau might have occurred due to endoplasmic reticulum stress, and

vice versa creating a pathological feedback loop (55). Furthermore, mitochondrial ROS might also be involved in tauopathies. The balance between ROS and antioxidants within the neurons is certainly fragile, in which any slight disruption to their equilibrium can lead to neuronal death (56). However, the link between ROS production and tau hyperphosphorylation seen in Alzheimer's disease still need to be elucidated within the heart.

G. Autophagy vs. Apoptosis in Diabetic Cardiomyopathy

Autophagy is a cellular degradation process responsible for the turnover of unnecessary or dysfunctional organelles and cytoplasmic proteins and has been studied extensively in lower organisms such as yeast, *Caenorhabditis elegans*, and *Drosophila* (57, 58, 59, 60). Autophagy involves sequestration of cytosolic constituents, including proteins and organelles, in autophagosomes. The autophagosomes fuse with lysosomes to form autolysosome. Beclin-1 (Atg5) is needed for the vesicle (called isolation membrane) nucleation step of autophagy (61, 62). LC3-II is used as a marker of autophagy.

Autophagy has been suggested to be an essential function for cell homeostasis and cell defense and adaptation to an adverse environment (58, 60, 63). Autophagy is typically activated by starvation, when the cytoplasmic proteins or organelles are delivered to the lysosome and degraded (58-60, 64). Autophagy allows the cell not only to recycle amino acids but also to remove damaged organelles, thereby eliminating oxidative stress and allowing cellular remodeling for survival (60, 65). It can also

prevent accumulation of misfolded and aggregated proteins in Parkinson's, Huntington's, and Alzheimer's diseases (66). However, unrestrained stimulation of autophagy might induce a pathway for programmed cell death, known as type II cell death. Indeed, excessive autophagic activity is capable of destroying major proportions of the cytosol and organelles, most noticeably the mitochondria and the endoplasmic reticulum (ER), leading finally to the total collapse of all cellular functions (67, 68). Given its role in cell survival, autophagy in dying cells seems to be a sign of failed repair rather than a way for cells to commit suicide by consuming themselves.

In the heart, autophagy is a homeostatic mechanism for the maintenance of normal cardiac function and morphology under baseline conditions. Under normal or mildly stressed conditions, autophagy degrades and recycles cytoplasmic components, such as long-lived proteins and organelles, and selectively removes damaged mitochondria as a cytoprotective mechanism (69). As damaged mitochondria release pro-apoptotic factors such as cytochrome *c*, (70) autophagy can prevent activation of apoptosis (71, 72). Ultra-structural analyses of Atg5-deficient hearts reveal a disorganized sarcomere structure, misalignment and aggregation of mitochondria, and aberrant concentric membranous structures. Inactivation of Atg5 causes the accumulation of abnormal proteins and organelles and promotes ER stress and apoptosis. These results indicate that constitutive cardiomyocyte autophagy is required for protein quality control and normal cellular structure and function under the basal state. Accumulation of abnormal proteins and organelles, especially mitochondria, may directly cause cardiac dysfunction. It is clear that autophagy is essential for maintaining healthy myocytes in the myocardium. In contrast, some reports suggest that

upregulation of autophagy can be detrimental to the heart. Increased numbers of autophagosomes have been observed in cardiac tissues from patients with cardiovascular disorders such as dilated cardiomyopathy (73). However, the functional significance of autophagy in such diseases is still not clear.

H. Hypothesis and Aim of the Study

Based on literature, diabetes can lead to cardiovascular complications through a complex mechanism leading to cardiac dysfunction. In a diabetic heart, the CYPs are altered, such that the levels of CYP4A protein expression, which synthesizes 20-HETE, increases, while that of CYP2C, which synthesizes EETs, decreases. An increase in 20-HETE levels is detrimental to the heart, but an increase in EETs is cardioprotective. According to other studies, reducing 20-HETE production through inhibiting CYP4A or increasing EET levels through inhibiting sEH reverses myocardial injury. In this project, we assumed that administering HET0016 (CYP4A inhibitor) or AUDA (sEH inhibitor) will reverse the hyperglycemia-induced damage to the heart. Thus, we hypothesized that altered levels of CYPs under diabetic conditions are the major source of oxidative stress, which will eventually lead to reinduction of “fetal gene program”, cardiac fibroblast and cardiomyocyte injury, and autophagy. We further hypothesize that the tau found within the heart is hyperphosphorylated similar to what is seen in Alzheimer’s disease, and may be a possible signaling pathway through which altered CYP450 act to induce damage within the heart. In order to test our hypothesis, the present study aims to:

1. Examine whether systolic/diastolic function and hypertrophy are altered in the myocardium of diabetic, HET0016 and AUDA treated rats.
2. Visualize cardiac remodeling in terms of protein glycosylation and fibrosis in the diabetic, HET0016 and AUDA treated rats.
3. Detect whether the “fetal gene program” is inhibited by treating the diabetic rats with either HET0016 or AUDA.
4. Study the effect of diabetes, HET0016 and AUDA treatments on autophagy.
5. Check whether the treatment with HET0016 or AUDA will reduce oxidative stress established by diabetes.
6. Assess the protein levels of CYP2C11 and CYP4A1/A2/A3 in diabetic, HET0016- and AUDA-treated rats.
7. Test whether tau protein is altered in the heart under diabetic conditions similarly to what is observed in diabetic patients with Alzheimer’s disease.

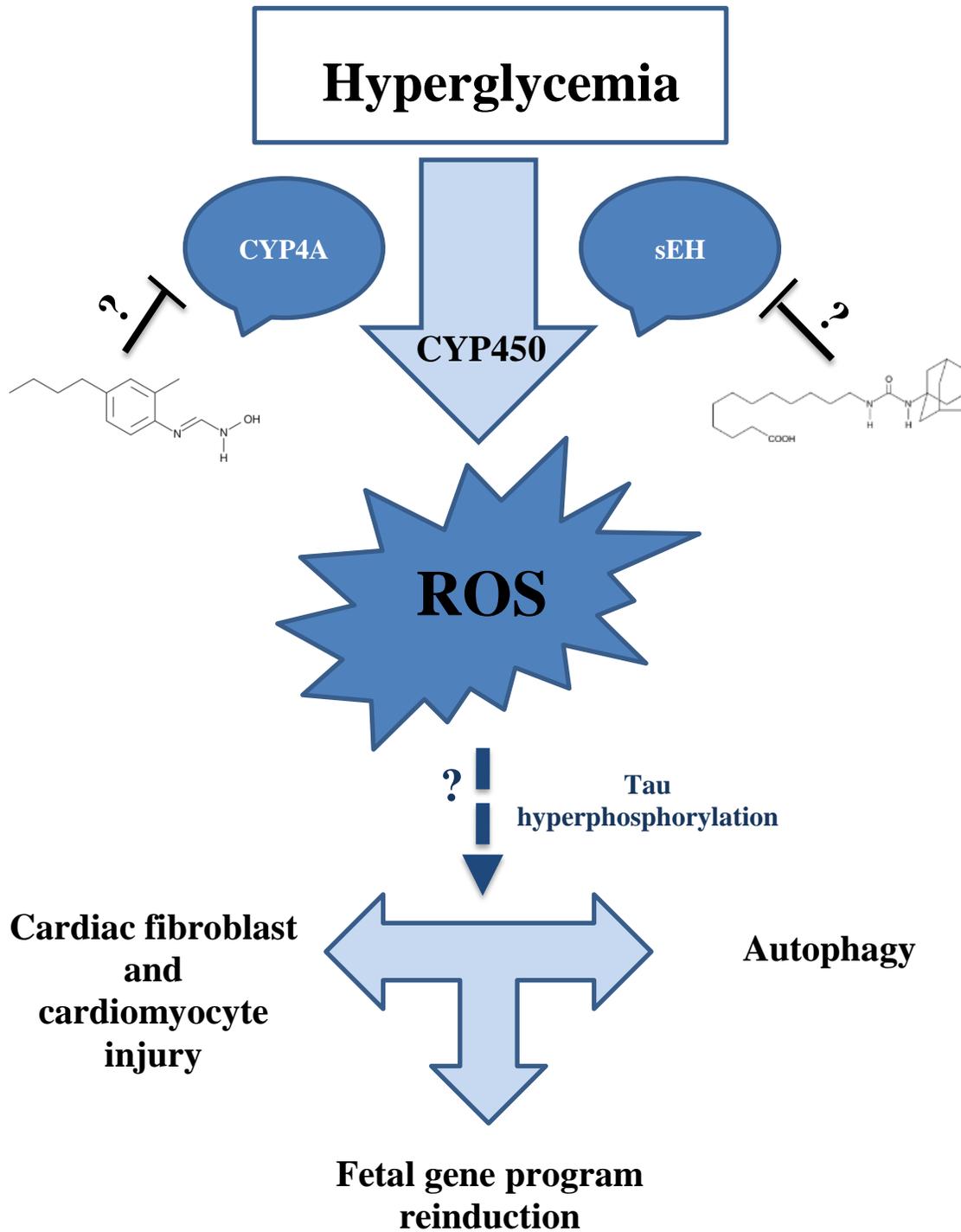


Figure 1: Hypothesis. Hyperglycemia leads to oxidative stress through activation of CYP450 enzymes. Increased ROS production causes fetal gene program reinduction, cardiac cell injury, and increased autophagy through tau hyperphosphorylation.

