THE UNIVERSITY OF HULL

Impact of Dietary Manipulation on Cardiac Hypertrophy

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by

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Abstract

Left ventricular hypertrophy (LVH) is a significant risk factor for the development of heart failure (HF), the incidence of which is increased by obesity. Diets high in fat and sugar have been linked with the development of the metabolic syndrome and obesity, and may expose the heart to a unique environment via the differential actions of dietary macronutrients. The main objectives of this study were to determine the effect of differing dietary regimens upon (i) the progression of LVH and whole organism morphology (ii) function and metabolism in the hypertrophied heart, and (iii) cardiac ceramide content.

Cardiac hypertrophy was surgically induced in male Sprague-Dawley rats via abdominal aortic constriction (AC). Animals were assigned to either a diet containing 5% sucrose/7% fat (standard diet, SD), 9 % sucrose/45 % fat (high-fat diet, HFD), or 14% sucrose/44% fat (western diet, WD) for 9 weeks. LVH was observed in all AC groups but was greatest in those fed a SD or WD. Both HFD and WD resulted in a significant increase in abdominal fat mass, which was positively associated with serum concentrations of leptin.

In vitro cardiac function was unaltered by any dietary regimen alone, but was significantly enhanced in hypertrophied hearts from HFD and WD-fed animals, consistent with a compensated phase of hypertrophic remodelling. This was accompanied by a small reduction in palmitate oxidation and increased reliance upon lactate, an effect which was exacerbated in hearts from WD-fed animals. In WD-fed animals, there was a substantial increase in cardiac triglyceride (TG), which was not affected by AC. PPARα protein was reduced following AC in the hearts of animals fed a SD or WD, whereas the HFD prevented this decline. CD36 protein expression was not different between control and AC animals, but was highest in those fed a WD.

In addition to elevated TG, WD hearts also exhibited a significant accumulation of long-chain ceramide species (C16-C24) compared with other dietary groups; consistent with metabolic remodelling. This effect was observed independent of AC. In order to simulate a model of HF, WD animals were treated with adriamycin (ADR), and cardiac ceramide content was further increased with the specific accumulation of C16 and C18 ceramide.

These findings suggest that dietary macronutrient composition can have a profound effect upon the progression of LVH. Furthermore, the enhanced ceramide content in WD hearts indicates that the macronutrient composition of this dietary profile is most deleterious to the hypertrophied heart. Prolonged exposure of the hypertrophied heart to the WD may lead to increased apoptosis and accelerate the transition to HF.

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Equations

Abbreviations

AC.....Aortic constriction
ACC.....Acetyl-CoA carboxylase
ACO..... Acyl-CoA oxidase
ACS..... Acyl-CoA synthetase
ADR..... Adriamycin
AFE..... Atwater Fuel Energy
Akt.....Protein kinase B
AMP.....Adenosine monophosphate
AMPK.....AMP-activated protein kinase
ATP..... Adenosine triphosphate
BMI.....Body mass index
BSA..... Bovine Serum Albumin
Bpm.....Beats per minute
BW..... Body weight
CAT.....Carnitine acylcarnitine transferase
CE.....Cardiac Efficiency
CFR..... Coronary flow rate
CoA.....Co-enzyme A
CPT..... Carnitine Palmitoyltransferase
CRP..... C-reactive protein
CS..... Citrate synthase
DCA..... Dichloroacetic acid
DHA..... Docosahexaenoic acid
DSS rat.....Dahl salt-sensitive rat
DTNB..... 5,5 dithio-bis-2-nitrobenzoic acid
EAT..... Epididymal adipose tissue
ECM.....Extracellular matrix
EDP.....End diastolic pressure
EDTA.....Ethylenediaminetetraacetic acid
EGTA.....Ethylene glycol tetraacetic acid
EPA.....Eicosapentaenoic acid
ER..... Endoplasmic reticulum
F1,6BP..... Fructose 1,6-bisphosphate
F2,6BP..... Fructose 2,6-bisphosphate
FA..... Fatty acid
FABP..... Fatty acid binding protein
FAO.....Fatty acid oxidation
FAT..... Fatty acid translocase
FFA..... Free fatty acid
GAPDH.....Glyceraldehyde-3-phosphate dehydrogenase
GI.....Glycaemic index
GLUT1/GLUT4..... Glucose transporter 1/4
HDL.....High-density lipoprotein

HF..... Heart failure
HFD.....High-fat diet
HK.....Hexokinase
HPLC..... High-performance liquid chromatography
HW..... Heart weight
ICM..... Intracellular membrane
IL..... Interleukin
IR..... Insulin resistance
iv..... Intravenous
JAK..... Janus kinase
KH.....Krebs-Henseleit (buffer)
KO.....Knock out
LCAD.....Long-chain acyl-CoA dehydrogenase
LCFA..... Long-chain fatty acid
LC-MS..... Liquid chromatography mass spectrometry
LC-MS/MS..... Liquid chromatography tandem mass spectrometry
LDL..... Low-density lipoprotein
LPL.....Lipoprotein lipase
LVDP.....Left ventricular developed pressure
LVH.....Left ventricular hypertrophy
MAPK..... Mitogen activated protein kinase
MAT.....Mediastinal adipose tissue
MCAD..... Medium-chain acyl-CoA dehydrogenase
MCT.....Monocarboxylate transporter
MI.....Myocardial infarction
MMP..... Matrix metalloproteinase
MS..... Mass spectrometry
MUFA..... Mono-unsaturated FA
NAD.....Nicotinamide adenine dinucleotide
PCA.....Perchloric acid
OGDH..... Oxoglutarate dehydrogenase
ORO.....Oil Red O
PAGE..... Polyacrylamide gel electrophoresis
PDH..... Pyruvate dehydrogenase
PDK.....PDH kinase
PDP.....PDH phosphatise
PEP..... Phosphoenol pyruvate
PFK..... Phosphofructokinase
PGC-1..... PPARγ coactivator-1
PI3K..... Phosphatidylinositol 3-kinase
PKC..... Protein kinase C
PPAR.....Peroxisome proliferator-activated receptor
PPRE..... Peroxisome proliferator response element
PUFA.....Poly-unsaturated FA
Retinoid-X-Receptor.....RXR
ROS.....Reactive oxygen species

RPAT..... Retroperitoneal + perirenal adipose tissue
RPP..... Rate pressure product
s/c..... Subcutaneous
SD..... Standard diet
SDS..... Sodium dodecyl sulphate
SFA..... Saturated FA
SHHF rat..... Spontaneously hypertensive heart failure rat
SL..... Sphingolipid
SM..... Sphingomyelin
SNA..... Sympathetic nerve activation
SOCS..... Suppressor of cytokine signalling
S1P..... Sphingosine-1-phosphate
SP..... Systolic pressure
SPT..... Serine Palmitoyltransferase
SR..... Sarcoplasmic reticulum
SREBP1c..... Sterol response element-binding protein-1c
STAT..... Signal transducer and activators of transcription
TAC..... Transverse aortic constriction
TCA cycle..... Tri-carboxylic acid cycle
TEP..... Triethyl phosphate
TG..... Triglyceride
TIC..... Total Ion Current
TNFα..... Tumour necrosis factor α
TOF..... Time of flight
TPP..... Thiamine pyrophosphate
TZD..... Thiazolidinedione
UCP..... Uncoupling protein
VLCAD..... Very long-chain acyl-CoA dehydrogenase
WD..... Western diet
ZDF rat..... Zucker diabetic fatty rat

Chapter 1 Introduction

1.1 Cardiac metabolism in the healthy heart

Cardiac function is dependent upon a continuous, rapid supply of energy due to a very high ATP demand (Ingwall and Weiss, 2004). Furthermore, this ATP can be derived from the reactions of glycolysis, oxidative phosphorylation, and from the small but crucially important conversion of phosphocreatine (PCr) to creatine (Cr). The predominant source of energy in the healthy heart is derived from the oxidation of fatty acids (FAs) (van der Vusse et al., 2000), with other major contributions from carbohydrate, predominantly glucose and lactate (Stanley et al., 2005). The numerous substrates utilised liken the heart to a metabolic "omnivore" (Taegtmeyer and Salazar, 2004). As such, maintaining a high level of ATP turnover and the ability to metabolise different substrates permits metabolic plasticity, crucially important in maintaining cardiac performance (Stanley et al., 2005).

1.1.1 Lipid uptake and metabolism

Cardiac tissue is greatly dependent upon the concentration of circulating FAs in order to meet energetic demand. FAs exist as either albumin-bound complexes or esterified into the formation of triglyceride (TG) in circulating chylomicrons and lipoproteins (van der Vusse et al., 2000). For the heart to utilise these endogenous sources of FAs they must be liberated via the action of lipoprotein lipase (LPL) upon the surface of vascular endothelial cells, or be endocytosed followed by intracellular hydrolysis and release of free fatty acids (FFAs) (Goldberg, 1996). Indeed, recent studies have indicated the importance of chylomicron- and VLDL-derived TGs in working heart preparations (Hauton et al., 2001, Niu et al., 2004).

The fatty acid translocase (FAT) CD36 was identified as a key protein in the uptake of long chain fatty acid (LCFA) species by Abumrad et al. (1993). The evidence for the necessity of CD36 is derived from studies which show that CD36 knockout (KO) in mice reduces the rate of 15-(p-iodophenyl)-3-(R,S)-methyl pentadecanoic acid (BMIPP) uptake – a FA analogue – with the greatest impairment in highly oxidative muscle such as heart and diaphragm (Coburn et al., 2000). In humans, similar effects on LCFA uptake are observed with CD36 mutations (Hirano et al., 2003). In addition to CD36, intracellular LCFA transport requires heart-type fatty acid-binding protein (H-FABP) (Stremmel, 1988, Binas et al., 1999). Indeed, H-FABP-null cardiomyocytes exhibit reduced uptake of palmitate when electrically stimulated, even though the capacity for oxidation remains unchanged (Schaap et al., 1999). Thus, binding of FAs to FABPs is a crucial step for their intracellular transport and utilisation.

LCFAs are not freely permitted to cross the outer mitochondrial membrane and consequently rely upon a carnitine-dependent transport system involving carnitine palmitoyltransferase 1 and 2 (CPT1 and 2), and carnitine acyltransferase (CAT) (figure 1.1).

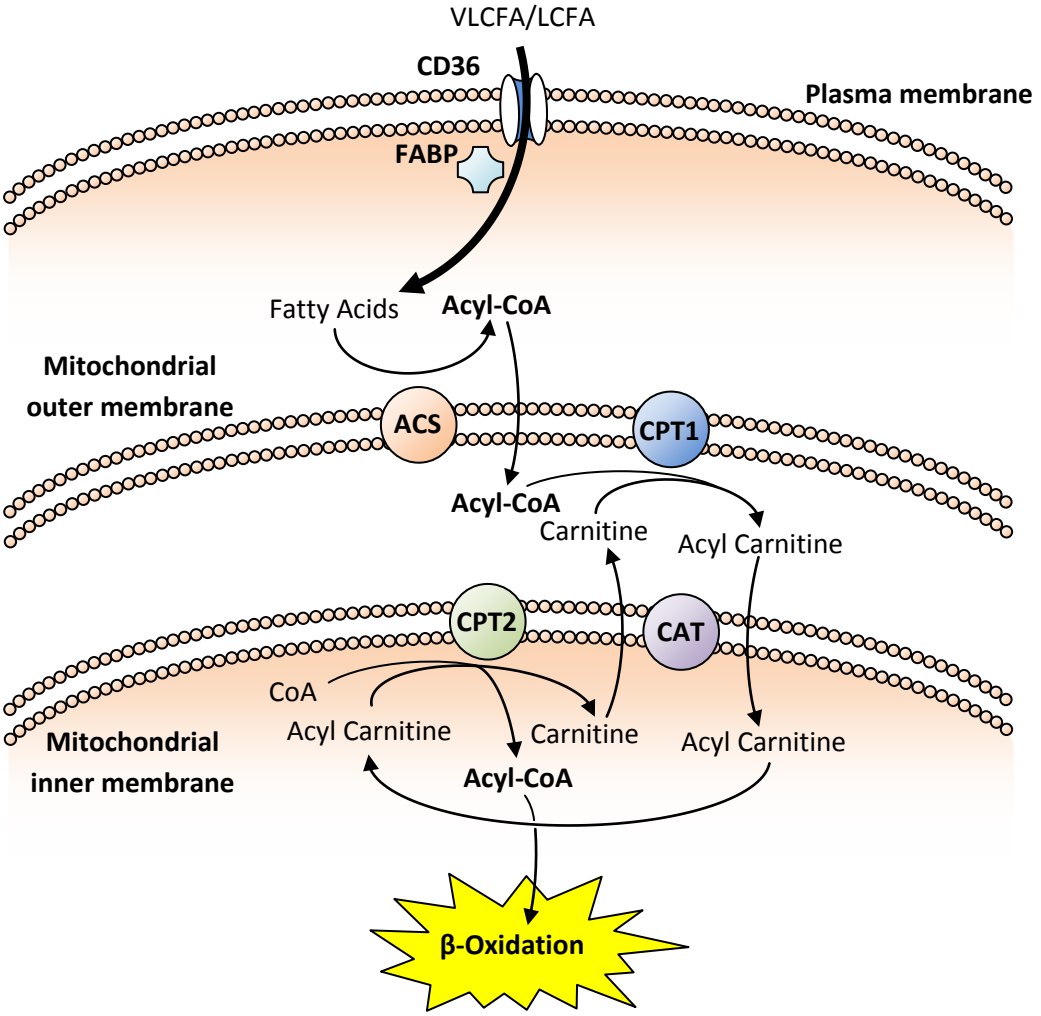

Figure 1.1 Cardiac fatty acid uptake and oxidation. Fatty acids are transported across the plasma membrane via CD36. LCFAs intracellular transport relies on FABPs. The fatty acid species is converted to an acyl-CoA species via acyl-CoA synthetase (ACS). CPT1 replaces CoA with carnitine and transports the newly formed acyl-carnitine into the inter membraneous space. CAT transports the acyl carnitine into the mitochondrial matrix whereby the carnitine moiety is replaced by a CoA group via CPT2. The products of this reaction are an acyl-CoA species and carnitine, which is removed from the matrix via CAT. Adapted from Kodde et al. (2007).

CPT1 activity is regulated by malonyl CoA (McGarry et al., 1983, Murthy and Pande, 1987), as well as FAs through increased activation of peroxisome proliferator-activated receptor (PPAR) α (Brandt et al., 1998). These workers also showed that the level of muscle CPT1 (mCPT1) gene transcription was increased both by FA concentration and the length of the acyl chain, with longer chain lengths increasing transcription (Brandt et al., 1998). As an inhibitor of CPT1, elevated levels of malonyl-CoA inhibit the entry of long-chain acyl-CoA species into the mitochondria. During fasting, the intramuscular malonyl CoA concentration of the heart falls by 71%, facilitating increased mitochondrial oxidation of fatty acid species (McGarry et al., 1983). High levels of malonyl-CoA are maintained by the enzyme acetyl-CoA carboxylase (ACC) in particular the ACC2 isoform. ACC is regulated by AMP-activated protein kinase (AMPK). Increased activity of AMPK decreases the activity of ACC, and subsequently malonyl-CoA synthesis falls.

FA chain length dictates the first enzyme of β-oxidation due to chain-length specificity. Indeed, fatty acyl-CoA species with a FA chain length of 16 carbons or more are degraded by very long-chain acyl-CoA dehydrogenase (VLCAD) (Roe et al., 2002). Progressive shortening of the carbon chain leads to recruitment of additional dehydrogenases with varying chain length specificities., including, long-chain acyl-CoA dehydrogenase (LCAD) (maximal activity with 10-12 carbons), medium-chain acyl-CoA dehydrogenase (MCAD) (maximal activity with 6-8 carbons), and short-chain acyl-CoA dehydrogenase (SCAD) (maximal activity with 4-6 carbons) (Ghisla and Thorpe, 2004). Consequently, the numerous acyl-CoA dehydrogenases facilitate the oxidation of many different acyl-CoA species. Importantly, deficiency of VLCAD and LCAD have been shown to reduce β-oxidation dramatically and have been associated with the development of paediatric cardiomyopathy (Roe et al., 2002).

An often over-looked pathway of FAO is the peroxisomal β-oxidation pathway. VLCFAs have been demonstrated to be preferentially oxidised in peroxisomes, with hexacosanoic acid being oxidised only in peroxisomes (Wanders, 2004). Furthermore, 3-day starvation in mice leads to an increase in cardiac acyl-CoA oxidase (ACO) and catalase activity suggesting enhanced peroxisomal oxidation (Crescimanno et al., 1989). Additionally, peroxisomal β-oxidation in the heart has been shown to be an important source of acetyl moieties for malonyl-CoA synthesis (Reszko et al., 2004), and therefore endogenous FA synthesis. An important difference

between mitochondrial and peroxisomal β-oxidation is the by-product of the initial reaction. Because the initial reaction of β-oxidation in the peroxisome is catalysed by ACO, large amounts of reactive oxygen species (ROS) are produced in the form of H_2O_2 (Schönfeld et al., 2009). To eliminate ROS species, peroxisomes contain the enzyme catalase. Acetyl-carnitine generated from peroxisomal β-oxidation is transported from the peroxisome via carnitine acetyltransferase (van Roermund et al., 1999), reconverted to acetyl-CoA via cytosolic CAT and transported into the mitochondria for oxidation in the tri-carboxylic acid (TCA) cycle, or the synthesis of FAs.

1.1.2 Glucose metabolism

Glucose uptake is regulated by the transmembrane glucose concentration and the ability of glucose to cross the sarcolemmal membrane, mediated by glucose transporters (Stanley et al., 2005). The glucose transporter GLUT1 is important for the uptake of glucose under basal conditions due to its expression being insulin independent, and can it be stimulated to translocate from the intracellular pool to the plasma membrane in response to ischaemia and increased cardiac work (Young et al., 1997). GLUT4 expression is increased by contractile work (Till et al., 1997), ischaemia, and insulin (Young et al., 1997) which cause translocation of GLUT4 from the intracellular pool to the plasma membrane. Two models of the GLUT4 translocation have been proposed, including the “dynamic retention” and the “static retention” hypotheses. The former suggests that the rate of GLUT4 exocytosis is equal to endocytosis, and that this rate of cycling is increased by insulin, whereas the latter describes the movement of GLUT4 from an intracellular pool to the plasma membrane (figure 1.2) (Augustin, 2010).

Within the cell, glucose is rapidly converted to glucose 6-phosphate, mediated by hexokinase (HK). Binding of HK to the outer mitochondrial membrane has been shown to enhance activity when compared to the cytosolic enzyme (Russell et al., 1992). The first regulatory step in glycolysis is catalysed by phosphofructokinase (PFK)-1, catalysing the formation of fructose 1,6-bisphosphate (F1,6BP), and is activated by ADP, AMP, Pi, and fructose 2,6-bisphosphate (F2,6BP) (Schaftingen and Hers, 1981), and inhibited by ATP, citrate, and decreasing pH (Stanley et al., 2005). Indeed, F2,6BP has been demonstrated to be the main feed forward activator of PFK-1 (Depre et al., 1993).

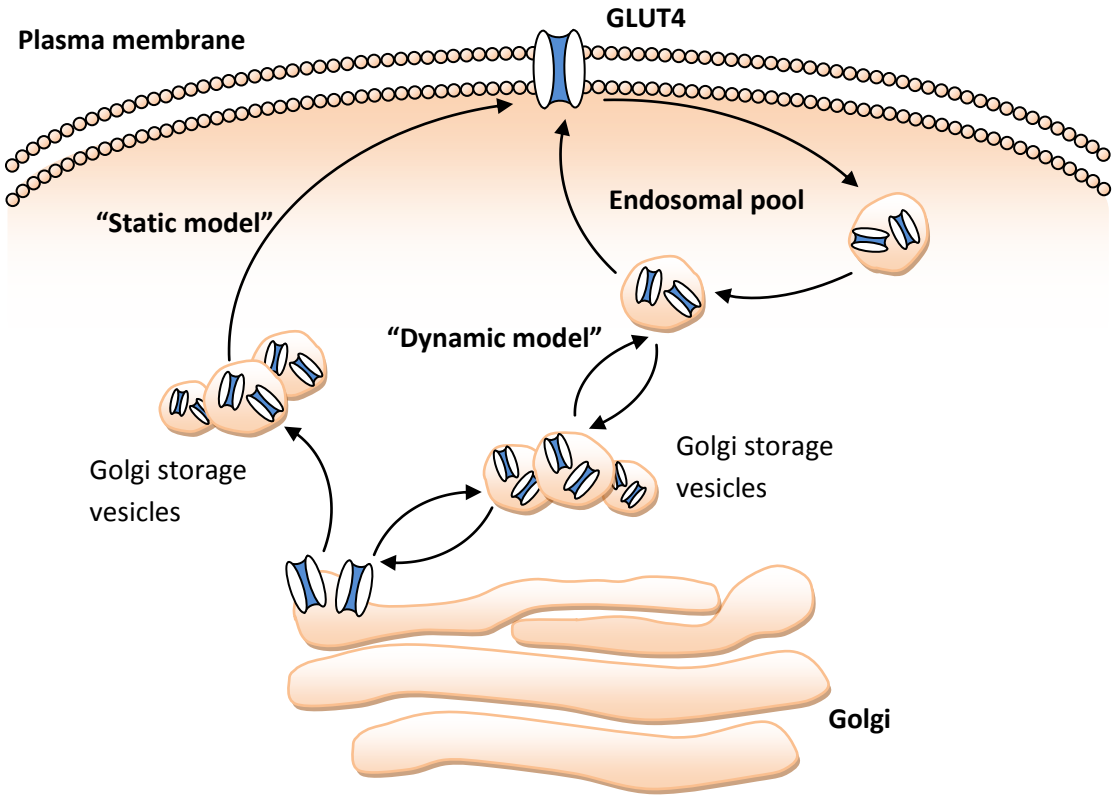

Figure 1.2 GLUT4 trafficking pathways. During the static model hypothesis, the golgi storage vesicles are only translocated to the plasma membrane in response to insulin. The dynamic hypothesis suggest that GLUT4 containing Golgi storage vesicles are cycled between the endosomal pool and the golgi network in the absence of insulin. Adapted from Augustin (2010).

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is responsible for catalysing the reaction forming 1,3-diphosphoglycerate from glyceraldehydes-3-phosphate, liberating the NADH molecules associated with glycolysis. GAPDH is negatively regulated by increasing cytosolic NADH, with NAD^+ stimulating the reaction (Stanley et al., 2005). The final generation of pyruvate from phosphoenol pyruvate (PEP) is catalysed by pyruvate kinase, combining the phosphoryl group of PEP with ADP to liberate ATP and pyruvate. Pyruvate kinase represents an important enzyme in the glycolytic pathway due to its positive regulation by F1,6BP and PEP (Kiffmeyer and Farrar, 1991, Jurica et al., 1998), therefore facilitating coupling of increased glycolytic flux with pyruvate formation and avoiding glycolytic intermediate accumulation.

Under aerobic conditions, pyruvate generated from glycolysis is converted to acetyl-CoA via the action of pyruvate dehydrogenase (PDH), located within the mitochondrial matrix (figure 1.3). The flow of pyruvate through PDH is controlled in part by phosphorylation and dephosphorylation of PDH via the PDH kinases (PDKs) and PDH phosphatases (PDPs), respectively (Fuller and Randle, 1984). The inhibitory properties of the PDK isoenzymes (PDK1-4) vary, although all PDKs phosphorylate the active site within PDH (Kolobova et al., 2001).

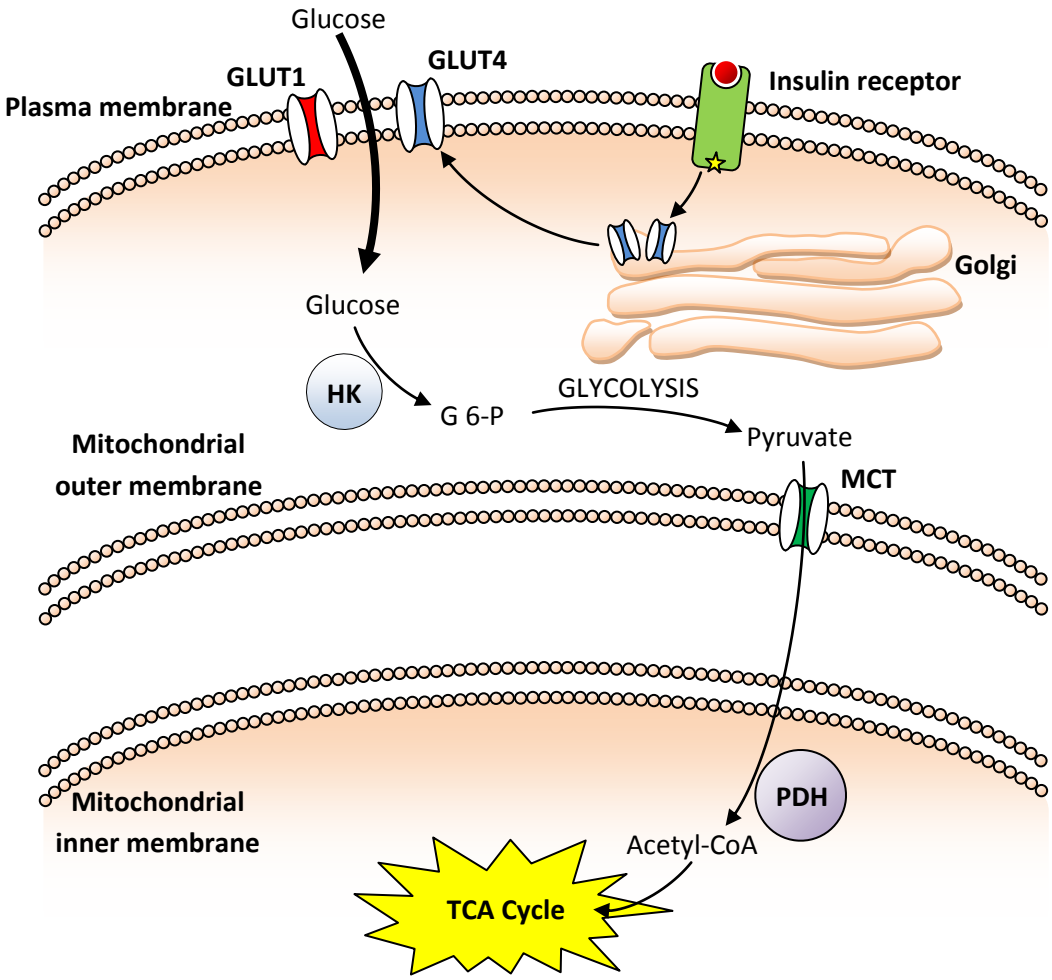

Figure 1.3 Cardiac glucose uptake and oxidation. Glucose is transported across the plasma membrane via GLUT1 and GLUT4 and trapped within the cell as glucose 6-phosphate via the actions of hexokinase (HK). The reactions of glycolysis result in the production of pyruvate which is transported across the outer mitochondrial membrane via the monocarboxylate transporter (MCT). Pyruvate dehydrogenase (PDH) catalyses the formation of acetyl-CoA from pyruvate which can subsequently feed into the TCA cycle. Adapted from Kodde et al. (2007).

The PDK isoenzymes exhibit tissue-specific distribution, with 1,2, and 4 expressed in cardiac tissue (Sugden and Holness, 2003). PDKs are regulated by several cellular substrates, including pyruvate, CoA and NAD^+, NADH, ATP, and ADP (Stanley et al., 2005). PDKs are stimulated by ATP, acetyl-CoA, and NADH, leading to phosphorylation and inactivation of PDH (Sambandam et al., 2002). Indeed, increased FAO leads to an elevated expression of PDK4 mRNA (Wu et al., 1998), and therefore would be anticipated to reduce the activity of PDH. Increases in cellular unbound CoA concentrations, NAD^+, and pyruvate lead to inhibition of PDKs, reduced phosphorylation of PDH and subsequent enhanced pyruvate flux through PDH. Opposing the actions of PDK, PDP dephosphorylates and activates PDH in response to Mg^{2+} and Ca^{2+} (Denton et al., 1972, Depre et al., 1999). Consequently, the balance between phosphorylation and dephosphorylation of PDH determines the rate of the conversion of pyruvate to acetyl-CoA (Sambandam et al., 2002).

1.1.3 Lactate metabolism

Lactate is transported across the sarcolemmal membrane by the monocarboxylate transporter (MCT) (Baker et al., 1998). The oxidation of both glucose and lactate yield pyruvate and as such, the final metabolic pathway of glucose or lactate metabolism is the same. Studies have shown the importance of lactate as a substrate in the heart. Indeed, increased generation of lactate from skeletal muscle during exercise is reflected by heightened myocardial lactate consumption (Stanley, 1991), therefore demonstrating the intimate relationship between circulating lactate concentrations and the rate of oxidation. Furthermore, evidence suggests that lactate metabolism is compartmentalised within the heart, with exogenous lactate being oxidised and glycolytically derived lactate released (Chatham et al., 2001).

