Effects of Conjugated Linoleic Acid on Cardiomyocyte Abnormalities in Diabetic Cardiomyopathy

By

Basma Aloud

A Thesis submitted to the Faculty of Graduate Studies of

the University of Manitoba

In the partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Faculty of Pharmacy

University of Manitoba

Winnipeg

ABSTRACT

Diabetic cardiomyopathy is defined as changes in the structure and function of the myocardium that occur in diabetic patients in the absence of other cardiovascular risk factors. Our laboratory has shown that conjugated linoleic acid (CLA - a naturally-occurring polyunsaturated fatty acid with multiple health benefits) prevents endothelin-1-induced myocyte hypertrophy *in vitro,* as well as cardiac hypertrophy *in vivo* using a rodent model of spontaneously hypertensive heart failure. These cardioprotective effects of CLA were mediated through activation of peroxisome proliferator activated receptors (PPAR isomers α and γ) and stimulation of diacylglycerol kinase ζ (DGKζ). Thus, the aims of this study were to (i) determine the effect of CLA on hyperglycemia-induced structural and functional abnormalities of cardiomyocytes, and (ii) assess the role of PPAR-γ and DGK**.**

High glucose treatment induced hypertrophy of primary adult cardiomyocytes, as indicated by augmented cell size and protein synthesis compared to untreated cardiomyocytes. The hyperglycemia-induced hypertrophy was attenuated by pretreatment with CLA (30 μM). The ability of CLA to prevent hyperglycemia-induced hypertrophy was suppressed by GW9662 (1 μM) and R59022 (10 μM), pharmacological inhibitors of PPAR-γ and DGK, respectively. In addition to structural abnormalities, high glucose impaired contractile function of adult cardiomyocytes as measured by maximal velocity of shortening, maximal velocity of relengthening, and peak shortening. Hyperglycemia-induced contractile dysfunction was likewise prevented by pretreatment with CLA (30 μM). Collectively, these findings support the idea that hyperglycemia is an independent risk factor for the development of diabetic cardiomyopathy. Hypertrophy

and contractile dysfunction elicited by high glucose were prevented by CLA. The antihypertrophic actions of CLA are mediated, at least in part, by activation of PPAR-γ and DGK.

ACKNOWLEDGEMENTS

I would first like to express my gratitude and appreciation to my supervisor, Dr. Hope Anderson, for her assistance, guidance, and continuous support and encouragement during the course of my research program. This dissertation would not have been possible without her persistent help and generous time.

I would like to extend my sincerest thanks to my committee members, Dr. Thomas Netticadan and Dr. Lavern Vercaigne, for their help, guidance, and valuable comments. I really appreciate their helpful suggestions and advice during my study period.

I am also grateful for the generous financial support received through the Libyan-North American Scholarship Program and University of Manitoba - Faculty of Graduate Studies.

Last but not least, I would like to give my special thanks to my husband and my family back home in Libya for their love, encouragement, and support during my program.

Table of Contents

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACEI	angiotensin converting enzyme inhibitors
AGEs	advanced glycation end products
ARB	angiotensin II receptor blockers
ALT-T11	alagebrium chloride
AngII	angiotensin II
ATP	adenosine triphosphate
BFM	body fat mass
CLA	conjugated linoleic acid
DAG	diacylglycerol
DGKs	diacylglycerol kinases
DMSO	dimethylsulfoxide
ET1	endothelin-1
GLC	glucose
GLUT	glucose transporters
GPCR	G-protein-coupled receptor
HbA1C	glycated hemoglobin
ICM	idiopathic cardiomyopathy
IRS-1	insulin receptor substrate-1
LVEF	left ventricular ejection fraction
LVH	left ventricular hypertrophy
MARCKS	myristoylated alanine-rich C-kinase substrate
NCX	Na^{+}/Ca^{2+}exchanger
PA	phosphatidic acid

PBS	phosphate-buffered saline
PI3K	phosphatidyl inositol 3-kinase
PKC	protein kinase C
PLC	phospholipase C
PPAR	peroxisome-proliferator activated receptor
PPRE	peroxisome-proliferator activated receptor response element
PS	peak shortening
RAGE	receptors for advanced glycation end products
ROS	reactive oxygen species
RXR	retinoid X receptor
SERCA2a	sarcoplasmic reticulum calcium pump
SHHF	spontaneously hypertensive heart failure
SR	sarcoplasmic reticulum
STZ	streptozotocin
TZDs	thiazolidinediones

CHAPTER I

INTRODUCTION

1. Diabetic cardiomyopathy

1.1. Introduction to diabetic cardiomyopathy

Diabetes mellitus is one of the most common chronic diseases affecting a large population worldwide. The prevalence of diabetes mellitus is increasing, and it is estimated that the number of people affected with diabetes in the world will increase to 552 million by 2030 compared to 366 million in 2011.[1] Patients with diabetes mellitus exhibit increased risk of morbidity and mortality due to the increased incidence of cardiovascular risk factors such as coronary heart disease, peripheral arterial disease and congestive heart failure. In the past, this high incidence of heart failure and mortality in diabetic patients was attributed to hypertension and coronary artery disease. However, several follow-up studies have shown that diabetes is a strong cardiovascular disease risk factor independent of other risk factors typically associated with diabetes such as hypertension and coronary artery disease.[2] This condition is known as *diabetic cardiomyopathy*. Diabetic cardiomyopathy is therefore defined as deterioration of the structure and the function of the myocardium which is associated with diabetes in the absence of other known concomitant cardiovascular risk factors that are frequently associated with diabetes mellitus such as hypertension, dyslipidemia, and coronary artery disease.[3] The existence of diabetic cardiomyopathy as a distinct clinical entity was described for the first time by Rubler *et al*. in 1972 on the basis of post-mortem findings of four diabetic patients. Despite the lack of evidence of coronary artery disease or hypertension, the autopsy dissection of their hearts showed fibrosis and an increase in left ventricular mass.[4] Diabetic cardiomyopathy has since been the subject of a number of clinical, epidemiological, animal, and post-mortem studies.

1.2. Epidemiology

The association between diabetes and heart failure was well-established in older cardiovascular epidemiological studies. The Framingham Study confirmed the strong link between diabetes and heart failure. This study showed that in diabetic men aged 45-74 years, the risk of congestive heart failure was increased by 2.36-fold compared to non-diabetic subjects. The risk of developing heart failure was even higher among diabetic women (5-fold increase). When subjects with coronary or rheumatic heart disease were excluded, the relative risk of congestive heart failure was higher still (3.8-fold and 5.5-fold in diabetic men and women, respectively).[5] Patients with idiopathic cardiomyopathy (ICM) also present with higher prevalence of diabetes (26.6%) compared to 17.2% in non-ICM subjects. This association between diabetes and ICM was independent of age, race, income, or hypertension.[6] Moreover, the incidence of diabetes detected in heart failure patients is very high. In the Organized Program to Initiate Lifesaving Treatment in Hospitalized Patients with Heart Failure, among patients studied, 42% had diabetes.[7] Left ventricular diastolic dysfunction, an early indicator of diabetic cardiomyopathy, was evident in 32% of normotensive diabetic patients.[8] However, this prevalence was based on standard echocardiography testing which was frequently unable to detect mild and early diastolic dysfunction.[9] Using Doppler echocardiography to assess the prevalence of pre-clinical diabetic cardiomyopathy in a diabetic population with no previous evidence of heart failure, coronary or other structural heart disease, diabetic cardiomyopathy was evident in 48% of diabetic patients. Diastolic function was abnormal in 38% of the patients.[10]

1.3. Physical characteristics

1.3.1. Structural remodeling

1.3.1.1. Left ventricular hypertrophy

The 2003 European Society of Cardiology criterion for left ventricular hypertrophy (LVH) defines LVH as left ventricular mass that exceeds 125 g/m^2 for men and 110 g/m^2 for women.[11] The Strong Heart Study indicated that type 2 diabetic patients exhibited increases in left ventricular mass and wall thickness independently of body mass index and arterial blood pressure compared to non-diabetic subjects.[12] In a multiethnic population study including 443 patients with type 2 diabetes, transthoracic echocardiographic measurements showed an approximate 1.5-fold increased risk of LVH in diabetic patients compared to their age-matched, non-diabetic controls.[13] Several factors have been implicated in the risk of LVH in diabetic patients. In the Strong Heart Study, LVH was positively correlated with microalbuminuria.[14] Another study on normoalbuminuric type 2 diabetic patients showed that 43% of the patients had LVH that correlated with raised body mass index, poor glycemic control, and elevated urinary albumin excretion rate.[15]

1.3.1.2. Interstitial fibrosis

Increased collagen accumulation and interstitial fibrosis in human diabetic hearts contributes to diastolic left ventricular stiffness. Ventricular myocyte hypertrophy and interstitial fibrosis were demonstrated in biopsy samples from diabetic patients without hypertension or coronary artery disease.[16] Collagen remodeling in hearts of type 2 diabetic patients was due to accumulation of collagen type III in the perimysium and perivascular regions.[17] Accelerated formation of glucose-derived advanced glycation

end products (AGEs), as a result of hyperglycemia,[18] also contributes to the development of left ventricular diastolic stiffness in diabetic hearts. AGEs cross-link to collagen, thereby increasing the tensile strength of collagen. AGEs can also augment collagen formation.[19] These findings show the potential role of fibrosis and AGEs in the pathogenesis of heart failure in diabetic patients.[20]

1.3.2. Diastolic dysfunction

Diastole is the interval of the cardiac cycle during which the myocardium produces no active tension and returns to its relaxed length and force.[20] Diastolic dysfunction occurs when the left ventricle exhibits abnormal mechanical properties including abnormal diastolic compliance, impaired filling, and slow diastole.[21] Impaired left ventricular diastolic dysfunction is the earliest evidence of functional abnormalities in diabetic cardiomyopathy.[22, 23] Romano *et al*. reported that left ventricular diastolic dysfunction was evident in 42% of patients within an otherwise cardiovascular-asymptomatic population with type 2 diabetes. Of note, left ventricular systolic function was not yet impaired in these patients.[22] The incidence of diastolic dysfunction in diabetic patients correlates with duration of diabetes and levels of glycated hemoglobin (HbA1C).[24] This association between glycemic control and diastolic dysfunction in diabetes may be explained by increased AGEs accumulation in the diabetic myocardium resulting in increased collagen deposition and cardiac fibrosis.[19] Since diastolic dysfunction is an early manifestation of diabetic cardiomyopathy, regular assessment of diabetic patients for the detection of subclinical diastolic dysfunction is important to limit the deterioration of cardiac function.[25]

1.3.3. Systolic dysfunction

Systole is defined as the interval of the cardiac cycle during which the myocardium contracts and blood is ejected from the ventricles. The relationship between diabetes and systolic dysfunction has been confirmed in a number of clinical studies. In a study of 1046 asymptomatic diabetic patients without known coronary artery disease, 16.7% of the patients had reduced left ventricular ejection fraction (LVEF) and the annual mortality rate for this group was 7%.[26] Many diabetic patients have normal left ventricular systolic function at rest and exhibit abnormalities during exercise. For example, the effect of exercise was investigated in 30 diabetic men without coronary artery disease or other cardiovascular diseases and with normal LVEF at rest. LVEF was reduced during exercise in 17% of the patients, and increased normally in only 56% of the patients. No significant change in LVEF was observed in 27% of the patients.[27]

Several clinical trials have shown that diabetic patients have deteriorated diastolic function and normal systolic function; this may be due to the lower sensitivity of systolic function evaluation vs. diastolic function assessment.[28] Techniques such as strain, strain rate, and myocardial tissue Doppler velocity have since been shown to be more sensitive in detecting preclinical systolic dysfunction in diabetic patients.[29]

1.4. Pathophysiological mechanisms

1.4.1. Metabolic aberrations

1.4.1.1. Fatty acid transport and metabolism

Under normal physiological conditions, 60-90% of ATP necessary to maintain the contractile function of the heart is generated through beta-oxidation of fatty acids, whereas glucose and lactate account for 10-40% of the energy provided to the heart.[30]

However, the diabetic heart is characterized by enhanced fatty acid oxidation and decreased carbohydrate metabolism.[31] Furthermore, in type 1 and type 2 diabetes mellitus, glucose uptake, glycolysis, and pyruvate oxidation are impaired.[31] The augmented fatty acid metabolism in the diabetic heart arises from enhanced lipolysis and fatty acid release from adipose tissue as a consequence of depressed insulin signaling with associated abnormalities in glucose uptake and utilization. Accordingly, the diabetic heart rapidly switches to an exaggerated reliance on fatty acid metabolism as the exclusive source for ATP generation.[31] Fatty acids are supplied to the heart either through lipolysis of endogenous triglycerides or from the circulation as free fatty acids bound to albumin.[32] This leads to an abnormal increase in the oxygen requirement for catabolism and intracellular accumulation of toxic intermediates of fatty acid metabolism such as long chain acyl-CoA and acylcarnitine.[32] Compared to glucose oxidation, enhanced fatty acid oxidation is less efficient at generating ATP since hearts utilize more oxygen for ATP production during fatty acids metabolism vs. glucose.[31] In addition, increased fatty acid plasma concentration induces insulin resistance by activating protein kinase C-θ (PKC-θ), a serine/threonine kinase that phosphorylates and activates IkB kinase. IkB kinase then phosphorylates insulin receptor substrate-1 (IRS-1), which decreases activation of IRS-1-associated phosphatidyl inositol 3-kinase (PI3K), impairing insulin signal transduction (Figure 1).[33, 34] This mechanism occurs in skeletal muscle and adipose tissue; however, its role is not known in cardiac muscle.[35] Free fatty acid overload can directly impair myocardial function and cause lipotoxic heart disease.[36] An animal model of type 2 diabetes, the ob/ob mouse, exhibits increased expression of cardiac genes that enhance cardiac fatty acid uptake and

triglyceride storage. Furthermore, lipid accumulation in the myocardium is associated with diastolic dysfunction.[37] Collectively, these findings identify a critical role for enhanced fatty acid utilization in the development of diabetic cardiomyopathy by inducing myocardial lipid toxicity, insulin resistance, and by affecting cardiac contractility.

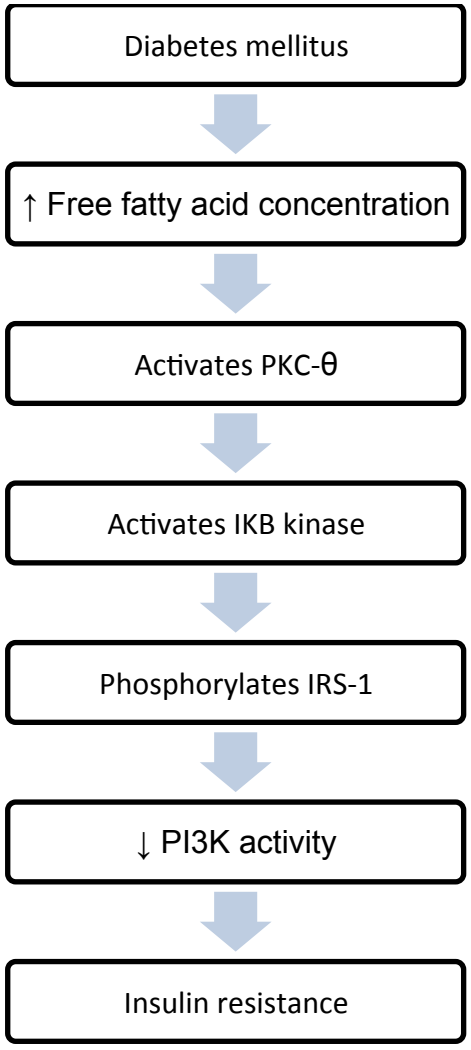

Figure 1. Proposed mechanism of free fatty acid-induced insulin resistance.

