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Lipid Metabolism in the Diabetic Heart

By

Andrew Scott Neitzel

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ABSTRACT

The metabolism of chylomicrons and palmitate was measured in *ex vivo* perfused working hearts from 12-week old diabetic mice, using both type 1 (streptozotocin induced insulin deficiency) and type 2 (genetic *db/db*) models of diabetes mellitus. Chylomicrons are a lipoprotein produced by the intestine and released into the circulation from the intestinal lymphatics. The triacylglycerol (TG) core of the chylomicron is hydrolyzed by the enzyme lipoprotein lipase (LPL), releasing fatty acids (FA) for uptake by the heart. The amount of LPL on the endothelium of the perfused mouse heart, was measured along with residual tissue activity, neither of which were different in the type 1 (STZ induced) or 2 (*db/db*) diabetic hearts compared to control hearts.

The utilization (oxidation and esterification) of chylomicrons (LPL-derived FA) and palmitate was significantly increased in the type 1 diabetic hearts. Chylomicron utilization was measured in the type 2 diabetic hearts and was also found to be significantly elevated. Since LPL levels were not different between the diabetic hearts and control hearts, increases in chylomicron metabolism must be the result of downstream metabolic alterations, which produce a similar increase in palmitate utilization. Increases in fatty acid metabolism were accompanied by reductions in cardiac contractile function in both models of diabetes.

This is the first study to measure chylomicron metabolism in either a type 1 or 2 diabetic mouse model. In addition, palmitate metabolism in the type 1 diabetic mouse model has not been previously reported. These findings are novel as they provide evidence that there is a cardiomyopathy associated with a dysregulation of metabolism in the diabetic heart.

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LIST OF ABBREVIATIONS

ACC	-	Acetyl CoA Carboxylase
ACTH	-	Adrenocorticotropin
ALB	-	Albumin
AMPK	-	5'-AMP-Activated Kinase
APO	-	Apolipoprotein
BSA	-	Bovine Serum Albumin
BW	-	Body Weight
CAT	-	Carnitine Acyl Transferase
CE	-	Cholesterol Esters
CM	-	Chylomicron
CO	-	Cardiac Output
CPT	-	Carnitine palmitoyltransferase
DG	-	Diacylglycerol
DGAT	-	Diacylglycerol Acyl Transferase
EDTA	-	Ethylenediamine Tetraacetic Acid
ER	-	Endoplasmic Reticulum
FA	-	Fatty Acid
FABP	-	Fatty Acid Binding Protein
FAT	-	Fatty Acid Translocase
FATP	-	Fatty Acid Transport Protein
FC	-	Free Cholesterol
GPAT	-	Glycerol-3-Phosphate Acyl Transferase
HDL	-	High Density Lipoprotein
HSL	-	Hormone Sensitive Lipase
HSPG	-	Heparan Sulphate Proteoglycans
IDDM	-	Insulin Dependent Diabetes Mellitus
IRS	-	Insulin Receptor Substrate
KHB	-	Krebs Henseleit Bicarbonate Buffer
LCAS	-	Long Chain Fatty Acyl CoA Synthase
LDL	-	Low Density Lipoprotein
LP	-	Lipoprotein
LPL	-	Lipoprotein Lipase
MCD	-	Malonyl CoA Decarboxylase
MG	-	Monoacylglycerol

MTP	-	Microsomal Triglyceride Transfer Protein
2-MG	-	2 – Monoacylglycerol
NEFA	-	Non-Esterified Fatty Acid
NIDDM	-	Non-Insulin Dependent Diabetes Mellitus
NADH	-	Nicotinamide Adenine Dinucleotide (reduced form)
PA	-	Phosphatidic Acid
PDH	-	Pyruvate Dehydrogenase
PI3K	-	Phosphatidylinositol-3-Kinase
PKC	-	Protein Kinase C
PL	-	Phospholipid
PPAR	-	Peroxisome Proliferator-Activated Receptor
PPH	-	Phosphatidate Phosphatase
RER	-	Rough Endoplasmic Reticulum
SER	-	Smooth Endoplasmic Reticulum
STZ	-	Streptozotocin
TG	-	Triacylglycerol
TLC	-	Thin Layer Chromatography
TZD	-	Thiazolidinediones
VLDL	-	Very Low Density Lipoprotein
WHO	-	World Health Organization

INTRODUCTION.

1. Diabetes Mellitus:

The last two decades have brought about an explosion in the number of individuals diagnosed with diabetes mellitus. The global increase in the incidence of this metabolic disorder has been ascribed to an increase in the prevalence of obesity, associated with a pronounced change in human behavior and lifestyle. Estimates predict that by 2010 the number of individuals with diabetes will have increased from the current 150 million to 221 million (Zimmet et al. 2001).

Diabetes mellitus is a metabolic disorder in which there is an inability to metabolize carbohydrates due to a dysfunction in the action of insulin. There are two main forms of diabetes, type 1 (insulin dependent) and type 2 (non-insulin dependent). Type 1 diabetes (IDDM) is due primarily to the autoimmune destruction of pancreatic β-cells resulting in reduced pancreatic insulin production and secretion. Individuals with type 1 diabetes must take exogenous insulin to regulate plasma glucose levels to prevent the development of potentially fatal ketoacidosis . Greater than 90 % of all cases of diabetes mellitus are of the type 2 form (Zimmet et al. 2001). Type 2 diabetes (NIDDM) is characterized by tissue insulin resistance in which plasma insulin levels are initially elevated (hyperinsulinemia) to maintain normoglycemia. Subsequently, a beta-cell secretory defect results in the development of hyperglycemia (Saltiel and Kahn 2001). People with type 2 diabetes are not dependent upon the exogenous administration of insulin to control metabolic homeostasis but may require insulin if serum glucose levels cannot be controlled through lifestyle measures or hypoglycemic agents.

2. Maintenance of Blood Glucose:

Maintaining a normal (4-7 mmol/L) blood glucose level is necessary for life. The predominant tissue responding to signals that indicate reduced or elevated blood glucose levels is the liver (Saltiel and Kahn 2001). One of the most important functions of the liver is to produce glucose for extra-hepatic tissues. Both reduced and elevated levels of blood glucose trigger hormonal responses to initiate pathways designed to restore blood glucose levels to normal (Saltiel and Kahn 2001). Low blood glucose triggers release of glucagon from pancreatic α-cells. High blood glucose triggers release of insulin from pancreatic β-cells. Additional signals such as ACTH and growth hormone, which are released from the pituitary, increase blood glucose by inhibiting uptake by extra-hepatic tissues. Glucocorticoids also act to increase blood glucose levels by inhibiting glucose uptake into tissues. Cortisol, the major glucocorticoid released from the adrenal cortex, is secreted in response to an increase in circulating ACTH levels. The adrenal medullary hormone epinephrine stimulates production of glucose by activating glycogenolysis in response to stressful stimuli.

Glucagon binds to receptors on the surface of liver cells triggering an increase in cyclic AMP production leading to an increase in the rate of glycogenolysis by activating glycogen phosphorylase via a protein kinase A (PKA) mediated cascade (Nesher et al. 2002). This is the same response hepatocytes have to epinephrine release. The resultant glucose-6-phosphate is hydrolyzed by glucose-6-phosphatase producing glucose, which then diffuses into the blood (Nesher et al. 2002). Glucose will then enter extra-hepatic cells where it is re-phosphorylated by hexokinase into glucose-6-phosphate which will continue through glycolysis producing pyruvate (Kantor et al. 2001).

Insulin stimulates extra-hepatic uptake of glucose from the blood and in both hepatic and extra-hepatic tissue insulin inhibits glycogenolysis and stimulates glycogen synthesis. One major response of non-hepatic tissues to insulin is the recruitment, to the cell surface, of glucose transporter complexes (Kantor et al. 2001). Glucose transporters comprise a family of five members, GLUT-1 to GLUT-5 (Kantor et al. 2001). GLUT-1 is ubiquitously distributed, GLUT-2 is found primarily in intestine, kidney and liver. GLUT-3 is also found in the intestine and GLUT-5 predominantly in the brain. Insulin-sensitive tissues such as skeletal muscle, cardiac muscle and adipose tissue contain GLUT-4 (Kantor et al. 2001). When the concentration of blood glucose increases in response to food intake, pancreatic GLUT-2 mediates an increase in glucose uptake which leads to increased insulin secretion.

Hepatocytes, unlike most other cells, are freely permeable to glucose (low-affinity GLUT2 transporter) and are, therefore, essentially unaffected by the action of insulin at the level of increased glucose uptake (McGarry 2002). When blood glucose levels are low, the liver does not compete with other tissues for glucose since the extra-hepatic uptake of glucose depends on insulin levels. Conversely, when blood glucose levels are high extra-hepatic needs are satisfied and the liver takes up glucose for conversion into glycogen for future needs. Under conditions of high blood glucose, liver glucose levels will be high and the activity of glucokinase will be elevated. The glucose-6-phosphate produced by glucokinase is rapidly converted to glucose-1-phosphate by phosphoglucomutase, and can then be incorporated into glycogen (McGarry 2002).

3. Diabetic Cardiomyopathy:

There are many complications associated with diabetes such as kidney failure, blindness and at the extreme the loss of gangrenous limbs (Zimmet et al. 2001). The presence of diabetes is in itself a risk factor for cardiovascular disease as patients with diabetes show an increase in relative mortality from various cardiovascular causes (Zimmet et al. 2001). The three major factors contributing to diabetic heart disease are coronary artery disease, autonomic neuropathy resulting in cardiac denervation and diabetic cardiomyopathy (Bell 1995; Zimmet et al. 2001). Many factors contribute to the etiology of diabetic cardiovascular disease including atherosclerosis, hypertension, obesity, insulin resistance and dyslipidemia (Bell 1995).

The term cardiomyopathy describes a disease of heart muscle (Bell 1995). Autopsy, experimental animal and clinical studies have provided evidence for the existence of a diabetic cardiomyopathy that is not due to coronary atherosclerosis or vascular disease (Bell 1995). The first report indicating that diabetes was associated with a cardiomyopathy was in 1881 by Leyden et al. Thirty years ago a study conducted by Rubler et al. (1972) provided the first experimental evidence that diabetes may trigger a cardiomyopathy independent of vascular disease. Over the last three decades many studies have been conducted in an attempt to elucidate this pathology. Today, it is believed that this cardiomyopathy may at least in part be due to a metabolic dysfunction (Rodrigues et al. 1992; Zhou et al. 2000; Unger 2002). For example, Belke et al. (2000) provided evidence that the metabolism of exogenous substrates is altered in the type 2 diabetic heart; glucose utilization (glycolysis and glucose oxidation) was reduced whereas fatty acid (FA) oxidation was elevated. This metabolic derangement was

accompanied by decrease in cardiac contractile function. Belke et al. (2000) were able to correct glucose metabolism through over-expression of the GLUT-4 glucose transporter, improving cardiac contractile function significantly. This result, in combination with other studies, provides strong evidence that the cardiomyopathy identified some 30 years ago is a result of, or may include, a metabolic derangement.

The etiology of this metabolic derangement remains to be defined but studies suggest that it may be a consequence, either direct or indirect, of elevated fatty acid metabolism (Zhou et al. 2000; Unger 2002). This theory has led to the description of a condition termed lipotoxicity, or lipotoxic heart disease.

Lipotoxicity is a process that results from elevated fatty acid utilization and the accumulation of fat deposits in non-adipose tissue such as pancreatic β-cells and cardiac muscle (Zhou et al. 2000; Unger 2002). The elevated fatty acid levels in these tissues are thought to stimulate apoptotic pathways such as that mediated by ceramide, a lipid second messenger (Zhou et al. 2000; Chiu et al. 2001; Finck et al. 2002). Cellular apoptosis will result in the loss of myocytes and β-cells eventuating in a reduction in cardiac contractile function and β-cell insulin production. Studies have been conducted recently on mouse models that are not diabetic but have elevated levels of fat utilization. Chiu et al. (2001) developed a mouse model in which long-chain acyl CoA synthase is over-expressed exclusively in heart. In this model, the esterification of cellular fatty acids with coenzyme A (CoA) is elevated. This is the initial step in fatty acid metabolism and as a consequence oxidation and esterification proceed at an accelerated rate. These hearts showed a significant increase in ceramide production and a subsequent significant reduction in cardiac function as assessed through echocardiography. Finck et al. (2002)

developed a mouse model that over-expresses the receptor peroxisome proliferator activated receptor α (PPARα) in heart selectively. Long chain fatty acids and a variety of related compounds serve as PPARα ligands. Genes involved in fatty acid metabolism have been identified as targets for PPARα. PPARα levels have been shown to be elevated in both a type 1 (streptozotocin-induced) and type 2 (*db/db*) diabetic mouse model. The study by Finck et al. (2002) showed an increase in the expression of genes associated with fatty acid metabolism such as carnitine palmitoyl transferase-1 (CPT-1), acyl CoA oxidase, fatty acid transport protein, and uncoupling proteins 2 and 3 (UCP 2/3). Echocardiographic measurements indicated that cardiac function was significantly depressed in these PPARα over-expressing hearts.

4. Cardiovascular Metabolism:

Normal cardiac function includes regular and forceful contraction propelling blood throughout the circulation. The metabolic pathways that supply energy to the heart are very complex, illustrating the extent to which cellular regulation in the myocardium has developed. Cellular regulation is seen in the control of substrate availability, product inhibition and dependence on a wide variety of cofactors such as substrates for phosphorylation and coenzymes for oxidation and reduction reactions. Together the many mechanisms of metabolic control in the heart fine-tune the pathways of energy production so as to optimize the utilization of substrate in attempt to meet the specific demands of the muscle at any given moment. It is of great importance that regulation acts to ensure that at all times the amount of ATP produced is equal to the amount of ATP

required by the functioning myocardium. It is this continuous and regulated supply of ATP which allows for the uninterrupted beating of the heart.

The heart is able to metabolize a broad variety of substrates (Kantor et al. 2001). These include carbohydrates, namely glucose, lactate and pyruvate. Ketone bodies are also important substrates for the heart. Work by Jeffrey et al. (1995) using NMR isotopomer analysis found that, as expected, fatty acids provide a significant proportion of oxidized substrate under normal conditions, but acetoacetate (a ketone body) was utilized to a larger extent than expected, providing as much as 23% of total energy supply. Fatty acids (FA) are the preferred metabolic substrate of the heart (Kantor et al. 2001). There are two main sources of FA for cardiac metabolism (Figure 1), non-esterified fatty acids (NEFA) and triacylglycerols (TG). The NEFAs are derived through lipolysis in adipose tissue and are found circulating in the blood bound to albumin (Kantor et al. 2001). Triacylglycerols circulate in association with phospholipids, cholesterol and proteins in lipoprotein complexes. Circulating triacylglycerol-rich lipoproteins include chylomicrons generated by absorption of dietary lipids, and very-low-density lipoproteins (VLDL) synthesized in the liver. Fatty acids are generated from lipoprotein-triacylglycerol complexes through hydrolysis by the enzyme lipoprotein lipase (LPL), which is bound to the coronary endothelium (Kantor et al. 2001).

4.1 Carbohydrate Metabolism:

Dietary carbohydrates enter the body in complex forms such as disaccharides and the polymers of starch and glycogen (Mathews and Van Holde 1996). The first step in metabolism of digestible carbohydrate is the conversion of higher polymers to simpler,

soluble forms that can be transported across the epithelial wall and delivered to tissues including the heart. The breakdown of polymeric sugars begins in the mouth and continues through the stomach into the small intestine. Within the small intestine the action of the pancreatic enzyme α-amylase produces disaccharides and trisaccharides (Mathews and Van Holde 1996). Further degradation by saccharidases results in the almost complete conversion of digestible carbohydrate to monosaccharides that are transported across the intestinal wall to the hepatic portal vein and then to liver parenchymal cells and to the heart and other tissues (Mathews and Van Holde 1996).

Nearly all carbohydrates ingested in the diet are converted to glucose following transport to the liver. Catabolism of dietary or cellular proteins generate carbon atoms that can be utilized for glucose synthesis via gluconeogenesis. Additionally, other tissues besides the liver that incompletely oxidize glucose (predominantly skeletal muscle, cardiac muscle and erythrocytes) provide lactate that can be converted to glucose via gluconeogenesis (Mathews and Van Holde 1996).

Intracellular glucose utilization occurs through both oxidative and non-oxidative mechanisms. The oxidative pathway results in the complete breakdown of glucose with CO_2 production, or the conversion of glucose into fatty acids in lipogenic tissues (liver, adipose tissue). The non-oxidative pathway (storage) leads primarily to the production of glycogen. The glycolytic breakdown of glucose or glycogen provides a limited amount of ATP. It is the subsequent entry of pyruvate into the mitochondria, its conversion into acetyl-CoA and entry into the citric acid cycle that provides the majority of the energy obtained from glucose metabolism (Mathews and Van Holde 1996).

4.2 Fat Sources and Mobilization:

The primary sources of fatty acids for utilization by tissue are dietary and those mobilized from cellular stores (Mathews and Van Holde 1996). Fatty acids from the diet are delivered from the small intestine to peripheral cells via transport in the blood. Fatty acids are stored in the form of triacylglycerol complexes primarily within adipocytes. The fatty acids which are stored as triacylglycerols can then be mobilized for use by peripheral tissues through a complex series of interrelated cascades that result in the activation of hormone sensitive lipase (HSL) (Mathews and Van Holde 1996).

Within adipose tissue (Figure 1) this cascade can be activated by numerous stimuli including glucagon and epinephrine (Mathews and Van Holde 1996). These hormones bind to cell-surface receptors that are coupled to the activation of adenylyl cyclase. Activation of adenylyl cyclase results in an increase in cyclic AMP levels and subsequent activation of hormone sensitive lipase. Hormone sensitive lipase hydrolyzes fatty acids from the 1 and 3 positions of triacylglycerols (Mathews and Van Holde 1996). The remaining monoacylglycerols are substrates for monoacylglycerol lipase. The net result of the action of these enzymes is the production of three moles of free fatty acid and one mole of glycerol. The free fatty acids diffuse out of the adipose cells, combine with albumin in the blood and are thereby transported to peripheral tissues (Mathews and Van Holde 1996).

The mobilization of fatty acids from adipose tissue can be inhibited by numerous stimuli, the most significant of which is insulin. In the post prandial state insulin is released from the pancreas and prevents the inappropriate mobilization of fat from adipose tissue by inhibiting adenylyl cyclase.

For the body to utilize dietary lipids they must first be absorbed through the intestine (Figure 2). The major dietary lipid is TG; other lipids in the diet include cholesterol and phospholipids. TG within the intestine is first emulsified by bile salts, which are synthesized by the liver and secreted from the gall bladder. The emulsified TG is then degraded by pancreatic lipase generating free fatty acids and 2-monoacylglycerols; these lipolysis products are then taken up by enterocytes and re-esterified to TG.

4.3 Lipoproteins:

Most lipid absorption occurs in the first one third (duodenum and jejunum) of the small intestine. Within the intestinal cells TGs are then packaged with phospholipids and proteins forming a chylomicron (CM), which will later be secreted (Ginsberg 1998). Another major class of TG-rich lipoproteins are very-low-density lipoproteins (VLDL), mainly produced by the liver. The core TG component of both of these lipoproteins, CMs and VLDLs, is hydrolyzed by the enzyme lipoprotein lipase (LPL) (Ginsberg 1998). The intestine synthesizes CMs to transport dietary fat and fat-soluble vitamins into the blood. CMs are very large with diameters ranging from 75-450 nm; VLDLs range in diameter from 30-80 nm. CMs are spherical consisting of TG (85-92%), phospholipids (6-12%) and protein (1-2%). The generic structure of the chylomicron includes a core containing TG and cholesterol ester with little ordered structure (Ginsberg 1998). The surface of the particle is a monolayer containing phospholipid (mainly phosphatidylcholine), free cholesterol and protein (Ginsberg 1998).

The production of a CM within the enterocyte is an assembly process in which proteins, phospholipids and cholesterol are added to the newly-esterified dietary TG (Figure 2). An essential structural protein of the CM is apolipoprotein B48 (Ginsberg 1998). Apolipoproteins provide structural stability and have critical roles in regulating lipoprotein metabolism. ApoB48 is translated from an apoB100 mRNA that is post-transcriptionally edited (Ginsberg 1998). This involves the enzymatic deamination of cytosine to uracil at nucleotide 6666 (Hussain et al. 1996; Hussain 2000). During chylomicron assembly within the enterocyte apoB48, which is synthesized in the rough endoplasmic reticulum (RER), moves as a membrane component to the smooth endoplasmic reticulum (SER). It is in the SER that TGs are synthesized by DGAT (diacylglycerol acyl transferase) (Hussain et al. 1996; Hussain 2000). ApoB48 and TG are transferred into the lumen in a microsomal triglyceride transfer protein (MTP) dependent process. From the SER the chylomicrons move to and accumulate in the Golgi lumen before release across lateral borders of the enterocytes (Hussain et al. 1996; Hussain 2000). Once chylomicrons cross the lateral borders they enter the lymph on passage to the circulation.

Upon entry into the circulation chylomicrons acquire additional apolipoproteins (Ginsberg 1998). There are as many as ten major apolipoproteins found on the surface of lipoproteins. Chylomicrons acquire specifically apoC-I, apoC-II, apoC-III and apoE (Figure 3), from the surface of high-density lipoproteins (HDL). The transfer of free and esterified cholesterol and phospholipids also occurs at this time (Hussain et al. 1996; Hussain 2000). There is evidence that ApoE may become a surface component somewhat later than apoC-II and apoC-III (Ginsberg 1998). ApoC-I may act to inhibit the hepatic

uptake of CMs and apoE acts as a ligand for the binding of several lipoproteins to the LDL (low density lipoprotein) receptor. ApoC-II and apoC-III play a critical role in the interaction of the chylomicron with lipoprotein lipase, the enzyme which hydrolyzes the triacylglycerol core. ApoC-II is the critical activator of lipoprotein lipase while apoC-III may act as an inhibitor of LPL, in addition to modulating the interaction of the lipoprotein with the endothelial cell surface (Ginsberg 1998).

The cellular uptake of fatty acids, stored within the core of the lipoprotein, requires that esterified fatty acids in the triacylglycerol first be cleaved from their glycerol backbone (Hussain et al. 1996). The cleaving of ester bonds linking the fatty acids to glycerol requires hydrolysis. The capillary endothelium bares an enzyme, lipoprotein lipase, which hydrolyses the position 1 and 3 ester bonds, releasing free fatty acids and 2-monoacylglycerol (2-MG) which can then be taken up by the myocardium (Figure 1).

4.4 Lipoprotein Lipase:

The initial discovery of lipoprotein lipase occurred in 1943 when Hahn noted that the injection of heparin into dogs led to a decrease in post-prandial lipemia. This 'clearing factor' was later identified as the enzyme lipoprotein lipase that is released into the circulation by heparin. Campos et al. (1992) demonstrated that post-heparin plasma from humans with severe hyperchylomicronemia (type 1 hyperlipoproteinemia) did not hydrolyze chylomicrons *in vitro*, linking lipoprotein lipase with this disorder and presenting an essential role for LPL in initiating chylomicron metabolism. Further, hypertriacylglycerolemia develops to a significant extent in both humans and animals that

are lipoprotein lipase deficient (Santamaria-Fojo and Dugi 1994). Various studies have demonstrated consistently that LPL-mediated hydrolysis of lipoprotein triacylglycerols is a necessary step in lipoprotein metabolism (Santamaria-Fojo and Dugi 1994; Goldberg 1996).

LPL is an enzyme that is produced predominantly in adipose and muscle tissue and is key in the metabolism of plasma lipoproteins by the myocardium. In addition, abnormal LPL activity plays a central role in the development of various pathophysiological conditions such as obesity, insulin resistance and diabetes (Ranganathan et al. 2000).

Lipoprotein lipase activity can be altered in a tissue-specific manner although there is only one gene for LPL (Ranganathan et al. 1995). Muscle and adipose LPL are regulated inversely in response to influences such as feeding, exercise, insulin and catecholamines. This is physiologically important as this will direct fatty acid utilization according to the metabolic demands of the individual tissue.

The human LPL protein is 475 amino acids (aa); the mature protein contains 448 amino acids with a hydrophobic leader of 27 amino acids (Braun et al. 1992). The molecular mass of LPL is 50,394 daltons (Braun et al. 1992). The human LPL cDNA is characterized by a long 3′-untranslated region that has two polyadenylation signals (Ranganathan et al. 1995). The human LPL gene has been mapped to the short arm (p22 region) of chromosome 8 (Ranganathan et al. 1995). The gene spans approximately 30 kb and contains ten exons and nine introns (Braun et al. 1992). Six functional sites have been identified in the monomer: 1) the 27 aa signal peptide, 2) a catalytic site, 3) a site for interaction with the apo-CII cofactor, 4) an interfacial binding site, 5) a polyanion

binding site for interaction with heparan sulphate proteoglycans, and 6) a site for non-covalent subunit-subunit interactions (Braun et al. 1992). Although the mechanism for tissue specific transcriptional regulation is not fully understood, four transcription initiation sites, two promoter elements and several enhancer motifs have been identified in the 5′ upstream region of the LPL gene (Braun et al. 1992).

LPL is a glycoprotein with its overall structure including 8-12 % carbohydrate (Santamarina-Fojo and Dugi 1994). Glycosylation of LPL involves the transfer of a lipid-linked oligosaccharide [Glc_3-$Man_9(GlyNAc)_2$] to specific arginine residues of the nascent LPL polypeptide (Braun et al. 1992). The three terminal glucose residues are removed by glucosidases I and II in the ER. This is followed by the removal of one mannose by a α-mannosidase resulting in a mannose structure [Man_8-$(GlcNAc)_2$-protein] which is then transferred to the Golgi. Within the Golgi three mannose molecules are removed by the action of mannosidase I. The further addition of GlcNAc and the removal of another two mannose residues by mannosidase II results in the structure [GlcNAc-$Man_3(GlcNAc)_2$-protein]. This structure is then modified further within the Golgi by a series of transferase reactions resulting in the addition of GlcNAc, galactose and sialic acid residues. Studies have shown that N-linked glycosylation of LPL is required for LPL catalytic activity (Braun et al. 1992).

The physiological site of action for LPL is the luminal surface of blood vessels, yet vascular endothelial cells do not synthesize LPL (O'Brien et al. 1994). *In situ* hybridization studies indicate that endothelial LPL originates, in the case of the adult heart, within the myocyte (O'Brien et al. 1994). It appears that the metabolic demands of

the cardiomyocyte govern the production of LPL, its regulation and possibly the translocation of LPL to the endothelial surface (Olivecrona et al. 1997).

Following transcription of the LPL gene, and subsequent translation and processing, LPL is maintained within vesicles in cardiac myocytes (O'Brien et al. 1994). Both constitutive and regulated secretion of LPL is observed. Very little information is available with regards to the translocation of LPL from sites of synthesis across interstitial spaces to the luminal surface of capillary endothelial cells.

LPL is a dynamic molecule that plays numerous roles in the catabolism of CMs and to a lesser extent VLDLs (Hultin and Olivecrona 1998). First, LPL is necessary for the hydrolysis of TGs in the core of the lipoprotein particles. Hydrolysis of TGs results in the delivery of free fatty acids to the myocardium (Figure 1). As a second function in lipoprotein catabolism, LPL helps to anchor the lipoprotein to the cell surface (coronary endothelium) and proteoglycans (ligand function), increasing their retention by the sub-endothelial matrix and increasing FA uptake into the heart. In addition, LPL may act as a ligand binding to various members of the low-density lipoprotein (LDL) receptor family, playing a role in the cellular endocytosis of lipoproteins (Hultin and Olivecrona 1998).

LPL performs these numerous functions by direct physical interactions with lipoproteins, proteoglycans and receptors (Hussain et al. 1996). LPL favors binding to the apoB-containing lipoproteins as compared with apoA1-containing lipoproteins, yet the biochemical basis for the binding of LPL to apoB containing proteins is not completely understood (Santamarina-Fojo and Dugi 1994). It is known that the N-terminal region of apoB contains multiple binding sites for ionic interactions with LPL. In addition, it is

speculated that LPL may contain a surface-exposed positively charged amino acid cluster which may be important for various physiological interactions (Hussain et al. 1996).

LPL mediated hydrolysis occurs while lipoproteins are temporarily bound to the endothelial binding lipolysis sites (Ginsberg 1998). At these sites heparan sulphate proteoglycans extend their oligosaccharides through the glycocalyx of endothelial cells. LPL is bound to these chains mainly through electrostatic forces. Additional interactions may occur between clusters of positively charged amino acid residues on apoE and/or apoB48 and heparan sulphate chains (Ginsberg 1998).

LPL mediated chylomicron-TG hydrolysis reduces the core volume and surface area of the chylomicrons. In addition, there is a transfer of phospholipid, free cholesterol, apoC-II and apoC-III back to high-density lipoproteins (Hussain et al. 2000). After hydrolysis the remaining remnant chylomicron particles (Figure 3), which are cholesterol and apoE enriched, continue on their passage through the circulation. When they reach the hepatic circulation they interact with receptors on hepatocytes that direct their rapid removal from the circulation (Hussain et al. 2000). The uptake of chylomicron remnants by the liver involves LDL receptors that recognize apoE, specific apoE receptors such as the LDL receptor related protein (LRP), and cell surface proteoglycans that bind apoE (Hussain et al. 1996).

4.5 Cellular Fatty Acid Uptake:

Under normal physiological conditions fatty acids are an important oxidizable substrate for the heart, serve as building blocks for cellular membranes after their

esterification into phospholipids and are involved in signal transduction pathways (Kantor et al. 2001).

Fatty acids have a low solubility in water and it is because of this that they travel in the circulation bound to plasma albumin or in the TG core of lipoproteins. Within the vasculature of the target tissue the fatty acid will dissociate from albumin or, through LPL-mediated lipoprotein hydrolysis, be generated from the lipoprotein triacylglycerol core. Fatty acids are then transferred from the capillary lumen through the capillary endothelium into the cardiac muscle cell (van der Vusse et al. 2000) (Figure 4).

The process by which the fatty acids cross the endothelium is poorly understood. Theory suggests that the transport may be in combination with albumin either through the endothelial cell, or between adjacent endothelial cells (van der Vusse et al. 2000). But based upon the diffusion rate of albumin both of these routes appear unlikely. Another suggestion is that the fatty acid dissociates from albumin at the endothelial luminal membrane and passes through the endothelial cytoplasm in combination with a fatty acid binding protein. The most likely route for both LPL-derived and albumin delivered fatty acid passage through the endothelial cell is by transport across the endothelial luminal membrane as a fatty acid, through the endothelial cytoplasm as either an unbound fatty acid or a fatty acid bound to a fatty acid binding protein, and finally across the abluminal membrane (van der Vusse et al. 2000). Once the fatty acids have reached the interstitial compartment they likely bind to interstitial albumin and are delivered to the cardiomyocyte.