1.2 Control of cardiac metabolism

PPARs play an important role in the regulation of cardiac metabolism. PPARs belong a family of nuclear hormone receptors which share homology with thyroid hormone-, retinoic acid-, and vitamin D receptors (Aranda and Pascual, 2001). PPARs form a heterodimer with the retinoid-X-receptor (RXR), which facilitates binding to DNA, in particular regions labelled as peroxisome proliferator response elements (PPREs). The PPAR/RXR complex is known to be influenced by co-activators and co-repressors. Such co-activators include steroid receptor coactivator-1 (SRC-1), CREB binding protein (CBP)/p300 and peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) (Viswakarma et al., 2010), in addition to FA species (Moya-Camarena et

al., 1999, van der Lee et al., 2000, Armstrong and Towle, 2001). Co-repressors of PPAR function include COUP transcription factor 2 (COUP-TF2) and nuclear receptor corepressor (NCoR) (Sack et al., 1997, Treuter et al., 1998, Yu et al., 2005).

PPARα expression is highest in tissues with enhanced FAO capacity such as heart, liver, and muscle (Escher et al., 2001). Increased activation of PPARα has been shown to augment the mRNA transcript levels of various PPAR responsive genes implicated in FAO, including H-FABP, ACS, and LCAD (Moya-Camarena et al., 1999, van der Lee et al., 2000), and CD36 (Akki and Seymour, 2009). Furthermore, loss-of-function models have shown that PPARα is a crucial determinant of cardiac metabolic gene expression (Watanabe et al., 2000, Muoio et al., 2002). Watanabe et al. (2000) demonstrated repression of CPT1, LCAD, MCAD, and ACO mRNA in hearts from PPARα KO mice, thus confirming the importance of PPARα in regulating FAO.

PPARβ and γ have been less extensively researched in the heart. PPARβ KO mice exhibit reduced basal rates of FAO (Cheng et al., 2004). This suggests that PPARβ may have a similar role to PPARα. Importantly, such hearts exhibit substantial lipid accumulation and lipoapoptosis despite being fed a normal, rodent chow. Unlike PPARα and β, PPARγ does not have a direct role in regulating cardiac FAO (Gilde et al., 2003). Furthermore, when cardiomyocytes are incubated with both palmitic and oleic acid no observable increase in PPARγ mRNA was observed, in contrast to PPARα and β (Gilde et al., 2003). However, it is widely known that a class of drugs termed thiazolidinediones (TZDs) are PPARγ agonists, with their main effects being improving insulin sensitivity, lowering of circulating FAs, and the partitioning of FAs into the adipocyte (for review see Spiegelman, 1998). As consequence, modulating PPARγ in non-cardiac tissues may indirectly regulate myocardial metabolism due to altering blood metabolites.

1.3 Cardiac hypertrophy

Mammalian hearts exhibit a limited response to stress, with the primary response being that of hypertrophy or enlargement. As such, the initial response is regarded as a beneficial adaptation to normalise wall tension and maintain stroke volume. Importantly, however, if the stress is chronic, the enlargement and accompanying remodelling can become maladaptive and decompensation can occur (Heineke and Molkentin, 2006). The decompensation of the

hypertrophied heart reflects a decrease in the functional capability, compromising cardiac output and producing signs of heart failure (HF). Indeed, cardiac hypertrophy has long been recognised as a significant independent risk factor for the development of congestive HF (Levy et al., 1990, Carabello, 2006, Rame and Dries, 2007).

1.3.1 Stages of hypertrophy

Experimentally, the development of pathological hypertrophy proceeds via 3 distinct stages. Initially, the sudden demand placed upon the heart can lead to a depression in myocardial function, or can alternatively lead to no change. However, there is marked increased protein synthesis, which is responsible for the transition into stage 2, the compensated phase. This second stage is characterised by ventricular remodelling with preserved or mild hyperfunction, potentially due to increased contractile protein synthesis. Progression of the underlying insult causes this compensated phase to eventually decompensate, whereby the heart is no longer able to maintain function due to death of myocytes. This final stage (stage 3) represents the decompensation observed HF (Wang et al., 2003).

1.3.2 Structural and cellular remodelling

Left ventricular hypertrophy (LVH) was originally thought to be the only response as cardiomyocytes are believed to be terminally differentiated cells, although this has been challenged (Anversa and Kajstura, 1998). What is well-known is that there are two clear processes which occur in the heart in response to stress – concentric and eccentric remodelling. Classically, eccentric hypertrophy, whereby myocytes exhibit a disproportionate increase in cell length versus cell width due to sarcomere additions in series, is common in the left ventricle of post-myocardial infarction (MI) hearts and is associated with an increased end diastolic volume (EDV) and reduced stroke volume (Anversa et al., 1986, Kleaveland et al., 1988). Conversely, concentric remodelling is characterised by an increase in myocyte width relative to length, with sarcomeres being added in parallel. Such hearts show an increase in ventricular mass and a smaller ventricular cavity (Hein et al., 2003, Carabello, 2006). Pregnancy and chronic exercise increase cardiac dimensions uniformly (Shapiro, 1984, Iemitsu et al., 2001), producing a physiological hypertrophy. This frequently referred to “athletic heart” remains compliant despite an increase in mass (figure 1.4) (Woodiwiss and Norton, 1995).

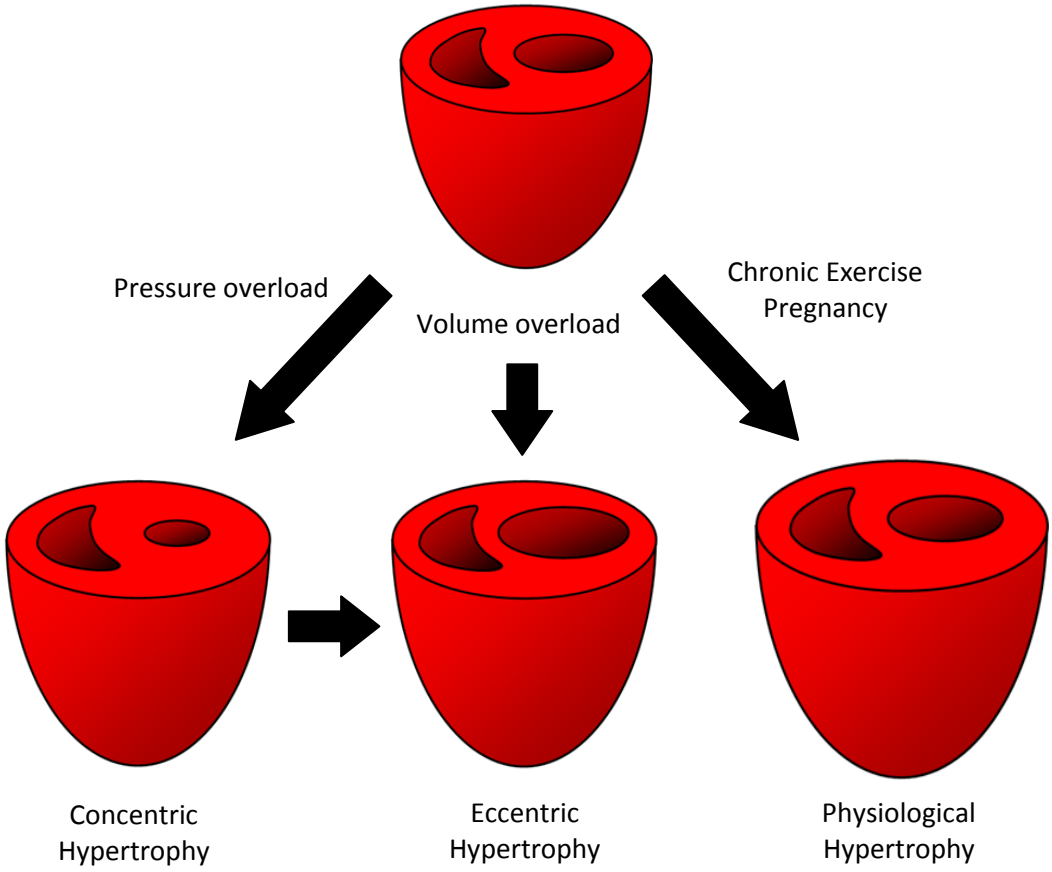

Figure 1.4 Ventricular structure following physiological and pathological stimuli. MI, myocardial infarction. Adapted from Heineke and Molkentin (2006).

It has been stated that such an increase in myocyte – and hence ventricular wall – thickness is beneficial and can be explained in part by the law of La Place, suggesting that wall tension is directly proportional to a given pressure and chamber radius, and inversely proportional to wall thickness (Katz, 2011). Although the remodelling processes for eccentric and concentric hypertrophy are different, macroscopic and ultrastructural changes have been well documented in both concentric and eccentric hypertrophy (Hess et al., 1984, Schaub et al., 1997), suggesting similarities between the two processes. In addition to changes in cardiomyocyte structure, remodelling also affects other components of the ventricular wall, most importantly collagen (Fujita et al., 2008, Moens et al., 2008), blood vessels (Shiojima et al., 2005, Miyachi et al., 2009), and hyperplasia of endothelium (Dämmrich and Pfeifer, 1983).

Collagen in the heart is mainly comprised of type I (50-85%) and type III (10-45%) (Weber et al., 1993), referred to frequently as the extracellular matrix (ECM) and provides the scaffolding from which myocyte contraction is translated globally. Such collagens are extremely stable yet are increased in the hypertrophied human (Caspari et al., 1977), rat (Weber et al., 1990), and mouse heart (Bradshaw et al., 2009). The initial stage of hypertrophic remodelling is associated with an increase in type III collagen and a reduction in ventricular stiffness, whereas chronic hypertrophy is accompanied by an increase in necrosis and type I collagen content (Weber et al., 1988). Hein et al. (2003) have shown increased fibrosis in hearts from patients undergoing aortic valve replacement. Indeed, in this study patients were subdivided into 3 categories based on ejection fraction, the lowest of which being <30%. Fibrosis was significantly increased in patients with an ejection fraction of 30-50% and <30% compared to the control group, and was significantly correlated with the percentage of dead myocytes. This suggests that as myocytes are lost from the hypertrophied heart they can be replaced with collagen, impeding cardiac function.

In addition to increased accumulation of collagen in the hypertrophied heart, the rate of collagen degradation via the action of matrix metalloproteinases (MMPs) may also be important (Spinale, 2007). This process of degradation is important as changes to the rate of both collagen synthesis and proteolysis may contribute to the ventricular stiffness and dilated nature of diastolic HF and systolic HF, respectively. MMPs are regulated by their tissue inhibitors, termed tissue inhibitors of metalloproteinases (TIMPs) and therefore changes to the MMP/TIMP ratio will alter the ECM components. These alterations may be important in understanding the ECM changes observed in eccentric and concentric ventricular remodelling. Addressing this issue, Lopez et al. (2006) studied a small population of HF patients (16 systolic HF and 23 with diastolic HF) for changes in the distribution and process of collagen degradation by analysing MMP-1 and TIMP-1 in cardiac tissue. Using various approaches, they demonstrated that the profile of collagen deposition differed between systolic and diastolic HF, with a reduction in interstitial collagen fibres in the former group.

1.3.3 Metabolic remodelling in left ventricular hypertrophy (LVH)

Although the link between chronic LVH and HF is well known (Rame and Dries, 2007), the mechanisms which worsen the progression of LVH to failure remain largely incompletely understood. Importantly, the cardiac remodelling process during the development of hypertrophy is characterised by alterations in metabolism, principally of carbohydrate and fat.

Indeed, when adult hearts are unloaded, FAO declines (Depre et al., 1998) with this metabolic phenotype not being dissimilar to the hypertrophied heart. However, because hypertrophied hearts are still "loaded", the oxidation of carbohydrate is much greater in an attempt to provide sufficient ATP for contraction.

1.3.3.1 Fatty acid metabolism in the hypertrophied heart

Metabolic remodelling is an early feature in the development of cardiac hypertrophy (Akki et al., 2008). Importantly, this metabolic remodelling is observable in both experimental models (Okere et al., 2006b, Chess et al., 2007, Akki et al., 2008) and patients with LVH and failure (Sack et al., 1996). Two major models of hypertrophy – the aortic constriction (AC) rat and the MI rat – exhibit decreased LCFA oxidation and increased reliance on glucose and an associated reduction in protein expression associated with FA uptake and oxidation (Akki et al., 2008, Rosenblatt-Velin et al., 2001).

In spontaneously hypertensive HF (SHHF) rats with LVH there is a specific down-regulation of the mRNA for LCAD and MCAD, which were matched by a significant decrease in respective enzyme activity at 16 months of age. Similarly Sack et al (1996) showed that the failing human ventricle was characterised by a decrease in the activity of MCAD and LCAD. Further remodelling has demonstrated reduced CD36 expression in pressure-overload hypertrophied hearts (Doenst et al., 2010), suggesting impairment to LCFA uptake. Indeed supplementing a diet with medium chain TG (MCTGs) improves function in the hypertrophied heart and reverses protein changes associated with hypertrophic remodelling (Iemitsu et al., 2008). In addition, carnitine depletion may also limit the ability of the hypertrophied and failing heart to oxidise LCFAs (el Alaoui-Talibi et al., 1992, Allard et al., 1994a). Using octanoate in combination with palmitate, Allard et al. (2007) observed a significant increase in FAO rates in hypertrophied hearts when compared to hearts perfused with palmitate alone. Together, these observations suggest specific disruption to the uptake and oxidation of LCFA in the hypertrophied heart.

1.3.3.2 Glucose metabolism in the hypertrophied heart

High rates of glucose uptake are achieved via an insulin-dependent translocation of GLUT4 from the sarcoplasmic vesicles. Although basal, non insulin-dependent glucose uptake is via GLUT1, cellular glucose uptake via this membrane transporter is upregulated during

hypertrophic growth (Montessuit and Thorburn, 1999), in part due to an impaired ability to increase GLUT4 expression suggesting reduced insulin sensitivity (Paternostro et al., 1999).

Metabolic activity associated with glycolysis is significantly higher in hypertrophied hearts and increased rates of glycolysis have been demonstrated by several groups (Bishop and Altschuld, 1970, Allard et al., 1994b, Wambolt et al., 1999, Leong et al., 2002, Nascimben et al., 2004), although this has been shown to be dependent upon the severity of LVH (Degens et al., 2006, Akki et al., 2008). However, an uncoupling of glycolysis and glucose oxidation has been suggested and may represent a left ventricular dysfunction (Lydell et al., 2002).

Increased oxidation of glucose would be speculated to be a compensatory response to decreased rates of FAO (Randle, 1986), and would be anticipated to be increased following higher rates of glycolysis. Thus, the observations of uncoupled glycolysis and deranged FAO may reflect substantial differences in the severity of experimental models used, as well as the duration of the experiment.

1.3.3.3 Endogenous substrates usage in the hypertrophied heart

Alternatively, substrates for the TCA cycle may be derived from anaplerosis. This mechanism of substrate generation is often overlooked in discussing cardiac metabolism in favour of exogenous glucose and FA metabolism, yet it is widely known that the heart has an endogenous store of both glycogen and TG. Gibala et al. (2000) provide an excellent review of the anaplerotic potential of the heart and skeletal muscle. What is striking from this is that the removal of the anaplerotic capabilities of the heart produces substantial and significant reductions in function, which can be improved by anaplerotic substrate (Gibala et al., 2000). Interestingly, Sorokina et al. (2007) demonstrated that in LVH with reduced palmitate oxidation there is a compensatory increase in anaplerosis. Importantly, it was observed that ^{13}C enrichment of glutamate proceeds despite no increase in the enrichment of acetyl-CoA derived from glycolytic pyruvate (Sorokina et al., 2007). Thus, anaplerotic substrate usage may be important in maintaining TCA cycle intermediates in the hypertrophied heart. This suggests that under normal conditions, anaplerosis may be an important source of TCA intermediates when PDH activity is reduced (Seymour and Chatham, 1997).

1.3.4 Experimental models of LVH and failure

Several key models have been used to study the development of LVH and transition to HF and have been reviewed extensively in terms of their advantages and limitations (Berry et al., 2007, Patten and Hall-Porter, 2009, Doggrell and Brown, 1998) (figure 1.5).

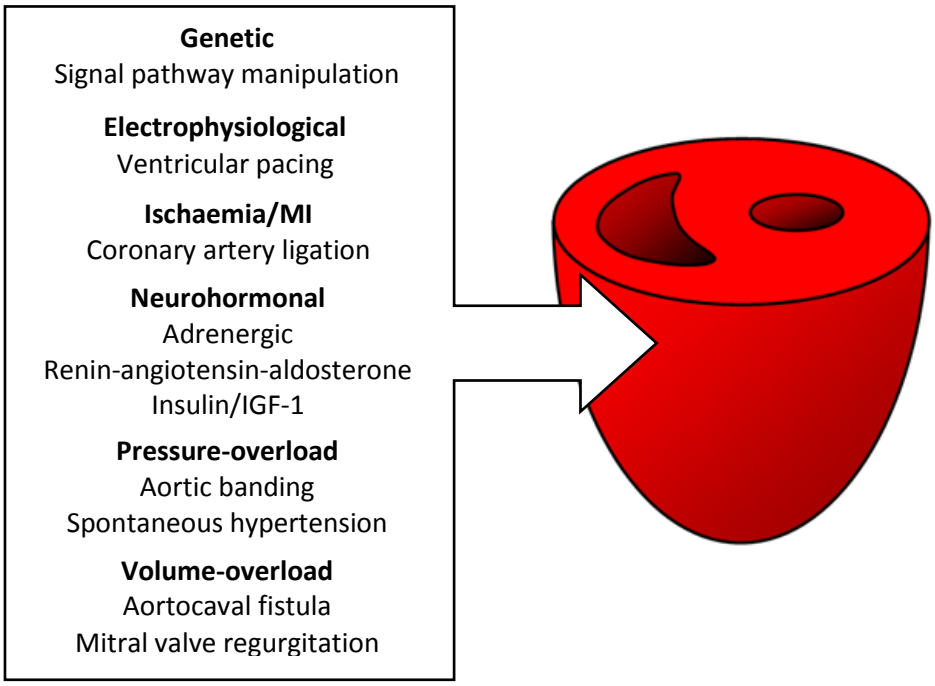

Figure 1.5 Mechanisms for inducing cardiac hypertrophy. Numerous pathways exploit the limited ability of the heart to respond to stress. IGF-1, insulin-like growth factor-1. Adapted from Berry et al. (2007).

Increasing aortic blood pressure leads to the development of left ventricular concentric remodelling and hypertrophy (Boateng et al., 1997, Barrick et al., 2007, Akki et al., 2008, Chess et al., 2008). Importantly, the site of the AC has been shown to lead to very different phenotypes. In rats, constriction of the aorta at the level of the renal arteries has been shown to induce compensated hypertrophy with no dysfunction by 9 weeks post surgery (Akki et al., 2008, Akki and Seymour, 2009). Constriction of the aorta at the supravalvular or transverse level can induce a more severe phenotype characterised by impaired diastolic filling (Chess et al., 2008, Litwin et al., 1995). Other models for the study of pressure-overload hypertrophy include the Dahl salt-sensitive (DSS) and the SHHF rat. LVH is observable in DSS rats after 6 weeks of salt treatment (Okere et al., 2005), and HF develops following 12 weeks of salt treatment (Kang et al., 2004).

The model of ascending AC developed by Litwin and colleagues (1995) raises an important concept that the duration and age at which the intervention is imposed is important. In this model, banding of the ascending aorta in weanling rats led to the development of hypertrophy by 6 weeks post-banding with no evidence of dysfunction. By 18 weeks post-banding there was evidence of HF, with tachypnoea, oedema, and systolic deterioration. This model has the advantage that the pressure changes exerted upon the heart are gradual due to the procedure being performed in young animals, and hence as the animals age the pressure difference across the aortic band increase. This principle applies to all models whereby the induction for hypertrophic growth is performed in immature animals. Similar to rats, transverse aortic constriction (TAC) in mice is a widely used method of inducing LVH and HF, although the response has been shown to be partly strain-specific (Barrick et al., 2007). These models develop HF within a period of 2 weeks (Patten et al., 2008) and thus are not suitable for evaluating the early or compensated hypertrophic phase of remodelling. Both spontaneously hypertensive and DSS rats develop hypertrophy gradually due to the steady onset of hypertension, similar to AC in young rats. However, both AC and TAC have the additional advantage of providing the opportunity to study immediate changes in cardiac gene and protein expression due to the sudden elevation in arterial blood pressure .

1.4 Obesity

Recent UK statistics suggest that 22% of men and 24% of women aged 16 and over are obese (The NHS Information Centre, 2011). Furthermore, in 2009 16% of boys and 15% of girls aged 2 to 15 were obese (The NHS Information Centre, 2011), potentially due to high contribution of dietary fat to the total daily calories (Bender, 2008) (table 1.1). Obesity is defined as a body mass index (BMI) of ≥30 kg/m^2 (The NHS Information Centre, 2011). BMI remains a powerful tool in identifying obesity although other indexes such as body fat percentage, waist circumference, waist-to-hip ratio, and weight-to-height ratio are also useful (Litwin, 2008). Being overweight predisposes the individual to other co-morbidities including hyperglycaemia, hypertension, dyslipidaemia and insulin resistance [IR; a reduced sensitivity to the metabolic actions of insulin upon glucose disposal (Houben et al., 2012)] (Grundy, 2008, Despres, 1998, Harris et al., 2000, Lemieux et al., 2000).

Table 1.1 Average UK dietary macronutrient profile

Average UK Diet	
Dietary Component	**% of Energy Intake**
Carbohydrate	45.0
Starch	23.0
Sucrose	14.0
Lactose	3.0
Glucose	2.0
Protein	15.0
Fats	37.0
Saturated	17.0
Monounsaturated	12.0
Polyunsaturated	6.0
Trans	2.0
Alcohol	3.0
Fibre	-

Adapted from Bender (2008).

Although it has been argued that diets high in fat induce obesity – due to fat containing a greater number of calories per gram, high-sugar diets have also been shown to be detrimental to health and increase adiposity (Swarbrick et al., 2008, Stanhope et al., 2009). Importantly, obesity is strongly associated with hypertension, coronary artery disease and diabetes, all of which are significant risk factors for HF. Given that LVH is regarded as an important independent risk factor alongside hypertension, it is perhaps unsurprising that there is a close link between obesity and LVH.

1.4.1 Atherogenic effects of fatty acids

Excessive dietary fat consumption can have an important role on other factors which may alter cardiac function in obesity, including atheroma formation. To this end, studies have reported a deleterious action of increasing saturated FA (SFAs) consumption upon endothelial function (Fuentes et al., 2001, Keogh et al., 2005, Krogmann et al., 2011), and lipoprotein synthesis (Skeaff and Miller, 2009). Indeed, human coronary artery endothelial cells express a large platform of inflammatory genes in response to treatment with palmitate and stearate

(Krogmann et al., 2011). Furthermore, specific SFAs exert differential effects upon the cholesterol fractions of lipoproteins, with lauric, myristic, and palmitic acid augmenting low-density lipoprotein (LDL) cholesterol production, and stearic acid having no effect (Skeaff and Miller, 2009).

Greater focus has been placed upon understanding the relationship between dietary mono-unsaturated FAs and poly-unsaturated FAs (MUFAs and PUFAs, respectively) and the development of atherosclerosis, in particular n-3 and n-6 species. C5B7BL/6 mice fed a diet rich in n-3 eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) exhibit reduced aortic whole LDL and LDL-derived cholesterol ester uptake in comparison to littermates fed a high SFA diet (Chang et al., 2009). In addition, serum concentrations of EPA are inversely associated with anti-oxidised-LDL antibodies, with oxidation of LDL an important factor in the development of atherosclerosis (Garrido-Sanchez et al., 2008). Human populations which traditionally consume greater quantities of n-3 FAs show a reduction in atherosclerosis and carotid intima-media thickness compared to those which do not (Sekikawa et al., 2008). Furthermore, patients with hyperlipoproteinaemia exhibit reduced carotid intima-media thickness following 6 g/day of n-3 PUFAs (Baldassarre et al., 2006), suggesting a beneficial effect of dietary PUFA in regulating atheroma formation. Similar to EPA and DHA, α-linolenic acid has favourable implications in most epidemiological studies examining the impact of FAs upon coronary heart disease (Mozaffarian, 2005). Moreover, n-3 fatty acids may also be direct ligands for transcription factors (Lengqvist et al., 2004), and also regulate inflammatory cell function (Caughey et al., 1996). The anti-inflammatory properties of n-3 FAs may relate to the partial displacement of arachidonic acid (a precursor to eicosanoids synthesis) from phospholipid membranes (Healy et al., 2000), therefore resulting in reduced inflammation and attenuated progression of atherosclerotic disease (Kinsella et al., 1990).

The n-6 FA linoleic acid is the primary dietary n-6 PUFA, and has a strong association with reduced cardiovascular disease (Ascherio, 2002). Following ingestion, linoleic acid can be desaturated and elongated to form further n-6 species, most importantly γ-linolenic and subsequently arachidonic acid, itself an important precursor for eicosanoid biosynthesis (Kelley et al., 1998). Importantly, western dietary patterns dictate an n-6:n-3 ratio of between 15:1 and 20:1 (Simopoulos, 2002, Simopoulos, 2006), demonstrating a low level of n-3 consumption in comparison to n-6. In vascular endothelial cells, n-6 FAs exert anti-inflammatory properties, suppressing the atherogenic activation and expression of adhesion

molecules and chemokines (De Caterina et al., 2000). Furthermore, a recent Japanese study demonstrated that high arachidionic acid supplementation for 4 weeks did not negatively impact upon platelet aggregation or any biochemical parameter measured (Kusumoto et al., 2007), an observation previously supported by a large-scale study by Ferrucci et al. (2006). Additionally, Pischon et al. (2003) showed that the most substantial effect upon inflammatory markers was observed with combined n-6 and n-3 PUFAs. However, it is impossible to ignore the large body of research which suggests that lowering n-6 PUFA consumption – and therefore reducing the n-6:n-3 ratio – is beneficial in terms of limiting diseases with a large inflammatory component such as atherosclerosis (Simopoulos, 2008). The favourable effect of linoleic acid upon reducing cardiovascular events may relate largely to its well-known cholesterol-lowering effects. A large meta-analysis revealed that substitution of carbohydrate for n-6 PUFAs had the greatest positive effect on total:high-density lipoprotein (HDL) cholesterol (Mensink et al., 2003). Furthermore, isocaloric replacement of 10% SFA with n-6 PUFAs leads to a reduction in LDL cholesterol of 0.47 mM (Mensink and Katan, 1992). This relationship has also been observed in primates fed a high n-6 PUFA diet for up to 5 years, with no evidence of harm (Wolfe et al., 1994). This may explain the positive association between high linoleic acid consumption and low rates of cardiovascular disease. This however, raises the question of whether the ratio of n-6:n-3 or the absolute amounts of these FAs are the important regulator of cardiovascular disease.

Dietary MUFAs have shown variable roles in the regulation of atherosclerotic disease. Indeed, a large meta-analysis of 11 studies in both America and Europe revealed that consumption of MUFA instead of SFA had no impact upon prevention of coronary heart disease (Jakobsen et al., 2009). Conversely, other large-scale studies have suggested a positive effect of MUFA consumption on coronary heart disease as part of a Mediterranean-style diet (Fung et al., 2009). Indeed, similar to PUFAs, MUFAs reduce total and LDL-cholesterol by 10% and 14%, respectfully, with olive oil ingestion reducing cardiovascular risk by 25% (Kris-Etherton et al., 1999). Furthermore, oleic acid enhances the ability of LDL to resist oxidative modification (Berry et al., 1991, Bonanome et al., 1992), potentially reducing the atherogenic effect of these particles, and does not lead to changes in LDL size in comparison to SFA consumption (Rivellese et al., 2003). However, as reviewed by Brown et al. (2007), when atheroma formation was assessed following MUFA intervention, both animal and human studies suggest no protection in comparison to SFAs. As such, the beneficial effects of MUFA consumption within the Mediterranean-style diet may relate more closely to their involvement in

inflammation and endothelial function. Indeed, such a diet reduces C-reactive protein (CRP), interleukin (IL)-6, and improves endothelial function (Esposito et al., 2004).

Trans FAs (TFAs) are produced via partial hydrogenation of vegetable oils, yielding a substance that compared to SFA, MUFAs, and PUFAs, has a long shelf life thus making it appealing to the food industry. In comparison to an equal percentage of calories derived from either SFA or MUFAs/PUFAs, TFAs increase LDL concentration with a concomitant decrease in HDL, therefore augmenting the total cholesterol:HDL ratio (Mensink et al., 2003). Additionally, compared to MUFAs or PUFAs, TFAs increase fasting TG concentrations and apoprotein (Apo)B levels, reduce ApoA-I (a key apolipoprotein associated with HDL) (Mozaffarian and Clarke, 2009), decrease LDL size (Mauger et al., 2003), and increase the activity of cholesterol ester transfer protein (CETP) (van Tol et al., 1995). TFAs induce endothelial cell apoptosis (Zapolska-Downar et al., 2005), and impair flow-mediated dilation in the brachial artery after chronic (4 weeks) consumption. (de Roos et al., 2001). TFAs have also demonstrated substantial pro-inflammatory properties. In a 5 week crossover study, Baer et al. (2004) showed that TFAs increased IL-6 concentrations in comparison with MUFA, and also elevated levels of CRP. Combined, these studies suggest an important role of TFAs in potentially regulating the pathogenesis of atherosclerosis.

