GLP-1R Agonists & Cardiac Energy Metabolism in Type 2 Diabetes

By

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Abstract

Type 2 diabetes (T2D) is associated with an increased risk for cardiovascular disease. Of interest, liraglutide, a therapy for T2D that activates the glucagon-like peptide-1 receptor (GLP-1R) to augment insulin secretion, reduces cardiovascular-related death in people with T2D. However, it remains enigmatic as to how liraglutide may reduce cardiovascular death in patients with T2D. Importantly, the GLP-1R is not expressed in ventricular cardiac myocytes, so it is likely that indirect actions independent of the myocardium are involved. We hypothesized that augmented insulin secretion is a key factor contributing to liraglutide-induced cardioprotection, which thereby increases myocardial glucose oxidation. C57BL/6J male mice were fed either a low-fat diet for 10-weeks (lean) or were subjected to experimental T2D (10-weeks of high-fat diet supplementation plus a single injection of streptozotocin at 75 mg/kg 4-weeks into the protocol) and treated with either saline or liraglutide (30 g/kg via subcutaneous injection) 3x over a 24-hr period. 2-hr following the final injection, all mice were euthanized and had their hearts perfused in the working mode to assess myocardial energy metabolism. In a separate cohort of mice subjected to our experimental model of T2D, animals were randomized to receive either vehicle control or liraglutide treatment for 2-weeks, and cardiac function was assessed via ultrasound echocardiography prior to and upon completion of the study. Systemic treatment of lean mice with liraglutide increased myocardial glucose oxidation rates without affecting glycolysis rates. Conversely, direct treatment of the isolated working heart with liraglutide had no effect on glucose oxidation. These findings were recapitulated in mice with experimental T2D and associated with increased circulating insulin levels. Furthermore, Liraglutide treatment attenuated declining diastolic function in mice with experimental T2D. Our data demonstrates that liraglutide augments myocardial glucose oxidation via indirect mechanisms, which may mechanistically explain how liraglutide improves cardiovascular outcomes in people with T2D.

Preface

This thesis document is an original work by Malak Almutairi under the supervision of Dr. John Ussher. The work of had been conducted in the Ussher lab (2-055 Katz) in the Faculty of Pharmacy and Pharmaceutical Sciences. Some of the experiments were also conducted at various core facilities present at the University of Alberta

Dedication

This thesis is dedicated to my parents Musead Almutairi and Ahlam Alshahid, my sisters Rania, Saeedah, Zainab, Shuruq, Rana, Ghafran, my brothers Sultan and Abdualziz Almutairi, my nephew and nieces Yazzan, Hala, and Celine, and my brothers-in-law Alaa Ali and Sami Alomar , who have always been a constant source of support and encouragement during my journey. Special thanks to my partner Massi Akrour for his help, love, support and inspiration.

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List of Abbreviations

ACCORD	Action to Control Cardiovascular Risk in Diabetes
ADVANCE	Action in Diabetes and Vascular disease Preterax and Diamicron-MR Controlled evaluation
Akt	Protein kinase B
AMPK	5’ Adenosine monophosphate-activated protein kinase
ANP	Atrial natriuretic peptide
ANOVA	Analysis of variance
AS160	Akt substrate of 160 KDa
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BW	Body weight
CACT	Carnitine-acylcarnitine translocase
cAMP	Cyclic adenosine monophosphate
CO_2	Carbon dioxide
CPT-1	Carnitine palmitoyl transferase 1
cTn	Cardiac troponin
CVD	Cardiovascular disease
CRP	C-reactive protein
DAG	Diacylglycerol
DBP	Diastolic blood pressure
DCA	Dichloroacetate
DIO	Diet induced obesity
DIRKO	Double incretin receptor knockout
DPP4	Dipeptidyl peptidase 4
EC	Endothelial cell
ECG	Echocardiography
EDTA	Ethylenediaminetetraacetic acid
EF	Ejection fraction
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
ESC	European Society of Cardiology

ETC	Electron transport chain
ELIXA	Evaluation of Lixisenatide in Acute Coronary Syndrome
FAs	Fatty acids
FAD	Flavin adenine dinucleotide
FFA	Free fatty acid
FS	Fractional shortening
GIK	Glucose-insulin-potassium
GIP	Glucose-dependent insulin tropic polypeptide
GIPR	GIP receptor
GLP-1	Glucagon like peptide -1
GLP-1R	GLP-1 receptor
GLUT1 / GLUT4	Glucose transporters 1 and 4
GPCRs	G protein-coupled receptors
GSIS	Glucose-stimulated insulin secretion
GTP	Guanosine triphosphate
HbA1C	Glycated hemoglobin A1C
HCAECs	Human coronary artery ECs
HFD	High fat diet
HPR	Horseradish peroxidase
HR	Heart rate
HUVECS	Human umbilical vein ECs
IGT	Impaired glucose tolerance
IL-1	Interleukin-1
IP	Intraperitoneal
IR	Insulin receptors
IRS	Insulin receptor substrate
IV	Intravenous
K_{ATP}	Adenosine triphosphate sensitive potassium channel
LAD	Left anterior descending
LEADER	Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome Results
LPS	Lipopolysaccharide
LV	Left ventricular
MACE	Major adverse cardiovascular events

mRNA	Messenger ribonucleic acid
MI	Myocardial infarction
NAD	Nicotinamide adenine dinucleotide
NYHA	New York Heart Association
OGTT	Oral glucose tolerance test
PDC	Pyruvate dehydrogenase complex
PDH	Pyruvate dehydrogenase
PDHK	PDH kinase
PDHP	PDH phosphatase
PI_3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PET	Positron emission tomography
PPAR-γ	Peroxisome proliferator-activated receptor-γ
SBP	Systolic blood pressure
SDS	Sodium dodecyl sulfate
SGLT2	Sodium glucose co-transporter 2
SMCs	Smooth muscle cells
SQ	Subcutaneous
STZ	Streptozotocin
SUR1	Sulfonylurea receptors 1
SV	Stroke volume
T2D	Type 2 diabetes
TAG	Triacylglycerol
TMB	Tetramethylbenzidine
TNF-α	Tumor necrosis factor alpha
TZD	Thiazolidinedione
UKPDS	United Kingdom Prospective Diabetes Study
VADT	Veteran's Affairs Diabetes Trial
VDCCS	Voltage-dependent Ca^{2+} channels
VLDL	Very low-density lipoprotein
VSMCs	Vascular smooth muscle cells
WHO	World Health Organization
WT	Wild type

Chapter One

- **Introduction**

Part of this chapter includes content previously published in the following reviews;

Al Batran, R., M. Almutairi, and J.R. Ussher, Glucagon-like peptide-1 receptor mediated control of cardiac energy metabolism. Peptides. 2018. 100: p. 94-100.

Almutairi, M., R. Al Batran, and J.R. Ussher, Glucagon-like peptide-1 receptor action in the vasculature. Peptides. 2019. 111: p. 26-32.

Type 2 Diabetes

The prevalence of type 2 diabetes (T2D) continues to increase at an endemic rate, as there are currently over 422 million people living with diabetes worldwide as determined World Health Organization (WHO) [1, 2]. Of these, ~90% have T2D, with the majority resulting from underlying obesity and different risk factors such as family history, age, polycystic ovary syndrome and metabolic genetic factors [2, 3]. T2D is chronic metabolic disease associated with hyperglycemia. It is also characterized by irregular glucose and lipid metabolism, impaired insulin sensitivity and insulin resistance. In addition to genetic predisposition and environmental risk factors, obesity is the most significant risk factor for the development of T2D and is strongly associated with insulin resistance. However, the molecular mechanisms responsible for the development of insulin resistance in obesity are complex and not fully characterized. There are several well-documented hypotheses that attempt to explain how obesity causes insulin resistance [2]. These hypotheses involve different mechanisms including inflammation, endoplasmic reticulum (ER) stress, oxidative stress, hyperinsulinemia and lipotoxicity [4-7]. Statistics show that weight gain in early adulthood is associated with a higher risk of earlier onset of T2D than weight gain between the ages of 40 and 55 years [8]. There are different factors that can delay or prevent T2D, including adopting a healthy life style and reduction in body weight by either dietary measures or physical activity, which can contribute to a marked decline in glycated hemoglobin levels and improve cardiovascular disease risk factors [8]. Obesity-induced derangements in glucose metabolism such as impaired glucose tolerance (IGT) can appear early in obese children and adolescents. As a result, obese young people with IGT are characterized by peripheral insulin resistance and a relative β cell dysfunction [9]. Insulin resistance is defined as a condition whereby insulin-induced glucose uptake and subsequent glucose metabolism are impaired in insulin-sensitive tissues (e.g. skeletal muscle, adipose tissue, etc.). As a result, inhibition of the insulin signalling

pathway forces the islet β-cells to produce and secrete large amounts of insulin (hyperinsulinemia) to control high blood glucose levels [10]. Impaired insulin signaling also causes glucotoxicity and lipotoxicity, which may be related to inflammation, a physiological process associated with elevation of white blood cells and pro-inflammatory cytokines such as interleukin (IL)-1 and tumour necrosis factor (TNF) α levels in the circulation or tissue [11]. A study has also shown that lipotoxicity and glucotoxicity can contribute to dysregulated glucose-stimulated insulin secretion (GSIS) in the pancreatic β-cells. By growing INS-1 (832/13) cells in conditions with a high concentration of glucose and palmitate for ten weeks, it resulted in increased oxidative stress and impaired GSIS in these β-cells [12]. It is important to note that dysregulation of free fatty acid (FFA) metabolism is a key event responsible for insulin resistance which can lead to lipotoxicity. The balance between the production of lipids, and their oxidation or transport is required to maintain normal cell function [13]. However, in lipotoxic β-cells the insulin secretory response to high glucose level is inhibited. Moreover, previous studies have shown in muscle biopsies from people with T2D muscle that increases in intramyocellular triacylglycerol (TAG) by ^{1}H-NMR positively correlate with insulin resistance [14]. Findings regarding mechanisms of lipotoxicity include the accumulation of intramyocellular ceramide, which is associated with development of insulin resistance [15]. Data shows that in C57BL/6 mice when the synthesis of ceramide is inhibited with myriocin (serine palmitoyl transferase inhibitor), insulin resistance caused by diet induced obesity (DIO) can be reversed [15, 16]. Previous studies have postulated that this accumulation of lipid-derived metabolites results from an impaired ability of the mitochondria to oxidize fatty acids, which interferes with insulin signaling. Indeed, human and animal data have shown that lipid accumulation in non-adipose tissues is associated with insulin resistance in muscle and liver, and also promotes impaired β-cell and cardiac function in animal models [17]. In obese Zucker Diabetic Fatty rats, TAG, insulin and ceramide levels were high compared to their lean littermates, thus linking lipotoxicity with insulin resistance [18]. Moreover, the utilization of lipids as fuel in states of overnutrition increases both fatty acid oxidation and TAG storage,

leading to increased formation of diacylglycerol (DAG). Moreover, the accumulation of DAG and intramyocellular acyl CoAs activates different isoforms of protein kinase C (PKC), which can phosphorylate and inhibit IRS-1, preventing the activation of PI3K, thereby reducing insulin signaling and subsequent glucose uptake [19]. In male Wistar rats infused with lipid for 5-hr, activation of PI3K was impaired as a result increased DAG levels, which was associated with decreased insulin-stimulated glucose uptake. These results indicate that increases in fatty acids induce a defect in the insulin signalling pathway. Additional molecular mechanisms of insulin resistance arising from lipid accumulation hypothesis include tissue build up of ceramides, which induce insulin resistance by inhibiting the phosphorylation of Akt. The result of this inhibition is decreased translocation of GLUT4 to the plasma membrane and hence decreased uptake of glucose (see figure 1-4).

Complications of T2D

Chronic hyperglycemia plays a major role in the initiation of diabetic vascular complications. Importantly, diabetes related complications are associated with long-term damage and failure of various organ systems, with macrovascular and microvascular complications being major contributors to the morbidity and mortality seen in diabetic patients [22]. Macrovascular complications affect the cardiovascular system and include coronary artery disease, peripheral arterial disease, and stroke, whereas microvascular complications are often dependent on the duration and severity of the uncontrolled elevations in blood sugar and include diabetic nephropathy, neuropathy, and retinopathy [23]. Diabetic retinopathy affects the macula and the peripheral retina which can cause visual disability and can be influenced by existing hypertension [24] People with diabetes also frequently have diabetic neuropathy which is defined as the nerve damage, that occurs with a poor glycemic control. Approximately one-half of people with diabetes

have peripheral neuropathy that typically affects the extremities [25]. Moreover, the prolonged duration of uncontrolled blood sugar can cause diabetic nephropathy, and it is characterised by microalbuminuria which is defined as albumin excretion of 30-299 mg/24 hours in the urine. Moreover, untreated diabetic nephropathy leads to proteinuria and eventually to renal failure [26]. According to the UK Prospective Diabetes Study (UKPDS), successful control of blood glucose with a HbA1C target of 6.5% can greatly reduce the development of these microvascular causes in T2D people [27, 28].

Diabetes increases the risk for an individual to develop macrovascular complications, thus people with T2D are at higher risk of accelerated atherosclerosis, which is the leading cause of cardiovascular disease (CVD). In normal physiological conditions, endothelial cells produce and release adequate amounts of pro- and anti-inflammatory cytokines to maintain normal vascular functions [29]. However, atherosclerosis disrupts this physiological balance by promoting the production of proinflammatory cytokines. These manifestations can modulate the inflammatory response in the vascular system. Atherosclerosis also increases the risk for myocardial infarction (MI) and stroke. MI is associated with a low but significant risk of stroke which substantially increases following an ischemic event. MI is classified into various types and the type that is related to atherosclerosis is referred to as type 1 and is characterized with atherosclerotic plaque rupture, ulceration or erosion that can result in embolus to travel to one or more of the coronary arteries, leading to reduced myocardial blood flow [30, 31]. Furthermore, stroke and MI share many risk factors including, dyslipidemia, hypertension, and diabetes. The term MI is commonly known as a “heart attack” and is defined as myocardial cell death due to prolonged ischemia. Symptoms of MI can be detectable by different signs such as abnormalities in both Echocardiography (ECG) and cardiac enzymes in the circulation. Besides, early detection of a neglectable amount of myocardial injury or necrosis can be done by assessment of sensitive and myocardial tissue-specific cardiac biomarkers cardiac troponin (cTn), and more sensitive imaging

techniques [31, 32]. Although stroke shares some similarity with MI as it can happen suddenly under ischemic conditions, it is mostly caused by rapid blockage of arteries within the brain [33, 34]. It's also important to note that microvascular and macrovascular complications develop simultaneously in diabetes and both complications can be influenced by the severity and duration of the diseases. Moreover, hyperglycemia, is likely to contribute to excessive cardiovascular risk in patients with T2D [35-37].

Diabetes and Cardiovascular Disease

The high rate of deaths from CVD is a major challenge in T2D management [21]. Importantly, the risk of CVD increases continuously along with high levels of fasting plasma glucose, even in prediabetic people [38] .The Framingham heart study indicated that the percentage of people who were diagnosed with T2D their risk of CVD increased by 8.7 % in the period from 1952–1998 [38]. Moreover, recent data published by the European Society of Cardiology (ESC) indicates that the duration and the progression of diabetes are important factors that contribute to the increasing risk of CVD [39]. Studies also suggested that people with T2D without previous MI are more susceptible to have MI compared to nondiabetic people with a history of MI [40]. As a result, there has been an increased focus on the management of T2D as it pertains to their risk for CVD. Pharmaceutical companies developing new therapies for T2D not only have to prove that their agent improves hyperglycemia, but they must also provide evidence on their cardiovascular safety via completion of large-scale multicenter cardiovascular outcomes trials [1].

Pharmacotherapy of T2D

Prevention and treatment strategies are necessary to minimize the complications of T2D. T2D is one of the major causes of morbidity and mortality worldwide and is a significant economic burden

[1]. The pharmaceutical industry has introduced a number of therapeutic agents to control glycemia mainly and improve complications of T2D. T2D is primarily characterized by insulin resistance and a defect in insulin secretion, subsequently hepatic glucose production increases and decreased peripheral uptake of glucose both lead to hyperglycemia. Setting glycemic targets is also an important factor to take into consideration when people with T2D receive pharmacotherapy, which is achieved by monitoring their (HbA1C) levels. The ADA recommends that a reasonable HbA1C goal for adults with T2D should be less than 7% as shown in (ACCORD, ADVANCE, VADT and UKPDS international clinical trials) [28, 41-43]. However, this target might change for a specific population such as in the pregnant and elderly. Therefore, an individualized treatment approach is recommended [44].

Oral Antidiabetic Agents

Biguanides

The first line therapy for T2D is metformin, which is an older medication that belongs to the biguanides class of drugs. Metformin is also a drug of choice for obese, prediabetic, and T2D patients [45, 46]. These drugs target hepatic glucose production by inhibiting gluconeogenesis. However, metformin's mechanism of action for inhibiting gluconeogenesis remains unclear and is frequently debated [45]. Proposed mechanisms include activation of 5' adenosine monophosphate-activated protein kinase (AMPK), which may occur indirectly via inhibition of the mitochondrial respiratory chain complex 1 [47, 48]. However, another study indicated that metformin has a different mechanism by antagonizing the action of glucagon, which decreases the production of cyclic adenosine monophosphate (cAMP) within hepatocytes, thereby reducing activation of cAMP response element binding protein and decreasing transcription of key genes involved in gluconeogenesis [47].

Thiazolidinediones (TZD)

A major factor contributing to the development of T2D is insulin resistance, which is the inadequate response of liver, adipose tissue, skeletal muscle, and other insulin responsive organs to the actions of insulin [49]. Therefore, another therapeutic approach that reverses this condition, involves the use of insulin sensitizing agents such as drugs in the TZD class (2). TZDs such as (pioglitazone and rosiglitazone) exert their antidiabetic effects by enhancing glucose uptake by the skeletal muscle, β-cell, adipose tissue, skeletal muscle and liver. Their mechanism which involves activation of peroxisome proliferator-activated receptor (PPARγ) which play an essential role in glucose and lipid metabolism. PPARγ is expressed in many tissues but is most abundant in adipocytes [50]. By increasing the fat storage in adipose tissue, this will reduce excess lipid storage in the muscle and liver, which improves muscle and liver insulin sensitivity and subsequent glucose uptake in these organs. However, with TZDs it may take to 2-4 months to exert their effect on the adipose tissues to increase fat storage, and to decrease the excess fat levels in muscle and liver [51] .

Sodium Glucose Co-transporter 2 (SGLT2) Inhibitors

SGLT2 inhibitors, such as empagliflozin and canagliflozin, facilitate glucose excretion in urine by preventing glucose reabsorption in the proximal convoluted tubules of the kidney [52] ;Thus, plasma glucose level decrease in the blood leading to an improvement in HbA1C levels, thereby reducing hyperglycemia. Some studies have also indicated that empagliflozin has several

mechanisms of action that contribute to glucose lowering, including reductions in body weight [53].

α-Glucosidase Inhibitors

Agents in this drug class (e.g. acarbose) inhibit the α-glucosidase enzyme that is responsible for the breakdown of disaccharides into monosaccharides; therefore, the intestinal absorption of monosaccharides (e.g. glucose) is delayed, and results in the lowering of blood glucose levels. Since these drugs don't affect insulin secretion or insulin sensitivity, they for most part do not increase the risk for hypoglycemia [54, 55].