1.4.1.2. Glucose transport and metabolism

In the diabetic myocardium, glucose supply and utilization are impaired at several levels. A reduction in glucose oxidation was observed in cardiomyocytes isolated from the streptozotocin (STZ)-induced diabetic rat.[38] Similarly, myocardial glucose utilization rates in type 2 diabetic patients were reduced compared to normal subjects.[39] Impaired glucose utilization in the diabetic heart is probably the result of diminished rates of glucose transport across the myocardial sarcolemmal membrane due to depletion of glucose transporters (GLUT1 and GLUT4).[40, 41] However, impaired glucose transport and utilization can be corrected by insulin therapy[40] and forced overexpression of GLUT4.[42] Reduced glucose oxidation in the diabetic heart is also caused in part by the inhibitory effect of increased circulating free fatty acid levels.[43] When free fatty acid supply to the heart increases, free fatty acid oxidation rate increases and glucose oxidation decreases.[43] Again, fatty acid oxidation requires more oxygen per ATP produced compared to glucose oxidation. Therefore, the net result of enhanced fatty acid oxidation and reduced glucose oxidation in the diabetic heart is decreased cardiac efficiency. In other words, the amount of work produced per oxygen molecule consumed by the heart is reduced.[43]

1.4.1.3. Calcium homeostasis

Regulation of intracellular calcium concentration is a critical determinant of contractile performance of the heart. Calcium influx triggered by activation of voltage-dependent L-type calcium channels upon membrane depolarization induces the release of calcium via calcium release channels (ryanodine receptors) of sarcoplasmic reticula through a calcium-induced calcium release mechanism.[44] Calcium ions then diffuse through the

cytosolic space to contractile proteins to bind to troponin C resulting in the release of the inhibitory troponin I. Calcium binding to troponin C triggers sliding of thin (actin, troponin, tropomyosin) and thick (myosin) filaments resulting in cardiac force generation and contraction. Calcium is then returned to diastolic levels by activation of the sarcoplasmic reticulum (SR) calcium pump (SERCA2a), the sarcolemmal (SL) Na^{+}/Ca^{2+} exchanger (NCX), and the SL Ca^{2+} pump.[44] Impaired calcium homeostasis in the diabetic heart can result from diminished expression of NCX[45] or SERCA2a.[46] In the STZ-induced diabetic rat, defective calcium signaling was attributed to decreased expression of SR Ca^{2+} transport and NCX proteins,[47] and was associated with systolic and diastolic dysfunction.[47] Overexpression of SERCA2a in diabetic hearts protected from contractile dysfunction, possibly by improving the Ca^{2+} sequestration of the SR.[48] Impaired SR function in the diabetic myocardium may compromise cardiac performance in two ways. The depressed Ca^{2+} uptake by SR causes a slower rate of cardiac relaxation.[49] Furthermore, as a result of decreased Ca^{2+} release from the SR, Ca^{2+} levels available for force generation decrease, and this in turn lowers indices of tension generation in the diabetic myocardium.[49]

1.4.2. Hyperglycemia-induced aberrations

1.4.2.1. Advanced glycation end products

Advanced glycation end products (AGEs) are formed mainly from the non-enzymatic reaction of reducing sugars with amino acids in proteins, lipids, or DNA.[50] Chronic hyperglycemia promotes accumulation of AGEs[51] and may contribute to the development of diabetic cardiomyopathy.[52] AGEs affect proteins in the extracellular matrix such as collagen and elastin by creating cross-links, and excessive cross-linking

increases the rigidity of the extracellular matrix proteins. This may contribute to the development of diastolic dysfunction in the heart.[53] AGEs also enhance reactive oxygen species production by binding to the receptor for advanced glycation end products, RAGE.[54] A study by Ma and colleagues showed increased methylglyoxal, AGEs, and receptors for AGEs (RAGE) levels in the STZ-rat heart, particularly in cardiomyocytes.[55] These effects were blocked by *in vivo* short interfering RNA knockdown of RAGE expression.[55] Furthermore, prolongation of time to peak shortening and time to relengthening of cardiomyocytes were blocked by an antibody to RAGE.[55] Alagebrium chloride (ALT-T11), an AGE cross-link breaker, improved SR Ca^{2+} reuptake in cardiomyocytes, and prevented diastolic dysfunction *in vivo*.[56] Thus, the AGE-RAGE axis clearly contributes to the pathogenesis of diabetic cardiomyopathy.

1.4.2.2. Oxidative stress

Oxidative stress is imbalance between free radical production and elimination by protective antioxidant systems.[57] Continuous generation of reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical, and hydrogen peroxide is normal, but ROS levels are regulated by antioxidant enzymes such as superoxide dismutase, glutathione, peroxidase, catalase, and thioredoxin.[58] During pathological conditions, ROS production becomes excessive, resulting in oxidative stress and harmful effects of ROS on different body tissues.[58] Hyperglycemia induces oxidative stress by elevating ROS levels through glucose autoxidation, AGEs formation, and activation of the polyol pathway. The elevated free fatty acid levels in diabetes also contribute to the increased formation of ROS.[59] Excess free fatty acids enter the citric acid cycle and generate acyl-CoA to produce excess NADH. This in turn increases mitochondrial superoxide

production.[59] In patients with uncomplicated type 1 diabetes of short duration, impaired total plasma antioxidant capacity and increased oxidative stress were detectable compared to control subjects.[60] Activation of the renin-angiotensin system also increases oxidative stress, and in diabetes,[61] hyperglycemia upregulates the local (myocyte) renin-angiotensin system to increase angiotensin II (AngII) levels.[62] AngII induces oxidative damage and contributes to cardiac cell death.[63] In the diabetic heart, enhanced ROS formation is associated with reduced antioxidant capacity of the heart; the resultant oxidative stress contributes to the onset of structural and functional abnormalities.[58] Increased ROS formation as a result of high glucose levels in diabetes induces cardiomyocyte apoptosis through the cytochrome C-activated caspase-3 pathway.[64] These findings suggest that myocardial oxidative stress plays a major role in the pathogenesis of diabetic cardiomyopathy.

1.4.2.3. Activation of the diacylglycerol-protein kinase C pathway

In the hyperglycemic or diabetic state, diacylglycerol (DAG) levels are chronically elevated due to *de novo* synthesis through the glycolytic pathway.[65] Augmented DAG levels occur in the aorta and hearts of STZ-induced diabetic rats,[66] and high glucose treatment increased DAG levels in cultured endothelial and vascular smooth muscle cells. These data suggest a relationship between DAG levels in diabetes and hyperglycemia.[66] The high levels of DAG activate PKCs aberrantly in diabetes. In the myocardium from diabetic rats, PKC translocation (activation) to the membrane fraction is elevated,[67] and enhanced activity of PKC in the membrane fraction was attributed to translocation of PKC from the cytosolic to the membrane fraction. Of the six PKC

isoforms, the PKC-β2 isoform is predominantly up-regulated in diabetes; moreover, increased PKC-β2 activity is associated with cardiac hypertrophy and fibrosis.[68]

1.4.2.4. Experimental evidence of glucose-induced cardiomyopathy

To examine the direct effect of hyperglycemia on cardiac structure and function, an *in vitro* model has been developed in primary cardiomyocytes derived from healthy animals to stimulate the hyperglycemic milieu of diabetes. A typical experimental paradigm is exposure to concentrations of D-glucose (25 to 30 mM or 450 to 540 mg/dL vs. 5 mM or 100 mg/dL) to represent high and normal glucose levels, respectively, for 12-72 hours.[69-71] The effect of hyperglycemia on the structure of isolated cardiomyocytes has been assessed in many *in vitro* studies. In neonatal rat cardiomyocytes, the presence of high glucose (25 mM) for 48 hours induced hypertrophy as indicated by increases in cell size and mRNA levels of hypertrophic genes (atrial natriuretic peptide, brain natriuretic peptide, and beta-myosin heavy chain).[70] As hyperglycemia increases the intracellular content of DAG, in turn leading to increased expression and/or activity of PKC-α, PKC-β2, nuclear factor (NF)-kB and c-Fos, these signals may contribute to the development of diabetic cardiomyopathy.[72]

High glucose levels also impair contractile function of cardiomyocytes isolated from healthy animals. In adult rat cardiomyocytes, high glucose (25.5 mM) altered excitation-contraction coupling by prolonging action potential duration, calcium transients, and relaxation after only a 24 hour exposure.[73] Similar effects were observed in cardiomyocytes isolated from STZ-induced diabetic animals after only 4-6 days of STZ injection.[74] Others also reported that adult rat cardiomyocytes exposed to high glucose exhibit abnormal mechanical function including decreased peak

shortening, maximal velocity of shortening/relengthening, prolonged time-to-peak shortening, time-to-90% relengthening, and intracellular calcium clearance. Furthermore, sarcoplasmic reticulum Ca^{2+}-ATPase activity was inhibited in response to high glucose.[75] These high glucose-mediated contractile abnormalities of cardiomyocytes were inhibited by increased extracellular calcium.[75]

1.5. Management of diabetic cardiomyopathy

1.5.1. Glycemic control

As discussed previously, hyperglycemia causes abnormalities in the heart, including aberrant substrate supply and utilization, free fatty acid elevation, oxidative stress, and lipotoxicity. Therefore, glycemic control is an important goal to reduce the cardiovascular risk in diabetic patients. Elevated fasting plasma glucose and glycated hemoglobin (HbA1c) levels are associated with increased risk of myocardial infarction and angina.[76] Improved glycemic control in patients with type 1 diabetes mellitus is associated with reduced cardiovascular risk. In the Diabetes Control and Complication Trial, 1441 type 1 diabetic patients received intensive diabetes therapy (>3 daily injections of insulin or treatment with an external insulin pump) or conventional therapy (1-2 daily injections of insulin) over 6.5 years. The number of major macrovascular events in the conventionally-treated group was 40 compared to 23 in the intensive-treatment group; however, the differences were not statistically significant.[77] 93% of the 1441 patients were subsequently followed in the Epidemiology of Diabetes Intervention and Complications study. After 17 years of follow up, intensive diabetes treatment reduced the risk of cardiovascular events by 42% and the risk of myocardial infarction, stroke or death from cardiovascular disease by 57%. These beneficial effects of

intensive therapy were associated with decreases in the glycosylated hemoglobin levels.[78] However, even though optimal glycemic control is epidemiologically associated with a lower risk of cardiovascular events, many major prospective clinical trials, such as the Action to Control Cardiovascular Risk in Diabetes (ACCORD),[79] Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE),[80] and Veterans Affairs Diabetes Trial (VADT),[81] failed to confirm that tight glycemic control improves the macrovascular outcomes in patients with type 2 diabetes.

1.5.2. Sulfonylureas

Sulfonylureas are widely used in the management of type 2 diabetes mellitus. These drugs exert their hypoglycemic action by stimulating insulin release from pancreatic beta cells. They bind to the SUR subunit of ATP– sensitive potassium channels in the pancreatic beta cells.[82] This maintains the channels in closed position and stimulates the influx of calcium ions into the cells resulting in insulin release by exocytosis.[82] Few clinical studies have examined the outcome of sulfonylurea use in diabetic heart failure. Although one study indicated that sulfonylureas are not associated with increased risk of mortality compared with other insulin secretagogues,[83] there is evidence that they may increase the risk of cardiovascular events in diabetic patients.[84] Traditional sulfonylurea drugs are not specific for pancreatic beta cells. For example, they also bind to ATP – sensitive potassium channels in cardiomyocytes and in vascular smooth muscle cells.[84] A case – control study revealed that sulfonylurea drug use is associated with increased risk of in-hospital mortality among diabetic patients undergoing coronary angioplasty for acute myocardial infarction.[85] This increased risk may reflect the

deleterious effects of these drugs on myocardial tolerance for ischemia and reperfusion.[85] For surviving patients, sulfonylurea drug use was not associated with increased incidence of serious late adverse events.[85] Due to their inhibitory effect on ATP – sensitive potassium channels found in the heart, the use of sulfonylureas in diabetic patients also attenuates echocardiographic ST-segment elevation during acute myocardial infarction which may delay its diagnosis.[86] These findings suggest that another anti-diabetic agent should be considered in this high risk population.[86] Generally, the use of traditional sulfonylurea drugs, such as glibenclamide, should be avoided. Instead, the use of more pancreas-specific agents that have less effect on myocardial ATP-sensitive potassium channels, such as glimepiride, glicizide and nateglinide, should be considered.

1.5.3. Biguanides

Biguanides belong to a class of anti-diabetic drugs of which metformin is the only drug still used in most countries. Metformin exerts its anti-diabetic effects by inhibiting hepatic gluconeogenesis, stimulating peripheral glucose uptake, and reducing glucose absorption from the gastrointestinal tract. Metformin also decreases glycated hemoglobin (HbA1c) and improves blood lipid profile.[87] However, metformin use in diabetic patients with cardiovascular disease is strongly cautioned. This is due to the theoretical risk of lactic acidosis, particularly in patients with conditions such as heart failure or myocardial infarction which increase the risk of lactic acidosis.[87] Nevertheless, data from 3 cohort studies showed that metformin use is associated with a decrease in mortality and was not associated with risk in patients with heart failure compared to other anti-diabetic agents.[83] Metformin was recently shown to be as safe as other anti-

diabetic agents in diabetic patients with heart failure, even in patients with chronic kidney disease or reduced left ventricular ejection fraction.[88] Furthermore, metformin decreased all-cause hospitalization without increased risk of lactic acidosis.[88]

1.5.4. Thiazolidinediones (TZDs)

TZDs are a class of oral anti-diabetic agents used in the treatment of type 2 diabetes mellitus. They act by increasing insulin sensitivity in skeletal muscle and adipose tissue through peroxisome-proliferator activated receptor (PPAR) – γ. PPAR-γ is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors that regulate gene transcription.[89] Additionally, these agents have beneficial effects on lipid profile, blood pressure, inflammation, and peripheral and coronary endothelial function.[89] However, TZDs contribute to fluid retention by PPAR-γ-mediated stimulation of sodium reabsorption through sodium channels in the renal collecting tubule cells,[90] and therefore increase the risk of heart failure.[91] This increased risk of heart failure in patients randomized to TZDs compared with placebo occurred at high and low doses after a treatment duration of 24 weeks and was not limited to the elderly.[91] Current evidence suggests that TZDs have equivalent efficacy to other anti-diabetic agents with significant safety concerns and contraindications. TZDs are contraindicated in patients with congestive heart failure, bladder cancer, and severe osteoporosis.[92]

1.5.5. Renin angiotensin system inhibition

Angiotensin converting enzyme inhibitors (ACEI) and angiotensin II receptor blockers (ARB) are also beneficial in diabetic cardiomyopathy. Administration of ramipril (2.5 mg/day), or telmisartan (40 mg/day), or their combination to asymptomatic patients with type 2 diabetes for 3 months improved echocardiographic indices of left ventricular

diastolic function and reduced plasma BNP levels.[93] The possible beneficial effect of losartan in patients with type 2 diabetes was investigated in the Losartan Intervention For Endpoint reduction (LIFE) and Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) studies. Losartan reduced the incidence of first hospitalization for heart failure compared to placebo in the RENAAL study and compared to atenolol in the LIFE study.[94]

1.5.6. β-blockers

β-blockers are now well-established in the treatment of heart failure. Due to concerns regarding dyslipidemia and worsening insulin resistance, diabetic patients with heart failure are less likely to be discharged from the hospital on a β-blocker.[95] However, given that β-adrenoceptors modulate vasoactive substance release and that β-blockers improve left ventricular function and symptoms in chronic heart failure,[96] β-blockers are now accepted as a well-tolerated treatment in patients with heart failure and diabetes.[97] Chronic treatment with metoprolol (β1-selective inverse agonist) has been shown to ameliorate the decline in cardiac function in the STZ–induced diabetic cardiomyopathy.[98] However, the effect of β-blockers in patients specifically with diabetic cardiomyopathy has not yet been investigated in clinical trials.

1.5.7. Antioxidants

As the progression of diabetic cardiomyopathy involves oxidative stress and accumulation of ROS, antioxidants have received considerable interest as potential therapeutic agents against diabetic cardiomyopathy. However, clinical trials have been unable to demonstrate any protective effects from the use of antioxidants in patients at high risk for cardiovascular events. For example, in the Heart Outcomes Prevention

Evaluation (HOPE) Study that included patients at high risk for cardiovascular events, treatment with 400 IU of vitamin E daily for a mean of 4.5 years had no detectable beneficial effects on cardiovascular outcomes compared to placebo.[99]

2. Conjugated Linoleic Acid (CLA)

The term conjugated linoleic acid (CLA) refers to a mixture of 28 positional and geometric isomers of unconjugated linoleic acid, an 18-carbon carboxylic acid with 2 *cis* double bonds located at carbons 9 and 12.[100, 101] As distinct from unconjugated linoleic acid, in CLA double bonds are conjugated (separated by a single double bond) and are in *cis* or *trans* configurations.[102] Most of the physiological effects of CLA have been reported to be produced by isomeric mixtures of CLA in which *cis* 9, *trans* 11 and *trans* 10 *cis* 12 isoforms (Figure 2) are the main constituents.[102] CLA is found in dairy products from ruminant animals and is mainly produced in rumen (the first part of stomach of ruminant animals) during the microbial biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*, or from biohydrogenation of transvacceninc acid in mammary tissue.[103] CLA has been reported to have numerous health benefits including effects to reduce carcinogenesis,[102] atherosclerosis,[104] and body fat mass.[105]

Figure 2. Structures of unconjugated linoleic acid (top), c-9, t-11 CLA (middle) and t-10, c-12 CLA isomers (bottom).[106] This figure was used with permission from the Turkish Journal of Veterinary and Animal Sciences.

2.1. Health properties of CLA

2.1.1. Anticarcinogenic activities of CLA

Data from many studies demonstrate that dietary CLA inhibits cancer in experimental models, including papilloma,[107] stomach neoplasia,[108] and mammary tumors.[109] In addition, CLA was shown to inhibit tumor progression of prostatic carcinoma cells in the severe combined immunodeficient (SCID) mouse model.[110] An epidemiological study investigated the association between dairy products consumption and breast cancer, and showed that milk consumption reduced the incidence of breast cancer in women during a 25 year follow-up period.[111]

Even though the anti-tumor activity of CLA is well-established in different experimental models of cancer, some studies did not find any significant effect from CLA supplementation on tumorigenesis. For example, CLA did not inhibit oncogenesis

in the Apc Min mouse, a genetic model of intestinal tumorigenesis,[112] and did not affect the development of an invasive mammary tumor.[113]

The exact mechanism by which CLA inhibits tumorigenesis is not fully understood. However, it has been proposed that CLA exerts its anticarcinogenic effects by inhibiting carcinogen activation,[105] inhibiting DNA adduct formation,[108] or by inducing phase I detoxification pathways.[105]

2.1.2. Fat reducing activity of CLA

Dietary CLA reduces adipose tissue fat accumulation in experimental animals. CLA reduced subcutaneous fat deposition and increased lean body mass in pigs fed a diet containing 2% CLA compared to pigs fed a control diet containing 2% sunflower oil.[114] In addition, CLA was able to reduce body fat mass (BFM) in humans. A randomized, double blind study to determine the effect of supplementation with CLA or placebo on BFM in overweight or obese subjects (body mass index 25-35 kg/m^2) was conducted.[105] CLA supplementation (3.4 and 6.8 g/day for 12 weeks) decreased BFM compared with the control group (received 9 g olive oil). No added beneficial effects on BFM were achieved with doses exceeding 3.4 g/day.