1.4.2 Adipose tissue as an endocrine organ

In health, the sum total of the visceral adipose tissue mass is normally much smaller than that of subcutaneous (s/c) adipose tissue. Visceral adipose tissue is an important factor in influencing blood glucose and IR (Tulloch-Reid et al., 2004, Preis et al., 2010), although others have shown a similar correlation between s/c or visceral adipose tissue and IR (Abate et al., 1996, Kelley et al., 2000). The classic visceral deposition of TG within the abdomen involves the omental, mesenteric, and retroperitoneal fat depots (Wajchenberg, 2000). Bergman et al. (2006) proposed the size of visceral and s/c fat was linked to hepatic IR, which precedes the development of peripheral IR. This hypothesis is derived from studies on liver insulin receptor knockout (LIRKO) mice, which exhibit hyperinsulinaemia due to reduced receptor-mediated endocytosis (Michael et al., 2000). Importantly, in the fasted state these mice show substantial hyperglycaemia in the face of hyperinsulinaemia, indicating muscle and adipose tissue IR (Michael et al., 2000). Indeed, removal of omental adipose tissue improves glycaemia and reduces both hepatic and peripheral IR (Gabriely et al., 2002). Furthermore, removal of s/c adipose tissue via liposuction in obese women significantly improves glycaemia, insulin

concentrations, and IR (Giugliano et al., 2004). Ultimately, these studies suggest that both s/c and visceral fat depots contribute to the regulation of glycaemia and IR (Wajchenberg, 2000).

The destination of dietary fat is dependent upon existing adipose deposits. Increasing dietary fat in a lean individual primarily enhances deposition within the visceral depot, with continuing increases in dietary fat eventually over-spilling from visceral adipose tissue into s/c adipose tissue (Bergman et al., 2006). A recent area of interest in relation to ectopic fat accumulation and ventricular enlargement is the storage of fat in and around the myocardium, termed epicardial adipose tissue. This is a significant storage depot for dietary fat as there is no fascia between the adipose tissue and (underlying) myocardium, allowing adipocyte products – including FAs and adipokines – to diffuse readily between the two (Sacks and Fain, 2007). This characteristic of epicardial adipose tissue may be of benefit to the myocardium as it may serve as a "filter" for circulating fatty acids, converting them to TG, and therefore limiting the exposure of the myocardium to high levels of FAs (Sacks and Fain, 2007). Importantly for obesity research, many rodent models of obesity exhibit no or very little epicardial adipose tissue (Marchington et al., 1989), compared to rabbits or larger mammals (Carroll et al., 1996), with this finding having important ramifications for comparison with human studies. Epicardial adipose tissue has also been shown to correlate strongly with left ventricular mass (Iacobellis et al., 2004) and peripheral vascular resistance in patients (Kankaanpää et al., 2006), although a stronger relationship has been observed between FFAs and ventricular mass (Kankaanpää et al., 2006). The source of such FFAs may be derived from high rates of lipolysis observed in epicardial, visceral, and s/c adipose tissue (Mattacks and Pond, 1988, Marchington et al., 1989, Koutsari and Jensen, 2006). Interestingly, higher rates of lipolysis are observed in obese subjects (Nielsen et al., 2004) and experimental models of obesity (Bairras et al., 2007, Wueest et al., 2009), and may, in part, responsible for elevated FFAs in obesity (Koutsari and Jensen, 2006).

Adipose tissue is obviously the most an important tissue quantitatively for the storage of TG. However, storage of TG also occurs in non-adipose tissue (ectopic storage) in obese individuals, including the liver, skeletal muscle, and heart (Forouhi et al., 1999, Guzzaloni et al., 2000, Goodpaster et al., 2000). Recent evidence has identified adipose tissue as not solely a storage tissue but an important endocrine organ which can play an important role in regulating adiposity and thus obesity through release of key adipocyte hormones including leptin and adiponectin (Ahima, 2006, Hajer et al., 2008).

1.4.2.1 Leptin

Leptin is a 16-kDa peptide encoded by the obesity gene (ob) and predominantly synthesised and released from adipocytes (Tartaglia, 1997). Leptin concentrations are substantially greater in women than men (Saad et al., 1997). Control of metabolism, in particular fat oxidation via AMPK activation (Unger et al., 1999, Minokoshi et al., 2002) and neural tone (Rahmouni et al., 2005) is intimately linked with leptin via leptin-receptor interactions within the hypothalamus and other organs, including kidney, liver and lungs (Sader et al., 2003), as well as the heart (Purdham et al., 2004). Food consumption ultimately leads to the transfer of TG and glucose from the blood into the adipocyte for storage. The increase in adipocyte mass leads to the release of leptin into the circulation where it crosses the blood-brain barrier (BBB) in the hypothalamus and inhibits the activity of neurones containing neuropetide Y (NPY) and agouti-related peptide (AgRP) (Sader et al., 2003).

Leptin additionally stimulates those neurones which produce pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) (figure 1.5) (Sader et al., 2003). NPY-containing neurones are crucial for the feeding stimulus, evidenced by the feeding response elicited following NPY injection into the hypothalamus of rats (Stanley and Leibowitz, 1985). Both sides of the control mechanism are mediated by melanocortin-4 (MC4) responsive neurones via interactions with agonsits (POMC) and antagonists (AgRP) (Sader et al., 2003).

The leptin receptor family consists of six different members (Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re, and Ob-Rf) due to the variability produced during pre-mRNA transcript splicing of the diabetes gene (db) (Sweeney, 2002). In the rat heart, leptin, Ob-Ra, -Rb, and -Re are globally expressed (Purdham et al., 2004). Additionally, it has been shown that leptin is produced from cardiomyocytes, suggesting that in the heart leptin may operate in an autocrine-paracrine manner (Purdham et al., 2004).

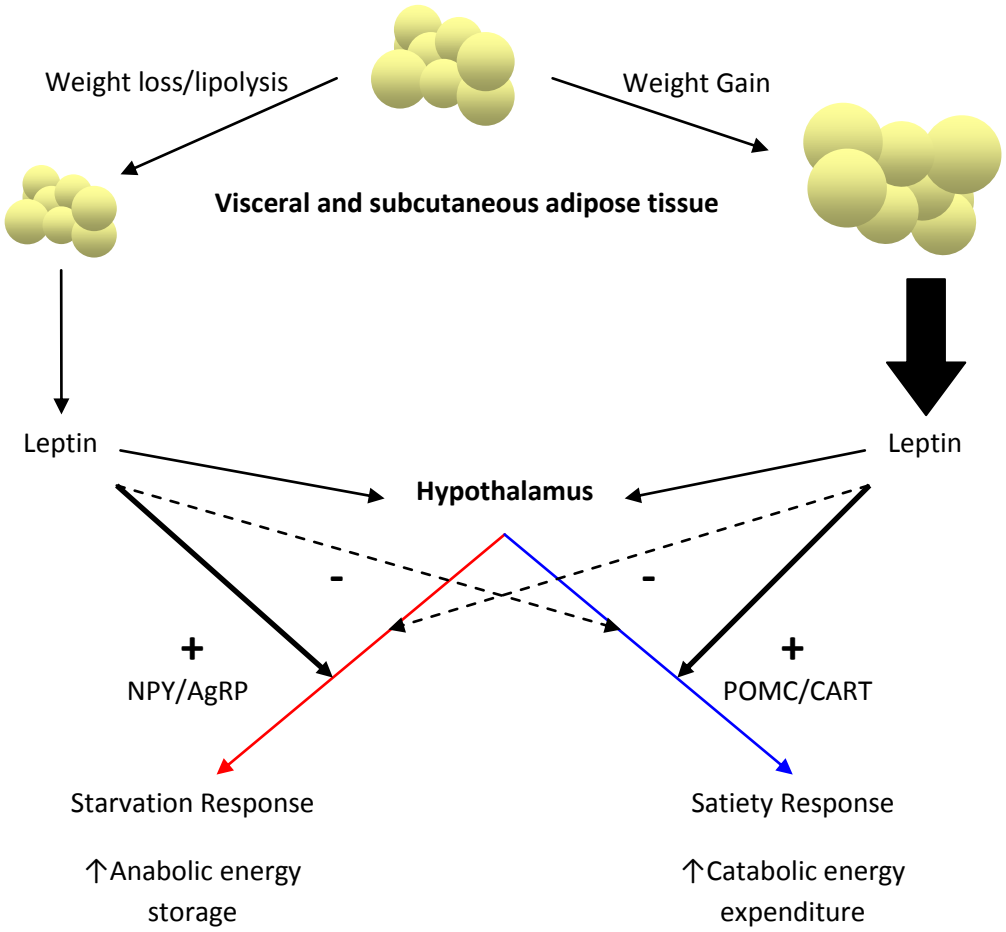

Figure 1.6 Regulation of feeding behaviour by leptin. In the balanced system, stimulation of NPY- and AgRP-containing neurones occurs during periods of fasting and increased lipolysis, as a direct result of reduced leptin release from adipocytes. This ultimately leads to an increased feeding signal and promotes energy storage. Conversely, increased fat storage leads to inhibition of NPY/AgRP neurones and stimulation of those containing POMC/CART, signalling satiety and increasing energy expenditure. Adapted from Friedman (2011).

Binding of leptin to Ob-Rb results in janus kinase (JAK) activation, phosphorylation and activation of signal transducer and activators of transcription-3 (STAT3) and increased transcription of target genes, in particular, suppressor of cytokine signalling 3 (SOCS3), itself a negative regulator of leptin signalling (figure 1.7) (Sweeney, 2002). The leptin signal is also potentially able to cross-talk with insulin signalling via the phosphorylation and activation of insulin receptor substrate (IRS) and phosphatidylinositol 3-kinase (PI3K), respectively (Münzberg et al., 2005), and phosphodiesterase 3B (PDE3B) (Sahu and Metlakunta, 2005).

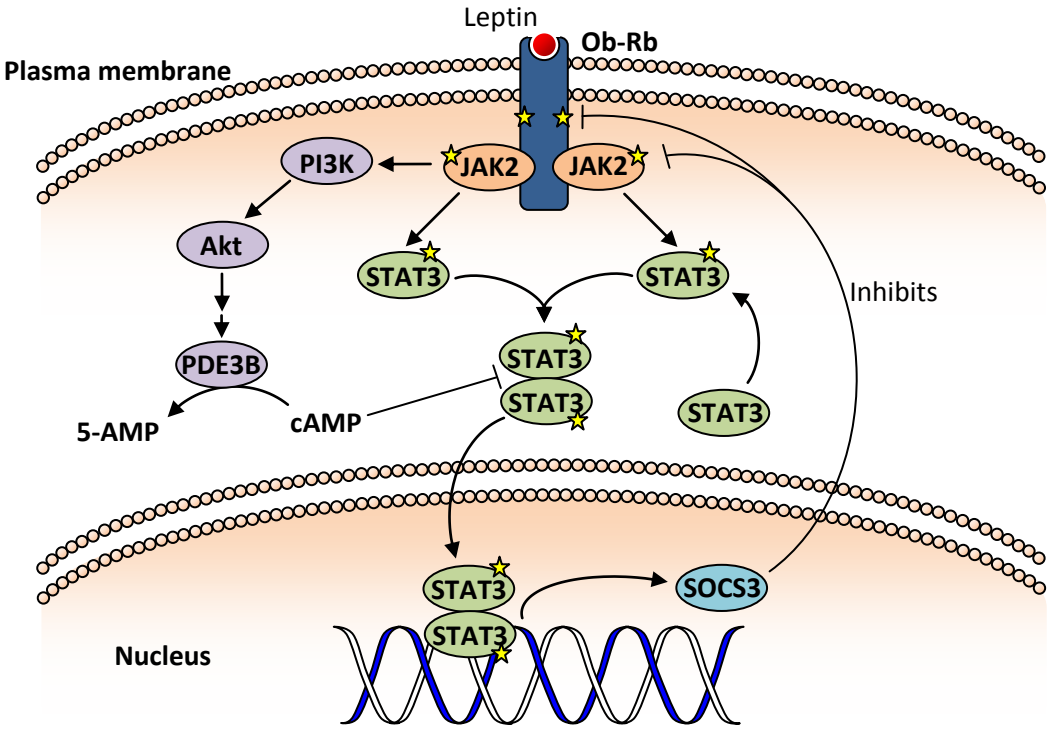

Figure 1.7 Intracellular leptin signalling. Upon binding to the Ob-Rb receptor, janus kinase (JAK) phosphorylates STAT3 which translocates to the nucleus and activates SOCS3 transcription, itself a negative regulator of the leptin cascade, acting by blocking leptin-induced JAK phosphorylation. Leptin also activates the PI3K and PDE3B, reducing the inhibitory action of cAMP upon STAT3. Stars denote phosphorylation. Adapted from Sahu (2003).

Obese humans and rodents exhibit hyperleptinaemia (Caprio et al., 1996, Hassink et al., 1996, Rahmouni et al., 2005). Rahmouni et al. (2005) demonstrated that in healthy weight rats fed a standard rodent chow, leptin administration via the femoral vein or carotid artery reduces food consumption, a pathway absent in obese rats. In mice, mean arterial pressure increases with the dose of leptin administered (Shek et al., 1998) and plasma leptin levels in obese subjects with weight gain-associated hypertension are significantly higher than obese normotensive, weight-matched controls, (Masuo et al., 2000). Moderately obese male C57BL/6J mice show no response to peripherally administered and intracerebroventricular-administered leptin in terms of appetite suppression, but exhibit preserved renal sympathetic nervous activity in response to leptin (Rahmouni et al., 2005). Taken together, the above results imply that hypothalamic leptin sensitivity is impaired in obesity, yet peripheral actions may still remain.

1.4.2.2 Adiponectin

Scherer et al. (1995) first discovered the 247 amino acid peptide secreted from white adipose tissue, which was found to consist of four distinct domains; an N-terminus signal sequence, a variable region, a collagenous domain, and a globular region at the C-terminus (Scherer et al., 1995). Translation of mRNA coding for adiponectin results in generation of the adiponectin monomer, a structure which can undergo cleavage forming globular adiponectin. Alternatively, monomers can oligomerise to initially form low molecular weight (LMW) and then form the high molecular weight (HMW) structures (Suzuki et al., 2007, Ahima, 2006) (figure 1.8).

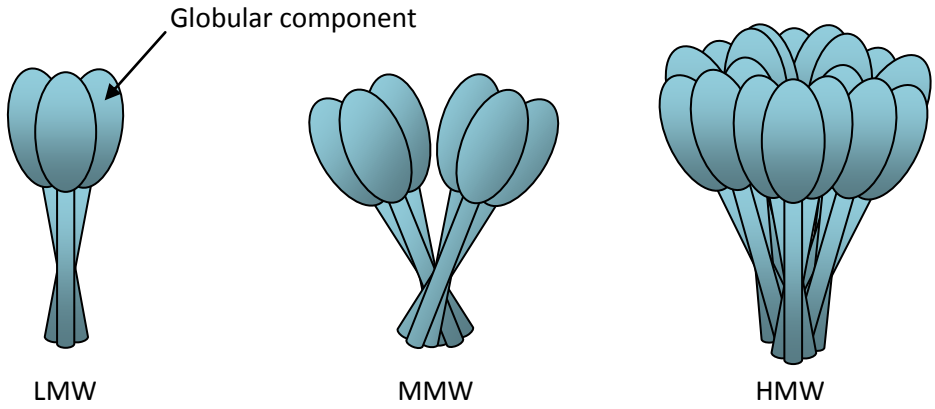

Figure 1.8. Polymerisation of adiponectin. Adiponection exists as trimers [low molecular weight (LMW)] which can form dimers [middle molecular weight (MMW), or high molecular weight (HMW) complexes. Adapted from Ahima et al. (2006).

Full length or globular adiponectin heightens the action of insulin in wild-type and *ob*/*ob* mice, and increases FAO via PPARα and PPARγ (Yamauchi et al., 2001, Berg et al., 2002), acting through the specific receptors AdipoR1 and AdipoR2. Both receptors show a high degree of homogeneity (>96%) but differing abundance, with AdipoR1 being primarily found in skeletal muscle and AdipoR2 within the liver (Yamauchi et al., 2001). Furthermore, adiponectin has been shown to stimulate glucose oxidation in C2C12 myocytes - as well as *in vivo* – via AMPK activation (Yamauchi et al., 2002). Deletion of the AdipoR1 receptor reduces FAO associated with globular adiponectin, but this effect does not extend to full length adiponectin, presumed to be signalling via AdipoR2 (Yamauchi et al., 2001). Adiponectin is reduced in obesity and shows a strong negative correlation with abdominal adiposity (Cnop et al., 2003, Pajvani et al., 2003), IR, and hyperlipidaemia (Maeda et al., 2002). Indeed, activation of PPARγ in IR humans and rodents by thiazolidinediones (TZDs) elevates plasma adiponectin levels, with this increase

abolished by TNFα (Yu et al., 2002b, Maeda et al., 2001, Phillips et al., 2009). As such, pathological conditions which disrupt the level of circulating adiponectin may be implicated in the obesity-linked IR.

Similar to leptin, adiponectin exhibits significant sexual dimorphism, with females showing higher concentrations (Kos et al., 2007, Pajvani et al., 2003). Specifically, in the female mouse total serum adiponectin was approximately 250% greater than that of males, with females showing a significant increase in the proportion of total serum adiponectin in the HMW form. (Pajvani et al., 2003). Similarly, the sexual dimorphism of adiponectin was found later to exist within the cerebrospinal fluid (CSF) of normal C57BL/6J mice, albeit at a much lower concentration than that of serum (Qi et al., 2004). Indeed, this finding has been observed in human subjects (Kos et al., 2007), suggesting that adiponectin diffuses from the serum across the BBB into the brain. Interestingly, CSF adiponectin consists of LMW and trimer structures, with HMW adiponectin being absent (Kusminski et al., 2007), suggesting that adiponectin transport across the BBB is size-dependent. Indeed, since most of the biological actions of adiponectin are through the HMW form (Pajvani et al., 2004), it remains controversial as to the function of the LMW and trimer complexes in the CSF.

1.4.2.3 Insulin

Insulin is synthesised from the progressive degradation of preproinsulin to proinsulin within the β-cells of the pancreas. Secretion of insulin is promoted by increased blood glucose, amino acids (van Loon et al., 2003), and FFAs (Itoh et al., 2003), and promotes protein and lipid anabolism in liver, muscle, and adipose tissue. Importantly, insulin concentrations are increased in obese subjects, suggesting potential resistance to its actions.

In obese persons, insulin-mediated glucose entry into liver, muscle, and adipose tissue is reduced. Several mechanisms have been proposed for the decreased sensitivity to the action of insulin, including reduced expression of the insulin receptor (Soli et al., 1975), decreased phosphorylation of tyrosine kinase and IRS (Hotamisligil and Spiegelman, 1994), and changes in the expression of glucose transporters (Kahn and Pedersen, 1993, Zierath et al., 1997, Brennan et al., 2004). Down-regulation of skeletal muscle and adipose tissue GLUT4 expression has been shown to occur in obesity, and may be responsible for the attenuated response to intravenous (iv) glucose tolerance test (Brennan et al., 2004). Additionally, Zierath et al. (1997)

observed that diet-induced obesity reduces insulin-stimulated glucose transport in muscle with specific impairment of membrane recruitment of GLUT4, accompanied with an attenuated interaction between IRS1 and PI3K. Thus, alterations the expression of GLUT4 via disruption to the translocation mechanism may play an important role in potentiating hyperglycaemia and IR.

The adipokines TNFα and adiponectin (Yamauchi et al., 2001, Cnop et al., 2003, Giugliano et al., 2004), and FAs (Yu et al., 2002a) have been linked to reduced insulin sensitivity and the development of IR. Increased adiposity is also linked with IR (Indulekha et al., 2011). Tulloch-Reid et al. (2004) demonstrated that IR is inversely related to both s/c and visceral adipose tissue depots. In this study group consisting of 78 African-Americans, both s/c and visceral adipose tissue showed differing degrees of correlation with insulin sensitivity depending on sex. Furthermore, increased s/c and visceral adiposity, as well as plasma TG, was associated with decreased insulin sensitivity in men and women (Tulloch-Reid et al., 2004). Taken together, the hormonal and metabolic alterations in obesity may expose the heart to a unique environment which may impact upon cardiac function.

1.4.3 Obesity and cardiac function

Obesity increases the risk of developing hypertension and associated LVH, with this process ultimately leading to HF (Hubert et al., 1983, Kenchaiah et al., 2002). Indeed, Kenchaiah et al. (2002) demonstrated that for every 1 increment in BMI there was a 5 percent and 7 percent increase in the risk of developing HF in both men and women, respectively. An expanded fat mass increases circulating blood volume and leads to haemodynamic overload (Alpert, 2001). In addition to the increased preload derived from an augmented circulating volume, afterload is also increased due to hypertension. Consequently, obesity can impose a unique environment for cardiac growth due to both a pressure- and volume-overload stimulus.

Both concentric and eccentric remodelling patterns are evident in obesity (Chakko et al., 1991, Avelar et al., 2007), occurring with left atrial enlargement (Sasson et al., 1996, Movahed and Saito, 2008). Despite the ventricular remodelling, cardiac output was significantly increased in a population of obese patients when compared to lean subjects (Karason et al., 1998), potentially due to increased heart rate. In contrast to the positive effect upon cardiac output, ejection fraction is reduced (Karason et al., 1998, Alpert, 2001), unchanged (Grandi et al.,

2000, Peterson et al., 2004), or increased (Pascual et al., 2003) following obesity. Importantly, the observed unchanged and increased ejection fraction should be interpreted cautiously as ejection fraction alone does not indicate more subtle changes in the myocardium such as fractional shortening. Indeed, both obese rats (Zhou et al., 2000) and patients (Alpert et al., 1985) show reduced fractional shortening, suggesting that ejection fraction alone may be an insufficient indicator of cardiac function in the obese subject. In addition to changes in systolic performance (fractional shortening), diastolic function has been shown to be impaired in hearts from obese patients (Sidana et al., 2005, Powell et al., 2006) and animal models (Carroll et al., 1999, Christoffersen et al., 2003).

Contrary to the finding that obesity increases the risk of developing cardiac dysfunction and HF, a greater BMI may be cardioprotective in HF patients, referred to as the "obesity paradox" (Anker and von Haehling, 2011). Horwich et al. (2001) showed in a large study of HF patients (n=1203) that 5-year mortality is significantly reduced in obese patients compared to lean equivalents. Furthermore, the authors postulated that this could be due in part to the ability of the obese population to tolerate more robust afterload-reducing agents such as angiotensin converting enzyme (ACE) inhibitors, and the fact that obese patients may present at a clinically earlier stage due other co-morbidities. Additionally, in a large population of obese hypertensive patients, mortality was 30% lower than a normal weight group (Uretsky et al., 2007), although this is not without challenge (Kenchaiah et al., 2002). Increased adiposity may offer protective effects due to increased metabolic reserve during the end stages of HF characterised by cachexia (Oreopoulos et al., 2008). Furthermore, the elevated blood pressure in obesity may actually be beneficial in end-stage HF by maintaining adequate pressure in the face of declining cardiac function (Oreopoulos et al., 2008). As such, advocating weight-loss in HF patients may be detrimental to health (Anker and von Haehling, 2011).

1.4.4 Genetic models of obesity and LVH

1.4.4.1 ob/ob and db/db mice

Unlike humans, animals are able to regulate body weight (BW) closely and hyperphagia is uncommon unless genetically predisposed. Indeed, several genetic models of hyperphagia have exploited the intimate relationship between food consumption and body mass, most notably those that disrupt leptin signalling.

Both ob/ob and db/db mice exhibit disruption to the leptin signalling axis via either abrogation of leptin production (ob/ob) or the presence of a non-functional leptin receptor (db/db). ob/ob and db/db mice exhibit signs of substantial LVH and cardiac remodelling (Barouch et al., 2003). Importantly, in the hearts of ob/ob mice the ventricular remodelling process occurs independently of changes in systolic blood pressure or heart rate, suggesting a direct effect of leptin upon cardiac mass (Mascareno et al., 2009). Furthermore, leptin administration to ob/ob mice completely reverses the observed LVH (Barouch et al., 2003). Cardiomyocytes from ob/ob mice also show characteristics similar to those of the hypertrophied heart, including alterations to the cardiac β-adrenergic response, reduced sarcomere shortening, and attenuated sarcoplasmic reticulum Ca^{2+} storage (Minhas et al., 2005). However, strain-specific differences exist with ob/ob mice, and this itself is an important consideration when interpreting data from these models. Indeed, ob/ob mice from a C57BLK/SJ background exhibit signs of diabetes by 5 weeks of age (Buchanan et al., 2005), whereas the more commonly used strain (C57BL/6J) develops hyperglycaemia, hyperinsulinaemia, and IR by 8-12 weeks of age (Buchanan et al., 2005, Drel et al., 2006, Van den Bergh et al., 2008).

In contrast to ob/ob mice, db/db equivalents exhibit signs of cardiac dysfunction, with an increased end diastolic pressure (EDP), and decreased cardiac output and cardiac power despite an absence of LVH (Belke et al., 2000, Aasum et al., 2003). In this regard, these studies highlight a direct role of leptin signalling in cardiac physiology. Leptin is progressively elevated due to a non-functional leptin receptor and the hyperphagia observed in these mice (due to an absence of the satiety stimulus) ensures worsening of the leptin profile due to accumulation of adipose tissue and further synthesis of leptin. Both the ob/ob and db/db mouse show signs of metabolic disturbances, including dyslipidaemia, hyperglycaemia, and hyperinsulinaemia (Kobayashi et al., 2000, Aasum et al., 2003, Dong et al., 2006, Van Den Hoek et al., 2008), mirroring features commonly found in obese subjects. Normal levels of leptin may serve to maintain cardiac geometry, and deviation away from a normal serum leptin may induce a reversible structural hypertrophy. However, in obese humans, serum levels of leptin are elevated. As such, the changes in cardiac geometry in response to leptin-deficient obesity (arguably not a true obese phenotype) may not relate directly to a known cardiac pathology.

1.4.4.2 Zucker diabetic fatty (ZDF) rat (fa/fa)

The Zucker diabetic fatty (ZDF) rat exhibits severe obesity as a product of a dysfunctional leptin receptor, similar to that of the db/db mouse. ZDF rats exhibit hypertension (Alonso-Galicia et

al., 1996), cardiac hypertrophy (Fredersdorf et al., 2004), serum disturbances including hyperglycaemia, hyperinsulinaemia and hypertriglycerideaemia (Zhou et al., 2000, Coort et al., 2004). These rats also exhibit more severe signs of obesity including respiratory dysfunction (reduced total lung volume) (Yilmaz et al., 2010) further replicating the severely obese phenotype.

Although hearts from ZDF rats are generally larger than age-matched controls, Zhou et al. (2000) found that only the left ventricle was significantly hypertrophied when compared to lean +/+ littermates. ZDF rats develop an increase in end diastolic chamber size, reduced contractile dysfunction by 20 weeks of age, and augmented deposition of TG (Zhou et al., 2000). The significance of the left ventricle becoming hypertrophied may relate to the findings of Sista et al. (2005), who observed that the aorta of ZDF rats exhibited increased stiffness and fibrosis, thus increasing arterial hypertension and potentially causing enlargement of the left ventricle.

1.5 Objectives

Although initially beneficial, cardiac hypertrophy is a significant and independent risk factor for the development of HF, and may have an important role in the progression of the disease. Additionally, besides being an additional risk factor for the development of HF, obesity produces a substantial challenge for the heart, both in terms of functional adaptation and metabolism. Human LCAD deficiency and animal models of diabetes and obesity (Zhou et al., 2000, Sharma et al., 2004) suggest that discord between the uptake and oxidation of LCFA can lead to accumulation of intracellular TG. Furthermore, this increased lipid accumulation may lead to enhanced cellular apoptosis via a ceramide-dependent mechanism (Park et al., 2008). Thus, the hypothesis that diminished rates of FAO in the hypertrophied heart may predispose cardiomyocytes to lipid accumulation when confronted with increased supply, forms the central tenet of this thesis. Consequently, the objectives of this study were to:

1. Determine the extent of lipid accumulation and contractile function in the hypertrophied heart under conditions of increased fat and sugar supply.
2. Examine the impact of increasing dietary fat and sugar upon myocardial substrate oxidation under basal and pressure-overload conditions.

3. Develop a robust method for the quantification of cardiac ceramide content and investigate potential alterations in its level following dietary intervention and pressure-overload hypertrophy.

Chapter 2 Materials and methods

2.1 Materials

A list of all chemicals and their suppliers are given in table 2.1. Reagents were AnalaR grade unless otherwise stated.