Insulin Secretagogues

Sulfonylureas

Sulfonylureas are anti-diabetic drugs classified into 2 generations; the first generation (e.g. chlorpropamide) agents are not as commonly used to treat T2D as the second generation (e.g. glipizide). Both drug classes decrease circulating glucose levels, whereby they augment insulin secretion from islet β-cells [56, 57]. Sulfonylureas bind to the sulfonylurea receptor 1 (SUR1) and induce closure of ATP-sensitive potassium (K_{ATP}) channels. However, sulfonylureas can act as partial antagonists, so that high-affinity of sulfonylurea inhibition is incomplete, even at saturating drug concentrations [57, 58]. Their insulinotropic action is mediated through closure of K_{ATP} channels, which results in membrane depolarization and calcium influx through voltage-gated calcium channels [59, 60]. These events lead to an increase in intracellular calcium and subsequent exocytosis of insulin-containing granules. Sulfonylureas are contraindicated for

overweight people with T2D, since insulin is a potent anabolic hormone that promotes weight gain [56].

Glinides

Glinides are rapid-onset and insulinotropic agents that restore early prandial insulin secretion in a glucose-dependent manner, and thereby target explicitly postprandial hyperglycemia [61]. Their insulinotropic action is mediated through closure of K_{ATP} channels, which results in membrane depolarization and calcium influx through voltage-gated calcium channels [60, 62]. These events lead to subsequent exocytosis of insulin-containing granules. Although the glinides mechanism of action is similar to the sulfonylureas, the glinides binds to the SUR1 receptor rapidly as partial antagonists, and also dissociate from the SUR1 receptor rapidly [63, 64]. This rapid association and dissociation provide a unique "fast-on/fast-off" effect. Thus, Glinides such as nateglinide have an early onset and short duration of action stimulating insulin secretion in vivo and its control of postprandial hyperglycemia in a glucose-dependent manner [64].

Incretin Based treatments

Glucagon-Like Peptide-1 (GLP-1) Receptor (GLP-1R) Agonists

GLP-1 is an insulinotropic hormone that potentiates glucose-stimulated insulin secretion following nutrient ingestion, actions mediated via the GLP-1 receptor (GLP-1R) present on islet β-cells [65]. Therefore, GLP-1R agonists (e.g. exenatide and liraglutide) are used to treat T2D via potentiating insulin secretion, and for most part do so in a glucose-dependent manner, thereby the risk for hypoglycemia is much lower with agents in this drug class versus those in the sulfonylureas drug class [66, 67]. The vast majority of GLP-1R agonists are modified peptides of GLP-1 that have

their second amino acid residue mutated so that they are resistant to degradation by dipeptidyl peptidase 4 (DPP4, see below) [68-70].

Dipeptidyl Peptidase (DPP4) Inhibitors

One of the therapeutic approaches that have been pursued for T2D treatment is inhibition of the dipeptidyl peptidase-4 (DPP-4), which is an enzyme that degrades peptides that have a position 2 alanine or proline amino acid residue. As both the incretin hormones GIP and GLP-1 have an alanine position 2 residue, DPP4 inhibitors increase the plasma levels of intact GLP-1 and GIP by inhibiting circulating DPP-4 and preventing their degradation [71]. By increasing intact circulating GLP-1/GIP, DPP-4 inhibitors thereby potentiate β-cell mediated insulin secretion following meal intake (see above section on GLP-1R agonists)

Table 1-1 Antidiabetic drugs overview

Class	*Drug (Generic name)*	*Mechanism of action*
Biguanides	Metformin	Reduces hepatic glucose production
Thiazolidinediones (TZD)	Pioglitazone Rosiglitazone	PPARγ agonist that increases insulin sensitivity
Sodium glucose cotransporter 2 (SGLT2) inhibitors	Empagliflozin Canagliflozin	Increases urinary glucose excretion
α-glucosidases inhibitor	Acarbose	Slows the absorption of ingested carbohydrates
Sulfonylureas	Glipizide Glimepiride	Augments islet β-cell insulin secretion
Glinides	Nateglinide Repaglinide	Augments islet β-cell insulin secretion
Glucagon-like peptide-1 receptor (GLP-1R) agonist	Exenatide Liraglutide	Augments glucose-stimulated islet β-cell insulin secretion
Dipeptidyl peptidase-4 (DPP-4) inhibitors	Saxagliptin Sitagliptin	Prevents degradation mediated inactivation of incretin hormones (GLP-1 & GIP)

Incretins hormones

The GIP receptor (GIPR) and the GLP-1 receptor (GLP-1R), represent the receptors for GIP and GLP-1, respectively, which belong to the class B family of G protein coupled receptors. Of importance, it has been demonstrated that defective release of incretin hormones in patients with T2D leads to delayed release of insulin [72]. After incretin secretion, both GIP and GLP-1 are rapidly cleaved by DPP-4 [73]. The inactivation of incretin hormones by DPP-4 led to the pharmacological development of DPP-4 inhibitors to augment insulin secretion indirectly, via preserving intact GIP and GLP-1 following their secretion from enteroendocrine cells during nutrient/meal ingestion. As a result, the half-life of the native incretin hormones GIP and GLP-1 increases. Supporting the importance of GLP-1 and GIP in improving glycemia following DPP-4 inhibition. Preclinical findings illustrate that when double knockouts for both incretin receptors (GIPR and GLP-1R) in mice (DIRKO mice) are treated with a DPP4 inhibitor, no effect on circulating glucose or insulin levels is observed following an oral glucose tolerance test (OGTT). In contrast, in wild-type (WT) mice, glucose levels are reduced significantly and associated with increased plasma insulin levels after administration of a DPP4 inhibitor for 7-weeks [74]. These findings demonstrate that the actions of DPP4 inhibitors are abolished in DIRKO mice, which strongly shows the critical role for GIP and GLP-1 in augmenting insulin secretion and controlling glycaemia in response to DPP-4 inhibitors [75]. GLP-1 also reduces glucagon secretion, and combined with increases in circulating insulin, blood glucose levels are appropriately controlled in people with T2D. Moreover, these two hormones insulin and glucagon act on the vasculature, and their vascular actions may contribute to how GLP-1-based drugs modify cardiovascular function and CVD progression in people with T2D.

The GLP-1 Receptor (GLP-1R)

The GLP-1R is a member of the class B1 family of G protein-coupled receptors (GPCRs), which are seven transmembrane domain receptors [74]. This family also includes several receptors such as GLP-2, GIP, and glucagon receptors. GLP-1R signaling also relies on GLP-1R-mediated intracellular pathways, by coupling to G proteins; as a result, it promotes the production of cAMP) through increasing enzymatic activity of adenylate cyclase. This subsequently stimulates both PKA (protein kinase A) and Epac2 (exchange protein activated by cAMP-2), which is directly involved in enhancing proinsulin gene transcription. As a result, GLP-1R activation promotes membrane depolarization of β cells by inhibiting K^+ channels, which allow voltage-dependent Ca^{2+} channels (VDCCs) to open and accelerate Ca^{2+} influx to occur, resulting in the exocytosis of insulin from β-cells. Beside the insulin secretory effect, the activation of the GLP-1R stimulate β cell proliferation and neogenesis and it also has an antiapoptotic impact which is critical for the maintenance of a proper β cell mass (see figure 1-1) [76]. The proliferative effects of GLP-1 activated via activation of the epidermal growth factor receptor (EGFR) which promotes phosphoinositide 3 kinase (PI3K) and protein kinase B (Akt). Moreover, activation of PI3K/Akt signaling leads to cell proliferation and growth by regulating cyclinD1 expression via insulin receptor substrate 2 (IRS) as well as activation of cAMP/PKA. However, the specific mechanisms involved in GLP-1–dependent β cell differentiation/neogenesis are not fully understood [77]. Activation of the GLP-1R occurs in a glucose-dependent manner and GLP-1 hormone acts directly on β cells to stimulate insulin secretion [76]. The GLP-1R was found initially in β-cells of the pancreatic islets and is also widely expressed in different tissues such as the brain, kidney, lung, stomach, and major blood vessels [78]. On the other hand, liver, adipose tissues, and muscle do not express the GLP-1R, suggesting that any actions mediated via GLP-1 on these tissues would be indirectly mediated [76]. Moreover, a few studies demonstrated that GLP-1 has direct action on hepatocytes. In human hepatocytes treatment with 10nM GLP-1 or exendin-4 for

12-hr increased phosphorylation of Akt and PKC ζ, which are related indirectly with the reduction of TAG stores in the hepatocytes compared with the vehicle control treated cells [80]. By acting on these multiple organ systems GLP-1 not only controls islet hormone secretion, but also gastrointestinal motility, food intake, and overall energy homeostasis [79, 81].

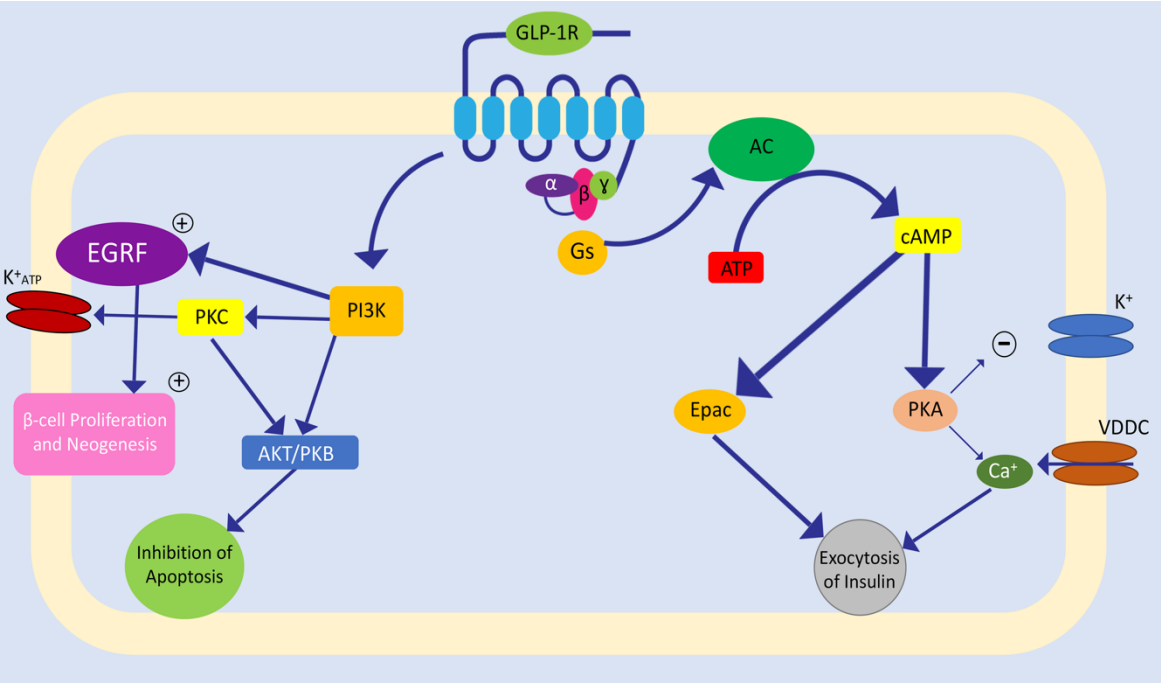

***Figure 1-1 GLP-1R* signaling overview**

GLP-1R-mediated intracellular pathways can be coupled to activation of Gs proteins; as a result, it promotes the production of cAMP through increasing enzymatic activity of AC. This subsequently stimulates both PKA and Epac2. As a result, GLP-1R activation promotes membrane depolarization of β cells by inhibiting K+ channels, which accelerate Ca2+ influx via voltage-dependent Ca2+ channels (VDCCs), resulting in the exocytosis of insulin. Activation of the GLP-1R also activates EGFR and promotes PI3K/Akt signaling and stimulates β cell proliferation, neogenesis and it inhibit apoptosis which is critical for the maintenance of a proper β cell mass.

Of relevance to my specific thesis, accumulating evidence illustrates that ventricular cardiac myocytes do not express the GLP-1R, therefore it is highly likely that GLP-1R agonist-induced cardioprotection is mediated via indirect actions on other peripheral tissues that subsequently improve cardiac function in patients with T2D [73]. Despite the recognized benefits of GLP-1R agonists on ischemic heart disease and heart failure in preclinical and clinical studies, it is now accepted that previous immunohistochemistry studies indicating that the GLP-1R is expressed in ventricular cardiac myocytes are incorrect as the commercially used GLP-1R antibodies have been demonstrated to be non-specific [82]. Furthermore, cardiac myocyte-specific GLP-1R knockout mice demonstrated equivalent cardioprotection versus their myosin heavy chain α-Cre littermates following treatment with liraglutide (30 mg/kg i.p. twice) for 1 week prior to undergoing a left anterior descending (LAD) coronary artery ligation to induce MI. An improvement in cardiac hypertrophy and a decrease in cardiac infarct scar formation were noticed in both genotypes treated with liraglutide [83]. These findings indicate that the cardiac (atrial) GLP-1R is not crucial for the cardioprotective effects of liraglutide [82]. With regards to other cell types within the cardiovascular system where the GLP-1R may be expressed, specific localization of GLP-1R expression in different primate tissues using a ^{125}I GLP-1 monoclonal antibody demonstrated GLP-1R expression in smooth muscle walls of the arteries and arterioles in the heart, as well as localization in the sinoatrial node [84]. GLP-1R protein expression was initially identified in both mouse mesenteric artery smooth muscle cells (SMCs) and heart coronary SMCs [1]. Moreover, evidence for vascular smooth muscle cell (VSMC) GLP-1R expression was also demonstrated by Richards et al., who showed co-expression of *Glp1r*-promoter driven yellow fluorescent protein and smooth muscle actin in cells within ventricular blood vessels [85, 86]. Moreover, GLP-1R expression was detected in mouse thoracic artery SMCs via immunohistochemistry, utilizing a NOVUS Biologicals antibody that remains to be validated for its specificity towards the GLP-1R [87]. In contrast, studies in human heart samples acquired from the University of Pennsylvania Heart Biobank failed to detect full-length GLP-1R mRNA transcripts in coronary artery SMCs.

With regard to endothelial cell (EC) GLP-1R expression, both human coronary artery ECs (HCAECs) and human umbilical vein ECs (HUVECs) demonstrate positive GLP-1R protein expression via western blotting [88, 89], though validity of the GLP-1R antibodies used in these studies remains to be determined, or has been shown to be non-specific, respectively. Conversely, coronary artery ECs in human heart samples from the University of Pennsylvania Heart Biobank once again did not demonstrate full-length *GLP1R* mRNA transcript expression [90]. Taken together, while species-specific findings may explain some of the discrepancies regarding VSMC/EC GLP-1R expression, it remains to be conclusively determined whether the GLP-1R is expressed within these cell-types in the vascular endothelium of animals or humans. In addition, whether specific vascular beds express the GLP-1R (e.g. coronary vasculature versus the renal vasculature), and whether this may change in the presence of underlying disease (e.g. coronary artery disease, renal failure, etc.) are important questions that future studies will need to address. Therefore, based on the broad distribution of where the cardiac GLP-1R is localized, the mechanisms and cell types by which GLP-1R agonists improve CVD in people with T2D remains incompletely understood and is an active area of intense investigation [91].

Cardiac Energy Metabolism

The heart is a highly metabolic flexible organ that has the ability to adapt to a variety of physiological states to maintain its required metabolic requirements to sustain contractile function [92]. In the healthy heart, energy metabolism is primarily met by oxidative metabolism, which accounts for > 90% of cardiac ATP production, with the remaining ATP production derived primarily from anaerobic glycolysis [1, 93]. The vast amounts of oxidative metabolism are accounted for by the mitochondrial oxidation of long-chain fatty acids (FAs) glucose, lactate, however, amino acids and ketone bodies can also contribute to cardiac oxidative ATP production

depending on the physiological state (e.g. starvation) [93, 94]. The adult heart obtains typically 50 – 70% of its ATP from fatty acid oxidation. Moreover, fatty acid use by the heart is influenced at many levels and is dependent on several elements such as the source, concentration, and type of fatty acids delivered to the heart, as well as the presence of competing energy substrates (e.g. (glucose, lactate, ketones, and amino acids) [93]. When the FFA concentration in the blood is high, the heart increases its FA uptake and allows FAs to become the major source of energy. When the FAs cross the cell by protein-mediated mechanisms, FAs are esterified to CoA by the acyl CoA synthetase. In order to traverse the mitochondrial membrane, the acyl CoA requires the carnitine shuttle, which consists of carnitine palmitoyltransferase 1 (CPT-1), which converts the acyl CoA into an acylcarnitine. Carnitine-acylcarnitine translocase (CACT) then exchanges free carnitine for the acylcarnitine, which can now enter the mitochondria and is reconverted to acyl-CoA via a CPT-2 isoform and subjected to β-oxidation (see figure 1 for overview of fatty acid oxidation). The acyl CoAs that undergo β-oxidation generate acetyl CoA and the reducing equivalents NADH and $FADH_2$. The acetyl CoA is oxidized within the Krebs Cycle to generate additional NADH and $FADH_2$. The NADH and $FADH_2$ that are generated by both fatty acid β-oxidation and the Krebs cycle subsequently donate their electrons to the complexes of the ETC, which drives ATP production via oxidative phosphorylation [14, 93, 95].

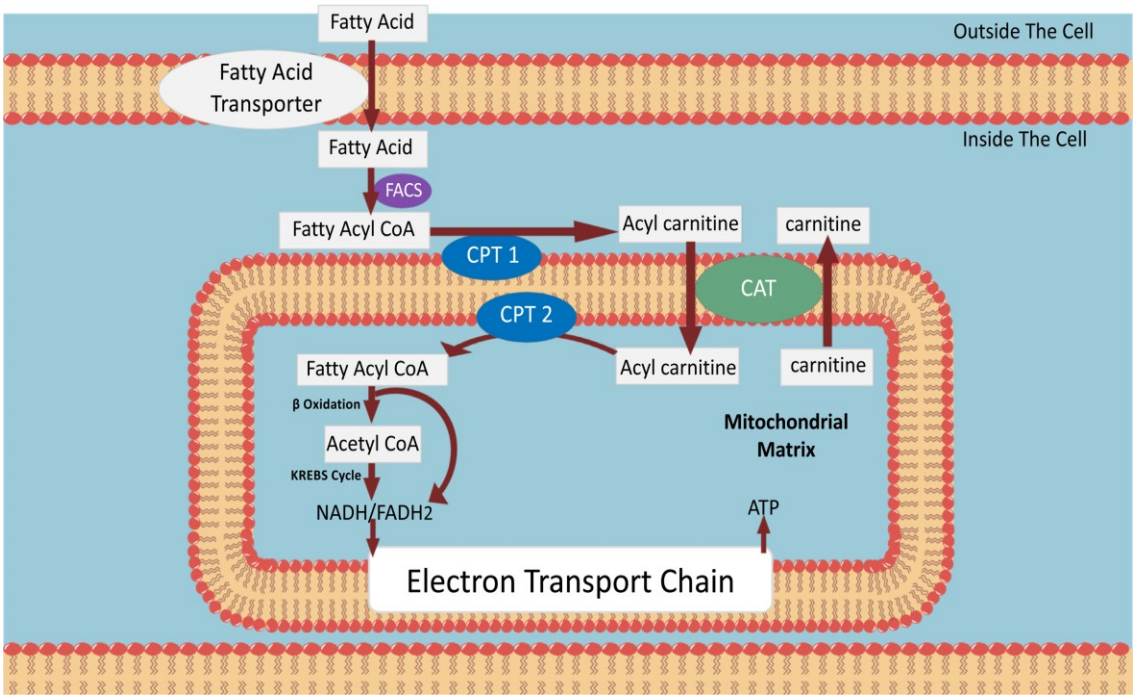

Figure 1-2 Fatty acid oxidation overview

FAs are esterified to CoA by the acyl CoA synthetase. In order to traverse the mitochondrial membrane, the acyl CoA requires the carnitine shuttle, where CPT1 converts the long-chain acyl-CoA to long-chain acylcarnitine. The acylcarnitine is transported by CAT across the inner mitochondrial membrane, where CPT2 converts it back to long-chain acyl-CoA. The acyl CoAs that undergo β-oxidation generate acetyl CoA and the reducing equivalents NADH and $FADH_2$. This acetyl-CoA then enters the Krebs cycle. The NADH and $FADH_2$ produced by both β-oxidation and the Krebs cycle donate their electrons to the electron transport chain to produce ATP. FACS, fatty acyl-CoA synthase; CPT1, Carnitine palmitoyl transferase 1; CPT2, Carnitine palmitoyl transferase 2; CAT, carnitine translocase.