CHAPTER II

MATERIALS AND METHODS

A. Animal Models

Male Sprague-Dawley rats weighing between 200 and 225 g were divided into 4 groups of five animals each. Rats in *group 1* were injected with sodium citrate buffer (0.01 M, pH 4.5) alone. Group 2 rats were injected with one single dose of 55 mg/kg body weight Streptozotocin (STZ) in sodium citrate buffer intravenously via the tail vein to induce type 1 diabetes. After 6 weeks of inducing diabetes, rats in group 3 were treated subcutaneously with HET0016 (2.5mg/kg/b.w.), a CYP 4A inhibitor, and group 4 rats were treated with an sEH inhibitor (AUDA, 25mg/L of drinking water).

All rats were kept in a temperature controlled room and on a 12/12 dark/light cycle and had free food and water access. Blood glucose levels were measured every two days (3 times per week) using a glucometer. All protocols were approved by the Institutional Animal Care and Use Committee of the American University of Beirut. After 5 weeks of treatment, all rats were anesthetized, and echocardiography was performed. Afterwards, the heart was isolated and weighed. The left ventricle was snap-frozen in liquid nitrogen and stored at -80°C , or was fixed with formaldehyde and embedded in paraffin for biochemical and histological analysis.

B. Echocardiography

Rats were weighed and anesthetized using 1–2% isoflurane. Echocardiography was performed with a linear 25-MHz transducer connected to a Sonix Touch Ultrasound System. The anterior chest wall was shaved, rats were secured in the supine position, and normal body temperature was maintained. M-mode and two-dimensional echocardiography images were acquired in the parasternal long- and short-axis views. LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), LV end-diastolic volume (LVEDV), and LV end-systolic volume (LVESV) were measured. The percentage of LV fractional shortening and LV ejection fraction was calculated as follows: $[(LVEDD - LVESD)/LVEDD] \times 100$ (%) and $[(LVEDV - LVESV)/LVEDV] \times 100$ (%), respectively. All measurements were performed on three distinct cardiac cycles, and the values were averaged at each time point.

C. Western Blot Analysis

Homogenates from the LV of the heart were prepared in 250 μ L of radioimmunoprecipitation assay (RIPA) buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 150 mM sodium chloride, 50 mM Tris-hydrochloride, 100 mM EDTA, 1% Tergitol (NP40), and 1% of the protease and phosphatase inhibitors using a dounce homogenizer. They were then incubated for 2h at 4 °C, and subsequently centrifuged at a speed of 13,600 rpm for 30 minutes at 4 °C. The supernatant containing the proteins was collected, and proteins were quantified using Bradford protein assay.

For immunoblotting, 50µg of proteins were separated on 10% or 15% Polyacrylamide gel Electrophoresis (Bio-Rad Laboratory, CA, USA), depending on their molecular weights, and were then transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The membranes were blocked with 5% low fat milk in Tris-buffered saline and then incubated with the following primary antibodies:

- rabbit polyclonal anti-CYP 4A1/A2/A3 (1:1000, abcam)
- rabbit polyclonal anti-CYP 2C11 (1:2000, abcam)
- goat polyclonal anti- α -MHC (1:500, Santa Cruz Biotechnology)
- mouse monoclonal anti- β -MHC (1:250, Santa Cruz Biotechnology)
- rabbit polyclonal anti-beclin-1 (1:1000, Cell Signaling Technology)
- rabbit polyclonal anti-LC3B (1:1000, Cell Signaling Technology)
- rabbit polyclonal tau (phospho-S214) antibody (1:250, abcam).

The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:7000, Bio-Rad). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software.

D. Detection of Intracellular ROS

Dihydroethidium (DHE), which is relatively specific for superoxide anion measurement, is an oxidative fluorescent dye that undergoes a two-electron oxidation to form the DNA-binding fluorophore ethidium bromide. The DHE staining for superoxide was carried out as previously described (74). Briefly, LV frozen samples were cut into 5 µm thick sections and placed on glass slides. DHE (20 µmol/l) was

applied to each tissue section, and the slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Fluorescent images of ethidium-stained tissue were obtained with a laser-scanning confocal microscope (Zeiss, LSM 710) at t=30 mins. Fluorescence was detected at 561 nm long-pass filter. Superoxide generation was demonstrated by red fluorescent labeling. The average of three sections for different samples of same group stained with DHE was taken as the value for each group. Quantification was done using Zen light Software.

E. Histological Analysis

Upon sacrifice, a piece of the left ventricle was cut and put in 4% formaldehyde for 48 hours. Afterwards, the tissues were embedded in paraffin blocks and cut into sections that are 4-5µm in thickness and mounted on glass slides. Tissues were stained with Periodic acid Schiff (PAS) reagent to quantify the amount of glycosylated proteins to assess for glycogen deposition. A quantitative measurement for three randomly picked LV sample tissues from three different rats of each group was performed using Image J software.

F. TUNEL Assay

Upon sacrifice, a piece of the left ventricle was snap frozen with liquid nitrogen. The tissue was then cut into sections of 8µm in thickness and mounted on glass slides. Apoptotic nuclei are detected and quantified within the tissue section using an apoptosis detection kit (Trevigen's TACS® TdT In Situ Apoptosis Detection Kit).

The samples were first dried at 45°C for at least 2 hrs. Next, they were rehydrated with ethanol, washed with 1X PBS, and covered with Cytonin for around 45 mins. Afterwards, the samples were covered with the following in their respective order: Labeling Buffer, Labeling Reaction Mix, Stop Buffer and Strep-Fluor solution. Drops of a fluorescent mounting medium (DAPI) was applied on the tissue sections. The samples were stored overnight in the dark. The tissue sections were viewed under a confocal microscope.

The apoptotic nuclei, which are characterized by chromatin condensation, were quantified using ZEN Software. Furthermore, TUNEL assay images were used for counting the number of nuclei per unit area measured using ZEN software in order to detect hypertrophy.

G. Statistical Analysis

Results are represented as Mean \pm 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 and diabetic (*) is used when $p < 0.05$, and when comparing diabetic to diabetic treated (#) is used when $p < 0.05$.

CHAPTER III

RESULTS

In this study, STZ-induced type 1 diabetic rats were used. Control animals were treated with citrate buffer. In parallel experiments, STZ-induced type 1 diabetic rats were treated with HET0016, a specific inhibitor of 20-HETE production, or AUDA, an sEH inhibitor. sEH molecule decreases EET levels by converting them into a less active form known as DHETs. Our results show an increase in CYP4A and a decrease in CYP2C11 in the left ventricle of diabetic animals' myocardium. Treatment with HET0016 blocks the increase in CYP4A protein levels, but on the other hand enhances the increase in CYP2C11 protein levels. Treatment with AUDA had no effect on either, and this is because it acts on sEH enzyme rather than on CYP450. Taken together, these results suggest that our treatments achieved the desired effects.

A. Diabetes is accompanied by systolic dysfunction and reduced ejection fraction.

Pertinent characteristics and hemodynamic variables of the four groups of rats studied are shown in (**Table 1**). Untreated diabetic rats and diabetic rats treated with either HET0016 or AUDA had equivalently elevated blood glucose concentration compared with the control rats. Body weight was reduced in the diabetic rats and to a similar extent in the diabetic rats treated with either HET0016 or AUDA. There was a significant change in heart weight to body weight ratio in the diabetic group when compared to the control group, which is correlated with the presence of hypertrophy. Treatment with either HET0016 or AUDA did not reverse CH. Also, during the study period, diastolic and systolic thicknesses of the interventricular septum and of the posterior wall did not differ significantly among the four groups. LV internal dimension (in mm) and LV internal volume (in μl) during systole were significantly increased in diabetic rats compared with control animals ($P < 0.05$). This was accompanied by a significant decrease in percent fractional shortening (**Figure 2A**) and percent ejection fraction (**Figure 2B**), suggesting an impaired systolic function. These parameters were reversed following HET 0016 or AUDA treatment ($P < 0.05$).