Table 2.1 Materials and suppliers

Suppliers	Materials
Abbott Laboratories **Kent, UK**	Isofluorane
Agilent Technologies **Berkshire, UK**	Screw cap vials Screw vials with fixed insert
Apollo Scientific Ltd **Stockport, UK**	Deuterated water Deuterated chloroform
Avanti Polar Lipids **AB, USA**	C8, C16, C17, C18, C20, C24 Ceramide
Biorad Laboratories **Munich, Germany**	Bio-rad protein assay
British Oxygen Corporation **Manchester, UK**	95% oxygen/5% carbon dioxide
Cambridge Isotope Laboratory **Andover, USA**	[U-^{13}C] Sodium palmitate [3-^{13}C] Sodium lactate
Chromacol **Hertfordshire, UK**	10ml screw top vial, clear round base
Fisher Scientific **Leicester, UK**	Glutamine Polysine® Slides
Harvard Apparatus **Edenbridge, UK**	Anaesthetic apparatus (gaseous) – Fluovac vacuum, veterinary fluosorber and ohmeda fluotec vaporizer
Intervet UK, Ltd **Cambridge, UK**	Amphipen

Johnson & Johnson **North Yorkshire, UK**	**Ethicon 3-0 (Vicryl braided)** **Ethicon 3-0** **Isoflurane**
Millipore **Cork, Ireland**	0.45µm filter (HA type) 5.0µm filter (SVPP type)
Nestle **UK**	Marvel milk powder
Novartis Animal Health **Hertfordshire, UK**	Thiovet (sodium thiopentone)
Sigma-Aldrich **Poole, UK**	Acrodisc® (0.2µm syringe filter)
Pfizer Ltd **Sandwich, UK**	Rimadyl (5% w/v carprofen)
Proliant Health Biologicals **Iowa, USA**	Bovine serum albumin (fatty acid free <0.001%)
Roche Diagnostics Limited **Sussex, UK**	Complete® protease inhibitor cocktail
Sakura Finetek **Zoeterwoude, Netherlands**	Tissue-Tek OCT compound

All biological and chemical enzymes supplied by Sigma Aldrich (Poole, UK) unless otherwise specified.

2.2 Methods

2.2.1 Surgical induction of pressure-overload hypertrophy

Pressure-overload hypertrophy was surgically induced as described previously (Boateng et al., 1997, Akki and Seymour, 2009). All animal experimentation conformed to the UK Animals (Scientific Procedures) Act (ASPA) of 1986.

Prior to surgery, food intake was restricted for 12 hours. Male Sprague Dawley rats (240-280g) were anaesthetised using 3.5% isoflurane in 3L O_2. A s/c injection of Rimadyl (4mg/Kg BW, Pfizer Ltd, Sandwich, UK) was administered for post-operative pain relief. Anaesthesia was maintained using 2.5% isoflurane in 1L O_2 and anaesthetic depth assessed using the pedal withdrawal and tail-pinch reflexes. A laparotomy was performed and the abdominal aorta exposed at the level of the renal vessels. Non-absorbable 3-0 Mersilk® suture thread (Johnson & Johnson, UK) was passed underneath the aorta and tied around a 0.5mm outer diameter blunted needle. Ligation of the aorta induced temporary blanching of the left kidney which recovered following removal of the needle (figure 2.1). Control (Con) animals underwent an identical procedure but without constriction of the aorta. Sterile isotonic saline (Animal Care, York, England) was administered directly into the abdominal cavity to replace fluid loss. The abdominal muscle layer was closed with braided absorbable sutures (Ethicon Vicryl, Johnson & Johnson, UK) and the dermal layer using non-absorbable monofilament sutures (Ethilon, Johnson & Johnson, UK). Animals received a s/c injection of the antibiotic Amfipen (42mg/kg, Intervet UK Ltd, Cambridge UK).

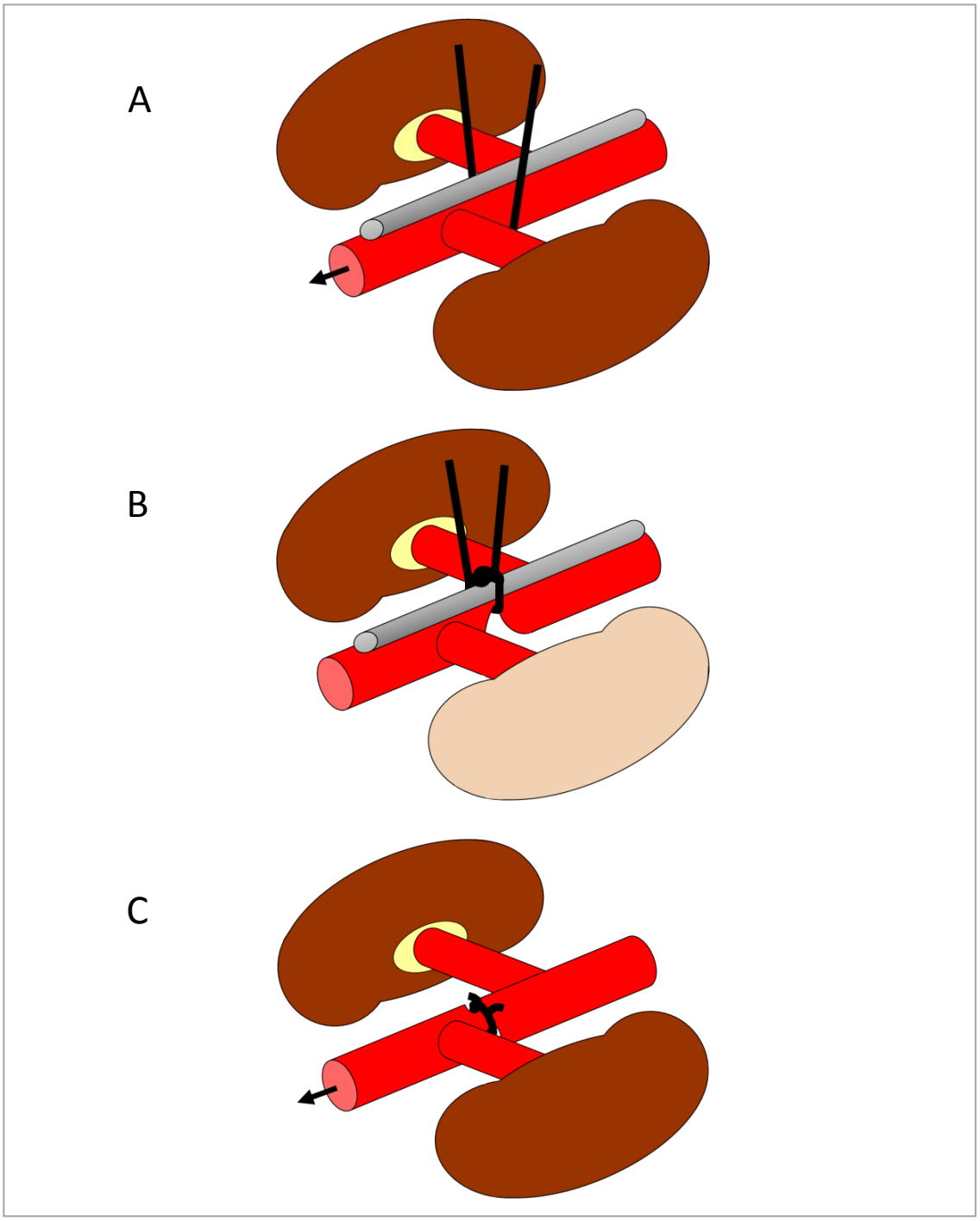

Figure 2.1 Surgical Induction of cardiac hypertrophy. A ligature was passed around the needle and aorta (A), tied (B), and the needle subsequently withdrawn (C).

2.2.2 Dietary regimen

Animals were allowed to recover for 48 hrs with food and water *ad libitum* before being assigned to diets containing 7% fat and 5% sucrose (standard diet, SD); 45% fat and 9% sucrose (high-fat diet, HFD); or 44% fat and 14% sucrose (western diet, WD) (table 2.2). The contribution of carbohydrate, protein, and individual FA species were calculated using heat of combustion values of 4, 4, and 9 kcal/g, respectively. Animals were maintained on each of the diets for 9 weeks. The WD was formulated to mimic western dietary patterns (table 1.1).

Table 2.2 Dietary constituents

	SD 3.3 kcal/g		HFD 4.5 kcal/g		WD 4.6 kcal/g	
Dietary Component	g/100g	AFE	g/100g	AFE	g/100g	AFE
Carbohydrate	61.7	74.8	39.8	35.4	40.3	35.0
Starch	45.0	54.5	28.3	25.2	19.6	17.0
Sucrose	4.1	5.0	10.5	9.3	16.0	13.9
Other	12.6	15.3	1.0	0.9	4.6	4.0
Protein (Casein)	14.4	17.5	23.0	20.4	23.0	20.0
Fats (Lard + Soya Oil)	2.7	7.4	22.6	45.2	22.5	44.0
Saturated	0.5	1.4	6.5	13.0	6.5	12.7
C12:0	0.0	0.0	0.1	0.2	0.1	0.2
C14:0	0.1	0.3	0.4	0.8	0.4	0.8
C16:0	0.3	0.8	4.1	8.2	4.1	8.0
C18:0	0.1	0.3	1.9	3.8	1.9	3.7
Monounsaturated	0.9	2.5	6.4	12.8	6.4	12.5
C14:1	0.0	0.0	0.0	0.0	0.0	0.0
C16:1	0.1	0.3	0.1	0.2	0.1	0.2
C18:1 n-9	0.8	2.2	6.3	12.6	6.3	12.3
Polyunsaturated	0.9	2.5	3.5	7.0	3.5	6.8
C18:2 n-3	0.1	0.3	0.4	0.8	0.4	0.8
C18:3 n-6	0.7	1.9	3.1	6.2	3.1	6.1
C20:4 n-6	0.1	0.3	0.0	0.0	0.0	0.0
Other	0.4	1.1	6.2	12.4	6.2	12.1
Fibre	4.7	-	4.6	-	4.6	-

SD, Standard Diet; HFD, high-fat diet; WD, western diet; AFE; Atwater Fuel Energy (% total calories).

2.2.3 Preparation of Krebs-Henseleit (KH) buffer

Krebs-Henseleit (KH) buffer was prepared using Milli Q 18MΩ ultra-pure water gassed with 95%O_2/5%CO_2 containing (in mM): NaCl (118), $NaHCO_3$ (25), KCl (4.8), KH_2PO_4 (1.2), $MgSO_4.7H_20$ (1.2), $CaCl_2.2H_2O$ (1.25), glucose (5), glutamine (0.5), lactate (1.0), palmitate (0.3), pyruvate (0.1), and insulin (0.1mU/ml) and filtered using 0.45µm filter paper prior to use.

2.2.4 Preparation of bovine serum albumin (BSA) buffer containing palmitate

2.2.4.1 Preparation of dialysis tubing

Lengths of dialysis tubing (approximately 30cm) were boiled in 500ml containing 2% w/v $NaHCO_3$ and 1mM EDTA for 10 minutes before being rinsed with ultra-pure water and boiled for a further 10 minutes in 500ml of 1mM EDTA. All tubing was thoroughly rinsed with ultra-pure water prior to BSA dialysis.

Prior to dialysis a 30% (w/v) BSA solution was prepared using ultra-pure water 18MΩ containing (in mM); NaCl (118) and $CaCl_22H_2O$ (2.5) and dialysed against 20L of NaCl (118) and $CaCl_22H_2O$ (2.5), at 4°C for 48 hours. Following dialysis, the total volume of BSA was recorded, aliquoted and frozen at -20°C until use.

2.2.4.2 Preparation of 3% BSA buffer containing substrates

Sodium palmitate (0.3mM) was dissolved in hot ultra-pure water before addition to 30% w/v BSA and then diluted appropriately to obtain a final concentration of 3% w/v BSA. Correction factors were applied to account for NaCl and $CaCl_2$ already present (equation 1) and resultant KH/BSA buffer filtered through a 5µm filter before use.

Mass of NaCl or $CaCl_2$ = Vol. of Albumin/L KH Buffer x MW of chemical x Molarity (M)

Equation 1: Calculation for NaCl and $CaCl_2$ present per litre of BSA solution.

For example, if the aliquoted BSA has a volume of 160ml, the amount of NaCl present in the BSA is equal to 0.160 x 58.44 x 0.118 = 1.10g/L. This value can be subsequently substracted from the amount of NaCl required in the K-H buffer (6.89 -1.10 = 5.79g).

2.2.5 Isolated heart perfusion

Following 9 weeks of dietary regimen, animals were terminally anaesthetised via an intraperitoneal (i/p) injection of sodium thiopentone (0.5ml/100g BW). Hearts were rapidly excised and placed in ice-cold KH buffer containing 5mM glucose and 0.1 ml 20IU Heparin (LEO Laboratories Limited, Buckinghamshire, UK). Hearts were cannulated via the aorta and perfused in a modified isovolumic Langendorff mode (figure 2.2) (Sample et al., 2006) at a constant flow rate of 14ml/min (MHRE/22 Mk3 Flow Inducer, Watson-Marlow pump, Leeds, UK). Buffer was oxygenated with 95% O_2/5% CO_2 (BOC, Surrey, UK) using a specially constructed oxygenator (Gamcsik et al., 1996). The apex of the heart was pierced using a 21G needle to prevent accumulation of fluid within the thebesian circulation.

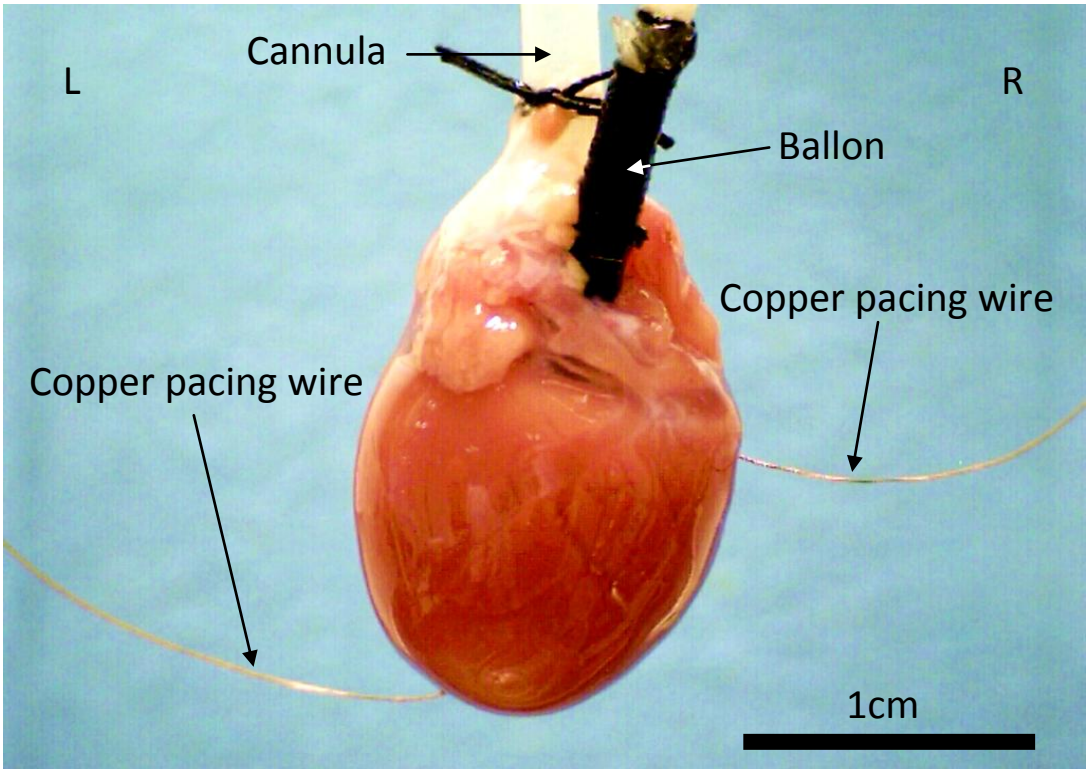

Figure 2.2 Isolated Langendorff heart perfusion. L, left; R, right.

Copper pacing wires were inserted into the right ventricle and apex of the heart using a 25G microlance (BD, Ireland) and stimulated at a frequency of 5Hz and a voltage range of 0.6-1.5V.

2.2.5.1 Perfusion protocol

All hearts were equilibrated for an initial 15 minutes with unlabelled BSA buffer and perfused for a total of 65 minutes (figure 2.3). Buffers were either switched to those containing ^{13}C-labelled substrates or continued with unlabelled substrates. CFR was measured every 15 min. For mass spectrometry studies, hearts were perfused with KH buffer for 10 minutes to remove blood from the ventricles and the atria removed. At the end of the perfusion period hearts were freeze-clamped using liquid nitrogen-cooled Wollenberger tongs and stored at -80°C until use.

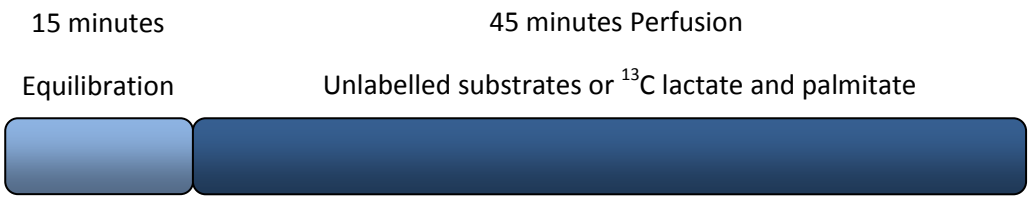

Figure 2.3 Perfusion protocols used in the current studies.

2.2.5.2 *In vitro* cardiac function

Left ventricular function was monitored using a balloon constructed of Saran wrap (Dow, UK) inserted into the left ventricle via the mitral valve. The balloon was attached via fluid-filled line to a physiological pressure transducer (SensorNor, Horten, Norway) and in turn was connected to a MacLab bridge amplifier (Powerlab (4/30) and ADInstruments Chart® 5.5 reading system (ADInstruments, Hastings, UK). The balloon was inflated using a 2ml micrometer syringe (Gilmont Instruments, Barrington, USA) to achieve an end diastolic pressure (EDP) of approximately 7mmHg. Heart rate (HR), systolic pressure (SP), and EDP were recorded continuously throughout the perfusion (figure 2.4). Left ventricular developed pressure (LVDP) was calculated by subtracting the EDP from SP. Rate pressure product (RPP) was calculated as an index of cardiac function (equation 2).

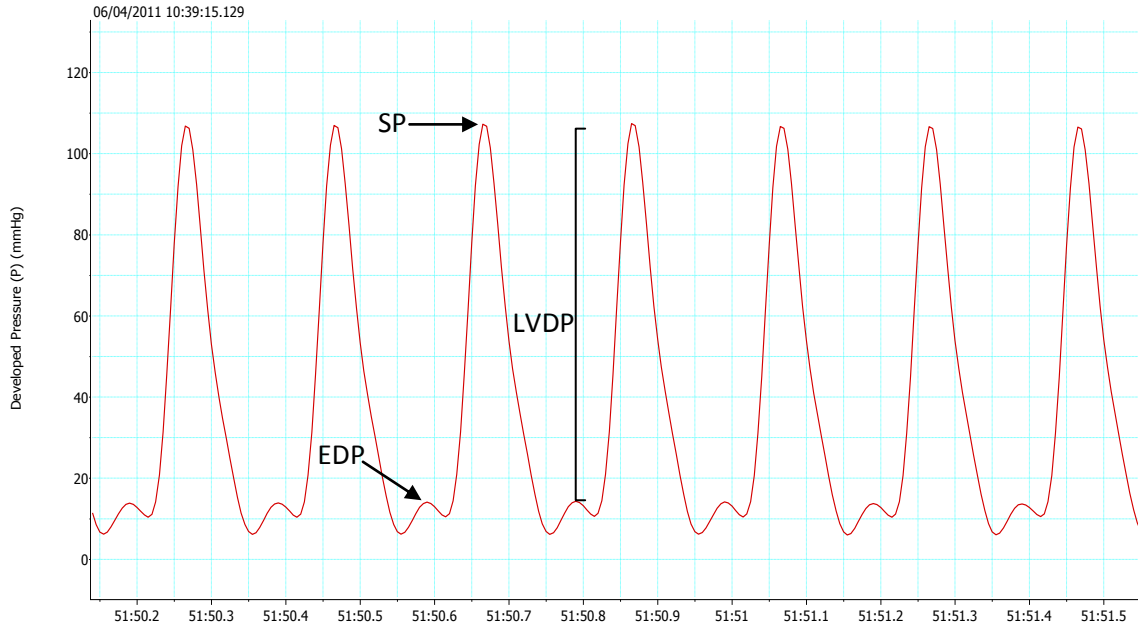

Figure 2.4 Recorded function obtained during isolated heart perfusion. EDP, end diastolic pressure; SP, systolic pressure; LVDP, left ventricular developed pressure.

$$RPP = LVDP \times HR$$

Equation 2: Calculation for determining RPP.

2.2.5.3 Myocardial oxygen consumption (MVO_2)

Cardiac oxygen consumption was calculated from measurements of perfusion buffer and coronary effluent analysed using an ABL blood gas analyser (ABL77, Radiometer Medical, USA), and MVO_2 calculated described by Neely et al. (1967) (equation 3).

$$MVO_2 = \frac{\left[\left[\frac{pO_2 \text{ perfusate} - pO_2 \text{ effluent (mmHg)}}{760\text{mmHg}}\right] \times 0.199 \times CFR\right]}{\text{Wet heart weight (g)}}$$

Equation 3: Calculation of myocardial oxygen consumption. Where 0.199 as the solubility of oxygen at 37°C (µmol/ml); CFR, coronary flow rate (14 ml/min).

Cardiac efficiency (CE) was calculated by dividing RPP by MVO_2.

2.2.5.4 Indices of cardiac hypertrophy

The extent of LVH was determined using the heart weight (HW)-to-tibia length (HW:TL) ratio (Yin et al., 1982) and HW-to-BW (HW:BW) ratios. Lung water content was used as an indicator of pulmonary congestion. Left:right kidney ratios were used to assess the severity of aortic constriction.

2.2.5.5 Assessment of model adiposity

Retroperitoneal + perirenal, epididymal, mediastinal, adipose tissue (RPAT, EAT, MAT, respectively) deposits were resected and weighed.

2.2.5.6 Haematocrit and serum analysis

Blood was collected from the thoracic cavity following excision of the heart and haematocrit determined using the ABL77 Blood Gas Analyser (Radiometer Medical, USA). Serum was obtained by centrifugation at 4000xg, 4°C for 10 minutes and stored at -20°C.

2.2.6 Metabolite assays

2.2.6.1 Tissue extraction of glycogen

Approximately 300mg of ventricular tissue was digested with 0.5ml of 30% (w/v) KOH for 30min at 100°C using a heating block (Stuart Scientific Co LTD, UK) and 0.2ml of 2% (w/v) Na_2SO_4 added when cooled . A sufficient volume of 100% (v/v) ethanol was added to give a final concentration of 75% (v/v). Samples were subsequently transferred to sealed centrifuge tubes and stored overnight at 4°C to allow glycogen to precipitate. The pellet was washed then centrifuged at 27000xg (Sorvall RC-58, USA) for 10 minutes before the supernatant was removed and the pellet washed again with 80% (v/v) ethanol and disaggregated with a glass rod. This solution was then centrifuged at 27000xg for 10 minutes and the pellets dried at 37°C.

Gycogen was digested using 0.5ml acetate buffer (1M sodium acetate/acetic acid buffer, pH 5.0), 0.1mM amyloglucosidase (500µg/ml amylo α-1,4 α-1,6 glucosidase in acetate buffer), 1.5ml ultra-pure water, at 37°C for 1 hour prior to the addition of 1.4ml ultra-pure water. The

suspension was centrifuged at 27000xg for 20minutes, the supernatant decanted and used in the assay of glucose as described below.

2.2.6.1.1 Glucose assay

Glucose concentrations were determined using a modified method of Huggett and Nixon (1957) based on the following reactions:

$$\text{Glucose} + O_2 \xrightarrow{\text{GOD}} \text{D-Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-AAP} + \text{4-HBS} \xrightarrow{\text{POD}} \text{Quinoneimine} + 4H_20$$

GOD, glucose oxidase; POD, peroxidase; 4-AAP, 4-aminoantipyrine; 4-HBS, 4-hydroxybenzene sulphonic acid.

Glucose reagent was prepared on the day of analysis and contained (in mM) NaH_2PO_4 (100), pH 7.0, 4-aminoantipyrine (0.4), 4-hydroxybenzene sulphonic acid (10), 0.8U/ml Peroxidase, and 10U/ml glucose oxidase. Samples (0.1ml) were mixed with 3.5ml glucose reagent and incubated at 30°C for 30 minutes and absorbances determined at 510nm. Glucose standard solutions (0.5-10mM) were analysed in parallel to the sample measurements. Sample concentrations were calculated using the straight line relationship (figure 2.5).

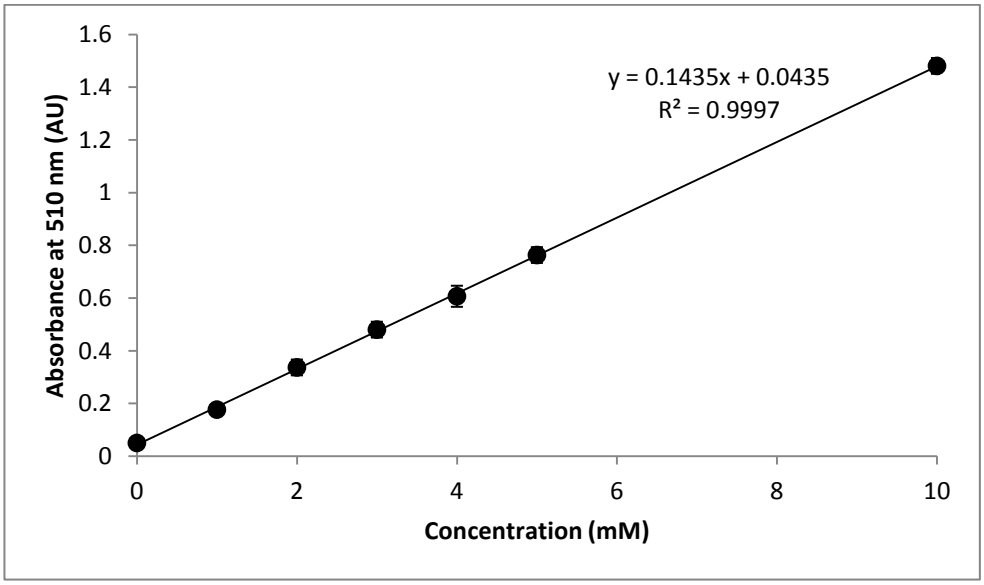

Figure 2.5 Glucose standard curve. Values represent mean of triplicates ± SEM.

2.2.6.2 Tissue extraction of triglyceride and free fatty acids

Cardiac TG and FFA species were extracted using a modified method of the Bligh and Dyer (1959). Approximately 300mg of ground cardiac tissue was homogenised in 1ml chloroform/methanol/ultra-pure water (2:1:0.8 v/v/v) for 4 x 30 seconds using an Ultra Turrax T25 homogeniser (Janke & Kunkle GmbH and Co KG IKA Laboratechnik, Stauffen, Germany) at maximum speed. Chloroform/ultra-pure water (1ml, 2:1 v/v) was added and samples subsequently centrifuged at room temperature for 15 minutes at 764xg. The lower organic layer was removed and transferred to clean glass tubes, evaporated under a nitrogen stream and reconstituted in 0.5ml propan 2-ol.

2.2.6.2.1 Triglyceride assay

Tissue and serum TGs were analysed using a Sigma-Aldrich kit (TR0100,) according to the manufacturer's instructions based on the following reactions:

$$\text{TG} \xrightarrow{\text{LPL}} \text{Glycerol} + \text{FA}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{GK}} \text{G-1-P} + \text{ADP}$$

$$\text{G-1-P} + O_2 \xrightarrow{\text{GPO}} \text{DAP} + H_2O_2$$

$$H_2O_2 + \text{4-AAP} + \text{ESPA} \xrightarrow{\text{POD}} \text{Quinoneimine} + H_2O$$

TG, triglyceride LPL, lipoprotein lipase; FA, fatty acid; GK, glycerol kinase; ATP; adenosine-5'-triphosphate; ADP, adenosine-5'-diphosphate; G-1-P, glycerol-1-phosphate; GPO, glycerol phosphate oxidase; DAP, dihydroxy-acetone phosphate; 4-AAP, 4-aminoantipyrine; ESPA, N-ethyl-N-(3-sulphopropyl) m-anisidine; POD, peroxidase.

All components were reconstituted with ultra-pure water. Briefly, reconstituted glycerol reagent (0.8ml) was mixed with 10µl of sample and incubated at 37°C for 5 minutes. Absorbance (A_1) was read at 540nm using a Philips PU 8720 UV/VIS spectrophotometer (Philips, Spectroninc Analytical Equipments, Leeds, UK) and 0.2ml of a reconstituted triglyceride reagent was added and incubated for a further 5 minutes at 37°C and absorbance (A_2) measured again. The linearity of the assay was determined by analysis of standards to the same time as samples (figure 2.6).

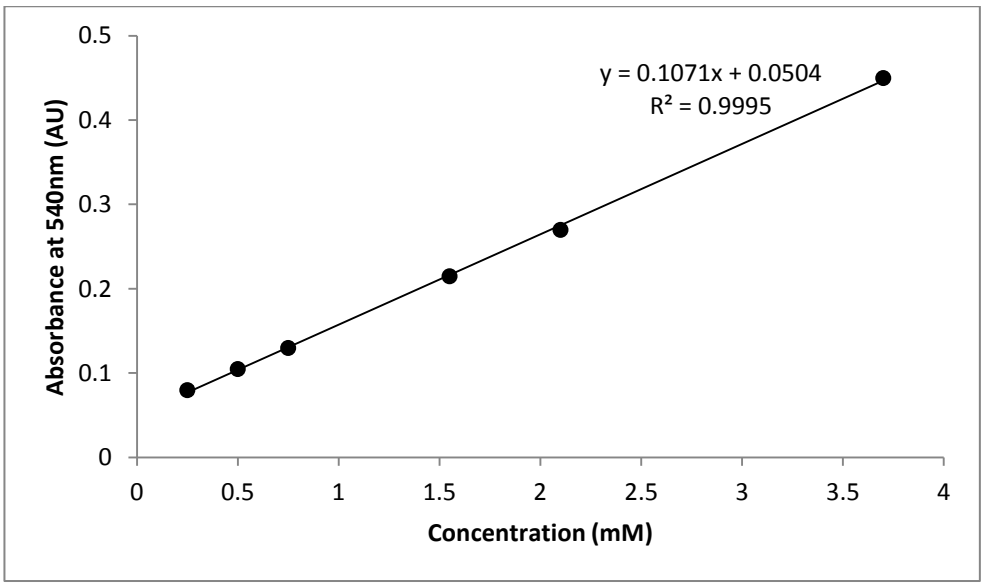

Figure 2.6 Triglyceride standard curve. Values represent mean of triplicates ± SEM.