Fuel preference in the heart is also determined by oxygen demand/availability. When the oxygen availability is low, oxidation of carbohydrate is a more efficient substrate in terms of ATP produced per mole of oxygen consumed [96]. However, studies have demonstrated that during ischemia of the heart, fatty acids still account for the majority of residual oxidative ATP production despite being the less oxygen efficient fuel [97, 98]. Glucose accounts for ~20-30% of the energy production in the healthy heart, and the majority of glucose that enters the cardiac myocyte is derived from the circulation. Glucose enters the cardiac myocyte via facilitated diffusion through glucose transporters 1 and 4 (GLUT1 and GLUT4), which are the most abundant isoforms in the heart. GLUT1 is insulin-independent and regulates basal glucose uptake, whereas GLUT4 translocates to the plasma membrane in an insulin-dependent manner and is responsible for the majority of glucose transport into insulin sensitive cells/tissues [93, 99]. The glucose that enters the cardiac myocyte is rapidly phosphorylated by hexokinase to glucose-6-phposphate, which can then be directed to either glycogen synthesis or the rest of the metabolic pathway for glycolysis. Glycolysis converts glucose to 2 pyruvate molecules for production of a net gain of 2 ATP and 1 NADH molecule [100]. In order to completely oxidize the glucose for large amounts of ATP production, the pyruvate produced from glycolysis will enter the mitochondria, where it is converted into acetyl-CoA, NADH and CO_2 by the PDC. From here, the acetyl-CoA enters the Krebs Cycle, where the reducing equivalents NADH and $FADH_2$ are formed, which then serve as electron donors to the ETC, in order to drive ATP synthase to produce ATP via oxidative phosphorylation (See figure 1-2 for overview)

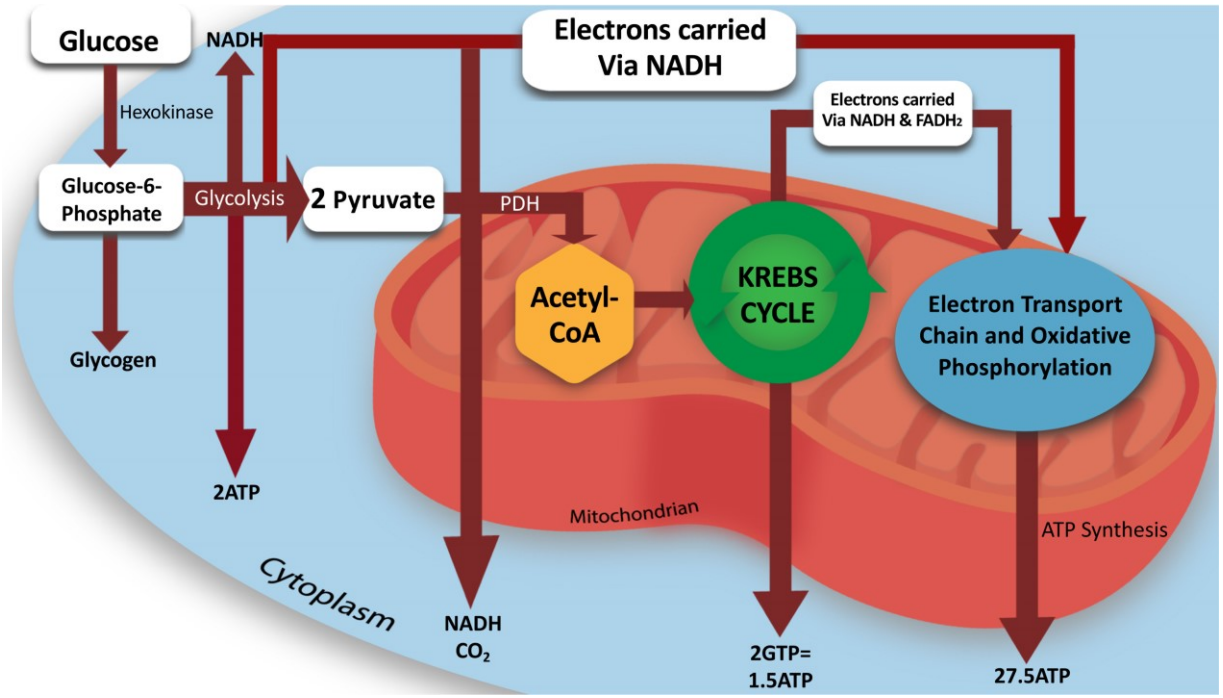

Figure 1-3 Glucose Metabolism overview

This figure depicts overall glucose metabolism. Glucose enters the cell and is immediately phosphorylated by hexokinase to glucose-6-phposphate, which can then be directed to glycolysis. Glycolysis converts glucose to 2 pyruvate molecules for production of a net gain of 2 ATP and 1 NADH molecule. In order to completely oxidize the glucose, the pyruvate produced from glycolysis will enter the mitochondria, where it is converted into acetyl-CoA, NADH and CO_2 by the pyruvate dehydrogenase (PDH) complex. From here, the produced acetyl-CoA enters the Krebs Cycle, where the reducing equivalents NADH and $FADH_2$ are formed, which then serve as electron donors to the ETC, in order to drive ATP synthase to produce ATP via oxidative phosphorylation.

Several factors regulate ATP production in the heart, including alterations in hormonal changes, energy substrate supply and oxygen supply to the heart, as well as energy demand [101]. During starvation or prolonged fasting the heart relies primarily on fatty acids and ketone bodies as an energy source to support contractile function. However, in response to feeding and ingestion of carbohydrates, the heart will switch its metabolism to rely more on carbohydrates as a primary fuel source [93, 102].

Insulin and Glucagon Signalling

Glucoregulatory hormones such as insulin and glucagon also play an important role that can affect fuel metabolism in the heart. Since the heart is an insulin-dependent and energy-consuming organ, insulin and glucagon control most of the signaling that integrates the regulation of cardiac metabolism, growth and survival. Insulin promotes glucose metabolism, including increases in glucose uptake, glycolysis, and glucose oxidation, whereas it reduces both fatty acid uptake and fatty acid oxidation [103, 104]. In addition, insulin indirectly lowers circulating free fatty acids by inhibiting lipolysis in adipocytes, which will decrease myocardial fatty acid oxidation rates [105].

Insulin Receptors

The insulin receptor is a member of the ligand-activated receptor and tyrosine kinase family of transmembrane signaling proteins that are essential regulators of cell differentiation, growth, and metabolism. Insulin binding to insulin receptors (IR) to activate receptor autophosphorylation of tyrosine residues in the intracellular domain which triggers signalling two major phosphorylation cascades MAPK (mitogen-activated protein kinase) for cell growth and survival and PI3K (phosphoinositide 3-kinase) that promote glucose uptake. Signalling via the PI3K pathway involves the interaction of phosphorylated IRS-1 with the p85 regulatory subunit of PI^3K, which activates PI3K and promotes the translocation of the Akt to the cell membrane once it's phosphorylated by phosphoinositide-dependent kinase (PDK) (see figure 1-3) [106, 107]. Akt

activation is necessary for the translocation of GLUT-4 glucose transporters to the cell membrane. However, there is less information about the connection between Akt activation and GLUT4 translocation, some studies described the relationship between Akt and GLUT4 by the Akt substrate of 160 KDa (AS160) protein that might mediate this connection [108]. AS160 becomes phosphorylated in response to insulin stimulation to promotes the GLUT4 translocation. Moreover, phosphorylated Akt can turn off Guanosine triphosphate activating proteins (GAPs) that result in terminating insulin signalling. However, a cell contains active GAPs that are able to prevent GLUT4 translocation, by preventing the accumulation Rab Guanosine triphosphate (RabGTP) in GLUT4 endosomes. The inactivation of GAPs such as AS160 via insulin-stimulated PI3K/Akt cascade results in accumulation of RabGTPs and facilities the GLUT4 translocation [109]. In parallel to the stimulation of glucose uptake, insulin also inhibits FA uptake in cardiomyocyte. Insulin signalling pathways and actions are modified in various pathological states such as myocardial ischaemia and T2D [100]. Moreover, insulin also improves cardiac contractility in male Wistar rats by activating the myocardial PI3K/Akt signaling pathway [110]. These finding have been recapitulated clinically in patients undergoing coronary artery bypass graft (CABG), as insulin was administered at (5 mU/kg/min) during surgery and produced an improvement in cardiac function, especially when blood glucose was maintained between 4.0 - 6.0 mM [111].

Glucagon is the counter-regulatory hormone to insulin and thus also important to glucose homeostasis. This includes promoting glucose mobilization in the liver via glycogenolysis and gluconeogenesis, which maintains normoglycemia during fasting/starvation. Besides its actions on glucose metabolism, glucagon has a number of other actions. In the brain glucagon regulates appetite, the central control of energy expenditure and body weight [112]. It also regulates hepatic lipid metabolism by stimulating extracellular cAMP, via AMPK-mediated PPARα activation [113]. Glucagon may also influence cardiac energy metabolism, as glucagon treatment (1 ng/mL) significantly increases fatty acid oxidation rates in isolated working rabbit hearts [114].

Metabolomics studies in hearts from mice systemically treated with glucagon following permanent LAD coronary artery occlusion, revealed marked increases in a broad range of long-chain acylcarnitine species, consistent with elevations in cardiac fatty acid oxidation.

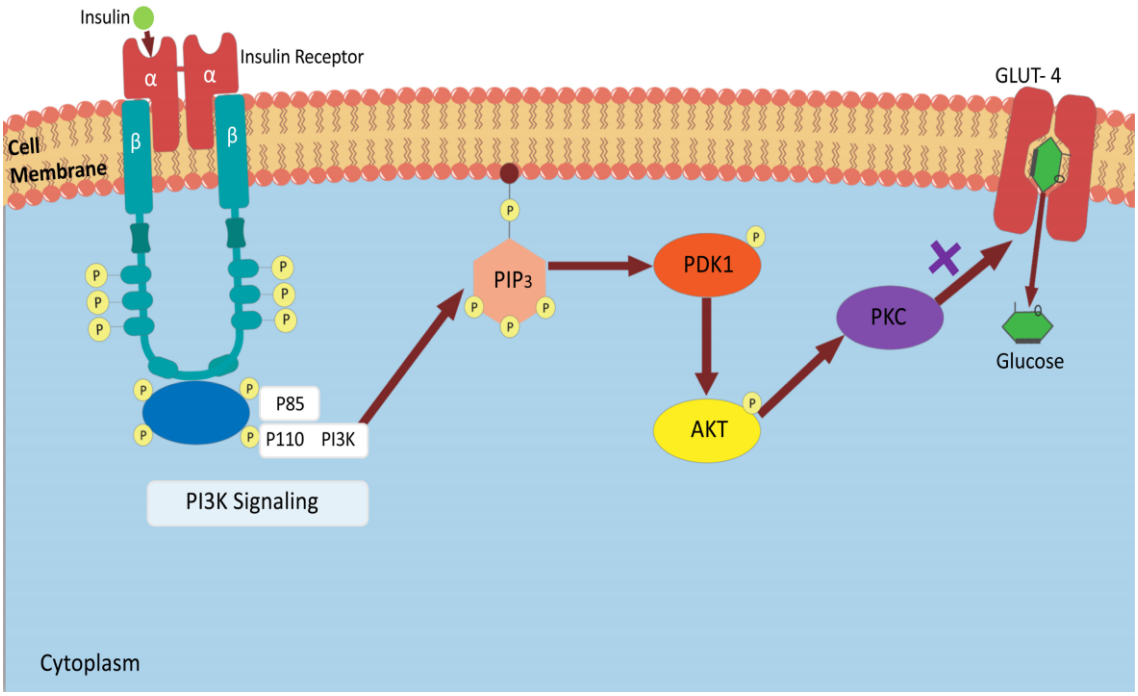

Figure 1-4 Insulin signaling overview

Insulin binding to insulin receptors (IR) activates receptor autophosphorylation of tyrosine residues to trigger phosphorylation and subsequent activation of PI3K (phosphoinositide 3-kinase) that promotes glucose uptake. Signaling via the PI3K pathway involves the interaction of phosphorylated IRS-1 with the p85 regulatory subunit of PI3K, which activates PI3K and promotes the translocation of the serine/threonine protein kinase B (Akt) to the cell membrane once it's phosphorylated by phosphoinositide-dependent kinase (PDK). Akt activation is followed by translocation of GLUT-4 glucose transporters to the cell membrane.

Cardiac Energy Metabolism Alterations in Obesity/T2D

In the setting of obesity and/or T2D, circulating FFAs and very low-density lipoprotein triacylglycerol (VLDL-TAG) levels are markedly elevated, which increases fatty acid delivery to the myocardium, similar to what is observed during prolonged starvation. At the same time, due to underlying insulin resistance, insulin's ability to promote myocardial glucose metabolism is impaired [115]. Hence, fatty acids also account for the majority of the heart's oxidative energy production in the setting of obesity/T2D, which comes at the expense of glucose/carbohydrate oxidation as a result of Randle's glucose-fatty acid cycle (Figure 1- 4), which describes the inverse relationship between fatty acid and glucose oxidation as a fuel source for the heart [93, 116, 117]. Indeed, mice supplemented with a chronic high fat diet (60% kcal from lard) for 12-weeks to induce experimental obesity, increases the heart's overall reliance on fatty acid oxidation for energy production [118]. Likewise, isolated working hearts from 12 to 13-week-old mice with T2D due to deficiency of the leptin receptor (db/db), demonstrate a 6–7-fold increase in fatty acid oxidation rates in comparison to their wild-type nondiabetic littermates [119]. These findings have been recapitulated by many in the field using similar animal models [120-122], and such observations have translated into the clinical setting. For example, reported findings from a cohort of obese women from Linda Petersen and colleagues using positron emission tomography (PET) imaging, revealed marked increases in cardiac fatty acid oxidation rates [123]. In addition, the increase in cardiac fatty acid oxidation rates strongly correlated with the degree of glucose intolerance and insulin resistance in these obese female subjects. Similarly, PET imaging studies from Rijzewijk and colleagues have demonstrated in T2D patients with diastolic dysfunction, significant increases in cardiac fatty acid oxidation rates and a decrease in cardiac glucose uptake [124].

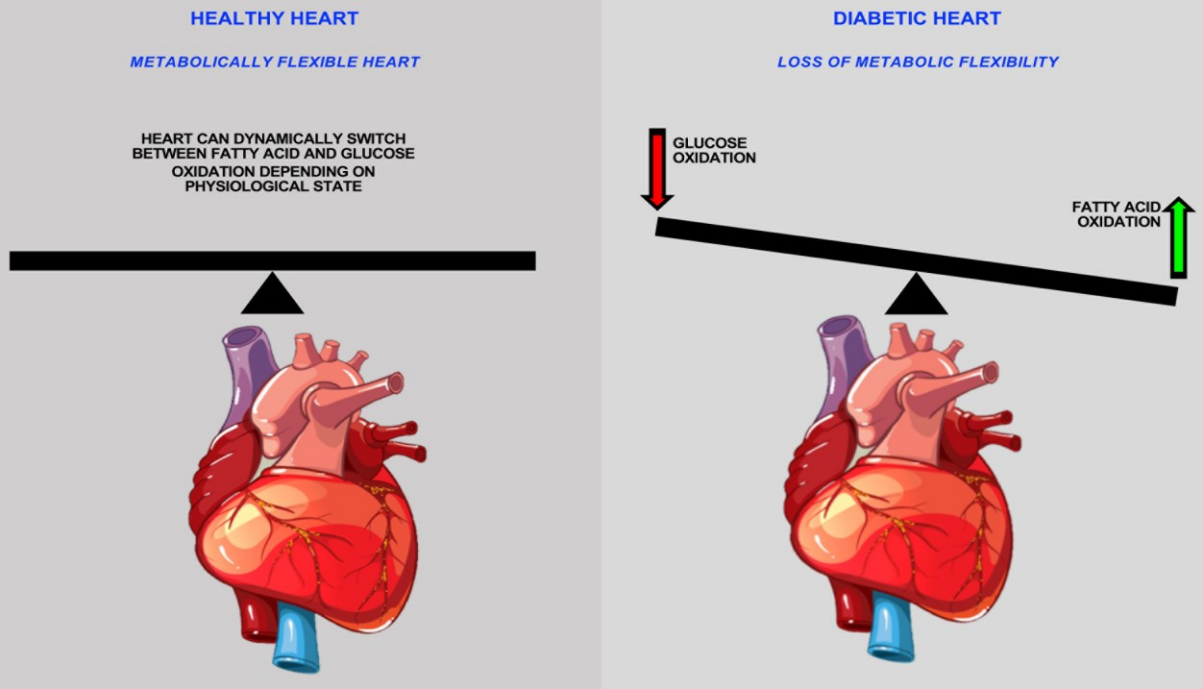

Figure 1-5 Cardiac energy metabolism in T2D

The healthy heart is metabolically flexible and thus able to switch between fatty acids as its primary oxidative fuel source in the fasted state, with carbohydrates becoming its primary oxidative fuel source in the fed state. However, this metabolic flexibility is lost in T2D, as fatty acid oxidation rates are markedly elevated, which comes at the expense of glucose oxidation [1].