	Control	Diabetic	AUDA	HET0016
Heart Weight, g	1.60±0.22	1.53±0.15	1.36±0.11	1.24±0.19
Body Weight, g	616.00±13.31	385.75±50.64	342.50±60.00	314.50±88.85
HW/BW Ratio	0.0026±0.0003	0.0041±0.0003*	0.0042±0.0005	0.0043±0.0004
Glucose, mg/dL	117.50±5.19	515.00±75.95	424.5±59.34	482.75±117.25
EDD, mm	7.42 ± 0.21	7.73 ± 0.42	7.54± 0.34	7.53± 0.39
ESD, mm	4.03± 0.1 1	4.98± 0.34*	4.10± 0.12 [#]	4.04± 0.10 [#]
FS, %	46.10 ± 0.2 1	34.12± 0.82*	48.17±0.27 [#]	47.32 ± 0.30 [#]
EDV, <u>ul</u>	330.21 ± 25.65	358.32±49.28	345.24±45.25	348.52±47.23
ESV, <u>ul</u>	91.65 ± 6.54	157.43 ± 17.22*	98.15 ± 9.21 [#]	89.97 ± 12.43 [#]
EF, %	71.65 ± 2.19	58.95 ± 2.22*	70.54 ± 2.35 [#]	71.18 ± 2.19 [#]

Table 1. Diabetes is accompanied by systolic dysfunction and reduced ejection fraction.

Values are means ± SE; *n* = 4. EDD, end-diastolic diameter; ESD, end-systolic diameter; FS, fractional shortening; EDV, end-diastolic volume; ESV, end-systolic volume; EF, ejection fraction. ***P<0.05 vs. control rats**, [#]**P<0.05 vs. diabetic rats**

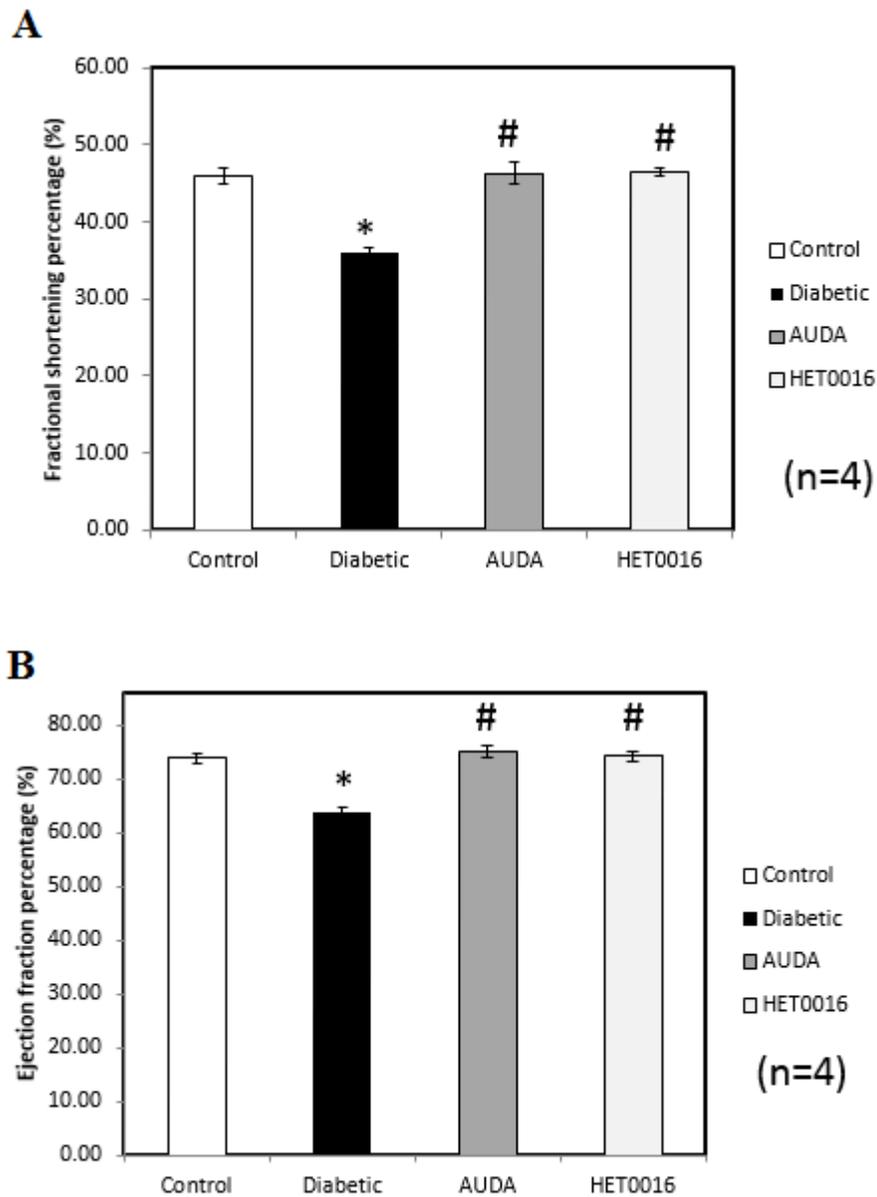


Figure 2: Diabetes is accompanied by systolic dysfunction and reduced ejection fraction.

A) Fractional shortening % and B) ejection fraction % exhibited improved cardiac function in HET 0016 and AUDA treated rats. (n=4) *P<0.05 vs. control rats, #P<0.05 vs. diabetic rats

B. Hyperglycemia increases protein glycosylation within the left ventricle.

Hyperglycemia serves as a major risk factor in the development of glycogen deposition. PAS stain is used to detect glycosylated proteins within the left ventricular tissue. In the diabetic rat's left ventricle, glycosylation is significantly increased when compared to the control group. Treatment with either AUDA or HET0016 significantly decreased glycoprotein deposition when compared to the diabetic group. (**Figure 3, Table 2**)

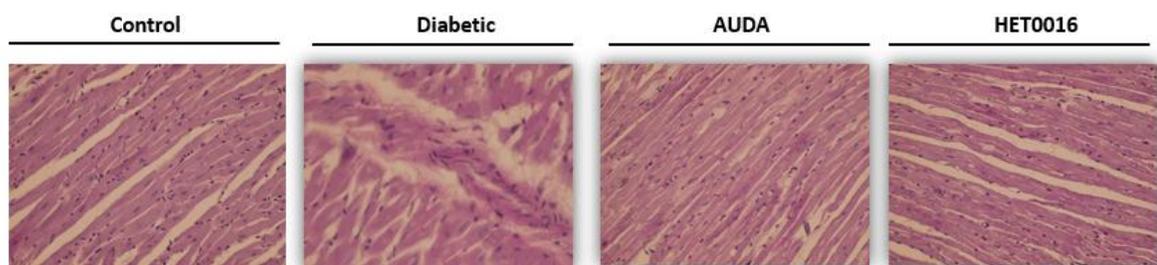


Figure 3: Hyperglycemia increases protein glycosylation within the left ventricle.

In the diabetic group, there is an increase in the amount of glycosylated proteins and nuclear condensation when compared to the control group. Treatment with AUDA or HET0016 significantly reduced protein glycosylation when compared to the diabetic group.

Rats	Scoring
Control	1
Diabetic	3.4 ± 0.7*
AUDA	1.7 ± 0.5#
HET0016	1.8 ± 0.6#

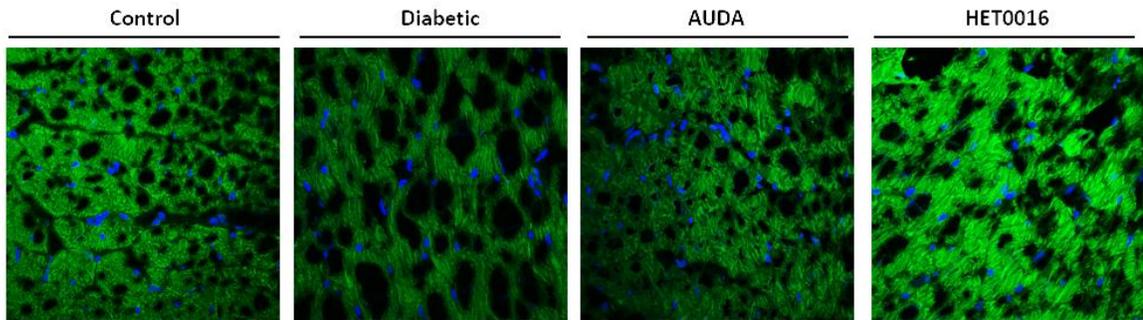
Table 2. Hyperglycemia increases protein glycosylation within the left ventricle, while treatment with AUDA or HET0016 significantly reduced it.