The means by which the fatty acid will pass across the sarcolemmal membrane in cardiomyocytes is a point of debate. It appears that there may be both non-protein

mediated and protein mediated fatty acid transport acting in concert (van der Vusse et al. 2000). Transport of protonated fatty acyl moieties may occur by rapid flip-flop across the phospholipid bilayer, whereas proteins (FATP, FAT / CD36) are involved in the transport of ionized fatty acyl moieties (Hamilton and Kamp 1999).

Once the fatty acids have crossed the sarcolemma and are in the cytoplasm they bind to transport ligands, known collectively as intracellular fatty acid binding proteins (van der Vusse et al. 2000). There are as many as five identified fatty acid binding proteins, the heart containing one designated the H-FABP. The fatty acid binding proteins have been shown to be significant in fat metabolism by the heart based upon a strong correlation between H-FABP presence and fatty acid oxidation capacity (Kantor et al 2001). Once in combination with a FABP the fatty acids are delivered to the mitochondria for oxidation.

4.6 Mitochondrial Fatty Acid Oxidation:

Prior to the fatty acid entering the mitochondria for oxidation or being committed to triacylglycerol synthesis, the fatty acid must have its carboxylic acid terminus converted to a CoA thioester producing a fatty acyl CoA (Kantor et al 2001). This enhances the activity of the fatty acid molecule and is the result of the action of an enzyme on the outer mitochondrial membrane. This enzyme, long chain acyl CoA synthase (LCAS) (Figure 4), is a member of a family of acyl CoA synthetases. This activation consumes ATP and is a non-reversible absolute step in cellular fatty acid utilization (Kantor et al 2001).

The transport of the fatty acyl CoA into the mitochondria is accomplished via a acyl-carnitine intermediate which is generated by carnitine palmitoyl transferase I (CPT-I), an enzyme that resides on the outer mitochondrial membrane (Figure 4). CPT-I generates the acyl-carnitine intermediate while at the same time bringing the intermediate across the outer mitochondrial membrane into the intra membrane space. A carnitine acyl translocase, located in the inner mitochondrial membrane, transports the acyl carnitine intermediate into the inner mitochondrial matrix. Within the mitochondrial matrix the enzyme carnitine palmitoyl transferase II (CPT-II) converts the acyl-carnitine intermediate back into fatty acyl CoA.

The transport of fatty acyl CoA into the mitochondria is a point of regulation for fatty acid oxidation. Specifically, CPT-I activity is governed by the presence of malonyl CoA (Figure 4). Malonyl CoA is a three carbon activated compound formed by the action of acetyl CoA carboxylase (ACC) (Kantor et al 2001). ACC activity is modulated by AMP activated protein kinase (AMPK). AMPK has been defined as a low fuel warning system that is activated by low ATP levels and concomitant increases in AMP. It appears that as the AMP/ATP ratio increases, indicating changes in the cellular energy level, AMPK kinase is activated. AMPK kinase phosphorylates and activates AMPK which then phosphorylates and inactivates ACC (Kantor et al 2001). Inactivating ACC reduces the production of malonyl CoA from acetyl CoA thereby reducing the inhibitory effect of malonyl CoA on CPT-I. Increases in the activity of CPT-I may play a role in increasing fatty acyl CoA oxidation.

The fatty acyl CoA that has been transported into the mitochondria will enter the β-oxidation pathway. The process of fatty acid oxidation is termed β-oxidation since it

occurs through the sequential removal of 2-carbon units by oxidation at the β-carbon position of the fatty acyl CoA molecule (Mathews and Van Holde 1996). A 16-carbon fatty acid such as palmitate will undergo 7 cycles of β-oxidation producing 8 molecules of acetyl CoA. Each round of β-oxidation produces 1 mole of NADH, $FADH_2$ and acetyl CoA. The acetyl CoA then enters the citric acid cycle where it is further oxidized to CO_2 with the concomitant generation of three moles of NADH, one mole of $FADH_2$ and one mole of ATP. The NADH and $FADH_2$ generated during the fatty acid oxidation process enter the respiratory pathway for the production of ATP (Mathews and Van Holde 1996).

4.7 Fatty Acid Esterification:

Fatty acids can be stored in the heart as intracellular TG for future use (Figure 4). Two molecules of fatty acyl CoA are esterified to glycerol-3-phosphate to yield 1,2-diacylglycerol phosphate (phosphatidic acid) by two acyltransferases (GPAT, MGAT). The phosphate is then removed by phosphatidate phosphatase to yield 1,2-diacylglycerol. A third fatty acyl CoA is finally added, again through the action of DGAT (Mathews and Van Holde 1996).

The stored triacylglycerol can exist as cytoplasmic droplets or as membrane associated lipid particles (Kantor et al. 2001). Cytosolic fat droplets in cardiac myocytes tend to be smaller than those in other cell types (Jodalen and Rotevatn 1982). Presumably large fat droplets in cardiac myocytes would be deleterious to the functional properties of the heart. The triacylglycerol pool is in constant turnover providing a source of fatty acid for oxidation when the exogenous supply is low (Kantor et al. 2001) and as much as 2 % of this pool can be turned over per minute (Saddik and Lopaschuk 1991). As much as

11% of total ATP production can be generated, as shown in isolated perfused hearts, from endogenous triacylglycerol stores (Saddik and Lopaschuk 1991).

Steatosis is the storage of excess fat as triacylglycerol in a cell. Other than adipocytes, most cells have a limited capacity for fat storage. Excess triacylglycerol levels have been shown to be an indicator or marker for the development of potential defects such as reductions in the contractility of the cardiomyocyte, hypertrophy and cell death (Veniant et al. 1999; Zhou et al 2000). There are several strategies to reduce lipid overload in cardiac tissue; a recent suggestion is that the heart may unload surplus lipids on lipoprotein particles (Veniant et al. 1999). The expression of lipoprotein assembly genes, apoB and microsomal triglyceride transfer protein (MTP), has been shown in the heart (Figure 4). Further to this Bjorkegren et al. (2001) have shown that isolated perfused hearts release lipoproteins into a re-circulating perfusion buffer.

5. Diabetic Cardiovascular Metabolism:

Diabetes and the ensuing alterations in metabolism stem essentially from a defect in the action of insulin (Kantor et al. 2001). As described above the defect in insulin action in the type 1 diabetic is generally a lack of insulin, while in the type 2 diabetic it is resistance to the action of insulin in peripheral tissues. Insulin plays a role in many aspects of both carbohydrate and fat metabolism such as increasing cell permeability to glucose, increasing glycolysis, glycogen and triglyceride synthesis as well as protein, DNA and RNA synthesis while decreasing gluconeogenesis, lipolysis and protein degradation (Mathews and Van Holde 1996; Kantor et al 2001). Therefore a defect in the action of insulin will result in a metabolic deregulation that is multi-faceted with a

complicated etiology. Identifying the pathologies in diabetic metabolism has been and will continue to be the focus of research initiatives. There are as many potential mechanisms through which a metabolic dysfunction may result as there are actions of insulin.

5.1 Diabetic Carbohydrate Metabolism:

All of those processes which are dependent upon insulin such as glucose transport, glycolysis and glucose oxidation will be reduced in the insulin deficient diabetic state (Kantor et al. 2001). Glycolysis is reduced in the diabetic cardiomyocyte as a result of a depression in the transport and uptake of glucose into cells and a reduction in the rate of glucose phosphorylation (Randle et al. 1994). Depressed glucose phosphorylation is likely the result of elevated fatty acid metabolism (Randle et al. 1963; Randle et al. 1994). This interaction was first described by Randle et al. in 1963 and has been named the Randle Cycle, or the Glucose Fatty Acid Cycle. This cycle describes a relationship between glucose and fatty acid metabolism in which the oxidation of fatty acids and ketone bodies inhibit the catabolism of glucose. The oxidation of fatty acids mediate this inhibitory effect through reducing the activity of phosphofructokinase-1, hexokinase and the pyruvate dehydrogenase complex (PDH) (Randle et al. 1963; Randle et al. 1994). It is believed that an increase in fatty acid oxidation will elevate the mitochondrial ratio of acetyl-CoA/CoA, which in turn inhibits the PDH complex. This is followed by an elevation in citrate levels which will lead to the inhibition of phosphofructokinase-1 and hexokinase (Randle et al. 1963; Randle et al. 1994).

Shulman (2000) suggest additional mechanisms through which free fatty acids may impair glucose oxidation. For example, an increase in fatty acid oxidation may lead to an increase in intracellular fatty acid metabolites such as diacylglycerol, fatty acyl CoA and ceramides. The metabolites diacylglycerol and fatty acyl CoA may activate a serine/threonine kinase cascade leading to phosphorylation of serine/threonine sites on insulin-receptor substrates (IRS). Phosphorylation of the IRS's may lead to inhibition of phosphatidylinositol 3-kinase, attenuating the downstream insulin-signaling cascade, consequent glucose uptake and metabolism (Shulman 2000). Ceramide may activate apoptotic pathways leading to cell death and subsequent cardiac dysfunction (Zhou et al. 2000).

5.2 Diabetic Fatty Acid Metabolism:

As a consequence of drastically reduced levels of glucose utilization the diabetic heart becomes almost entirely dependent upon fatty acid metabolism to supply its energy needs (Stanley et al. 1997). As described earlier, it is this elevated level of fatty acid metabolism that is thought to be responsible, directly or indirectly, for diabetic cardiovascular disease.

Animal studies have been conducted in order to investigate changes in diabetic cardiovascular fat metabolism. Early studies used chemically induced type 1 diabetic rat models in which the administration of a β-cell specific necrotic agent, streptozotocin, would cause diabetes (Tomlinson et al. 1992). These studies assessed levels of various fatty acid oxidation specific enzymes and proteins (O'Looney et al. 1983; Saddik and Lopaschuk 1991). Later studies were conducted with spontaneously type 2 diabetic rats

such as the Zucker ZDF (*fa/fa*) or corpulent JCR:LA (*cp/cp*) rat (Luiken et al. 2001). Both of these type 2 diabetic conditions are the result of a leptin receptor mutation in which these animals are essentially leptin resistant. These type 2 diabetic models are obese and hyperinsulinemic but their degree of hyperglycemia is only mild to moderate.

A mouse model of type 2 diabetes was discovered at Jackson Laboratories that is today known as the *db/db* mouse (C57BL/KsJ-$lepr^{db}/lepr^{db}$). This spontaneous mutation, resulting in type 2 diabetes, has been identified as a G - T point mutation in mouse chromosome number 4 producing a frame shift mutation that selectively eliminates the long form of the leptin receptor, resulting in deficient leptin signaling (Chen et al. 1996). This model of type 2 diabetes is obese, hyperinsulinemic, hyperlipidemic and hyperglycemic (Chen et al. 1996). The metabolic features of this *db/db* mouse model of type 2 diabetes are quite similar to the human diabetic condition. This mouse model has become a popular investigative tool because current knowledge of the mouse genome enables investigators to create gene specific alterations.

Diabetes-induced metabolic changes have been investigated in this *db/db* mouse model. The results of Belke et al (2000) indicate that the oxidation of NEFA (palmitate) by the diabetic heart is increased dramatically, from 0.35 to 0.74 $\mu mol \cdot min^{-1} \cdot g\ dry\ wt^{-1}$. Myocardial TG stores are also significantly elevated (Aasum et al. 2002), as well as serum FA-albumin and CM-TG complexes. Along with increased levels of fatty acid oxidation was a concomitant decrease in glucose oxidation, from 0.49 to 0.08 $\mu mol \cdot min^{-1} \cdot g\ dry\ wt^{-1}$.

The mechanism through which fatty acid oxidation is elevated in the diabetic heart requires further clarification but a few potential mechanisms have been identified.

As discussed earlier, studies have been conducted on mouse models that are not diabetic but have elevated levels of fat utilization, simulating the diabetic condition. The mouse model developed by Chiu et al. (2001) over-expressed long-chain acyl CoA synthase. As a consequence the esterification of cellular fatty acids with coenzyme A (CoA) is elevated. As this is the initial step in fatty acid metabolism oxidation and esterification proceed at an accelerated rate. The first step in the transport of the activated fatty acid into the mitochondria is the responsibility of CPT-I. Malonyl CoA is synthesized by acetyl CoA carboxylase (ACC) and degraded by malonyl CoA decarboxylase (MCD; Figure 4). A significant decrease in ACC activity has been observed in diabetic hearts and has been suggested to occur through either phosphorylation control (elevated levels of AMPK) or allosteric inactivation (Kantor et al. 2001). Work with insulin-deficient hearts, in which diabetes was induced through administration of streptozotocin, have shown a decrease in malonyl CoA levels (Hall et al. 1997). Malonyl CoA is an inhibitor of CPT-I, therefore a reduction in the presence of malonyl CoA may produce a stimulation of CPT-I activity and consequently an acceleration of fatty acid oxidation.

Once within the mitochondrial matrix the fatty acid enters the β-oxidation pathway as described earlier. An early study investigated the activity of an enzyme involved in the β-oxidation pathway. They found that the activity of β-hydroxylacyl-CoA dehydrogenase is elevated in the diabetic rat heart (Chen et al. 1984). Elevated levels of circulating fatty acids, increased rates of transport into the mitochondria and enhanced levels of enzymes associated with β-oxidation will in combination act to increase fatty acid oxidation and the delivery of acetyl CoA to the tricarboxylic acid cycle (TCA cycle).

After the TCA cycle the next step in ATP production is the entry of NADH and $FADH_2$ into the electron transport chain. Some evidence indicates that there may be a defect in mitochondrial respiration, although overall ATP production levels are not compromised (Stanley et al. 1997). Recent work suggests that there may be an uncoupling of the ATP generating ATPase from the respiratory chain. This uncoupling is essentially a shunt through which protons can flow back into the mitochondrial matrix through an uncoupling protein (UCP) (Dalgaard and Pederson 2001). The presence or enhanced activity of an uncoupling protein may result in an inefficiency in the electron transport chain and as a consequence greater quantities of substrate (fat) have to be metabolized to produce the required quantity of ATP.

6. The *Ex Vivo* Perfused Heart:

The isolated perfused heart has been recognized as a valuable tool to investigate cardiovascular substrate metabolism, oxygen consumption and mechanical activity since the pioneering work of Langendorff in 1895. The isolated perfused heart allows the investigator to measure the aforementioned variables while retaining the tissue architecture and an intact vascular system. This means that metabolic substrates can be delivered to tissue by their normal physiological route; this is important for studies in which LPL-derived fatty acid metabolism is investigated. Retrograde Langendorff perfusion was the method of isolated heart perfusion until the work of Neely and Morgan in 1966 (Neely et al. 1966). They developed the *ex vivo* working heart model in which the left atrium was perfused at a pre-load pressure and the heart pumped against an after-

load. This preparation offered the opportunity to vary and measure ventricular pressure development, substrate metabolism, oxygen consumption and external work.

Classically the vast majority of isolated heart perfusion studies have been conducted on rats. Ng et al. (1991) published one of the first studies using an isolated perfused working mouse heart and Grupp et al. (1993) examined various functional parameters with this model. A major complication with the perfused mouse heart was handling the very small heart size (about 170 mg wet weight). In the late 1990's work by several labs, including that of Larsen et al. (1999), contributed to optimizing the isolated *ex vivo* perfused mouse heart model (Figure 5). Interest in using the mouse as an investigative tool arose as the potential for manipulating genes and developing transgenic models in mice developed.

Over the last decade several investigations into the metabolism of the murine heart have been conducted. The majority of theses studies have assessed carbohydrate metabolism, while very few have included fat as a metabolic substrate for the functioning heart. Belke et al. (1999) characterized glycolysis, glucose oxidation and fatty acid oxidation in the Swiss-Webster mouse. Subsequently, Belke et al. (2000) investigated fat and carbohydrate metabolism in the type 2 diabetic *db/db* mouse. They were able to demonstrate altered metabolism of both glucose (decreased utilization) and fatty acid (increased palmitate oxidation) in this diabetic model as well as a dysfunction in myocardial mechanical activity. With the metabolism of glucose and free fatty acids defined (Belke et al. 2000) in the diabetic mouse heart, the next logical step in investigating alterations in diabetic cardiovascular metabolism is to look at another fat source, namely lipoproteins. Recently, Mardy et al. (2001) measured the metabolism of

chylomicrons by perfused working hearts from Swiss Webster mice. Perfused mouse hearts had active endothelium-bound LPL that could be displaced into the perfusate by heparin (heparin-releasable LPL activity). Perfusion of mouse hearts with radiolabeled chylomicrons was used to determine the fate of LPL-derived FA; rates of oxidation and esterification were approximately equal (Mardy et al. 2001). Therefore, it is feasible to determine if chylomicron metabolism is altered in perfused hearts from diabetic mice, using the *db/db* model (Belke et al 2000) as well as a type 1 insulin-deficient model produced by administration of streptozotocin to mice.

7. Statement of Objectives:

- To assess the level of lipoprotein lipase activity in type 1 and 2 diabetic mouse hearts.
- To compare the metabolism of two lipid sources, chylomicrons and NEFA (palmitate) in type 1 and 2 diabetic mouse hearts.
- To determine alterations in cardiac contractile function in type 1 and 2 diabetic hearts.

METHODS.

1. Animals:

All experiments were approved by the University of Calgary Health Sciences Animal Welfare Committee in accordance with the regulations of the Canadian Council on Animal Care. Adult male mice were used for all experiments. Animals were housed in the animal facility at the University of Calgary for at least 5 days prior to experiments, maintained under a 12 hour light-dark cycle and fed standard laboratory chow and water *ad libitum*.

1.1 Type 1 Diabetic Model:

Swiss Webster mice (30-40 grams) were acquired from local breeding sources provided by the University of Calgary. Mice (10 weeks of age) were treated with streptozotocin which produces selective necrosis of pancreatic β-cells, resulting in a well-characterized model of insulin deficiency that has been used extensively (Tomlinson et al. 1992). Streptozotocin was prepared in 100 mM citrate buffer (pH 4.5) at a concentration of 30 mg/ml. Streptozotocin was administered to animals by intraperitoneal injection over a three day protocol (Kennedy and Zochodne 2000). Control animals received only the citrate buffer. On the first day, mice received 85 mg/kg streptozotocin, on the second day 75 mg/kg and on the third 55 mg/kg, for a total cumulative dose of 215 mg streptozotocin per kg mouse body weight. Metabolic studies were conducted two weeks after the three-day streptozotocin injection protocol (at 12 weeks of age). Insulin-deficient (type 1) diabetes was also produced by administration of streptozotocin to

C57BL/KsJ-*lepr*^{db}/*lepr*$^{+}$ (db/+) mice, for direct comparison to the type 2 (db/db) model of murine diabetes.

1.2 Type 2 Diabetic Model:

Genetically diabetic C57BL/KsJ-*lepr*^{db}/*lepr*^{db} (*db/db*) mice were purchased from Jackson Laboratories (Bar Harbour ME, USA). All experiments were conducted on animals at 12 weeks of age, when plasma insulin levels are at peak values with concomitant hyperglycemia (Coleman 1982; Belke et al 2000). These *db/db* mice have two mutant copies of the leptin receptor gene (Leibel et al 1997) and exhibit an obese type 2 diabetic phenotype with hyperinsulinemia and hyperglycemia. The lean heterozygote control (*db/+*) mice possess one mutant and one normal copy of the leptin receptor gene, and are phenotypically normal with respect to body weight and plasma concentrations of glucose, lipids and insulin.

1.3 Blood Glucose:

Diabetes was confirmed through measurements of blood glucose levels. Whole blood for analysis of blood glucose was acquired from each animal through a tail tip slice. Blood glucose was measured with a One Touch Ultra® blood glucose meter (Johnson and Johnson Company, Milpitas CA, USA).

2. Heart Isolation and Perfusion Conditions:

The isolated *ex vivo* working heart preparation (Larsen et al 1999; Belke et al 1999, 2000) is an invaluable model for investigating cardiac metabolism in a variety of

physiological and pathological states. The perfusion technique allows substrate to be delivered by a normal physiological route to cardiac muscle cells whose absorption, transport, and biochemical mechanisms have not been chemically or mechanically abused. Maintaining an intact vascular system is vital in experiments directed at measuring the metabolism of LPL-derived FA as this enzyme is bound to the endothelial surface of the coronary vasculature. The use of working mode allows metabolic measurements to be performed under controlled conditions of energy demand.

Mice were anesthetized with a 10 mg intraperitoneal injection of sodium pentobarbital. Once under anesthesia the chest and abdominal cavity were opened. Prior to excising the heart 200 µl of 10 mM EDTA was injected into the abdominal aorta as an anti-coagulant. Heparin could not be used as an anti-coagulant as it displaces endothelium-bound lipoprotein lipase (Elkes and Williams 1974). Once excised the heart was placed into a chilled (4°C) Krebs-Henseleit bicarbonate (KHB) buffer solution. The Krebs-Henseleit buffer (pH 7.4) used for the initial Langendorff retrograde perfusion consisted of (in mM): 118.5 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 0.5 EDTA and 11 glucose and was gassed with 95% O_2 and 5% CO_2.

Within the chilled KHB bath the aorta was cannulated with an 18 gauge steel cannula. Once cannulation was complete the heart was removed from the chilled KHB and retrograde (Langendorff) perfusion was initiated; the KHB perfusion buffer was oxygenated (95% O_2 and 5% CO_2) and warmed to 37°C. The heart was perfused in retrograde fashion for 10 minutes prior to the start of an experiment, allowing for heart function to stabilize and for extraneous tissue to be removed.

2.1 Langendorff Heart Perfusions:

Langendorff hearts were perfused in retrograde fashion at a perfusion pressure of 50 mmHg. This perfusion method was used for the measurement of heparin-releasable lipoprotein lipase activity (HR-LPL). In addition, all hearts were stabilized in Langendorff mode. The perfusion buffer was not re-circulating. Heparin displaces the endothelium-bound enzyme lipoprotein lipase (LPL) from its heparan sulphate proteoglycan binding sites on the luminal surface of capillary endothelial cells of the heart, releasing LPL into the perfusate (Elkes and Williams 1974; Mardy et al 2001). The buffer used for these experiments was a modified KHB with the addition of 3% bovine serum albumin (BSA).

At time 0, after the 10-minute stabilization period, the KHB perfusion buffer was switched to one that contained 3% BSA and 5 U/ml heparin (gassed, pH 7.4). Hearts were perfused with this non re-circulating buffer for the duration of the 20-minute experiment. At given time points, 0.5 ml samples of perfusate were collected and flash frozen. At the end of the experiment, the heart was removed from the perfusion apparatus and was flash frozen so that tissue LPL activity could be measured at a later time.

2.2 Working Heart Perfusions:

Hearts were perfused in working mode as described by Larsen et al. (1999). Isolation and aortic cannulation followed the same steps as described above for Langendorff perfusions, but following the removal of extraneous tissue the pulmonary vein was cannulated with a 16 gauge steel cannula. Once the pulmonary vein was cannulated the heart was ready to be switched into working mode. In working mode the

left atrium was perfused at a constant pre-load pressure of 15 mmHg, while the left ventricle pumped against a constant after-load pressure of 50 mmHg. Switching the heart from Langendorff into working mode entailed opening the atrial pre-load and the aortic after-load lines. The 40 ml working heart perfusion buffer was a modified KHB including 3% BSA and either a lipoprotein (chylomicron) or NEFA (palmitate) lipid source at a concentration of 0.4 mM. This perfusion solution was re-circulating and the perfusion apparatus was sealed such that infused oxygen exited the system at only one point.

Heart contractility (left ventricular pressure) was measured with a pressure transducer in the aortic after-load line (Millar Micro-Tip, Millar Instruments). Aortic pressure traces were recorded with CView Software (University of Calgary, Calgary AB, Canada) every 15 minutes for the 90 minute perfusion period. As aortic pressure traces were recorded, aortic and coronary flow rates were measured by timed collections of the flow in the afterload line and the buffer dripping from the bottom of the heart, respectively (Belke et al 2000). Every 30 minutes for the 90-minute perfusion period a 2.5 ml buffer sample was removed through an injection port for metabolic measurements (see below).

3. Isolation of Radiolabeled Chylomicrons:

Although perfused hearts from control and diabetic mice were used for studies of chylomicron metabolism, it was not technically feasible to isolate chylomicrons from mice due to their small size. Therefore, rats were the chylomicron source, as first described by Bollman et al. (1948), essentially using the procedures described by Mardy et al. (2001).

3.1 Intestinal Lymphatic Duct Cannulation and Chyle Collection:

Chylomicrons were collected from adult male rats (300-400 grams) acquired from local breeding sources provided by the University of Calgary. Rats were fasted the night prior to the lipoprotein isolation procedure. Approximately two hours prior to surgery, rats were fed a high-fat liquid meal (Ultra Shake; Abbott Laboratories Ltd, Sainte-Laurent, QC). Feeding the animals the high-fat meal stimulated the production of chyle which flowed through the otherwise transparent intestinal lymphatics, allowing better visualization for cannulation.

Prior to anesthetizing the rat it was important to prepare the tubing which was used to cannulate the intestinal lymphatic. The silastic tubing (1.19 mm OD, Dow, Midland, MI) was bathed in TDMAC solution (Tridodecylmethylammonium chloride with 2% heparin). TDMAC is a petroleum-based product that encourages the tubing to swell, allowing the heparin within the TDMAC solution to specifically bind with the tube surface. This reduced the potential of the chyle clotting. Once the tubing is removed from the TDMAC, it will take on its original dimensions. The tubing was bathed next in 75% alcohol for sterilization and efforts were made to keep the tubing clean. With a syringe, the now sterile heparinized tubing was infused with sterile phosphate buffer (PBS) solution. The PBS washed out the 75% alcohol and any remaining TDMAC. The silastic tubing was kept on the syringe so that immediately prior to cannulation PBS could be infused through the tube to remove air bubbles and reduce potential clotting.

Rats were anesthetized with ether. Ether is a better anesthetic then halothane as it is much easier on the animal, and rats come out of anesthesia much more comfortably. The abdomen of the rat was shaved, including the animals left side and the shaved area

was swabbed with 75% alcohol to disinfect and remove detritus. A mid-line incision was made from the lower abdomen to the xyphoid process (~ 5 cm). The abdomen was spread open with a retractor and sterile gauze was placed around the incision upon which the intestine was temporarily placed. The sterile gauze was kept moist with PBS solution. The liver was reflected to the top right (animal left) and the stomach and intestine to the left (animal right). The left kidney and renal vein was exposed; the adrenal vein could then be clearly observed entering the renal vein. The abdominal aorta was identified and the branches of the mesenteric and siliac arteries were found. The intestinal lymphatic duct lies adjacent to the mesenteric artery and between the mesenteric and siliac arteries (Figure 6). The lymphatic duct was separated from the mesenteric artery, very gently with the tip of a hemostat so as to not damage the lymphatic duct. Once the duct and artery were separated, a small hole was made in the connective tissue on the siliac side of the lymphatic duct. A tie was passed around the lymphatic duct for securing the tubing in place once it had been inserted into the duct. Anchor stitches (6.0 silk with BV-1 needle) were placed into the lymphatic duct in line (parallel) with the direction of the duct itself. The anchor ties were used to hold open the lymphatic duct once it had been cut to allow for the insertion of the tubing. Once all the above was complete and in place, the lymphatic duct was cut open with micro-scissors. Lymph flowed out of the severed lymphatic. With the anchor stitches holding the duct open, the tubing was inserted and secured with the previously placed tie. Once lymph was flowing, the silastic tubing was secured to muscle within the abdomen of the animal. A small hole (puncture) was made in the side of the animal. The tubing exited through this hole. The tubing was secured to the abdominal wall at the point where the tubing passed through the side of the animal.

The animal was injected with an antibiotic. Optimum draw of lymph from the animal occured when the tubing extended below the animal to a depth of 10 cm (Bollman et al. 1948).

To aid in preserving the chyle as it is isolated from the animal, three preservatives were included in the collecting tube: EDTA to prevent clotting, glutathione as an anti-oxidant and gentamycin as a preservative (40, 19.6 and 6.1 mg respectively). This combination was added to the collection tube for every 10 ml of chyle collected.

Once the surgery was complete and the silastic tubing secure, the rat was placed into a restraining cage for the duration of the collection protocol. The restraining cage allowed the animal to move forward and backward to access food and water but minimized movement which might dislodge or damage the silastic tubing. The rat was acclimatized to the restraining cage prior to the day of surgery.

3.2 Chylomicron Radiolabeling and Purification:

Chylomicrons must be labeled *in vivo* as an attempt to radiolabel isolated unlabeled chylomicrons with [^{3}H]triolein would result in the label only transiently associating with the exterior of the lipoprotein particle. *In vivo* labeling incorporates the radiolabeled triacylglycerol into the non-polar core of the chylomicron particle. *In vivo* labeling of chylomicrons was accomplished by feeding the rat a radiolabeled high-fat liquid meal containing [^{3}H]palmitate (approximately 600 μCi). The [^{3}H]palmitate was taken up by the intestines, esterified into triacylglycerol and incorporated into the core of the CM particle (CM-[^{3}H]TG) in the chyle.

In order to remove extraneous proteins and contaminating [^{3}H]palmitate, [^{3}H]chylomicrons in the collected chyle were isolated and purified by a series of centrifugations. The chyle to be purified was combined with an equal volume of saline and mixed very gently. The chyle saline mixture was then centrifuged at 25,000 rpm for 30 minutes. After centrifugation, the chylomicrons rested at the top of the mixture. The lower phase was removed and the remaining chylomicrons combined with a 4% BSA solution to bind any remaining free [^{3}H]palmitate. The chylomicrons were then isolated by a second centrifugation at 30,000 rpm for 30 minutes, followed by a third wash in saline to remove any remaining BSA (30,000 rpm for 40 minutes). Again the lower phase was removed after centrifugation and the remaining chylomicrons combined with a saline solution and stored at 4°C.