Regulation of Pyruvate Dehydrogenase (PDH)

The PDC consists of 3 different enzymes (pyruvate dehydrogenase (E1), dihydrolipoamide acetyltransferase (E2), and dihydrolipoamide dehydrogenase (E3) [125], though we will refer to it throughout this Thesis as PDH. Mammalian PDH catalyzes the oxidative decarboxylation of pyruvate, which couples the glycolytic pathway to the oxidative pathway to produce acetyl CoA in the mitochondria for the Krebs Cycle. Hence, PDH is critical in coupling glycolysis to complete mitochondrial oxidation of a glucose molecule and therefore acts as the rate-limiting enzyme

controlling glucose oxidation rates. The activity of PDH is primarily regulated at the level of post-translational modification (Figure 1-5), which involves phosphorylation-induced inactivation by 4 isoforms of PDH kinase (PDHK), or dephosphorylation-induced activation by 2 isoforms of PDH phosphatase (PDHP). During fasting/starvation, fatty acid oxidation rates are high and subsequently glucose oxidation rates are low as a result of Randle's glucose-fatty Acid Cycle. At a molecular level, high fatty acid oxidation rates also increase the acetyl CoA/CoA and NADH/NAD^+ ratios in mitochondria, which activate PDHK to phosphorylate and inhibit PDH, further contributing to how increases in fatty acid oxidation reduce glucose oxidation rates in the heart [126]. Importantly, these same mechanisms take place in the hearts of individuals with obesity/T2D, due in part to the chronic elevation in circulating FFAs and TAGs, thereby making it so that the obese/T2D heart is heavily dependent on fatty acids as its primary fuel source to support contractile function.

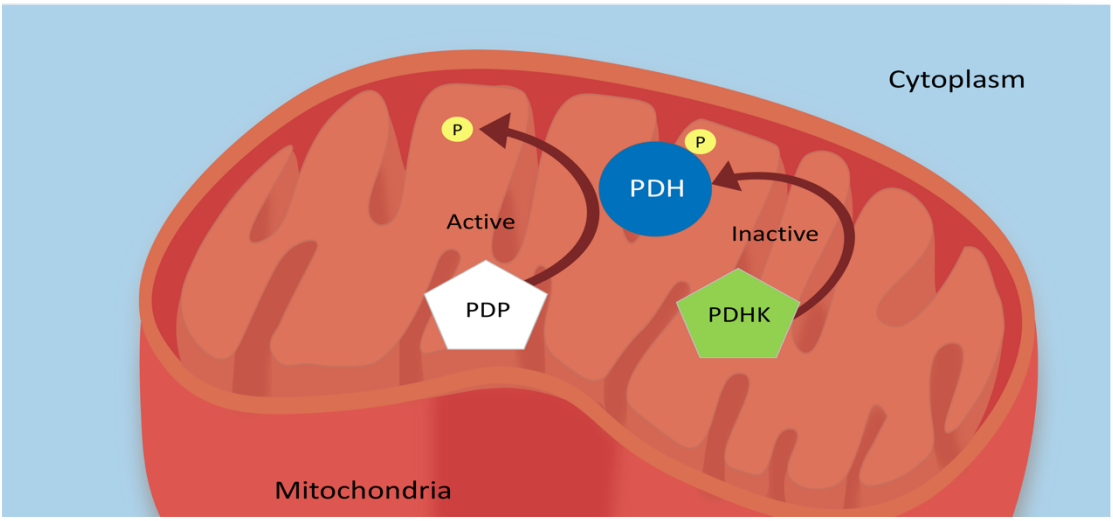

Figure 1-6 Regulation of PDH

Diagram depicts the regulation of the enzymatic activity of PDH, whereby PDP dephosphorylates and activates PDH, while PDHK4 phosphorylates and inhibits PDH activity. PDH, pyruvate dehydrogenase; PDP, pyruvate dehydrogenase phosphates; PDK, pyruvate, dehydrogenase kinas.

Optimizing Cardiac Energy Metabolism to Improve Cardiac Function In Obesity/T2D

Of relevance to this Thesis proposal, a number of preclinical studies and clinical studies have demonstrated that normalizing cardiac energy metabolism can mitigate obesity and/or T2D-related cardiac dysfunction. In brief, trimetazidine is a clinically approved antianginal in over 80 countries used to treat angina, which reduces fatty acid oxidation rates secondary to an inhibition of 3-ketoacyl CoA thiolase [93, 120]. Preclinical studies in middle-aged obese C57BL/6J mice demonstrate that treatment with trimetazidine can prevent the development of obesity-induced cardiomyopathy and diastolic dysfunction [127]. Moreover, therapy with trimetazidine for 3-months in a small number of overweight and/or obese patients with dilated cardiomyopathy, or 6-months in a small number of diabetic patients with dilated cardiomyopathy, both lead to significant improvements in left ventricular (LV) systolic function [128, 129]. Conversely, normalizing the depressed cardiac glucose oxidation rates observed in obesity and/or T2D has also been shown to have salutary actions on cardiac function. Dichloroacetate (DCA) is a pharmacological inhibitor of PDHK, thereby increasing glucose oxidation via preventing phosphorylation-induced inhibition of PDH [47,48], and DCA supplementation in the drinking water (final concentration of 1 mM) abrogates diastolic dysfunction and cardiomyopathy in a rat model of experimental T2D [130].

GLP-1R agonist-induced cardioprotection

Numerous studies have demonstrated cardioprotective effects of GLP-1 and GLP-1R agonists in both animals and humans with cardiovascular disease [65]. For example, male Sprague Dawley rats given a bolus infusion of native GLP-1 (4.8 pmol• kg^{-1}• min^{-1}) intravenously (IV) throughout a 30-min ischemia and 2-hr reperfusion period significantly reduced infarct size [131]. Moreover, adult male Wistar rats exhibited improvements in cardiac function and reductions in infarct size

when treated with the GLP-1R agonist lixisenatide (10 μg/kg/d S.Q) when administered long-term (~10 weeks) following ischemia-reperfusion injury via left anterior descending artery (LAD) occlusion for 45-min and heart reprefused for 120-min [132].These cardiac actions attributed to GLP-1/GLP-1R agonists are also applicable to large animal models, as treatment with exendin-4 (10 μg IV and SQ 5-min before the onset of reperfusion) decreased infarct size and improved LV systolic function in pigs following a 75-min ischemia (via ligation of the left circumflex artery) and 72-hr reperfusion period [65]. In a canine heart failure model involving rapid pacing of the right ventricle for 28-days, native GLP-1 infusion (1.5 pmol• kg^{-1} •min^{-1}) for 48-hr markedly improved LV systolic function (stroke volume, cardiac output, ejection fraction, and LV mechanical efficiency [133, 134]. GLP-1R agonist-induced cardioprotection extends to models of chronic ischemia-induced heart failure, as pretreatment with liraglutide for 1-week prior (75 μg/day twice daily via SQ injection) to permanent occlusion of the left anterior descending (LAD) coronary artery markedly improves survival and adverse LV remodeling in both lean and T2D C57Bl/6J mice [135]. Furthermore, it was demonstrated 9-month-old spontaneously hypertensive, heart failure-prone rats that are also obese, that a 3-month continuous infusion of native GLP-1(1.5 pmol• kg^{-1} • min^{-1}) reduces cardiac myocyte apoptosis, prevents cardiac hypertrophy and improves LV function [50]. GLP-1R agonist treatment with liraglutide (30 μg/day twice daily via SQ injection) for 1-week also produces beneficial actions on LV function and attenuates cardiomyopathy in C57BL/6J mice fed a high fat diet for 20-weeks, as determined by invasive hemodynamic pressure-volume loop recordings [136]. Similarly, a 40-day infusion of exendin-4 (24 nmol • kg^{-1} • day^{-1}) via subcutaneous osmotic pump implants reduced cardiac myocyte size, mitochondrial oxidative stress, and cardiac steatosis in chronically high fat fed and obese mice [137].

Until recently, the majority of human studies investigating the cardiovascular properties of GLP-1 and GLP-1R agonists had been carried out in small samples sizes with various limitations.

However, many have reported cardioprotective actions, as a 72-hr infusion of GLP-1 (1.5 pmol • kg^{-1} • day^{-1}) initiated ~3.5-hr after angioplasty in patients suffering from an acute MI enhanced LV ejection fraction and infarct zone-related regional wall motion [138]. Moreover, native GLP-1 infusion (1.2 pmol • kg^{-1}• day^{-1}) prevents myocardial stunning in coronary artery disease patients undergoing either dobutamine stress echocardiography or coronary balloon occlusion during percutaneous coronary intervention [54,55]. In patients undergoing percutaneous coronary intervention for ST-segment-elevation MI, treatment with exenatide (10 μg SQ and IV bolus 5-min prior to reperfusion, followed by 10 μg SQ for 2 days) reduced infarct size and improved LV systolic function as determined by cardiac magnetic resonance and echocardiography, respectively [77]. Conversely, a 6-h exenatide infusion (average circulating concentration of 0.177 nM) initiated 15-min prior to onset of reperfusion in patients with an ST-segment-elevation MI did not improve LV systolic function but did reduce infarct size and improve the myocardial salvage index [28,29]. Although the aforementioned clinical studies indicate that GLP-1 and GLP-1R agonists may confer cardioprotective actions in human subjects, it was the completion of the cardiovascular outcomes trials for liraglutide and semaglutide, which provided meaningful evidence that GLP-1R agonists may reduce cardiovascular risk in patients with T2D [139, 140]. Results from the LEADER trial involved 9340 patients with a median follow-up of 3.8 years, and demonstrated that liraglutide treatment (median daily dose of 1.78 mg SQ) reduces major adverse cardiovascular events (MACE) in T2D patients with high cardiovascular risk (4.7% versus 6.0% death rate from cardiovascular causes) [140]. Furthermore, this was associated with a trend (P=0.14) to reduce hospitalization rates for heart failure. Similarly, results from the SUSTAIN 6trial demonstrated in 3297 patients with T2D that once-weekly treatment with semaglutide (0.5 or 1.0 mg SQ) for 2 years reduced rates in their primary outcome, which involved a composite of cardiovascular death, nonfatal MI, or nonfatal stroke [35]. Whether this represents a drug-class effect or is specific to liraglutide and semaglutide remains to be determined. However, results

from the ELIXA (Evaluation of Lixisenatide in Acute Coronary Syndrome) cardiovascular outcomes trial demonstrated that once-daily lixisenatide treatment (maximum dose of 20 μg SQ) did not impact MACE rates in 6068 patients with T2D that had a previous MI or were hospitalized for unstable angina within the previous 180-days prior to randomization.

Rationale

Of direct relevance to this Thesis proposal, it still remains unknown at a mechanistic level, how GLP-1R agonists improve cardiovascular outcomes in people with T2D. Previous studies have suggested that GLP-1R agonists produce direct actions in the myocardium and may reduce cardiac myocyte apoptosis [73]. However, the GLP-1R is not expressed in ventricular myocytes in multiple species [90], and it is therefore crucial to understand potential indirect mechanisms linking GLP-1R signaling to ventricular cardioprotection. Moreover, evidence clearly demonstrating that GLP-1R agonists improve cardiac function independent of directly acting on the myocardium, mice with a specific of cardiac/atrial-specific deletion of the GLP-1R undergoing permanent ligation of the LAD coronary artery to induce MI, exhibit equivalent cardioprotection following treatment for 1-week with liraglutide (30 μg/kg i.p. BID) as their control littermates [83]. This included similar adverse LV remodeling and overall survival post-MI. As previously discussed, GLP-1/GLP-1R agonists also modify insulin and glucagon secretion. Since insulin and glucagon may influence cardiac energy metabolism, which is significant altered and may contribute to cardiac dysfunction in people with obesity/T2D, it is plausible that optimization of cardiac energy metabolism may represent another potential indirect mechanism by which GLP-1/GLP-1R agonists improve cardiovascular outcomes in people with T2D.

In support of this notion, Sprague-Dawley rats demonstrated increases in relative myocardial carbohydrate versus fat oxidation as determined via ^{1}H magnetic resonance spectroscopy (MRS) measurements following treatment with the GLP-1R agonist albiglutide [141]. These metabolic modifications were associated with cardioprotection, as seen by reduced infarct size and improved cardiac function assessed via ultrasound echocardiography. However, it is important to note that these actions were initially attributed to albiglutide acting directly on the heart. Although more recent evidence indicates that the GLP-1R is not expressed in ventricular cardiac myocytes, the principle cell-type within the heart responsible for the heart's enormous metabolic demand, some studies have demonstrated that direct treatment of the isolated rodent heart increases glucose uptake. Studies from Shannon and colleagues suggest that GLP-1 has insulinomimetic actions in the heart, and direct treatment of the Langendorff perfused rat heart with native GLP-1 (0.5 nM) increased glucose uptake to a similar extent as insulin (100 μU/mL). Using healthy canines or canines with heart failure, Shannon and colleagues have also consistently demonstrated that native GLP-1 infusion increases glucose uptake in the heart, which involves a p38-mitogen-activated protein kinase pathway that increases nitric oxide production and promotes GLUT1 but not GLUT4 translocation [138, 142, 143]. Although some studies have investigated the role of GLP-1 in regulating cardiac glucose uptake, very few studies to date have assessed the role of GLP-1 and GLP-1R agonists in modifying glucose and fatty acid oxidation.

Conversely, positron-emission tomography (PET) imaging studies in castrated male Ossabaw swine made obese by eating ~8000 kcal/day for 16-weeks demonstrates that GLP-1 infusion (1.5 $pmol•kg^{-1}•min^{-1}$) for 5h only increases cardiac glucose uptake in lean but not obese swine [144]. These authors also demonstrated in a clinical study that GLP-1 infusion (1.5 $pmol•kg^{-1}•min^{-1}$) for 13-h fails to increase glucose uptake in patients with T2D versus healthy volunteers, though a placebo vehicle control treated group with T2D was not included in these studies. Similarly,12-weeks of treatment with albiglutide (30 mg SQ once weekly) in human subjects with New York

Heart Association (NYHA) Class II or III heart failure did not improve cardiac glucose uptake as determined via PET imaging [145]. It should be noted though that we are limited in our ability to confirm a role for increased glucose oxidation in GLP-1/GLP-1R agonist-induced cardioprotection, as we do not have sophisticated and validated tools to quantify glucose oxidation rates in human hearts noninvasively.

Taken together, a major goal of my Thesis proposal is to confirm whether GLP-1R agonists modify cardiac energy metabolism, and whether these effects are mediated via direct actions on the heart, or through indirect actions on other peripheral tissue systems.

Hypothesis

- Because ventricular cardiac myocytes do not express the GLP-1R, our goal was to elucidate the indirect mechanisms by which GLP-1R agonists improve cardiovascular outcomes in people with T2D, with a specific focus on changes in cardiac energy metabolism (Figure 1- 6).
- We hypothesised that GLP-1R agonists will improve cardiac function in people with T2D by improving cardiac glucose oxidation rates, which would be due to increases in insulin secretion.

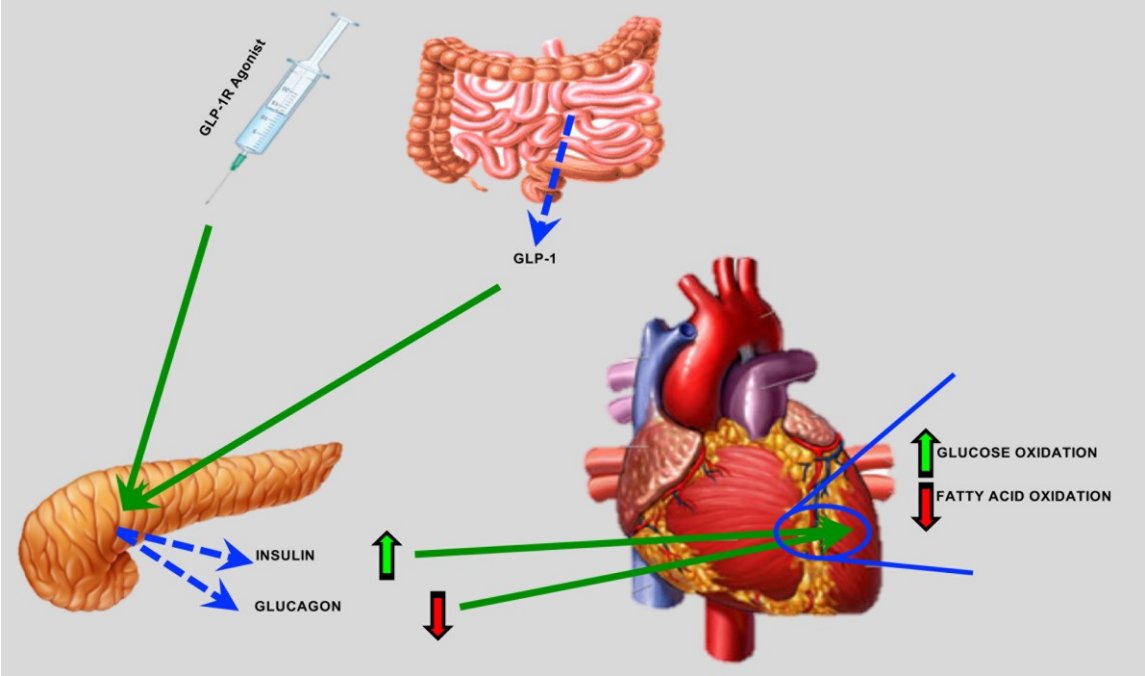

Figure 1-7 GLP-1 and GLP-1R agonist effects on cardiac energy metabolism

Studies to date demonstrate that GLP-1 and GLP-1R agonists increase cardiac glucose oxidation and decrease cardiac fatty acid oxidation, which may be explained by their indirect actions to enhance insulin and impair glucagon secretion, respectively [1].

Objectives

- To determine whether direct liraglutide treatment of the isolated working heart modifies cardiac energy metabolism (glycolysis, glucose oxidation, fatty acid oxidation).
- To determine whether isolated working hearts from mice systemically treated with liraglutide would exhibit alterations in cardiac energy metabolism (glycolysis, glucose oxidation, fatty acid oxidation), to determine if liraglutide is still able to modify cardiac energy metabolism in mouse model of experimental T2D.
- To determine if liraglutide can mitigate cardiomyopathy in mice with experimental T2D, and whether this is associated with improved PDH activity/glucose oxidation rates.

Chapter Two

- **Methodology**

Animal studies

All animals received care in accordance with the regulations of the Canadian Council on Animal Care and with the Alberta Health Sciences Animal Welfare Committee. 8-week old C57BL/6J mice from the Jackson Laboratory were housed under a 12-hour light/dark cycle at the U of A Health Sciences Laboratory Animal Services facility with free access to food and water. The mice were placed on a standard chow/low-fat diet (10% kcal from lard, Research Diets; D12450J) or high-fat diet (60% kcal from lard, Research Diets; D12492) for 4-weeks. Upon study completion, all mice were euthanized. For extraction or isolated heart perfusions (see below)

In Vivo Experiments:

Experimental Model of T2D

12-week-old male C57BL/6J mice were placed on an HFD for 4 weeks, following which they received a single intraperitoneal injection of streptozotocin (STZ, Sigma S013-1G, 75 mg/kg). STZ was prepared in a sodium citrate buffer solution with molarity of 0.1M and pH of (5.0-5.5) immediately before injection as STZ comes out of solution rapidly (within 10-min). Blood glucose levels were measured weekly to confirm whether the mice were progressing towards T2D.

Exclusion Criteria

Mice with low body weight (30 g or less) were excluded from our study since STZ causes destruction of the pancreatic β cells that leads to a lack of insulin production, which can reduce body weight gain due to excess lipolysis [103]. Moreover, a major characteristic of T1D animal models is low body weight. However, in our T2D model, the aim was to cause mild β cell destruction without affecting the body weight to mimic a T2D model assessed with obesity.