2.1.3. CLA and atherosclerosis

Several animal studies have found that CLA can protect against atherosclerosis development. For example, CLA reduced serum triglycerides and low density lipoprotein levels in rabbits fed a pro-atherosclerotic diet for 22 weeks.[104] Similar findings on cholesterol metabolism were reported in another study conducted in hamsters fed a pro-atherosclerotic diet containing different levels of CLA. Hamsters fed

CLA had decreased levels of plasma total cholesterol and aortic atherosclerosis compared with hamsters fed a diet without CLA.[115] Regardless of these beneficial effects of CLA on atherosclerotic plaque formation in rabbits and hamsters, a study by Munday *et al.* conducted in C57B1/6 mice showed that CLA promoted the development of aortic fatty streaks.[116] Further studies are required to demonstrate conclusively whether CLA exerts beneficial or harmful effects against the development of atherosclerosis.

3. Peroxisome proliferator-activated receptors (PPARs)

PPARs are nuclear hormone receptors that respond to xenobiotic stimulation with peroxisomal proliferation in the liver.[117] Three types of PPARs have been identified: PPAR-α, PPAR-β, and PPAR-γ,[117] which are encoded by 3 distinct genes and have different tissue distributions and functions. PPARs heterodimerize with the retinoid X receptor (RXR) and bind to the promoter region of target genes involved in controlling fatty acid metabolism and storage.[118] The main functional domains in the PPAR structure include an N-terminal domain that regulates PPAR activity, a DNA-binding domain that binds to the PPAR response element (PPRE) in the promoter region of target genes, and a C-terminal region which regulates ligand selectivity.[119] When PPARs are stimulated by a specific ligand, they heterodimerize with RXR and bind to PPRE to regulate gene transcription.[120] In the inactivated state, PPARs are bound to co-repressor proteins. However, when PPARs are activated, they detach from co-repressors and recruit transcriptional co-activator complexes such as PPAR-binding protein and steroid receptor coactivator-1 to stimulate gene expression.[121]

3.1. Peroxisome proliferator activated receptor-α

PPAR-α is activated by natural ligands such as fatty acids and eicosanoids, and by synthetic ligands, the lipid lowering fibrates.[122] PPAR-α is mainly expressed in tissues with high demand for fatty acid catabolism such as hepatocytes, cardiomyocytes, the kidney cortex, and skeletal muscles.[123] PPAR-α has a central role in fatty acid metabolism by regulating gene expression of enzymes involved in fatty acid metabolism, with examples that include fatty acid binding protein and acyl-CoA oxidase.[122]

In the myocardium, the expression of PPAR-α is relatively high and it is responsible for modulation of cardiac fatty acid metabolism by regulating expression of enzymes directly involved in fatty acid oxidation.[124] The expression of PPAR-α decreases as a part of the adaptation process to switch cardiac energy metabolism from fatty acids to glucose utilization. This metabolic shift is mediated by the inhibitory effect of high glucose on free fatty acid metabolism by downregulating PPAR-α, and is particularly important in pathological conditions such as hypertrophy and ischemia.[125]

The use of PPAR-α-knockout mouse model has advanced our understanding of the physiological role of this isoform of PPARs.[126] For example, in the heart, a reduced fatty acid oxidative capacity was reported in the PPAR-α-deficient mouse model. This effect was accompanied by decreased expression of at least seven mitochondrial fatty acid-metabolizing enzymes. Thus, these data establish a central role for PPAR-α in cardiac fatty acid metabolism. Furthermore, abnormalities in the expression of fatty acid-metabolizing enzymes are associated with myocardial damage and fibrosis.[127]

In addition to its role in fatty acid metabolism, PPAR-α activation has favorable effects on reducing insulin resistance in nutritional (high-fat diet), genetic (Zucker obese *fa/fa* rat), or lipoatrophic (A-ZIP/F-1) models of insulin resistance.[128-130] This beneficial effect of PPAR-α activation on insulin sensitivity is explained by the fact that intracellular fatty acids and their derivatives inhibit insulin-mediated glucose metabolism either through metabolic competition or through a direct effect on insulin signaling pathway. Therefore, these protective effects of PPAR-α activation are mediated by increased fatty acid oxidation, thus decreasing tissue lipid accumulation and minimizing insulin resistance and lipotoxicity.[131]

3.2. Peroxisome proliferator activated receptor-β

PPAR-β is widely expressed in many tissues including the heart.[132] The main function of PPAR-β is to induce fatty acid oxidation by modulating transcriptional programs involved in fatty acid metabolism.[133] The role of PPAR-β in myocardial fatty acid oxidation was investigated by cardiomyocyte-restricted PPAR-β knockout mice. In these mice, cardiac-specific deletion of PPAR-β expression downregulates the expression of fatty acid oxidation genes and inhibits myocardial fatty acid oxidation. Furthermore, these mice exhibit cardiac dysfunction, progressive myocardial lipotoxicity, cardiac hypertrophy and increased mortality rate.[133] In STZ-induced diabetes, cardiomyopathy was associated with a decrease in cardiac expression of PPAR-β. The reduction in PPAR-β during hyperglycemia was associated with an increase in reactive oxygen species, cardiomyocyte hypertrophy, and augmented protein synthesis.[134] Further studies are needed to understand the precise mechanistic function of PPAR-β ligands in diabetes mellitus and cardiovascular disease.

3.3. Peroxisome proliferator activated receptor-γ

PPAR-γ is expressed in many tissues including, adipose tissues, the heart, mucosa of colon, immune cells, and in the placenta.[131, 135] PPAR-γ plays a critical role in modulating glucose metabolism and lipogenesis and has been shown to have beneficial effects on inflammation, hypertrophy, and atherosclerosis.[128] Natural ligands for the PPAR-γ isoform include unsaturated fatty acids such as oleate, linoleate, eicosapentaenoic and arachidonic acids.[122] In addition, TZDs, insulin sensitizing medications used in the treatment of type 2 diabetes, are PPAR-γ-selective ligands.[136]

Even though TZDs use is associated with fluid retention and weight gain, particularly when used in combination with insulin,[137] TZDs have been shown to have several health benefits in diabetic cardiomyopathy and hypertension. For example, rosiglitazone may have an antiapoptotic effect in diabetic cardiomyopathy.[138] Rosiglitazone also reduced cardiac fibrosis and left ventricular diastolic dysfunction via inhibiting receptors for advanced glycation end products and connective tissue growth factor in the diabetic myocardium.[139] Rosiglitazone treatment was shown to reverse high-fructose diet-induced hypertension in rats,[140] prevent the development of hypertension and protect against impaired endothelial function in Zucker fatty rats.[141] These protective effects of rosiglitazone on blood pressure and vessel function are mediated, at least in part, by its direct effect on blood vessels.[142]

4. Diacylglycerol kinases (DGKs)

Diacylglycerol (DAG) is a lipid second messenger, which is generated by hydrolysis of intracellular phospholipids by phospholipase C enzymes (PLC).[143] DAG is known to be

involved in different biological processes by acting as an endogenous ligand for many important proteins including protein kinase C (PKC) isoforms,[144] RasGRP nucleotide exchange factors,[145] and some transient receptor potential channels.[146] DAG is mainly metabolized by a group of nine enzymes collectively known as diacylglycerol kinases (DGKs).[147] DGKs belong to a family of intracellular lipid kinases that catalyze the phosphorylation of DAG to generate phosphatidic acid (PA), thereby regulating DAG-PKC signaling and the subsequent hypertrophic response.[148, 149] The structural differences in DGK isoforms in mammals indicate that these enzymes have critical roles in higher vertebrates, including advanced neural functions, immune surveillance, or organogenesis.[150] All of the DGK isoforms have two common structural subunits: a catalytic domain that is necessary for kinase activity, and two cysteine rich regions, the C1 domains, that bind to DAG and play a critical role in its subcellular localization.[150, 151] In addition to the catalytic domains and C1 domains, DGKs contain other structural motifs that have different regulatory functions. Based on the existence of these structural motifs, DGK isoforms are divided into five subfamilies.[152]

Type I DGKs (isoforms α, β, and γ) contain calcium binding EF hand motifs that increase their activity in the presence of calcium.[153] Type II DGKs (isoforms δ and η) have pleckstrin homology (PH) domains,[154] which have been demonstrated to bind weakly and non-selectively to phosphatidylinositols.[155] The type III enzyme (DGKε) has specificity towards acyl chains of DAG, particularly arachidonate-containing DAG.[154] Members of the type IV group (isoforms ζ and ι) have C-terminal ankyrin repeats and a PDZ-domain binding sequence, as well as myristoylated alanine-rich C-kinase substrate

(MARCKS) homology region upstream of the catalytic region.[147] The type V enzyme, DGKθ, has three cysteine-rich domains and a PH domain.[150]

4.1. DGKζ

DGKζ is a type IV DGK with ankyrin repeats, which are known to play a critical role in protein-protein interaction.[148] In addition, DGKζ contains a MARCKS homology domain and a carboxy-terminal PDZ binding domain that play an important role in its translocation (localization) in different cellular compartments, such as the nucleus, plasma membrane, and cytoplasm.[148]

Role of DGKζ in cardiac structure and function

Of the nine DGK isoforms, only DGKζ, ε, and α have been demonstrated to be expressed in the heart, with DGKζ being the predominant isoform.[156] Several reports have shown that DGKζ has beneficial actions on cardiac structure and function in animal models. For example, its overexpression in transgenic mice prevented cardiac hypertrophy, fibrosis, and left ventricular systolic dysfunction four weeks after thoracic transverse aortic constriction.[157] These cardioprotective effects were speculated to be achieved by attenuating G-protein-coupled receptor (GPCR) signaling, thereby inhibiting pathological activation of DAG-PKC signaling.[157] The cardiac-specific DGKζ transgene also attenuated cardiac dysfunction and fibrosis in the STZ-induced diabetic mouse model compared to diabetic wild type mice, and this was associated with inhibition of PKC-β and δ isoforms.[156] These findings are consistent with those from another study showing that diabetic mice exhibit decreased expression of DGKζ with upregulation of PKC-β2 and myocardial injury.[71]

5. Current study

This study is based on previous findings from our laboratory on the antihypertrophic effects of CLA.[158] CLA was shown to suppress endothelin-1 (ET1)-induced cardiomyocyte hypertrophy. These antihypertrophic effects of CLA were mediated by the activation of PPARα and γ. Furthermore, CLA upregulated the expression and activity of the antihypertrophic enzyme, DGKζ, and attenuated the activity of PKCε.[158] To our knowledge, no study has investigated the protective potential of CLA on the structural and functional abnormalities of cardiomyocytes in diabetic cardiomyopathy. Therefore, this study was designed to examine the effect of CLA on the structure and contractile function of cardiomyocytes maintained in normal or high glucose environments. The involvement of PPARγ and DGK in the actions of CLA was also investigated.

CHAPTER II

STUDY PHASE ONE:

EFFECTS OF CLA ON HYPERGLYCEMIA-INDUCED STRUCTURAL AND FUNCTIONAL ABNORMALITIES OF CARDIOMYOCYTES

1. Rationale and hypothesis

The development of diabetic cardiomyopathy and the increased risk of heart failure and poor prognosis in diabetic patients have been linked directly to poor glycemic control.[159] The strong association between glycemic control in diabetic patients and the development of diabetic cardiomyopathy has been demonstrated in major clinical trials.[160] For example, the Diabetes Control and Complications Trial (DCCT) demonstrated that diabetic patients who are treated with conventional diabetes therapy (1-2 insulin injections/day) have an approximately double prevalence of diabetic cardiomyopathy as compared to the intensively treated diabetic patients (received 3 or more insulin injections/day or an external insulin pump).[77] The United Kingdom Prospective Diabetes Study (UKPDS) demonstrated that for each 1% decrease in glycated hemoglobin (HbA1C), a measure of glycemic control over a prolonged period of time, there is a 14% decrease in the incidence of myocardial infarction.[160] Furthermore, patients with mild pre-diabetic hyperglycemia have an increased risk of diabetic cardiomyopathy, suggesting that impaired glucose tolerance is a risk for diabetic cardiomyopathy.[160] Thus, hyperglycemia is an independent risk factor for diabetic cardiomyopathy.[64] Experimental models for diabetic cardiomyopathy show that treatment of isolated cardiomyocytes with high glucose leads to the development of structural and functional abnormalities that characterize diabetic cardiomyopathy. For example, treatment of neonatal rat cardiomyocytes with high glucose (25 mM) for 48 hours causes myocyte enlargement (i.e. hypertrophy).[161] Furthermore, high glucose impairs contractile function of adult rat ventricular myocytes and causes both diastolic and systolic dysfunction.[162]

This research project is based on previous findings regarding the cardiovascular effects of conjugated linoleic acid (CLA) from Dr. Hope Anderson's laboratory. Dr. Anderson previously reported in the Journal of Biological Chemistry[158] that CLA prevented indicators of cardiomyocyte hypertrophy induced by endothelin-1 (ET1), including cell size augmentation, *de novo* protein synthesis, and fetal gene activation. Dietary supplementation with CLA also inhibited the development of cardiac hypertrophy *in vivo* in the spontaneously hypertensive heart failure (SHHF) rat, a genetic model of essential hypertension that would normally exhibit cardiac hypertrophy.

The effect of CLA on cardiomyocytes in the context of diabetic cardiomyopathy remains undetermined. The specific aim of this study was therefore to investigate the protective potential of CLA in high glucose-induced structural and functional abnormalities of adult rat cardiomyocytes. Since cardiomyocyte hypertrophy and contractile dysfunction are the two main aberrations of diabetic cardiomyopathy at the cardiomyocyte level,[75, 161] we investigated whether CLA exerts protective effects on hyperglycemia-induced cardiomyocyte hypertrophy and contractile dysfunction in isolated adult rat cardiomyocytes.

To achieve this specific aim, an *in vitro* model of hyperglycemia in isolated cardiomyocytes was developed to test the following hypotheses:

i. hyperglycemia is an independent risk factor for the development of diabetic cardiomyopathy. Accordingly, treating cardiomyocytes with high levels of glucose would lead to cardiomyocyte hypertrophy and contractile dysfunction.

ii. pretreatment with CLA would prevent the development of these structural and functional aberrations.

2. Experimental design and methodology

2.1. Adult rat cardiomyocyte isolation

The experimental procedures used in this study were approved by the Animal Care Committee of the University of Manitoba and the Canadian Council of Animal Care. In brief, adult male Sprague Dawley rats (200-250 g) were anesthetized with 3% isoflurane and injected with heparin into the saphenous vein (1000 U/ml at 1 ml/Kg body weight). The heart was immediately removed and placed into a perfusion chamber and cannulated through the aorta. The heart was washed of blood with calcium-free buffer (mM: NaCl 90, KCl 10, KH_2PO_4 1.2, $MgSO_4.7H_2O$ 5.0, $NaHCO_3$ 15, taurine 30, glucose 20, pH 7.4) for 5 minutes. The heart was then perfused for 20 minutes (at 37°C) with calcium free buffer containing 179 U/Ml collagenase II. After perfusion, ventricles were removed, minced, and incubated for five minutes at 37°C with re-circulated collagenase buffer for further digestion. Isolated cardiomyocytes were then plated on plates precoated with laminin (10 µg/ml) and maintained for 2 hours at 37°C and 5% CO_2 in a medium consisting of medium 199 containing 5% fetal bovine serum, 5% horse serum, and 1% penicillin/streptomycin. After two hours, the medium was replaced with medium 199 supplemented with 5 mM taurine, 2 mM L-carnitine, 1 mM creatine, 2 µM insulin, and 100 IU/ml penicillin/streptomycin.

2.2. Treatments

As previously mentioned, the aim of this study was to investigate the protective potential of CLA on hyperglycemia-induced cardiomyocyte hypertrophy and contractile dysfunction. Vehicle (0.05% dimethylsulfoxide (DMSO)) or CLA (30 μM) were added to cultured cardiomyocytes 1 hour before the addition of glucose. The CLA preparation used in this study contained a mixture of CLA isomers (39.1% c9,t11 and 40.7% t10,c12 CLA); this preparation, according to the manufacturer, also included the following isomers: 1.8% c9,c11 CLA; 1.3% c10,c12 CLA; 1.9% t9,t11 and t10,t12 CLA; 1.1% c9,c12 linoleic acid; and 14% remainder.