Lipid incorporation into isolated chylomicrons was measured by extraction and thin layer chromatography, essentially as described by Mardy et al. (2001). Chylomicron lipids were extracted by mixing 0.4 ml with 2 ml of a chloroform/methanol (2:1 v/v) mixture and 0.3 ml water. The mixture was centrifuged for 10 minutes at 3000 rpm. The upper aqueous phase was discarded while the lower organic phase was dried down under nitrogen. This lipid extract was then resuspended in 200 µl of the chloroform/methanol mixture with 5 µl of a lipid carrier solution containing monacylglycerol (MG), diacylglycerol (DG), FA and TG as markers. The resuspended lipid extract was then streaked onto a thin layer chromatography plate and run in a solvent of diethyl ether/heptane/acetic acid (75:25:1, v/v/v) for approximately 1 hour. The plate was allowed to dry and the bands were visualized by placing the plate in iodine vapor. The lipids migrated in the order of PL (origin), MG, DG, FA and TG (near the solvent front).

The lipid bands were added to scintillation vials and mixed with 0.5 ml ethanol and 3 ml of scintillation fluid (Ecolite, ICN Biomedical Research Products, Costa Mesa CA, USA). More than 90% of radioactivity incorporated into the chylomicron preparation was in TG.

In order to measure chylomicron metabolism by perfused mouse hearts, it was necessary to determine the total TG concentration of the radiolabeled chylomicrons so that the specific activity of LPL-derived FA could be calculated. This was accomplished with a triglyceride assay kit (GPO-Trinder) from Sigma Diagnostics (St. Louis, USA). To facilitate the activity of LPL in metabolizing the lipoprotein substrate, 1% serum (rat) was included in the assay mixture. The TG concentration of the stock chylomicron preparation used in these experiments was 55 mM.

4. Chylomicron Metabolism by Perfused Working Mouse Hearts:

Lipoproteins have accessory proteins on their surface called apolipoproteins (Ginsberg 1998). As described earlier these apolipoproteins have specific functions and some are necessary for lipoprotein metabolism. Chylomicrons require an apolipoprotein, apoCII, for LPL catalyzed hydrolysis of core TGs; apoCII is normally acquired in the blood stream by exchange with other lipoproteins (HDL; Figure 3). Chylomicrons were isolated directly from the lymph, prior to their entrance into the blood stream, because they have a very short half-life once they have entered the blood (approximately 10 minutes). Therefore, the required apoCII (LPL activator) was added *in vitro*. This was done by incubating (37 °C) the isolated chylomicrons with 3% heat-inactivated rat serum (10 minutes) prior to their addition to the working heart perfusion buffer.

The chylomicron substrate buffer was the KHB described above with the addition of 3% BSA. At time 0 of the experiment the chylomicron/serum mixture (0.4 mM TG; specific activity 1594 dpm/nmol LPL-derived FA) was added through an injection port into the re-circulating perfusate for metabolism by the isolated perfused mouse heart.

The metabolism of chylomicrons was measured over a 90-minute perfusion protocol. The heart was allowed to stabilize in working mode for 2.5 minutes before the chylomicron/serum mixture was added. The chylomicrons were allowed to mix with the perfusion buffer for another 2.5 minutes before the experiment was initiated. Throughout the 90-minute experiment, functional measurements (aortic flow, coronary flow, heart rate and peak systolic pressure) were taken every 15 minutes. With these functional measurements, cardiac output (ml/min) and the rate-pressure-product (mmHg/min x 10^{-3}) were calculated. Perfusate samples (2.5 ml) were withdrawn from the re-circulating buffer every 30 minutes through an in-line injection port and immediately flash frozen for later analysis. At the end of the experiment the heart was cut down and flash frozen.

4.1 Oxidation of LPL-derived FA:

Chylomicron oxidation was determined by measuring the amount of ^3H_2O released during the 90-minute perfusion period, as outlined by Saddik and Lopaschuk (1991) and Mardy et al. (2001). During β-oxidation [^{3}H] is transferred from LPL-derived [^{3}H]FA (9 and 10 positions of 9,10 [^{3}H] palmitate) to nicotinamide adenine dinucleotide (NADH) and flavine adenine dinucleotide ($FADH_2$). These reduced coenzymes then deliver [^{3}H] to the electron transport chain where it is combined with oxygen to form water at complex IV (Belke et al. 1999). The ^3H_2O is released from the heart into the re-

circulating perfusate buffer; ^3H_2O content was measured in perfusate samples removed at 30-minute intervals during the 90 minute perfusion.

An aliquot (0.5 ml) of the perfusate sample was combined with 1.9 ml of a chloroform methanol mixture (1:2 v/v), followed by an additional 0.625 ml of chloroform and 0.625 ml of a KCl / HCl solution (1.1:0.9 M ratio). The chloroform will draw the unhydrolyzed lipids into the lower phase while all the protein (BSA) will rest between the upper and lower phases. The upper aqueous phase contains the ^3H_2O produced by the metabolism of LPL-derived [^{3}H]FA. The solution was mixed vigorously and then centrifuged for 15 minutes at 2700 rpm resulting in an aqueous upper and chloroform lower phase. The lower phase was removed and discarded. To the remaining aqueous phase 2 ml of a chloroform methanol (1:1 v/v) solution was added followed by 0.9 ml of the KCl / HCl solution. The mixture was again mixed vigorously and centrifuged for 15 minutes at 2700 rpm. Triplicate samples (0.5 ml) of the upper phase of each sample tube were added to scintillation vials, scintillation fluid (3 ml, Ecolite, ICN) was then added and [^{3}H] radioactivity was measured. The amount of LPL-derived [^{3}H]FA oxidized to ^3H_2O was calculated as described by Belke et al. (1999). It was assumed that all three fatty acids of the triacylglycerols in the chylomicron core were labeled for the calculation of LPL-derived [^{3}H]FA specific activity. Rates of fatty acid metabolism are reported as the average of three time points (0-30, 30-60 and 60-90 minutes) unless otherwise stated.

4.2 Esterification of LPL-derived FA:

Ex vivo perfused hearts frozen at the end of the 90-minute perfusions were thawed, sliced into small pieces and homogenized 2x30 (4°C) sec in 3 ml of

chloroform/methanol (2:1, v/v) (Polytron #6 setting). The homogenate was diluted in 1 ml water, vortexed and centrifuged for 10 minutes at 3500 rpm. The upper phase was discarded and the lower dried down under nitrogen. The lipid extract was then resuspended in 1 ml chloroform; 50 µl was spotted onto the TLC plate along with the lipid standards MG, DG, FA, TG and PL. The plate was run in 100 ml solvent of diethyl ether/heptane/acetic acid (75:25:1, v/v/v) for approximately 1 hour. The bands were visualized by placing the plate in iodine vapor and then added to scintillation vials with 0.5 ml methanol and scintillation fluid (Ecolite, ICN). Through measuring the amount of radioactivity in each of these lipid categories and the specific radioactivity of the lipid substrate (chylomicron), the total amount of radioactivity incorporated into the lipid classes in the heart over the perfusion period was determined.

4.3 Calculation of Metabolic Rates:

All metabolic rates are presented in relation to heart dry weight in order to account for slight variations in heart size. The dry mass of the heart was calculated by cutting off a piece of each frozen heart and allowing it to thaw, and then dry. The dry to wet ratio of this piece was used to calculate the dry weight of the whole heart.

5. NEFA (Palmitate) Metabolism by Perfused Working Mouse Hearts:

The study of NEFA metabolism required that [^{3}H]palmitate be bound to BSA. As described by Belke et al. (1999), this was accomplished by dissolving palmitic acid in 25 ml of a water/ethanol mixture (60:40 v/v) containing 0.55 g Na_2CO_3/g palmitate. The water/ethanol mixture was boiled until all the ethanol had evaporated and then poured

rapidly into a mixing KHB/BSA solution (glucose free). This solution was then dialyzed (8000-12000 mw cutoff, SPECTRAPOR, Spectrum Medical Industries, LA, USA) overnight in a 10x (vol) of KHB at 4°C. The next morning glucose (11 mM) was added to the dialyzed solution which was finally filtered (0.45 μm, COSTAR, Fischer Scientific, Edmonton AB, Canada) and frozen for future use.

The perfusion procedure was very similar to that for the chylomicron substrate. After the 10-minute stabilization period hearts were switched into working mode. The hearts were stabilized in working mode for 5 minutes before the experiment was initiated. Functional measurements were taken every 15 minutes and perfusate samples (2.5 ml) every 30 minutes. Hearts were flash frozen at the end of the experiment for later analysis of esterification into tissue lipids.

5.1 Palmitate Oxidation:

The oxidation of [^{3}H]palmitate (0.4 mM in the perfusate) was determined by measuring the release of ^3H_2O into perfusate samples collected at 30 minute intervals during the 90-minute working heart perfusion, exactly as described above for the oxidation of LPL-derived FA.

5.2 Palmitate Esterification:

At the end of the perfusion, the incorporation of radiolabel into tissue lipids (TG) was determined by lipid extraction and thin layer chromatography, as described above.

5.3 Metabolic Rates:

Rates of [^{3}H]palmitate oxidation and esterification were corrected for dry heart weights, as described for chylomicron metabolism.

6. LPL Assay:

As described by Carroll et al. (1995), this routine *in vitro* assay measures LPL activity by using an artificial triacylglycerol substrate, radiolabeled [^{3}H]triolein (glycerol tri-[9,10(n)-^{3}H] oleate). Lipoprotein lipase will hydrolyze [^{3}H]triolein (0.6 mM, 1 Ci/mol) into free radiolabeled [H^3]oleate. The level of LPL hydrolysis is determined by a simple one step liquid-liquid partition system for isolation of the free fatty acid product from any unhydrolyzed [^{3}H]triolein substrate.

The assay entails the sonication of triolein into vesicles in the presence of 0.05% BSA (w/v) and 25 mM Pipes, pH 7.5. This solution is then incubated with 3% heat inactivated chicken serum (apoCII source), 50 mM $MgCl_2$ and the LPL source (perfusate sample from Langendorff perfusions or heart homogenates) for 30 minutes at 37 °C (final volume of 400 µl).

The reaction was stopped and the hydrolyzed fatty acids extracted by the addition of 3 ml of fatty acid extraction solution (FAES) to the assay tubes followed by 0.1 ml of 1.0 M NaOH (to bring the pH to >12). The FAES solution consists of methanol, heptane, chloroform and oleic acid in a 1410:1000:1250:0.01 (v/v/v/v) ratio. The mixture was then centrifuged for 10 minutes at 2700 rpm. The FAES solution extracts the hydrolyzed [^{3}H]oleate (sodium salt) into the upper aqueous phase, free from the unhydrolyzed

[^{3}H]triolein substrate in the lower chloroform phase. A 0.5 ml sample of the upper phase was then counted to determine the [^{3}H]oleate generated by LPL hydrolysis.

Tissue samples were prepared for the LPL assay by first removing the atria and homogenizing (2x30 sec, Polytron) the remaining tissue in a lysis buffer (2 ml). The lysis buffer contained 25 mM NH_4Cl, 5 mM EDTA (pH 8.2), 0.8% Triton X-100, 0.04% sodium dodecyl sulphate, 33 μg/ml heparin and 10 μg/ml leupeptin. The role of the lysis buffer was to breakdown the tissue and solubilize LPL. The homogenate was centrifuged at 3000 rpm for 15 minutes at 4°C. The low-speed supernatant was diluted 1:10 (v/v) with a buffer (pH 7.5) containing (in mM) 250 sucrose, 10 Hepes, 1 EDTA and 1 dithiothreitol. The diluted supernatant was then assayed for LPL activity, using the standard LPL assay.

All assays were performed in duplicate, under conditions where the LPL assay was linear with respect to time of incubation and quantity of LPL enzyme. LPL activity is routinely expressed as nmol oleate released per hour per ml of perfusate or per mg of tissue homogenate protein, measured using the Coomassie Protein Assay Reagent kit (Pierce, Rockford IL).

In some experiments, a [^{3}H]chylomicron (0.6 mM in the final assay volume) preparation replaced [^{3}H]triolein as the assay substrate. This was done to establish the validity of using [^{3}H]chylomicrons as a substrate for endothelium-bound LPL in perfused mouse hearts.

7. Statistics:

Where applicable, the unpaired t-test was used to determine significant differences between groups. A p value of less than 0.05 was considered significant.

8. Materials:

i) [9,10(n)-^{3}H]Palmitic acid (specific radioactivity 2.00 TBq/mmol) was obtained from Amersham Pharmacia Biotech (Buckinghamshire, England).

ii) Glycerol tri [9,10(n)-^{3}H]oleate ([^{3}H]triolein, specific radioactivity 740 GBq/mmol) was obtained from Amersham Life Science (Buckinghamshire, England).

iii) TDMAC-heparin (Tridodecylmethylammonium heparinate), 2 % (w/w) solution (~ 1000 USP units/ml) obtained from Polysciences Inc. (Warrington PA, USA).

iv) Heparin-sodium (1000 iu/ml) (Porcine Intestinal Mucosa) from Leo Pharma Inc. (Ajax ON, Canada).

v) Bovine Serum Albumin , Fraction V (minimum 96 %) obtained from Sigma Chemical Company (St. Louis MO, USA).

RESULTS.

1. Lipoprotein Lipase Activity - In Vitro Assays:

The *in vitro* assay typically utilizes an artificial lipoprotein substrate, radio-labeled [^{3}H]triolein (glycerol tri-[9,10(n)-^{3}H] oleate) as the LPL substrate. This artificial substrate is hydrolyzed by LPL, producing [^{3}H]oleate; LPL-catalyzed hydrolysis is measured by a simple one step liquid-liquid extraction technique (Carroll et al. 1995). The LPL assay was used to quantify the amount of LPL released by heparin (5 U/ml) from the *ex vivo* Langendorff perfused mouse heart (HR-LPL as an index of endothelium-bound LPL) and the level of tissue LPL activity. Therefore, it was necessary to characterize the LPL assay and confirm its linearity with respect to time, serum (apoCII source) and lipoprotein lipase amount. These three variables were assessed using both radio-labeled [^{3}H]triolein and [^{3}H]chylomicron substrates *in vitro* in order to test the validity of using isolated [^{3}H]chylomicrons to determine the metabolism of LPL-derived FA by isolated perfused working hearts from control and diabetic mice.

1.1 Time Dependence:

The LPL assay typically consisted of a 30-minute incubation period. Therefore, it was important to show that the assay was linear for the entire 30-minute period. The LPL assay was performed with both [^{3}H]triolein and [^{3}H]chylomicron substrates, both at 0.6 mM. For both lipid substrates, the LPL assay was linear with time with an R^2 value of 0.99 (Figure 7).

1.2 ApoCII Dependence:

Lipoprotein lipase is activated by apoCII. The artificial substrate created by sonicating [^{3}H]triolein does not have the apoCII protein, as a lipoprotein substrate would *in vivo*. In addition, the chylomicrons isolated from the intestinal lymphatic of the rat may not contain sufficient apoCII as it is in the blood stream that the majority of this activator is acquired (Ginsberg 1998; see Figure 3). Therefore, it was necessary to include an exogenous source of apoCII (heat inactivated chicken serum) in the *in vitro* LPL assay. To ensure the LPL assay contained the appropriate quantity of apoCII to activate LPL maximally, the activity of LPL was measured with different serum quantities.

With the artificial [^{3}H]triolein substrate LPL activity was increased dramatically upon the addition of serum and reached its maximal level of activity at 0.5 % serum (v/v) (Figure 8a). The activity of LPL remained at a constant level from 0.5 to 3.0 % serum, and then declined. This reduction in LPL activity is most likely the result of other proteins and complexes impairing LPL catalytic function. At 0 % serum LPL activity was approximately 1/3 of its maximal activity at 3 % serum. This suggests that some LPL hydrolyzing activity with the [^{3}H]triolein substrate can be measured in the absence of the apoCII activator.

The chylomicron substrate appeared to be a good substrate as characterized by the *in vitro* LPL assay. LPL activity was not serum (apoCII) dependent using the chylomicron substrate; activity was constant from 0-3 % serum (Figure 8b). Again by 6 % serum, LPL activity began to decline, most likely for the same reasons as discussed above. Overall, LPL activity measured with a [^{3}H]chylomicron substrate was comparable to apoCII-stimulated activity based on [^{3}H]triolein hydrolysis.

1.3 Enzyme Dependency:

The LPL assay requires an enzyme source, either a post-heparin perfusate or a heart tissue homogenate. To draw conclusions about the validity of assessing endothelium-bound LPL activity by assay of post-heparin perfusate, it was necessary to show that the assay was linear through various LPL amounts as represented by post-heparin perfusate volume.

The LPL source for this experiment was the post-heparin perfusate from an isolated perfused mouse (Swiss-Webster) heart. Perfusate volumes ranging from 0-100 µl for [^{3}H]triolein assays and 0-160 µl for the [^{3}H]chylomicron substrate were included in the assay (total assay volume was constant at 400 ul).

The LPL assay for both lipid substrates was linear through all LPL amounts with R^2 values of 0.99 (Figure 9). This clearly indicates that the assay is an effective tool for measuring LPL activity.

In contrast to other lipases such as hepatic lipase, the catalytic activity of LPL is inhibited by high ionic strength. To ensure that the hydrolyzing activity in the post-heparin perfusate was due only to LPL, the assay was conducted in the presence of 1 M NaCl. In the presence of this high ionic strength solution, all hydrolyzing activity was inhibited in an assay with the [^{3}H]chylomicron substrate (Figure 10). This result further establishes the validity of the LPL assay.

2. Chylomicron Characterization:

Before beginning metabolic experiments, the chylomicron preparation was characterized thoroughly.

After the washing and purification procedure, [^{3}H]chylomicrons had an average TG concentration of approximately 55 mM, with a specific activity (approx 1594 dpm/nmol FA) sufficient to ensure that the perfused hearts would metabolize enough radio-active TG to allow for the detection of ^3H_2O production (oxidation of LPL-derived FA) and incorporation of radioactivity into tissue lipids (esterification).

Thin layer chromatography was conducted to determine the lipid profile of the chylomicron substrate. Results indicated that the radiolabel in the chylomicron preparation was over 93 % TG with the remaining 6.75 % of the label distributed in phospholipid, diacylglycerol, fatty acid and monoacylglycerol (Figure 11).

Further characterization included varying the concentration of [^{3}H]chylomicrons in the *in vitro* LPL assay. This was done to ensure that the concentration (0.4 mM) of substrate included in the perfusion buffer of mouse hearts would be metabolized optimally. Concentrations of 0 - 1.0 mM were investigated, and the results followed simple Michaelis-Menten kinetics (Figure 12). Therefore, a chylomicron concentration of 0.4 mM lays well within the range of optimal hydrolysis by LPL as indicated by the *in vitro* LPL assay.

As discussed earlier, the chylomicrons collected from the lymphatic system are not considered mature as they lack certain apolipoproteins such as apoCII. The *in vitro* LPL assay indicated that LPL activity with the [^{3}H]chylomicron was not increased by serum as an apoCII source (Figure 8b). To investigate whether this was also the case for endothelium-bound LPL in the perfused heart, isolated heart perfusions were conducted with 0, 1 and 3 % heat inactivated rat serum (v/v) added to the [^{3}H]chylomicron substrate. Results from heart perfusions were similar to what had been observed *in vitro*.

Specifically, the activity of endothelium-bound LPL, as evidenced from the oxidation of LPL-derived FA (^3H_2O production) was not increased by addition of 1% or 3% rat serum (Figure 13). Nevertheless, it seemed reasonable to include 1 % serum to measure the metabolic fate of LPL-derived FA, in part to be consistent with the previous work by Mardy et al. (2001) on chylomicron metabolism by isolated perfused Swiss-Webster mouse hearts.

3. Characterization of Diabetic Mouse Models:

This research investigated the impact of both a type 1 and type 2 diabetic condition on the metabolism of two lipid substrates, a lipoprotein source (chylomicrons) and a NEFA source (palmitate).

3.1 Body Weight:

Type 1 (insulin-deficient) diabetes was chemically induced in experimental mice by the administration of streptozotocin (STZ). Swiss-Webster (SW) mice groups were approximately 37 g at the time of STZ or carrier (citrate buffer) administration (Table 1). A second type 1 diabetic model was used to control for strain differences between the SW and *db/db* mouse. C57BL/KsJ *db/+*mice were also treated with STZ through the same procedure as described for the SW mice. The SW control group gained weight (approximately 2 g) over the 2-week period. In contrast, the SW diabetic group lost a significant amount of weight (approximately 5 g), and thus had significantly lower body weights than the control group (31.9 +/- 0.7* vs. 39.2 +/- 1.27 g) after 2 weeks. The type 1 diabetic STZ-treated *db/+* mice also lost a significant amount of weight (approximately

8 g) over the two week period, and had significantly lower body weights than their control *db/+* counterparts (19.8 +/- 0.64* vs. 30.0 +/- 0.69 g). These findings of significant weight loss after induction of insulin-deficient type 1 diabetes are consistent with other reports in the literature for both mice (George et al. 2002) and rats (Sheperd et al. 1998, Rodrigues et al. 1997) treated with streptozotocin.

The type 2 diabetic *db/db* animal has a leptin receptor defect in which the long form of the leptin receptor is not present (Leibel et al 1997). These animals are therefore leptin resistant and as a consequence hyperphagic and obese. The *db/db* mice were significantly heavier than their *db/+* littermates (52.6 +/- 1.08* vs. 30.0 +/-0.69 g).

3.2 Heart Weight:

As described above, the type 1 diabetic models (SW and *db/+*) had significantly lower body weights compared to their littermate controls. Further to this, heart weights in both the SW (29.0 +/- 1.28* vs. 33.3 +/- 0.9 mg) and *db/+* (22.6* +/- 1.39 vs. 28.0 +/- 1.83 mg) diabetic groups were significantly lower as well (Table 1). It has been reported in the literature that STZ-induced type 1 diabetes may result in reductions in heart weight (Howarth and Qureshi 2001) associated with depressed cardiac contractility. Heart weights for type 2 *db/db* mice were the same as for non-diabetic littermates (Table 1).

3.3 Blood Glucose:

As expected, both the type 1 and 2 diabetic mouse models had significantly higher levels of blood glucose than their non-diabetic controls (SW, 30.1 +/- 0.81 vs. 8.6 +/-

0.48; *db/+stz*, 28.5 +/- 1.43 vs. 10.2 +/- 0.56; *db/db*, 31.6 +/- 1.08 vs. 10.2 +/- 0.56 mmol/L).

4. Lipoprotein Lipase Activity in Hearts from Control and Diabetic Mice:

Lipoprotein lipase (LPL) is a capillary endothelium-bound enzyme in the heart which hydrolyzes the TG core of circulating lipoproteins (Goldberg 1996). The hydrolysis of lipoprotein-TG by LPL releases free FA for uptake by the cardiomyocyte. Thus, LPL plays a regulatory role in the delivery of lipoprotein-derived FA to metabolically active tissues like the heart. For this reason LPL activity is of interest in the diabetic state as it has been shown that FA metabolism (increased palmitate oxidation) is altered in db/db hearts (Belke et al. 2000). LPL is bound to heparan sulphate proteoglycans (HSPG) on the endothelial surface. LPL can be displaced from HSPG binding sites by heparin, releasing LPL into the perfusate. By including heparin within the heart perfusion buffer and collecting the coronary perfusate, the amount of LPL bound to the capillary endothelium (functional enzyme) of hearts from control and diabetic mice can be measured.

4.1 Post Heparin Perfusate (Heparin-Releasable LPL Activity):

Lipoprotein lipase activity was measured over a 20-minute Langendorff perfusion period, with buffer samples taken at given time points. There was a very low basal level of LPL release prior to the administration of heparin (Figure 14). LPL release from the heart was greatest within the first 1-2 minutes of perfusion with heparin, declining to almost basal levels by approximately 5 minutes. Peak heparin-induced LPL release in the

type 2 diabetic model was not significantly different from the non-diabetic controls (Table 2 and Figure 14). But both type 1 diabetic models (STZ induced) showed a significantly higher level of peak release compared to the peak type 2 diabetic *db/db* release (SW, 2462 +/- 231*; *db/+stz*, 2468 +/- 286*; *db/db*, 1912 +/- 146 nmole/hr/ml). However, the total cumulative amount of LPL released by the diabetic animals compared to their littermate controls, or between type 1 and type 2 diabetic models, was not significantly different (SW, 10477 +/- 832 vs. 9650 +/- 1102; *db/+stz*, 9584 +/- 1250 vs. 9085 +/- 829; *db/db*, 10522 +/- 653 vs. 9085 +/- 829 nmole/hr). This suggests that the delivery of LPL-derived fatty acids is not altered in the type 1 or type 2 diabetic state.

4.2 Tissue (Residual) LPL Activity:

The heparin perfusion buffer releases LPL bound to the capillary endothelial surface. As described earlier, LPL is synthesized and processed within the myocyte and then is translocated to its endothelial site of action based upon metabolic demand. Total heparin-induced LPL release from the endothelium over a 20-minute perfusion period was not different in diabetic hearts as compared to control hearts. To complete this investigation, the amount of LPL retained within the cardiac tissue at the end of the 20-minute perfusion period was also measured. Results indicate that there is no significant difference in the amount of residual LPL activity measured in post-heparin tissue homogenates, for both type 1 and 2 diabetic models compared to their non-diabetic controls (Table 2).

If the three pools of LPL activity that have been measured are compared, ~ 82 % of LPL is retained within the tissue, ~ 17 % is released in the post-heparin perfusate and < 1 % is released basally into the pre-heparin perfusate.

5. Chylomicron Metabolism by Working Hearts from Control and Diabetic Mice:

As discussed earlier data indicates that the metabolism of both glucose and NEFA (palmitate) is altered in *db/db* hearts (Belke et al. 2000). Results presented above indicate that the supply of LPL-derived FA will not be different for diabetic hearts, since functional endothelium-bound LPL activity was unchanged (Figure 14 and Table 2). Therefore, the following experiments were conducted to determine if the metabolic fate of LPL-derived FA would be altered in perfused diabetic hearts.

Hearts were perfused with the [^{3}H]chylomicron substrate at a concentration of 0.4 mM for 90 minutes, during which time cardiac metabolism and function were measured. Cardiac metabolism of LPL-derived FA was measured by analyzing two metabolic fates, oxidation and esterification (storage) into tissue lipids. Oxidation was assessed through measuring the production of ^3H_2O in the perfusion buffer, and is presented as the average of three perfusion periods; 0-30, 30-60 and 60-90 minutes. At the end of each perfusion (90 minutes), incorporation of radioactivity into tissue lipids was measured by thin layer chromatography.

5.1 Oxidation of LPL-derived FA:

The metabolism of chylomicrons and the consequent accumulation of ^3H_2O in the perfusion buffer was reasonably linear throughout the 90-minute perfusion period for all

hearts, control and diabetic. For the type 1 diabetic hearts (STZ-treated SW and *db/+* mice), the level (μmol ^3H_2O/g dry wt) of chylomicron oxidation was significantly higher throughout the 90-minute perfusion period (Figure 15a and 16a), compared to control hearts. Consequently, the average rate of oxidation for hearts from both type 1 diabetic models was significantly greater than their respective controls (SW, 0.438 +/- 0.09* vs. 0.132 +/- 0.03; *db/+stz*, 0.441 +/- 0.06* vs. 0.139 +/- 0.02 μmol ^3H_2O/min/g dry wt) (Table 3).

A similar trend was observed in the type 2 diabetic *db/db* model. At all time points the cumulative oxidation of LPL-derived FA was significantly greater in perfused working *db/db* hearts as compared to the control *db/+* hearts (Figure 18a). As seen with the type 1 diabetic hearts, the *db/db* hearts also had a significantly larger average rate of oxidation than the control db/+ hearts (0.321 +/- 0.037 vs. 0.139 +/- 0.02 μmol/min/g dry wt) (Table 3).

5.2 Esterification of LPL-derived FA:

The incorporation of radiolabeled FA into cardiac tissue lipids was determined by the separation of the lipid fractions of heart homogenates by thin layer chromatography. Both type 1 diabetic hearts had a significantly higher rate of fatty acid esterification into TG than the non-diabetic controls (SW, 0.197 +/- 0.04* vs. 0.079 +/- 0.014; *db/+*, 0.181* +/- 0.02 vs. 0.101 +/- 0.02 μmol/min/g dry wt) (Figure 15b and 16b). This was also the case for the type 2 diabetic db/db hearts (0.168 +/- 0.03* vs. 0.101 +/- 0.02 μmol/min/g dry wt) (Figure 18b and Table 3).

Through measuring the distribution of lipid fractions (TG, PL, FA, DG and MG) in heart tissue by thin layer chromatography, the vast majority (~ 75 %) of radioactivity appeared to be incorporated into TG (Figure 19a).

6. NEFA (Palmitate) Metabolism by Working Hearts from Control and Diabetic Mice:

Belke et al. (2000) has reported that palmitate oxidation was increased in perfused *db/db* hearts. Therefore, these experiments looked at the metabolism of radio-labeled palmitate in only the type 1 diabetic models.

6.1 Palmitate Oxidation:

The oxidation of [^{3}H]palmitate by perfused hearts from type 1 diabetic Swiss-Webster mice followed a similar trend as that observed for chylomicron metabolism (oxidation of LPL-derived FA). The accumulation of ^3H_2O in the perfusion buffer was linear through the 90-minute perfusion period and significantly greater in the diabetic hearts at all time points (Figure 20a). The average rate of metabolism over the perfusion period was significantly higher in the diabetic hearts from the STZ-treated SW mice (1.118 +/- 0.09* vs. 0.541 +/- 0.11 μmol/min/g dry wt) (Figure 21a and Table 3).

Perfused hearts from the second type 1 diabetic model (STZ-treated *db/+* mice) also oxidized palmitate in a linear fashion for the 90-minute perfusion period (Figure 22a). The cumulative oxidation of [^{3}H]palmitate was significantly greater by these diabetic hearts at all time points other than at 60 minutes (p=0.056). The average rate of metabolism over the perfusion period was significantly higher in the diabetic animals as compared to their littermate controls (1.01 +/- 0.09* vs. 0.471 +/- 0.172 μmol/min/g dry

wt) (Figure 21a and Table 3). The observed rate of oxidation for the control (*db/+*) hearts is similar (0.35 μmol/min/g dry wt) to what has been reported in the literature (Belke et al. 2000).

These results also indicate that the oxidation of [^{3}H]palmitate is significantly greater than the metabolism of chylomicrons (LPL-derived FA) by both control and diabetic experimental groups (Table 3).

6.2 Palmitate Esterification:

The methods for analyzing the incorporation of radio-labeled palmitate into tissue lipids is the same as that described for the chylomicron substrate. In both type 1 diabetic models, the esterification of [^{3}H]palmitate into tissue lipids was greater in the diabetic hearts compared to the non-diabetic control hearts (SW, 0.247 +/- 0.02* vs. 0.171 +/- 0.03; *db/+stz*, 0.231* +/- 0.06 vs. 0.159 +/- 0.04 μmol/min/g dry wt) (Figure 20b, Figure 21b and Table 3). Again the vast majority (~ 75 %) of radioactivity was incorporated into TG (Figure 19b), as was observed for the esterification of LPL-derived FA.