Treatment with Liraglutide

We had two experimental protocols; either acute treatment or chronic treatment. Both groups were treated with either liraglutide or vehicle control (30 μg/kg BW via SQ injection twice daily, Novo Nordisk). For the chronic treatment mice were injected daily twice a day with either liraglutide (30 μg/kg BW via SQ injection) or vehicle control (saline) between 8-9 am to 4-5 pm weight and blood glucose level measurements were taken weekly). Acute studies involved 3x injections over 24-hr. prior to animal euthanasia, whereas chronic studies involved twice daily treatment for 2-weeks.

Glucose Tolerance Test

Mice were transferred to clean cages and fasted overnight for approximately 16 h with free access to drinking water. Subsequently, fasting blood glucose levels were measured immediately prior to injecting the mice with glucose (1 mg/kg via IP injection). Whole tail-blood was collected at 15, 30, 60, 90 and 120 min for the measurement of blood glucose levels using the Contour Next (Bayer) glucose monitoring system.

Ultrasound Echocardiography

Mice were anaesthetized with ~2-3 % of isoflurane (Isoflurane USP), following which their chests were shaved and ultrasound gel (Sigma gel electrode Aquasonic) was applied with a cotton swab. A MX 550S Probe (transducer) was used. Images were acquired with a VisualSonics Vevo 3100 imaging system. During image acquisition, full monitoring of body temperature, electrocardiography and respiratory rate of mice took place while situated on a heating station to maintain body temperature. Several in vivo parameters were measured to assess cardiac function in mice, such as left ventricular functions such as (ejection fraction (EF%), fractional shorting (FS%), cardiac output (CO) LV Mass, stroke volume (SV), and heart rate (HR).

Equations

Parameters of cardiac functions were determined using the following equations:

Equation 2. 1. Cardiac output (CO)

CO = Heart Rate (bpm) x Stroke Volume (mL)

Equation 2. 2. Stroke Volume (SV)

SV (mL) = End diastolic volume (EDV) (mL) – end systolic volume (ESV) (mL)

***Equation 2. 3. Left ventricular ejection fraction* (LVEF%)**

LVEF% = ((EDV – ESV)/EDV) x 100

Equation 2. 4. Fractional shortening (FS%)

FS% = ((LV end-diastolic diameter (LVEDD) – LV end-systolic diameter (LVESD))/LVEDD)) x 100

Euthanasia and Tissue Extraction

Pentobarbital in sterile saline was administered IP at a dose of 12 mg. Once the euthanasia was performed, collection of tissues was initiated in this procedure, all materials used were sterilized before euthanizing the animals. Scissors and thumb forceps (one for the skin and one for the internal tissues), 70 % alcohol, sterile gloves paper towels, liquid nitrogen ($LN_{2)}$, 2ml Eppendorf clean tubes (some of the tubes were coated with EDTA for blood collection), and aluminum foils (for storing tissues). Whole blood was collected after the removal of the hearts to assess insulin level, all tissues were immediately frozen and kept at -80^{o}C collection for further analysis.

Ex Vivo Experiments

Measurement of Glycolysis, Glucose Oxidation and Palmitate Oxidation

Hearts were extracted from euthanized mice and perfused in the working mode aerobically for 40 min. Oxygenated Krebs-Henseleit solution consisting of 5.5 mM glucose and 0.8 mM palmitate bound to 3% bovine serum albumin (BSA) with the appropriate radiolabeled tracers for measuring glycolysis rates ([5-^{3}H] glucose), glucose oxidation rates ([U-^{14}C] glucose), and fatty acid oxidation rates ([9,10-^{3}H] palmitate). This method involves adding 500 μl of water into 5ml scintillation vials. A 200ul perfusate sample was then added to the microcentrifuge tube, and the scintillation vial capped. Scintillation vials were then stored initially at 50°C for 24-hr. and then at 40 °C for 24-hr. following storage the microcentrifuge tube was removed, scintillation fluid (Ecolite, ICN) was added and the vials were counted for radioactivity in a liquid scintillation counter. Glucose oxidation rates were determined by quantitative measurement of $^{14}CO_2$ production including $^{14}CO_2$ released as a gas in the oxygenation chambers and $^{14}CO_2$ dissolved as $H^{14}CO^{3-}$ in the perfusate the (gaseous $^{12}CO_2$ which exits the perfusion system via exhaust was trapped in hyamine hydroxide solution). The dissolved $^{14}CO_2$ as $H^{14}CO^{3-}$ was released and trapped on filter paper saturated with hyamine hydroxide in the central well of 25 ml stoppered flasks after perfusate samples were acidified by the addition of 1 ml of $9NH_2SO_4$. At the end of perfusion hearts were immediately snap frozen in LN_2 with Wollenberger tongs and stored at −80°C for further biochemical analyses.

In Vitro Experiments:

Plasma Insulin Determination

A 96-well microplate and all reagents (ALPCO) were equilibrated to room temperature (18-25°C) prior to use. 5 μl of each standard, low- and high-insulin control, and sample were pipetted into individual wells, following which 75 μl of provided working conjugate buffer was added. The

microplate was then incubated for 2-hr at room temperature and placed on a microplate shaker at 700-900 round per minute. After the incubation was completed, washing buffer was applied to each well 6x and the liquid discarded by firmly tapping it and inverted to ensure that all the wash buffer was completely removed, while all air bubbles were removed. 100 μl of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added to each well and the microplate was then incubated for 30 min at room temperature at 700-900 RPM. From here, 100 μl of stop solution was added to each well and the microplate gently shaken to mix the contents, while any remaining air bubbles were removed. The microplate was then read and plasma insulin levels ng/ml were measured using a spectrophotometer at a wavelength of 450 nm.

Powdering of Frozen Hearts for Extraction of RNA/Protein

Frozen hearts were powdered using liquid nitrogen pre-cooled ceramic mortar and pestle, following which a funnel and spatula were used to collect the powdered tissues into liquid nitrogen cooled 2-ml cryovials. ~20-25 mg of powdered hearts samples were subsequently weighed out into 2-ml Eppendorf tubes for the extraction of RNA or protein (see below).

Tissue Protein Extraction for Immunoblot Analysis

~20-25 mg of frozen powdered myocardial tissue contained within 2-mL Eppendorf tubes was homogenized in lysis buffer containing 50 mM Tris HCl, (pH 8 at 4°C),1mM ethylenediaminetetraacetic acid (EDTA),10% glycerol weight/volume 0.02% Brij-35 weight/volume 1mM dithiothreitol (DTT), protease and phosphate inhibitors (Sigma). All samples were kept on ice during homogenization and after homogenization for ~20 minutes prior to centrifugation at 10,000 x g for 10 minutes. The supernatant was collected and stored in 1.5 ml Eppendorf tubes at -80°C for further analysis. Protein concentrations were determined in the extracted lysates by utilizing a Bradford protein assay kit (Bio-Rad). 300 μl of protein lysate

contains 2.5 - 3 μg/μl of protein. Next, samples were denatured in sample denaturation buffer containing Tris 1.5M (pH 6.8), 6.25 mL β-mercaptoethanol or 0.25 M DTT, SDS sodium dodecyl sulfate; 10%, glycerol 100 % (50 % final) and bromophenol Blue (0.025 % final), followed by heating for 5 minutes at (94^{o}C).

Sodium Dodecyl Sulfate (SDS) Gel Electrophoresis

Protein samples were subjected to SDS gel electrophoresis on 10% SDS acrylamide gels. 2.5-3μg/μl of the samples were loaded, and the gel run at 80 at lower voltage and 100 as maximum voltage 80V through the stacking gel, following which the voltage was upped to 100V to run the samples through the separating gel until the loading dye clearly ran through the entire separating gel . After all the protein were once all protein samples underwent sufficient separation through the separating gel, samples were transferred onto 0.45 μm nitrocellulose membranes in a transfer tank (Bio-Rad) with transfer buffer for 2-hr. Following successful protein transfer, membranes were blocked in 10% fat-free milk for 1-hr. The membranes were then probed overnight with a variety of antibodies (see Table 2 - 1 for antibody dilution, antibody manufacturer, and catalog #) prepared in 5% BSA overnight at 4^{0}C. The following morning the nitrocellulose membrane was washed 3x 10 min with 0.05% tris-buffered saline with tween™ 20 detergent (TBS-TWEEN) and then probed with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse or anti-rabbit depending on the species the primary antibody was raised against) at a 1:2000 dilution in 1% fat-free milk for 2-hr. From here, membranes were washed again 3x 10 min with 0.05% TBS-tween and subsequently had the secondary-primary antibody reaction visualised using chemiluminescent reagent (Thermos Scientific) which the kit enables you to visualize once the chemiluminescent reagent added to the membrane and photographed with a Gel-Doc imager (Image quant LAS 900 mini bioscience). Detected protein bands were quantified and analyzed using the Gel-Doc imager's associated software (ImageQuant TL).

Table 2. 2. Antibodies Used During Experiments

Antibody	*Dilution*	*Company*	*Catalog number*
Anti-Akt	1/1000 dilution	Cell Signaling Technology	9272S
Anti-P-Akt	1/1000 dilution	Cell Signaling Technology	4060L
Anti-GS	1/1000 dilution	Cell Signaling Technology	3886S
Anti-P-GS	1/1000 dilution	Cell Signaling Technology	3891S
Anti-GSK3	1/1000 dilution	Cell Signaling Technology	5676S
Anti-P-GSK3	1/1000 dilution	Cell Signaling Technology	9327S
Anti-Hsp90	1/1000 dilution	Biosciences	610418
Anti-PDH	1/1000 dilution	Cell Signaling Technology	3205S
Anti-P-PDH	1/1000 dilution	Cell Signaling Technology	50045

Statistical Analysis

All values are presented as means ± standard error of the mean (SEM). Significant differences were determined by the use of an unpaired, two-tailed Student's *t*-test, or a one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis where appropriate. Differences were considered significant when $P < 0.05$.

Chapter Three

- The GLP-1R Agonist Liraglutide Increases Myocardial Glucose Oxidation Rates via Indirect Mechanisms and Mitigates Diabetic Cardiomyopathy

My role in the described work involved all the experiments except for the isolated working heart perfusions, which were done by Cory Wagg and Dr. Manoj Gandhi, and the ultrasound echocardiography was done with the assistance of Dr. Keshav Gopal.

Manuscript status: This manuscript is currently in preparation to be submitted to Circulation: Heart Failure.

Abstract

Background: type 2 diabetes is associated with an increased risk for cardiovascular disease. Of interest, liraglutide, a therapy for T2D that activates the GLP-1R to augment insulin secretion, reduces cardiovascular-related death in people with T2D. However, it remains enigmatic as to how liraglutide may reduce cardiovascular death in patients with T2D. Importantly, the GLP-1R is not expressed in ventricular cardiac myocytes, so it is likely that indirect actions independent of the myocardium are involved. We hypothesized that optimization of cardiac energy metabolism is a key factor contributing to liraglutide-induced cardioprotection. Methods and Results: C57BL/6J male mice were fed either a low-fat diet (lean) or were subjected to experimental T2D (high-fat diet for 10-weeks plus a single injection of streptozotocin at 4-weeks (75 mg/kg)) and treated with either saline or liraglutide (30 μg/kg via subcutaneous injection) 3x over a 24-hr period. 2-hr following the final injection, all mice were euthanized and had their hearts perfused in the working mode to assess myocardial energy metabolism. In a separate cohort of mice subjected to our experimental model of T2D, animals were randomized to receive either vehicle control or liraglutide treatment for 2-weeks during weeks 9-10 of our model, and cardiac function was assessed via ultrasound echocardiography prior to and upon completion of the study. Systemic treatment of lean mice with liraglutide increased myocardial glucose oxidation rates without affecting glycolysis rates. Conversely, direct treatment of the isolated working heart with liraglutide had no effect on glucose oxidation. These findings were recapitulated in mice with experimental T2D and associated with increased circulating insulin levels. Furthermore, Liraglutide treatment attenuated declining diastolic function in mice with experimental T2D.

Conclusions: Our data demonstrates that liraglutide augments myocardial glucose oxidation via indirect mechanisms, which may mechanistically explain how liraglutide improves cardiovascular outcomes in people with T2D.

Introduction

The prevalence of T2D continues to rapidly increase, as data from the American Diabetes Association indicates that there are currently over 420 million people living with diabetes worldwide. Of these individuals, ~90% have T2D, the majority of whom will likely die from macrovascular cardiovascular disease (e.g. myocardial infarction, heart failure, etc.), as T2D increases one's risk for cardiovascular disease by 32.2%, even if their glycemia is adequately controlled with pharmacotherapy [38].

Because of this increased risk for cardiovascular disease, there is now intense scrutiny to delineate the potential cardiovascular benefits and consequences of current and novel therapies in development for T2D[146]. Of relevance, multiple preclinical studies have demonstrated that the (GLP-1R) agonist drug class for T2D, agents that improve glycemia via potentiating insulin secretion in a glucose-dependent manner [1]. exert robust cardioprotective actions. This includes reductions in infarct size in response to in vivo ischemia/reperfusion injury in rodents and swine [1], as well as attenuating the adverse remodeling associated with chronic ischemia-induced heart failure [1]. These salutary actions appear to translate to human T2D patient populations, as reults from the LEADER trial (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcome) demonstrated that liraglutide treatment significantly reduced rates of death via cardiovascular causes (4.7%) when compared to the placebo-treated T2D subjects receiving standard of care (6.0%). Despite a plethora of studies demonstrating that GLP-1R agonists exert cardioprotective actions in both preclinical and clinical studies, the mechanisms of action responsible remain enigmatic. It has been postulated that increased vasodilation and decreased cardiac myocyte apoptosis may both make independent contributions to GLP-1R agonist-induced cardioprotection following ex vivo or in vivo ischemia/reperfusion [131]. In the setting of obesity, it has also been proposed that activation of 5'AMP activated protein kinase contributes to GLP-1R agonist-induced cardioprotection [147]. Of interest, both obesity and T2D result in profound

alterations to myocardial energy metabolism, primarily characterized by marked increases in fatty acid oxidation and corresponding decreases in glucose oxidation [118]. Conversely, studies in rats have suggested that the GLP-1R agonist albiglutide increases and decreases myocardial glucose oxidation rates and fatty acid oxidation rates, respectively [141].

Accordingly, we proposed that GLP-1R agonist mediated improvements in myocardial energy metabolism may contribute to how this drug class improves cardiomyopathy in the setting of obesity and/or T2D. However, since rodent ventricular cardiac myocytes do not express the GLP-1R [148, 149], it is likely that GLP-1R agonist mediated changes in myocardial energy metabolism are mediated via myocardial-independent actions. In support of this, we demonstrate herein that the GLP-1R agonist liraglutide exerts no actions on myocardial energy metabolism when directly applied to the perfusate of isolated working mouse hearts, whereas systemic treatment of both lean and T2D mice with liraglutide produces robust increases in myocardial glucose oxidation. In addition, we demonstrated that a 2-week treatment regimen with liraglutide in mice with experimental T2D improved diastolic function but not systolic function, which was associated with augmented myocardial pyruvate dehydrogenase (PDH) activity, the rate-limiting enzyme of glucose oxidation [130].

Methods

Animal Care and Experimentation

All animals received care in accordance with the regulations of the Canadian Council on Animal Care and with the Alberta Health Sciences Animal Welfare Committee. 8-week-old weeks male C57BL/6J (Jackson Laboratory mice) were fed a HFD 60% kcal from lard, Research Diets D12492) for 4-weeks, following which they received a single injection with the β cell toxin streptozotocin (STZ, 75 mg/kg) dissolved in sodium citrate (0.1M) and pH of (5.0-5.5) via intraperitoneal injection to induce experimental T2D as previously described (Figure 3 -1A). All

T2D mice remained on the HFD for an additional 6-weeks, whereas a separate group of mice were fed a LFD (10% kcal from lard, Research Diets D12450J) for the 10-week duration (lean group). On the final day of the study, all mice were injected with either with liraglutide (30 μg/kg) or saline 3x over 24-hr (each treatment separated by 12-hr). Animals were subsequently euthanized via an IP injection of sodium pentobarbital (12 mg), following which the hearts were extracted and perfused in the isolated working mode for the assessment of energy metabolism. A separate cohort of lean 8 to 10-week-old C57BL/6J mice were euthanized as described above and had their hearts extracted/perfused in the isolated working mode for the assessment of energy metabolism, with liraglutide (10 nM) added directly to the Krebs Henseleit perfusate. Last, in an independent cohort of T2D mice, during the final 2-weeks of the 10-week study protocol, all mice received twice daily treatment with either saline or liraglutide (30 μg/kg at 8:00 am and 5:00 pm) (Figure 3-1B). On the final day of the study, all mice were euthanized via IP injection of sodium pentobarbital following either a 20 hr fast, or a 16 hr fast-4 hr refeed, prior to extraction of the heart and other peripheral tissues, which were immediately snap frozen in LN_2 using LN_2 cooled Wollenberger tongs.

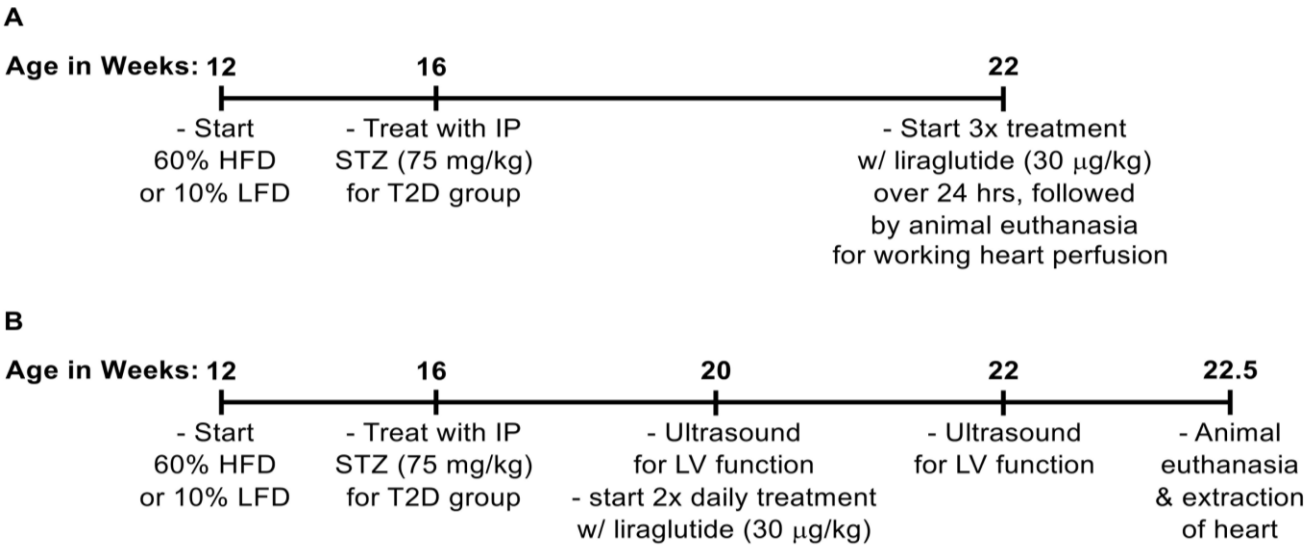

Figure 3-1 Experimental protocol to generate T2D mice

Ultrasound Echocardiography

Mice were anaesthetized with 2-3 % of isoflurane (Isoflurane USP), following which the chest was shaved and ultrasound gel (Aquasonic) applied. All ultrasound images were acquired using a MX 550S probe and VisualSonics Vevo 3100 rodent ultrasound imaging system in T2D mice at 4-weeks post-STZ (prior to receiving 2-week treatment) and 6-weeks post-STZ (upon completion of 2-week treatment) (Figure 3-1B). During ultrasound image acquisition, we performed constant monitoring of body temperature, electrocardiography ECG and respiratory rate in all mice. Several parameters were assessed to provide a complete profile for LV systolic function and diastolic function, including but not limited to; % LVEF, % LVFS, CO, LV mass, SV, HR, LV systolic volume, LV diastolic volume, and the mitral E/A ratio as previously described [150].