To mimic pathophysiological hyperglycemia, cells were treated with incremental concentrations of D-glucose (5, 15, 25 mM) for 24 or 48 hours. These concentrations reflect normal, moderately high, and severely high plasma glucose levels respectively.[163] In addition, the use of 25 mM D-glucose is based on previous studies performed on neonatal and adult rat cardiomyocytes.[75, 161] A potential confounding factor is the increased osmolarity produced by high concentrations of glucose. To isolate the effect of high glucose from any artifactual effect of increased osmolarity, normal glucose treated cells were treated with 5 mM D-glucose and 10 or 20 mM L-glucose, to adjust the osmolarity to match that of groups treated with 15 or 25 mM D-glucose, respectively (Table 1).

Group	Descriptor	CLA: 12-24 or 48 h Time = -1 h	Glucose: 12-24 or 48 h Time = 0 h
1	Normal glucose	-	5 mM D-glc + 10 mM L-glc (for moderately high glucose levels)
2	Normal glucose	-	5 mM D-glc + 20 mM L-glc (for severely high glucose levels)
3	Moderately high glucose	-	15 mM D-glc
4	Severely high glucose	-	25 mM D-glc
5	Normal glucose + CLA	30 μM	5 mM D-glc + 10 mM L-glc (for moderately high glucose levels)
6	Normal glucose + CLA	30 μM	5 mM D-glc + 20 mM L-glc (for severely high glucose levels)
7	Moderately high glucose + CLA	30 μM	15 mM D-glc
8	Severely high glucose + CLA	30 μM	25 mM D-glc

Table 1: Treatment paradigms. Groups 1, 2, 5 & 6 were treated with normal D-glucose (osmotic control). Groups 3 & 7 were treated with moderately high D-glucose. Groups 4 & 8 represent the severely high D-glucose treated groups. Groups 5, 6, 7, & 8 were pretreated (1 h) with CLA before adding glucose.

2.3. Measurement of ventricular myocyte hypertrophy

Hypertrophic growth was detected by 2 distinct parameters: augmentation of myocyte size and *de novo* protein synthesis. The presence of these 2 events provides evidence of myocyte hypertrophy.

2.3.1. Cell size

Cardiomyocyte size was determined with immunofluorescence microscopy. Adult rat ventricular myocytes were cultured on 12-well plates (2×10^5 cells/well), changed to

serum free medium, cultured at 37°C and 5% CO_2 for 24 hours, and then subjected to the treatments indicated in Table 1 for 48 hours. Cells were then washed twice with phosphate-buffered saline (PBS) at room temperature and fixed with 1 ml 4% paraformaldehyde for 1 hour at room temperature. The cells were permeabilized using 0.1% triton X-100 in PBS for 5 minutes and blocked with a blocking solution containing 2% milk in 0.1% triton X-100 for 1 hour. Cardiomyocytes were then incubated with primary antibody (anti-alpha actinin) at a 1:800 dilution in 2% milk for 2 hours at room temperature. Cells were washed with PBS3 times (5 minutes each) and incubated with Alexa 488-conjugated goat anti-mouse secondary antibody at room temperature for 1 hour. Cells were visualized using fluorescence microscopy. Cell surface areas of individual cardiomyocytes were determined using ImageJ software.

2.3.2. Protein synthesis

De novo protein synthesis in cardiomyocytes was studied by [^{3}H]-leucine incorporation assay. Myocytes were cultured on 12-well plates (2 x 10^5 cells/well), changed to serum free medium, cultured at 37°C and 5% CO_2 for 24 hours, and then subjected to the treatments indicated in Table 1 (groups 2, 4, 6, & 8) for 48 hours. After treatments, cells were pulsed with [^{3}H]-leucine (1 µCi/ml) in leucine-free medium for 4 hours. The radioactive medium was removed and the cells were washed with ice-cold PBS. 10% trichloroacetic acid was added and incubated at 4°C for 30 minutes. Cells were lysed with 0.25 N NaOH for 2 hours. 2.5 mM HCl in 1 mM Tris-HCl was added to neutralize the reaction. Radioactivity of the neutralized lysate was measured in a liquid scintillation counter.

2.4. Measurement of ventricular myocyte contractile function

Contractile properties of ventricular myocytes were assessed using a video-based edge-detection system (the Ionoptix HyperSwitch Myocyte System; Figure 3). Cardiomyocytes were cultured on coverslips (1 x 10^6 cells/coverslip), changed to serum free medium, and then treated as indicated in table 1 (groups 2, 4, 6, & 8) for 12-24 hours. To measure the mechanical properties of the cells, coverslips were placed on a chamber mounted on the stage of an inverted microscope and perfused with a buffer containing (in mM): 131 NaCl, 4 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose,10 HEPES, at pH7.4. Cells were stimulated to contract using the IonOptix Myopacer at a frequency of 0.5 Hz. Cardiomyocytes were displayed on a monitor display using an IonOptix Myocam camera. SoftEdge software (IonOptix) was used to compare changes in cell length during shortening (contraction) and relengthening (relaxation). Indices used to evaluate cell contractility included maximal velocity of shortening (+dL/dt), maximal velocity of relengthening (-dL/dt), and peak shortening (PS). These are representations of systolic contraction, diastolic relaxation, and peak force of contraction, respectively.

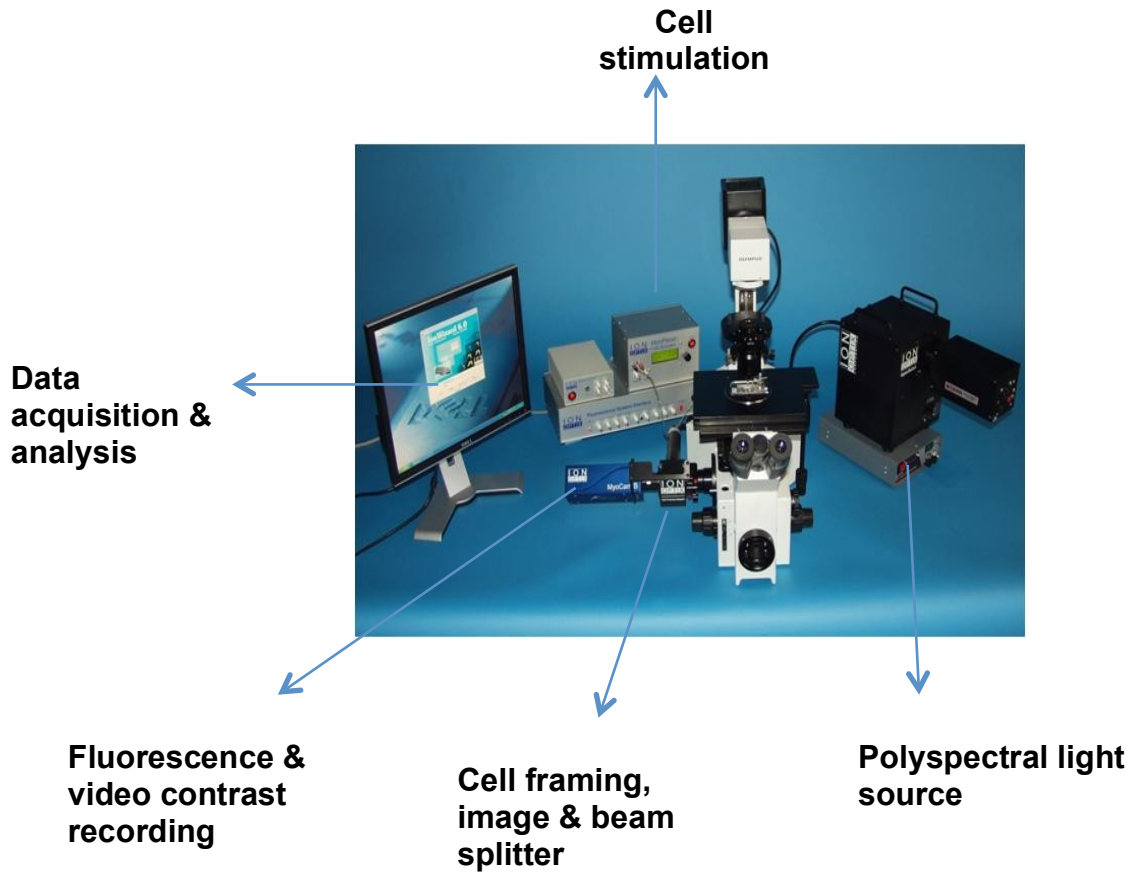

Figure 3. The Ionoptix HyperSwitch Myocyte System. This figure was used with permission from the IonOptix Corporation.

2.5. Statistics

Data analysis was performed with a statistical software package (GraphPad Prism 4.0.) All data are expressed as means ± SEM. Data were subjected to one-way ANOVA and the Newman-Keuls multiple comparison test. For peak shortening data, one-way ANOVA followed by Bonferroni's test were performed. A difference of $p<0.05$ was considered significant.

3. Phase one results

3.1. CLA inhibits high glucose-induced hypertrophy of adult rat cardiomyocytes

Previous studies have shown that hyperglycemia induces hypertrophy in isolated neonatal rat cardiomyocytes.[161] Here, we first reproduced the hyperglycemia model in adult rat cardiomyocytes, since this is a key structural aberration in diabetic cardiomyopathy. Compared to the normoglycemic cells, treatment with moderately high D-glucose resulted in a significant increase in cardiomyocyte size (Figure 4). Treatment with CLA inhibited moderately high glucose–induced cardiomyocyte hypertrophy (Figure 4). Likewise, exposing adult rat cardiomyocytes to severely high D-glucose levels caused enlargement of myocytes (Figure 5), and this was prevented by CLA (Figure 5). Furthermore, [^{3}H]-leucine incorporation into protein was increased by severely high D-glucose (Figure 6), and this was prevented by pretreatment with CLA (Figure 6).

3.2. CLA inhibits high glucose-induced cardiac contractile abnormalities in cultured adult rat cardiomyocytes

Cardiomyocytes maintained in severely high D-glucose displayed reduced +/- dL/dt and PS compared to normal glucose treated myocytes (Figures 7, 8, and 9). High glucose-induced contractile dysfunctions were inhibited by CLA (Figures 7, 8, and 9).

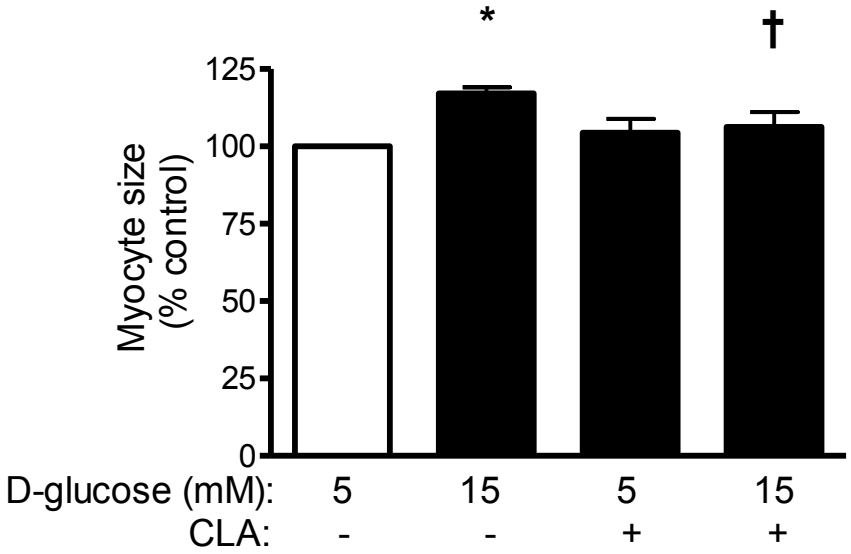

Figure 4. **CLA inhibits moderately high D-glucose-induced cardiomyocyte hypertrophy.** Cell surface areas are presented as percent of myocyte size (μm^2) of the control group. *p<0.05 compared to normoglycemic controls. †p<0.05 compared to untreated hyperglycaemic myocytes. Each bar represents the mean ± SEM from 5 independent experiments.

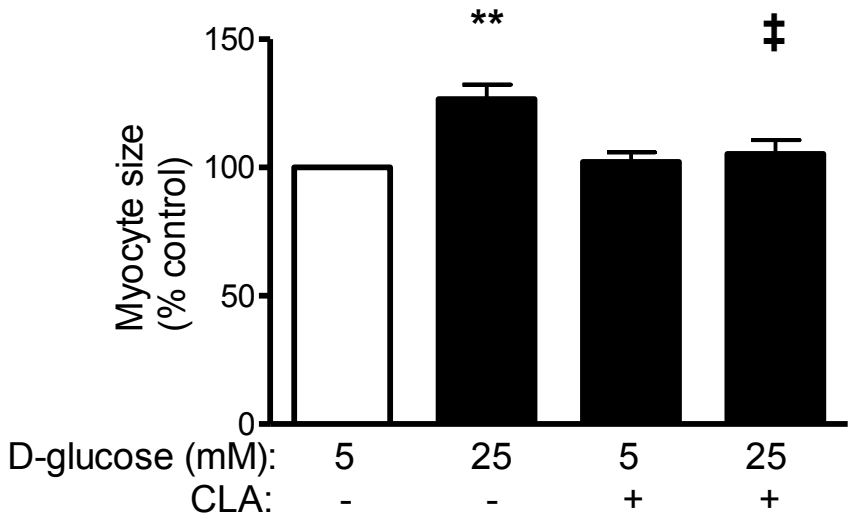

Figure 5. **CLA inhibits severely high D-glucose-induced cardiomyocyte hypertrophy.** Cells surface areas are presented as percent of myocyte size (μm^2) of the control group. ***p*<0.01 compared to normoglycemic controls. ‡*p*<0.01 compared to untreated hyperglycemic myocytes. Each bar represents the mean ± SEM from 4 independent experiments.

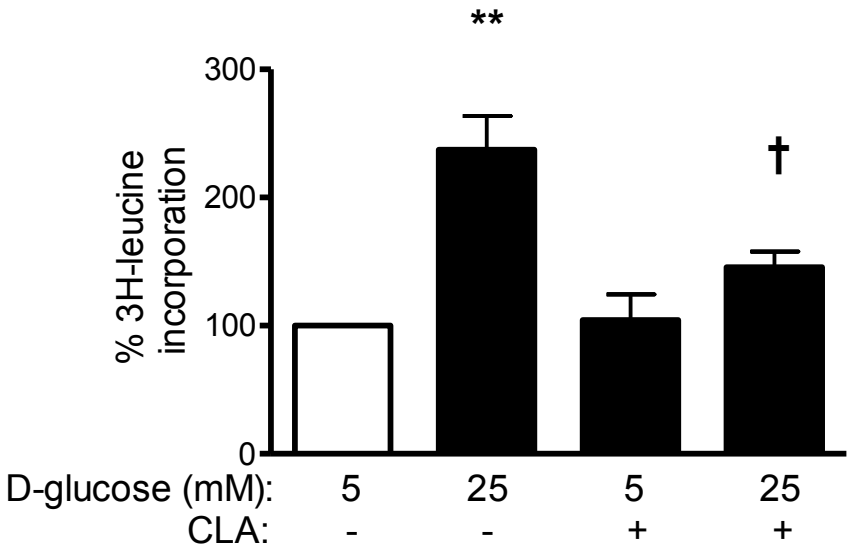

Figure 6. CLA inhibits severely high glucose-induced protein synthesis. 3H-Leucine incorporation into acid insoluble protein is presented as vehicle-treated controls. ** $p<0.01$ compared to normoglycemic controls. †$p<0.05$ compared to untreated hyperglycemic myocytes. Each bar represents the mean ± SEM from 4 independent experiments.

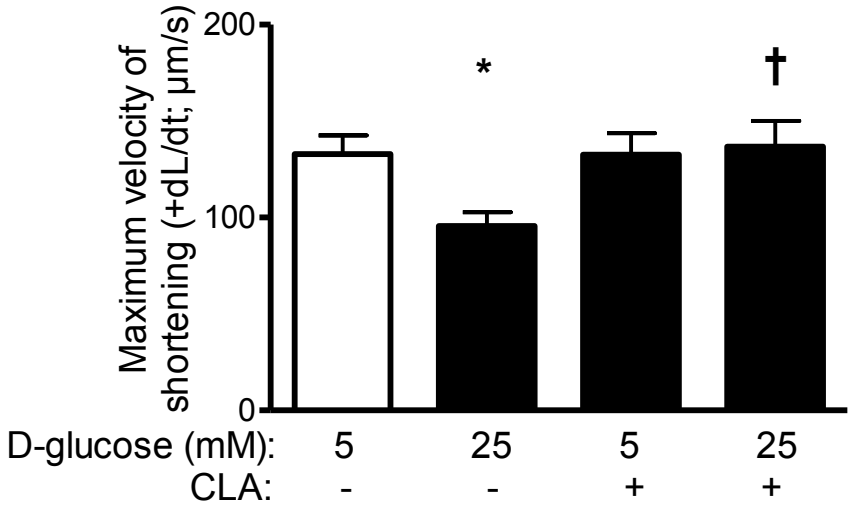

Figure 7. CLA inhibits severely high D-glucose-induced abnormalities in maximum velocity of shortening. Treatment with severely high (25 mM) D-glucose impaired contractile function of adult rat myocytes as measured by maximum velocity of shortening, a representation of systolic contraction. Hyperglycemia-induced contractile dysfunction was prevented by pretreatment with CLA. *p<0.05 vs. untreated normoglycemic myocytes. †p<0.05 compared to untreated hyperglycemic myocytes. Each bar represents the mean ± SEM from 9 independent experiments.