Semi-quantification of periodic acid Schiff (PAS) staining using a score 0-4: 1, normal histology; 2, cell swelling and nuclear condensation, with loss of up to one-third of the nuclei; 3, same as for score 2, but greater than one-third and less than two-thirds of the cells profile showing nuclear loss, and 4, greater than two-thirds of the cells profile showing nuclear loss. The total score per left ventricle was calculated by addition of all the calculated scores of each section/rat. (n=3) *P<0.05 vs. control rats, #P<0.05 vs. diabetic rats

C. Hyperglycemia induces cardiomyocyte hypertrophy.

Left ventricular hypertrophy is defined as the thickening of the left ventricular wall. It is considered as a compensatory mechanism of the myocardial cells, and cardiomyocytes in specific, to resist increased work. In a hypertrophied myocardium, the area within the left ventricle chamber is reduced and so does the amount of blood occupying that space. Hence, more force is required to pump more blood to the rest of the body. TUNEL assay was performed to assess apoptosis as well as cellular hypertrophy. The number of nuclei per unit area was calculated using ZEN Software. The number of nuclei per unit area was calculated as an indicator of hypertrophy and chromatin condensation was used as an indicator for apoptosis. Our results indicate that hyperglycemia significantly increased hypertrophy of cardiomyocytes within the left ventricle of the diabetic animals when compared to their control littermates. Treatment with AUDA or HET0016 did not have any effect on hypertrophy. No apoptosis was seen in the diabetic myocardia when compared to the control myocardia. (**Figure 4**)

A



B

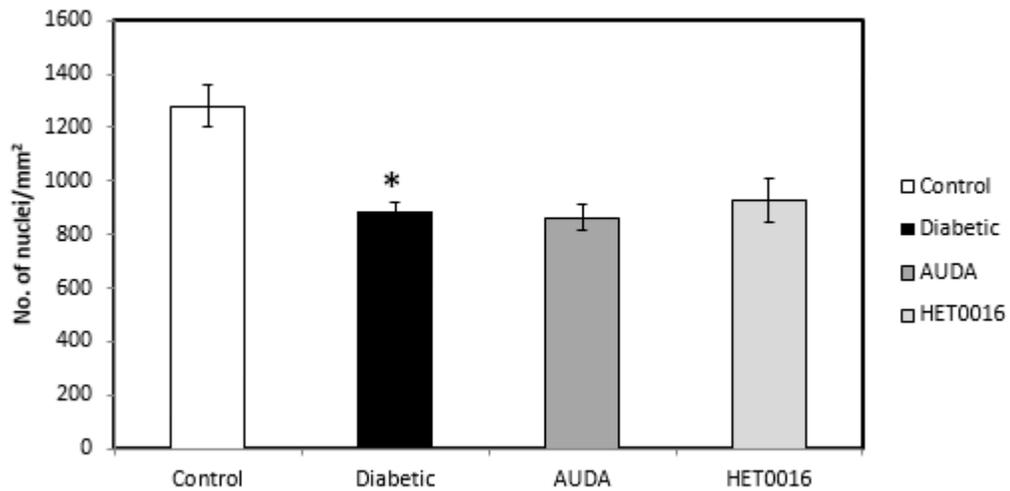


Figure 4: Hyperglycemia induces myocyte hypertrophy, while treatment with AUDA or HET0016 did not significantly reverse hypertrophy.

A) TUNEL assay images seen under the confocal microscope showing a higher number of nuclei per unit area (blue stain). B) Histogram showing the ratio between the number of nuclei and the area it occupies. (n=3) *P<0.05 vs. control rats, #P<0.05 vs. diabetic rats

D. Change in EET levels and 20-HETE production mediate the effect of hyperglycemia on “fetal gene program”.

An induction of the “fetal gene program” has been described in the diabetic heart (75). This is reflected by a decrease in alpha-MHC and a decrease in beta-MHC. Nevertheless, the role of cytochromes and their metabolites in “fetal gene program” induction is not yet studied.

We examined whether increased EET levels through the blockage of sEH, an enzyme that metabolizes EETs into a less active form, will reverse hyperglycemia-induced “fetal gene program”. Alpha-MHC and beta-MHC protein expressions were assessed by western blot. Our data show an increase in alpha-MHC and a decrease in beta-MHC in AUDA-treated group.

We also assessed whether reducing 20-HETE by inhibiting CYP4A through treating the rats with HET0016 will switch off the “fetal gene program”. Alpha-MHC in HET0016-treated group increased, while beta-MHC did not considerably change.

(Figure 5, Figure 6)

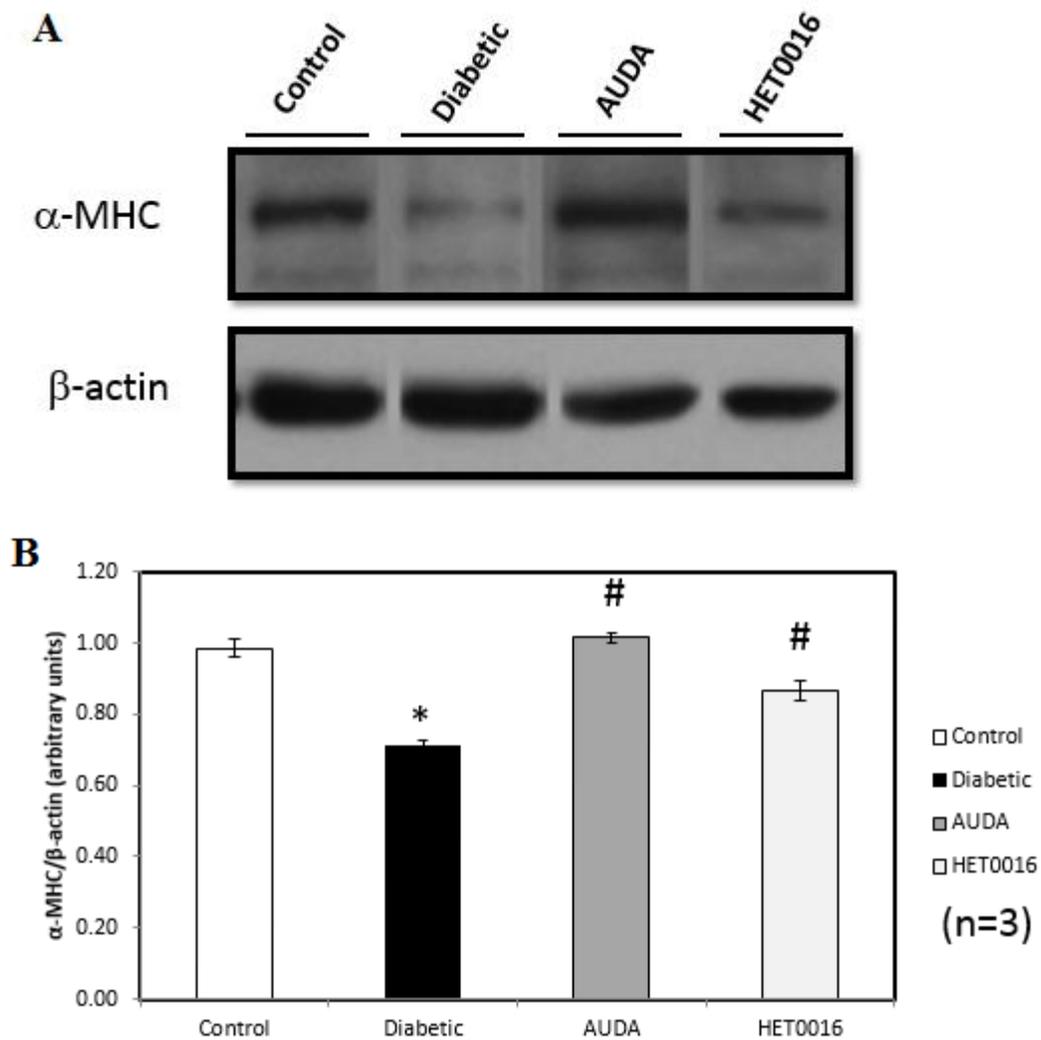


Figure 5: Hyperglycemia alters α -MHC protein expression.