7. Oxidation / Esterification Ratio:

Total metabolic fate was calculated as a relative percentage for both oxidation and esterification. Approximately 65 % of LPL-derived FA was oxidized and 35 % esterified (Figure 23). These percentage oxidation results are slightly higher than those observed by Mardy et al. (2001) who found that chylomicron oxidation was approximately 50 % of total lipid utilization for control SW hearts. In contrast, metabolism of [^{3}H]palmitate provided different results in that the ratio of oxidation to esterification was approximately

80:20 %. These results are similar to that found by Mardy et al. (2001) and Evans et al. (2001).

There does appear to be a difference in the ratio of oxidation to esterification for the lipid sources, chylomicrons and palmitate. This difference was significant for two of the experimental groups, hearts from the type 1 diabetic (STZ-treated SW) and the non-diabetic control SW hearts (Figure 23), where the percentage oxidation of palmitate was significantly higher than for LPL-derived FA oxidation.

8. Cardiovascular Function:

Studies have suggested that the diabetic heart suffers from contractile dysfunction (Lopaschuk 1996; Belke et al. 2000). This dysfunction is expressed as depressed cardiac contractility and reductions in recovery from ischemic insults. Recently, changes in lipid metabolism (lipotoxicity) have been implicated as the cause for contractile dysfunction in diabetic hearts (Zhou et al. 2000).

Results presented above indicate that there is a significant elevation in the extent of lipid metabolism in the diabetic heart, for perfusions with both chylomicrons and palmitate. To investigate the potential of a reduction in cardiovascular function, several functional parameters were measured including aortic flow, coronary flow, heart rate and peak systolic pressure. These functional measurements were taken every 15 minutes during the 90-minute perfusion protocol.

Aortic and coronary flow rates (ml/min) were not significantly reduced in diabetic hearts as compared to controls (Table 4). Cardiac output (aortic flow + coronary flow) was as well not significantly different in diabetic hearts versus controls. This was true for

all experimental groups with the exception of the type 1 diabetic stz-treated *db/+* animals. In this case aortic flow and consequently cardiac output was significantly lower than the db/+ control hearts. However, these hearts were also significantly smaller (Table 1) as indicated by heart mass (mg dry weight) than controls. When normalized for heart weight, the chylomicron-metabolizing diabetic hearts were no longer significantly different, although hearts metabolizing the NEFA (palmitate) substrate remained significantly lower than controls. This significance stems from the very high flow rates observed in the *db/+* control animals as the STZ-treated *db/+* hearts produced similar flows to the other experimental groups (Table 4).

Peak systolic pressure generation was measured with a Millar pressure transducer and recorded by CView software. Results indicate that peak systolic pressure (mmHg) was not significantly different between the diabetic and control experimental groups. Heart rates (bpm) were calculated based upon the aortic pressure trace. All diabetic hearts showed a significant reduction in heart rate compared to non-diabetic control hearts (Table 4).

The product of heart rate and peak systolic pressure (rate pressure product) provides a measure of myocardial mechanical performance (Hauton et al. 2001). Consequently, the rate pressure product (RPP) of each heart was calculated every 15 minutes for the perfusion period. The RPP (mmHg/min x 10^{-3}) for hearts metabolizing the chylomicron substrate was quite constant over the 90-minute perfusion period with a small decline in function appearing at approximately 60-75 minutes. The diabetic hearts on average had a significantly lower RPP over the perfusion period indicating a significant reduction in cardiovascular mechanical performance (Figure 24).

The type 1 diabetic groups metabolizing palmitate showed a much more significant reduction in myocardial performance over the perfusion period in both control and diabetic hearts. When the average RPP for the 90-minute perfusion period is calculated, the diabetic heart performance was significantly lower than control (Figure 25).

These functional measurements indicate that there is a significant reduction in cardiovascular function associated with the type 1 and 2 diabetic condition; the extent of this dysfunction is influenced by the nature of the lipid substrate presented to the diabetic heart.

9. Summary of Results:

The supply of LPL-derived FA was not altered in diabetic mouse hearts, as the amount of LPL measured at the luminal surface of capillaries and within tissue was unchanged. However, results indicate that both oxidation and esterification of chylomicrons was elevated in the diabetic hearts. Therefore, the fate of LPL-derived FA was altered in hearts from both type 1 and type 2 diabetic mice. In addition, the metabolism of a NEFA substrate (palmitate) was significantly elevated in hearts from type 1 diabetic mice. In concert with elevated levels of lipid metabolism (Table 3), a significant reduction in myocardial mechanical performance was observed in diabetic hearts (Figure 25).

DISCUSSION.

The *ex vivo* perfused heart model allows for the investigation of various cardiac contractile parameters including aortic flow, coronary flow and heart rate, as well as the concomitant measurement of carbohydrate and fat metabolism. In addition, this preparation allows for the control of mechanical work through stimulating heart rate as well as controlling pre-load and after-load pressures. It is for these reasons that this model is used extensively (Grupp et al. 1993; Jeffery et al. 1995; Belke et al. 1999, 2000; Mardy et al. 2001) and was the model of choice for our investigation into cardiac metabolism and function.

1. Lipoprotein Lipase:

Lipoprotein lipase, originally named 'clearing factor lipase', was identified as an enzyme intrinsic to the metabolism of lipoproteins almost 60 years ago (Hahn 1943). Throughout the ensuing years many studies were conducted to characterize this enzyme (Borensztajn et al. 1970; Elkes and Williams 1974; O'Looney et al. 1983; Rodrigues et al. 1997). Work in the early 1960's brought fat metabolism into the picture with respect to the functional and metabolic cardiovascular defects identified in the diabetic state (Randle et al. 1963). Recognizing that fat metabolism played a role in the cardiovascular complications associated with diabetes lead investigators to explore various aspects of fat and lipoprotein metabolism, including lipoprotein lipase activity (Elkes and Williams 1974; O'Looney et al. 1983; Mardy et al. 2001).

The majority of studies investigating the activity of lipoprotein lipase over the last 25 years have used a diabetic rat model. The type 1 diabetic model was generally

induced through the administration of streptozotocin (Tomlinson et al. 1992), although early studies also used the compound alloxan. There is very little agreement within the literature as to the changes in heparin-releasable or tissue LPL activity that result from diabetes. To our knowledge there have been no studies that have investigated LPL activity in a type 1 diabetic murine model, and only one in the type 2 diabetic *db/db* mouse model (Kobayashi et al. 2000). The methodology of the Kobayashi study was significantly different than that utilized in this thesis research as they did not investigate heparin-releasable LPL activity in the heart itself. Whole body LPL activity was measured in the plasma 5 minutes after the injection of heparin (100 u/kg body weight) into the tail vein. They showed a significant reduction in heparin-releasable LPL activity. In this study Kobayashi did investigate residual cardiac tissue LPL mRNA levels and found a significant decrease in the diabetic heart compared to controls. Due to the significant differences in methodology the results of Kobayashi do not provide a strong point of reference or comparison with the results in this thesis.

The first objective of this thesis research project was to determine if endothelium-bound (heparin-releasable) HR-LPL activity was altered in hearts from STZ-treated (type 1) or *db/db* (type 2) diabetic mice. Possible mechanisms that could change HR-LPL activity in diabetic hearts include: (i) altered synthesis and/or processing of LPL in cardiomyocytes; (ii) altered translocation from cardiomyocytes to functional sites on the surface of capillary endothelial cells; and (iii) altered binding to the endothelium because of changes to heparan sulphate proteoglycans on the cell surface (Ebara et al. 2000).

The experimentally determined activity of cardiac LPL appears to be influenced by many different factors such as animal strain, type of diabetes, route of STZ

administration, dose of STZ (severity of diabetes) and the duration of diabetes (Rodrigues et al. 1997). This variability is illustrated by the summary of studies presented in Table 5.

Results obtained from both mouse models of type 1 and type 2 diabetes indicate that LPL activity, as represented by the total release of LPL by heparin from Langendorff perfused hearts, was not different between diabetics and non-diabetic controls (Figure 14), nor was LPL activity different between the type 1 or 2 diabetic conditions (Table 2). These results are similar to those found by Rodrigues and Severson (1993) with STZ-treated Wistar-Kyoto rats. Analysis of cardiac tissue homogenates also indicated no changes in LPL activity (Table 2). A similar result has been found in both Sprague-Dawley (Lui and Severson 1995) and Wistar rats (Inadera et al. 1992).

The release of lipoprotein lipase from the isolated perfused hearts followed a similar time course to that observed by Liu and Severson (1995) and Mardy et al. (2001). The peak of heparin induced LPL release occurred within the first minute and by 5 minutes the release of LPL reached a steady state low level. As described in the results, the majority of LPL was found in tissue (~ 82 %) with the remainder in the post heparin perfusate (~ 17 %) and basal (pre-heparin) release (> 1 %). These results are very similar to the report by Blanchette-Mackie et al. (1989). This group described LPL distribution in mouse hearts through immunocytochemistry and found that 78 % of LPL was within myocytes, 18 % was bound to the capillary endothelium and 3-6 % in extracellular spaces.

In conclusion, our results suggest that the supply of LPL-derived fatty acids does not change in the diabetic condition. Therefore, any alterations observed in lipoprotein

metabolism by the diabetic heart will be the result of changes occurring at the intracellular level involving the fate of LPL-derived FA.

2. Lipid Substrates:

Neutral lipids, such as TG and cholesteryl esters, are insoluble in aqueous solution and therefore must be transported in the blood stream within a lipoprotein complex (Ginsberg 1998). Hydrophobic TG and cholesteryl ester molecules form the core of the lipoprotein and are surrounded by an amphipathic monolayer of phospholipids, free cholesterol and apoproteins. Several major classes of lipoproteins have been identified and defined by their physical and chemical characteristics: chylomicrons, VLDLs, LDLs, IDLs and HDLs.

Chylomicrons were the choice of lipoprotein for this investigation for several reasons. First, chylomicrons have the highest TG content at 80-95 % (Ginsberg 1998), 30-40 % greater than VLDLs. It seems reasonable that in order to measure the metabolism of lipoprotein-TG in the heart you would provide the most TG-rich substrate. Second, chylomicrons are the preferred substrate of LPL compared to VLDL (Goldberg 1996). This has been based on several pieces of information. Chylomicrons have the shortest half-life (<10 min) of all the lipoproteins suggesting faster hydrolysis of the TG component. It has been suggested that this is because chylomicrons are the largest lipoprotein and as a result will be hydrolyzed faster due to increased interaction with LPL molecules (Goldberg 1996). As many as 44 LPL molecules can interact with a single chylomicron (Blanchette-Mackie et al. 1989). There have also been competition experiments that have shown that in the presence of chylomicrons, VLDL hydrolysis is

reduced. Finally, chylomicrons are much easier to obtain than VLDLs, the other lipoprotein candidate. The method was quite simple; once the intestinal lymphatic was cannulated, the rat was simply fed [^{3}H]palmitate and chyle collected for the isolation of [^{3}H]TG containing chylomicrons.

Although the use of rat chylomicrons for metabolism by a mouse heart is not ideal, the ability of mouse LPL to metabolize rat chylomicrons was demonstrated by the *in vitro* LPL assay. Including heat-inactivated rat serum as an apoCII source will enhance the physiological relevance of this model. Chylomicrons were incubated with the apoCII source at 37 °C for 10 minutes prior to exposure to the heart. Previously, the metabolism of chylomicrons by working perfused rat hearts has been measured primarily in the absence of an apoCII source (Wang et al. 1998; Hauton et al. 2001). However, Wang and Evans (1997) showed that the addition of an apoCII source ('starved' heat-inactivated rat serum) did increase chylomicron metabolism in the isolated perfused rat heart.

The NEFA substrate (palmitate) that was used in our series of experiments is not the most soluble fatty acid available. Oleate is more soluble and is the fatty acid of preference in some labs (Wang et al. 1998). Palmitate was used in this investigation because our lab is familiar with it through use in previous studies (Belke et al. 1999, 2000; Mardy et al. 2001).

3. Metabolism of Chylomicrons:

In comparison to the number of studies which have investigated the metabolism of a non-esterified source of fatty acids, there are relatively few investigations into lipoprotein metabolism. Of these, VLDLs have been the subject of most interest

(O'Looney et al. 1983; Wang et al. 1998), with very few studies exploring the metabolism of lipoproteins by diabetic hearts (Kreisberg 1966; O'Looney et al. 1985). Kreisberg (1966) and O'Looney et al. (1985) observed a decrease in the utilization of lipoproteins, as indicated by lipoprotein clearance from a re-circulating buffer, in the *ex vivo* perfused diabetic heart compared to controls (40 % and 47 % respectively). The only study to date investigating lipoprotein metabolism in the mouse heart was conducted by Mardy et al. (2001).

As the work by Belke et al. (2000) has characterized the metabolism of a NEFA (palmitate) substrate in the diabetic db/db mouse heart, it was natural to continue investigating lipid metabolism in the diabetic mouse model but with another lipid source. As lipoproteins are an important energy source for the heart (Ginsberg 1998) they were the natural substrate of choice. As described above, chylomicrons are the preferred lipoprotein substrate for LPL and were therefore the lipid choice for these investigations.

Results clearly indicate an increase in chylomicron oxidation in the diabetic hearts (db/db and STZ-treated mice) compared to their littermate controls. Staprans et al. (1992) found no change in the oxidation of chylomicrons but this study was conducted in the whole animal in which the radiolabeled lipoprotein was added to the blood and the blood was sampled at given time intervals. The work of Kreisberg (1966) and O'Looney et al. (1983) both showed a decrease in lipoprotein oxidation in the diabetic (type 1) rat heart, although the latter investigated the oxidation of VLDLs, not chylomicrons. Further, O'Looney et al. (1983) observed a decrease in heparin-releasable cardiac LPL activity, limiting the supply of LPL-derived FA to the heart. The work of Kreisberg et al. (1966) was conducted with a Langendorff isolated heart preparation on alloxan treated Sprague-

Dawley rats. As discussed earlier the activity of cardiac LPL appears to be dependent upon many factors (Table 5). Although Kreisberg did not assess cardiac LPL activity, other studies have shown that in the Sprague-Dawley rat cardiac LPL activity is reduced (Liu and Severson 1995; Table 5). Therefore, as in the O'Looney et al. (1983) study, the reduction in chylomicron oxidation may be a consequence of reduced cardiac LPL levels.

The rate of chylomicron oxidation observed in control hearts is very similar to that published by Wang et al. (1998) and Wang and Evans (1997) with perfused working rat hearts. Their results are published as a function of wet weight. Our results are expressed as a function of dry weight but if a ratio of 4:1 (as indicated by our results) wet to dry weight is applied to express our results as a function of wet weight (~ 16.5 nmole/min/g wet wt - *control hearts*), this rate is essentially the same as the two Wang publications (Wang and Evans 1997, ~ 15.5; Wang et al. 1998, ~ 14 nmole/min/g wet wt).

In addition to an increase in oxidation, esterification of LPL-derived fatty acids into cardiac tissue lipids was also increased. These results are supported by O'Looney et al. (1983) and Staprans et al. (1992). Despite the fact that they did not observe an increase in the oxidation of lipoproteins, both of these studies showed an increase in lipid esterification. Kreisberg (1966) showed a decrease in the incorporation of [^{3}H]TG into tissue lipids.

There was no significant difference in the rate of oxidation or esterification between the type 1 and 2 diabetic heart models.

4. Metabolism of NEFA:

The area of non-esterified fatty acid metabolism has received much attention. The majority of these studies have been conducted on rat models and many of these studies have investigated changes in metabolism resulting from diabetes (Christe and Rodgers 1995; Shepard et al. 1998; Chatham et al. 1999). The principal investigations into carbohydrate and lipid metabolism in the type 2 diabetic *db/db* mouse heart have been conducted by Belke et al. (2000).

Results indicate that the oxidation of NEFAs was significantly higher in *db/db* and STZ-treated hearts compared to control hearts. This is supported by work on type 1 diabetic (STZ induced) rats (Sprague-Dawley and Spontaneous Hypertensive) (Christe and Rodgers 1995) and on the *db/db* mouse (Belke et al. 2000). The rate of NEFA oxidation observed in *db/+* hearts (0.471 +/- 0.172 is similar to that (0.35 μmol/min/g dry weight) reported by Belke et al. (2000).

An increase in lipid esterification was observed in the diabetic hearts compared to control hearts. This is supported by the results of Zhou et al. (2000) who observed an increase in TG content in diabetic ZDF rat hearts. There are few studies within the literature to which we can compare our esterification results, although the chylomicron data presented in Section 3 supports an increase in fatty acid (NEFA) esterification in diabetic hearts.

5. Cardiovascular Function:

The early study of Rubler et al. (1972) on post-mortem findings and the clinical records of 27 patients led to the suggestion that there is a model of cardiac dysfunction in

diabetic patients without significant disease of the coronary arteries. The study of this vascular-independent disease ensued with various rodent models. Rodents are good models as they are resistant to the development of atherosclerotic disease, allowing the investigator to study the non-vascular defects (i.e. a diabetic cardiomyopathy).

The rat is the most popular rodent model, although other models such as the guinea pig have been used. It has become quite clear over the last 20 years that cardiac performance is compromised during diabetes mellitus. Most of this work has been done on insulin-dependent models such as the streptozotocin- or alloxan-treated rat. The non-insulin dependent model has been investigated less thoroughly but key studies conclude that this model of diabetes is as well associated with a vascular independent cardiac defect (Schaffer et al. 1985; Pierce et al. 1997).

Our type 1 and type 2 diabetic models both present a similar degree of cardiovascular dysfunction as represented by the rate-pressure product. The rate-pressure product has been described as an effective means of measuring myocardial mechanical performance (Hauton et al. 2001). Our results did not indicate a significant decline in cardiac output (ml/min), as has been previously shown with diabetic mouse (Belke et al. 2000) and rat (Pierce et al. 1997) hearts. There have however been publications in which a significant decline in cardiac flow or pressure development (Christe and Rodgers 1995) has not been observed but a dysfunction was apparent as indicated by a decrease in heart rate (Litwin et al. 1990). It is through a significant decrease in heart rate that we observed a decline in cardiac function. The STZ-treated *db/+* type 1 diabetic model did present a significantly lower cardiac output than their littermate controls but these diabetic hearts were also significantly smaller than controls. After normalization for heart weight, the

cardiac output of this diabetic group was no longer significantly lower than the control *db/+* hearts.

6. Substrate Preference:

The rate of fatty acid metabolism observed in our series of experiments was consistently higher for the NEFA source (palmitate) compared to chylomicron metabolism (LPL-derived FA). This is supported by the work of Kreisberg (1966) and Mardy et al. (2001), both of which found that NEFAs are metabolized at a higher rate than lipoproteins (1.67 and 1.85 times respectively). The work by Hauton et al. (2001) did not show a significant difference in the rates of oxidation between NEFAs and chylomicrons. Their experimental animals were fasted for 24 hours prior to experimentation. Wang and Evans (1997) have shown that with fasting, the metabolism of lipoproteins is increased as much as two-fold. Therefore, it seems reasonable that the reason Hauton et al. (2001) did not observe a difference in the rate of metabolism between NEFAs and lipoproteins is the metabolic state of their animals. The higher level of NEFA metabolism may be a consequence of the rate limiting LPL hydrolysis step required for chylomicron metabolism.

Cardiac function was maintained for a longer period of time when hearts were metabolizing chylomicrons as opposed to palmitate. At time 0, all hearts began the perfusion at a similar level of cardiac function as represented by the rate-pressure product, approximately 20 mmHg/min x 10^{-3}. By 60 minutes those hearts which were metabolizing chylomicrons had a significantly higher cardiac performance, compared to the palmitate metabolizing hearts, and continued to do so for the remainder of the

protocol. At the end of the 90-minute perfusion period, the function of chylomicron-metabolizing hearts was approximately 2 times that of the NEFA-metabolizing hearts (between the diabetic groups). Isolated heart perfusion studies typically show a decline in function over the perfusion period (Belke et al. 2000).

As it is the elevated level of fat metabolism that has been suggested as the cause of cardiac dysfunction in the diabetic heart (Shimabukuro et al. 1998; Zhou et al. 2000; Listenberger et al. 2001), it is plausible that this may be the reason a greater decline in cardiac function was observed with the NEFA substrate. It is interesting that substrate metabolism of the diabetic heart was approximately twice as high with a NEFA source and their subsequent function at the end of the perfusion period is only one half of the chylomicron perfused hearts. This observation may be attributed to increases in the rate of metabolizing palmitate specifically. It is likely that through the process of chylomicron production within rat enterocytes the triacylglycerols forming the core of the chylomicron are not exclusively composed of palmitate, a 16-carbon fatty acid. Most likely, the triacylglycerols in chylomicrons are composed of various length fatty acids. Work by Listenberger et al. (2001) suggests that fatty-acid induced apoptosis is specific for the saturated fatty acids palmitate and stearate (both 16 carbons in length or greater), and not for saturated fatty acids of length ranging from C4-C14 or with unsaturated fatty acids. Therefore, the lower function we observe in the NEFA metabolizing hearts may a consequence of simply higher rates of fatty acid metabolism and subsequently increased myocyte apoptosis, or may be more specific to the increased dependence and metabolism of a 16 carbon fat substrate.

7. Potential Mechanisms:

Our results quite clearly show that fatty acid oxidation and esterification is increased in diabetic hearts. This has been demonstrated in the literature (Belke et al. 2000). The increase in fatty acid metabolism in the diabetic heart is likely the result of many contributing factors.

Proton leaks constitute a considerable part of the resting metabolic rate (Dalgaard and Pedersen 2001). An uncoupling protein (UCP) was discovered in brown adipose tissue in the early 1980's and since then homologues of this UCP-1 have been discovered in other tissues such as the heart (Dalgaard and Pedersen 2001). The uncoupling proteins are found in the mitochondria, associated with the electron transport chain, shunting protons back into the mitochondrial matrix. Recently it has been shown that one of these homologues, uncoupling protein-3 (UCP-3), is found in elevated levels in the type 1 diabetic rat heart (Depre et al. 2000). An increase in cardiac tissue levels of UCP-3 may induce an increase fatty acid oxidation through reducing the efficiency of the electron transport chain (Figure 26). Essentially, through shunting protons back into the mitochondrial matrix across the inner mitochondrial membrane the amount of ATP produced per substrate molecule oxidized is reduced. Therefore, to maintain a biologically appropriate amount of ATP production through the mitochondrial matrix more substrate needs to be oxidized such that a greater number of protons can be delivered via NADH and $FADH_2$. With a greater number of protons shuttling through the electron transport chain ATP can be produced in sufficient quantity despite the elevated level of UCP-3 and proton shunting. Although UCPs may reduce the amount of ATP produced per substrate molecule oxidized, it is also possible that the diabetic heart suffers

from a pathology in which the capacity to utilize energy is compromised (Holmes et al. 2002). This means that the diabetic heart is less efficient, requiring a greater supply of energy (ATP) to perform the same amount of work as the non-diabetic heart. This may be observed as an increase in substrate utilization (energy production) while cardiac function is unchanged or reduced. Alternatively, UCPs increase FA oxidation by promoting export of fatty acid anion from mitochondria; this transport function for fatty acid anion, which is produced by the action of a mitochondrial thioesterase, provides free CoA-SH for the mitochondrial reactions involved in FA oxidation (Himms-Hagen and Harper 2001).

Another potential means by which fatty acid oxidation might be elevated is at the level of fatty acid transport into the mitochondria. This process is accomplished by the two enzymes carnitine palmitoyl transferase 1 and 2 (CPT-1, CPT-2; Figure 4 and 26). Elevated levels of CPT-1 and 2 (protein and message) have been shown in the type 1 diabetic rat (Kurtz et al. 2000) and the NOD mouse (Cook et al. 2001) heart. The NOD mouse is a non-obese type 1 diabetic model that results from early onset destruction of pancreatic β-cells. This suggests that elevated levels of fatty acyl CoA are being shuttled into the mitochondria and subsequently oxidized. The activity of CPT-1 is regulated by malonyl CoA, so changes in the amount of malonyl CoA in the diabetic heart may also be a potential point of regulation. Malonyl CoA is produced by acetyl CoA carboxylase and is an important inhibitor of CPT-1 acting at a site distinct from the catalytic site of CPT-1 (Sakamoto et al. 2000). Although the sensitivity of CPT-1 to the inhibitory effect of malonyl CoA has not been shown to change in the diabetic heart, a decrease in malonyl CoA content has been seen in type 1 diabetic porcine hearts. This would suggest reduced

inhibition of CPT-1 by malonyl CoA and elevated levels of fatty acid oxidation (Hall et al. 1996).

With an increase in oxidation, a concomitant increase in the esterification of fatty acids into tissue lipids was also observed. This implies that increased fatty acid metabolism in the diabetic heart is not exclusively due to alterations in mitochondrial function. There must be events occurring upstream of the mitochondria, for example increased levels of fatty acid uptake into the myocyte (Figure 4 and 26). Elevated levels of fatty acid uptake and storage have been reported by Zhou et al. (2000) and Turcotte et al. (2001). In addition, elevated levels of proteins involved in fatty acid transport such as the FABP and FAT/CD36 (Luiken et al. 2001) have been observed. In the ZDF *fa/fa* diabetic rat Luiken et al. (2001) found that FAT mRNA was significantly elevated (1.4-fold) and $FABP_{PM}$ mRNA was even more up regulated (2.5-fold). In addition, the sarcolemmal content of FAT/CD36 was 1.6-fold higher in obese Zucker rat hearts compared to lean littermate control hearts. They also measured palmitate uptake and found that uptake rates were 1.8-fold higher in obese and diabetic ZDF rat hearts.

These results suggest that fatty acid uptake into the myocyte is increased in the diabetic condition supporting our findings that fatty acid metabolism was elevated in the type 1 and 2 diabetic models. The mechanisms through which this elevation in fatty acid metabolism occurs is unclear but must include many changes in cellular fatty acid handling along with alterations in FA uptake.

There is evidence in the literature pointing towards changes in lipid metabolism and handling as the cause for depressed diabetic heart function. Many human and animal studies have been conducted in attempt to correlate depressed cardiac contractile function

in diabetes with altered plasma lipid levels and lipid metabolism. The Framingham study found that diabetics who had a higher incidence of cardiovascular disease also presented with higher lipoprotein TG levels (Kannel and McGee 1979). The World Health Organization (WHO) found that diabetic serum TG levels were the strongest indicator for cardiovascular disease (West et al. 1983). In addition, a study by Rosenstock et al. (1987) has reported that a reduction in cardiovascular risk may be confirmed through meticulous control of the type 1 diabetic condition, minimizing resultant changes in plasma lipid and lipoprotein levels.

Work by Shimabukuro et al. (1998) provided evidence that destruction of pancreatic β-cells may occur through a lipid-dependent ceramide-mediated pathway of cellular apoptosis. Their proposed pathway begins with elevated levels of intracellular long chain fatty acids and their conversion into long chain acyl CoA by acyl CoA synthase. The fatty acyl CoAs are than converted into ceramide by serine palmitoyltransferase. Elevated levels of cellular ceramide up-regulate the expression of inducible nitric oxide synthase (iNOS) thereby augmenting the production of nitrogen oxide (NO). Increases in NO result in elevated levels of peroxynitrite and finally cellular apoptosis.

Two years later, Zhou et al. (2000) published data using hearts from diabetic ZDF rats suggesting that this same mechanism might occur in cardiac myocytes resulting in cell death and depression of cardiac function. In this scenario the excessive deposition of TG in cardiac tissue, termed steatosis, enlarged the intracellular pool of fatty acyl CoA. The large pool of fatty acyl CoA provides substrate for non-oxidative pathways such as ceramide synthesis and eventual cellular apoptosis.

There is as well a potential means through which elevated levels of fatty acid metabolism can result in cellular apoptosis that is not dependent on ceramide. Listenberger et al. (2001) suggest a mechanism whereby palmitate can induce apoptosis through the production of reactive oxygen species. They showed through an *in vitro* cell preparation (CHO cells) and the use of inhibitors of ceramide production such as fumonisin (100 μM) or L-cycloserine (1 mM), that reductions in ceramide levels did not rescue cells from apoptosis. By comparison, anti-oxidants in the preparation were able to reduce cell death.

Troglitazone is a member of the thiazolidinedione group of insulin sensitizing drugs. Although thiazolidinediones have profound effects on glucose utilization, they are also associated with an improvement in lipid profiles (Sreenan et al 1999). Troglitazone has been shown by Sreenan et al. (1999) and Jia et al. (2000) to lower serum lipid levels, by greater than 50 %, and to prevent the onset of diabetes in the diabetic ZDF rat (Jia et al. 2000). The troglitazone results lend support to the theory that the cardiomyopathy associated with diabetes is the consequence of elevated levels of fatty acid utilization.

Although the mechanisms through which elevated levels of fatty acid metabolism in the diabetic condition induce myocyte death and depress cardiac function remain to be further clarified, there is little doubt that high levels of lipid metabolism play a significant role (Shimabukuro et al. 1998; Zhou et al. 2000; Listenberger et al. 2001).

8. Summary of Findings:

The first result of significance is that the delivery of LPL-derived fatty acids is not altered in the diabetic heart, in experiments with both type 1 and type 2 models of

diabetes in mice. There were no changes in the activity of LPL at the coronary endothelium, measured as a heparin releasable LPL activity, or LPL remaining within the tissue at the end of the perfusion period. Thus the changes observed in the metabolism of LPL-derived fatty acids are a consequence of intracellular alterations. Studies to date have been inconclusive as to the activity of LPL in the diabetic heart. As discussed earlier this is a consequence of several factors including the use of many different rodent strains, differences in the type or duration of diabetes and variations in measurement procedures. This set of experiments was conducted on two forms of diabetes mellitus, insulin dependent and non-insulin dependent. Although there was a strain difference between the type 1 and 2 diabetics this was controlled for by the STZ treated group of C57BL/KsJ-*lepr*^{db}/*lepr*^{db} (db/+). All hearts were subjected to the same experimental protocol minimizing the variability prevalent in early investigations. This set of experiments is the first to characterize the activity of heparin releasable and tissue levels of LPL in the hearts of both a type 1 and type 2 diabetic model, and the first to investigate the activity of LPL in a diabetic mouse heart.