TG concentration was calculated using equation 4:

$$\text{TG Concentration (mM)} = \frac{[\text{FA sample} - (\text{IA sample} \times F)]}{[\text{FA standard} - (\text{IA blank} \times F)]} \times \text{Standard concentration} \times 1.13$$

Equation 4: Calculation of triglyceride concentration. TG, triglyceride; FA, final absorbance; IA, initial absorbance; F, 0.8; standard concentration, 2.5mg/ml; and 1.13 a conversion factor from mg/ml to mM.

2.2.6.2.2 Free fatty acid assay

FFA samples (serum or extracted tissue) were analysed using a commercially available Half-micro test kit (11 282 175 001). In brief, 50µl of sample was mixed with 1 ml of solution A and incubated at 25°C for 10 minutes. N-ethylmaleimide solution (0.05ml; provided with kit) was added and the initial absorbance read at 546 nm. To this, 0.6 ml of a reaction mixture was added and incubated at 25°C for a further 15 minutes before the final absorbance was read.

$$\text{FFA + CoA + ATP} \xrightarrow{\text{Acyl CS}} \text{Acyl-CoA + AMP + pyrophosphate}$$

$$\text{Acyl-CoA + } O_2 \xrightarrow{\text{ACOD}} \text{Enoyl-CoA + } H_2O_2$$

$$H_2O_2 + \text{4-AAP + TBHB} \xrightarrow{\text{POD}} \text{Quinoneimine + } 2H_2O + \text{HBr}$$

FFA - free fatty acid; Acyl CS, acyl-CoA synthetase; ACOD, acyl-CoA oxidase; 4-AAP, 4-aminoantipyrine; TBHB, 3-hydroxy 2,4,5-tribromobenzoic acid; POD, peroxidase.

The concentration of FFA in the sample was determined using equation 5. A 0.35mM palmitic acid standard solution was used to produce a standard curve demonstrating the linearity of the assay (figure 2-7).

$$C = \frac{V}{(\varepsilon \times l \times v)} \times (\Delta As - \Delta Ab)$$

Equation 5: Calculation of free fatty acid concentration. V, total cuvette volume (1.15ml); ε, extinction coefficient for of red dye (19.3 l $mmol^{-1}$ cm^{-1}); l, light path (1cm); v, sample/standard volume (0.05ml); and ΔAs and ΔAb, change in sample and blank absorbance, respectively.

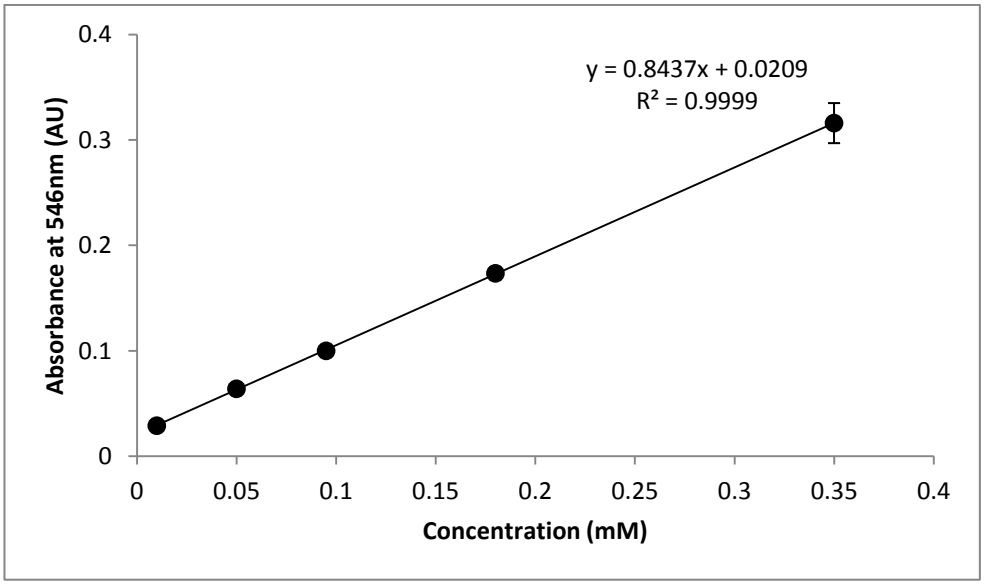

Figure 2.7 Free fatty acid standard curve. Values show mean of duplicates ± SEM.

2.2.7 Mitochondrial enzyme assays

2.2.7.1 Medium-chain acyl-CoA dehydrogenase (MCAD) assay

The MCAD assay was based upon the method of Lehman et al. (1990).

2.2.7.1.1 Preparation of ferricene salt

Ferricenium salt was kindly prepared by Mrs. Kath Bulmer. In brief, a solution of 5g ferrocene in 10ml conc H_2SO_4 was transferred to 150ml ultra pure water, filtered through two layers of Whatman no. 1 filter paper under vacuum. 5ml of a saturated sodium hexafluorophosphate ($NaPF_6$) was added to the filtrate and the solution stored on ice for 30min after which the crystals were collected, washed with ultra pure water, filtered under vacuum and dried in a desiccator for 3 hours and stored at -20°C until required. Prior to use, the ferricenium hexafluorophosphate (FC^+PF_6) was dissolved in 2.5ml 10mM HCl and centrifuged at 3000xg for 10 minutes at 4°C. The supernatant was decanted, absorbance measured at 300nm, and concentration calculated using an extinction coefficient of $4.3mM^{-1}cm^{-1}$.

2.2.7.1.2 MCAD assay procedure

Frozen heart tissue (50mg) was homogenised in 1ml of extraction buffer containing (in mM); KH_2PO_4 (50), EDTA (1), $MgCl_2$ (2) for 2x30s on ice at 2400rpm using an Ultra Turrax T25 homogeniser and centrifuged at 9600xg at 4°C for 5min using a microfuge (Eppendorf, Hamburg, Germany). The supernatant (20µl) was added to 900µl reaction buffer containing (in mM) KH_2PO_4 (100), EDTA (1), and $Na_2S_4O_6.2H_2O$ (0.5), pH 7.2, and octanoyl-CoA (50µM). The change in absorbance was measured over 3 minutes using a wavelength of 300 nm and activity calculated using an extinction coefficient of $4.3mM^{-1}cm^{-1}$ and equation 6.

$$\mu mol/min/g\ wet\ wt = \left(\frac{\text{Abs change/min}}{\text{Extinction Coefficient}}\right) \times \left(\frac{\text{Total cuvette vol (µl)}}{\text{Sample vol (µl)}}\right) \times \text{dilution factor}$$

Equation 6: Calculation of enzyme activity.

2.2.7.2 Citrate synthase (CS) assay procedure

CS activity was determined in extracted cardiac tissue by following the change in absorbance of 5,5 dithio-bis-2-nitrobenzoic acid (DTNB) (Morgan-Hughes et al., 1977). In brief, cardiac tissue (15mg) was homogenised in 1ml (in mM); imidazole (100), EGTA (1), and $MgCl_2$ (10), pH 7.2. Triton X- 100 (10µl) was added to the homogenates and kept on ice for 1 hour. Samples were centrifuged at 400xg for 10 minutes at 4°C and 20µl of supernatant was added to 1.98ml reaction buffer containing (in mM) tris [hydroxymethyl] aminomethane (50, pH 8.1), DTNB (0.2), acetyl CoA (0.1), oxaloacetate (0.5) and 0.05% w/v Triton X-100. The change in absorbance was measured at 412nm over 3 minutes and an extinction coefficient of 13.6 $mM^{-1}cm^{-1}$ used to calculate activity (equation 6).

2.2.7.3 Pyruvate dehydrogenase (PDH) assay

PDH was assayed using the method of Seymour and Chatham (1997). To determine the fraction of PDH in the active form (PDHa), inhibitors of PDH phosphatase (KH_2PO_4 and KF) and PDH kinase (ADP and DCA) were used. For determination of total PDH (PDHt), the enzyme was converted to its active form using $MgCl_2$ to stimulate the phosphatase and ADP to inhibit the kinase.

For both PDHa and PDHt, approximately 200mg of ground ventricular tissue was homogenised using an Ultra Turrax T25 homogeniser (IKA Laboratechnik, Stauffen, Germany) for 30 seconds at maximum speed. The homogenisation buffer for PDHt contained (in mM) HEPES (75), dichloroacetate (DCA) (5), $MgCl_2$ (5), ADP (1), DTT (1), Leupeptin (0.5), TPP (thiamine pyrophosphate) (0.2), and 1% Triton X-100 (pH 7). For PDHa, the homogenisation buffer was HEPES (25), KH_2PO_4 (25), DCA (1), EDTA (3), DTT (1), Leupeptin (0.5), and 1% Triton X-100 (pH 7). The sample was subsequently frozen in liquid nitrogen, thawed, and homogenised again for a total of 3 freeze-thaw cycles. Samples were centrifuged using a Microfuge (Eppendorf, Hamburg, Germany) for 13000xg at 4°C for 8 minutes after which the supernatant was assayed.

2.2.7.3.1 PDH assay procedure

PDH activity was determined spectrophotometrically (equation 2.8). The reaction buffer consisted of (in mM) 50mM HEPES (50), $MgCl_2$ (1), EGTA (0.08) , and rotenone (4µM) (pH 7.2), with the addition of 50µl of 1.67mM NAD, (1.67), CoA (0.1) , TPP (0.2), lactate (16.7), and 5µl

LDH (to give a final concentration of 2U) per 0.75ml and incubated at 37°C for 5 minutes. 50µl of sample was added to each cuvette and the reaction followed for 3 minutes. An extinction coefficient of 6.22mM^{-1}cm^{-1} was used for NADH to calculate PDH activity (equation 6).

2.2.7.4 Oxoglutarate dehydrogenase (OGDH) assay procedure

OGDH activity was determined based upon the method of Hansford et al. (1990) following the change in absorbance of NAD at 340nm. OGDH was measured in PDHa and PDHt extracts to correct for any differences in extraction efficiency. OGDH activity was measured by adding 20µl of supernatant to 1ml reaction buffer containing (in mM) KH_2PO_4 (50), $MgSO_4$ (10), $MgCl_2$ (5), EDTA (1), DTT (1) (pH 7.2), rotenone (2µm), NAD (2), 150 CoA (150 µm), TPP (20µm) 2-oxoglutarate (10), and ADP (2), and monitoring the change in absorbance for 3 minutes. An extinction coefficient of 6.22 mM^{-1}cm^{-1} was used for NAD.

2.2.8 Hormones

2.2.8.1 Leptin

Serum leptin was assayed using an ELISA kit (L1670-30J, US Biological). Serum samples were diluted 20-fold according to the manufacturer's instructions using a dilution buffer and 100µl added to each of the 96 well microplate and incubated for 1 hour at room temperature on a microplate shaker (300rpm). The plate was rinsed 3 times with a 10-fold dilution of a wash solution and biotin-labelled anti-mouse leptin (100 µl/well). Samples were then incubated for a further 1 hour at room temperature. The plate was rinsed a further 3 times and with Streptavidin-HRP conjugate (100µl/well) added after the final wash. The plate was shaken on a shaker (300rpm) for 30 minutes before being rinsed. Following rinsing, the plate was incubated for 10min with 100µl/well of substrate solution after which 100µl/well stop solution added and absorbance measured at 450nm. Serum leptin concentrations were determined using a standard curve generated with known leptin standards (figure 2.8).

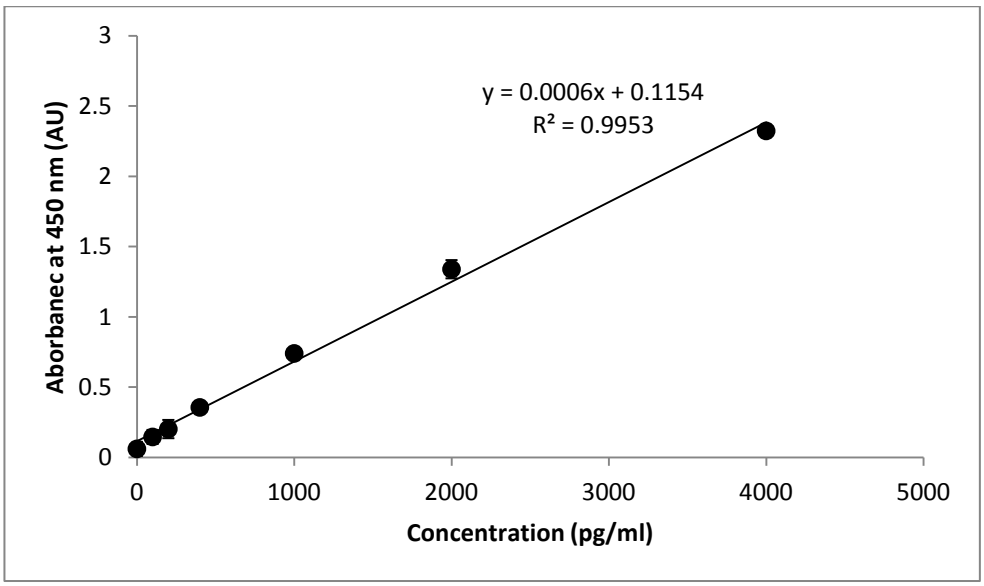

Figure 2.8 Leptin standard curve. Values show mean of triplicates ± SEM.

2.2.8.2 Insulin

Animals were fasted for 12 hours prior to the collection of serum. Fasted levels of insulin were assayed in serum using an Elisa kit (10-1250-01, Mercodia). Samples were diluted and 10µl of standard or sample loaded per well. Peroxidase conjugated mouse monoclonal anti-insulin was diluted 11-fold and 100µl added per well and the plate subsequently incubated on an orbital shaker at room temperature for 2 hours. The plate was rinsed with supplied wash solution and 20µl of 3,3',5,5'-tetramethylbenzidine (TMB) added per well and the plate incubated at room temperature for 15 minutes before the addition of 50µl of a stop solution and the absorbance at 450 nm read. Serum insulin concentrations were determined by using a standard curve generated with known insulin standards (figure 2.9).

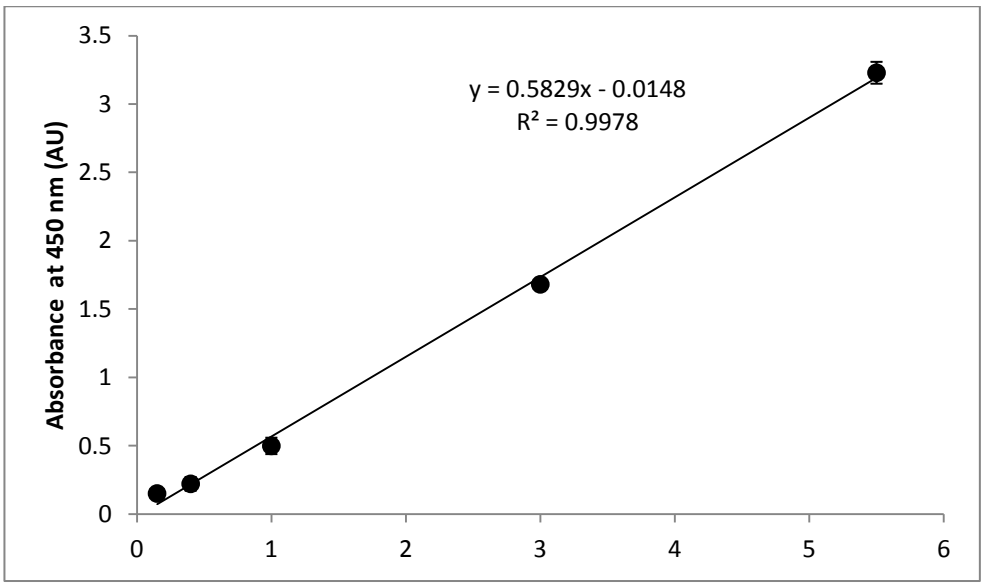

Figure 2.9 Insulin standard curve. Values show mean of triplicates ± SEM.

2.2.9 ^{13}C Nuclear magnetic resonance (NMR) spectroscopy

2.2.9.1 Perchloric acid (PCA) extraction

Frozen cardiac tissue was ground to a fine powder using a mortar and pestle cooled with liquid nitrogen. The extraction procedure followed was that of Seymour et al (1990). Approximately 1g of ground cardiac tissue was added to 6% PCA at a ratio of 1:5 (w/v). The suspension was left on ice for 10 minutes after which samples were centrifuged at 3000xg for 10 minutes at 4°C. The supernatant was neutralised to approximately pH 6.5 using 6M KOH and centrifuged again. The resultant supernatant was decanted and frozen in liquid nitrogen, after which samples were lyophilised at -60°C for 48hrs in a freeze dryer (Modulyo, BOC Edwards, UK).

KH_2PO_4 (50mM, pH 6.5) was prepared using ultrapure water and lyophilised for 48hrs using a freeze drier (Modulyo, BOC Edwards, UK). Lyophilised phosphate buffer was dissolved in 1ml of deuterium oxide (Apollo Scientific, Manchester, UK).

Tissue extracts were reconstituted in 0.95ml of deuterated phosphate buffer. To remove paramagnetic ions a small spatula of chelating resin (Chelex 100-sodium form, Sigma, Poole, UK) was added. The sample was subsequently filtered using a 0.2µm filter (Acrodisc® Pall Life Sciences, USA) into a 5mm NMR tube.

2.2.9.2 Spectral acquisition

High-resolution ^{1}H decoupled (WALTZ-16 sequence) ^{13}C NMR were acquired using an 11.7 Tesla vertical ultra-shielded superconducting Bruker Magnet (Bruker, Coventry, UK) and a 5mm broadband probe interfaced with a Bruker spectrometer. Free induction decays (FIDs) were acquired over a total of 36000 scans per sample at a 90°pulse. Pulse duration was 9.95 µs with a 1-second delay. Spectra were analysed after Fourier transform using Bruker Topsin (v.13).

2.2.9.3 ^{13}C NMR analysis

Glutamate isotopomer analysis were performed using the TCAcalc program provided by Dr. Mark Jeffrey (University of Texas, USA). Myocardial oxidative metabolism was examined using ^{13}C-labelled glutamate isotopic labelling. Determination of the peak intensities of the $^{13}C2$, C3 and C4 resonances of glutamate and the $^{13}C3/^{13}C4$ peak area ratio were used to calculate the relative contributions of exogenous ^{13}C-labelled substrates to the generation of ^{13}C-labelled acetyl-CoA and subsequent calculation of oxidative metabolism.

The TCAcalc program assumes that acetyl-CoA can become enriched from the following sources (Young et al., 1997):

Generated from unlabelled substrates producing no ^{13}C labelling (Fc0).

Generated from ^{13}C substrates labelling acetyl-CoA at the carbonyl carbon atom, (Fc1).

Generated from ^{13}C substrates labelling acetyl-CoA at the methyl carbon atom, (Fc2).

Generated from ^{13}C substrates labelling acetyl-CoA on both the carbonyl and methyl carbon atoms, (Fc3).

The total contribution of each enrichment varies, but by definition $\sum Fc0 + Fc1 + Fc2 + Fc3 = 1$.

The TCAcalc program is based upon the following assumptions (Seymour et al., 1990):

The entry of carbon into the TCA cycle is derived from acetyl-CoA or via anaplerosis, the latter assumed to be 0.1.

The TCA cycle is in a steady-state.

The exchange between α-ketoglutarate and glutamate is rapid.

The ^{13}C label is scrambled after fumarate owing to symmetry of molecules

The ^{13}C found endogenously does not influence the labelling pattern of glutamate.

2.2.9.3.1 Labelling pattern of 3-^{13}C lactate

Metabolism of 3-^{13}C lactate produces acetyl-CoA labelled at the C2 position of acetyl-CoA. Condensation of 2-^{13}C acetyl CoA with oxaloacetate produces citrate and subsequent α-ketoglutarate labelled at the C4 position. Progression through the first turn of the cycle sees the label scrambled between the C2 and C3 of malate and fumarate. When 2-^{13}C acetyl-CoA meets oxaloacetate labelled in the C2 or C3 position, citrate labelled at C2 and C4, or C3 and C4 is generated. Continuation of the cycle labels glutamate at the C2, C3 and C4 positions of glutamate (figure 2.10).

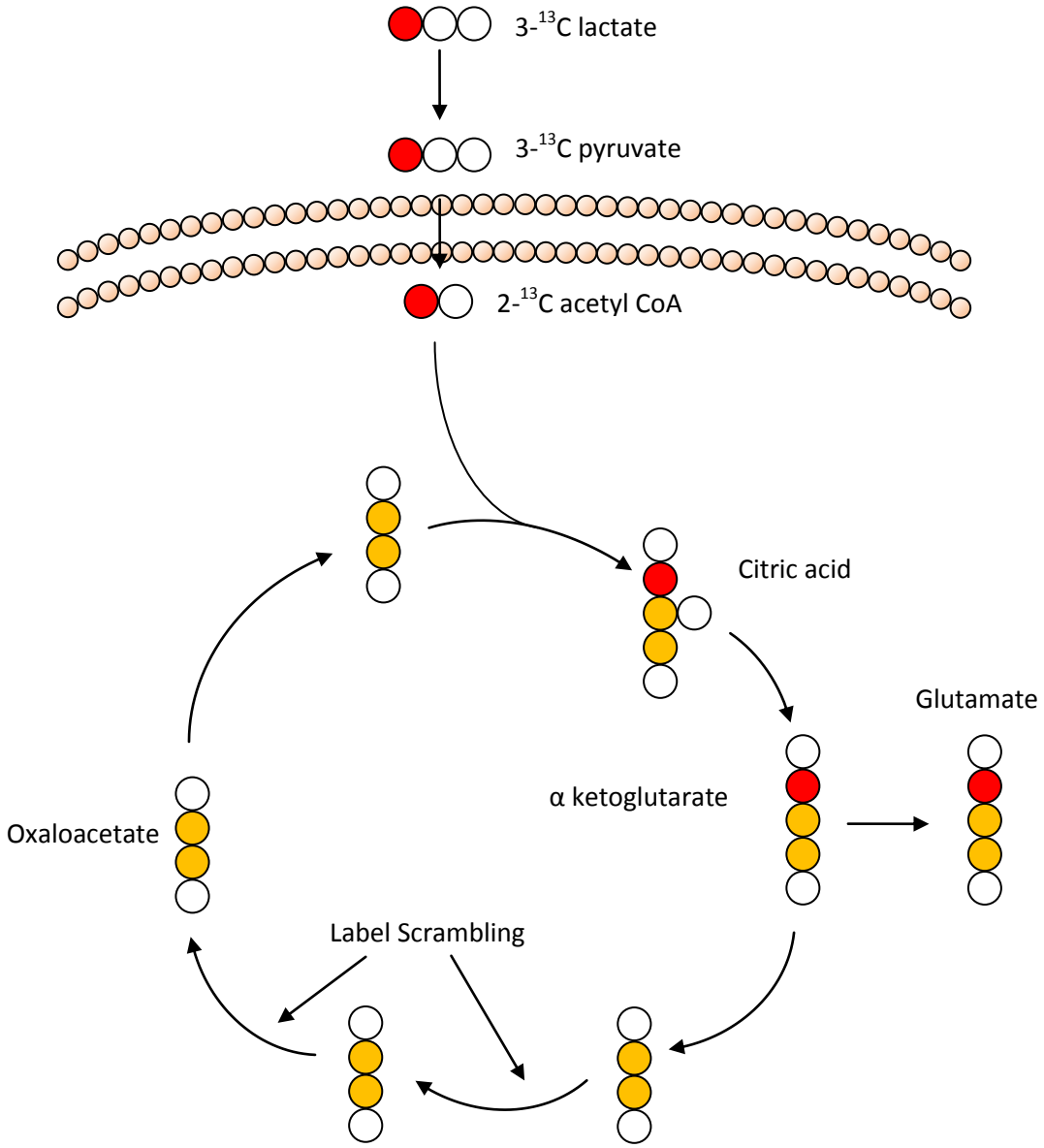

Figure 2.10 Profile of 3-^{13}C lactate. Red represents ^{13}C labelled carbon enrichment from the first turn. Orange represents ^{13}C labelled carbon from the previous turn.

2.2.9.3.2 Labelling pattern of U-^{13}C palmitate

Uniformly labelled ^{13}C palmitate gives rise to acetyl-CoA labelled in both C1 and C2. During the first turn of the cycle, the ^{13}C label is found on the C5 and C4 due to the condensation of 1, 2-^{13}C acetyl-CoA with unlabelled oxaloacetate and the subsequent formation of α-ketoglutarate and glutamate labelled at C5 and C4. Continuation of the first turn of the cycle sees the label incorporated into either C4 and C3, or C2 and C1 of malate and fumarate. Depending upon the

labelling pattern generated during the first turn of the cycle, two labelling patterns of glutamate are generated (figure 2.11).

^{13}C-labelled lactate produces one molecule of labelled acetyl-CoA. The relative contribution of palmitate to oxidative metabolism must be multiplied by two due to only 50% of the palmitate used contained the ^{13}C label (0.15mM unlabelled + 0.15mM labelled) due to expense. Hearts perfused with both lactate and glucose exhibit 9 peaks for both the C2 and C4 region, and 5 peaks for C3 (figure 2.12).

2.2.10 Lipid histology

Animals were anaesthetised via i/p sodium thiopentone (0.5ml/100g BW) and the heart rapidly excised. Hearts were perfused with KH buffer for 5 minutes to rinse blood from the ventricles and mounted on cork blocks using Tissue-Tek OCT®. Mounted hearts were placed in pre-cooled 2-methyl-butane for 1 minute then transferred to liquid nitrogen. Samples were stored at -20°C prior to sectioning.

Five 10µm sections were cut starting at the apex of the heart using a cryostat microtome (Microm HM 505 E Cryostat, Thermo Scientific, Walldorf, Germany) and moving inwards 500µm, then taking 5 10µm sections. This process was repeated until the whole heart had been sectioned. Lipid deposition was examined using Oil red O (ORO) stain. The stain was prepared by dissolving 1g ORO in 100ml 60% triethyl phosphate (TEP) until saturated. The solution was left for 24 hours before being filtered on the day of use using Whatman No.4 filter paper. Slides were stained for 30 minutes then washed in 60 % TEP, counter-stained using haematoxylin for 3 minutes, washed a further time with ultrapure water and fixed using glycergel.

Sections were analysed using a light microscope (x45 magnification, Leica labor lux5, Leica Microsystems, Milton Keynes, UK) and colour camera (coolSNAP-Pro, Meyer Instruments, Inc, Texas, UK) and lipid deposition quantified by using ImageJ software.

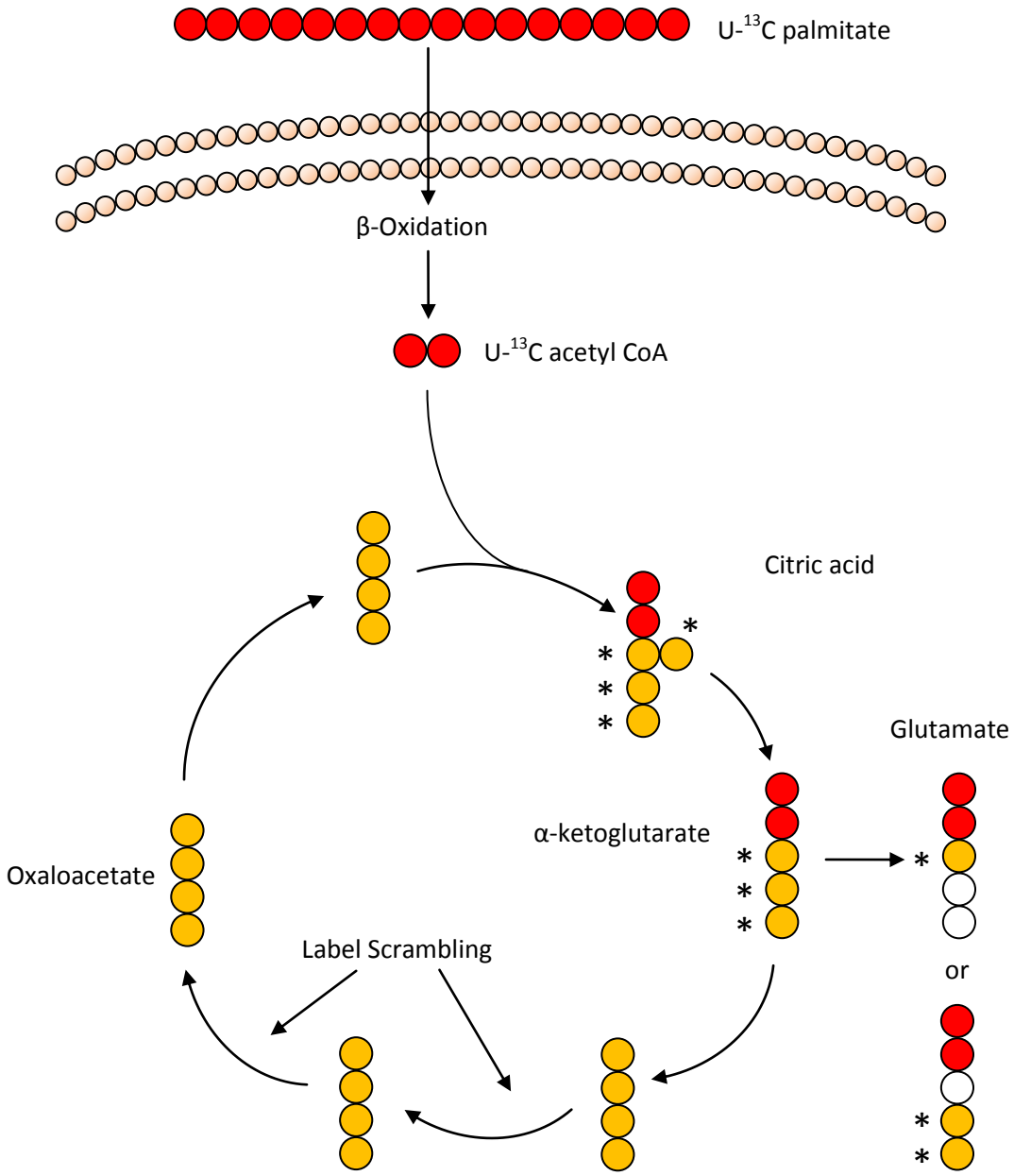

Figure 2.11 Profile of U-^{13}C palmitate. Red represents ^{13}C labelled carbon enrichment from the first turn. Orange represents ^{13}C labelled carbon from the previous turn.* denotes half levels of ^{13}C enrichment from label scrambling.