Western blot was performed to assess protein expression of α -MHC. (A) Representative western blot of α -MHC. (B) Histogram showing protein levels of α -MHC relative to protein levels of β -actin. (n=3) *P<0.05 vs. control rats, #P<0.05 vs. diabetic rats

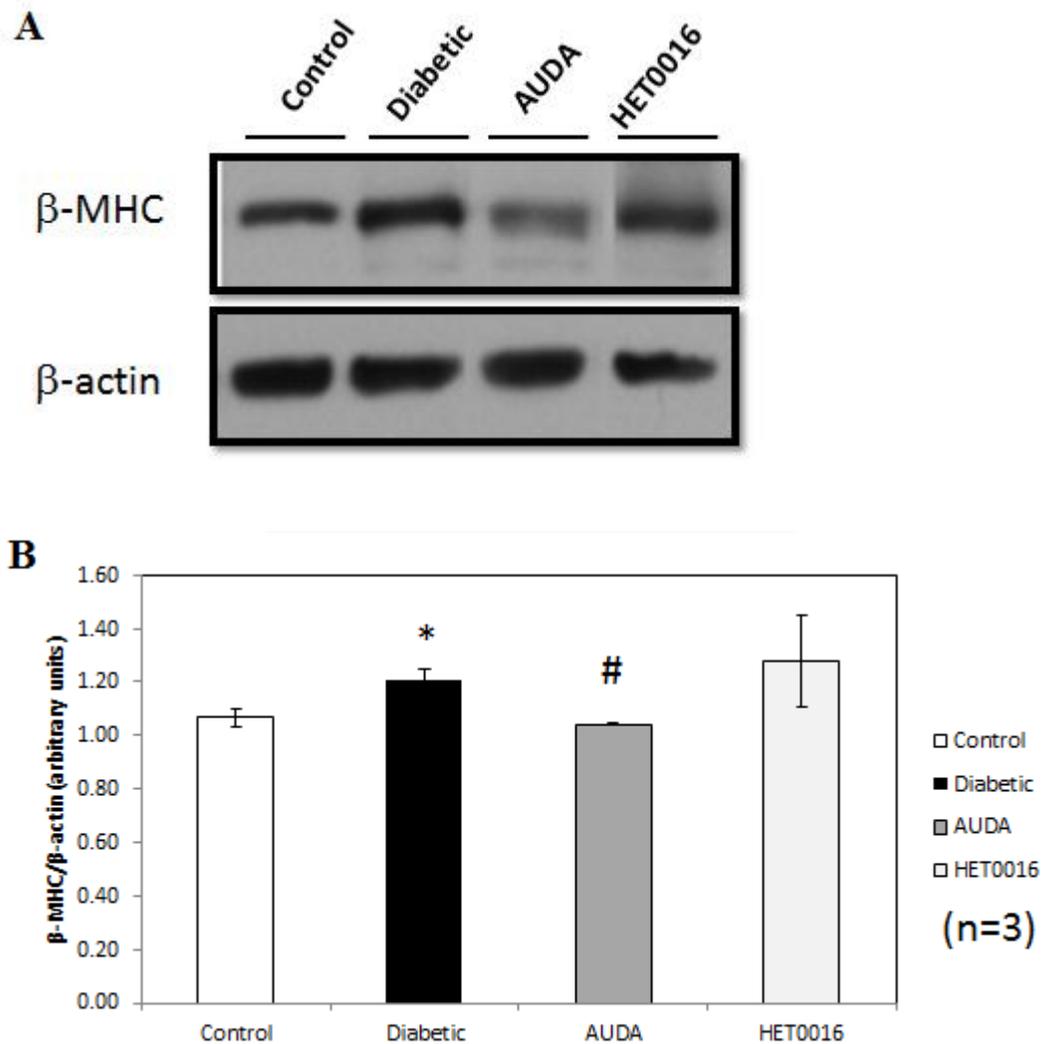


Figure 6: Hyperglycemia alters β -MHC protein expression.

Western blot was performed to assess protein expression of β -MHC. (A) Representative western blot of β -MHC. (B) Histogram showing protein levels of β -MHC relative to protein levels of β -actin. (n=3) * $P < 0.05$ vs. control rats, # $P < 0.05$ vs. diabetic rats

E. Hyperglycemia induces autophagy by increasing beclin-1 and reducing LC3II protein expressions.

Autophagy is a cytoprotective method that is activated under stressful conditions. One vital protein for autophagy is beclin-1. During the process of autophagy, autophagosomes are formed. LC3-B, a microtubule-associated protein, is linked to autophagosomes, and their levels are correlated with the number of autophagosomes formed. However, the process of autophagy is still debatable (76). Therefore, we assessed the protein levels of beclin-1 and LC3-B to determine whether autophagy is impaired under diabetic conditions.

Our results show that beclin-1 increased and LC3-B decreased in the diabetic group of rats. Treatment with AUDA was more effective in increasing LC3-B protein expression and slightly increasing beclin-1 protein levels. On the other hand, treatment with HET0016 significantly reduced beclin-1 protein levels bringing it back to near control levels, but there was no significant change in protein levels of LC3-B. (**Figure 7, Figure 8**)

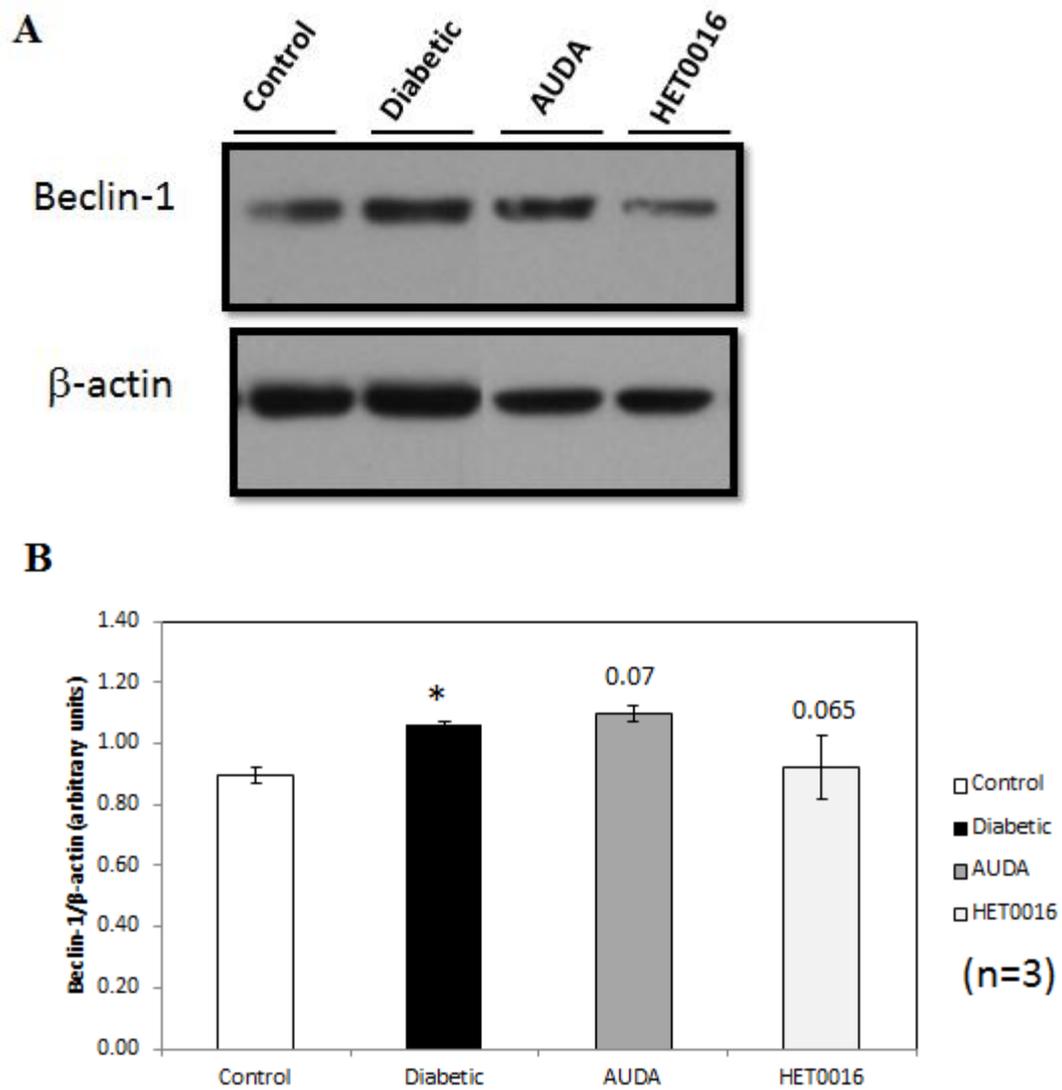


Figure 7: Hyperglycemia alters beclin-1 protein expression.