The majority of isolated perfused heart studies have not included fatty acids as a metabolic substrate for the heart. Of those few that have included a fat source there are again very few which have investigated the cardiac metabolism of lipoproteins, and only a fraction of these using a chylomicron substrate. The work of Mardy et al. (2001) is the only publication exploring the metabolism of chylomicrons in the mouse and there are no publications describing the metabolism of chylomicrons in the diabetic mouse heart. This series of experiments is the first to investigate the cardiac metabolism of chylomicrons in the insulin dependent and non-insulin dependent diabetic mouse, and the first to

investigate the metabolism of a NEFA (palmitate) substrate in an insulin dependent mouse model. We observed a significant increase in fatty acid utilization from both chylomicron catabolism and a non-esterified source (palmitate) in the STZ induced type 1 diabetic (IDDM) and the genetic type 2 diabetic (NIDDM) mouse models. Fatty acid utilization included both oxidation and incorporation (esterification) into tissue lipids.

Cardiac function has been quite clearly defined in the IDDM rat, but less clearly so in the NIDDM rat model. Cardiac function has been assessed in the type 2 diabetic mouse by Belke et al. (2000) and Aasum et al. (2002), but no work has been published measuring the cardiac function of type 1 diabetic mouse hearts, or type 2 diabetic mouse hearts metabolizing a lipoprotein substrate. With observed increases in fatty acid metabolism was a concomitant decline in cardiac function. This decrease in function, as indicated by the rate-pressure product, was significant for both lipid substrates and both diabetic models. It is most likely that the observed decline in function is a result of elevated levels of fatty acid metabolism and consequent lipid-induced toxicity (lipotoxicity).

This series of experiments are the first to clearly define the metabolism (oxidation and esterification) of a lipoprotein substrate (chylomicron) in the type 1 and type 2 diabetic mouse heart.

9. Limitations and Future Directions:

As this was the first series of experiments to be conducted with this experimental model the isolated perfused hearts were provided with concentrations of metabolic substrate that would be found in the healthy animal. This protocol would provide us with

base-line results based upon physiological as oppose to pathophysiological substrate levels. It has however been shown that altered levels of metabolic substrate impact rates of utilization. Saddik and Lopaschuk (1991) designed experiments with the isolated rat heart to investigate the metabolism of low (0.4 mM) and high (1.2 mM) levels of lipid (palmitate) and the consequent impact on glucose utilization and cardiac function. They found that elevated levels of fatty acid utilization (almost 2-fold) occurred as the isolated heart was supplied with the higher concentration of fat. With the increase in fat utilization they observed a significant decrease in glycolysis and glucose oxidation. Those hearts that metabolized the high fat also suffered a significant decrease in peak systolic pressure generation. These results of Saddik and Lopaschuk (1991) are supported by Chatham et al. (1999) who found that in the isolated perfused rat heart elevated levels of palmitate supply result in elevated levels of palmitate oxidation and reduced levels of lactate oxidation.

Future experiments should include elevated substrate levels of glucose, NEFA and lipoproteins. Elevated levels of glucose will most likely not impact glucose utilization as this seems to be determined by both fatty acid oxidation (Randle et al. 1963), and the presence of glucose transporters on the membrane (Belke et al. 2000). Increasing the fatty acid supply to perfused hearts will likely result in elevated levels of utilization, as was shown by Saddik and Lopaschuk (1991) and Chatham et al. (1999). Similar to the observations of Saddik and Lopaschuk (1991), our results suggest that higher levels of fatty acid oxidation may result in lower levels of cardiac function. Therefore, it seems reasonable to conclude that increasing the supply of fatty acids,

simulating the 'diabetic' condition, will result in elevated levels of fat metabolism with concomitant reductions in cardiac function.

Work by Jeffery et al. (1995) on the *ex vivo* perfused rat heart has shown through ^{13}C NMR isotopomer analysis that substrates such as lactate and ketone bodies are metabolically important to the heart. Under normal physiological fed conditions acetoacetate (a ketone body) contributed 23 % of total energy supply (contribution to acetyl CoA), lacate 15 % and lipid 49 %. Under these conditions the use of glucose was negligible. Under fasted conditions, the use of fatty acids was reduced to 12 % of total contribution to energy while acetoacetate increased to 78 %. To make the isolated heart preparation used in this series of experiments more physiologically relevant substrates such as lactate and ketone bodies should be included in the perfusion buffer. As indicated by the results of Jeffery et al. (1995), including in the perfusion buffer substrates such as lactate and ketone bodies may reduce the utilization of fatty acids. As described above fatty acid metabolism appears to impact cardiac function. Therefore, providing the heart with substrates it prefers, such as ketone bodies, while reducing fatty acid utilization may confer an increase in cardiac function.

Chylomicrons were isolated from a healthy rat for this series of experiments although studies have demonstrated a change in the apolipoprotein content of lipoproteins in the diabetic state (O'Looney et al. 1985; Callow and Redgrave 1993). O'Looney et al. (1985) observe an increase in VLDL apo B, AI and AIV and a decrease in apo E and CII. They further indicate that the metabolism of a "diabetic" VLDL by a diabetic heart was lower than the metabolism of 'non-diabetic' VLDL by a diabetic heart (1.1 +/- 0.2 vs. 2.5 +/- 0.1 μmol). Based upon these studies, it appears that the diabetic lipoprotein contains

different quantities of apolipoproteins than the normal heart and will therefore be metabolized at a different rate. Levy et al. (1985) looked at the composition and removal of chylomicrons in both normal and diabetic rats. Similar to the results of O'Looney et al. (1985), they found that the apolipoprotein content of the chylomicron is altered in the diabetic state. In addition, their observations indicate that the removal of 'diabetic' chylomicrons from the circulation (*in vivo*) was lower than the removal of 'non-diabetic' chylomicrons. The above studies suggest that the utilization of chylomicrons by the heart might be reduced if the chylomicrons were harvested from a diabetic animal source. But if the diabetic hearts are supplied with elevated levels of chylomicron (1.2 mM) the rate of utilization will likely increase. Therefore, if the chylomicrons were harvested from a 'diabetic' source and supplied at an above normal concentration, it seems reasonable that the final rate of utilization may be similar to the rates of utilization we have observed in this series of experiments.

10. Implications and Therapies:

The increased cardiovascular morbidity and mortality of diabetic patients was originally attributed solely to vascular disease until a small number of diabetic patients presented with heart failure in the absence of vascular complications (Rubler et al. 1972). In addition, these patients were without the other common causes of heart failure such as hypertension, alcoholism, congenital heart disease or valvular heart disease. These patients were identified as having a unique but unknown pathology with a propensity to cause heart failure. This seeded a plethora of studies and experiments over the last 30

years attempting to elucidate the clinical characteristics of this disease and its pathophysiology.

It has now been generally accepted that this cardiomyopathy is a consequence of metabolic alterations in the diabetic state (Randle et al. 1963; Rubler et al. 1972; Zhou et al. 2000). Clinicians and therapists have traditionally focused predominantly on the treatment of the atherosclerotic vascular disease. These treatments would include anti-hypertensive therapy such as thiazide diuretics, cardioselective beta-blockers and ACE inhibitors. The latter of these has been reported to be more effective than other antihypertensive agents with regard to the reduction of albuminuria and proteinuria, but equally effective with regard to their influence on reducing glomerular filtration rate in diabetic nephropathy (Inzucchi 2002). Cholesterol lowering therapy (hypolipemic therapy) has also been an area of intense investigation as total serum cholesterol has been shown to be a predictor of cardiovascular disease. By lowering serum cholesterol, the high levels of small dense atherogenic LDL particles will be reduced, as will the rates of lipoprotein oxidation and glycation, both of which contributing to the development of atherosclerosis. Recently, it has been demonstrated that treatment with HMG-CoA reductase inhibitors (statins) produces significant reductions in elevated cholesterol levels and can reduce the development of atherosclerosis (Betteridge 2001). Today, with the understanding that there is a vascular independent cause of diabetic heart disease clinicians and therapists now have to consider and develop treatment methods aimed at more than simply treating cardiac atherosclerosis.

It has become clear that through maintaining blood glucose levels within a healthy range it is possible to attenuate the development of many of the chronic complications

associated with diabetes (Moller 2001). This was observed indirectly by Belke et al. (2000) as the overexpression of the cardiac glucose transporter Glut-4 normalized the glucose and lipid profile and improved cardiac function. At present, metabolic therapies intended to reduce cardiac dysfunction in the diabetic have been designed to normalize hyperglycemia (Moller 2001).

The treatment of diabetes has improved due to the availability of new classes of metabolic anti-diabetic drugs and new insulin analogs. Three types of medication exert their anti-diabetic action without directly stimulating insulin release (Moller 2001). The α-glucosidase inhibitors (e.g. acarbose) were introduced in 1996 and function through interfering with the digestion and absorption of glucose and dietary glucose precursors (Inzucchi 2002). They delay the absorption of intestinal carbohydrates by competitive inhibition of the enzyme α-glucosidase effectively reducing post-prandial serum glucose excursions. Biguanides (e.g. metformin) act through increasing insulin sensitivity. Although the mechanism of their action remains unclear, they induce greater peripheral uptake of glucose while at the same time inhibiting hepatic gluconeogensis, thereby lowering fasting blood glucose concentrations (UKPDS 34. 1998). Improved glucose control is achieved without weight gain, a major side effect of many anti-diabetic agents. Biguanides also reduce concentrations of plasminogen-activator inhibitor type-1 (PAI-1) and thus may increase fibrinolytic activity. Thiazolidinediones (e.g. rosiglitazone) are pharmacological ligands for a nuclear receptor known as PPAR-γ. When activated the receptor binds with response elements on DNA, altering the transcription of a variety of genes that regulate carbohydrate and lipid metabolism. The most prominent effect of TZDs is increased insulin stimulated glucose uptake by muscle (i.e. reduced insulin

resistance). In contrast, there are two classes of anti-diabetic drugs that stimulate insulin release from pancreatic β-cells (Moller 2001). Sulphonylureas (e.g. glyburide) have been used successfully to treat diabetes since 1954 (UKPDS 33. 1998). Sulphonylureas bind to a sulphonylurea receptor, found on the surface of pancreatic β-cells. This interaction leads to the closure of voltage gated K_{ATP} channels, facilitating membrane depolarization, subsequent calcium entry into the cell and insulin secretion (UKPDS 33. 1998). Through this mechanism serum glucose levels are reduced. The main drawback of sulphonylurea therapy is that with prolonged use they may cause chronic hypoglycemia and weight loss. A new generation sulphonylurea, glimepiride, has a reduced number of side effects. Meglitinides (e.g. repaglinide) and D-phenylalanine derivatives (e.g. nateglinide) are powerful prandial insulin secretagogues having a similar mechanism of action to the sulphonylureas (Inzucchi 2002). They as well interact with the K_{ATP} channels on β-cells but are distinguished from sulphonylureas by their short metabolic half-life. Lastly, the administration of exogenous insulin itself is a therapeutic agent as it reduces glucose production and augments glucose utilization.

Although these therapies do provide benefit to the patient they are limited in their efficacy, tolerability and may have side effects. Of particular concern is the tendency of most treatments to induce weight gain. Diet, exercise and weight loss should be the focal point of any therapeutic program. These lifestyle modifications have many benefits including the reduction of blood glucose concentrations, but importantly they also ameliorate many of the frequently coexisting risk factors of cardiovascular disease. Many diabetics are unable to achieve complete control of their condition with lifestyle modifications and thus will also require pharmacological intervention. A problem

common to most therapies is that they do not adequately address the underlying defects such as obesity and the development of insulin resistance. The development of new therapies continues in earnest, some of them fantastical in their design. Riu et al. (2002) have engineered a mouse model in which skeletal muscle produces and secretes basal levels of insulin. These STZ-treated mice (IDDM) showed increased insulinemia and reduced hyperglycemia in the post-prandial condition, and normoglycemia and normoinsulinemia when fasted. As new therapeutic approaches are developed particular emphasis should be placed on finding and using mechanisms that are dependent on physiological responses (e.g. glucose-mediated insulin secretagogues) and encourage the maintenance or development of a healthy body weight.

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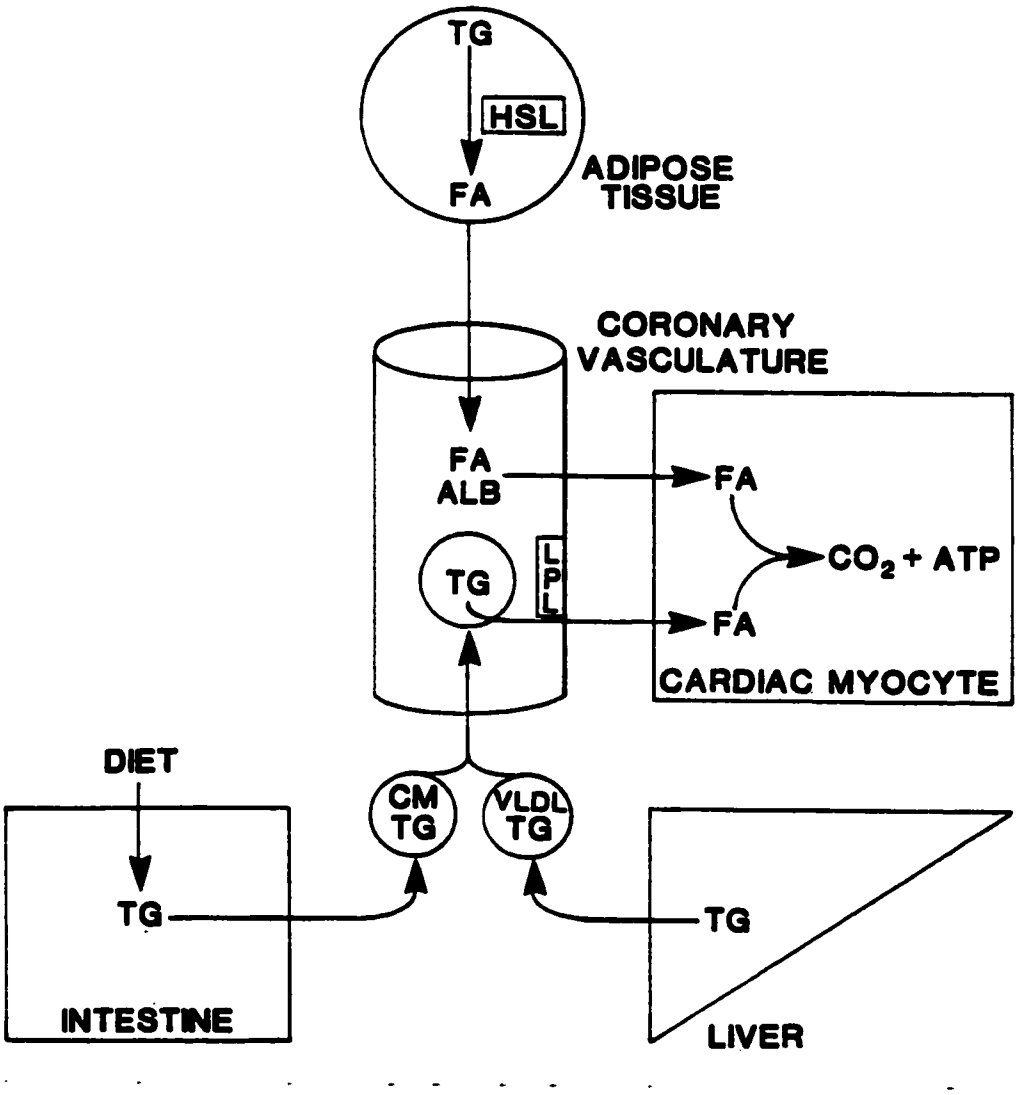

Figure 1: Sources of fatty acid for the heart. Within adipose tissue, TGs are hydrolyzed into fatty acids by hormone sensitive lipase (HSL). These fatty acids then enter the circulation combining with albumin to form a FA-Albumin complex, delivering NEFA to the heart. TGs are carried through the circulation in lipoprotein complexes, for example chylomicrons (CM) and very low density lipoproteins (VLDL). CMs are produced within the intestine and carry dietary TG, while VLDLs are produced by the liver. An enzyme is required for the mobilization of fatty acids from the lipoprotein complex. Lipoprotein lipase performs this function by hydrolyzing fatty acids from the TG glycerol backbone; the fatty acids are subsequently taken up by the myocardium. Alb, albumin; FA, fatty acids; TG, triacylglycerol.

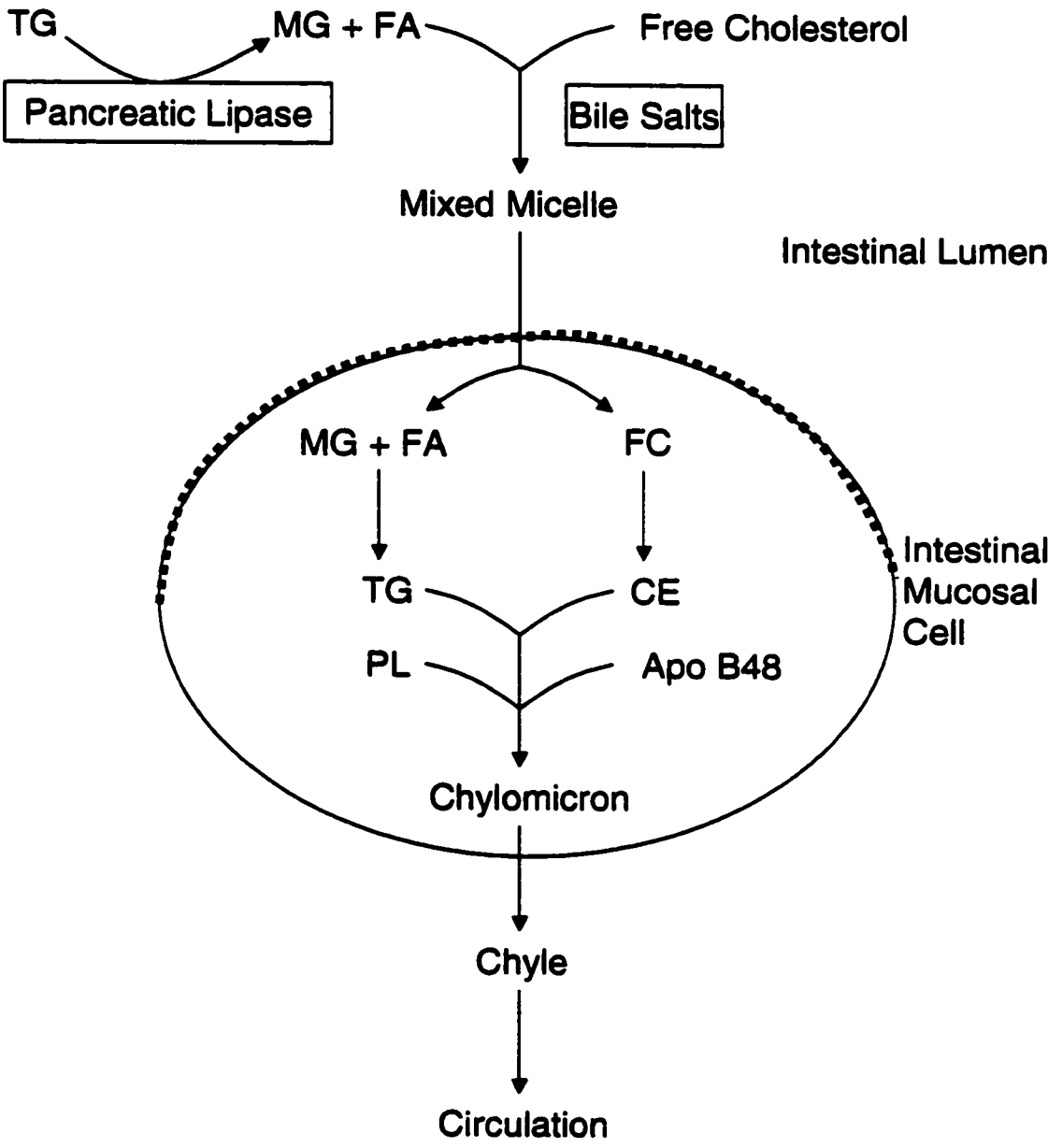

Figure 2: Chylomicron assembly within the intestine. Dietary TGs within the intestinal lumen are hydrolyzed into monoacylglycerols (MG) and fatty acids (FA) by pancreatic lipase. These lipolytic products combine with cholesterol (FC) and are emulsified into micelles by bile salts and subsequently taken up into the intestinal mucosal cell. CMs are formed within the enterocyte through the combination of lipids and the apolipoprotein apo B48. CMs are then secreted basolaterally into the lymph for transport to the circulation. CE, cholesterol ester; PL, phospholipid; TG, triacylglycerol.

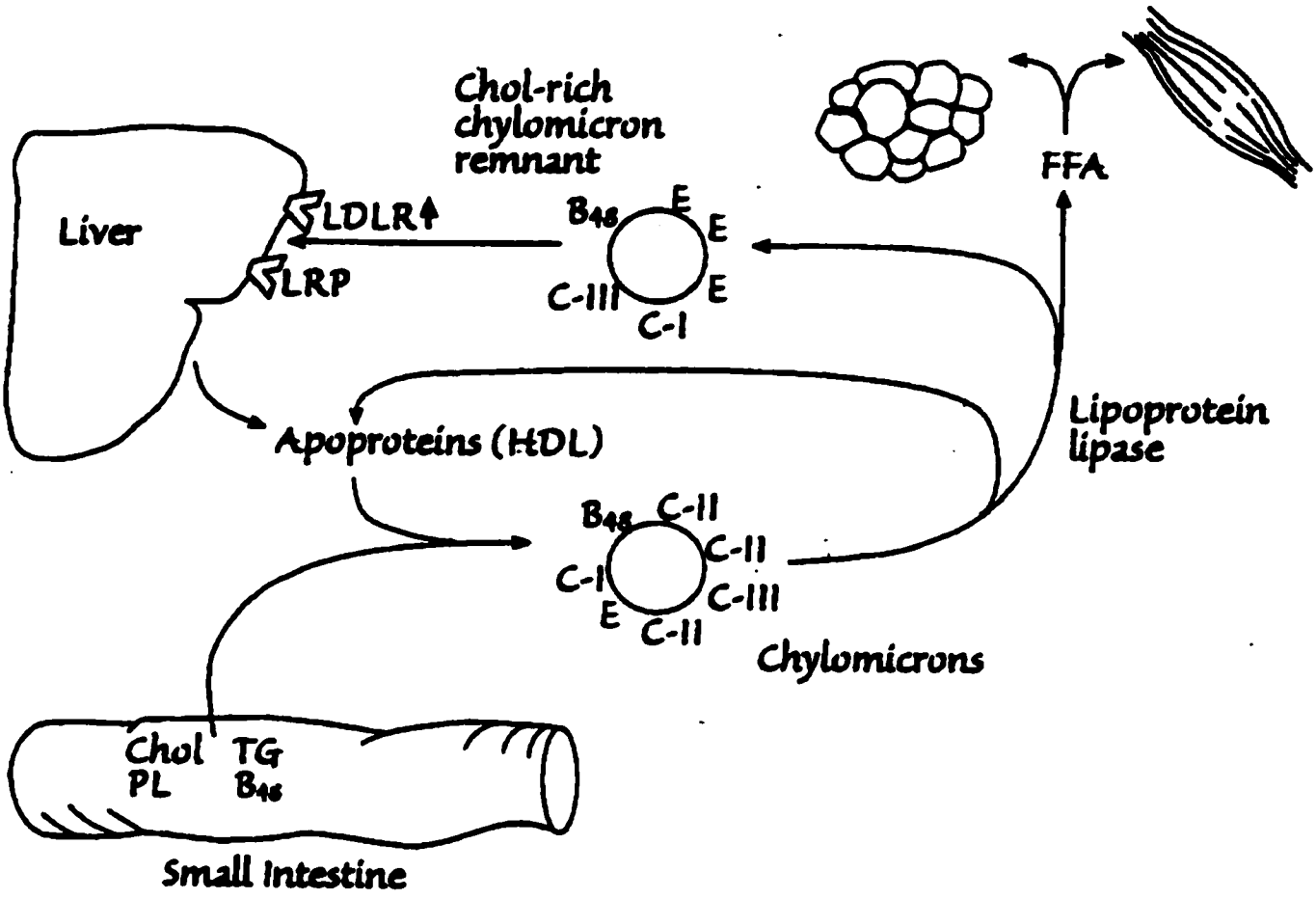

Figure 3: Transport of chylomicrons and chylomicron remnants. Chylomicrons enter the circulation from the lymphatic system as 'immature' lipoproteins, i.e. they do not yet have all of their accessory apoproteins. CMs interact with HDL in the circulation from which they acquire apo CI, CII, CIII and apo E. Transfer of free and esterified cholesterol and phospholipids also occurs at this time. After gaining apo CII, the activator of LPL, the CM interacts with LPL resulting in hydrolysis of the triacylglycerol core. LPL mediated triacylglycerol hydrolysis is accompanied by a reduction in the core volume and surface area of the chylomicron and by the transfer of phospholipid, free cholesterol, apo CII and CIII back to HDL. The remaining remnant CM particles are rapidly removed from the circulation by the liver. Figure from Ginsberg. (1998). HDL, high density lipoprotein; FFA, free fatty acid; LDLR, low density lipoprotein receptor; LRP, low density lipoprotein receptor-related protein; PL, phospholipid; TG, triacylglycerol.

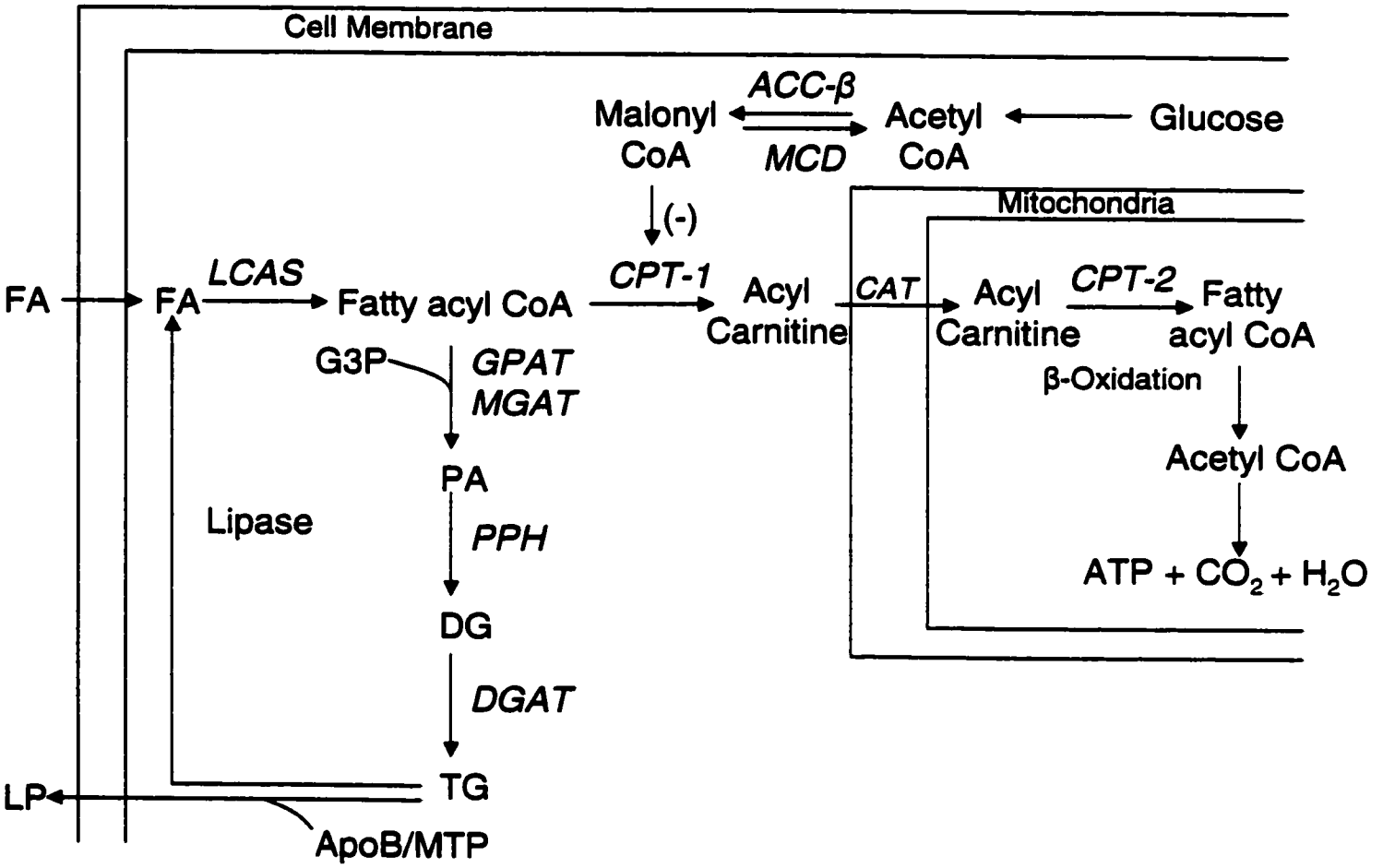

Figure 4: Cardiac fatty acid metabolism. Fatty acids have three potential fates: 1) oxidation and energy production within the mitochondria; 2) intracellular storage within a TG pool; or 3) export as a lipoprotein. The oxidation of fatty acids is regulated by the enzyme carnitine palmitoyltransferase 1 (CPT-1). The activity of CPT-1 is controlled by malonyl CoA which is synthesized from acetyl CoA by the enzyme acetyl CoA carboxylase (ACC). Triacylglycerol synthesis is catalyzed by glycerol-3-phosphate acyl transferase (GPAT), monoglycerol acyl transferase (MGAT), phosphatidate phosphatase (PPH) and diacylglycerol acyltransferase (DGAT). The mobilization of fatty acids from the TG pool is achieved via a myocardial lipase. Lipoprotein synthesis is dependent upon apo B and MTP expression, as described by Veniant et al. (1999). FA, fatty acid; CAT, carnitine acyl transferase; DG, diacylglycerol; G3P, glycerol 3 phosphate; LP; lipoprotein; MCD, malonyl CoA decarboxylase; MTP, microsomal transfer protein; TG, triacylglycerol.