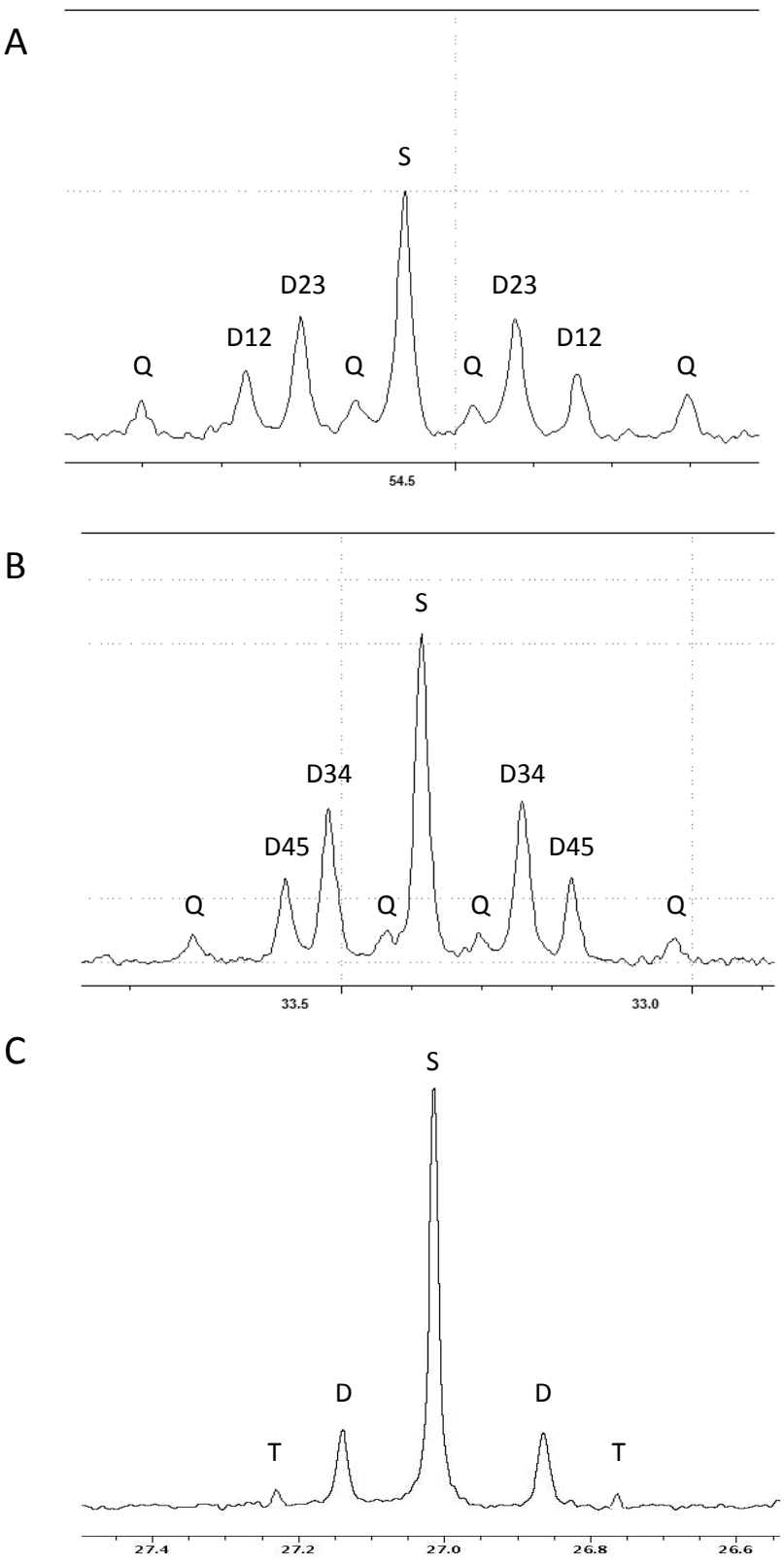

Figure 2.12 ^{13}C isotopomer labelling profile glutamate. A) C2; B) C4; and C) C3. Q, quartet; D, doublet; S, singlet.

2.2.11 Western blotting

Protein expression of PPARα, CD36 and actin was examined in ventricular tissue using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) followed by western blotting for CD36 (Bonen et al., 2000) and PPARα (Morgan et al., 2006a). GLUT4 and $\alpha_1Na^+K^+$ATPase were detected by methods based upon Aksentijević et al. (2009).

2.2.11.1 Sample preparation

200mg of ventricular tissue was homogenised (2 x 5 seconds at full speed) using a Ultra Turrax homogeniser in 1ml extraction buffer containing 50mM Tris Base (pH 7.4) , 1% SDS, and complete protease inhibitor cocktail® and centrifuged at 12100xg for 5 minutes at 4°C using a microcentrifuge (MicroCL 17R, Thermo Scientific, UK). Protein content in each sample was determined using the Bio-Rad assay method. Samples were subsequently diluted with the extraction buffer to a concentration of 10µg/µl. An equal volume of 45mM Tris Base (pH7.4), 10% glycerol, 1% SDS, 2.5% 2-Mercaptoethanol Blue and Bromophenol Blue) was added to achieve a final protein concentration of 5µg/µl. Samples were aliquoted, boiled for 3 minutes, and subsequently stored at -20°C.

2.2.11.2 Subcellular membrane isolation

Subcellular membrane fractions were purified using differential sucrose gradient centrifugation based on the method of Aksentijevic et al. (2009) and used to measure GLUT4 expression.

Membrane fractions [sarcoplasmic (SR) and intracellular (ICM)] were harvested and diluted 1/5 in $NaHCO_3$ (10mM)/NaN_3 (5mM). Suspensions were centrifuged at 190000xg for 1 hour at 4°C. Pellets were re-suspended in sucrose (250mM)/Tris Base (50mM), pH 7.4. Due to low protein yield in the fractions, samples were pooled together with a total of 12 hearts for each experimental group. Intracellular membranes (ICMs) were characterised by no expression of $\alpha_1Na^+K^+$ATPase. The sarcolemmal fraction was characterised by expression of $\alpha_1Na^+K^+$ATPase.

2.2.11.3 SDS-PAGE and blotting

Proteins were separated using SDS-PAGE. 50µg of protein (10µl) was loaded per well in a 3% stacking gel (5.55ml ultra pure water, 75µl 10% SDS, 0.94ml 1M Tris (pH 6.8), 0.95ml

acrylamide/Bis (30:0.8 w/v), 37.5µl ammonium persulphate (10%), and 15µl TEMED), and separated on a 12% running gel (5.02ml ultra pure water, 150µl 10% SDS, 3.75ml Tris [pH 8.8], 5ml 30:0.8 w/v acrylamide/Bis, 75µl ammonium persulphate, and 15µl TEMED). Proteins were transferred to nitrocellulose membranes for 2 hours at 4°C using a mini-transblot cell (BioRad laboratories, UK).

Membranes were blocked for 1 hour in the appropriate buffer (table 2.3) and washed with 0.25% TBS-Tween 3 x 10 minutes. Membranes were incubated with primary antibodies overnight at 4°C with gentle agitation. The primary antibody was removed with 3 x 10 min washes with 0.25% TBS-TWEEN and the membrane incubated with the secondary antibody at room temperature for 1 hour. The secondary antibody was removed with 5 x 5 minutes washes using 0.25% TBS-Tween (details given in table 2.3).

2.2.11.4 Visualisation using enhanced chemiluminescence (ECL)

Blots were visualised by exposing the membrane onto Kodak Biomex Light films (Sigma Aldrich, UK) using ECL reagents (Amersham, Uppsala, Sweeden. Scanned films were quantified using ImageJ® software.

2.2.12 Statistical analysis

All data are expressed as mean ± standard error mean (SEM). Multiple comparisons were carried out with Analysis of Variance (ANOVA), followed by the Scheffé test for post-hoc analysis using PASW Statistics 18 software. A value of $P < 0.05$ was considered significant.

Table 2.3 Primary and secondary antibodies, suppliers, and dilutions

Antibody		Host/Type	Manufacturer	Dilution	Diluent	Blocking Buffer
CD36	Primary	Rabbit/polyclonal	Abcam, Cambridge, UK	1:6000	0.25% Tween TBS with 1% milk	0.25% Tween TBS with 5% milk
	Secondary	Goat Anti-rabbit	Santa Cruz, USA	1:8000	As above	As above
PPARα	Primary	Rabbit/polyclonal	As above	1:1000	As above	As above
	Secondary	Donkeyt Anti-rabbit	As above	1:4000	As Above	As above
Actin	Primary	Rabbit/monoclonal	New England Biolabs, Ipswich, UK	1:1000	0.1% TBS-Tween with 5% BSA	As above
	Secondary	Anti-Rabbit	Santa Cruz, USA	1:1000	As above	As above
GLUT4	Primary	Goat/polyclonal	Santa Cruz, USA	1:5000	0.25% TBS-Tween with 1% milk	0.25% TBS-Tween with 7.5% milk
	Secondary	Donkey Anti-goat	As above	1:2000	As above	As above
$\alpha_1 Na^+K^+$ATPase	Primary	Mouse/polyclonal	Abcam, Cambridge, UK	1:5000	As above	As above
	Secondary	Rabbit Anti-mouse	As above	1:7000	As Above	As above

All secondary antibodies were HRP-conjugated.

Chapter 3 Characterisation of experimental hypertrophy

3.1 Introduction

3.1.1 Impact of diet upon morphology

Increases in BW in obesity are predominantly reflected by changes in fat mass. Indeed, studies of adipose tissue have demonstrated that it is not merely a "sink" for excess dietary calories, but rather a highly complex tissue capable of manipulating numerous signalling pathways through secretion of cytokines, termed adipokines (chapter 1, section 1.4.1).

Increasing fat consumption also leads to a significantly elevated BW in rats (Akiyama et al., 1996, Ren et al., 2008). Conversely, other HFDs containing 60% fat show no increase in BW, but still demonstrate an increase in adiposity (Chess et al., 2008). Importantly, these studies also demonstrate a wider effect upon morphology including increases in liver weight and HW (Akiyama et al., 1996, Chess et al., 2008, Ren et al., 2008). Consequently, this raises the important concept that increasing dietary lipid consumption can potentially lead to increased heart mass.

Although HFDs have been shown to be detrimental to cardiac morphology, recent focus has been upon the morphological impact of WDs. Indeed, WDs differ in composition by a reduction in fat content, but an increase in sugar, namely sucrose or fructose. WD feeding leads to increases in adiposity and BW, liver weight, and HW (Wilson et al., 2007, Matsuzawa-Nagata et al., 2008, Akki and Seymour, 2009). Thus, the implication from these studies is that the combination of dietary fat and sugar elicits a similar effect to high-fat consumption alone.

3.1.2. Dietary modulation of cardiac hypertrophy

The impact of dietary manipulation upon the regulation and development of cardiac hypertrophy has yielded conflicting data, primarily due to differing dietary constituents, duration of study and strain of animal. However, excessive consumption of fat leads to the development of cardiac hypertrophy, and hyperinsulinaemia (Ouwens et al., 2005, Relling et al., 2006, Ouwens et al., 2007, Ren et al., 2008). Indeed, diets rich in fructose exaggerate ventricular remodelling compared to more complex carbohydrates (Sharma et al., 2007). However, others have not observed this relationship (Wilson et al., 2007, Chess et al., 2008), despite increasing epididymal fat, serum concentrations of leptin, and with no change in

insulin concentration (Chess et al., 2008). Thus, the differing serological profiles generated by various dietary patterns may be an important determinant of cardiac mass.

Due to the relationship between adiposity, blood pressure, and hypertension (Doll et al., 2002, Tu et al., 2011), several groups have examined the impact of dietary manipulation upon LVH in response to pressure-overload. Similar to the effect of dietary manipulation upon the healthy heart, data show conflicting observations. Indeed, some have shown that HFDs can attenuate the development of LVH in an established model of cardiac hypertrophy (Okere et al., 2005, Okere et al., 2006b), whereas others have shown HFDs to exacerbate the remodelling process (Raher et al., 2008).

Interestingly, high carbohydrate consumption can lead to increases in LV mass when compared to HFDs (Okere et al., 2005). Taken further, diets higher in sugars such as fructose and sucrose can exacerbate ventricular remodelling when compared to those diets containing starch alone (Sharma et al., 2007, Bouchard-Thomassin et al., 2011). However, a criticism of these studies – in particular those investigating the impact of carbohydrate upon the ventricular remodelling process – is that frequently the increase in dietary carbohydrate is not physiological and does not truly represent an accurate WD (Wright and Wang, 2010).

3.1.3 Objectives

The aim of this study was to characterise the impact of dietary manipulation upon the normal and hypertrophied heart, and whole body morphology. Furthermore, the dietary impact upon the serum concentration of glucose, FFAs, and TG, as well as both insulin and leptin, was also investigated.

3.2 Materials and Methods

3.2.1 Induction of LVH

LVH was induced in male Sprague-Dawley rats as described in section 2.2.1.

3.2.2 Dietary Intervention

48 hours post-surgery, animals were assigned to the dietary regimens described in section 2.2.3, generating 6 experimental groups:

- Control + standard chow (Con-SD)
- Control + high-fat diet (Con-HFD)
- Control + western diet (Con-WD)
- Aortic Constriction + standard chow (AC-SD)
- Aortic Constriction + high-fat diet (AC-HFD)
- Aortic Constriction + western Diet (AC-WD).

Animals were studied at 9 weeks post-surgery. Hepatic histology was performed as described in section 2.2.10.

3.3 Results

3.3.1 Physiological data

All experimental groups demonstrated significant weight-gain following 9 weeks dietary manipulation. BW was significantly increased with HFD and WD feeding compared to the SD (figure 3.1A).

Over the 9 week period, BW increased in control (Con) and AC animals fed SD by 112% and 115%, respectively. Both groups fed the SD showed no significant difference in BW over the 9-week period (figure 3.1B). Con- and AC-HFD animals demonstrated a 150% and 124% increase in BW over the 9 week period, respectively (figure 3.1C). Similar increases in BW were observed for the WD group (161% and 117%, Con and AC respectively, figure 3.1D).

Con-HFD and Con-WD groups showed significantly greater BWs compared to SD counterparts at the end of the feeding protocol (table 3.1). Furthermore, the mean BW of the Con-WD group at this stage was significantly greater than the Con-HFD group. No significant difference was observed in BWs between AC groups by week 9 (table 3.1).

Haematocrit remained consistent in all groups (ranging from 41-44%) with no evidence of anaemia (table 3.1). Despite biological variation in the kidney response to AC in SD, HFD, and WD animals, left kidney:right kidney ratios were significantly reduced compared to respective control groups as expected (table 3.1). No difference between dietary groups was observed for AC animals. Furthermore, no group showed evidence of HF (dyspnoea, fatigue, or oedema), anaemia, or fluid accumulation in the lungs (table 3.1).

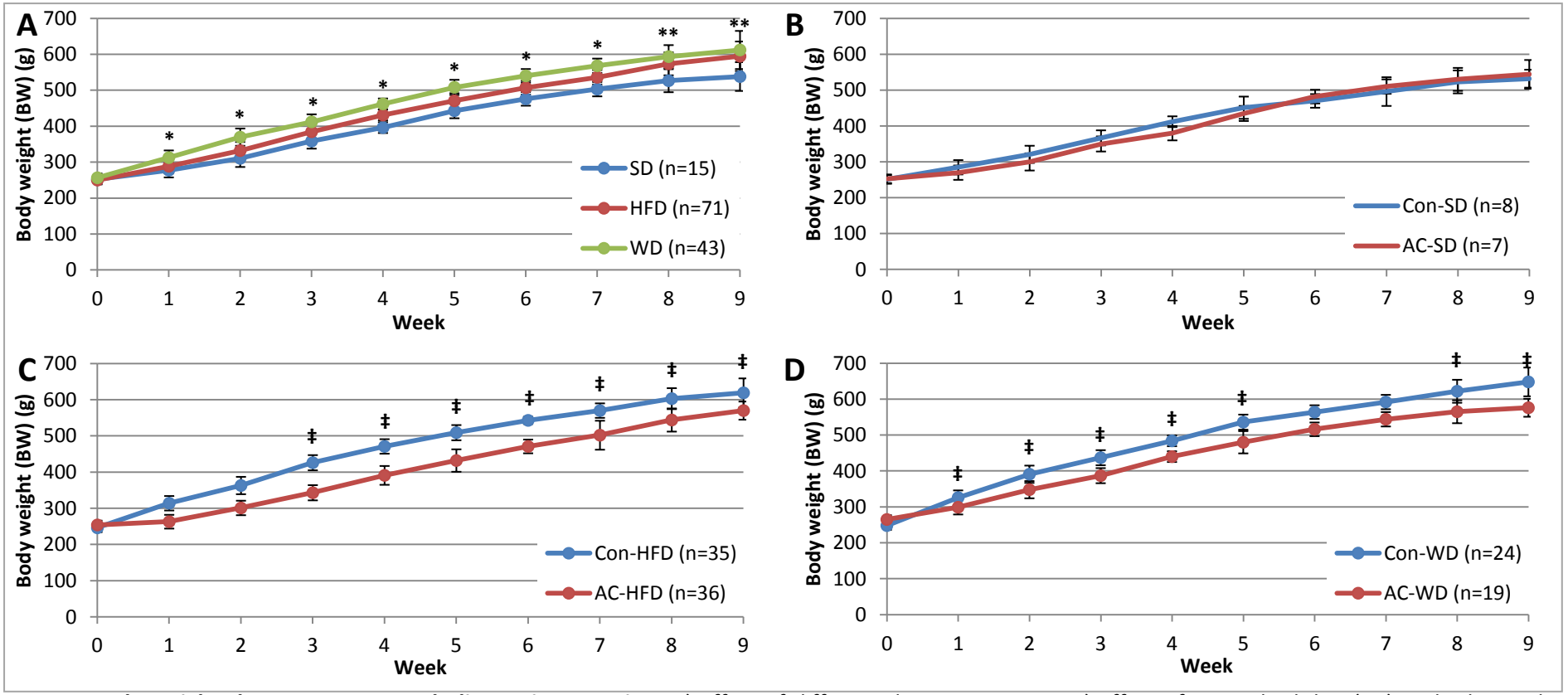

Figure 3.1 Body weight changes over 9 week dietary intervention. A) effect of different dietary regimens; B) effect of a standard diet (SD) on body weight; C) effect of a high-fat diet (HFD) on body weight; D) effect of a western diet (WD) on body weight. Con, control; AC, aortic constriction. * $P < 0.05$ WD vs. SD, HFD, ** $P < 0.05$ SD vs. HFD, WD, ‡ $P < 0.05$ vs. respective control. Data presented as mean ± SEM.

Table 3.1 Physiological changes following 9 weeks of dietary intervention

Group	Body weight (g)	Tibia Length (cm)	L K:R K	Lung wet wgt:dry wgt (%)
Con-SD (n=8)	532 ±24	4.44 ±0.03	1.0 ±0.0	76.4 ±0.8
Con-HFD (n=35)	619 ±9*	4.56 ±0.02	1.0 ±0.0	76.8 ±0.9
Con-WD (n=24)	645 ±19#	4.52 ±0.04	1.0 ±0.0	77.2 ±1.1
AC-SD (n=7)	544 ±14	4.54 ±0.01	0.6 ±0.2**	76.3 ±1.3
AC-HFD (n=36)	570 ±11**	4.31 ±0.03	0.7 ±0.3**	76.5 ±0.4
AC-WD (n=19)	576 ±18**	4.36 ±0.03	0.7 ±0.3**	77.6 ±0.8

Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet; L K:R K, left kidney:right kidney ratio. # $P < 0.05$ vs. Con-HFD, Con-SD. * $P < 0.05$ vs. Con-SD. ** $P < 0.05$ vs. respective control. Data presented as mean ± SEM.

HFD and WD groups exhibited a significant increase in EAT, RPAT, and MAT deposition compared to their SD counterparts (table 3.2). EAT and RPAT weights were reduced by 18.7% and 21.9 % in AC animals fed a HFD, and by 24.0% and 26.7% in AC animals fed a WD when compared to their corresponding controls (table 3.2) .Both EAT and RPAT showed a significant correlation with BW (example shown in figure 3.2).

Liver weight was significantly increased following HFD or WD feeding compared to SD counterparts but no significant difference was observed between Con and AC groups (table 3.2). Macroscopically, livers from HFD and WD groups showed evidence of lipid accumulation (figure 3.3), which was confirmed by histological staining using ORO (figure 3.4) but no significant difference in the density of staining from either Con or AC groups.

Table 3.2 Adipose tissue and hepatic physiology

Group	Abdominal		Thoracic	Liver weight (g)
	EAT (g)	RPAT(g)	MAT (g)	
Con-SD (n= 8)	7.0 ±0.9	9.6 ±1.6	0.15 ±0.02	12.3 ±1.2
Con-HFD (n=35)	15.0 ±0.8*	21.5 ±1.1*	0.22 ±0.03*	19.3 ±1.9†
Con-WD (n=24)	17.5 ±1.1#	23.2 ±2.0*	0.24 ±0.03*	19.7 ±1.1†
AC-SD (n= 7)	6.8 ±0.9	12.4 ±1.7	0.14 ±0.02	12.9 ±1.0
AC-HFD (n=36)	12.2 ±0.8†	16.8 ±1.0$†	0.21 ±0.03†	18.8 ±1.6†
AC-WD (n=19)	13.3 ±0.9$†	17.6 ±1.5$†	0.23 ±0.04†	19.2 ±1.7†

Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet; EAT, epididymal adipose tissue; RPAT, retroperitoneal + perirenal adipose tissue; MAT, mediastinal adipose tissue. tissue # $P < 0.05$ vs. Con-HFD, Con-SD. * $P < 0.05$ vs. Con-SD. $ $P < 0.05$ vs. respective control. † $P < 0.05$ vs. SD counterpart. Data presented as mean ± SEM.

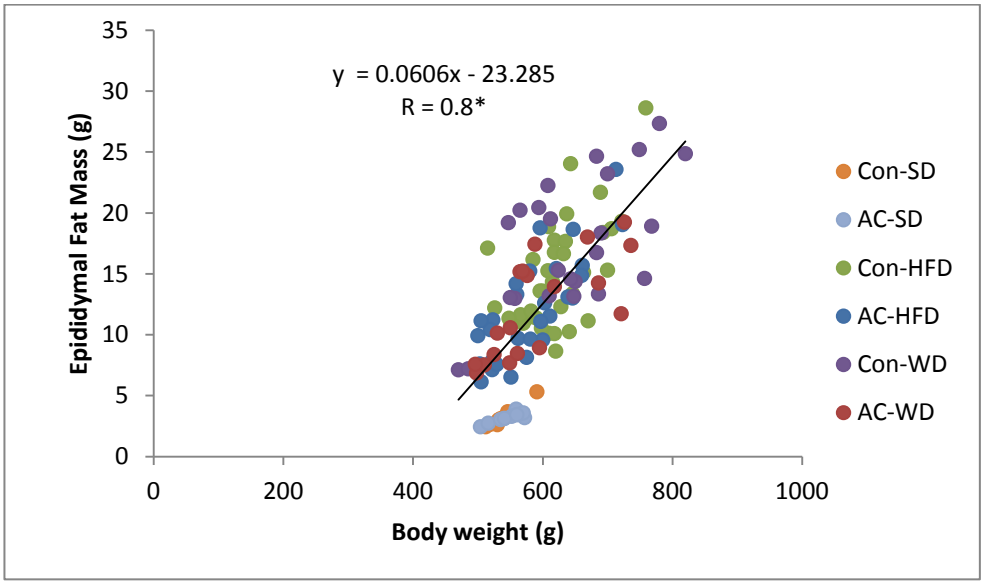

Figure 3.2 Correlation between body weight and epididymal fat mass. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet * $P < 0.01$.

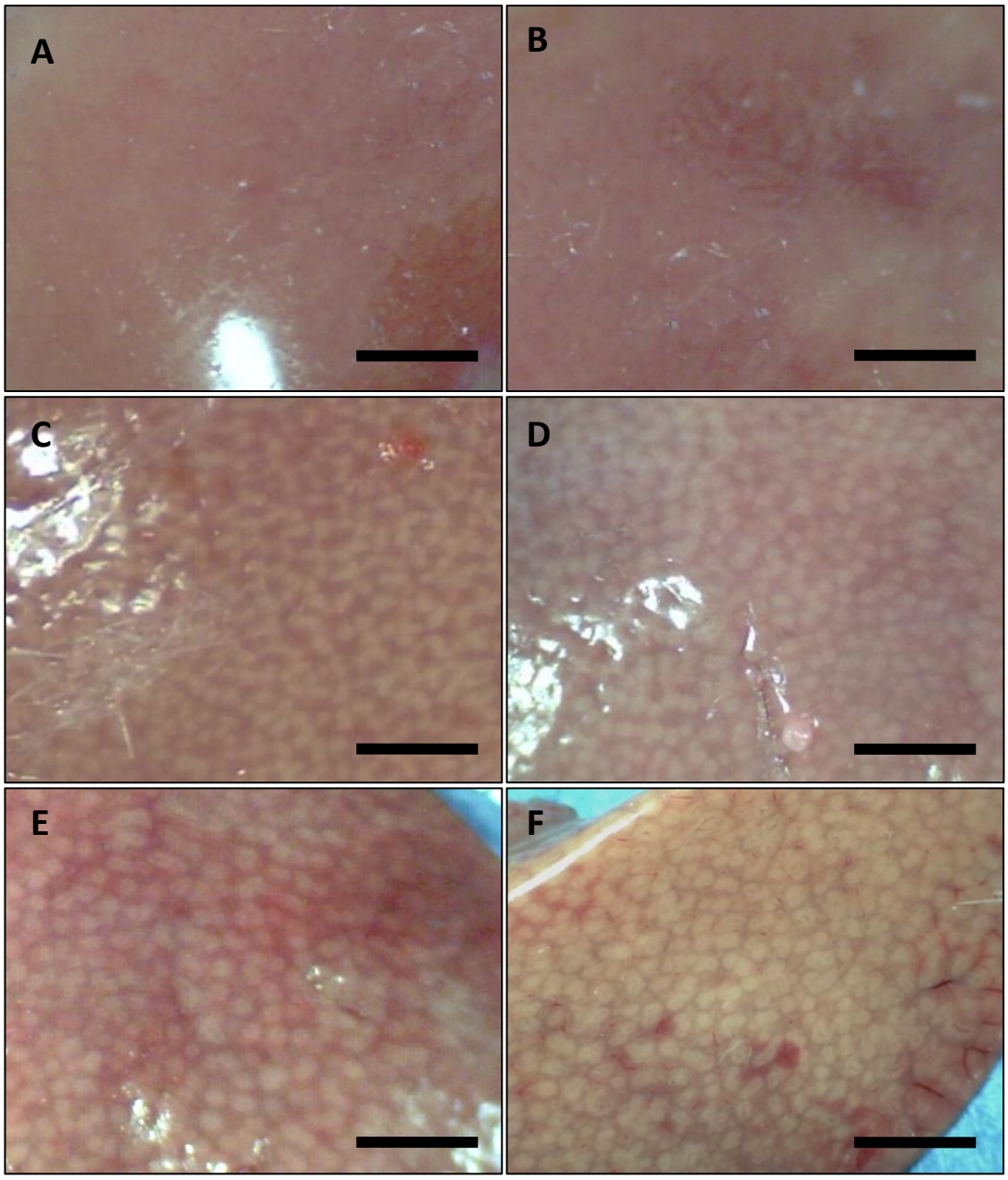

Figure 3.3 Macroscopic liver appearance following 9 weeks of dietary intervention. A) Con-SD; B) AC-SD; C) Con-HFD; D) AC-HFD; E) Con-WD; F) AC-WD. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. Scale bar represents 500µm.