Western blot was performed to assess protein expression of beclin-1. (A) Representative western blot of beclin-1. (B) Histogram showing protein levels of beclin-1 relative to protein levels of β-actin. (n=3) *P<0.05 vs. control rats, #P<0.05 vs. diabetic rats

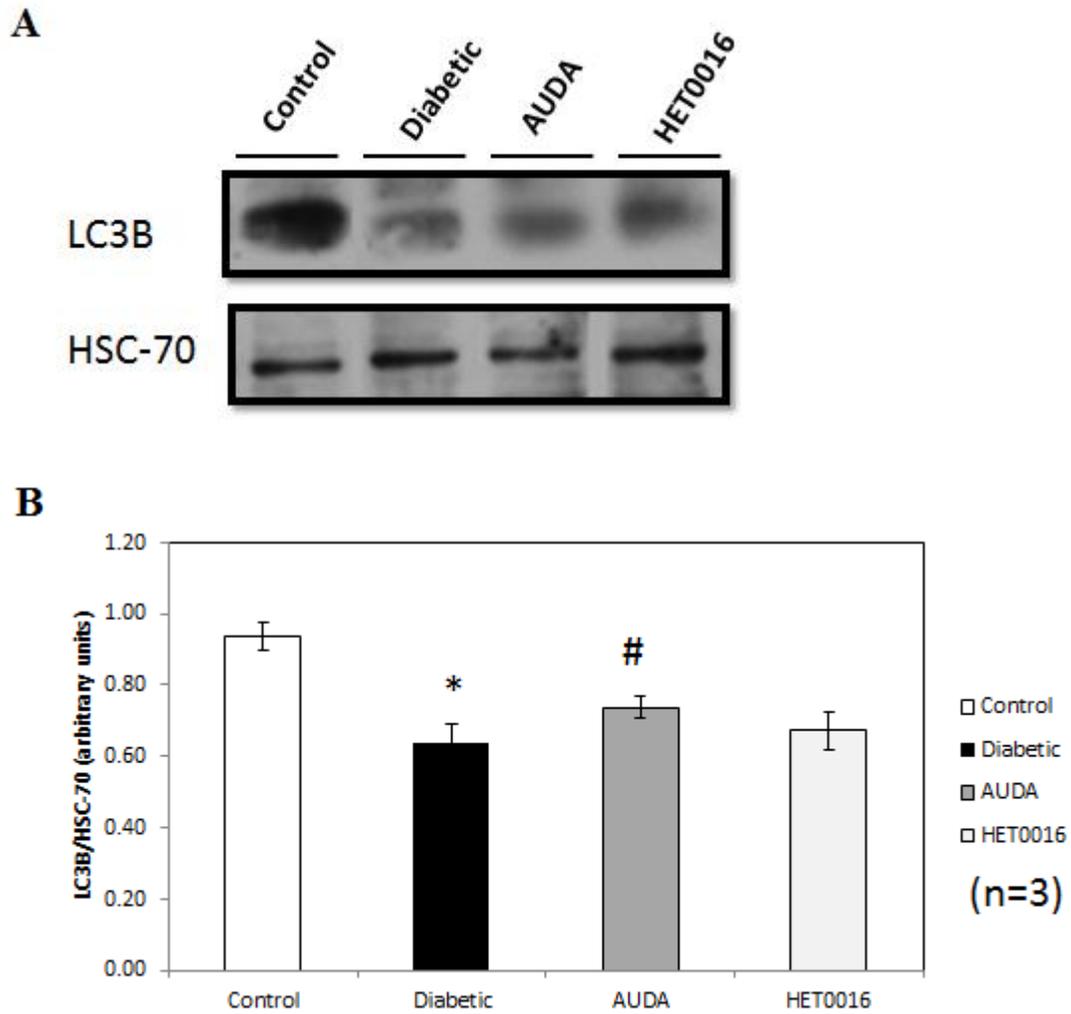


Figure 8: Hyperglycemia alters LC3B protein expression.

Western blot was performed to assess protein expression of LC3B. (A) Representative western blot of LC3B. (B) Histogram showing protein levels of LC3B relative to protein levels of HSC-70. (n=3) *P<0.05 vs. control rats, #P<0.05 vs. diabetic rats

F. Increase in EET levels and decrease in 20-HETE production attenuate hyperglycemia-induced oxidative stress.

Since diabetes and hyperglycemia are accompanied by oxidative stress, ROS production was evaluated by superoxide measurements for the different groups of rats. Intracellular ROS production was measured using DHE staining by laser-scanning confocal microscopy technique. As expected, hyperglycemia increased ROS production in the left ventricle of diabetic rats.

Our group has recently shown that CYPs are a major source of ROS in diabetic tissues (26). CYPs also serve as a vital machinery of ROS production within the myocardium. Our data reveal that hyperglycemia-induced ROS production within the left ventricle is brought back to near control levels in the diabetic rats when treated with AUDA or HET0016. **(Figure 9)**

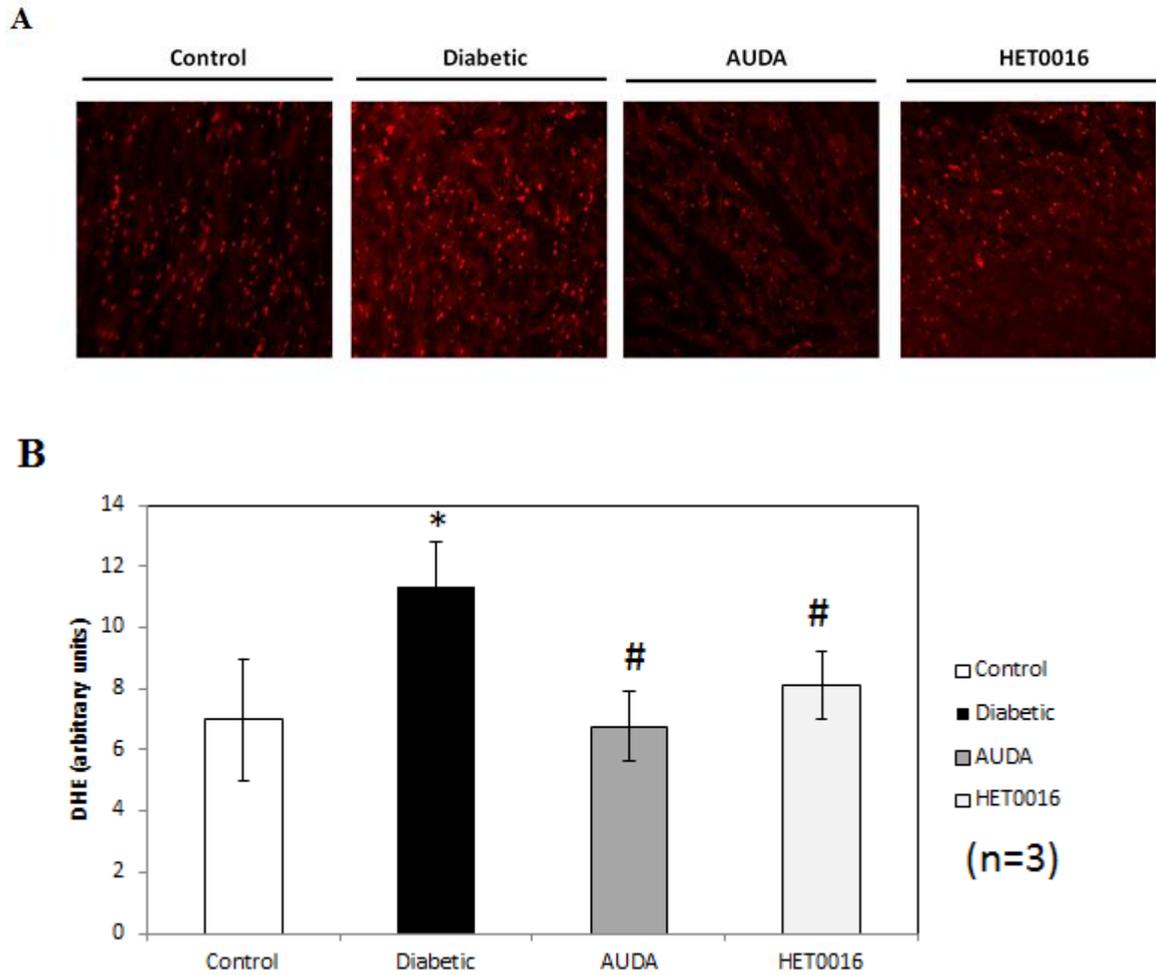


Figure 9: Hyperglycemia induces oxidative stress, while treatment with AUDA or HET0016 significantly decreased ROS production within the left ventricle of diabetic rats.

A) Super oxide production assessed by DHE staining. B) Histogram showing the quantification of ROS production in the left ventricle using ZEN light Software. (n=3)

*P<0.05 vs. control rats, #P<0.05 vs. diabetic rats

G. Hyperglycemia alters CYP4A and CYP2C11 protein expression in the left ventricle of type 1 diabetes rat model.

We next examined whether CYP4A contributes to hyperglycemia-induced oxidative stress in the left ventricle of diabetic rats. Western blot analysis was performed to assess the protein levels of CYP4A in the different groups of rats. In a previous study from our lab, Eid S. et al. demonstrated that HG increased ROS production and was associated with an upregulation of CYP4A (77). Similar results were observed in the left ventricle. CYP4A protein levels increased in the diabetic rats compared to the control group. In contrast, CYP4A protein levels were highly reduced upon HET0016 treatment (CYP4A inhibitor). This explains the decreased ROS levels that are very close to that of the control group. Furthermore, there was no significant change in CYP4A protein levels between the diabetic group and the AUDA-treated group.