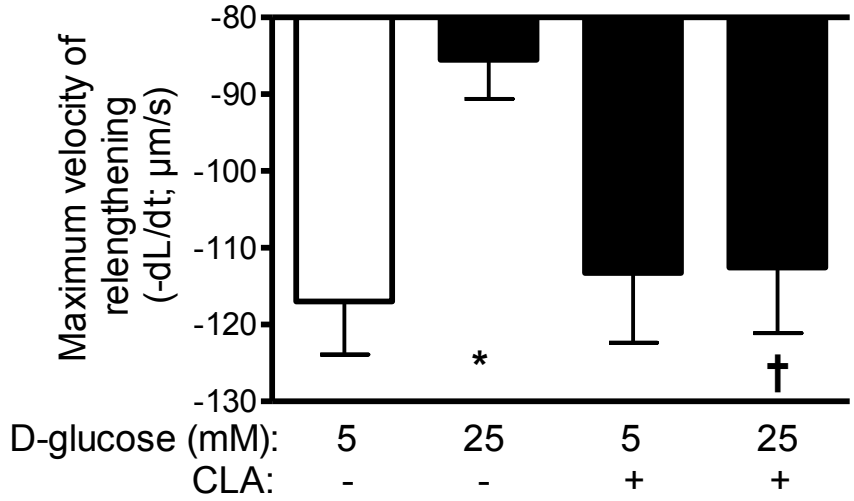

Figure 8. **CLA inhibits severely high D-glucose-induced abnormalities in maximum velocity of relengthening.** Treatment with severely high (25 mM) D-glucose impaired contractile function of adult rat myocytes as measured by maximum velocity of relengthening, a representation of diastolic relaxation. Hyperglycemia-induced relaxatory dysfunction was prevented by pretreatment with CLA. *p<0.05 vs. untreated normoglycemic myocytes. †*p*<0.05 compared to untreated hyperglycemic myocytes. Each bar represents the mean ± SEM from 9 independent experiments.

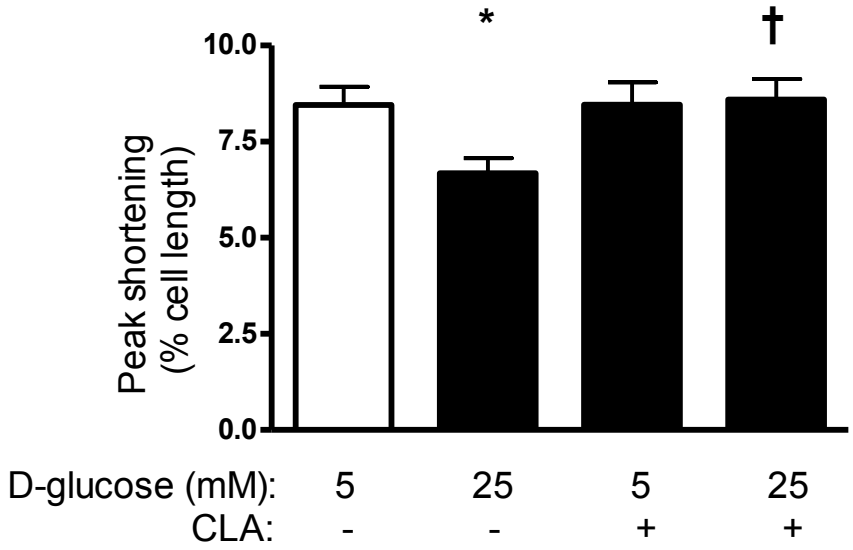

Figure 9. **CLA inhibits severely high D-glucose-induced abnormalities in peak shortening.** Treatment with severely high (25 mM) D-glucose impaired contractile function of adult rat myocytes as measured by peak shortening, a measure of peak force of contraction. Hyperglycemia-induced contractile dysfunction was prevented by pretreatment with CLA. *p<0.05 vs. untreated normoglycemic myocytes. †*p*<0.05 compared to untreated hyperglycemic myocytes. Each bar represents the mean ± SEM from 9 independent experiments.

4. Discussion

Hyperglycemia in patients with diabetes mellitus usually compromises energy metabolism, and it is implicated in myocardial damage, cardiac hypertrophy, overt fibrosis, and myocardial dysfunction.[75] Previous studies have investigated the direct effect of hyperglycemia on the structure of isolated ventricular myocytes, and indeed, hyperglycemia is associated with cardiomyocyte hypertrophy as evidenced by augmentation of myocyte size and activation of the hypertrophic gene program.[72, 161] However, these studies were performed using neonatal rat cardiomyocytes which are not truly representative of phenotypes of adult onset diseases.[164] Therefore, our study of the effects of CLA on high glucose-induced structural and functional abnormalities of cardiomyocytes isolated from adult rats, and the effects of CLA, are novel and important.

Since the development of diabetic cardiomyopathy is directly related to metabolic perturbations associated with diabetes such as hyperglycemia, hyperinsulinemia, and enhanced free fatty acid oxidation,[35] other experimental models to study diabetic cardiomyopathy may include exposing cardiomyocytes to high levels of insulin and free fatty acids. The main advantage of using the hyperglycemia model alone is that it allows us to understand the isolated effect of hyperglycemia on cardiac structure and function.

Our findings demonstrate that elevated extracellular glucose levels directly induce cardiomyocyte hypertrophy and contractile dysfunction as measured by depressed maximal velocity of shortening/relengthening and peak shortening. These findings are consistent with those of other studies in diabetic animals.[165] Our study

showed that the diabetes-like phenotype of cardiac contractile dysfunction of cardiomyocytes emerged early (12-24 hours after high glucose treatment). This rapid effect of high glucose on the mechanical function of cardiomyocytes is explained partly by experimental evidence suggesting that high glucose levels inhibit sarco/endoplasmic reticulum Ca^{+2} – ATPase, an intracellular Ca^{+2} regulatory protein, shortly (6-12 hours) after treatment.[75]

To stimulate the hyperglycemic milieu of diabetes, cardiomyocytes were exposed to two different concentrations of D-glucose, 15 and 25 mM, which reflect moderately high, and severely high plasma glucose levels respectively.[163] We first verified whether moderately high glucose levels (15 mM) could induce a significant increase in the size of cardiomyocytes, a key structural aberration in diabetic cardiomyopathy. However, based on previous experimental models of diabetic cardiomyopathy,[69-71] and the fact that adult rat cardiomyocytes in culture are characterized by short life span, subsequent experiments were carried out with 25 mM glucose to rapidly induce morphological and functional abnormalities in isolated cardiomyocytes.

Our findings determined that CLA prevented indicators of cardiomyocyte hypertrophy induced by high glucose, including cell size augmentation and *de novo* protein synthesis. In addition, CLA abolished the detrimental effects of high glucose on maximal velocity of shortening/relengthening and peak shortening, suggesting that CLA has direct protective effects on cardiomyocytes. These findings are consistent with the previous report from our laboratory indicating that CLA inhibits cardiac hypertrophy induced by ET1 *in vitro* as well as *in vivo* in the spontaneously hypertensive heart failure rat due to its direct actions on the heart instead of directly affecting blood pressure.[158]

Thus, taken together, these findings suggest that in addition to its protective effects against the development of cardiac hypertrophy in hypertension, CLA may attenuate diabetes-induced cardiac hypertrophy and contractile dysfunction.

It should be noted that a mixed preparation of CLA isomers was used in this study, with c9,t11 and t10,c12 are the main (80%) isomers at an approximate 1:1 ratio. Therefore, the important query that arises is whether the protective potential of CLA in diabetic cardiomyopathy is supported by one or more of the isomers found in the preparation. Based on experimental evidence, t10,c12 CLA is associated with adverse effects on insulin and glucose metabolism. For example, feeding t10,c12 CLA to mice has been demonstrated to induce insulin resistance.[166] These findings are consistent with those of another study showing that t10,c12 CLA induces isomer-specific insulin resistance in obese men with the metabolic syndrome.[167] In contrast, feeding mice c9,t11 CLA has been shown to improve insulin resistance and reduce hyperglycemia.[168] Likewise, the beneficial effects of CLA on insulin resistance in clinical trials were attributed to c9,t11 CLA with t10,c12 CLA is associated with adverse effects on insulin sensitivity.[169] Taken together, these findings suggest that the use of c9,t11 CLA should be considered in future studies investigating the impact of CLA on diabetic cardiomyopathy in animals and humans.

Based on the average dietary intake of CLA in non-vegetarian women and men (152-212 mg/d, respectively), the basal human plasma levels of CLA range from 10-70 μM.[170] Because the local concentration of CLA in tissues can increase up to 10 times in relation to its plasma levels[171] and CLA supplementation in healthy individuals (3 g/d)

causes 3-4-fold increases in plasma CLA levels, the concentration of CLA used in our study (30 μM) should be clinically relevant.[158]

In summary, in this study we demonstrated that hyperglycemia impairs cardiomyocyte structure and function following 48 and 12-24 hours of high glucose treatment, respectively. This difference may result from different pathophysiological mechanisms whereby high glucose induces these cardiomyocyte abnormalities. We also demonstrated that CLA prevents cardiomyocyte hypertrophy and contractile dysfunction induced by hyperglycemia. These findings suggest that dietary supplementation with CLA in diabetic patients might be an achievable strategy to prevent myocardial decompensation which leads to heart failure. Further studies are required to investigate the protective potential of CLA in animal models of diabetic cardiomyopathy as well as in diabetic patients.

CHAPTER III

STUDY PHASE TWO

CLA-INDUCED SIGNALING

1. Rationale and hypothesis

Accumulating evidence has indicated that peroxisome proliferator-activated receptors (PPARs) ligands protect against hypertrophic remodeling.[172] PPARs are ligand-activated nuclear hormone receptors belonging to the nuclear receptor superfamily of transcription factors, and their main function is to regulate genes involved in fatty acid and triglyceride metabolism.[130] A number of studies have indicated that activation of PPAR isoforms inhibits cardiomyocyte hypertrophy induced by endothelin-1 (ET1) or angiotensin II,[173-175] as well as cardiac hypertrophy *in vivo* in response to pressure overload.[176]

Based on the previously mentioned study from Dr. Anderson's laboratory, the antihypertrophic effects of conjugated linoleic acid (CLA) in neonatal rat ventricular myocytes are mediated by activation of PPAR isoforms α and γ,[158] and this was associated with upregulation of the antihypertrophic enzyme, diacylglycerol kinase zeta (DGKζ). DGKζ blocks cardiac hypertrophy induced by G protein coupled receptor agonists and pressure overload *in vivo*[177] by depleting diacylglycerol (DAG) levels via phosphorylative conversion, thereby inhibiting hypertrophic protein kinase C (PKC) signaling.[158] Therefore, DGKζ is critical in terminating DAG-PKC signaling to inhibit cardiac hypertrophy.

It has been reported that sustained hyperglycemia in diabetes mellitus stimulates hypertrophy of cardiomyocytes by increasing formation of DAG and subsequent activation of PKC isoforms.[72] In particular, PKCα and PKCβ2 isoforms have been shown to be activated in the early stage of diabetic cardiomyopathy, as well as in cardiomyocytes maintained in high glucose levels.[72]

The known antihypertrophic effects of CLA are mediated through activation of the PPAR-DGK signaling and subsequent attenuation of the pro-hypertrophic PKC signaling.[158] Therefore, we investigated whether PPAR-DGK signaling is involved in the mechanisms whereby CLA inhibits high glucose-induced cardiomyocyte hypertrophy. Specifically, we investigated the role of PPARγ and DGK activation by CLA on cardiomyocyte hypertrophy induced by high glucose.

To achieve this specific objective, pharmacological inhibitors of PPAR-γ and DGK were used to test the following hypothesis:

PPAR-γ-DGK signaling mediates the antihypertrophic effects of CLA in glucose-induced cardiomyocyte hypertrophy.

2. Experimental design and methodology

2.1. Adult rat cardiomyocyte isolation

Ventricular cardiomyocytes were isolated from adult male Sprague Dawley rats as previously described on page 32.

2.2. Treatments

2.2.1. Pharmacological inhibition of PPAR-γ

Isolated cardiomyocytes were plated on 12-well plates (2 x 10^5cell/well), serum-deprived for 24 hours, then pretreated with a selective pharmacological inhibitor of PPAR-γ (GW9662, 1 μM,[158] 1 hour before CLA treatment). Subgroups were subsequently treated with CLA and glucose as indicated in Table 1 (groups 2, 4, 6, & 8).

2.2.2. Pharmacological inhibition of DGK

Cardiomyocytes were plated on 12-well plates (2 x 10^5 cell/well) and serum-deprived for 24 hours. To study the participation of DGK (all isoforms) in the anti-hypertrophic actions of CLA, the non-selective DGK inhibitor, R59022 (10 μM[172]) was added to isolated cardiomyocytes 1 hour before adding CLA and glucose as indicated in Table 1 (groups 2, 4, 6, & 8).

2.3. Measurement of ventricular myocyte hypertrophy

2.3.1. Cell size

Cell size of ventricular myocytes was assessed by measuring surface areas of individual cardiomyocytes as described under measurement of ventricular myocyte hypertrophy on page 34.

2.3.2. Protein synthesis

Protein synthesis was measured using the [^{3}H]-leucine incorporation assay as previously described, and the Click-iT AHA Alexa Fluor 488 Protein Synthesis Assay. Click-iT AHA (L-azidohomoalanine) is an amino acid analog of methionine that is incorporated into proteins during active protein synthesis. After treating cardiomyocytes with the indicated treatments for 48 hours, cells were incubated with 50 μM AHA in pre-warmed methionine-free medium for 2 hours, washed once with PBS, and fixed with 3.7% formaldehyde in PBS at room temperature for 15 minutes. Cells were permeabilized with 0.5% Triton X-100 in PBS for 20 minutes at room temperature. The permeabilization solution was removed and the cells were washed with 3% albumin from bovine serum. Protein synthesis was detected following the click reaction with

Alexa Fluor 488 alkyne. Fluorescent activity measurement was performed using SpectraMax Gemini Fluorometer.

3. Phase two results

3.1. PPAR-γ mediates the anti-hypertrophic effects of CLA

Severely high glucose increased cell size and protein synthesis, and this was attenuated by CLA (Figures 5 and 6 in Chapter 2). GW9662 abolished the inhibitory effect of CLA on hyperglycemia-induced augmentation of cell size and protein synthesis (Figures 10 and 11). GW9662 treatment did not alter high glucose-induced cardiomyocyte hypertrophy in the absence of CLA.

3.2. DGK is involved in the anti-hypertrophic actions of CLA

The ability of CLA to inhibit high glucose-induced cardiomyocyte hypertrophy (Figures 5 and 6 in Chapter 2) was abolished by R59022, (Figures 12 and 13).

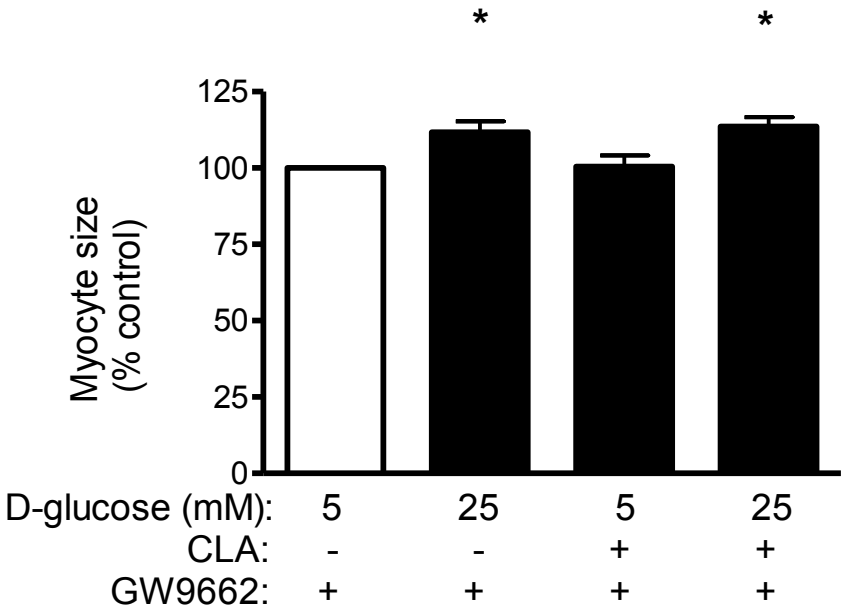

Figure 10. **The ability of CLA to prevent severely high D-glucose-induced cardiomyocyte hypertrophy is suppressed by the PPAR-γ antagonist, GW9662 (1 μM).** *p<0.05 vs. untreated normoglycemic myocytes. Each bar represents the mean ± SEM from 4 independent experiments.