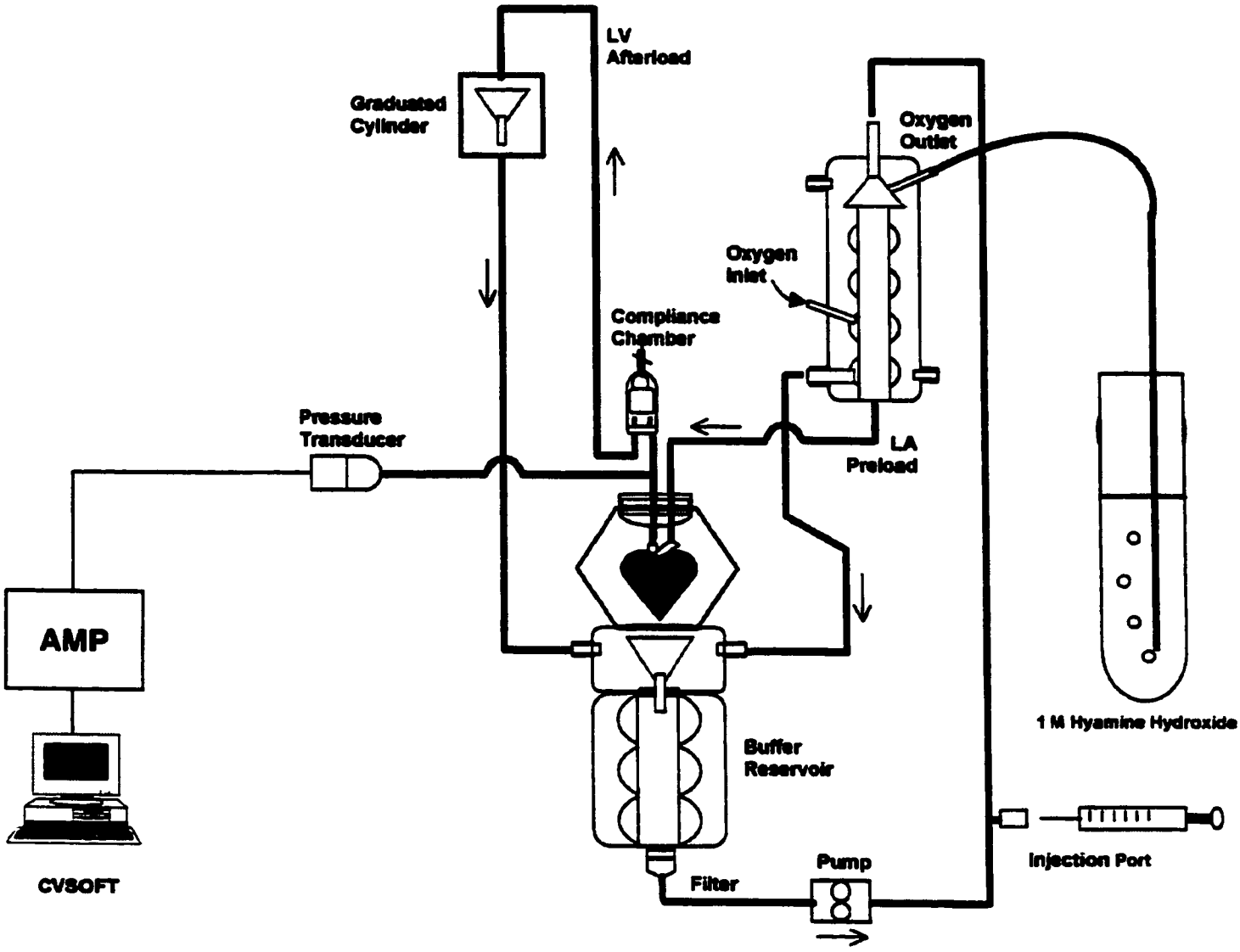

Figure 5: The working heart apparatus. Pressure is measured by a pressure transducer (Millar) within the aortic afterload line. Aortic and coronary flows are measured with graduated cylinders located at 68 cm (50 mmHg) above the heart in the afterload line and below the heart respectively. Chylomicron/serum substrate is added to the recirculating buffer, and buffer samples are removed through the injection port. Figure from Belke et al. (1999).

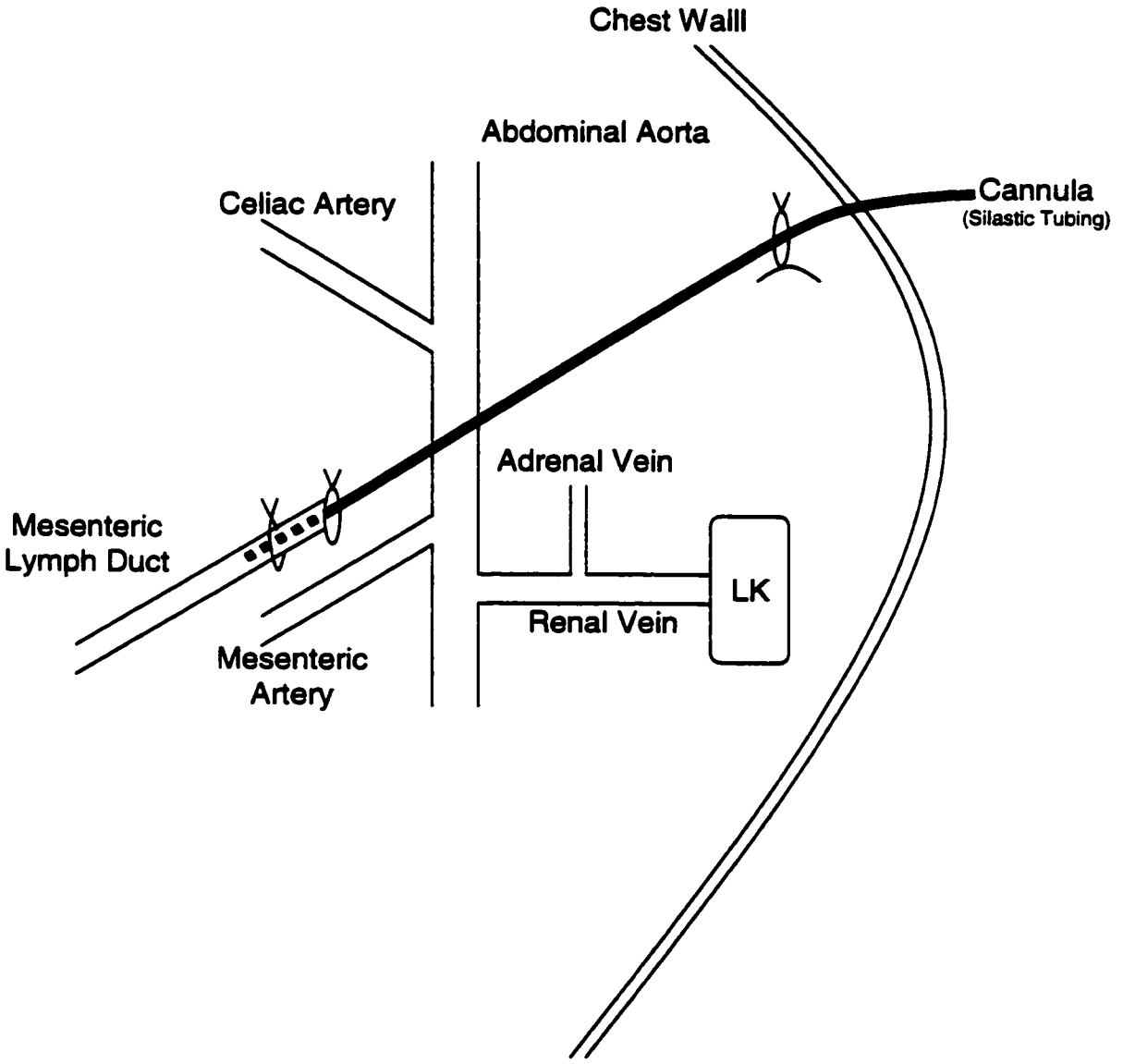

Figure 6: Chylomicron isolation. The mesenteric lymph duct lies adjacent to the mesenteric artery and is cannulated with a silastic tubing plastic cannula. The cannula is held within the lymph duct with two ties, and is secured to the inside of the abdominal wall with a stitch before it passes through the side of the rat. Chyle is collected from the cannula approximately 10 cm below the rat. LK, left kidney.

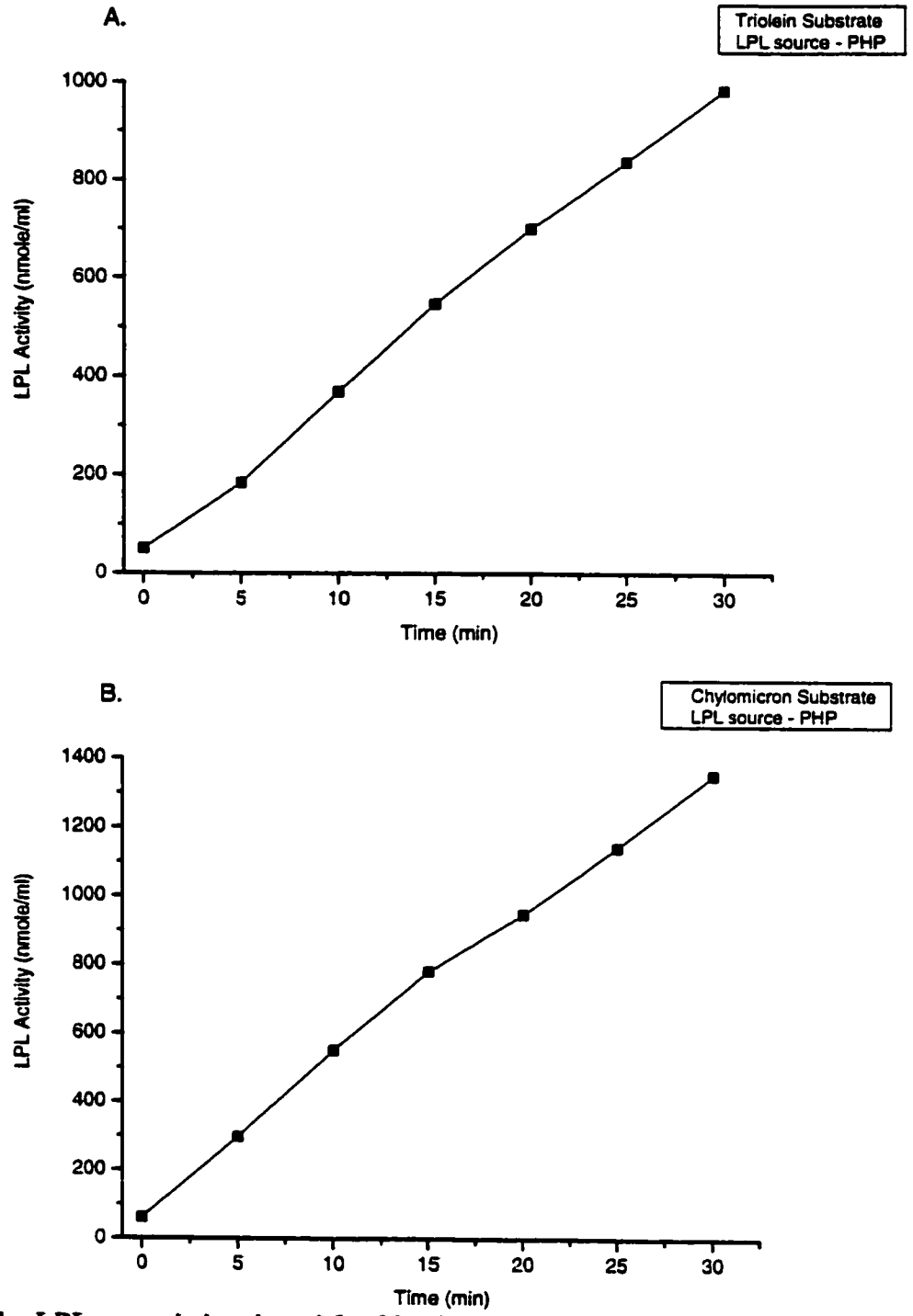

Figure 7: The LPL assay is incubated for 30 minutes. With a constant LPL source and lipid substrate the lipoprotein lipase assay is linear with time for both triolein (A) and chylomicron (B) substrates. LPL, lipoprotein lipase; PHP, post-heparin perfusate. Results are from a single experiment.

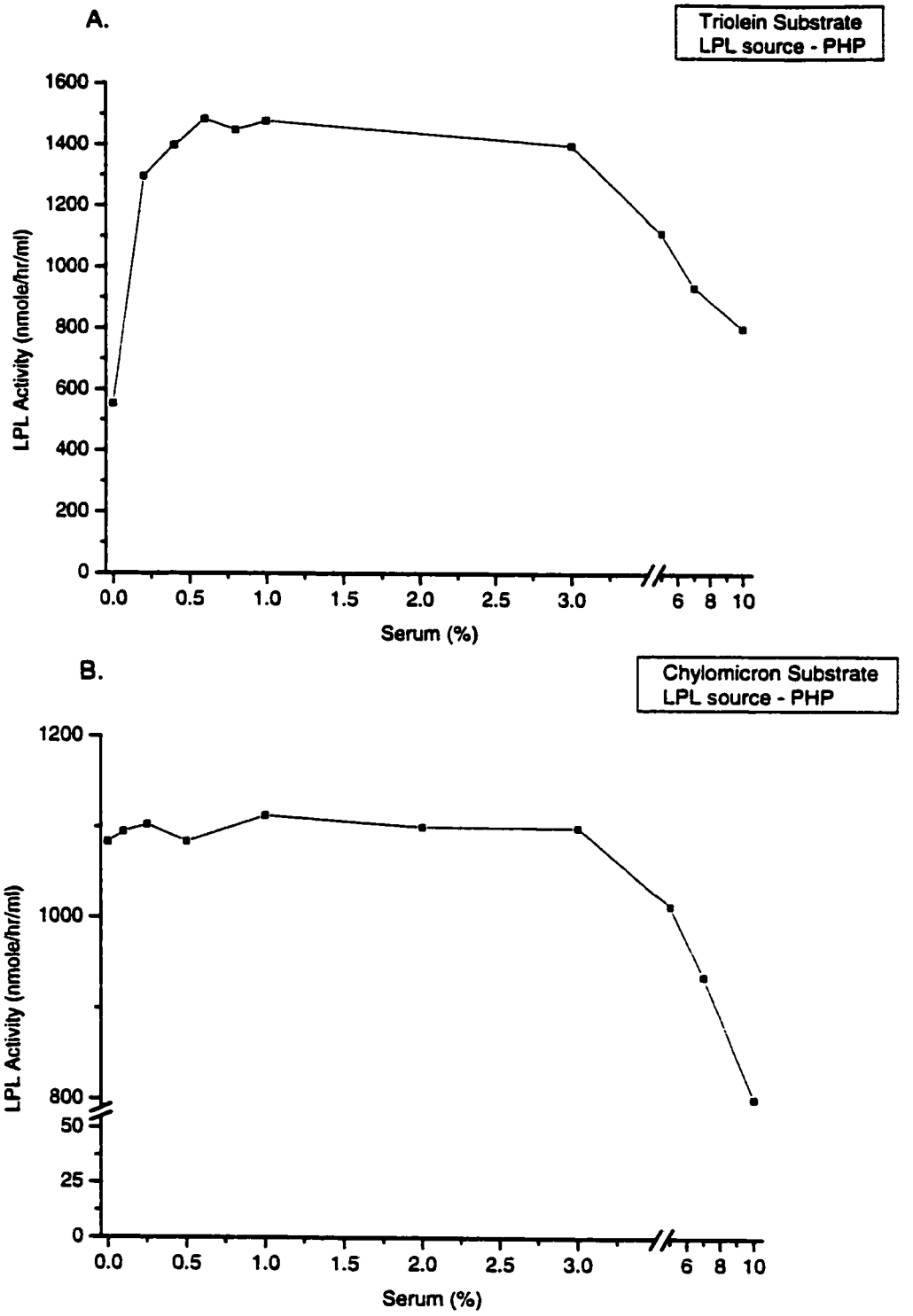

Figure 8: To examine the dependency of LPL on the activator protein apoCII. The assay was conducted with various chicken serum quantities. (A) the sonicated triolein substrate has a basal level of LPL activity present in the absence of apoCII. LPL activity increases with the addition of serum. At 0.50 % serum, the activity of LPL is maximal. (B) LPL activity is unchanged at serum additions of 0 – 3% indicating that hydrolysis of the chylomicron substrate does not increase with exogenous apoCII addition. LPL, lipoprotein lipase; PHP, post-heparin perfusate. Results are from a single experiment.

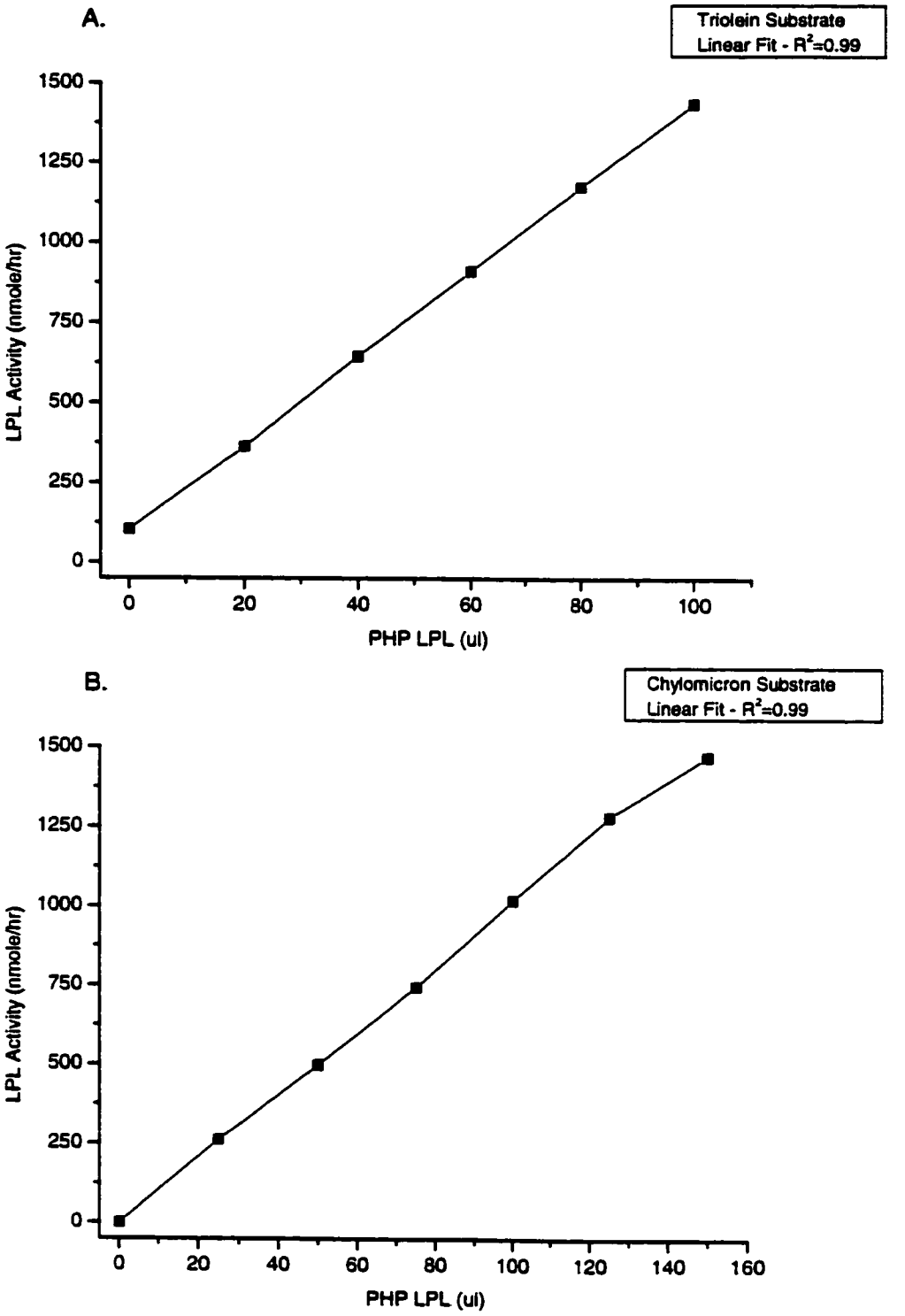

Figure 9: The LPL assay requires an LPL source which in this experiment is a post-heparin perfusate. The lipoprotein lipase assay is linear through a range of PHP amounts (ul) for both triolein (A) and chylomicron (B) substrates. LPL, lipoprotein lipase; PHP, post heparin perfusate. Results are from a single experiment.

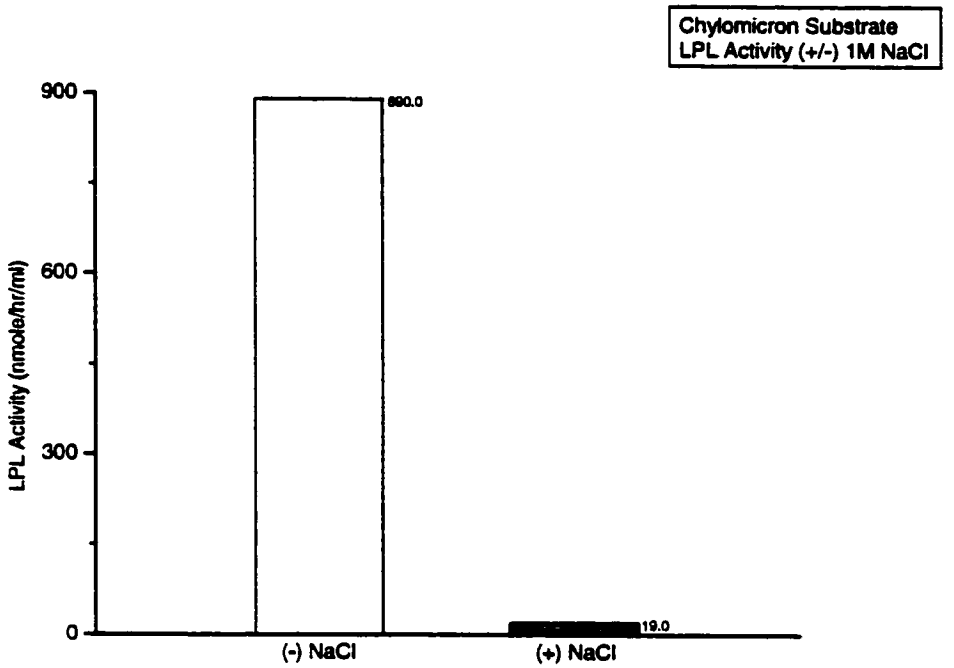

Figure 10: Unlike other lipases lipoprotein lipase is sensitive to high ionic strength environment. To confirm the action of LPL versus other lipases on a chylomicron substrate, 1 M NaCl was added to the assay; LPL activity was reduced by 98 %. LPL, lipoprotein lipase. Results are from a single experiment.

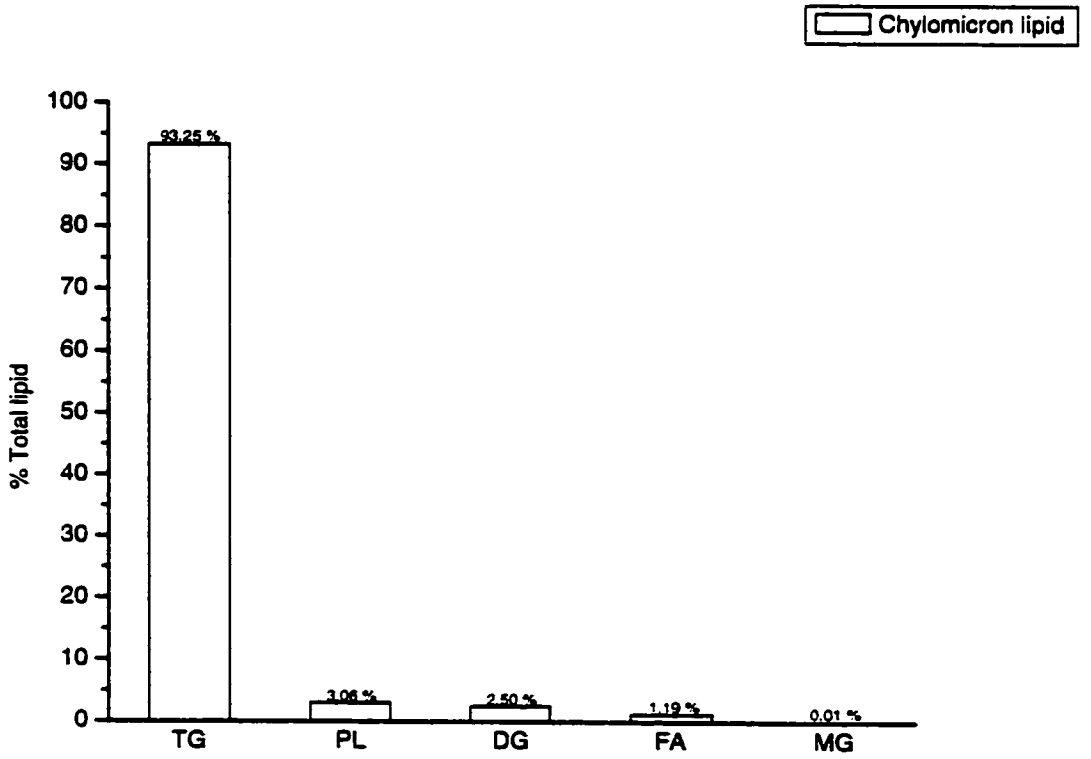

Figure 11: Radiolabeled chylomicron preparation (n=1). Lipids were extracted and subjected to thin-layer chromatography to define the lipid profile. Values are expressed as a percentage of the total lipid incorporation. As expected TG incorporation is greater than 90 %. FA, fatty acid; DG, diacylglycerol; MG, monoacylglycerol; PL, phospholipid; TG, triacylglycerol. Results are from a single experiment.

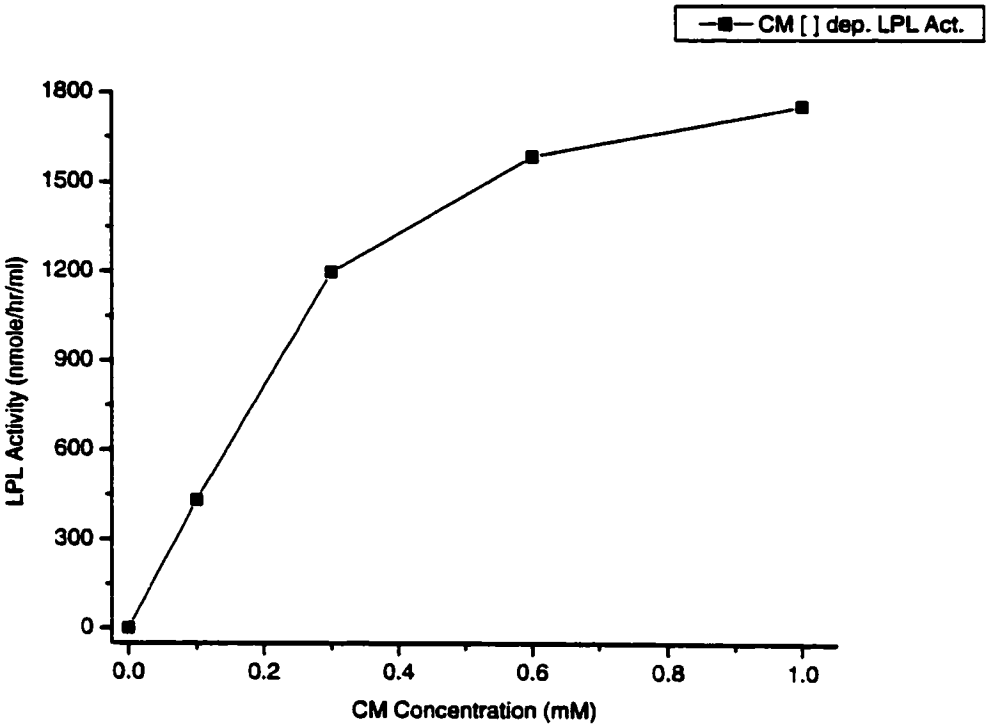

Figure 12: Mouse LPL enzyme hydrolysis of rat chylomicrons follows simple Michaelis-Menten kinetics. Results are from a single experiment.

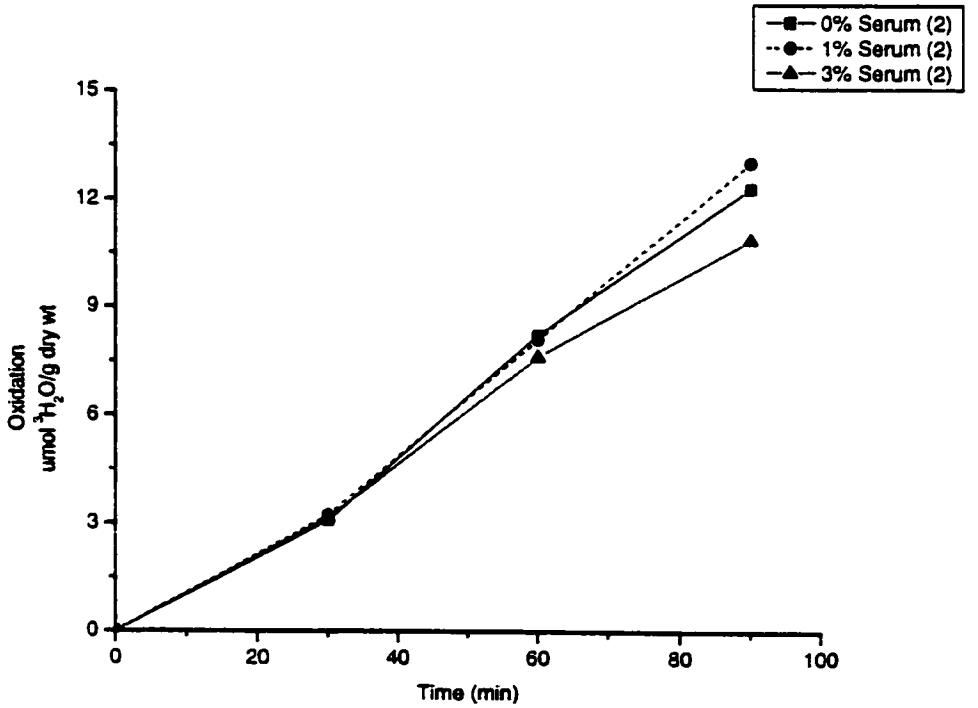

Figure 13: Isolated working mouse hearts perfused with [^{3}H]-chylomicrons. To assess the dependence of isolated heart endothelium-bound LPL on the LPL activator apoCII, rat serum was included at 0, 1 and 3 %. As shown by the oxidation of LPL-derived FA, LPL activity does not appear to depend upon the exogenous supply of Apo C II. Data are means, number of perfusions indicated in parentheses.