Glucose Tolerance Test and Determination of Plasma Insulin Levels

Mice were transferred to clean cages and fasted overnight for 16 hours with free access to drinking water. All mice subsequently received a single dose of glucose (1g/kg) via IP injection, while blood glucose levels from whole tail-blood were monitored using the Contour Next system (Bayer) at 0 min (immediately prior to IP glucose), 15 min, 30 min, 60 min, 90 min and 120 min post-glucose administration. Plasma was collected from whole tail-blood at the 0- and 20-min time points of the IP glucose tolerance test for the determination of circulating insulin levels using a commercially available kit (Alpco Diagnostics).

Isolated Working Heart Perfusions and Assessment of Energy Metabolism

Hearts were extracted from euthanized mice and perfused in the working mode aerobically for 40 min. Oxygenated Krebs-Henseleit solution consisting of 5.5 mM glucose and 0.8 mM palmitate bound to 3% bovine serum albumin (BSA) with the appropriate radiolabeled tracers for measuring glycolysis rates ([5-^{3}H]glucose), glucose oxidation rates ([U-^{14}C]glucose), and fatty acid oxidation rates ([9,10-^{3}H]palmitate) At the end of perfusion hearts were immediately snap frozen in LN_2 with Wollenberger tongs and stored at −80°C for further biochemical analyses.

Western Blotting

Powdered frozen heart samples (20 mg) were homogenized in protein lysis buffer containing 50 mM Tris HCl (pH 8 at 4ºC), 1 mM EDTA, 10% glycerol (w/v), 0.02% Brij-35 (w/v), 1 mM DTT, protease and phosphatase inhibitors (Sigma), following which extracted protein samples were denatured and subjected to western blotting protocols as previously described [151]. Protein kinase B (Akt) and phospho-Akt (9272S and 4060L (Cell Signaling)); glycogen synthase kinase

3β (GSK3β) and phospho-GSK3β (5676S and 9331L (Cell Signaling)); PDH and phospho-PDH (3205S (Cell Signaling)) were prepared in a 1/1000 dilution in 5% BSA.

Statistical Analysis

All values are presented as means ± standard error of the mean (SEM). Significant differences were determined by the use of an unpaired, two-tailed Student's *t*-test, or a one-way or two-way analysis of variance (ANOVA) followed by a Bonferroni post-hoc analysis where appropriate. Differences were considered significant when $P < 0.05$.

Results

Direct Treatment of The Isolated Working Heart with Liraglutide Has no Effect on Myocardial Energy Metabolism

Isolated working hearts from C57BL/6J male mice were aerobically perfused for 40 min in the absence of insulin and either saline or liraglutide (10 nM) was present within the Krebs Henseleit perfusate. Consistent with the absence of ventricular cardiac myocyte GLP-1R expression, the inclusion of liraglutide in the perfusate had no effect on glycolysis, glucose oxidation, or fatty acid oxidation rates (Figure 3-2).

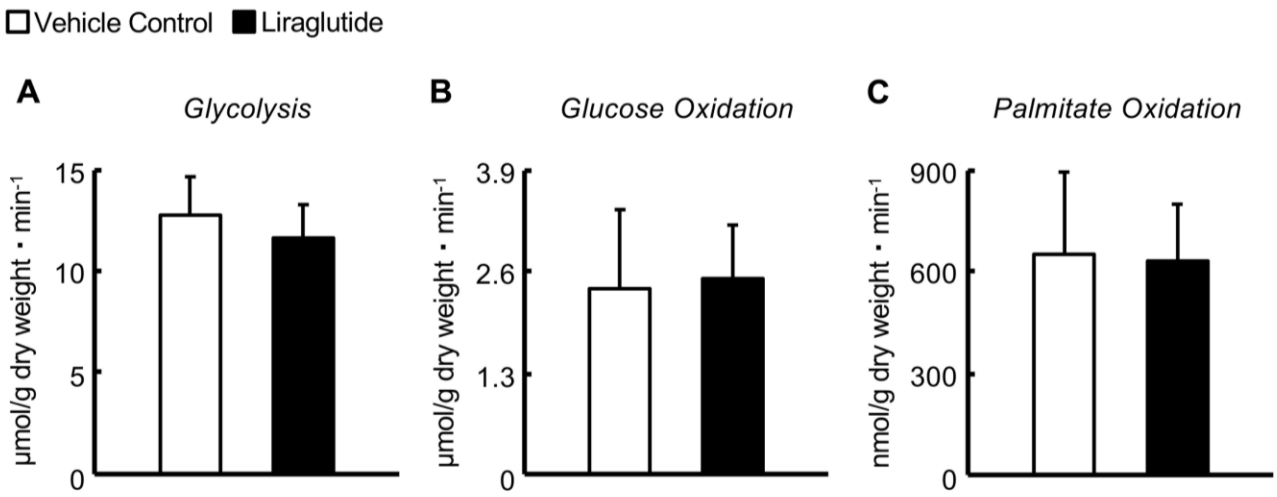

Figure 3-2 Assessment of cardiac energy metabolism ex vivo in lean mouse hearts directly treated with liraglutide

(A) *glycolysis rates* ***(B)*** *Glucose oxidation rates,* ***(C)*** *palmitate oxidation rates, during isolated aerobic working heart perfusions (n =6). Values represent means ± SEM.*

Systemic Treatment of Both Lean and T2D Mice with Liraglutide Increases Glucose Oxidation Rates During Isolated Aerobic Working Heart Perfusion

Similar to the aforementioned observations, systemic treatment of lean C57BL/6J mice with the GLP-1R agonist liraglutide (30 μg/kg) 3x over 24-hr had no effect on glycolysis rates when hearts from these animals were aerobically perfused for 40 min in the working mode in the absence of insulin (Figure 3-2A). Conversely, we now observed a marked increase in myocardial glucose oxidation rates, though this was not associated with a corresponding decrease in palmitate oxidation rates (Figure 3-2B/C). Importantly, these findings were recapitulated in mice subjected to experimental T2D, as T2D mice treated with liraglutide (30 μg/kg 3x over 24-hr) had similar glycolysis rates, increased glucose oxidation rates, and no change in palmitate oxidation rates when compared to their saline treated counterparts (Figure 3-2D-F). The systemic liraglutide treatment-induced increase in isolated working heart glucose oxidation rates did not augment

cardiac function or other ex vivo working heart parameters in both lean and T2D mice (Table 3-1). Plasma collected from mouse whole-blood following animal euthanasia revealed that as expected, treatment of both lean and T2D mice with liraglutide increased circulating insulin levels prior to removal of the heart for isolated working heart perfusions (Figure 3-4).

Table 3-1 Ex vivo cardiac function

	LEAN CONTROL	***LEAN LIRAGLUTIDE***	***HFD CONTROL***	***HFD LIRAGLUTIDE***
Heart rate (bpm)	221±9	238±5	230 ±32	237 ±25
Cardiac Output (ml/min)	10.4±0.14	9.0±0.95	8.2±0.91	8.9±0.76
Aortic out flow (ml/min)	8.7±0.24	7.1±0.91	6.3±0.84	6.1±0.93
Coronary flow (ml/min)	1.3±0.14	1.4±0.31	1.8±0.22	2.3±0.43
Cardiac work (joules/min/dry weight)	3.094±0.375	1.783±0.137	1.593±0.223	1.800±0.243
HR × PSP (mmHg × beats × min^{-1} × 10^{-3})	18±0.3	17±0.1	17±2.1	17±1.4

Ex vivo cardiac function parameter assessment during isolated aerobic heart perfusion in lean and HFD mice treated with vehicle or liraglutide (n=6). HR: heart rate; PSP: peak systolic pressure. Values represent means ±SEM.

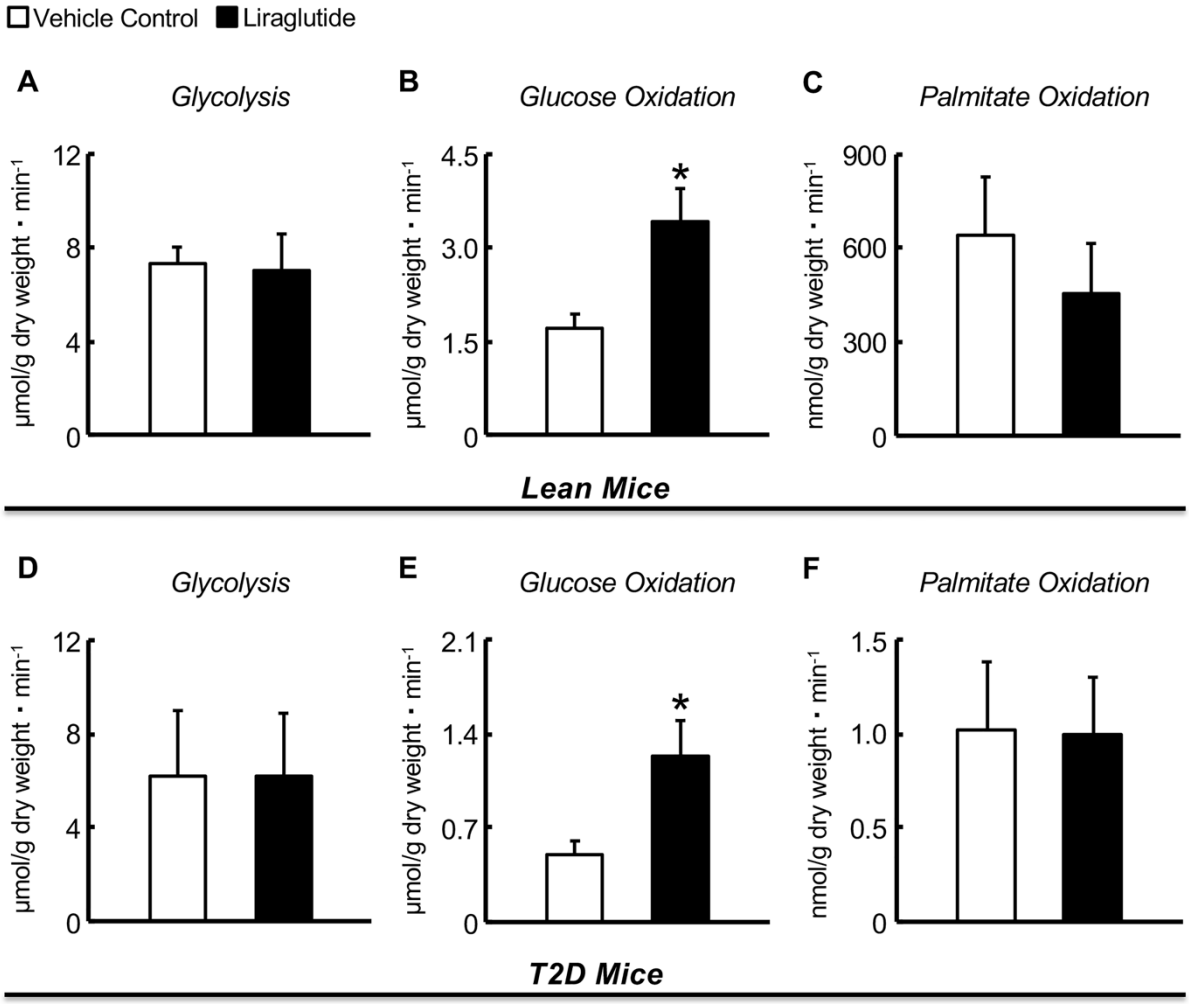

Figure 3-3 Myocardial energy metabolism ex vivo in lean and T2D mice

(A) *glycolysis rates* ***(B)*** *Glucose oxidation rates,* ***(C)*** *palmitate oxidation rates, in lean mice and their control.* ***(D)*** *glycolysis rates,* ***(E)*** *Glucose oxidation rates,* ***(F)*** *palmitate oxidation rates during isolated aerobic working heart perfusions (n =6). Values represent means ± SEM. Differences were determined using an unpaired, two-tailed Student's t-test. *P < 0.05.*

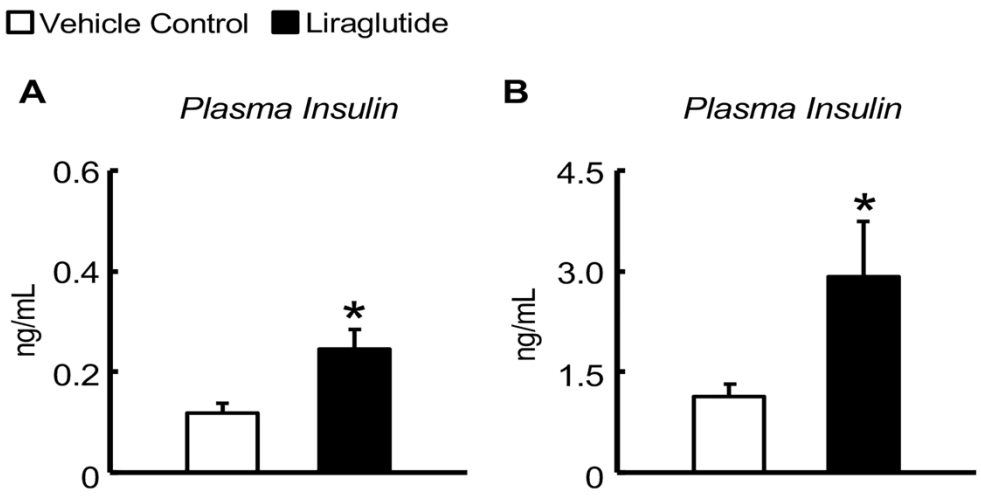

Figure 3- 4 Plasma insulin levels in lean and T2D mice treated with liraglutide

***(A**) plasma insulin levels in lean mice after the treatment with liraglutide or vehicle control **(B)** Plasma insulin level in T2D mice after the treatment with liraglutide or vehicle control (n=5,6)*

Liraglutide Treatment Mitigates Cardiomyopathy in Mice Subjected to Experimental T2D

To determine whether liraglutide treatment can attenuate cardiomyopathy in our experimental model of T2D, we treated T2D mice with either saline or liraglutide for the final 2-weeks starting at 4-weeks post-STZ injection (Figure 3-1B). Consistent with GLP-1R agonist mediated increases in insulin secretion, treatment with liraglutide improved IP glucose tolerance in T2D mice (Figure 3-5A). Serial ultrasound echocardiography assessments at 4-weeks and 6-weeks post-STZ injection, revealed that a 2-week treatment regimen with liraglutide had negligible impact on LV systolic functional parameters including LVEF and LVFS (Figure 3-5E/F & Table 3-2). On the contrary, we did observe a marked improvement in LV diastolic function, as liraglutide treatment prevented the T2D-induced decline in the mitral E/A and E/E' ratios (Figure 3-5H/I).

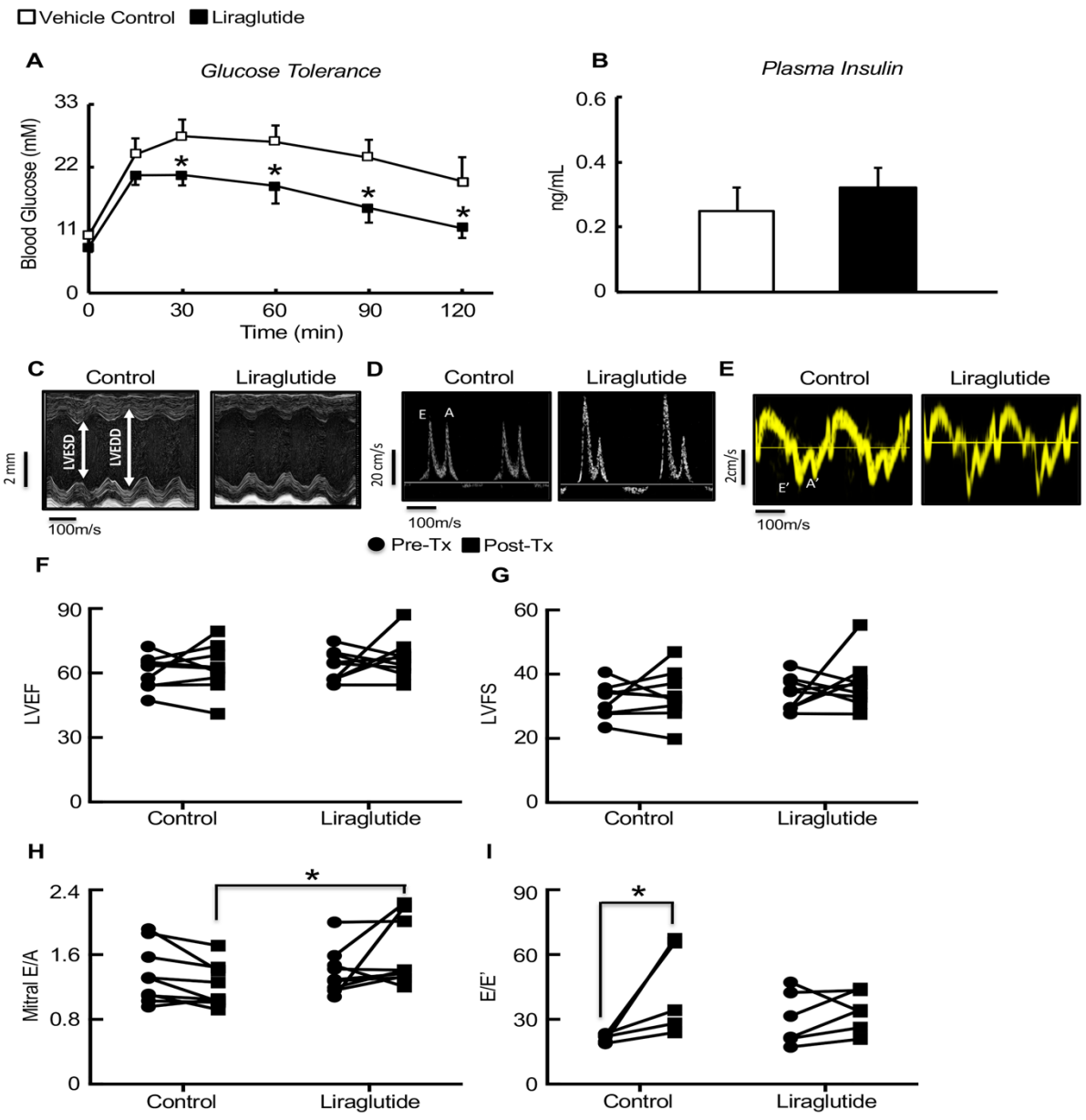

Figure 3-5 Treatment T2D mice with liraglutide improve LV diastolic dysfunction

***(A**)Glucose tolerance in T2D mice and their control (n = 4). normal systolic function with evidence of diastolic dysfunction ultrasound echocardiography was performed in T2D mice treated with liraglutide, vehicle control to assess heart function by showing **(B)** Plasma insulin levels **(C)** M-mode images and parasternal long-axis views **(D**) PW Doppler Mode(**E**) PW tissue Doppler Mode Doppler imaging systolic function; **(F)** left ventricular ejection fraction (LVEF), **(G)** left ventricular fractional shortening (LVFS), and **(H)** Diastolic function was assessed via measurement of the mitral E/A ratio. **(I)** Diastolic function was assessed via measurement of the mitral E/E' ratio Values represent means ± SEM (n = 6). Differences were determined using an unpaired, two-tailed Student's t-test. *P < 0.05.*