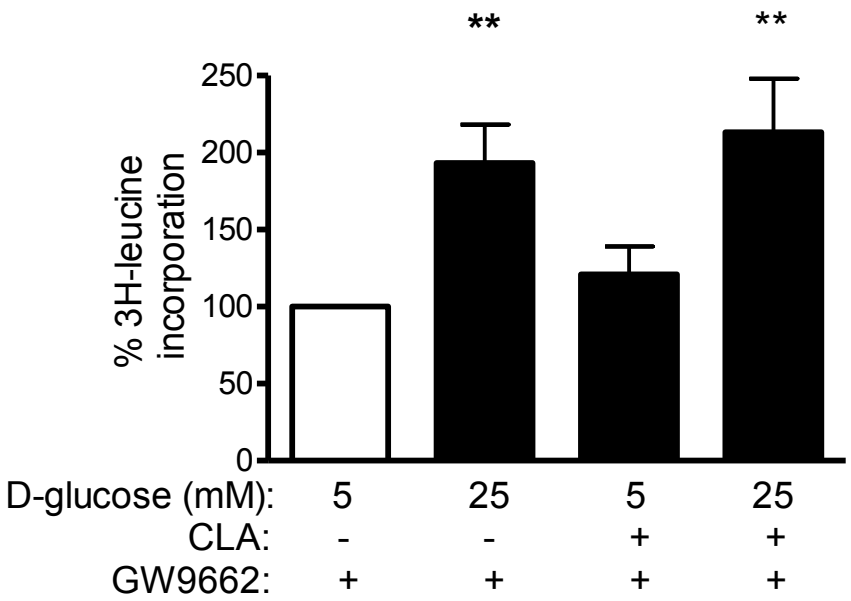

Figure 11. **The ability of CLA to prevent severely high D-glucose-induced augmentation of protein synthesis is suppressed by the PPARγ antagonist, GW9662 (1 μM). *** *p*<0.01 vs. untreated normoglycemic myocytes. Each bar represents the mean ± SEM from 4 independent experiments

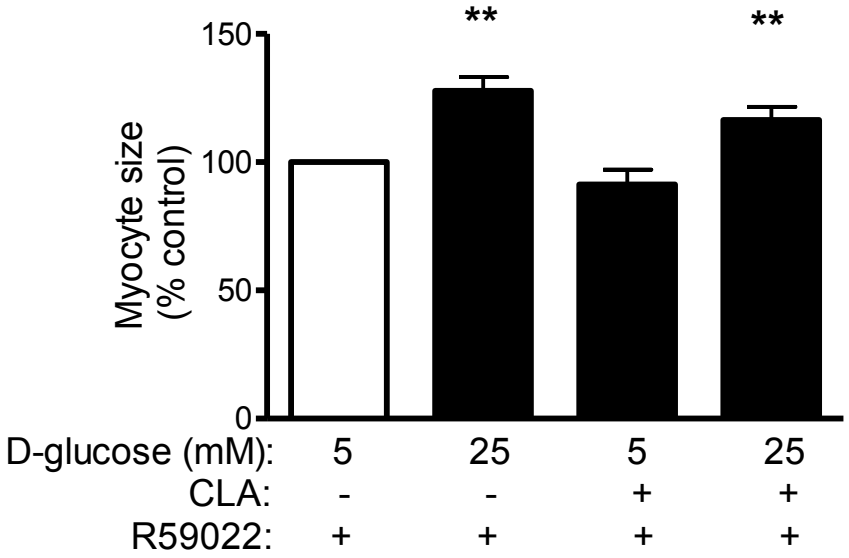

Figure 12. The ability of CLA to prevent severely high D-glucose-induced hypertrophy is suppressed by the non-selective DGK antagonist, R59022 (10 μM).

**p<0.01 vs. untreated normoglycemic myocytes. Each bar represents the mean ± SEM from 3 independent experiments.

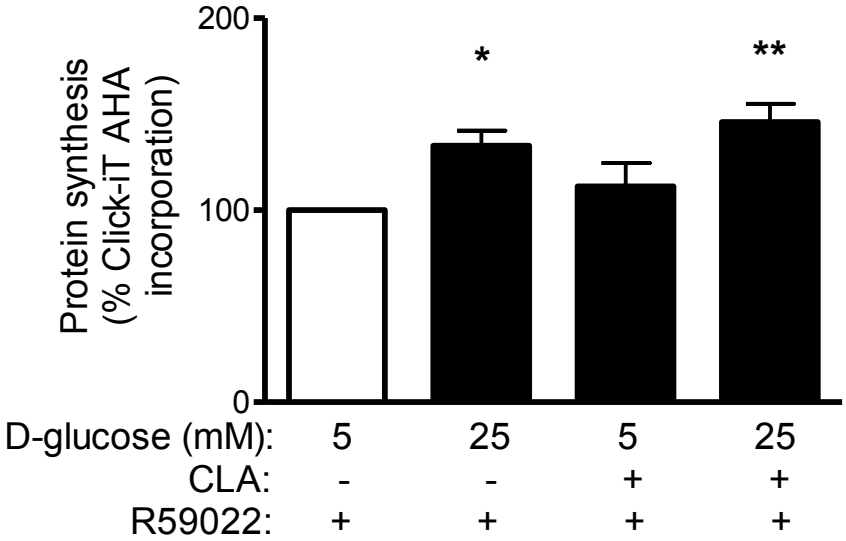

Figure 13. **The ability of CLA to prevent severely high D-glucose-induced augmentation of protein synthesis is suppressed by the non-selective DGK antagonist, R59022 (10 μM).** **p*<0.05 vs. untreated normoglycemic myocytes. *******p*<0.01 vs. untreated normoglycemic myocytes. Each bar represents the mean ± SEM from 5 independent experiments

4. Discussion of phase two results

Taken together, these findings suggest that CLA prevents cardiomyocyte hypertrophy induced by high glucose through activation of PPAR-γ and DGK. Therefore, this study shows that the ability of CLA to inhibit cardiomyocyte hypertrophy in the context of diabetic cardiomyopathy is associated with, at least in part, the activation of PPAR-γ by CLA which is speculated to induce upregulation of DGK and inhibit hyperglycemia-induced PKC activation. As previously mentioned, expression and activity of DGKζ (the predominant DGK isoform in the heart) are decreased in diabetes mellitus.[71] Furthermore, activity of PKCα and PKCβ2 isoforms has been shown to be upregulated in diabetes mellitus and in response to high glucose levels.[72] Therefore, we suggest that the inhibitory effect of CLA on glucose-induced cardiomyocyte hypertrophy is mediated via activation of DGKζ, leading to attenuation of PKCα/β2 signaling. The involvement of the other PPAR isoforms and the full mechanistic pathways in which high glucose induces cardiomyocyte hypertrophy and the effect of CLA on signal transduction pathways induced by high glucose have yet to be fully elucidated.

CHAPTER IV

CONCLUSIONS

1. Conclusion

Hyperglycemia is an independent risk factor for the development of diabetic cardiovascular complications. Our study showed that high glucose levels can directly affect the structure and the function of the myocardium and cause adult cardiomyocyte hypertrophy and contractile dysfunction.

CLA has received significant attention as a nutraceutical because of its numerous health benefits. The focus of this study was to investigate the cardioprotective effects of CLA on the structural and functional abnormalities of cardiomyocytes in diabetic cardiomyopathy. Our study demonstrated that CLA inhibits cardiomyocyte hypertrophy and contractile dysfunction stimulated by high glucose levels. The antihypertrophic effects of CLA were possibly related, at least in part, to the ability of CLA to activate PPAR-γ and DGK. This mechanistic contribution of DGKs in the antihypertrophic effects of CLA is likely due to the ability of DGKs to attenuate protein kinase C signaling by decreasing the availability of diacylglycerol, thereby inhibiting cardiomyocyte hypertrophy induced by hyperglycemia.

1.1. CLA – A clinical prospective

The findings of this study provide new insight for the potential use of CLA as a cardioprotective dietary supplement toward the prevention of heart failure in diabetic patients. However, despite the established beneficial health benefits of CLA, special caution should be taken when considering the use of CLA in diabetic patients with established heart disease. This is due to the ability of CLA to activate PPAR-γ which may contribute to fluid retention by stimulating sodium reabsorption by sodium channels in the renal collecting tubule cells. [90] Therefore, if CLA supplementation is to be used in

diabetic patients, it should be used as a preventative strategy against heart failure rather than therapeutic management in diabetic patients with heart failure.

1.2. Final remarks

The findings of this study indicate that CLA is effective in preventing cardiomyocyte hypertrophy and contractile dysfunction induced by high glucose. Future studies are required to fully investigate the mechanistic pathway in which hyperglycemia induces diabetic cardiomyopathy and the effect of pure CLA isoforms on signaling pathways induced by high glucose.

CHAPTER V

REFERENCES

References

1. Whiting DR, Guariguata L, Weil C, Shaw J. Idf diabetes atlas: Global estimates of the prevalence of diabetes for 2011 and 2030. *Diabetes Research and Clinical practice*. 2011;94:311-321
2. Stamler J, Vaccaro O, Neaton JD, Wentworth D. Diabetes, other risk factors, and 12-yr cardiovascular mortality for men screened in the multiple risk factor intervention trial. *Diabetes Care*. 1993;16:434-444
3. Goyal B, Mehta A. Diabetic cardiomyopathy: Pathophysiological mechanisms and cardiac dysfuntion. *Human & experimental toxicology*. 2012
4. Rubler S, Dlugash J, Yuceoglu YZ, Kumral T, Branwood AW, Grishman A. New type of cardiomyopathy associated with diabetic glomerulosclerosis. *The American Journal of Cardiology*. 1972;30:595-602
5. Kannel WB, Hjortland M, Castelli WP. Role of diabetes in congestive heart failure: The framingham study. *The American Journal of Cardiology*. 1974;34:29-34
6. Bertoni AG, Tsai A, Kasper EK, Brancati FL. Diabetes and idiopathic cardiomyopathy: A nationwide case-control study. *Diabetes Care*. 2003;26:2791-2795
7. Greenberg BH, Abraham WT, Albert NM, Chiswell K, Clare R, Stough WG, Gheorghiade M, O'Connor CM, Sun JL, Yancy CW, Young JB, Fonarow GC. Influence of diabetes on characteristics and outcomes in patients hospitalized with heart failure: A report from the organized program to initiate lifesaving

treatment in hospitalized patients with heart failure (optimize-hf). *American Heart Journal*. 2007;154:277 e271-278

8. Nicolino A, Longobardi G, Furgi G, Rossi M, Zoccolillo N, Ferrara N, Rengo F. Left ventricular diastolic filling in diabetes mellitus with and without hypertension. *American Journal of Hypertension*. 1995;8:382-389
9. Bell DS. Diabetic cardiomyopathy. *Diabetes care*. 2003;26:2949-2951
10. Kiencke S, Handschin R, von Dahlen R, Muser J, Brunner-Larocca HP, Schumann J, Felix B, Berneis K, Rickenbacher P. Pre-clinical diabetic cardiomyopathy: Prevalence, screening, and outcome. *European Journal of Heart Failure*. 2010;12:951-957
11. Hayat SA, Patel B, Khattar RS, Malik RA. Diabetic cardiomyopathy: Mechanisms, diagnosis and treatment. *Clinical Science*. 2004;107:539-557
12. Devereux RB, Roman MJ, Paranicas M, O'Grady MJ, Lee ET, Welty TK, Fabsitz RR, Robbins D, Rhoades ER, Howard BV. Impact of diabetes on cardiac structure and function: The strong heart study. *Circulation*. 2000;101:2271-2276
13. Eguchi K, Boden-Albala B, Jin Z, Rundek T, Sacco RL, Homma S, Di Tullio MR. Association between diabetes mellitus and left ventricular hypertrophy in a multiethnic population. *The American Journal of Cardiology*. 2008;101:1787-1791
14. Palmieri V, Tracy RP, Roman MJ, Liu JE, Best LG, Bella JN, Robbins DC, Howard BV, Devereux RB, Strong Heart S. Relation of left ventricular hypertrophy to inflammation and albuminuria in adults with type 2 diabetes: The Strong Heart Study. *Diabetes Care*. 2003;26:2764-2769

15. Sato A, Tarnow L, Nielsen FS, Knudsen E, Parving HH. Left ventricular hypertrophy in normoalbuminuric type 2 diabetic patients not taking antihypertensive treatment. *QJM : Monthly Journal of the Association of Physicians*. 2005;98:879-884

16. Nunoda S, Genda A, Sugihara N, Nakayama A, Mizuno S, Takeda R. Quantitative approach to the histopathology of the biopsied right ventricular myocardium in patients with diabetes mellitus. *Heart and Vessels*. 1985;1:43-47

17. Shimizu M, Umeda K, Sugihara N, Yoshio H, Ino H, Takeda R, Okada Y, Nakanishi I. Collagen remodelling in myocardia of patients with diabetes. *Journal of Clinical Pathology*. 1993;46:32-36

18. Berg TJ, Snorgaard O, Faber J, Torjesen PA, Hildebrandt P, Mehlsen J, Hanssen KF. Serum levels of advanced glycation end products are associated with left ventricular diastolic function in patients with type 1 diabetes. *Diabetes Care*. 1999;22:1186-1190

19. van Heerebeek L, Hamdani N, Handoko ML, Falcao-Pires I, Musters RJ, Kupreishvili K, Ijsselmuiden AJ, Schalkwijk CG, Bronzwaer JG, Diamant M, Borbely A, van der Velden J, Stienen GJ, Laarman GJ, Niessen HW, Paulus WJ. Diastolic stiffness of the failing diabetic heart: Importance of fibrosis, advanced glycation end products, and myocyte resting tension. *Circulation*. 2008;117:43-51

20. Brutsaert DL, Housmans PR, Goethals MA. Dual control of relaxation. Its role in the ventricular function in the mammalian heart. *Circulation Research*. 1980;47:637-652

21. Yturralde RF, Gaasch WH. Diagnostic criteria for diastolic heart failure. *Progress in Cardiovascular Diseases*. 2005;47:314-319

22. Romano S, Di Mauro M, Fratini S, Guarracini L, Guarracini F, Poccia G, Penco M. Early diagnosis of left ventricular diastolic dysfunction in diabetic patients: A possible role for natriuretic peptides. *Cardiovascular Diabetology*. 2010;9:89

23. Poirier P, Bogaty P, Garneau C, Marois L, Dumesnil JG. Diastolic dysfunction in normotensive men with well-controlled type 2 diabetes: Importance of maneuvers in echocardiographic screening for preclinical diabetic cardiomyopathy. *Diabetes Care*. 2001;24:5-10

24. Patil VC, Patil HV, Shah KB, Vasani JD, Shetty P. Diastolic dysfunction in asymptomatic type 2 diabetes mellitus with normal systolic function. *Journal of Cardiovascular Disease Research*. 2011;2:213-222

25. Schannwell CM, Schneppenheim M, Perings S, Plehn G, Strauer BE. Left ventricular diastolic dysfunction as an early manifestation of diabetic cardiomyopathy. *Cardiology*. 2002;98:33-39

26. Chareonthaitawee P, Sorajja P, Rajagopalan N, Miller TD, Hodge DO, Frye RL, Gibbons RJ. Prevalence and prognosis of left ventricular systolic dysfunction in asymptomatic diabetic patients without known coronary artery disease referred for stress single-photon emission computed tomography and assessment of left ventricular function. *American Heart Journal*. 2007;154:567-574

27. Vered A, Battler A, Segal P, Liberman D, Yerushalmi Y, Berezin M, Neufeld HN. Exercise-induced left ventricular dysfunction in young men with asymptomatic

diabetes mellitus (diabetic cardiomyopathy). *The American Journal of Cardiology*. 1984;54:633-637

28. Fang ZY, Prins JB, Marwick TH. Diabetic cardiomyopathy: Evidence, mechanisms, and therapeutic implications. *Endocrine Reviews*. 2004;25:543-567
29. Fang ZY, Yuda S, Anderson V, Short L, Case C, Marwick TH. Echocardiographic detection of early diabetic myocardial disease. *Journal of the American College of Cardiology*. 2003;41:611-617
30. Stanley WC, Chandler MP. Energy metabolism in the normal and failing heart: Potential for therapeutic interventions. *Heart Failure Reviews*. 2002;7:115-130
31. An D, Rodrigues B. Role of changes in cardiac metabolism in development of diabetic cardiomyopathy. *American journal of physiology. Heart and Circulatory Physiology*. 2006;291:H1489-1506
32. Rodrigues B, Cam MC, McNeill JH. Myocardial substrate metabolism: Implications for diabetic cardiomyopathy. *Journal of Molecular and Cellular Cardiology*. 1995;27:169-179
33. Kim JK, Fillmore JJ, Sunshine MJ, Albrecht B, Higashimori T, Kim DW, Liu ZX, Soos TJ, Cline GW, O'Brien WR, Littman DR, Shulman GI. Pkc-theta knockout mice are protected from fat-induced insulin resistance. *The Journal of Clinical Investigation*. 2004;114:823-827
34. Kim JK, Kim YJ, Fillmore JJ, Chen Y, Moore I, Lee J, Yuan M, Li ZW, Karin M, Perret P, Shoelson SE, Shulman GI. Prevention of fat-induced insulin resistance by salicylate. *The Journal of Clinical Investigation*. 2001;108:437-446

35. Poornima IG, Parikh P, Shannon RP. Diabetic cardiomyopathy: The search for a unifying hypothesis. *Circulation Research*. 2006;98:596-605

36. Unger RH, Orci L. Lipotoxic diseases of nonadipose tissues in obesity. *International journal of obesity and related metabolic disorders : Journal of the International Association for the Study of Obesity*. 2000;24 Suppl 4:S28-32

37. Christoffersen C, Bollano E, Lindegaard ML, Bartels ED, Goetze JP, Andersen CB, Nielsen LB. Cardiac lipid accumulation associated with diastolic dysfunction in obese mice. *Endocrinology*. 2003;144:3483-3490