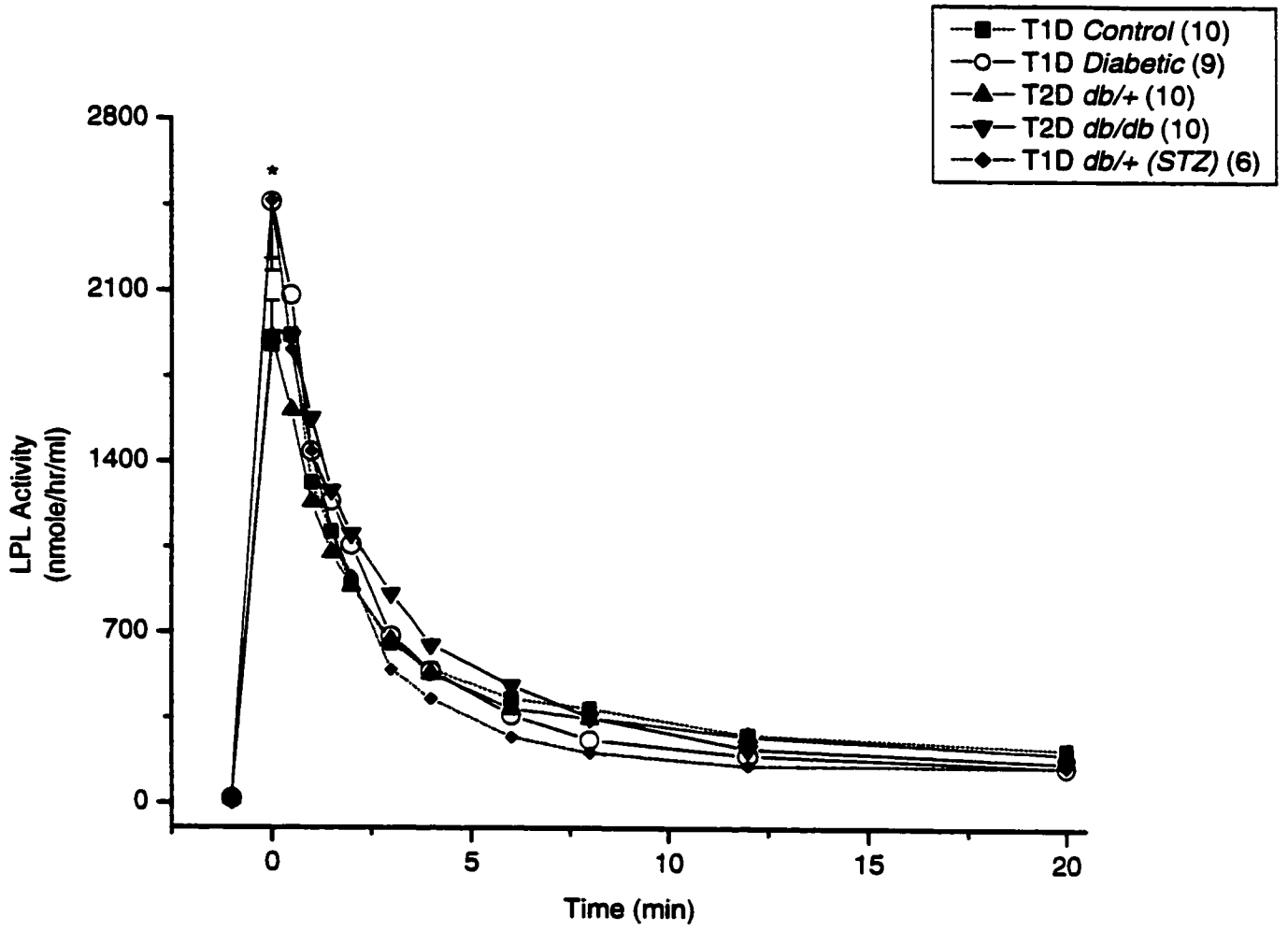

Figure 14: Heparin-releasable (functional) lipoprotein lipase activity in isolated Langendorff mouse hearts perfused with Krebs-Henseleit bicarbonate buffer containing bovine albumin (3 %) and heparin at 5 U/ml. Perfusate samples taken at the times indicated. Data are means, SE included for T1D *diabetic* and T2D *db/db*, number of perfusions indicated in parentheses. * T1D *diabetic* significantly different than T2D *db/db* ($P < 0.05$).

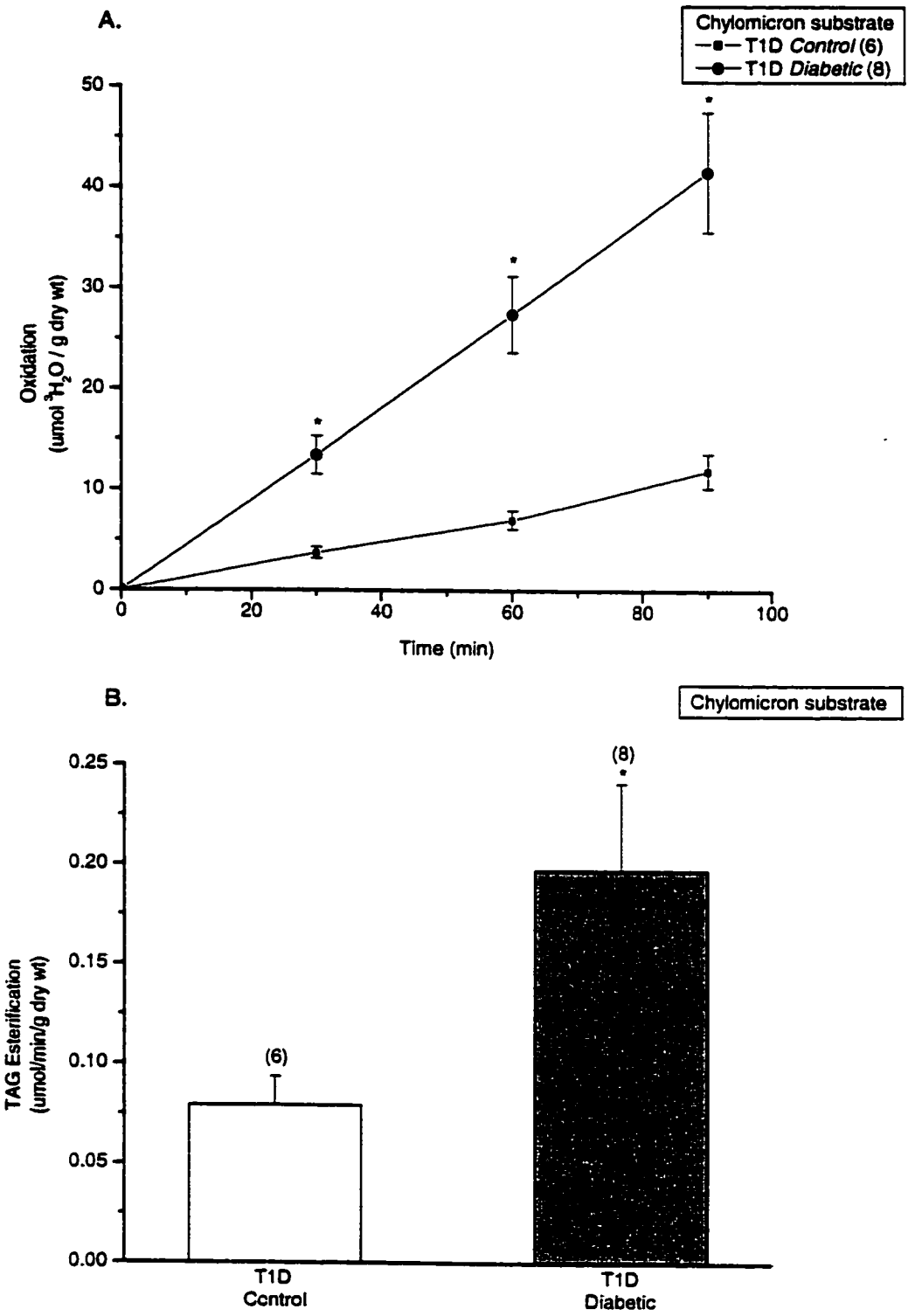

Figure 15: Chylomicron metabolism by perfused hearts from control *SW* and diabetic *SW + STZ* mice. Cumulative measurements of LPL-derived FA oxidation (A) measured with working hearts from T1D *control* and T1D *diabetic* mice over a 90 minute perfusion. (B) Total incorporation of LPL-derived FA into heart tissue lipids (TG). T1D, type 1 diabetes (SW mice +/- STZ (85, 75, 55 mg/kg/day; 2 weeks)). Data are means +/- SE, number of perfusions indicated in parentheses. * Significantly different than controls ($P < 0.05$).

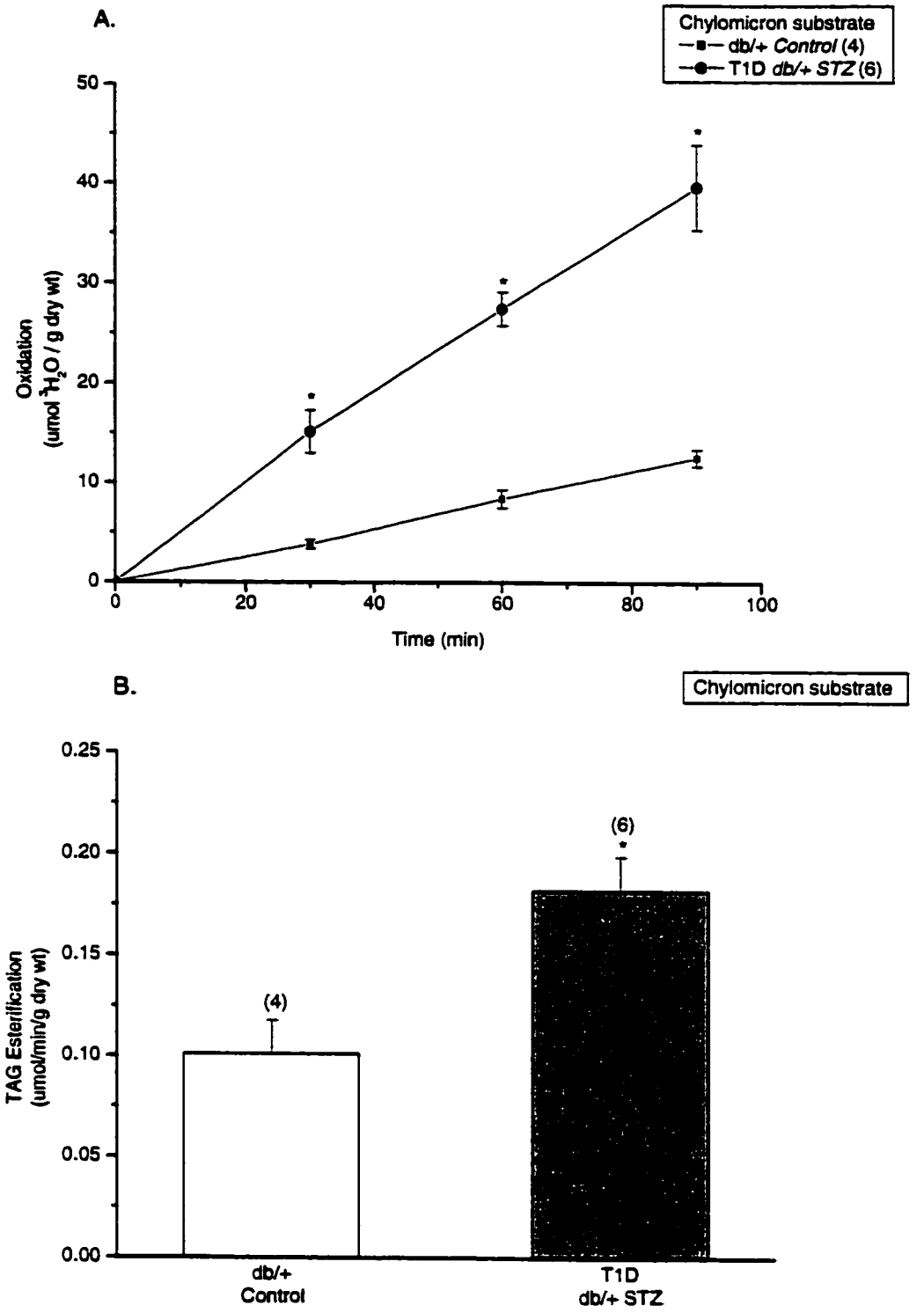

Figure 16: Chylomicron metabolism by perfused hearts from control *db/+* and diabetic *db/+stz* mice. Cumulative measurements of LPL-derived FA oxidation oxidation (A) measured with working hearts from db/+ *control* and T1D *db/+stz* mice over a 90 minute perfusion. (B) Total incorporation of LPL-derived FA oxidation into heart tissue lipids (TG). STZ, streptozotocin; T1D, type 1 diabetes (db/+ mice +/- STZ (85, 75, 55 mg/kg/day; 2 weeks)). Data are means +/- SE, number of perfusions indicated in parentheses. * Significantly different than controls ($P < 0.05$).

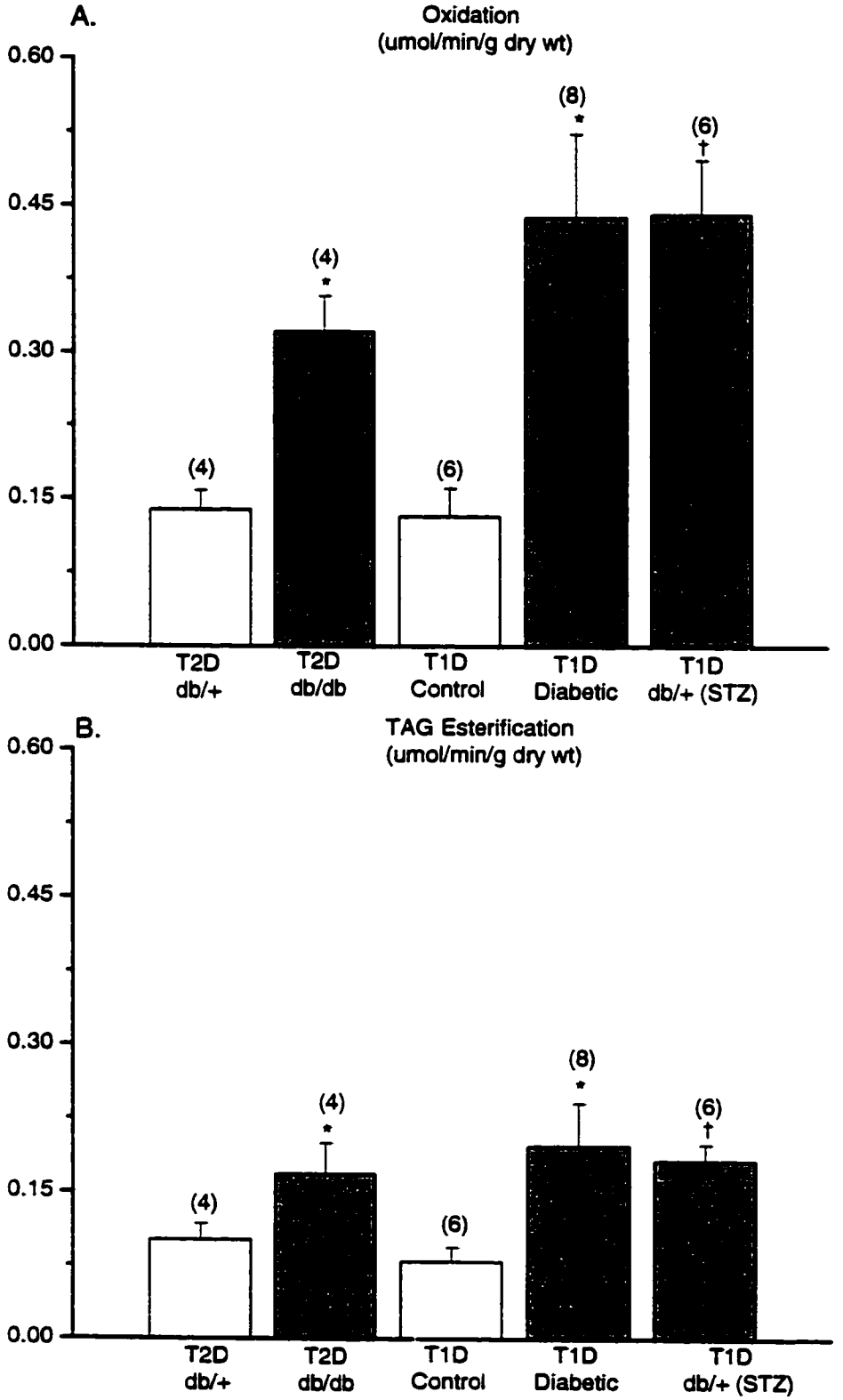

Figure 17: Isolated working hearts perfused with chylomicrons. A) Oxidation rates were calculated as the mean of three measurements, 0 – 30, 30 – 60 and 60 – 90 minutes. B) Lipids were extracted and subjected to thin-layer chromatography from myocardial tissue at the end of perfusion for estimation of TAG incorporation. TAG, triacylglycerol. Data are means +/- SE, number of perfusions indicated in parentheses. * Significantly different than controls, † significantly different than T2D *db/+* ($P < 0.05$).

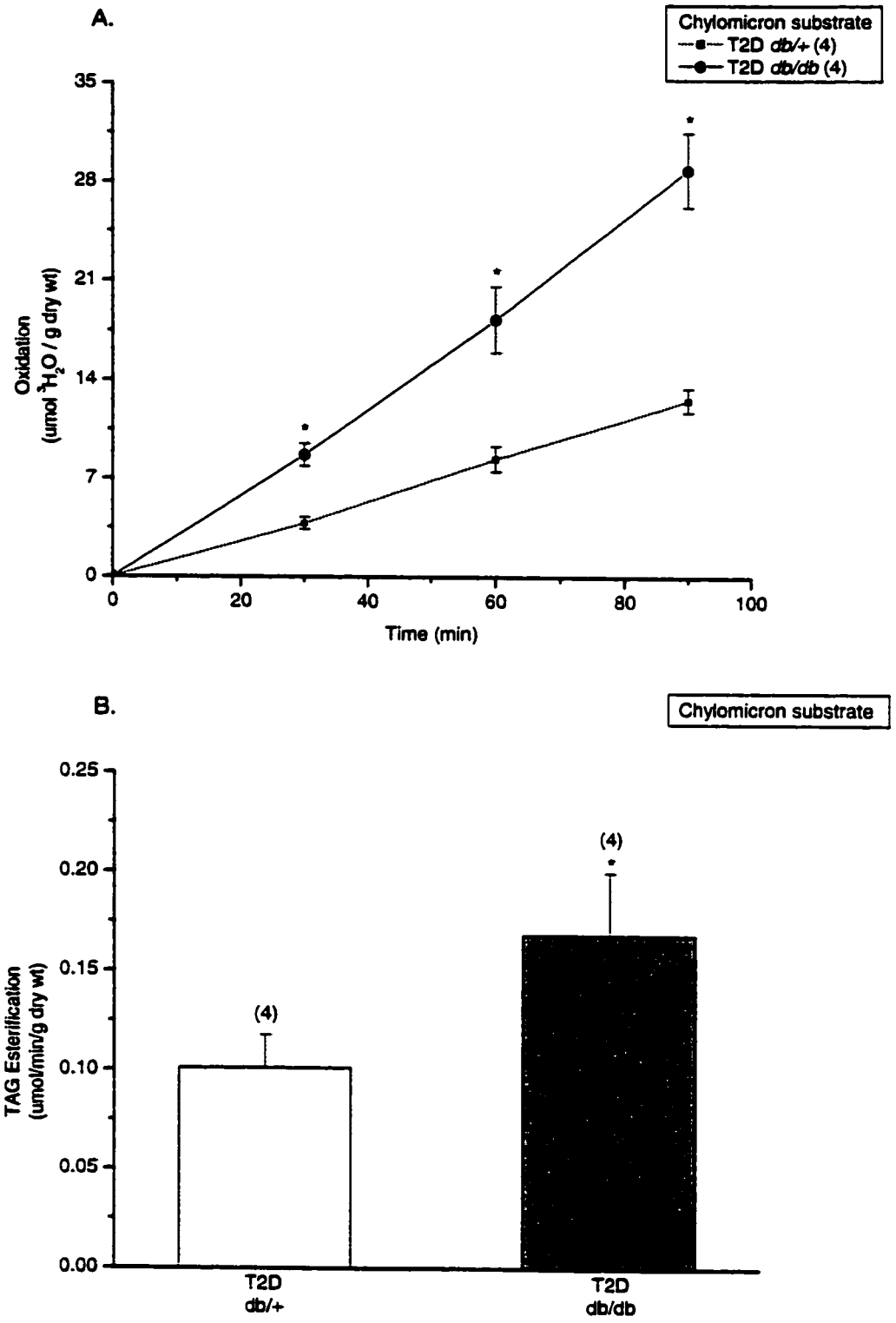

Figure 18: Chylomicron metabolism by perfused hearts from control *db/+* and diabetic *db/db* mice. Cumulative measurements of LPL-derived FA oxidation (A) measured with working hearts from T2D *db/+* and *db/db* mice over a 90 minute perfusion. (B) Total incorporation of LPL-derived FA into heart tissue lipids (TG). T2D, type 2 diabetes. Data are means +/- SE, number of perfusions indicated in parentheses. * Significantly different than controls ($P < 0.05$).

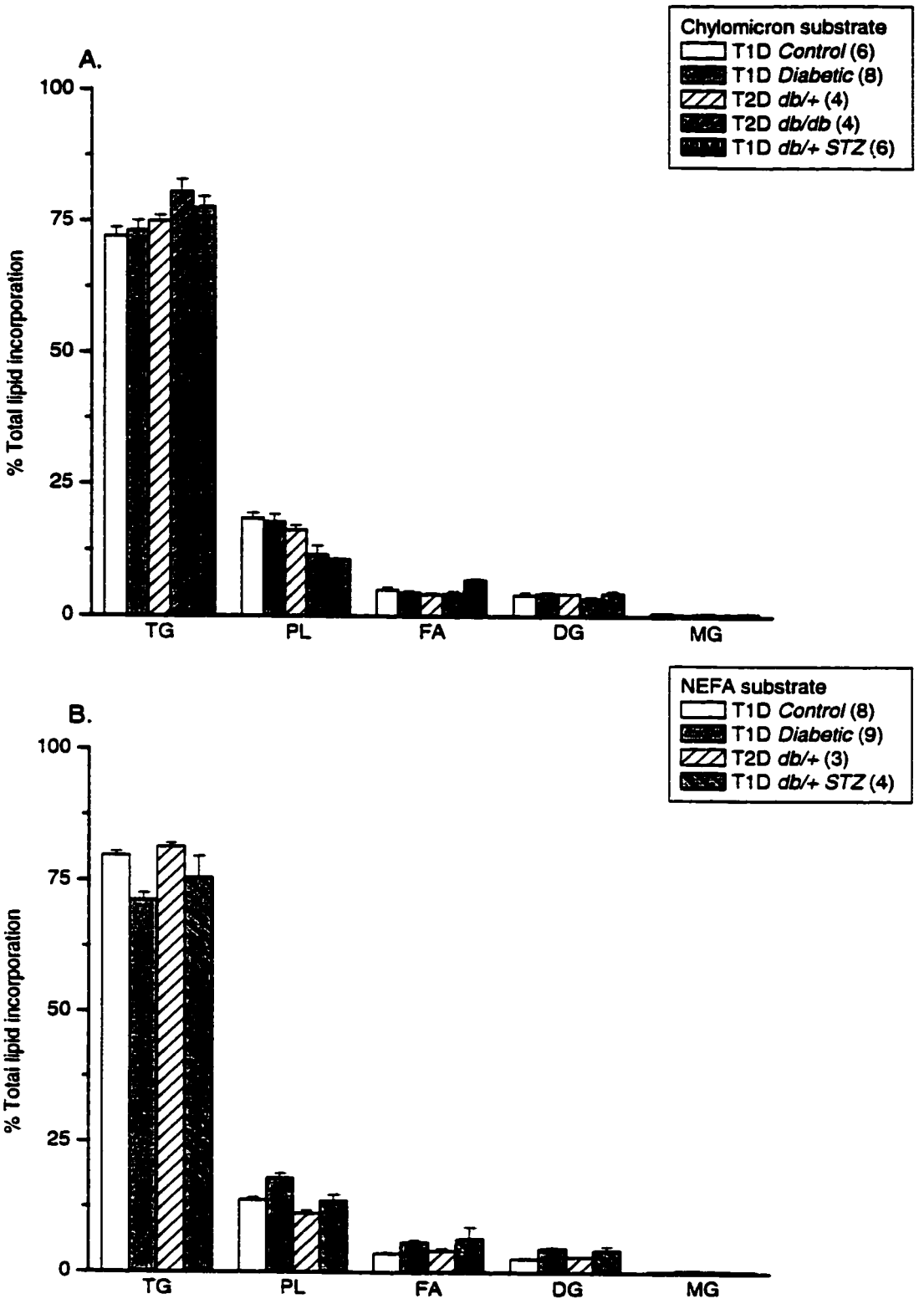

Figure 19: Isolated working hearts were perfused with chylomicrons (A) or NEFA (palmitate; B). Lipids were extracted through thin-layer chromatography from myocardial tissue for estimation of lipid incorporation. Values are expressed as the percent of each lipid fraction to the total lipid incorporation. FA, fatty acid; DG, diacylglycerol; MG, monoacylglycerol; PL, phospholipid; TG, triacylglycerol. Data are means +/- SE, number of perfusions indicated in parentheses.

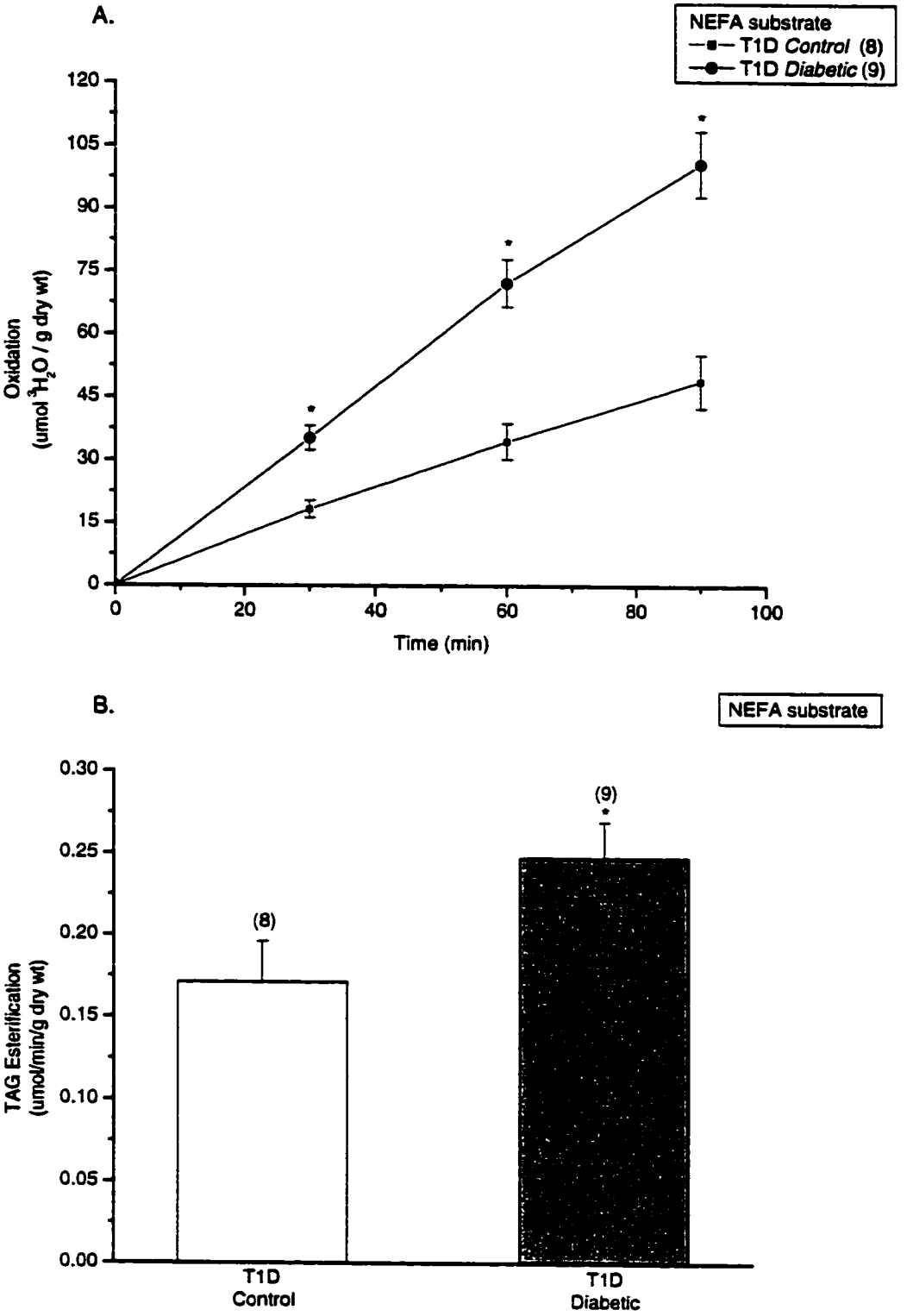

Figure 20: NEFA ([^{3}H]palmitate) metabolism by perfused hearts from control *SW* and diabetic *SW + STZ* mice. Cumulative measurements of [^{3}H]palmitate oxidation (A) measured with working hearts from T1D *control* and T1D *diabetic* mice over a 90 minute perfusion. (B) Total incorporation of [^{3}H]palmitate into heart tissue lipids (TG). T1D, type 1 diabetes (SW mice +/- STZ (85, 75, 55 mg/kg/day; 2 weeks)). Data are means +/- SE. * Significantly different than controls ($P < 0.05$).