Table 3-2 In vivo assessment of cardiac function and LV structure in T2D mice treated with liraglutide

	Before vehicle treatment	*After vehicle treatment*	Δ	*Before liraglutide treatment*	*After liraglutide treatment*	Δ
Diameter; s	2.67±10.7	2.50±0.2	-0.17±0.1	2.6 ± 1.5	2.4 ± 30.1	0.16 ± 0.2
Diameter; d	3.92±0.1	3.72±0.2	0.2 ± .01	3.9±0.1	3.8±0.1	-0.07±0.1
volume; s	27.25±0.1	24.13±4.2	-3.12±3.0	24.2±0.1	21.1±2.1	-3.10±3.4
volume; s	67.38±3.3	60.43±6.4	-6.95±4.3	65.1±2.0	63.0±4.5	-2.06±5.1
Stroke volume	40.13±4.8	36.30±3.2	-3.83±2.2	40.9±2.3	41.9±3.2	1.4±3.2
FE (%)	60.38±2.4	62.11±3.7	1.72±3.2	63.1±1.5	67.0±3.1	3.87 ± 4.3
FS (%)	31.97±2.6	33.32±2.6	1.34±2.4	33.9±2.3	37.1±2.6	3.23 ± 3.5
CO	15.24±1.7	13.81±1.4	-1.42±1.1	14.8±0.8	14.9±1.3	0.09±1.4
LV Mass	126.88±1.2	109.25±13.0	-17.64±9.7	127.8±0.8	104.8±12.4	-22.96±16.2
LVAW; s	1.27±8.9	1.25±0.1	-0.02±0.1	1.3±7.3	1.1±0.1	-0.15±13.0
LVAW; d	0.84±0.1	0.82±0.1	0.03±0.1	0.9±0.1	0.7	0.20±0.1
LVPW; s	1.23±0.1	1.13±0.	-0.10±0.1	1.3±0.1	1.2±0.1	0.00
LVPW; D	0.88±0.1	0.81±0.1	-0.07±0.1	0.9±0.1	0.9±0.1	-0.04±0.1
E'/A'	1.43±0.1	1.27±0.2	-0.16±0.2	1.2±0.1	1.4±0.3	0.20±0.1
MV E/A	1.69±0.1	1.51±0.1	-0.18±0.1	1.7±0.1	2.0±0.2	0.37±.02
MV E/E'	22.10±0.1	40.85±8.2	18.74±8.5	30.2±0.1	33.8±3.8	6.36±4.5

In vivo cardiac function and LV wall measurements were assessed via ultrasound echocardiography in isoflurane anesthetized T2D mice (n = 6). Indicates a significant difference from After liraglutide treatment**.** CO; Cardiac output, d; diastole, MV; mitral valve E; early diastole, A; late diastole, EF; ejection fraction, FS; fractional shortening, LV; left ventricular, LVAW; LV anterior wall, LVEDV; LV end diastolic volume, LVESV; LV end systolic volume, LVPW; LV posterior wall, s = systo

Table 3-3 Heart rate for In vivo assessment in T2D mice treated with liraglutide

	Before vehicle treatment	After vehicle treatment	Before liraglutide treatment	After liraglutide treatment
Heart rate	377.43±1.42	379.45±9.80	362.73±1.57	355.65±12.20

The Liraglutide Mediated Attenuation of Diabetic Cardiomyopathy is Associated with Increased Myocardial PDH Activity

All mice subjected to experimental T2D were euthanized following either a 20-hr fast, or a 16-hr fast-4-hr refeed upon completion of their 2-week treatment regimen with either saline or liraglutide. The insulin signaling pathway appeared to be activated in hearts from T2D mice treated with liraglutide, as reflected by the increase in myocardial Akt phosphorylation at serine 473 (Figure 3 - 6A). However, Liraglutide treatment did not increase the phosphorylation of myocardial glycogen synthase kinase 3 (GSK3) (Figure 3-7A) or glycogen synthase (GS) (Figure 3 -7B). Furthermore, myocardial PDH phosphorylation was reduced in liraglutide treated T2D mice during either the 20-hr fast or 16-hr fast-4-hr refeed (Figure 3 - 6B), indicative of increased PDH activity [151], and entirely consistent with our *ex vivo* observations that systemic treatment of both lean and T2D mice increases myocardial glucose oxidation rates (Figure 3 - 3B/E).

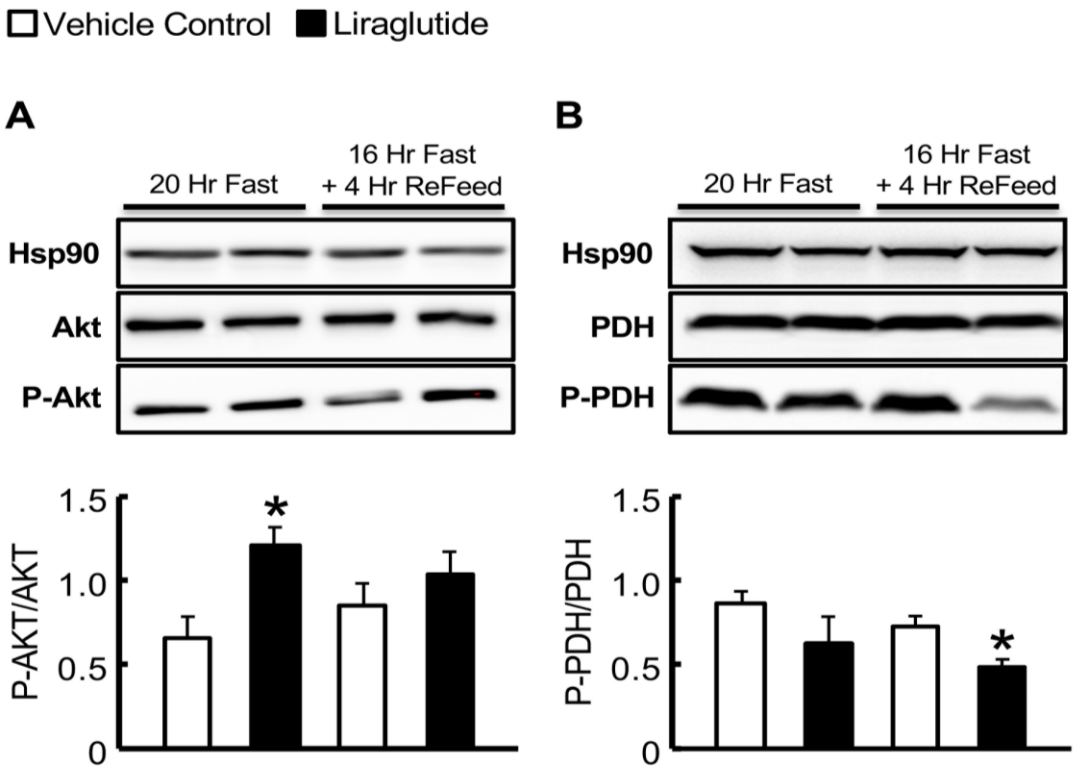

Figure 3-6 PDH and Akt phosphorylation in mouse hearts

*(**A-B**) comparing Akt and PDH expression in T2D mice treated with liraglutide and vehicle control (n = 3,4). Values represent mean ± SEM. Differences were determined using 2-way ANOVA *P < 0.05, significantly different*

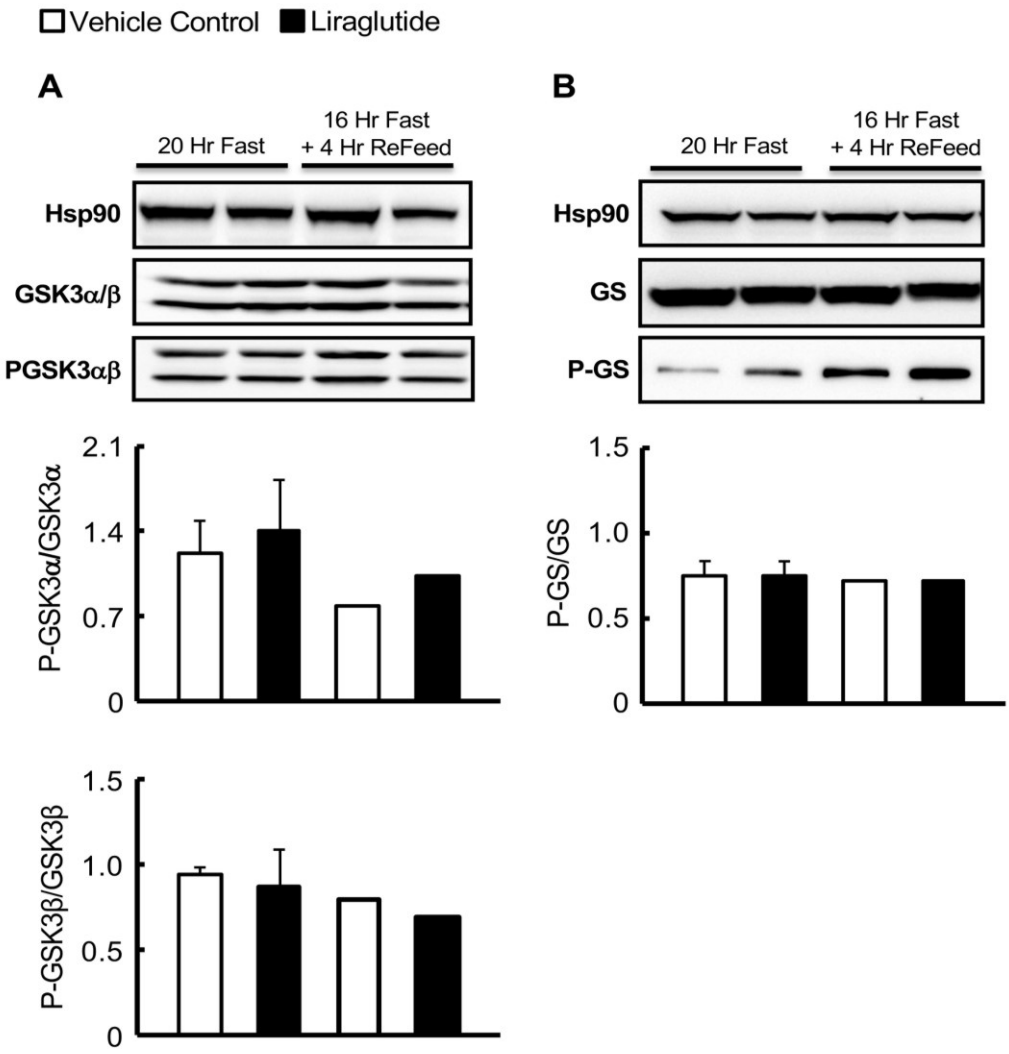

Figure 3-7 GSK3 and GS phosphorylation in mouse hearts

(A-B) comparing GSK3 α/β and GS expression in T2D mice treated with liraglutide and vehicle control (n = 1,3). Values represent mean ± SEM.

Discussion

Our study demonstrates that GLP-1R agonist-induced alterations in cardiac energy metabolism are indirectly mediated, as direct treatment of isolated working heart had no effect on glycolysis, glucose oxidation, or fatty acid oxidation rates, findings entirely consistent with the absence of GLP-1R expression in ventricular cardiac myocyte [152]. Conversely, we observed an increase in glucose oxidation rates during perfusion of isolated working hearts following systemic liraglutide treatment. Importantly, these effects were conserved in hearts from mice with experimental T2D, and a 2-week treatment with liraglutide attenuated experimental diabetic cardiomyopathy, which was associated with increased PDH activity, the rate-limiting enzyme of glucose oxidation [153].

Several preclinical and clinical studies have shown that obesity/T2D are associated with a distinct myocardial metabolic profile, characterized via increased reliance on fatty acids as an oxidative fuel source [154]. Moreover, both basal myocardial glucose oxidation and insulin stimulated glucose oxidation rates are severely impaired in obesity/T2D, due in part to the presence of insulin resistance [155], as well as the inverse relationship (Randle Cycle) between glucose and fatty acids for oxidative metabolism [118]. This profile is clearly seen in working hearts from both *ob*/*ob* and *db*/*db* mice working hearts from HFD fed obese mice [118, 154]. Rats subjected to experimental T2D via HFD supplementation and a low-dose injection of STZ [130], and in PET imaging studies in obese women [123, 156]. Intriguingly, interventions that reverse these obesity/T2D associated perturbations in myocardial energy metabolism have been shown to improve cardiac function in preclinical studies. For example, treatment with trimetazidine, a fatty acid oxidation inhibitor secondary to inhibiting the mitochondrial β-oxidation enzyme, 3-ketoacyl CoA thiolase, prevents the progression of cardiomyopathy and diastolic dysfunction in 26-week-old mice subjected to experimental obesity [157]. Furthermore, dichloroacetate, a PDH kinase inhibitor that increases glucose oxidation rates, also attenuates diabetic cardiomyopathy in Wistar

rats subjected to experimental T2D [130]. Because liraglutide improved both myocardial glucose oxidation rates and diastolic function in mice with experimental T2D, we therefore posit that these metabolic actions may mechanistically contribute to how liraglutide improves cardiovascular outcomes in people with T2D [158]. Although a number of studies have demonstrated that GLP-1 increases myocardial glucose uptake [134, 142, 152], few studies to date have assessed the role of GLP-1 and GLP-1R agonists in modifying myocardial glucose and fatty acid oxidation rates. Treatment of Sprague-Dawley rats with the GLP-1R agonist, albiglutide increases myocardial glucose oxidation rates and decreases fatty acid oxidation rates as assessed via ^{13}C nuclear magnetic resonance spectroscopy, which was associated with mitigation of acute ischemia/reperfusion injury [141]. It is worth noting that it was widely believed during this study's publication that these metabolic actions were mediated via direct actions on cardiac myocytes even though albiglutide was systemically administered, since dogma at the time indicated that ventricular cardiac myocytes expressed the GLP-1R [141]. Although our findings support the observations of the aforementioned study, we only observed an increase in myocardial glucose oxidation rates when isolated working hearts were perfused following systemic treatment of lean or T2D mice with liraglutide. If liraglutide was directly added to the perfusate of the isolated working heart, we failed to see an increase in glucose oxidation. As a number of studies have now demonstrated that ventricular cardiac myocytes do not express the canonical GLP-1R in mice [148, 149], the fact that we did not observe an increase in glucose oxidation rates in isolated working hearts directly treated with liraglutide is expected. In contrast, isolated working hearts from Sprague-Dawley rats have demonstrated that both native GLP-1 and the GLP-1R agonist, exendin-4, increase glucose oxidation rates, but only when fatty acids are not included in the perfusate [133]. Reasons for the discrepancy are unclear and could be due to species-specific differences, suggesting that further characterization of whether rat ventricular cardiac myocytes express the GLP-1R may be necessary. In addition, the increase in myocardial glucose oxidation

rates following treatment with exendin-4 was much milder (~15-20% increase) in comparison to what we observed in following systemic treatment of mice with liraglutide (~100% increase).

It has been proposed that increases in glucose oxidation improve cardiac function via improving the efficiency of contractile function, which is impaired in obesity/T2D [144]. Indeed, treatment of *db*/*db* mice with the thiazidinedione, rosiglitazone, increases glucose oxidation rates during isolated working heart perfusions, which is associated with a normalization of cardiac efficiency and subsequent improvement of cardiac function [118, 154]. With regards to GLP-1R agonists, recent studies in an obese swine model have demonstrated that liraglutide treatment for 4-weeks enhances cardiac efficiency, as indicated by significant increases in the relationship between cardiac power per pressure-volume area [159]. However, it was not considered by the authors whether changes in myocardial glucose oxidation were mechanistically involved. Furthermore, we demonstrated that liraglutide treatment of mice with experimental T2D was associated with reduced PDH phosphorylation (indicative of increased PDH activity) [125, 160], potentially explaining how liraglutide increases myocardial glucose oxidation. An element missing from our current study is that we have not explored the mechanism(s) by which systemic liraglutide administration increases myocardial glucose oxidation rates in both lean and T2D mice. It is likely that GLP-1R agonist-induced increases in insulin secretion are involved, as insulin is a potent stimulator of glucose oxidation in the mouse heart [102, 118], and insulin stimulated glucose oxidation rates are impaired in obese/T2D mice [118]. Moreover, we observed liraglutide mediated increases in myocardial Akt phosphorylation, a major regulatory node of the insulin signalling pathway [161, 162], which was associated with increased circulating insulin levels. Further highlighting that the increase in myocardial Akt phosphorylation is the result of increased circulating insulin levels and not direct actions on ventricular cardiac myocytes, systemic liraglutide treatment still produces robust increases in Akt phosphorylation in hearts from mice with a cardiac/atrial-specific deletion of the GLP-1R [83].

A limitation with our study is that our experimental model of T2D does not produce any significant systolic dysfunction (e.g. reduced LVEF and LVFS) but is rather characterized via reductions in diastolic function (e.g. reduced mitral E/A values). Nonetheless, a significant number of people with T2D and coexisting cardiovascular disease lack any detectable systolic dysfunction, though they present with numerous features of diastolic dysfunction and/or diabetic cardiomyopathy [155, 163]. As such, our findings demonstrate that liraglutide mitigates diastolic dysfunction in experimental T2D is clinically relevant, but it remains uncertain whether our observations would translate to people with T2D and systolic dysfunction. In addition, the LEADER (Liraglutide Effect and Action in Diabetes: Evaluation of Cardiovascular Outcomes Results) trial was not designed to study functional parameters of heart failure or compare responses in people with heart failure with reduced ejection fraction (HFrEF/systolic heart failure) or with heart failure and preserved ejection fraction (HFpEF/diastolic heart failure). Hence, it will be equally important to determine whether liraglutide-induced increases in myocardial glucose oxidation contribute to improvements in experimental models of systolic dysfunction, and to determine in clinical studies whether liraglutide can improve functional status in people with T2D and HFrEF or HFpEF. Of particular interest, we previously generated a cardiac-specific PDH deficient mouse model, which has a myocardial metabolic profile reminiscent of the heart in obesity/T2D [150]. These mice present with a diabetic cardiomyopathy-like phenotype, encompassing preserved LVEF and a decline in the mitral E/A ratio that is strikingly similar to what we observe in our mouse model of experimental T2D. Such observations suggest that cardiac-specific impairments in glucose oxidation are sufficient to induce diastolic dysfunction, and further support that liraglutide mediated increases in myocardial glucose oxidation mechanistically contribute to attenuating diabetic cardiomyopathy.