38. Chen V, Ianuzzo CD, Fong BC, Spitzer JJ. The effects of acute and chronic diabetes on myocardial metabolism in rats. *Diabetes*. 1984;33:1078-1084

39. Ohtake T, Yokoyama I, Watanabe T, Momose T, Serezawa T, Nishikawa J, Sasaki Y. Myocardial glucose metabolism in noninsulin-dependent diabetes mellitus patients evaluated by fdg-pet. *Journal of Nuclear Medicine : official publication, Society of Nuclear Medicine*. 1995;36:456-463

40. Garvey WT, Hardin D, Juhaszova M, Dominguez JH. Effects of diabetes on myocardial glucose transport system in rats: Implications for diabetic cardiomyopathy. *The American Journal of Physiology*. 1993;264:H837-844

41. Kainulainen H, Breiner M, Schurmann A, Marttinen A, Virjo A, Joost HG. In vivo glucose uptake and glucose transporter proteins glut1 and glut4 in heart and various types of skeletal muscle from streptozotocin-diabetic rats. *Biochimica et Biophysica Acta*. 1994;1225:275-282

42. Belke DD, Larsen TS, Gibbs EM, Severson DL. Altered metabolism causes cardiac dysfunction in perfused hearts from diabetic (db/db) mice. *American journal of physiology. Endocrinology and Metabolism*. 2000;279:E1104-1113

43. Natasha Fillmore GDL. Impact of fatty acid oxidation on cardiac efficiency. *Heart Metabolism*. 2011;53:33–37

44. Endoh M. Signal transduction and ca2+ signaling in intact myocardium. *Journal of Pharmacological Sciences*. 2006;100:525-537

45. Hattori Y, Matsuda N, Kimura J, Ishitani T, Tamada A, Gando S, Kemmotsu O, Kanno M. Diminished function and expression of the cardiac na+-ca2+ exchanger in diabetic rats: Implication in ca2+ overload. *The Journal of Physiology*. 2000;527 Pt 1:85-94

46. Belke DD, Swanson EA, Dillmann WH. Decreased sarcoplasmic reticulum activity and contractility in diabetic db/db mouse heart. *Diabetes*. 2004;53:3201-3208

47. Choi KM, Zhong Y, Hoit BD, Grupp IL, Hahn H, Dilly KW, Guatimosim S, Lederer WJ, Matlib MA. Defective intracellular ca(2+) signaling contributes to cardiomyopathy in type 1 diabetic rats. *American journal of physiology. Heart and Circulatory Physiology*. 2002;283:H1398-1408

48. Trost SU, Belke DD, Bluhm WF, Meyer M, Swanson E, Dillmann WH. Overexpression of the sarcoplasmic reticulum ca(2+)-atpase improves myocardial contractility in diabetic cardiomyopathy. *Diabetes*. 2002;51:1166-1171

49. Pierce GN, Russell JC. Regulation of intracellular ca2+ in the heart during diabetes. *Cardiovascular Research*. 1997;34:41-47

50. Luevano-Contreras C, Chapman-Novakofski K. Dietary advanced glycation end products and aging. *Nutrients*. 2010;2:1247-1265

51. Goh SY, Cooper ME. Clinical review: The role of advanced glycation end products in progression and complications of diabetes. *The Journal of Clinical Endocrinology and Metabolism*. 2008;93:1143-1152

52. Avendano GF, Agarwal RK, Bashey RI, Lyons MM, Soni BJ, Jyothirmayi GN, Regan TJ. Effects of glucose intolerance on myocardial function and collagen-linked glycation. *Diabetes*. 1999;48:1443-1447

53. Hartog JW, Voors AA, Bakker SJ, Smit AJ, van Veldhuisen DJ. Advanced glycation end-products (ages) and heart failure: Pathophysiology and clinical implications. *European Journal of Heart Failure*. 2007;9:1146-1155

54. Wang J, Song Y, Wang Q, Kralik PM, Epstein PN. Causes and characteristics of diabetic cardiomyopathy. *The Review of Diabetic Studies : RDS*. 2006;3:108-117

55. Ma H, Li SY, Xu P, Babcock SA, Dolence EK, Brownlee M, Li J, Ren J. Advanced glycation endproduct (age) accumulation and age receptor (rage) up-regulation contribute to the onset of diabetic cardiomyopathy. *Journal of Cellular and Molecular Medicine*. 2009;13:1751-1764

56. Kranstuber AL, Del Rio C, Biesiadecki BJ, Hamlin RL, Ottobre J, Gyorke S, Lacombe VA. Advanced glycation end product cross-link breaker attenuates diabetes-induced cardiac dysfunction by improving sarcoplasmic reticulum calcium handling. *Frontiers in Physiology*. 2012;3:292

57. Durackova Z. Some current insights into oxidative stress. *Physiological research / Academia Scientiarum Bohemoslovaca*. 2010;59:459-469

58. Watanabe K, Thandavarayan RA, Harima M, Sari FR, Gurusamy N, Veeraveedu PT, Mito S, Arozal W, Sukumaran V, Laksmanan AP, Soetikno V, Kodama M, Aizawa Y. Role of differential signaling pathways and oxidative stress in diabetic cardiomyopathy. *Current Cardiology Reviews*. 2010;6:280-290

59. Jay D, Hitomi H, Griendling KK. Oxidative stress and diabetic cardiovascular complications. *Free Radical Biology & Medicine*. 2006;40:183-192

60. Marra G, Cotroneo P, Pitocco D, Manto A, Di Leo MA, Ruotolo V, Caputo S, Giardina B, Ghirlanda G, Santini SA. Early increase of oxidative stress and reduced antioxidant defenses in patients with uncomplicated type 1 diabetes: A case for gender difference. *Diabetes Care*. 2002;25:370-375

61. Giacchetti G, Sechi LA, Rilli S, Carey RM. The renin-angiotensin-aldosterone system, glucose metabolism and diabetes. *Trends in Endocrinology and Metabolism: TEM*. 2005;16:120-126

62. Fiordaliso F, Li B, Latini R, Sonnenblick EH, Anversa P, Leri A, Kajstura J. Myocyte death in streptozotocin-induced diabetes in rats in angiotensin ii-dependent. *Laboratory Investigation; a Journal of Technical Methods and Pathology*. 2000;80:513-527

63. Frustaci A, Kajstura J, Chimenti C, Jakoniuk I, Leri A, Maseri A, Nadal-Ginard B, Anversa P. Myocardial cell death in human diabetes. *Circulation Research*. 2000;87:1123-1132

64. Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ. Hyperglycemia-induced apoptosis in mouse myocardium: Mitochondrial cytochrome c-mediated caspase-3 activation pathway. *Diabetes*. 2002;51:1938-1948

65. Xia P, Inoguchi T, Kern TS, Engerman RL, Oates PJ, King GL. Characterization of the mechanism for the chronic activation of diacylglycerol-protein kinase c pathway in diabetes and hypergalactosemia. *Diabetes*. 1994;43:1122-1129

66. Inoguchi T, Battan R, Handler E, Sportsman JR, Heath W, King GL. Preferential elevation of protein kinase c isoform beta ii and diacylglycerol levels in the aorta and heart of diabetic rats: Differential reversibility to glycemic control by islet cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*. 1992;89:11059-11063

67. Xiang H, McNeill JH. Protein kinase c activity is altered in diabetic rat hearts. *Biochemical and Biophysical Research Communications*. 1992;187:703-710

68. Gurusamy N, Watanabe K, Ma M, Zhang S, Muslin AJ, Kodama M, Aizawa Y. Inactivation of 14-3-3 protein exacerbates cardiac hypertrophy and fibrosis through enhanced expression of protein kinase c beta 2 in experimental diabetes. *Biological & Pharmaceutical Bulletin*. 2005;28:957-962

69. Wang M, Zhang WB, Zhu JH, Fu GS, Zhou BQ. Breviscapine ameliorates hypertrophy of cardiomyocytes induced by high glucose in diabetic rats via the pkc signaling pathway. *Acta Pharmacologica Sinica*. 2009;30:1081-1091

70. Duan Y, Zhou B, Su H, Liu Y, Du C. Mir-150 regulates high glucose-induced cardiomyocyte hypertrophy by targeting the transcriptional co-activator p300. *Experimental Cell Research*. 2013;319:173-184

71. Rui T, Zhang J, Xu X, Yao Y, Kao R, Martin CM. Reduction in il-33 expression exaggerates ischaemia/reperfusion-induced myocardial injury in mice with diabetes mellitus. *Cardiovascular Research*. 2012;94:370-378

72. Min W, Bin ZW, Quan ZB, Hui ZJ, Sheng FG. The signal transduction pathway of pkc/nf-kappa b/c-fos may be involved in the influence of high glucose on the cardiomyocytes of neonatal rats. *Cardiovascular Diabetology*. 2009;8:8

73. Ren J, Gintant GA, Miller RE, Davidoff AJ. High extracellular glucose impairs cardiac e-c coupling in a glycosylation-dependent manner. *The American Journal of Physiology*. 1997;273:H2876-2883

74. Ren J, Davidoff AJ. Diabetes rapidly induces contractile dysfunctions in isolated ventricular myocytes. *The American Journal of Physiology*. 1997;272:H148-158

75. Zhang RH, Guo H, Kandadi MR, Wang XM, Ren J. Ca+2/calmodulin-dependent protein kinase mediates glucose toxicity-induced cardiomyocyte contractile dysfunction. *Experimental Diabetes Research*. 2012;2012:829758

76. Adler AI, Neil HA, Manley SE, Holman RR, Turner RC. Hyperglycemia and hyperinsulinemia at diagnosis of diabetes and their association with subsequent cardiovascular disease in the united kingdom prospective diabetes study (ukpds 47). *American Heart Journal*. 1999;138:S353-359

77. Effect of intensive diabetes management on macrovascular events and risk factors in the diabetes control and complications trial. *The American Journal of Cardiology*. 1995;75:894-903

78. Nathan DM, Cleary PA, Backlund JY, Genuth SM, Lachin JM, Orchard TJ, Raskin P, Zinman B, Diabetes C, Complications Trial/Epidemiology of Diabetes I, Complications Study Research G. Intensive diabetes treatment and cardiovascular disease in patients with type 1 diabetes. *The New England Journal of Medicine*. 2005;353:2643-2653

79. Riddle MC. Effects of intensive glucose lowering in the management of patients with type 2 diabetes mellitus in the action to control cardiovascular risk in diabetes (accord) trial. *Circulation*. 2010;122:844-846

80. Group AC, Patel A, MacMahon S, Chalmers J, Neal B, Billot L, Woodward M, Marre M, Cooper M, Glasziou P, Grobbee D, Hamet P, Harrap S, Heller S, Liu L, Mancia G, Mogensen CE, Pan C, Poulter N, Rodgers A, Williams B, Bompoint S, de Galan BE, Joshi R, Travert F. Intensive blood glucose control and vascular outcomes in patients with type 2 diabetes. *The New England Journal of Medicine*. 2008;358:2560-2572

81. Duckworth W, Abraira C, Moritz T, Reda D, Emanuele N, Reaven PD, Zieve FJ, Marks J, Davis SN, Hayward R, Warren SR, Goldman S, McCarren M, Vitek ME, Henderson WG, Huang GD, Investigators V. Glucose control and vascular complications in veterans with type 2 diabetes. *The New England Journal of Medicine*. 2009;360:129-139

82. Ashcroft FM. Mechanisms of the glycaemic effects of sulfonylureas. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et Metabolisme*. 1996;28:456-463

83. Eurich DT, McAlister FA, Blackburn DF, Majumdar SR, Tsuyuki RT, Varney J, Johnson JA. Benefits and harms of antidiabetic agents in patients with diabetes and heart failure: Systematic Review. *Bmj*. 2007;335:497

84. Bell DS. Do sulfonylurea drugs increase the risk of cardiac events? *CMAJ : Canadian Medical Association journal = Journal de l'Association Medicale Canadienne*. 2006;174:185-186

85. Garratt KN, Brady PA, Hassinger NL, Grill DE, Terzic A, Holmes DR, Jr. Sulfonylurea drugs increase early mortality in patients with diabetes mellitus after direct angioplasty for acute myocardial infarction. *Journal of the American College of Cardiology*. 1999;33:119-124

86. Huizar JF, Gonzalez LA, Alderman J, Smith HS. Sulfonylureas attenuate electrocardiographic st-segment elevation during an acute myocardial infarction in diabetics. *Journal of the American College of Cardiology*. 2003;42:1017-1021

87. Fisman EZ, Tenenbaum A. A cardiologic approach to non-insulin antidiabetic pharmacotherapy in patients with heart disease. *Cardiovascular diabetology*. 2009;8:38

88. Eurich DT, Weir DL, Majumdar SR, Tsuyuki RT, Johnson JA, Tjosvold L, Vanderloo SE, McAlister FA. Comparative safety and effectiveness of metformin in patients with diabetes and heart failure: Systematic review of observational studies involving 34000 patients. *Circulation. Heart Failure*. 2013

89. McGuire DK, Inzucchi SE. New drugs for the treatment of diabetes mellitus: Part i: Thiazolidinediones and their evolving cardiovascular implications. *Circulation*. 2008;117:440-449

90. Guan Y, Hao C, Cha DR, Rao R, Lu W, Kohan DE, Magnuson MA, Redha R, Zhang Y, Breyer MD. Thiazolidinediones expand body fluid volume through ppargamma stimulation of enac-mediated renal salt absorption. *Nature Medicine*. 2005;11:861-866

91. Singh S, Loke YK, Furberg CD. Thiazolidinediones and heart failure: A teleo-analysis. *Diabetes Care*. 2007;30:2148-2153

92. Marks DH. Drug utilization, safety and clinical use of actos and avandia. *The International Journal of Risk & Safety in Medicine*. 2013;25:39-51

93. Symeonides P, Koulouris S, Vratsista E, Triantafyllou K, Ioannidis G, Thalassinos N, Katritsis D. Both ramipril and telmisartan reverse indices of early diabetic cardiomyopathy: A comparative study. *European journal of echocardiography : the journal of the Working Group on Echocardiography of the European Society of Cardiology*. 2007;8:480-486

94. Carr AA, Kowey PR, Devereux RB, Brenner BM, Dahlof B, Ibsen H, Lindholm LH, Lyle PA, Snapinn SM, Zhang Z, Edelman JM, Shahinfar S. Hospitalizations for new heart failure among subjects with diabetes mellitus in the renaal and life studies. *The American Journal of Cardiology*. 2005;96:1530-1536

95. Wlodarczyk JH, Keogh A, Smith K, McCosker C. Chart: Congestive cardiac failure in hospitals, an australian review of treatment. *Heart, lung & Circulation*. 2003;12:94-102

96. Lowes BD, Gill EA, Abraham WT, Larrain JR, Robertson AD, Bristow MR, Gilbert EM. Effects of carvedilol on left ventricular mass, chamber geometry, and mitral regurgitation in chronic heart failure. *The American Journal of Cardiology*. 1999;83:1201-1205

97. Giles TD. Use of beta-blockers for heart failure in patients with diabetes mellitus. *Postgraduate Medicine*. 2002;112:32-37

98. Sharma V, Dhillon P, Wambolt R, Parsons H, Brownsey R, Allard MF, McNeill JH. Metoprolol improves cardiac function and modulates cardiac metabolism in

the streptozotocin-diabetic rat. *American journal of physiology. Heart and Circulatory Physiology*. 2008;294:H1609-1620

99. Yusuf S, Dagenais G, Pogue J, Bosch J, Sleight P. Vitamin e supplementation and cardiovascular events in high-risk patients. The heart outcomes prevention evaluation study investigators. *The New England Journal of Medicine*. 2000;342:154-160

100. Pariza MW, Park Y, Cook ME. Mechanisms of action of conjugated linoleic acid: Evidence and speculation. *Proceedings of the Society for Experimental Biology and Medicine. Society for Experimental Biology and Medicine*. 2000;223:8-13

101. Pariza MW. Perspective on the safety and effectiveness of conjugated linoleic acid. *The American Journal of Clinical Nutrition*. 2004;79:1132S-1136S

102. Liew C, Schut HA, Chin SF, Pariza MW, Dashwood RH. Protection of conjugated linoleic acids against 2-amino-3- methylimidazo[4,5-f]quinoline-induced colon carcinogenesis in the f344 rat: A study of inhibitory mechanisms. *Carcinogenesis*. 1995;16:3037-3043

103. Kamlage B, Hartmann L, Gruhl B, Blaut M. Linoleic acid conjugation by human intestinal microorganisms is inhibited by glucose and other substrates in vitro and in gnotobiotic rats. *The Journal of Nutrition*. 2000;130:2036-2039

104. Lee KN, Kritchevsky D, Pariza MW. Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis*. 1994;108:19-25

105. Riserus U, Berglund L, Vessby B. Conjugated linoleic acid (cla) reduced abdominal adipose tissue in obese middle-aged men with signs of the metabolic syndrome: A randomised controlled trial. *International Journal of Obesity and*

Related Metabolic Disorders : Journal of the International Association for the Study of Obesity. 2001;25:1129-1135

106. AYDIN R. Conjugated linoleic acid: Chemical structure, sources and biological properties. *Turk J Vet Anim Sci*. 2005;29:189-195