In parallel, we assessed the expression of CYP2C11, a cytochrome present in a rat's heart. Our data show a significant decrease in CYP2C11 protein expression in the left ventricle of the diabetic rats. Interestingly, when compared to the diabetic group, CYP2C11 remains unaffected in AUDA-treated group. More importantly, when diabetic rats are treated with HET0016, we see an increase in CYP2C11 protein levels compared to the levels seen in the untreated diabetic group. (**Figure 10, Figure 11**)

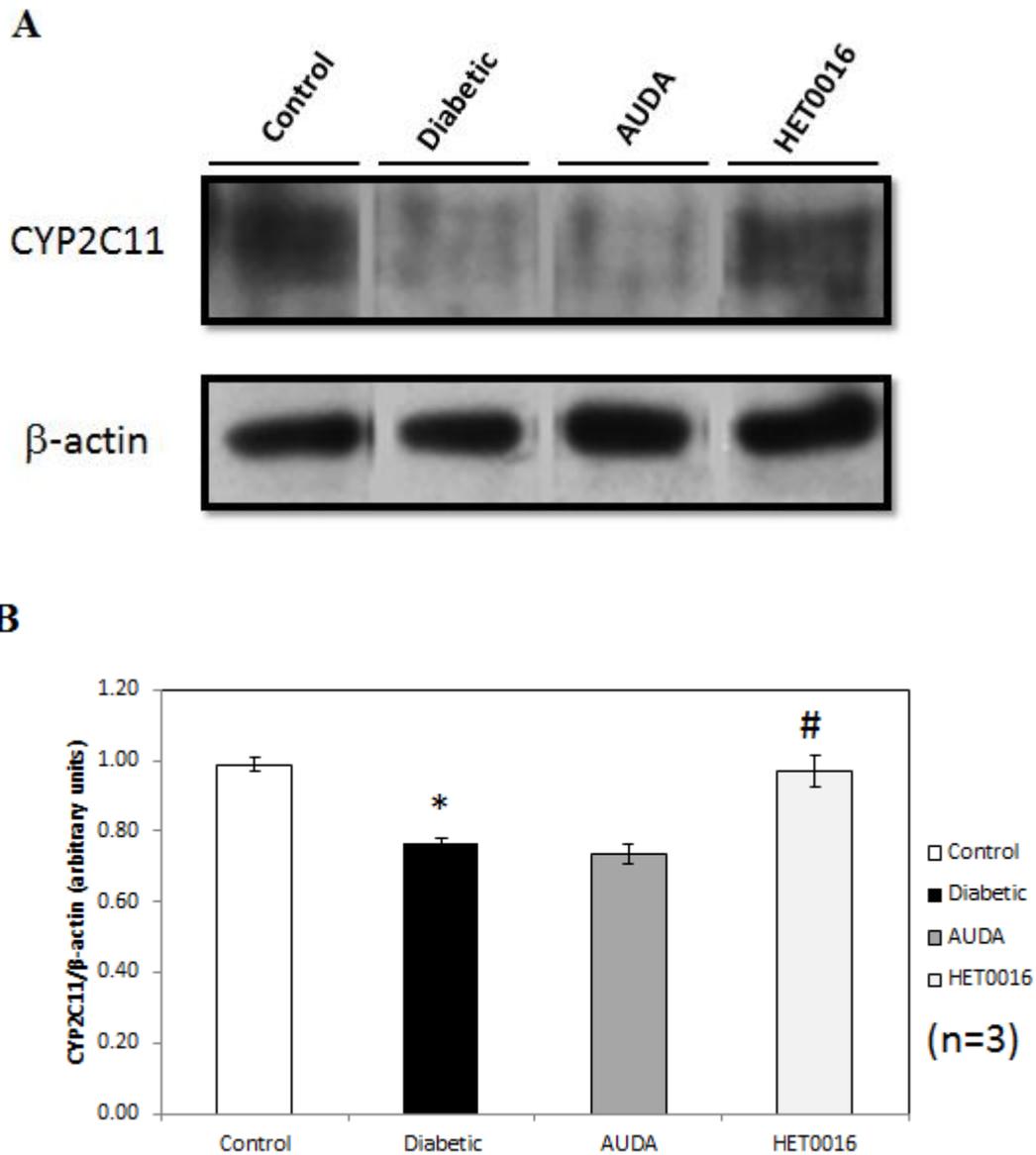
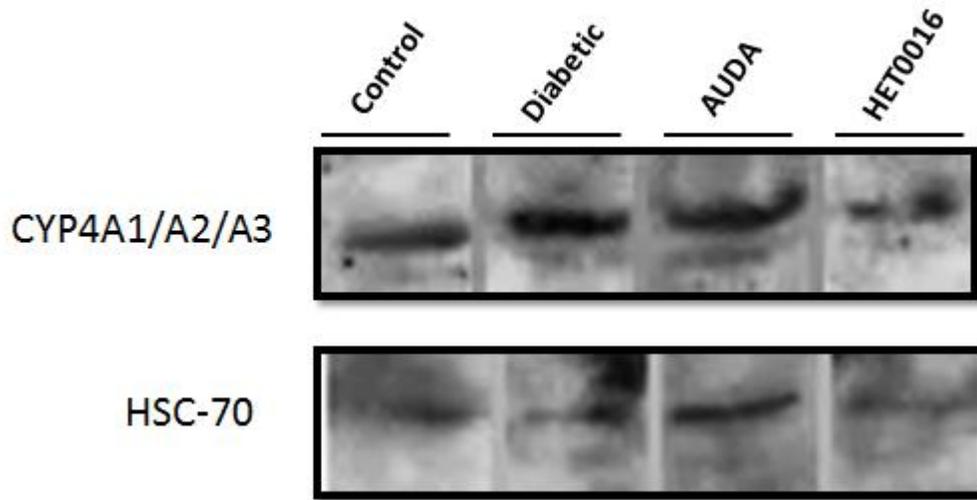


Figure 10: Hyperglycemia alters CYP2C11 protein expression.

Western blot was performed to assess protein expression of CYP2C11. (A) Representative western blot of CYP2C11. (B) Histogram showing protein levels of CYP2C11 relative to protein levels of β -actin. (n=3) * $P < 0.05$ vs. control rats, # $P < 0.05$ vs. diabetic rats

A



B

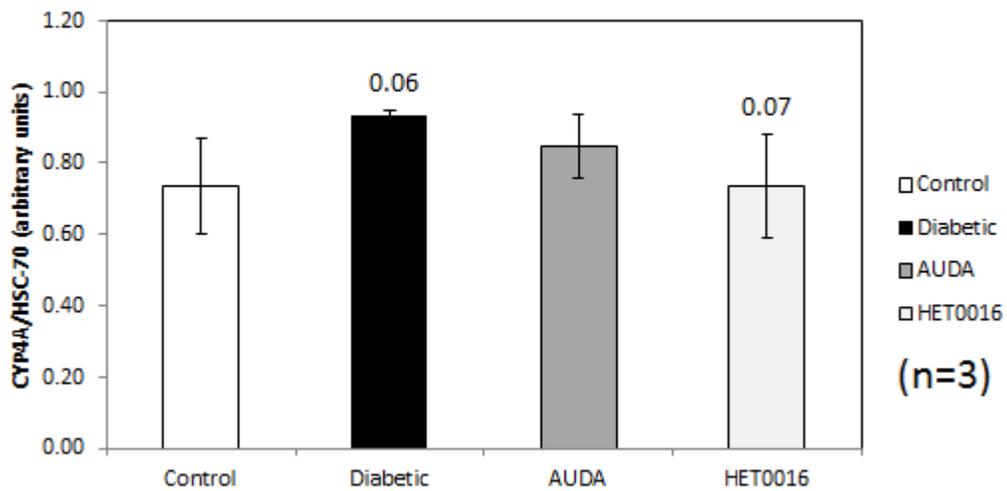


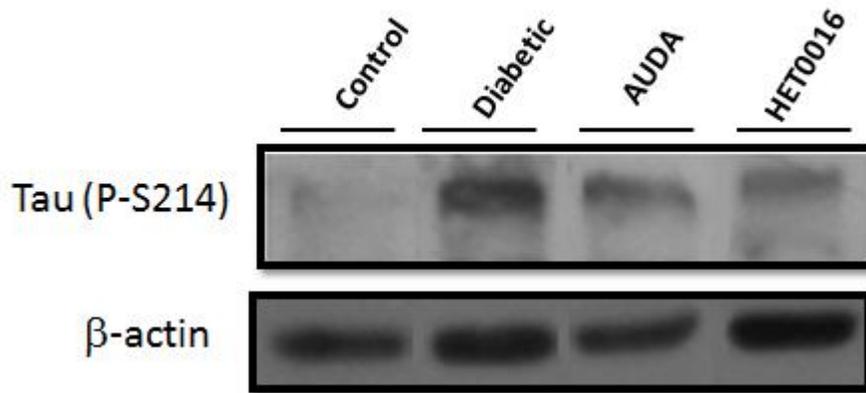
Figure 11: Hyperglycemia alters CYP4A1/A2/A3 protein expression.