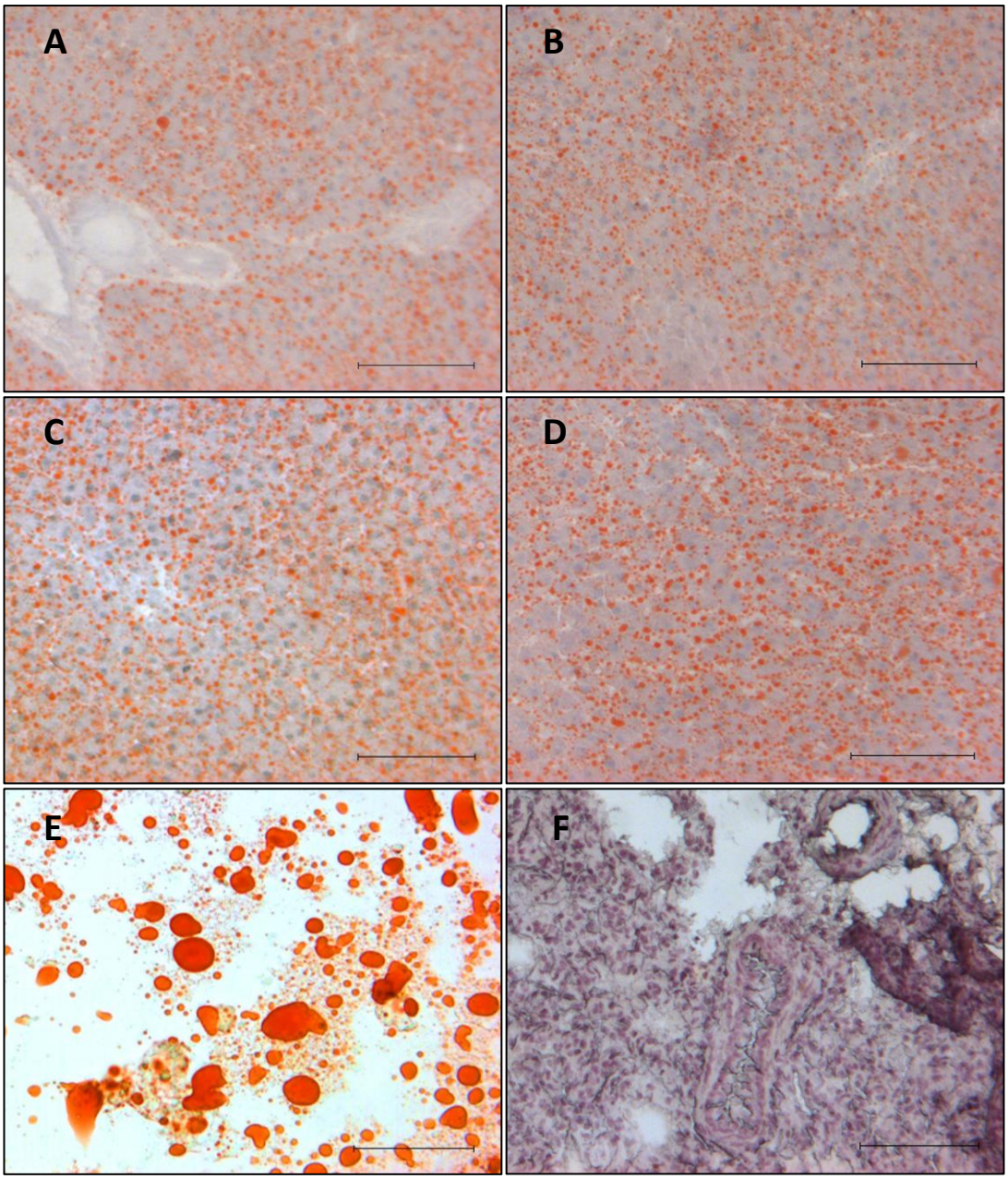

Figure 3.4 Example Oil red O staining of lipid accumulation in liver tissue. A) Con-HFD; B) AC-HFD; C) Con-WD; D) AC-WD; E) adipose tissue (positive control); F) lung (negative control). Con, control; AC, aortic constriction; HFD, high-fat diet; WD, western diet. Scale bar represents 100 µm.

3.3.2 Cardiac hypertrophy

A significant increase in HW was observed in AC animals fed a SD and WD compared to controls (figure 3.5). However, when HW was normalised to tibia length, all AC groups exhibited a significant increase compared to controls (figure 3.6). Similar results were observed when HW was normalised to BW (figure 3.7).

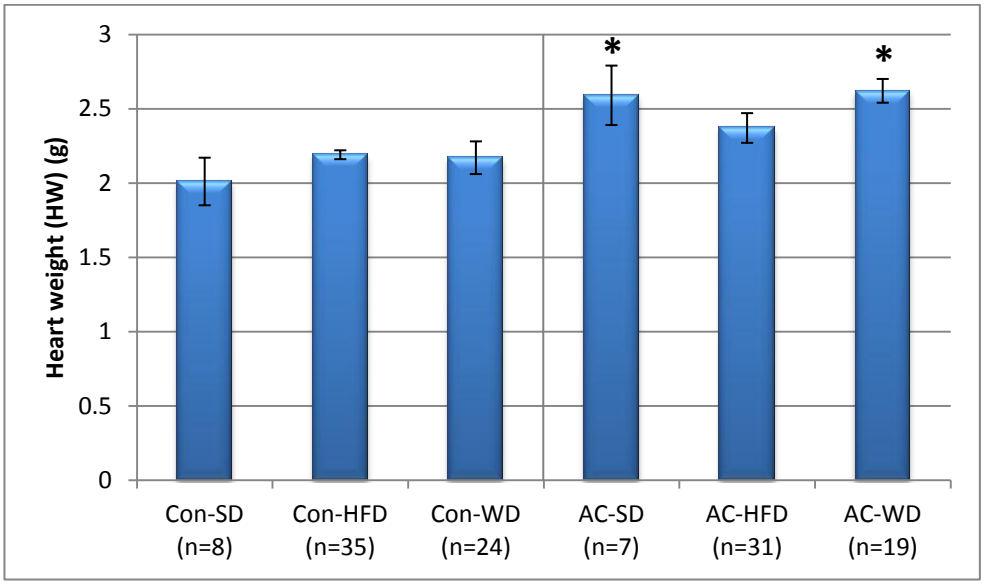

Figure 3.5 Heart weight following 9 weeks of dietary intervention. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet;. * $P < 0.05$ vs. respective control. Data presented as mean ± SEM.

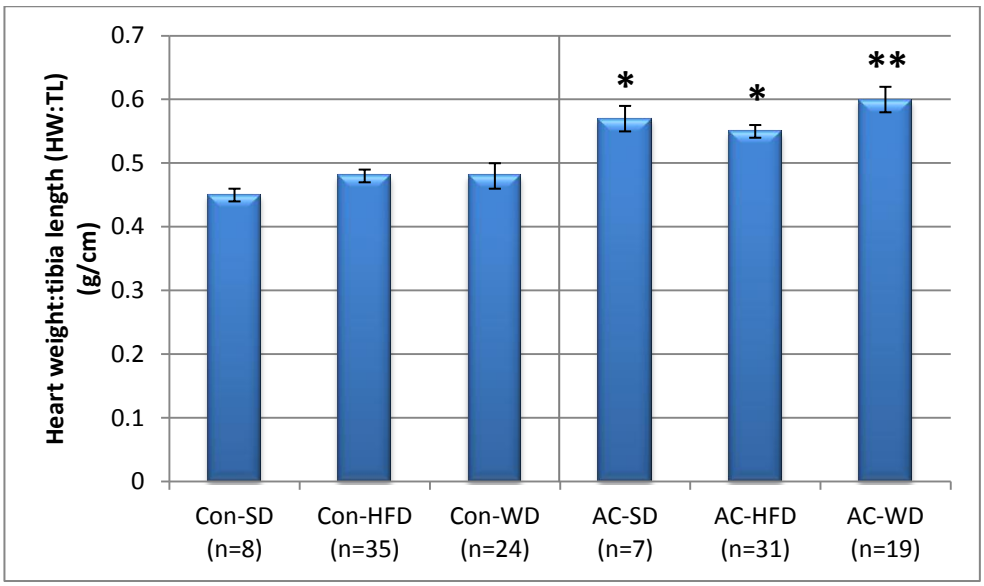

Figure 3.6 Heart weight-to-tibia length ratio following 9 weeks of dietary intervention. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. * $P < 0.05$ vs. respective control, ** $P < 0.001$ vs. Con-WD. Data presented as mean ± SEM.

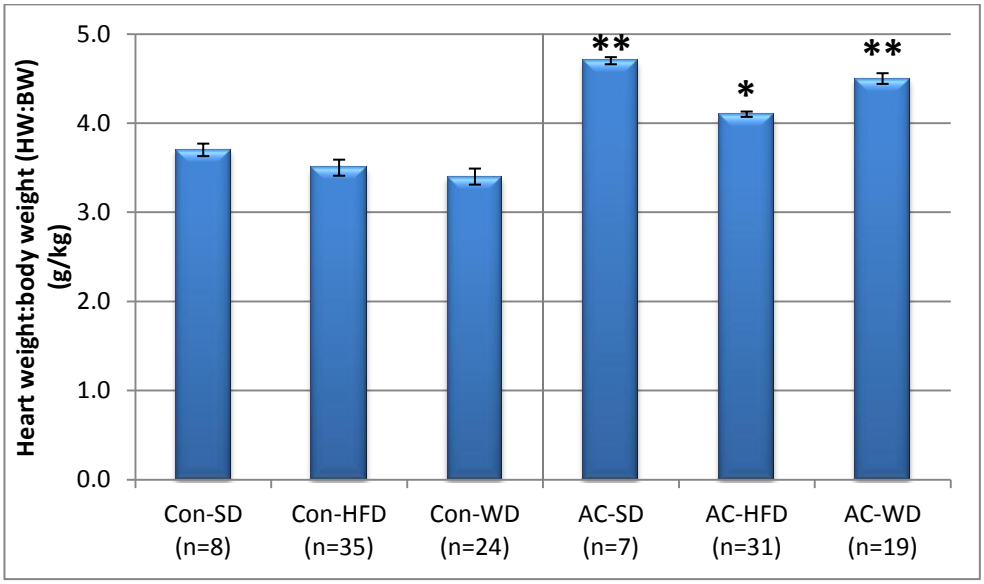

Figure 3.7 Heart weight-to-body weight ratio following 9 weeks of dietary intervention. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. * $P < 0.05$ vs. Con-HFD, ** $P < 0.001$ vs. respective control. Data presented as mean ± SEM.

3.3.3 Serum metabolic profile

Serum glucose concentrations were unchanged in all groups although there was a trend for increased glucose in HFD groups which was further elevated in the WD groups (table 3.3). Serum FFA levels were significantly increased following HFD and WD intervention but not further affected following AC. Although HFD and WD feeding increased serum TG in the Con groups, the magnitude of this increase did not reach significance. However, AC-HFD and AC-WD groups exhibited a significant increase in serum TG concentration over SD counterparts. The serum concentration of leptin was significantly increased following both HFD and WD feeding in Con and AC groups (table 3.3).

Table 3.3 Serum metabolites and hormone concentrations

	Glucose (mM)	Free Fatty Acid (mM)	Triglyceride (mM)	Leptin (ng/ml)
Con-SD (n= 8)	11.85 ±0.8	0.09 ±0.01	1.47 ±0.14	10.6 ±1.7
Con-HFD (n=20)	13.21 ±0.41	0.25 ±0.02*	2.01 ±0.26	51.0 ±4.2*
Con-WD (n=12)	13.73 ±0.44	0.26 ±0.03*	2.00 ±0.21	54.3 ±4.7*
AC-SD (n=7)	11.13 ±0.2	0.11 ±0.02	1.55 ±0.25	7.1 ±1.2
AC-HFD (n=20)	12.08 ±0.43	0.23 ±0.01*	2.42 ±0.21*	45.0 ±7.0*
AC-WD (n=12)	12.81 ±0.27	0.24 ±0.01*	2.34 ±0.23*	48.3 ±6.6*

Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. * $P < 0.05$ vs. SD counterpart. Data presented as mean ± SEM.

Fasting blood glucose concentrations did not differ in HFD and WD groups. AC had no impact (figure 3.4). Fasting concentrations of insulin were unaffected by WD feeding, or by AC (table 3.4).

Table 3.4 Fasting insulin and blood glucose concentrations

	Control-HFD (n=5)	Control-WD (n=5)	AC-HFD (n=5)	AC-WD (n=5)
Insulin (µg/L)	2.04 ±0.1	2.29 ±0.30	2.00 ±0.14	2.17 ±0.12
Glucose (mM)	12.63 ±0.55	12.62 ±0.31	12.26 ±0.75	12.23 ±0.27

Con, control; AC, aortic constriction; HFD, high-fat diet; WD, western diet. Data presented as mean ± SEM.

Serum concentrations of leptin showed a significant positive correlation with BW and EAT (figure 3.8, A and B, respectively). Leptin concentration also correlated strongly with RPAT (R=0.8, $P < 0.01$).

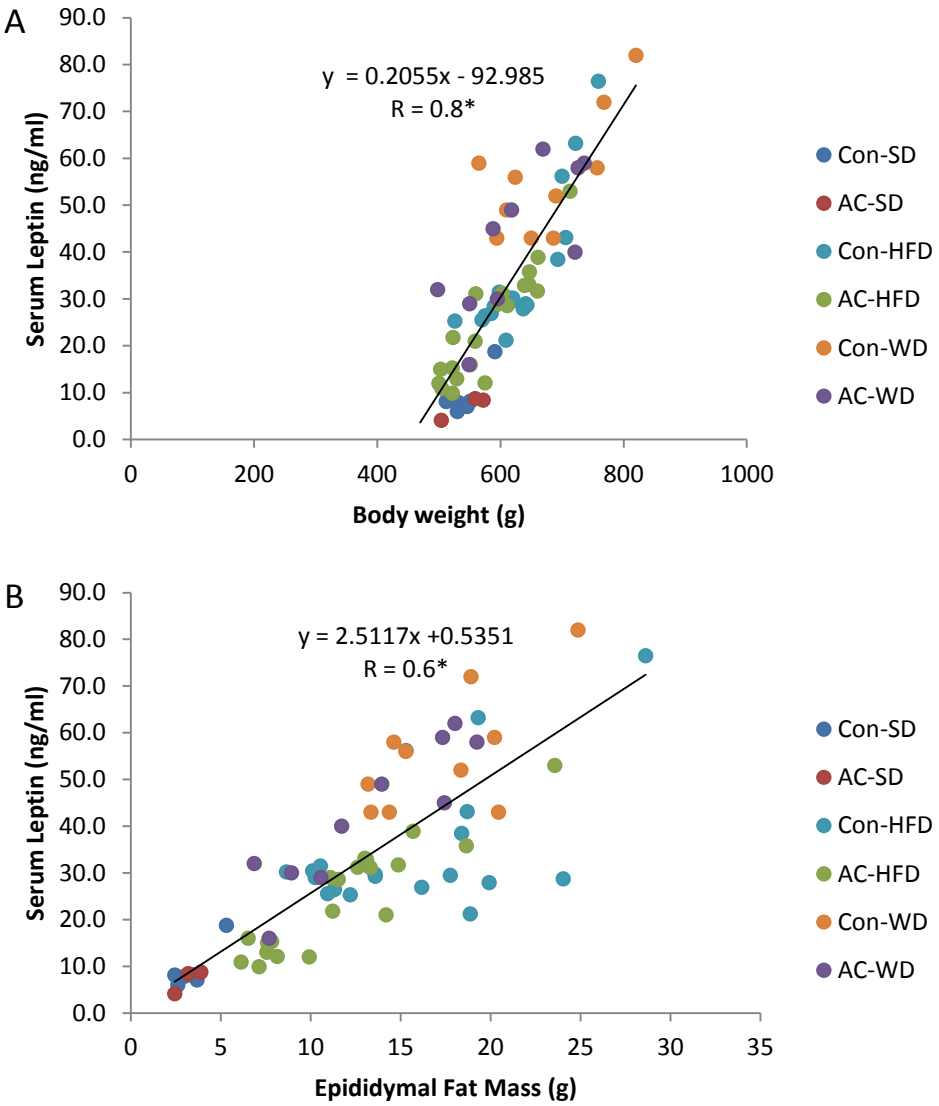

Figure 3.8 Leptin relationship with body weight and adiposity. Relationship between serum leptin and body weight (A) and epididymal fat mass (B). Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet * $P < 0.01$.

3.4 Discussion

The present study has shown that both HFDs and WDs have a substantial impact upon BW and adiposity, accompanied by an increase in serum leptin. These observations were greatest in animals fed a WD diet. In addition, animals from HFD and WD groups exhibited significant accumulation of lipid with the liver, demonstrated by histological staining. Importantly, dietary modification alone did not have any effect upon HW. By contrast, when combined with AC, WD feeding led to a greater degree of hypertrophy. These experiments suggest that increasing dietary carbohydrate consumption may exacerbate the development of cardiac hypertrophy in response to pressure-overload

3.4.1 Dietary impact upon body weight

Increasing dietary fat consumption to 45% of the daily calories (HFD and WD groups) led to a significant elevation in BW after 9 weeks in the Con groups (table 3.1), consistent with previous studies using a similar diet (Akki and Seymour, 2009) or those using a higher fat component (Chess et al., 2008). This finding is most likely attributable to the increased calorific content of HFD and WD diets (due to increased fat content). Furthermore, in Con animals fed a WD a greater increase in BW was observed giving the highest weight-gain in this group (table 3.1). This observation highlights the fact that although dietary lipid consumption is a potent stimulus for weight-gain, increasing carbohydrate with fat consumption can exacerbate this process (table 3.1). Indeed, Raben et al. (2002) have demonstrated that raising the percentage sucrose consumption increased BW over a 10 week period in humans with a significant elevation in fat-mass compared to a BMI-matched control group supplemented with artificial sweeteners. However, in contrast to the present study Wilson and colleagues (2007) have shown that a longer WD feeding period did not exacerbate BW gain compared to a 60% fat diet. Although difficult to account for these differences, palatability of the animal feed may in part account for changes in diet consumption and therefore terminal BW.

BW did not differ significantly between AC groups, irrespective of diet (table 3.1). AC animals fed a HFD or WD weighed consistently less than their respective controls after 9 weeks primarily due to reluctance to feed immediately following the surgical procedure. This effect was most pronounced in the HFD group (figure 3.1). Similarly, it has been shown that mice subjected to TAC and a HFD demonstrated similar BWs to low-fat diet fed controls (Chess et al., 2008). Furthermore, increased starch or fructose consumption did not cause changes in

BW in TAC mice (Chess et al., 2007), suggesting that the delay in weight gain observed in the current study may be attributable to surgical intervention and slower adaption to the HFD/WD.

The changes in BW observed in Con-HFD and Con-WD groups most likely reflect changes in fat EAT, RPAT, and MAT (table 3.2), supported by previous studies (Okere et al., 2006a, Chess et al., 2009). Similar to Con groups, AC groups maintained on the HFD or WD showed comparable patterns in abdominal adipose tissue accumulation (table 3.2). However, AC animals from these dietary groups demonstrated significantly less abdominal fat mass when compared to their respective control (table 3.2), potentially explaining the lower BWs observed. Importantly, however, the total fat content did not change substantially between the HFD and WD groups, despite the WD group exhibiting significantly increased EAT and RPAT (table 3.2). This may be explained by increased consumption of the diet (due to palatability), or increased partitioning of dietary sugar into the storage of fat (Stanhope et al., 2009, Jurgens et al., 2005). In addition, a study by Okere et al. (2006a) demonstrated that SFAs, MUFAs, and PUFAs could regulate changes in adipose mass. Specifically, it was observed that a 60% MUFA/PUFA diet increased abdominal adipose tissue whereas diets high (60%) in SFAs (mainly palmitate) induced greater adipose accumulation in the thoracic cavity (Okere et al., 2006a). Thus, the high levels of SFA, MUFA, and PUFAs in the HFD and WD of the present study may promote fat deposition in several different anatomical areas.

3.4.2 Liver physiology

Liver weight was significantly increased following both HFD and WD feeding (table 3.2), characterised by increased lipid accumulation, and was unaffected by AC (figures 3.3 and 3.4, respectively).

In support of these observations are the results of Milagro et al. (2006), where rats fed a cafeteria diet (9.3% protein, 31.5% carbohydrate, and 59.2% fat) supplemented with Pâté, bacon, chips, cookies and chocolate for 8 weeks, developed hepatomegaly (+33%) and increased production of malondialdehyde (MDA) indicative of oxidative stress. Furthermore, Buettner et al. (2006) showed that increased consumption of lipid led to accumulation of lipid droplets within the liver. Zheng and colleagues (2008) showed that a 45% fat diet induced

hepatomegaly and hepatic steatohepatitis after 7 months in C57BL/6J mice. Thus, enhanced fat consumption can lead to excessive hepatic lipid accumulation.

The hepatic lipid accumulation observed in response to the HFD and WD may be due to increased expression of fatty acid synthase (FAS) genes, under the control of sterol response element-binding protein-1c (SREBP-1c), and diminished expression of CPT1 via decreased expression of PPARα (Buettner et al., 2006). Interestingly, the expression of SREBP1c was increased following high-lard, -olive oil, or -coconut fat, but not with high-fish oil feeding (Buettner et al., 2006). Equally, in the study described by Buettner et al., fish oil was the only experimental diet which increased the expression of hepatic PPARα (Buettner et al., 2006). Consequently, under conditions of increased FA stress the liver may lose its capacity to oxidise dietary lipid fully, leading to lipid deposition.

Increased sugar consumption has also been linked to the development of liver pathology. Ryu and Cha (2003) showed that a diet high in sucrose led to a significant increase in hepatic TG and total cholesterol. Indeed, diets containing 40% (w/w) sucrose also induced lipid accumulation (Roncal-Jimenez et al., 2011). Furthermore, in Wistar rats, fructose (10% w/v in drinking water) combined with a HFD led to a significant increase in liver weight and hepatic lipid accumulation (Aragno et al., 2009). Moreover, high fat and fructose diets led to increased oxidative stress and activation of SREBP-1c (Aragno et al., 2009). Thus, the combined effect of increased lipid and sucrose consumption in the WD group may create a favourable environment for either enhanced hepatic synthesis of TG, or elevated uptake of adipose tissue-derived TG.

3.4.3 Dietary regulation of cardiac hypertrophy

The current study identified little impact of a HFD or WD upon cardiac mass or hypertrophy (figures 3.5-3.7), consistent with previous findings (Akki et al., 2008, Chess et al., 2008), and models of high starch and fructose consumption (Chess et al., 2007). In contrast, others have demonstrated a clear hypertrophy following high-fat and WD feeding in mice and rats (Aguila and Mandarim-de-Lacerda, 2001, Relling et al., 2006, Tikellis et al., 2008). Consequently, the differing responses to excessive dietary fat may be a reflection of the animal model used, the duration of the study, and perhaps more importantly, the source of dietary fat. Indeed, it was recently suggested that increased consumption of the n-3 PUFA, α-linoleic acid, was associated

with ventricular thickening in the absence of systemic changes in blood pressure (Jeckel et al., 2011). This clearly demonstrates the necessity for further research into the cardiac signalling pathways activated by dietary FAs.

The present study indicated that AC induced significant hypertrophy in all dietary groups, (figure 3.6 and 3.7). Furthermore, AC-SD and AC-WD groups showed a significant increase in HW compared to their controls, but not in the AC-HFD group (figure 3.5). Tibia length was used as an indicator of hypertrophy as this variable remains constant over a certain age (Yin et al., 1982). However, the increase in HW was so great following AC that all AC groups exhibited increased HW:BW ratios compared to their respective controls (figure 3.7) The greatest increases in HW, HW:TL , and HW:BW were observed in SD and WD groups (figure 3.6 and 3.7, respectively). Thus, supplementary dietary carbohydrate consumption exacerbates the development of cardiac hypertrophy in response to pressure overload. Additionally, the addition of sucrose to a HFD augmented the hypertrophic response compared to a HFD alone, suggesting an important role of dietary sugar in the progression of cardiac hypertrophy. This observation is supported by previous studies using a higher percentage fat in the diet (Okere et al., 2005, Okere et al., 2006b). Indeed, DSS rats maintained on a HFD (60% kcal as fat) exhibited a blunted hypertrophic response to salt treatment when compared to littermates fed a high-carbohydrate diet (Okere et al., 2005). Importantly, this effect was observed despite similar systemic blood pressures, indicating that dietary lipid may attenuate the development of LVH independently of blood pressure (Okere et al., 2005). Conversely, mice, subjected to TAC which were fed a HFD (60% kcal), still develop significant cardiac hypertrophy compared to low-fat fed controls (Chess et al., 2008). Consequently, the ability of HFDs to regulate the progression of cardiac hypertrophy may be dependent upon the severity of the hypertrophic stimulus, the duration of the study, and the time between the initiation of hypertrophic growth and commencement of the diet.

Heightened consumption of carbohydrate can augment LV mass in DSS rats in comparison to those maintained on a HFD (Okere et al., 2005). Furthermore, replacing standard carbohydrate starch (the main carbohydrate in many animal diets) with fructose (70% kcal) led to an increase in mortality in DSS rats when compared to those fed a high-starch (70% kcal), high-fat (60% kcal), or a WD (45% fat, 35% fructose) consumption, along with a concomitant increase in LVH (Sharma et al., 2007). This association between cardiac mass and dietary sugar has been observed with high-sucrose feeding (Sharma et al., 2008). Thus, diets high in carbohydrate may

be more detrimental to cardiac morphology than those containing a similar amount of lipid. This finding may account for the greater HWs observed in the AC-SD and AC-WD groups. As such, these data indicate that increasing dietary carbohydrate consumption leads to a more extensive LVH, whereas high lipid consumption could potentially attenuate this.

3.4.4 Serum alterations following dietary treatment and AC

The present study demonstrated no difference in TG concentration between Con and AC groups, although TG levels were slightly increased in both AC-HFD and AC-WD animals (table 3.3). Serum TG is elevated by diets containing 40% coconut fat, olive oil, and sunflower oil (Yaqoob et al., 1995, Ruiz-Gutierrez et al., 1999), and decreased or unchanged by high safflower or fish oil consumption (Yaqoob et al., 1995, Chalkley et al., 2002). This elevation in TG in the AC-HFD and –WD groups is difficult to account for as others have suggested that increasing fat consumption has little effect (Chess et al., 2008) or lowers serum TG in hypertensive animals (Okere et al., 2006b, Chess et al., 2009) and therefore require furthers investigation in the present model. However, these differences may be related to the strain of experimental animals, as it has previously been shown that both Wistar and Sprague Dawley rats have differing lipid metabolism (Galan et al., 1994), with additional strain changes observed following HFDs (Akiyama et al., 1996, Oliveira et al., 2009).

Serum glucose concentrations were marginally but not significantly elevated in HFD and WD groups when compared to SD controls (table 3.3 and 3.4), consistent with previous work from our laboratory (Akki and Seymour, 2009). Many groups have reported that increased lipid consumption and adiposity can produce hyperglycaemia (Akiyama et al., 1996, Gastaldelli et al., 2004, Srinivasan et al., 2004, Ouwens et al., 2005), although certain HFDs have little effect on blood glucose concentrations (Chess et al., 2008, Chess et al., 2009). Wilson and colleagues (2007) showed that chronic WD feeding did not cause hyperglycaemia, with similar findings observed by Aragno et al. (2009). Interestingly, a synergistic effect between dietary lipid and sugar may exist at a hepatic level (Coate et al., 2010). A combined effect of high-fat/high-fructose feeding led to reduced hepatic clearance of glucose and subsequent elevated blood glucose concentrations, in part due to desensitivity of the liver and skeletal muscle system to the actions of insulin (Coate et al., 2010).

Serum FFAs were significantly increased following a HFD and WD (table 3.3), and were higher than previously reported in animals fed a HFD (Akki and Seymour, 2009). Such a serological response to increased fat feeding has been documented elsewhere (Wilson et al., 2007, Chess et al., 2008). Increased circulating FFAs have been linked with obesity and type 2 diabetes (Reaven et al., 1988) and may lead to more severe IR (Coate et al., 2010).

Enhanced hepatic fat content, as observed in the present study, may occur as a result of increased resistance to insulin-stimulated suppression of lipolysis and consequently higher circulating FFAs (Seppälä-Lindroos et al., 2002). In addition, Buettner et al. (2006) have shown that the serum FFA profile mirrors the dietary FA constituents. However, other dietary components may alter the serum FFA profile. Modification of the digestibility of dietary starch has also been shown to influence the FFA content of blood. High GI starch derived from wheat increased plasma FFAs to a greater extent than low GI starch derived from mung beans (Lerer-Metzger et al., 1996). Similarly, increasing dietary sucrose consumption (39% daily calories) in low-fat fed WKA rats led to the accumulation of palmitic, palmitoleic, oleic, and a decrease in linoleic acid, arachadonic, and docosahexaenoic FA species (Fukuchi et al., 2004). Because the changes observed in the plasma FA profile did not mirror the dietary lipid constituents, endogenous synthesis of FAs was likely for the discordance between diet and plasma FFA in sucrose-fed rats (Fukuchi et al., 2004). Thus, the elevated FFA concentration in HFD and WD animals may be attributable to dietary origin, reduced partitioning into adipose tissue, or increased endogenous synthesis.