Taken together, our findings illustrate that GLP-1R agonist-induced modulation of cardiac energy metabolism is mediated via cardiac-myocyte independent mechanisms, entirely consistent with

negligible ventricular cardiac myocyte GLP-1R expression. Liraglutide treatment attenuated diabetic cardiomyopathy in mice and was associated with improved PDH activity in the heart.

Chapter Four

- **Discussion**

Part of this chapter includes content previously published in the following review; Almutairi, M., R. Al Batran, and J.R. Ussher, Glucagon-like peptide-1 receptor action in the vasculature. Peptides. 2019. 111: p. 26-32.

Discussion

Our study demonstrates that GLP-1R agonist-induced alterations in cardiac energy metabolism are indirectly mediated since direct treatment of the isolated working heart had no effect on glycolysis, glucose oxidation, or fatty acid oxidation rates, findings entirely consistent with the absence of GLP-1R expression in ventricular cardiac myocyte [152]. Conversely, we observed an increase in glucose oxidation rates during perfusion of isolated working hearts following systemic liraglutide treatment. Importantly, these effects were conserved in hearts from mice with experimental T2D, and a 2-week treatment with liraglutide attenuated experimental diabetic cardiomyopathy, which was associated with increased PDH activity, the rate-limiting enzyme of glucose oxidation [153]. We also assessed cardiac function in T2D mice and showed preserved LVEF and a decline in the mitral E/A ratio, and these observations are very similar to a cardiac-specific PDH deficient mouse model we recently characterized that have negligible myocardial glucose oxidation rates [150]. Such observations suggest that cardiac-specific impairments in glucose oxidation are sufficient to induce diastolic dysfunction, and further support that liraglutide mediated increases in myocardial glucose oxidation mechanistically contribute to attenuating diabetic cardiomyopathy, since insulin stimulated glucose oxidation rates are impaired in obese/T2D mice [118]. Moreover, we observed liraglutide improved PDH activity in the heart in the T2D mice and also liraglutide increases in myocardial Akt phosphorylation, a major regulatory node of the insulin signaling pathway. Taken together, our findings illustrate that GLP-1R agonist-induced modulation of cardiac energy metabolism is mediated via cardiac-myocyte independent mechanisms, entirely consistent with negligible ventricular cardiac myocyte GLP-1R expression. Liraglutide treatment attenuated diabetic cardiomyopathy in mice and was associated with improved PDH activity in the heart. These findings may represent a potential mechanism by which GLP-1/GLP-1R agonists confer cardioprotection and may even contribute to the improved cardiovascular outcomes seen in people with T2D in the LEADER cardiovascular outcomes trial.

As already extensively discussed, despite GLP-1R agonists conferring cardioprotective actions in patients with T2D, we currently have limited knowledge of the actual mechanisms of action responsible for these salutary actions, though strong evidence now supports that they are myocardium-independent [82]. While this Thesis has explored a potential role for optimization of cardiac energy metabolism as a mechanism, other mechanisms may also account for our observations that liraglutide mitigates experimental diabetic cardiomyopathy in mice. For example, as the GLP-1R does appear to be expressed in vascular smooth muscle cells, GLP-1R agonists may improve vascular/endothelial function, which reduces cardiovascular events in people with T2D. Using contrast-enhanced ultrasound, male Sprague Dawley rats receiving an intravenous infusion of native GLP-1 (30 pmol/kg/min) for 2-hr demonstrated significant improvements in both microvascular blood volume and microvascular blood flow [164]. This improvement resulted in increased muscle glucose utilization, insulin uptake, and interstitial oxygenation, as well as increases in circulating NO levels. Similar findings have been reported in male Sprague Dawley rats fed a high-fat diet for 4 weeks to induce insulin resistance. Moreover, Dong and colleagues demonstrated that GLP-1 infused (30 $pmol \bullet kg^{-1} \bullet min^{-1}$) for 120 min to male Sprague-Dawley rats increased microvascular blood flow in a PKA and NO-dependent manner, as these actions were blocked by either co-treatment with the PKA inhibitor H89, or the endothelial nitric oxide synthase inhibitor L-NAME [164, 165] The vascular/ endothelial actions of GLP-1/ GLP-1R agonists also demonstrate protection against hypertension and/ or atherosclerosis. For example, treatment with exendin-4 (20 nmol/kg twice daily) for 12 weeks significantly reduced systolic blood pressure (SBP) in hypertensive db/db mice provided with 2% salt in their drinking water [166]. Moreover, osmotic pump-mediated subcutaneous infusion of exendin-4 (1 μg/kg/ day) for 1 week reversed corticosterone-induced increases in SBP and diastolic BP (DBP) in rats, findings which were independent of changes in body weight and caloric intake [33]. These beneficial actions of GLP-1/GLP-1R agonists also translate to the angiotensin II-infusion model

of hypertension, as twice daily treatment with liraglutide (30 μg/kg) markedly reduced both SBP and DBP in angiotensin II-infused male C57BL/ 6 J mice. Of interest, these findings were dependent on liraglutide-induced atrial natriuretic peptide (ANP) secretion, since liraglutide treatment increased circulating ANP levels and failed to lower SBP/DBP in ANP deficient mice [167]. Clinical research conducted by Nystrom and colleagues showed an improvement of endothelial dysfunction was measured by post-ischemic endothelial-dependent vasodilation (FMD) of the brachial artery, using ultrasonography in T2D patients with coronary heart disease following infusion of GLP-1(2 pmol•kg^{-1}•min^{-1}) versus placebo. The treated group showed an improvement in endothelial dysfunction compared to the control group [89]. The vast majority of clinical studies have shown that chronic administration of GLP-1R agonists frequently lowers SBP/DBP in diabetic humans, which is consistent with preclinical studies, though the reported reductions are not as potent as those observed in their animal counterparts. In contrast, acute GLP-1/GLP-1R agonist treatment can transiently increase SBP/DBP, though reasons for these discrepant actions remain to be elucidated. With regards to chronic GLP-1R agonist administration, a 1-year treatment with exenatide significantly decreased SBP in subjects with T2D from the “Results from the Diabetes Therapy Utilization: Researching Changes in A1C, Weight and Other Factors Through Intervention with Exenatide Once Weekly (DURATION-1)” trial [10], with ~50% of patients that had a SBP≥ 130 mm Hg at baseline, achieving a normal SBP by week 52. Similarly, 314 overweight/ obese patients receiving exenatide (10 μg twice daily) for 82 weeks also demon started reductions in both SBP and DBP [168]. Finally, another study reported that a 20-week treatment with once-daily subcutaneous liraglutide (1.2, 1.8, 2.4 or 3.0 mg), followed by a non-blinded 2-year extension (final dose of 3.0 mg), produced an ~4.6 mm Hg decrease in SBP in 268 obese non-diabetic subjects [169] . Importantly, it is well established that reductions in SBP/DBP lead to marked reductions in cardiovascular risk in human subjects [170]. Moreover, the potential actions of the GLP-1R agonist on VSMC proliferation and/or oxidative stress would be anticipated to contribute to attenuate the progression of atherosclerotic lesion

progression as has been observed in preclinical studies [171-174], which would have favorable actions on cardiovascular outcomes. Increased microvascular recruitment and microvascular blood flow within the myocardium's coronary vasculature are likely to improve oxygen and nutrient delivery to the heart, which would be advantageous for a T2D subject comorbid for angina/ischemic heart disease and/or heart failure. Likewise, this could also improve insulin delivery to the cardiac myocytes within the myocardium, and thereby account for potential GLP-1R agonist-induced improvements in myocardial energy metabolism that augment contractile efficiency [1, 152]. Hence, the vascular/endothelial GLP-1R activity may potentially play a key role in GLP-1R agonist-induced cardioprotection, though further work is necessary to determine what cell types within the vascular endothelium express a full-length and functional GLP-1R that mediate such actions. In addition to glucose-lowering effects, anti-inflammatory actions may also contribute to how GLP-1/GLP-1R agonists reduce cardiovascular events in people with T2D. Inflammation is also known to be a risk factor for atherosclerotic cardiovascular disease, while the formation of atherosclerosis plaque may cause by pro-inflammatory stimuli in the vascular endothelial cells and is associated with increased plasma levels of TNF-α, IL-6, and circulating endotoxin such as lipopolysaccharide (LPS) [175]. These actions were observed in vitro with human aortic endothelial cells (HAECs). Prior to stimulation with TNF-α, the cells were incubated with liraglutide (30 nM) or vehicle for 30 min, and liraglutide attenuated the inflammatory responses to TNFα and PKCα stimulation, which were indicated by reduction of protein expression of the adhesion molecule VCAM-1 [176]. Importantly, reducing pro-inflammatory cytokines could be a potential therapy for limiting atherosclerotic events and therefore prevent cardiovascular events. Several studies in murine have demonstrated that elevated circulating levels of C-reactive protein (CRP) and other circulating inflammatory markers are associated with a high risk of future CVD events [175, 177].

In addition, a number of studies have associated CRP levels with the development of vascular disease in hypertensive patients, also it may contribute to carotid atherosclerosis, which can be a marker for a patient with hypertension developing arterial stiffness [178]. CRP levels are also associated with LV hypertrophy [179, 180]. Clinical studies suggest that the risk of CVD may be reduced following treatment with anti-inflammatory agents [181]. In a randomized, double-blind trial, canakinumab (150 mg once every 3 months), an anti-inflammatory agent used to target the inflammatory cytokine interleukin-1β, significantly decreased the rates of recurrent cardiovascular events when compared to placebo [181]. Therefore, it has been suggested that different immunomodulatory treatments would help to reduce the risk of CVD and the risk factors behind the progression of CVD, and that GLP-1R agonist-induced reductions in inflammation may also contribute to their cardioprotective profiles. Nevertheless, despite new preclinical studies demonstrating that GLP-1/GLP-1R agonists may improve vascular/endothelial function and/or reduce inflammation, or our own data in this Thesis demonstrating that GLP-1R agonists improve myocardial glucose oxidation rates, identifying the precise cardioprotective mechanism(s) of action through clinical studies will prove difficult. This is due in part to GLP-1R agonists affecting multiple organ systems following systemic administration. As such, sophisticated preclinical studies using cell/tissue-specific deficient mouse models for the *Glp1r* in experimental models of T2D and various cardiovascular disease, in response to treatment with GLP-1R agonists or placebo will be required and must continue, to shed light on whether vascular/endothelial mechanisms, or myocardial energy metabolism mechanisms, contribute to GLP-1R agonist-induced cardioprotection.

Future Directions

This thesis utilized a comprehensive framework to demonstrate that the GLP-1R agonist liraglutide increases myocardial glucose oxidation rates via indirect mechanisms and mitigates diabetic cardiomyopathy. These investigations have helped advance our understanding of the different aspects of the potential indirect actions by which GLP-1R agonists may confer cardioprotection in people with T2D.

Although we present strong evidence that liraglutide increases glucose oxidation in the heart via indirect mechanisms, we still do not know whether this truly contributes to GLP-1R agonist-induced cardioprotection. In order to confirm whether GLP-1R agonists such as liraglutide require an increase in myocardial glucose oxidation to confer cardioprotection in people with T2D, we have recently generated a cardiac-specific PDH (gene name *Pdha1*, *Pdha1*$^{\text{cardiac-/-}}$) deficient mouse model, which exhibits a phenotype very similar to that observed in T2D Indeed, *Pdha1*$^{cardiac-/-}$ mice exhibit diastolic dysfunction as seen by reduced mitral E/A ratios (Figure 4 -1), and they also exhibit a marked reduction in glucose oxidation and subsequent increase fatty acid oxidation in isolated working heart perfusion studies [150] (Figure 4-2).

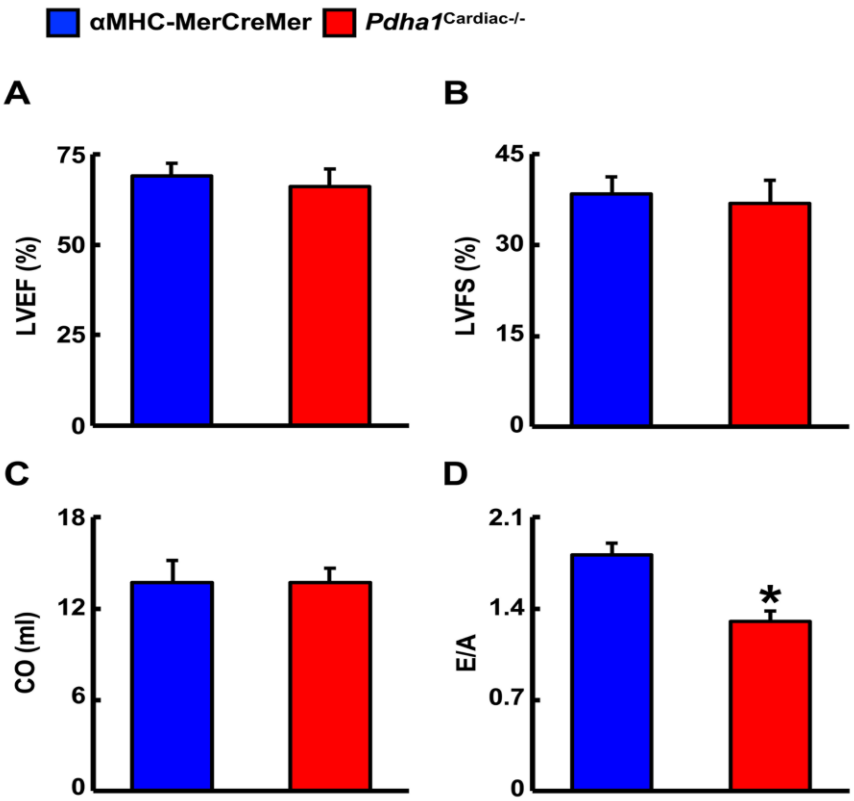

Figure 4-1 Pdha1$^{Cardiac-/-}$ mice exhibit normal systolic function with evidence of diastolic dysfunction Mice

*(**A**) left ventricular ejection fraction (**B**) left ventricular fractional shortening (**C**) cardiac output (**D**) Diastolic function was assessed via measurement of the mitral E/A ratio [150].*

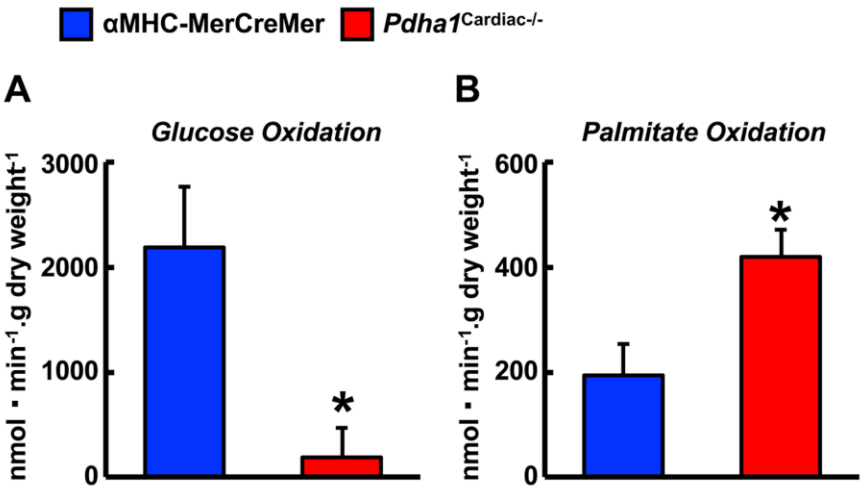

Figure 4-2 Altered myocardial energy metabolism in pdha1$^{Cardiac-/-}$ Mice

(A)*Glucose oxidation rate* ***(B)*** *palmitate oxidation rate*[150].

Since cardiac-specific *Pdha1*$^{cardiac-/-}$ mice have a severe defect in glucose oxidation and mimic cardiac metabolism/function profiles in T2D, if treatment with liraglutide fails to mitigate cardiac dysfunction in *Pdha1*$^{cardiac-/-}$ mice, it will indicate that increasing cardiac glucose oxidation is indispensable for GLP-1R agonist-induced cardioprotection. Conversely, if liraglutide still confers cardioprotection in *Pdha1*$^{cardiac-/-}$ mice subjected to our experimental model of T2D, it will suggest that other mechanisms, such as improvements in vascular/endothelial function, or reductions in inflammation, are more likely contributors to GLP-1R agonist-induced cardioprotection.

Because insulin stimulates myocardial glucose oxidation [118], we have proposed that our myocardial metabolism observations are due to liraglutide augmenting islet β-cell insulin secretion. Therefore, we assessed the insulin levels in all previous cohorts which were increased following systemic liraglutide administration and explains why we don't observe similar results following the direct treatment of the isolated heart, as insulin was not included in the perfusate, and is consistent with negligible ventricular cardiac myocyte GLP-1R expression. Moreover, our

findings with liraglutide on myocardial glucose oxidation should be conserved with other GLP-1R agonists, if dependent on enhanced islet β-cell insulin secretion. It is also possible that systemic GLP-1R agonist treatment influences other aspects of myocardial energy metabolism such as endogenous fuel source (e.g. glycogen, TAG) metabolism. Therefore, we will extensively investigate the extent to which systemic GLP-1R agonists affect myocardial energy metabolism, and the role of islet β-cell- secretion in these actions. To determine if GLP-1R agonist-induced insulin secretion is indeed responsible for the increase in myocardial glucose oxidation, we will collaborate with Dr. Jonathan Campbell (Duke University) and use a β-cell-specific deficient (*Glp1r*$^{\beta cell-/-}$) mouse model they have previously characterized, as these animals do not exhibit systemic GLP-1R agonist-induced increases in circulating insulin *Glp1r*$^{\beta cell-/-}$ mice and their control littermates will be systemically treated with a GLP-1R agonist such as liraglutide for the assessment of energy metabolism during isolated working heart perfusions in absences of insulin as previously described in Chapter 2 of this Thesis. We expect systemic GLP-1R agonist treatment to no longer increase glucose oxidation rates in hearts from (*Glp1r*$^{\beta cell-/-}$) mice that do not exhibit GLP-1R agonist-mediated increases in circulating insulin levels, despite PDH being fully intact. Taken together, the above experiments will support that a β-cell-insulin secretion/myocardial PDH axis explains how GLP-1R agonists increase myocardial glucose oxidation rates (Figuer1-5).

Previous evidence that suggests that lipotoxic intermediates contribute to insulin resistance and cardiac myocyte apoptosis (e.g. long-chain acyl CoA, ceramide, and diacylglycerol) [182]. As such, we will also assess whether this plays a role in our model of diabetic cardiomyopathy and whether liraglutide mediated improvements in cardiac function in mice with T2D involve changes in myocardial lipid accumulation. We will send myocardial samples from our study to the Metabolomics Innovation Centre (University of Alberta) core facilities, where they will quantify

these metabolites and a variety of other important (e.g. intermediates, organic acids, amino acids, etc.). This will allow us to determine whether systemic GLP-1R agonist-induced increases in myocardial glucose oxidation result in the modification of other vital elements influencing the progression of diabetic cardiomyopathy.

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