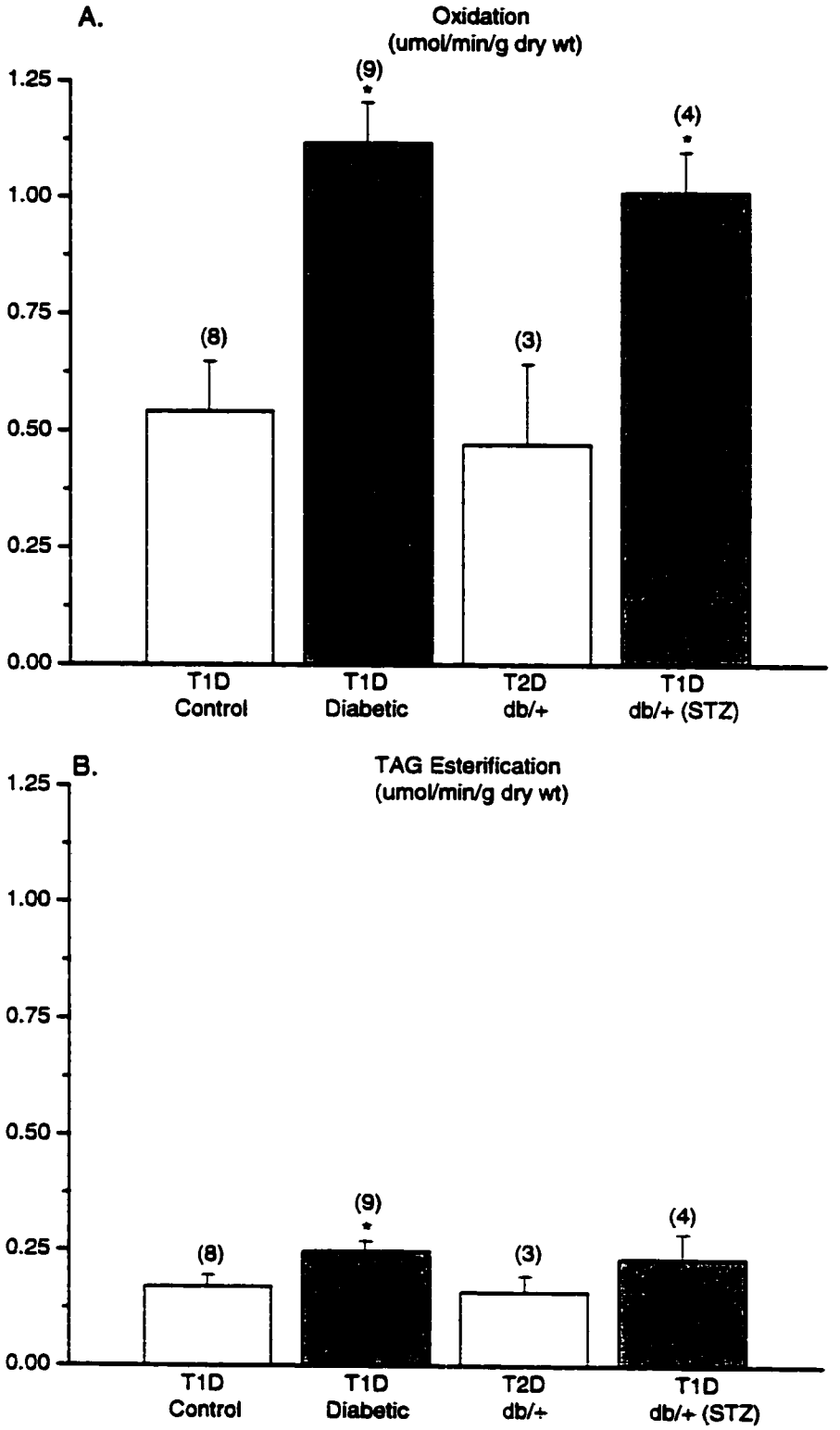

Figure 21: Isolated working hearts perfused with NEFA ([^{3}H]palmitate). A) Oxidation rates were calculated as the mean of three measurements, 0–30, 30–60 and 60–90 minutes. B) Lipids were extracted through thin-layer chromatography from myocardial tissue for estimation of TAG incorporation. NEFA, non-esterified fatty acid; TAG, triacylglycerol. Data are means +/- SE, number of perfusions indicated in parentheses. * Significantly different than controls ($P < 0.05$).

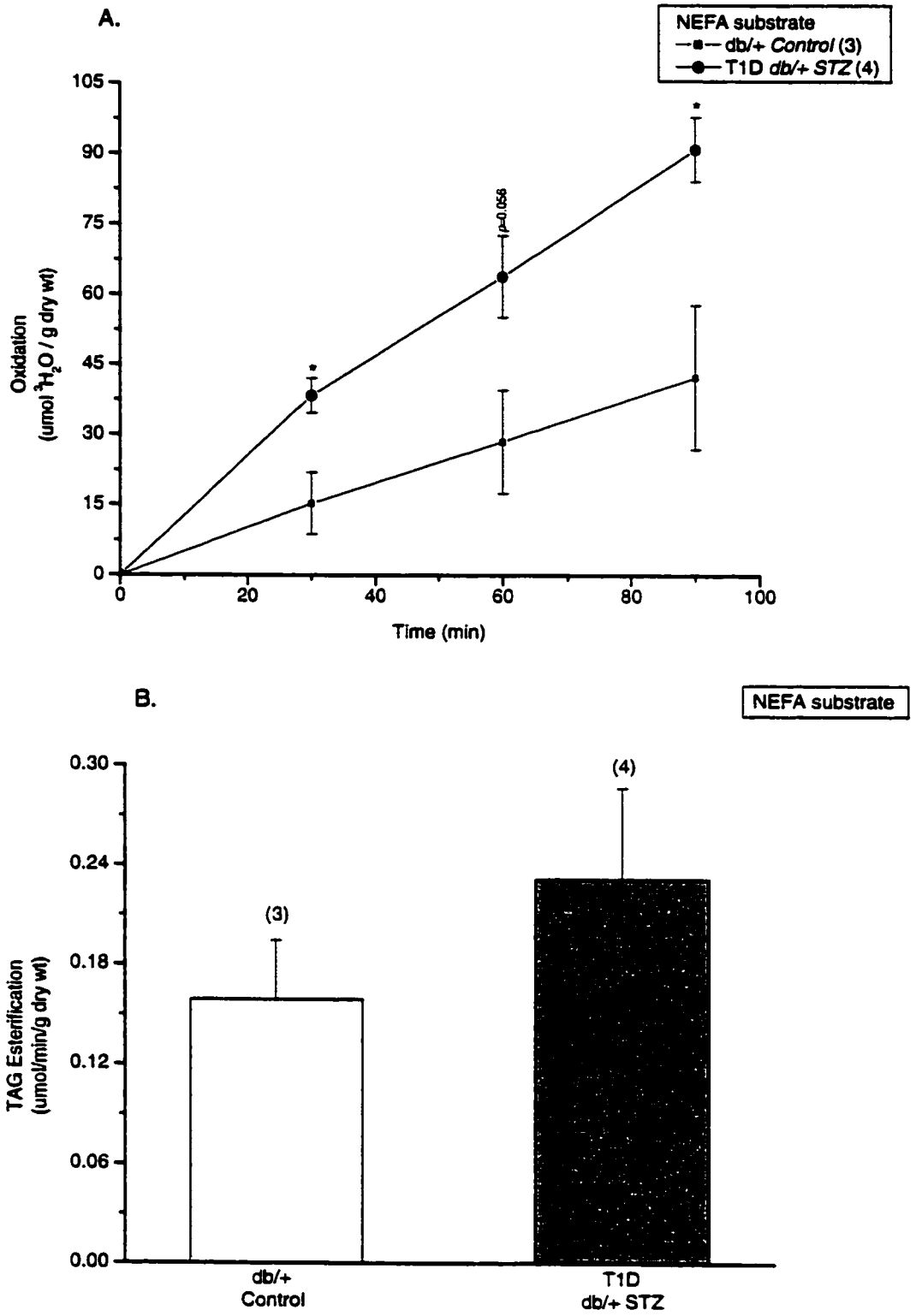

Figure 22: NEFA ([^{3}H]palmitate)metabolism by perfused hearts from control *db/+* and diabetic *db/+stz* mice. Cumulative measurements of [^{3}H]palmitate oxidation (A) measured with working hearts from db/+ *control* and T1D *db/+stz* mice over a 90 minute perfusion. (B) Total incorporation of [^{3}H]palmitate oxidation into heart tissue lipids (TG). STZ, streptozotocin; T1D, type 1 diabetes (db/+ mice +/- STZ (85, 75, 55 mg/kg/day; 2 weeks)). Data are means +/- SE. number of perfusions indicated in parentheses. * Significantly different than controls ($P < 0.05$).

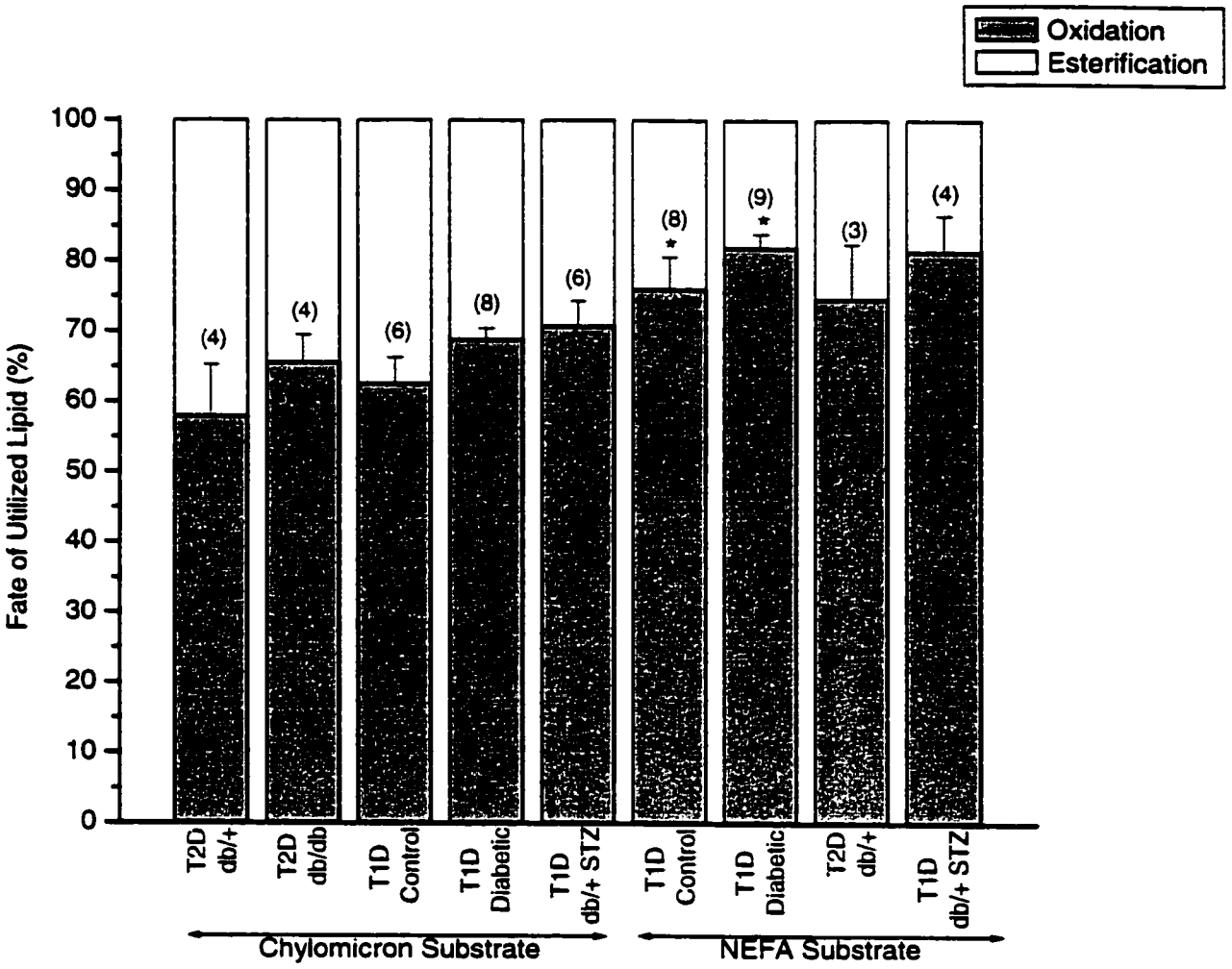

Figure 23: Lipid fate was calculated as a relative percentage of both oxidation and esterification to the total utilization of lipid. Fate for both chylomicron and NEFA source presented. Data are means +/- SE, number of perfusions indicated in parentheses. * Significantly different than hearts perfused with chylomicron substrate ($P < 0.05$).

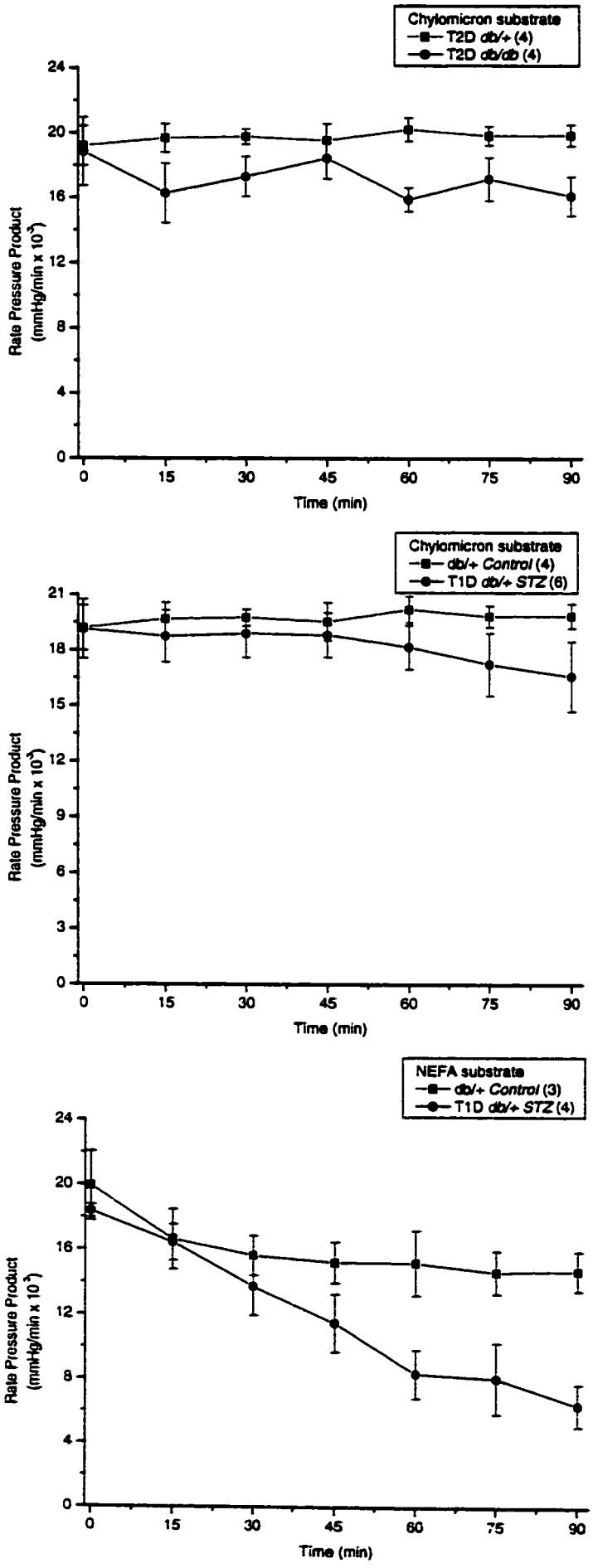

Figure 24: Myocardial mechanical performance (mmHg/min x 10-3) in type 1 and 2 diabetic isolated working mouse hearts perfused with chylomicrons (0.4 mM) or palmitate (0.4 mM). Data are means +/- SE, number of perfusions indicated in parentheses.

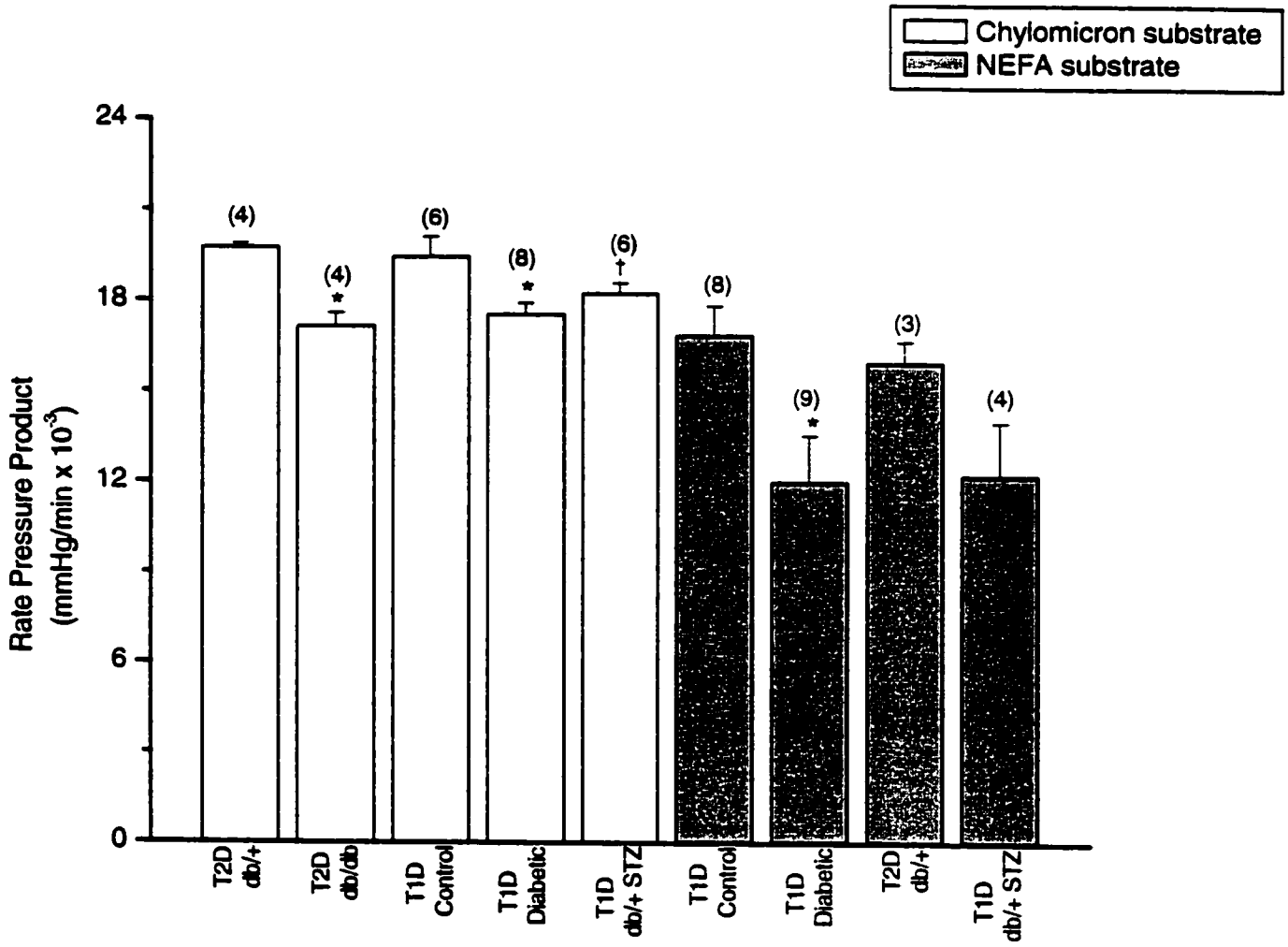

Figure 25: Mean myocardial mechanical performance in isolated working mouse hearts perfused with chylomicrons (0.4 mM) and palmitate (0.4 mM) for 90 minutes. Preload and afterload were fixed, hearts were not paced. Data are means +/- SE, number of perfusions indicated in parentheses. * Significantly different than controls, † significantly different than T2D *db/+* ($P < 0.05$).

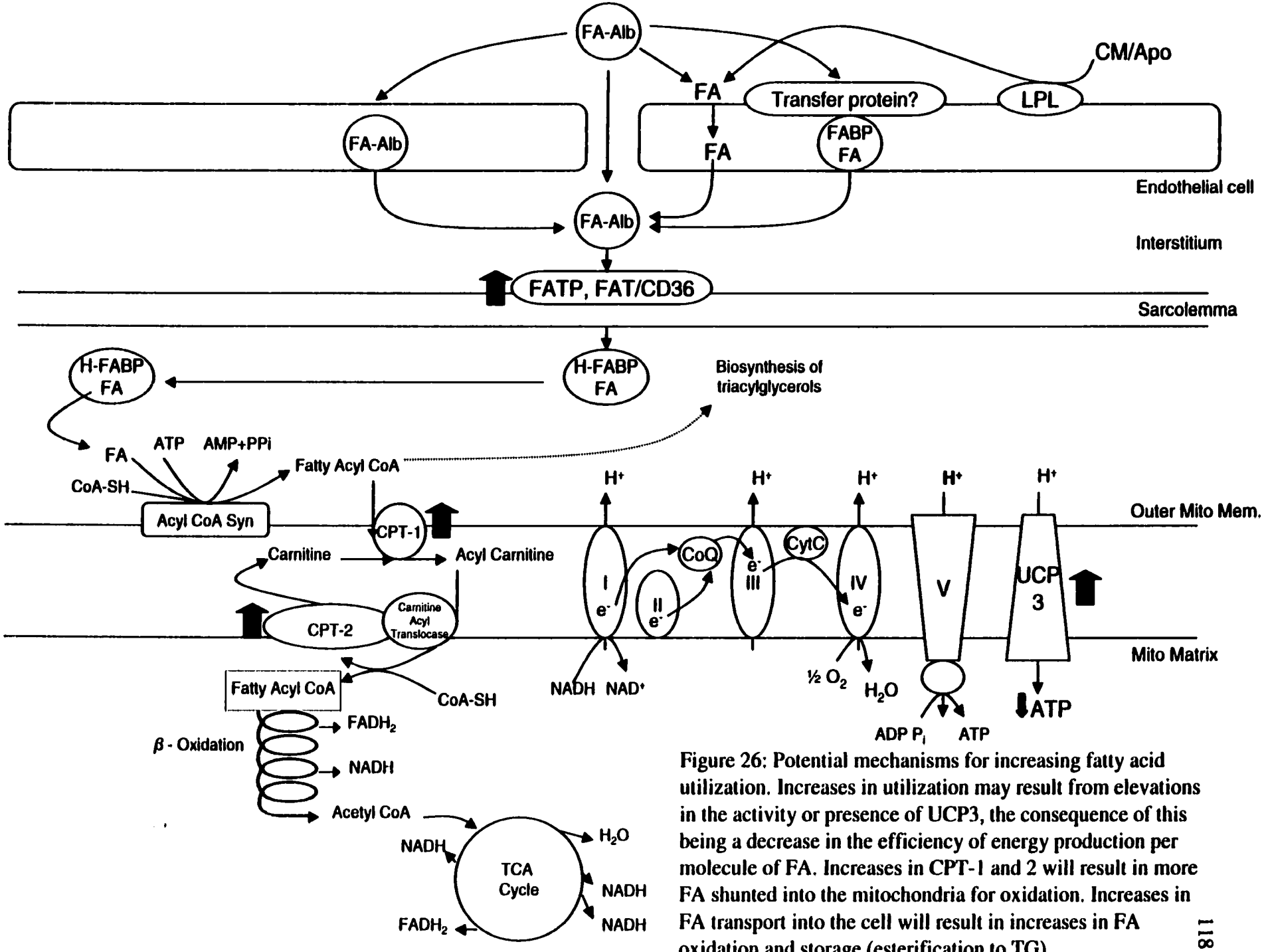

Figure 26: Potential mechanisms for increasing fatty acid utilization. Increases in utilization may result from elevations in the activity or presence of UCP3, the consequence of this being a decrease in the efficiency of energy production per molecule of FA. Increases in CPT-1 and 2 will result in more FA shunted into the mitochondria for oxidation. Increases in FA transport into the cell will result in increases in FA oxidation and storage (esterification to TG).

Table 1: Characteristics of Type 1 and 2 diabetic mice.

Mouse Type	Body Weight (g)	Heart Weight (mg)	Plasma Glucose (mmol/L)
T2D *(db/+)*	30.0 +/- 0.69	28.0 +/- 1.83	10.2 +/- 0.56
T2D *(db/db)*	52.6 +/- 1.08 *	29.3 +/- 3.2	31.6 +/- 1.08*
T1D *Control*	39.2 +/- 1.27 (Pre-CB 37.8 +/- 0.49)	33.3 +/- 0.90	8.6 +/- 0.48
T1D *Diabetic*	31.9 +/- 0.70 * † (Pre-STZ 37.0 +/- 0.59)	29.0 +/- 1.28 *	30.1 +/- 0.81*
T1D *(db/+ STZ)*	19.8 +/- 0.64 * † (Pre-STZ 28.3 +/- 0.28)	22.6 +/- 1.39 *	28.5 +/- 1.43 *

Data are means +/- SE for T2D *db/+* (n=10), T2D *db/db* (n=10), T1D *control* (n=10), T1D *diabetic* (n=10) and T1D *db/+ STZ* (n=6) mice; plasma samples were collected from fed mice. CB, citrate buffer; STZ, streptozotocin. * Significantly different than control ($P < 0.05$), † significantly different than pre-STZ treatment.

Table 2: Lipoprotein lipase activity in Type 1 and 2 diabetic mice.

Mouse Type	Peak perfusion (nmole/hr/ml)	Total HR LPL (nmole/hr)	Cardiac tissue (nmole/hr/mg)
T2D *(db/+)*	1908.2 +/- 126.34 (10)	9085 +/- 829 (10)	2571.7 +/- 108.67 (7)
T2D *(db/db)*	1912.6 +/- 146.43 (10)	10522 +/- 653 (10)	2588.1 +/- 114.71 (9)
T1D *Control*	1881.7 +/- 361.92 (10)	9650 +/- 1102 (10)	2245.9 +/- 119.67 (5)
T1D *Diabetic*	2462.9 +/- 231.93 * (9)	10477 +/- 832 (9)	2391.9 +/- 260.57 (5)
T1D *(db/+ STZ)*	2468.7 +/- 286.71 * (6)	9584 +/- 1250 (6)	2451.7 +/- 177.19 (6)

Data are means +/- SE, number of perfusions is indicated in parentheses. * Significantly different than T2D *db/db* ($P < 0.05$).

Table 3: Comparison of substrate metabolism in type 1 & 2 diabetic mice (umol/min/g dry wt).

	Chylomicron substrate		NEFA substrate	
	Oxidation	Esterification	Oxidation	Esterification
T2D *db/+*	0.139 +/- 0.020 (4)	0.101 +/- 0.017 (4)	0.471 +/- 0.172 (3)	0.159 +/- 0.035 (3)
T2D *db/db*	0.321 +/- 0.037 * (4)	0.168 +/- 0.032 * (4)	Belke et al. (2000)	-
T1D *Control*	0.132 +/- 0.029 (6)	0.079 +/- 0.014 (6)	0.541 +/- 0.107 (6)	0.171 +/- 0.025 (8)
T1D *Diabetic*	0.438 +/- 0.086 * (8)	0.197 +/- 0.044 * (8)	1.118 +/- 0.087 * (8)	0.247 +/- 0.022 * (9)
T1D *(db/+ STZ)*	0.441 +/- 0.056 * (6)	0.181 +/- 0.017 * (6)	1.01 +/- 0.088 * (4)	0.231 +/- 0.055 * (4)

Data are means +/- SE, number of perfusions is indicated in parentheses. STZ, streptozotocin; T1D, type 1 diabetes; T2D, type 2 diabetes. * Significantly different than controls ($P < 0.05$).

Table 4: Cardiac function in Type 1 and 2 diabetic perfused working mouse hearts.

		HR (bpm)	PSP (mmHg)	AF (ml/min)	CF (ml/min)	CO (ml/min)	CO (ml/min/g)
T2D *db/+*	CM (4)	357 +/- 9.1	55.4 +/- 1.28	6.69 +/- 0.05	1.64 +/- 0.01	8.30 +/- 0.06	296 +/- 2.6
	Palmitate (3)	300 +/- 21.8	53.1 +/- 0.97	7.86 +/- 0.27	2.03 +/- 0.07	9.89 +/- 0.32	353 +/- 11.6
T2D *db/db*	CM (4)	302 +/- 23.1 *	56.6 +/- 0.98	6.59 +/- 0.13	1.34 +/- 0.02	7.94 +/- 0.15	271 +/- 5.8
	Palmitate	277 +/- 13.0 †	57.0 +/- 1.0 †	6.80 +/- 0.50 †	2.10 +/- 0.10 †	8.90 +/- 0.50 †	-
T1D *SW*	CM (6)	370 +/- 22.2	54.3 +/- 0.97	7.48 +/- 0.14	2.04 +/- 0.03	9.52 +/- 0.15	285 +/- 4.6
	Palmitate (8)	317 +/- 23.3	62.4 +/- 5.63	8.25 +/- 0.56	1.85 +/- 0.07	10.10 +/- 0.62	303 +/- 18.7
T1D *SW + STZ*	CM (8)	330 +/- 19.7 *	53.8 +/- 0.72	7.59 +/- 0.17	1.89 +/- 0.06	9.48 +/- 0.22	326 +/- 8.6
	Palmitate (9)	241 +/- 23.6 *	58.3 +/- 3.13	7.04 +/- 0.58	1.56 +/- 0.06	8.60 +/- 0.64	296 +/- 22.1
T1D *db/+ + STZ*	CM (6)	332 +/- 6.37	55.1 +/- 0.06	5.06 +/- 0.09	1.36 +/- 0.02	6.42 +/- 0.11 *	284 +/- 4.9
	Palmitate (4)	227 +/- 31.8	53.8 +/- 0.32	5.01 +/- 0.34	1.42 +/- 0.04	6.43 +/- 0.37 *	284 +/- 16.6 *

Data are means +/- SE, number of perfusions is indicated in parentheses. AF, aortic flow; CF, coronary flow; CM, chylomicron; CO, cardiac output; HR, heart rate; PSP, peak systolic pressure. * Significantly different than controls ($P < 0.05$), † indicates data from Aasum *et al* (2002 in press).

Table 5: Variability in measurement of cardiac lipoprotein lipase activity in Type 1 and 2 diabetic models.

Model	PHP LPL	Tissue LPL	References
IDDM-S. Dawley (stz)	↓	n/c	Liu (1995)
IDDM-Wistar (stz)	↑	-	Sambandam (1999)
IDDM-W. Kyoto (stz)	n/c	↓	Rodrigues (1993)
NIDDM-Corpulent JCR	-	↓	Mantha (2002)
NIDDM-db/db	-	↓	Kobayashi (2000)
NIDDM-ob/ob	-	↑	Gasquet (1972)

Data are means +/- SE, number of perfusions is indicated in parentheses. * Significantly different than T2D *db/db* ($P < 0.05$).