Western blot was performed to assess protein expression of CYP4A1/A2/A3. (A) Representative western blot of CYP4A1/A2/A3. (B) Histogram showing protein levels of CYP4A1/A2/A3 relative to protein levels of HSC-70. (n=3) *P<0.05 vs. control rats, #P<0.05 vs. diabetic rats

H. Hyperglycemia induces tau phosphorylation, while CYP4A and sEH inhibitors reduce tau phosphorylation in rat model of type 1 diabetes.

There's emerging evidence that tau hyperphosphorylation is involved in cellular injury, especially in Alzheimer's disease (78). However, the role of tau in other diseases, especially diabetic cardiomyopathy, is still totally unknown. Under diabetic conditions, phosphorylation of tau at S214 site was significantly increased. Treatment with AUDA significantly decreased the level of phosphorylated tau. Similar results were obtained with HET0016 treatment. Our results suggest that EETs and 20-HETE might be playing a role in regulating phosphorylated tau levels through CYPs. (**Figure 12**)

A



B

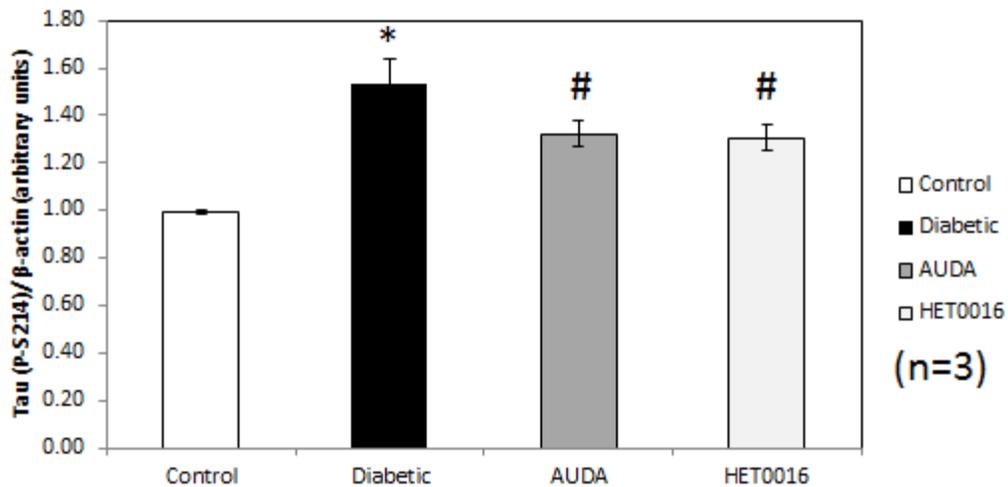


Figure 12: Hyperglycemia induces tau phosphorylation at S214 site in the myocardium of diabetic rats.

Western blot was performed to assess protein expression of tau (p-S214). (A) Representative western blot of tau (p-S214). (B) Histogram showing protein levels of tau (p-S214) relative to protein levels of β -actin. (n=3) *P<0.05 vs. control rats, #P<0.05 vs. diabetic rats

CHAPTER IV

DISCUSSION

Systolic and diastolic dysfunctions are an early sign of diabetic cardiomyopathy and progress with increased duration of T1DM (79). In this study, the diabetic group was characterized by a decrease in percent fractional shortening and percent ejection fraction, which are associated with an increase in LV dimension and volume at the end of systole when compared to the control group. These parameters were reversed following HET0016 or AUDA treatment. Neither the body weights nor the blood glucose levels were altered by the treatments when compared to the diabetic group.

Oxidative stress plays a role in the development and progression of cardiovascular complications (7). In addition to mitochondria and NADPH oxidase, CYP450 enzymes also serve as major source of ROS within the myocardium. Thus, targeting CYPs may reduce oxidative stress and further damage to the heart (80, 81). Preliminary data from our lab showed that HG-increased ROS generation in kidney podocytes was concomitant with sequential upregulation of CYP4A and an increase in 20-HETE (26). Also, the same correlation between ROS production and increased 20-HETE was discovered in pulmonary artery endothelial cells (82). Based on what is previously mentioned, sEH, an enzyme that metabolizes EETs into a less active form, and CYP4A, a monooxygenase that synthesizes 20-HETE, were targeted and blocked with AUDA and HET0016, respectively. In this study, we provide evidence that diabetic cardiomyopathy and oxidative stress are associated with a significant change in the expression of CYP450 and ultimately their AA metabolites,

20-HETE and EETs. Treatment with HET0016, a CYP4A inhibitor, prevented ROS production when compared to the diabetic group. Inhibition of sEH, an enzyme that metabolizes EETs into a less active form, also reduced oxidative stress presumably by increasing levels of EETs. This suggests that CYP450 enzymes are a major source of oxidative stress within the myocardium, and under diabetic conditions in particular.

The “fetal gene program”, which is made up of alpha- and beta-MHC, has also been described in literature to be re-induced in diabetes (9). Here, we demonstrate that hyperglycemia-induced “fetal gene program”, which may be through the production of ROS, was almost completely reversed with the AUDA treatment, while HET0016 only did significantly change the expression of alpha-MHC. It seems from our results that treatment with AUDA is more successful in not only reducing oxidative stress and bringing it back to near control levels, but also in reversing the “fetal gene program” back to normal.

Furthermore, contradicting literature papers were proposed about the link between beclin-1 and LC3B in autophagy. One paper mentioned that beclin-1 inhibition increases LC3B and hence, autophagy; another paper mentioned that increased beclin-1 levels is important for inducing autophagy (76, 83). Xianmin Xu et al. proposed that autophagy is inhibited in type 1 diabetes mouse model through a reduction in both beclin-1 and LC3B (84). In our study, beclin-1 was increased while LC3B was decreased in diabetes when compared to the control group. One possible explanation to the following phenomenon: the increase in beclin-1 indicates autophagy has occurred; but the decrease in LC3-B indicates lower number of autophagosomes, which can be due to sending autophagosomes for further degradation by lysosomes and the release of LC3 (85).

CYP4A protein expression was also studied. Its protein levels were increased in the diabetic hearts same as mentioned in literature (45); in HET0016-treated group, CYP4A was inhibited. Treatment with AUDA did not significantly change the protein levels of CYP4A when compared to the diabetic group. One possible reason: high levels of EETs do not affect CYP4A levels.

As for CYP2C11 protein expression, it is decreased in the diabetic left ventricle (45). Treatment with AUDA, which increases levels of EETs, did not affect CYP2C11 protein expression, similar to what is seen for CYP4A. This suggests that treatment with AUDA exerts its action on sEH rather than its effect on CYP450 enzyme expression. On the other hand, treatment with HET0016 enhanced CYP2C11 protein expression. This raises the question on whether there exists an interaction between CYP2C11 and CYP4A, since the former decreases and the latter increases under diabetic conditions; but when treated with HET0016 (CYP4A inhibitor), we see an increase in CYP2C11.

Altered CYP450 enzyme levels and induced oxidative stress also increased myocyte hypertrophy. Treatment with HET0016 or AUDA did not reverse hypertrophy. Also, protein glycosylation was increased in the diabetic heart, and the treatment with HET0016 or AUDA was successful in reducing glycosylated protein deposition.

Tau protein has been described in tauopathies such as Alzheimer's disease (78). It is expressed in a rat's heart and other organs in the body (54). No literature exists that describes the correlation between tau hyperphosphorylation and diabetic cardiomyopathy. Our study shows that tau hyperphosphorylation does occur under diabetic conditions. Treatment with HET0016 or AUDA significantly reduced tau hyperphosphorylation,

suggesting that increased levels of EETs and decreased levels of 20-HETE, as well as reduced oxidative stress might play a role in altering the state of phosphorylated tau.

Taken together, these observations may explain that in late diabetes, systolic dysfunction occurs, myocytes hypertrophy, and glycosylated proteins deposit within the left ventricle. Such biological outputs are perceived due to oxidative stress, which is secondary to altered CYP450 protein expressions. Furthermore, we showed that induced “fetal gene program” and increased autophagy are a cell’s way of survival under stressful conditions. Also, tau protein can be altered in the heart in type 1 diabetes, similar to what is observed in the brain of diabetic patients with Alzheimer’s disease. In conclusion, we have identified that CYP4A-dependent 20-HETE production is injurious to the heart, and that CYP4A inhibitors may be useful in designing effective therapies in the treatment of diabetic cardiomyopathy. Also, increased levels of EETs through inhibiting sEH and reducing further its metabolism truly does play a cardioprotective role in type 1 diabetes.

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