107. Ha YL, Grimm NK, Pariza MW. Anticarcinogens from fried ground beef: Heat-altered derivatives of linoleic acid. *Carcinogenesis*. 1987;8:1881-1887

108. Ha YL, Storkson J, Pariza MW. Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Research*. 1990;50:1097-1101

109. Ip C, Briggs SP, Haegele AD, Thompson HJ, Storkson J, Scimeca JA. The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet. *Carcinogenesis*. 1996;17:1045-1050

110. Cesano A, Visonneau S, Scimeca JA, Kritchevsky D, Santoli D. Opposite effects of linoleic acid and conjugated linoleic acid on human prostatic cancer in scid mice. *Anticancer Research*. 1998;18:1429-1434

111. Knekt P, Jarvinen R, Seppanen R, Pukkala E, Aromaa A. Intake of dairy products and the risk of breast cancer. *British Journal of Cancer*. 1996;73:687-691

112. Petrik MB, McEntee MF, Johnson BT, Obukowicz MG, Whelan J. Highly unsaturated (n-3) fatty acids, but not alpha-linolenic, conjugated linoleic or gamma-linolenic acids, reduce tumorigenesis in apc(min/+) mice. *The Journal of Nutrition*. 2000;130:2434-2443

113. Wong MW, Chew BP, Wong TS, Hosick HL, Boylston TD, Shultz TD. Effects of dietary conjugated linoleic acid on lymphocyte function and growth of mammary tumors in mice. *Anticancer Research*. 1997;17:987-993

114. M. E. R. Dugan JLA, A. L. Schaefer, and J. K. G. Kramer. The effect of conjugated linoleic acid on fat to lean repartitioning and feed conversion in pigs. *SHORT COMMUNICATION* 1997

115. Nicolosi RJ, Rogers EJ, Kritchevsky D, Scimeca JA, Huth PJ. Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. *Artery*. 1997;22:266-277

116. Munday JS, Thompson KG, James KA. Dietary conjugated linoleic acids promote fatty streak formation in the c57bl/6 mouse atherosclerosis model. *The British Journal of Nutrition*. 1999;81:251-255

117. Dreyer C, Krey G, Keller H, Givel F, Helftenbein G, Wahli W. Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell*. 1992;68:879-887

118. Feige JN, Gelman L, Michalik L, Desvergne B, Wahli W. From molecular action to physiological outputs: Peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. *Progress in Lipid Research*. 2006;45:120-159

119. Tugwood JD, Issemann I, Anderson RG, Bundell KR, McPheat WL, Green S. The mouse peroxisome proliferator activated receptor recognizes a response element in the 5' flanking sequence of the rat acyl coa oxidase gene. *The EMBO Journal*. 1992;11:433-439

120. A IJ, Jeannin E, Wahli W, Desvergne B. Polarity and specific sequence requirements of peroxisome proliferator-activated receptor (ppar)/retinoid x receptor heterodimer binding to DNA. A functional analysis of the malic enzyme gene ppar response element. *The Journal of Biological Chemistry*. 1997;272:20108-20117

121. Llopis J, Westin S, Ricote M, Wang Z, Cho CY, Kurokawa R, Mullen TM, Rose DW, Rosenfeld MG, Tsien RY, Glass CK. Ligand-dependent interactions of coactivators steroid receptor coactivator-1 and peroxisome proliferator-activated receptor binding protein with nuclear hormone receptors can be imaged in live cells and are required for transcription. *Proceedings of the National Academy of Sciences of the United States of America*. 2000;97:4363-4368

122. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: Nuclear control of metabolism. *Endocrine reviews*. 1999;20:649-688

123. Schiffrin EL, Amiri F, Benkirane K, Iglarz M, Diep QN. Peroxisome proliferator-activated receptors: Vascular and cardiac effects in hypertension. *Hypertension*. 2003;42:664-668

124. Lee TI, Kao YH, Chen YC, Huang JH, Hsiao FC, Chen YJ. Peroxisome proliferator-activated receptors modulate cardiac dysfunction in diabetic cardiomyopathy. *Diabetes Research and Clinical Practice*. 2013;100:330-339

125. Barger PM, Kelly DP. Ppar signaling in the control of cardiac energy metabolism. *Trends in Cardiovascular Medicine*. 2000;10:238-245

126. Aoyama T, Peters JM, Iritani N, Nakajima T, Furihata K, Hashimoto T, Gonzalez FJ. Altered constitutive expression of fatty acid-metabolizing enzymes in mice

lacking the peroxisome proliferator-activated receptor alpha (pparalpha). *The Journal of Biological Chemistry*. 1998;273:5678-5684

127. Watanabe K, Fujii H, Takahashi T, Kodama M, Aizawa Y, Ohta Y, Ono T, Hasegawa G, Naito M, Nakajima T, Kamijo Y, Gonzalez FJ, Aoyama T. Constitutive regulation of cardiac fatty acid metabolism through peroxisome proliferator-activated receptor alpha associated with age-dependent cardiac toxicity. *The Journal of Biological Chemistry*. 2000;275:22293-22299

128. Guerre-Millo M, Gervois P, Raspe E, Madsen L, Poulain P, Derudas B, Herbert JM, Winegar DA, Willson TM, Fruchart JC, Berge RK, Staels B. Peroxisome proliferator-activated receptor alpha activators improve insulin sensitivity and reduce adiposity. *The Journal of Biological Chemistry*. 2000;275:16638-16642

129. Ye JM, Doyle PJ, Iglesias MA, Watson DG, Cooney GJ, Kraegen EW. Peroxisome proliferator-activated receptor (ppar)-alpha activation lowers muscle lipids and improves insulin sensitivity in high fat-fed rats: Comparison with ppar-gamma activation. *Diabetes*. 2001;50:411-417

130. Chou CJ, Haluzik M, Gregory C, Dietz KR, Vinson C, Gavrilova O, Reitman ML. Wy14,643, a peroxisome proliferator-activated receptor alpha (pparalpha) agonist, improves hepatic and muscle steatosis and reverses insulin resistance in lipoatrophic a-zip/f-1 mice. *The Journal of Biological Chemistry*. 2002;277:24484-24489

131. Ferre P. The biology of peroxisome proliferator-activated receptors: Relationship with lipid metabolism and insulin sensitivity. *Diabetes*. 2004;53 Suppl 1:S43-50

132. Abbott BD, Wood CR, Watkins AM, Das KP, Lau CS. Peroxisome proliferator-activated receptors alpha, beta, and gamma mrna and protein expression in human fetal tissues. *PPAR Research*. 2010;2010

133. Cheng L, Ding G, Qin Q, Huang Y, Lewis W, He N, Evans RM, Schneider MD, Brako FA, Xiao Y, Chen YE, Yang Q. Cardiomyocyte-restricted peroxisome proliferator-activated receptor-delta deletion perturbs myocardial fatty acid oxidation and leads to cardiomyopathy. *Nature Medicine*. 2004;10:1245-1250

134. Yu BC, Chang CK, Ou HY, Cheng KC, Cheng JT. Decrease of peroxisome proliferator-activated receptor delta expression in cardiomyopathy of streptozotocin-induced diabetic rats. *Cardiovascular Research*. 2008;80:78-87

135. Lee TI, Kao YH, Chen YC, Chen YJ. Proinflammatory cytokine and ligands modulate cardiac peroxisome proliferator-activated receptors. *European Journal of Clinical Investigation*. 2009;39:23-30

136. Willson TM, Brown PJ, Sternbach DD, Henke BR. The ppars: From orphan receptors to drug discovery. *Journal of Medicinal Chemistry*. 2000;43:527-550

137. Yki-Jarvinen H. Thiazolidinediones. *The New England Journal of Medicine*. 2004;351:1106-1118

138. Baraka A, AbdelGawad H. Targeting apoptosis in the heart of streptozotocin-induced diabetic rats. *Journal of Cardiovascular Pharmacology and Therapeutics*. 2010;15:175-181

139. Ihm SH, Chang K, Kim HY, Baek SH, Youn HJ, Seung KB, Kim JH. Peroxisome proliferator-activated receptor-gamma activation attenuates cardiac fibrosis in type 2 diabetic rats: The effect of rosiglitazone on myocardial expression of

receptor for advanced glycation end products and of connective tissue growth factor. *Basic Research in Cardiology*. 2010;105:399-407

140. Sharabi Y, Oron-Herman M, Kamari Y, Avni I, Peleg E, Shabtay Z, Grossman E, Shamiss A. Effect of ppar-gamma agonist on adiponectin levels in the metabolic syndrome: Lessons from the high fructose fed rat model. *American Journal of Hypertension*. 2007;20:206-210
141. Walker AB, Chattington PD, Buckingham RE, Williams G. The thiazolidinedione rosiglitazone (brl-49653) lowers blood pressure and protects against impairment of endothelial function in zucker fatty rats. *Diabetes*. 1999;48:1448-1453
142. Ryan MJ, Didion SP, Mathur S, Faraci FM, Sigmund CD. Ppar(gamma) agonist rosiglitazone improves vascular function and lowers blood pressure in hypertensive transgenic mice. *Hypertension*. 2004;43:661-666
143. Rhee SG, Bae YS. Regulation of phosphoinositide-specific phospholipase c isozymes. *The Journal of biological chemistry*. 1997;272:15045-15048
144. Newton AC. Regulation of protein kinase c. *Current Opinion in Cell Biology*. 1997;9:161-167
145. Ebinu JO, Bottorff DA, Chan EY, Stang SL, Dunn RJ, Stone JC. Rasgrp, a ras guanyl nucleotide- releasing protein with calcium- and diacylglycerol-binding motifs. *Science*. 1998;280:1082-1086
146. Lucas P, Ukhanov K, Leinders-Zufall T, Zufall F. A diacylglycerol-gated cation channel in vomeronasal neuron dendrites is impaired in trpc2 mutant mice: Mechanism of pheromone transduction. *Neuron*. 2003;40:551-561

147. Topham MK, Epand RM. Mammalian diacylglycerol kinases: Molecular interactions and biological functions of selected isoforms. *Biochimica et Biophysica Acta*. 2009;1790:416-424

148. Hozumi Y, Ito T, Nakano T, Nakagawa T, Aoyagi M, Kondo H, Goto K. Nuclear localization of diacylglycerol kinase zeta in neurons. *The European Journal of Neuroscience*. 2003;18:1448-1457

149. Merida I, Avila-Flores A, Merino E. Diacylglycerol kinases: At the hub of cell signalling. *The Biochemical Journal*. 2008;409:1-18

150. Topham MK, Prescott SM. Diacylglycerol kinases: Regulation and signaling roles. *Thrombosis and Haemostasis*. 2002;88:912-918

151. Hurley JH, Newton AC, Parker PJ, Blumberg PM, Nishizuka Y. Taxonomy and function of c1 protein kinase c homology domains. *Protein science : a Publication of the Protein Society*. 1997;6:477-480

152. van Blitterswijk WJ, Houssa B. Properties and functions of diacylglycerol kinases. *Cellular Signalling*. 2000;12:595-605

153. Yamada K, Sakane F, Matsushima N, Kanoh H. Ef-hand motifs of alpha, beta and gamma isoforms of diacylglycerol kinase bind calcium with different affinities and conformational changes. *The Biochemical Journal*. 1997;321 (Pt 1):59-64

154. Sakane F, Imai S, Kai M, Wada I, Kanoh H. Molecular cloning of a novel diacylglycerol kinase isozyme with a pleckstrin homology domain and a c-terminal tail similar to those of the eph family of protein-tyrosine kinases. *The Journal of Biological Chemistry*. 1996;271:8394-8401

155. Takeuchi H, Kanematsu T, Misumi Y, Sakane F, Konishi H, Kikkawa U, Watanabe Y, Katan M, Hirata M. Distinct specificity in the binding of inositol phosphates by pleckstrin homology domains of pleckstrin, rac-protein kinase, diacylglycerol kinase and a new 130 kda protein. *Biochimica et Biophysica Acta*. 1997;1359:275-285

156. Bilim O, Takeishi Y, Kitahara T, Arimoto T, Niizeki T, Sasaki T, Goto K, Kubota I. Diacylglycerol kinase zeta inhibits myocardial atrophy and restores cardiac dysfunction in streptozotocin-induced diabetes mellitus. *Cardiovascular Diabetology*. 2008;7:2

157. Harada M, Takeishi Y, Arimoto T, Niizeki T, Kitahara T, Goto K, Walsh RA, Kubota I. Diacylglycerol kinase zeta attenuates pressure overload-induced cardiac hypertrophy. *Circulation journal : official journal of the Japanese Circulation Society*. 2007;71:276-282

158. Alibin CP, Kopilas MA, Anderson HD. Suppression of cardiac myocyte hypertrophy by conjugated linoleic acid: Role of peroxisome proliferator-activated receptors alpha and gamma. *The Journal of Biological Chemistry*. 2008;283:10707-10715

159. Grundy SM, Benjamin IJ, Burke GL, Chait A, Eckel RH, Howard BV, Mitch W, Smith SC, Jr., Sowers JR. Diabetes and cardiovascular disease: A statement for healthcare professionals from the american heart association. *Circulation*. 1999;100:1134-1146

160. Voulgari C, Papadogiannis D, Tentolouris N. Diabetic cardiomyopathy: From the pathophysiology of the cardiac myocytes to current diagnosis and management strategies. *Vascular Health and Risk Management*. 2010;6:883-903

161. Feng B, Chen S, Chiu J, George B, Chakrabarti S. Regulation of cardiomyocyte hypertrophy in diabetes at the transcriptional level. *American journal of physiology. Endocrinology and Metabolism*. 2008;294:E1119-1126

162. Colligan PB, Relling DP, Ren J. Ceramide attenuates high glucose-induced cardiac contractile abnormalities in cultured adult rat ventricular myocytes. *Cellular and Molecular Biology*. 2002;48 Online Pub:OL251-257

163. Anumah F, Ohwovoriole A. Serum biochemistry in nigerians with hyperglycemic emergencies. *Ethnicity & Disease*. 2008;18:26-30

164. Vlahos CJ, McDowell SA, Clerk A. Kinases as therapeutic targets for heart failure. *Nature reviews. Drug Discovery*. 2003;2:99-113

165. Li Q, Hueckstaedt LK, Ren J. The protease inhibitor ucf-101 ameliorates streptozotocin-induced mouse cardiomyocyte contractile dysfunction in vitro: Role of amp-activated protein kinase. *Experimental Physiology*. 2009;94:984-994

166. Poirier H, Shapiro JS, Kim RJ, Lazar MA. Nutritional supplementation with trans-10, cis-12-conjugated linoleic acid induces inflammation of white adipose tissue. *Diabetes*. 2006;55:1634-1641

167. Riserus U, Arner P, Brismar K, Vessby B. Treatment with dietary trans10cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. *Diabetes Care*. 2002;25:1516-1521

168. Moloney F, Toomey S, Noone E, Nugent A, Allan B, Loscher CE, Roche HM. Antidiabetic effects of cis-9, trans-11-conjugated linoleic acid may be mediated via anti-inflammatory effects in white adipose tissue. *Diabetes*. 2007;56:574-582

169. Wahle KW, Heys SD, Rotondo D. Conjugated linoleic acids: Are they beneficial or detrimental to health? *Progress in Lipid Research*. 2004;43:553-587

170. Kennedy A, Martinez K, Schmidt S, Mandrup S, LaPoint K, McIntosh M. Antiobesity mechanisms of action of conjugated linoleic acid. *The Journal of Nutritional Biochemistry*. 2010;21:171-179

171. Miller A, Stanton C, Devery R. Cis 9, trans 11- and trans 10, cis 12-conjugated linoleic acid isomers induce apoptosis in cultured sw480 cells. *Anticancer Research*. 2002;22:3879-3887

172. Huang Y, Zhang H, Shao Z, O'Hara KA, Kopilas MA, Yu L, Netticadan T, Anderson HD. Suppression of endothelin-1-induced cardiac myocyte hypertrophy by ppar agonists: Role of diacylglycerol kinase zeta. *Cardiovascular Research*. 2011;90:267-275

173. Liang F, Wang F, Zhang S, Gardner DG. Peroxisome proliferator activated receptor (ppar)alpha agonists inhibit hypertrophy of neonatal rat cardiac myocytes. *Endocrinology*. 2003;144:4187-4194

174. Planavila A, Rodriguez-Calvo R, Jove M, Michalik L, Wahli W, Laguna JC, Vazquez-Carrera M. Peroxisome proliferator-activated receptor beta/delta activation inhibits hypertrophy in neonatal rat cardiomyocytes. *Cardiovascular Research*. 2005;65:832-841

175. Ziouzenkova O, Perrey S, Marx N, Bacqueville D, Plutzky J. Peroxisome proliferator-activated receptors. *Current Atherosclerosis Reports*. 2002;4:59-64

176. Asakawa M, Takano H, Nagai T, Uozumi H, Hasegawa H, Kubota N, Saito T, Masuda Y, Kadowaki T, Komuro I. Peroxisome proliferator-activated receptor gamma plays a critical role in inhibition of cardiac hypertrophy in vitro and in vivo. *Circulation*. 2002;105:1240-1246

177. Takeishi Y, Goto K, Kubota I. Role of diacylglycerol kinase in cellular regulatory processes: A new regulator for cardiomyocyte hypertrophy. *Pharmacology & Therapeutics*. 2007;115:352-359