3.4.5 Leptin

In this study, a significant increase in leptin was observed following both HFD and WD feeding when compared to SD counterparts (table 3.3) in Con and AC groups. Furthermore, leptin was shown to correlate positively with BW (figure 3.9A) and fat mass (figure 3.9B), consistent with Frederich et al. (1995). Leptin levels are increased following high-fat and WD feeding (Woods et al., 2004, Chess et al., 2008), and also by high-fructose feeding (Shapiro et al., 2008) as a consequence of elevated fat mass.

The observations of progressive BW gain, increased adipose tissue mass, and elevated serum concentrations of leptin in the present study in HFD and WD animals would imply leptin

resistance. This has been previously documented in models of high-fat and WD feeding (Van Heek et al., 1997, Shapiro et al., 2008).

Serum leptin concentrations were decreased in AC animals fed a HFD and WD in comparison to their dietary controls (table 3.3) potentially due to reduced adiposity, and were also reported in hypertensive DSS rats (Okere et al., 2006b). Mascareno et al. (2009) have shown that TAC in ob/ob mice increased LV mass beyond that of TAC in C57BL6/J ob+/ob+ mice, and augmented collagen deposition, suggesting a potential anti-hypertrophic role of leptin. However, hyperleptinaemia observed following HFD and WD feeding might have important consequences for cardiac morphology. For example, hyperleptinaemia has been linked with increased sympathetic nerve activation (SNA), speculated to be an additive component in increasing blood pressure (Masuo et al., 2000). In addition, studies in neonatal rat ventricular myocytes (NRVMs) have shown that leptin stimulates hypertrophy via MAPK activation (Rajapurohitam et al., 2003), and also contributes to the hypertrophic actions of endothelin-1 (ET-1) and Angiotensin II (Ang II) (Rajapurohitam et al., 2006). More recent studies have shown that hypertrophy in NRVMs is also dependent upon activation of the JAK/STAT pathway, with this finding also being supported by murine post-MI studies (Abe et al., 2007). However, using tamoxifen-inducible leptin receptor knockout (ObR-KO) mice McGaffin et al. (2011) demonstrated that selective disruption of leptin signalling leads to increased ventricular remodelling and a worsening of the hypertrophic response, as well as inflammation and apoptosis. Consequently, the effect of hyperleptinaemia upon the heart remains unknown, but is important because of the relationship between leptin, obesity, and cardiac hypertrophy.

3.4.6 Insulin

Fasting levels of serum insulin did not differ between groups fed either a HFD or WD (table 3.4). However, there was a trend for WD animals to exhibit increased insulin concentrations despite similar fasting serum glucose concentrations (table 3.4).

Using a diet containing 60% SFA, Chess et al.(2008) demonstrated a similar response to the current study. Indeed, this insulin response has also been observed following diets high in PUFAs (Okere et al., 2006b). Conversely, others have demonstrated that a HFD leads to the development of IR (Matsuzawa-Nagata et al., 2008). Interestingly, these authors noted that prior to the onset of hyperinsulinaemia and IR, there was evidence of increased oxidative

stress via enhanced hepatic β-oxidation, activity of ACO, and cytochrome P450 2E1 (CYP2E1), and reduced ROS scavenging capability. Consequently, hepatic lipid metabolism may play a crucial role in regulating insulin sensitivity. Furthermore, mice fed a HFD demonstrated delayed glucose clearance following oral glucose administration and respond less rapidly to i/p insulin when compared to standard-chow fed controls, speculated to be a result of abrogated peripheral sensitivity following elevated serum FFAs (Sumiyoshi et al., 2006). Indeed, the present study has identified that FFAs were elevated following HFD and WD feeding, mirroring the obese phenotype (Mayer-Davis et al., 1997). Consequently, it may be inferred that the increased dietary fat gives rise to IR, therefore creating a favourable environment for elevated dietary sugar to promote hyperglycaemia.

Insulin has been implicated in the ventricular remodelling process with transgenic mice studies lending much weight to this hypothesis. Deletion of the cardiac insulin receptor leads to impaired cardiac growth and subsequent attenuated expression of protein kinase B (Akt) (Belke et al., 2002, Shiojima et al., 2002). Importantly, patients with LVH have a higher plasma insulin and greater degree of IR (Watanabe et al., 1999, Ilercil et al., 2002). Indeed, the relationship between LV mass and insulin still existed following correction for blood pressure and body mass (Ilercil et al., 2002). Interestingly, even when skeletal muscle exhibits IR, cardiac insulin sensitivity is preserved (Utriainen et al., 1998). As such, hyperinsulinaemia of obesity may be a physiological mechanism for maintaining skeletal muscle and liver insulin sensitivity but may lead to over-stimulation of the cardiac insulin cascade and potentially exacerbating LVH in response to hypertension.

3.4.7 Summary

In the current study, animals fed a HFD or WD showed progressive weight-gain over a 9 week period. Leptin concentrations were increased and correlated strongly with changes in adiposity and BW. A HFD or WD led to a subtle increase in blood glucose and TG, but a much greater increase in serum FAs, and substantial hepatic lipid accumulation. Limited impact was observed upon cardiac morphology following HFD or WD feeding in Con animals. Conversely, AC animals developed significant cardiac hypertrophy as anticipated, and demonstrated similar serum changes to their dietary matched controls. Furthermore, the hypertrophic response was greatest in animals fed either a SD or WD. This study demonstrates that the combination of dietary manipulation and LVH leads to differential cardiac responses but not HF within the time frame of the experiments.

Chapter 4: *In vitro* cardiac function and metabolism

4.1 Introduction

Although it has been argued that HFDs can induce obesity, high-sugar-containing diets can also be detrimental to health and increase adiposity (Swarbrick et al., 2008, Stanhope et al., 2009). Importantly, increased adiposity is strongly associated with hypertension and coronary artery disease, with hypertension a strong predictor of left ventricular mass and subsequent hypertrophy (Rame and Dries, 2007). Indeed, LVH is strongly associated with the development of heart failure (HF) (Rame and Dries, 2007). Understanding the mechanisms which regulate the transition from compensated LVH to HF remains an important research field. Moreover, recent studies have begun to examine the specific impact certain macronutrients have upon this remodelling process, frequently producing conflicting data.

4.1.1 Regulation of metabolism in cardiac hypertrophy

The crucial role PPARs play in regulating cardiac metabolism (chapter 1, section 1.2) lend themselves to study during hypertrophic growth, with the PPARα pathway being the most widely studied due to the relationship with cardiac lipid metabolism (Barger et al., 2000). In cardiomyocyte hypertrophy induced by phenylephrine stimulation, decreased PPARα mRNA expression has been observed concomitantly with decreased mCPT1 mRNA and reduced FAO (Barger et al., 2000, Kato et al., 2010). Similarly, models of LVH induced by TAC and abdominal AC also recapitulate this (Barger et al., 2000, Akki et al., 2008) and PPARα mRNA in the left ventricle decreases as ventricular mass increases (Sack et al., 1996). Indeed, expression of PPARα in LVH is reduced to a level expressed in the foetal phenotype the hypertrophied ventricle (Sack et al., 1997).

PGC-1α is a PPARα-coactivator induced under conditions related to increased energy production, such as adaptive thermogenesis (Puigserver et al., 1998), and is highly expressed in tissues with elevated rates of FAO, or tissues with high mitochondrial content such as the heart and brown adipose tissue (Puigserver and Spiegelman, 2003). Furthermore, an important role for PGC-1α in regulating the metabolic adaptation that accompanies hypertrophy has been deduced from its mitochondrial biogenic properties (Lehman et al., 2000). Data show that PGC-1α is decreased in pathological cardiac hypertrophy (Barger et al., 2000) and consequently result in a decline in mitochondrial content which may be a speculative cause for the energy depletion observed in HF (Neubauer, 2007).

4.1.2 Impact of diet upon ventricular function

The impact of diet upon ventricular remodelling has frequently produced conflicting data with studies identifying a positive (Okere et al., 2006a) (Okere et al., 2006b) or negative (Ouwens et al., 2005) impact upon function. Indeed, increased dietary fat consumption enhanced the expression of PPARα without a negative impact upon function (Okere et al., 2006b). Furthermore, data indicate that diets high in carbohydrate can lead to cardiac dysfunction (Duda et al., 2008, Deng et al., 2007). Furthermore, increasing dietary carbohydrate in combination with high fat content (mimicking a WD) led to a reduction in cardiac function and increased oxygen consumption when compared to low-fat fed controls (Wilson et al., 2007, Akki and Seymour, 2009).

4.1.3 Dietary impact upon function in LVH

Increased consumption of dietary lipid has yielded opposing data regarding the effect upon function in the hypertrophied heart. Several studies have indicated that increased fat consumption does not exacerbate deterioration in function (Morgan et al., 2006b, Chess et al., 2008). High-fat feeding in DSS rats attenuated ventricular dilation, preserves EDV, and maintains end systolic volume (ESV) when compared to low-fat fed littermates (Okere et al., 2006b). Conversely, Akki and Seymour (2009) have shown using *in vitro* perfusion studies that LVH induced by AC in rats fed a WD (45% kcal) reduced cardiac RPP and increased oxygen consumption. Hypertensive DSS rats fed a high-carbohydrate (starch) diet for 12 weeks show substantial systolic dysfunction when compared to hypertensive animals fed a HFD (Okere et al., 2006b), with a similar result observed for high-sucrose and high-fructose feeding (Chess et al., 2007, Sharma et al., 2008).

4.1.4 Dietary modification of hypertrophic metabolism

The impact of dietary manipulation on metabolic remodelling has been an area of considerable focus in recent years. Activating PPARα in the hypertrophied heart may preserve FAO and limit the switch to a foetal phenotype. High-fat feeding maintains both the expression of PPARα mRNA in the hypertrophied rat heart and the activity of MCAD (Okere et al., 2006b, Akki and Seymour, 2009). However, others have shown decreased PPAR mRNA expression in TAC mice fed a HFD, but elevated MCAD activity (Chess et al., 2008). Similarly, high-fat feeding in an infarct model of HF did not modulate either PPARα mRNA or protein expression, or its downstream targets (Morgan et al., 2006b). Combined, high fat and high sugar diets may

promote partial activation of PPARα and therefore lead to incomplete upregulation of FAO in the face of enhanced FA uptake (Wilson et al., 2007).

4.1.5 Lipotoxicity

Inappropriate cardiac lipid accumulation may be central to the contractile dysfunction observed in obese and/or diabetic persons, and can potentially accelerate the development of HF (Christoffersen et al., 2003). Enhanced lipid uptake can lead to lipid accumulation and dysfunction (Park et al., 2008). Furthermore, lipid accumulation can occur despite increased activation of PPARα (Finck et al., 2002). Indeed, if β-oxidation is decreased in the face of normal FA uptake, cardiomyopathy can develop (figure 4.1). Deficiency of very long-chain acyl-CoA dehydrogenase and long-chain acyl-CoA dehydrogenase (VLCAD and LCAD, respectively) manifest as cardiac hypertrophy in mice (Cox et al., 2009) and humans (Spiekerkoetter, 2010) primarily due to decreased energy provision. Furthermore, LCAD deficiency can enhance cardiac lipid accumulation in fasted mice (Bakermans et al., 2011) indicating that discord between FA uptake and oxidation is sufficient to induce lipid accumulation.

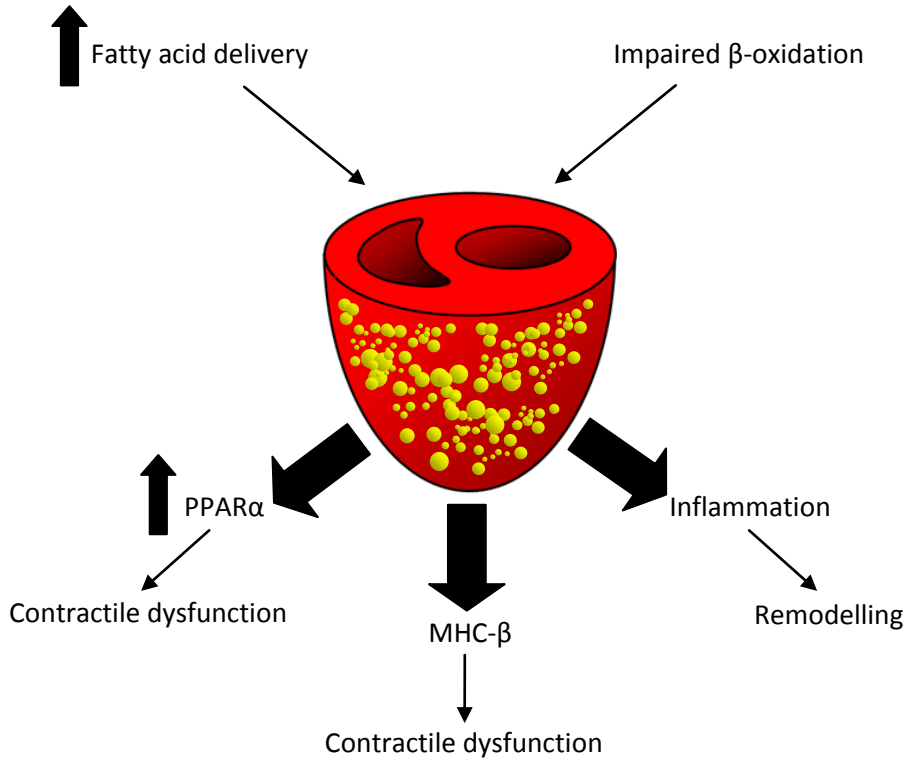

Figure 4.1 Mechanisms for cardiac lipotoxicity. MHC, myosin heavy chain; PPARα, peroxisome proliferator activated receptor-α. Adapted from Sharma et al. (2004).

4.1.6 Objectives

LVH is characterised by alterations to metabolism, with a decrease in the oxidation of FAs and an enhanced reliance on glucose. The combined effect of both increased supply of lipid and glucose to the heart due to high circulating concentrations as in obesity may promote ectopic lipid deposition if FA uptake is preserved.

The objective of this study was to determine the impact of increased dietary lipid and sucrose upon *in vitro* functional and metabolic cardiac remodelling associated with pressure-overload hypertrophy. Alterations in the profile of substrate usage were assessed using ^{13}C labelled substrates followed by ^{13}C NMR isotopomer analysis. Changes in the expression of key regulatory proteins as well as mitochondrial enzyme activities were determined in cardiac tissue extracts. In addition, the oxygen cost of contraction and basal metabolism was assessed to determine potential changes in efficiency.

4.2 Materials and Methods

4.2.1 Induction of LVH

LVH was induced in male Sprague-Dawley rats as described in section 2.2.1 with appropriate weight-matched controls. Animals were studied at 9 weeks post-surgery.

4.2.2 Dietary Intervention

48 hours post-surgery, animals were assigned to dietary regimens described in section 2.2.3, generating 6 experimental groups:

- Control + standard chow (Con-SD)
- Control + high-fat diet (Con-HFD)
- Control + western Diet (Con-WD)
- Aortic Constriction + standard chow (AC-SD)
- Aortic Constriction + high-fat diet (AC-HFD)
- Aortic Constriction + western Diet (AC-WD).

4.2.3 Experimental protocols

Hearts were excised, perfused and *in vitro* cardiac function and metabolism determined (as previously described in section 2.2.5). Perfusion protocols are shown in figure 4.2.

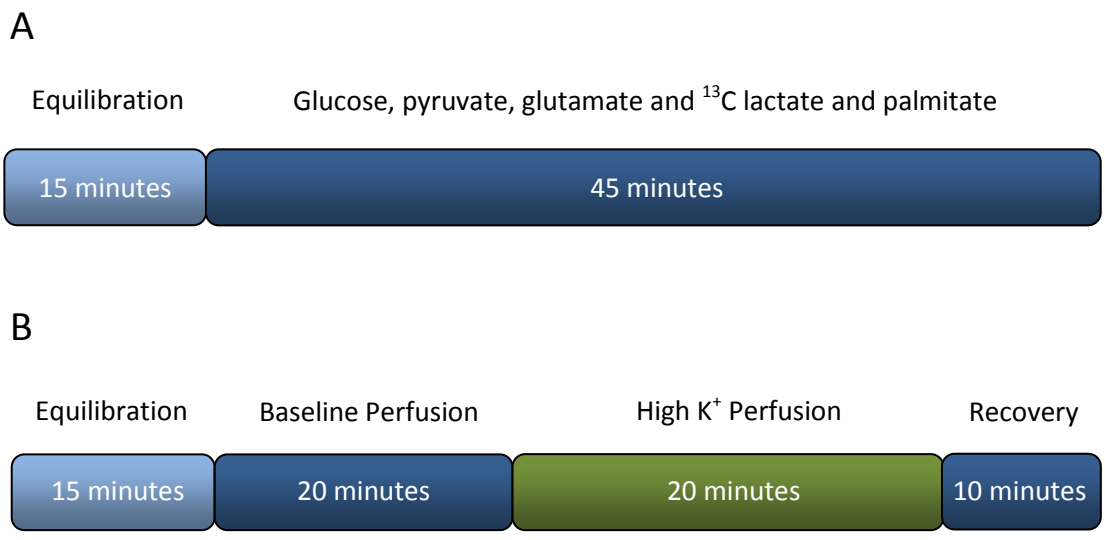

Figure 4.2 Perfusion protocols used in the current studies. A) Standard and ^{13}C labelled substrate perfusion; and B) oxygen consumption study perfusion protocol.

Western blotting was performed in freeze-clamped cardiac tissue as described in section 2.2.11. The activities of MCAD, CS, and PDH were determined as described in section 2.2.7. Cardiac glycogen and TG concentrations were determined at the end of experiments as described (section 2.2.6). Histological determination of myocardial neutral lipid content was performed as described in section 2.2.10. The oxygen cost of contraction and basal metabolism was determined by inhibiting contraction with 20mM potassium.

4.3 Results

4.3.1 *In Vitro* Cardiac Function

The effect of dietary manipulation on cardiac function in Con and AC hearts is given in table 4.1. SP was unaffected by diet alone. Equally, left ventricular developed pressure (LVDP), rate of contraction (dP/dtmax) and relaxation (dP/dtmin) were not altered following dietary intervention. Consequently, rate pressure product (RPP) was unaffected by any dietary intervention in controls (table 4.1 and figure 4.3).

SP was significantly increased in AC hearts from the WD group compared to SD counterparts (table 4.1). LVDP was significantly elevated by HFD and WD in animals subjected to AC compared to their respective controls, and also versus the SD group (AC-SD) (table 4.1). However, no alterations were observed in the rates of contraction (dP/dtmax) and relaxation (dP/dtmin) in AC groups, (table 4.1). AC-HFD and AC-WD groups showed a marked increase in RPP in comparison to the standard diet group.

Significant changes in MVO_2 were observed following dietary intervention (figure 4.5). This was significantly lower in the Con-WD group compared to its SD and HFD groups. Con-HFD and Con-WD groups exhibited a small increase in cardiac CE (figure 4.4) due to an elevated RPP and (figure 4.3) reduced myocardial oxygen consumption.

MVO_2 did not differ between AC-SD and AC-HFD hearts whereas the AC-WD group showed further reductions on oxygen consumption in comparison to the AC-HFD group. The only intra-group differences were observed between Con-SD and AC-SD hearts (figure 4.4). In the AC groups, CE was increased in both HFD and WD groups when compared to their SD counterparts (figure 4.5).

Table 4.1 *In vitro* cardiac function

	SP (mmHg)	LVDP (mmHg)	(dP/dt)max (mmHg/S)	(dP/dt)min (mmHg/S)
Con-SD (n=3)	128 ±6	118 ±4	3522 ±421	-2411 ±147
Con-HFD (n=20)	134 ±12	120 ±5	3622 ±211	-2354 ±122
Con-WD (n=15)	118 ±7	106 ±12	3654 ±153	-2657 ±192
AC-SD (n=4)	119 ±4	110 ±6	4206 ±156	-2673 ±202
AC-HFD (n=15)	151 ±16	145 ±11$^{\#\dagger}$	4365 ±457	-2781 ±256
AC-WD (n=12)	159 ±19†	148 ±9$^{\#\dagger}$	4491 ±184	-2891 ±132

Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet; SP, systolic pressure; LVDP, left ventricular developed pressure. # $P < 0.05$ vs. respective dietary control. † $P < 0.05$ vs. AC-SD. Data presented as mean ± SEM.

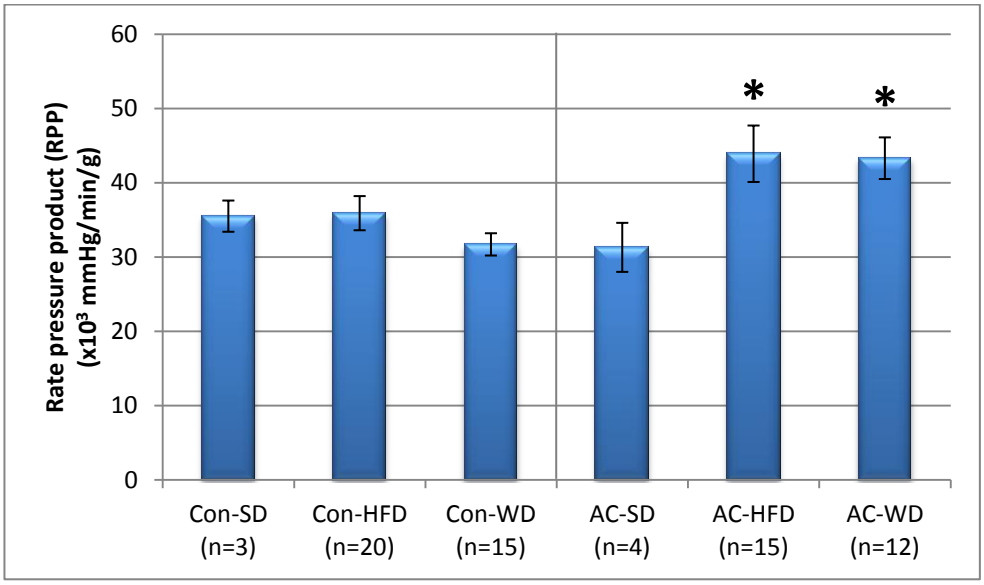

Figure 4.3. *In vitro* rate pressure product in perfused control or aortic constricted hearts after exposure to different dietary regiments for 9 weeks. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. * $P < 0.05$ vs. AC-SD, Data presented as mean ± SEM.

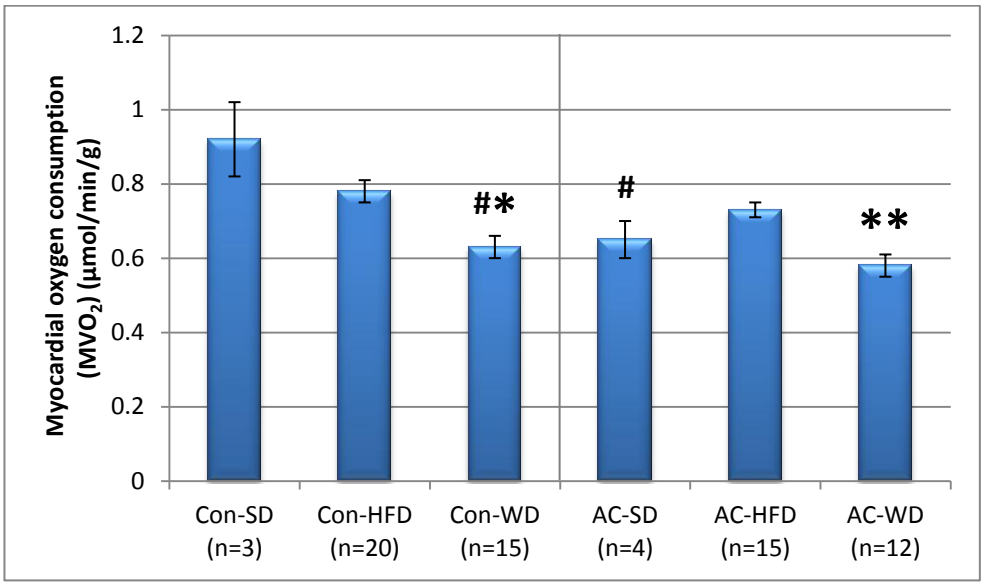

Figure 4.4 Myocardial oxygen consumption in perfused control and aortic constricted hearts exposed to different dietary regimens for 9 weeks. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. # $P < 0.05$ vs. Con-SD. * $P < 0.05$ vs. Con-HFD. ** $P < 0.05$ vs. AC-HFD. Data presented as mean ± SEM.

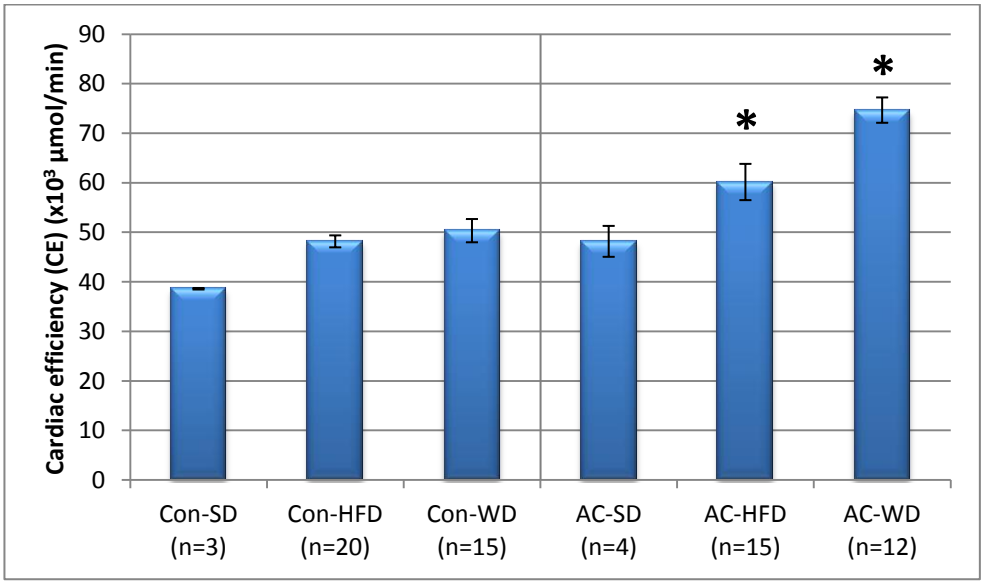

Figure 4.5 Cardiac efficiency in perfused control and aortic constricted hearts exposed to different dietary regimens for 9 weeks. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. * $P < 0.05$ vs. AC-SD. Data presented as mean ± SEM.

4.3.2 ^{13}C NMR analysis of oxidative metabolism

The oxidation of palmitate was reduced following WD feeding in the Con group and a small reduction in use of unlabelled substrates (including endogenous substrates). This was in part compensated by a corresponding increase in lactate oxidation (figure 4.6). Similarly, palmitate oxidation was reduced in AC groups compared to their controls (figure 4.6). Thus, WD had a greater impact upon the metabolic remodelling associated with cardiac hypertrophy.

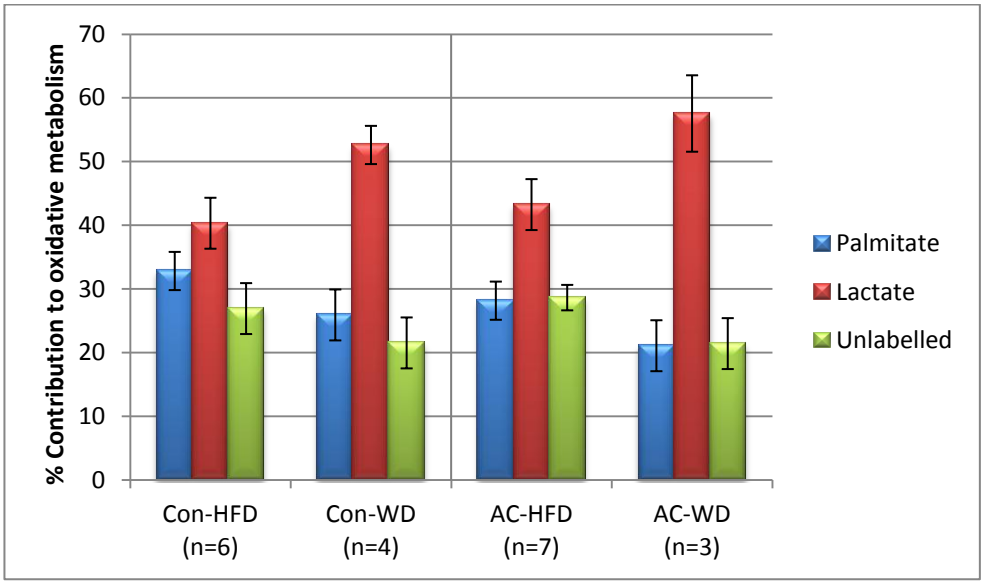

Figure 4.6 Percentage contribution of ^{13}C substrates to oxidative metabolism in perfused control and aortic constricted hearts exposed to different dietary regimens for 9 weeks. Con, control; AC, aortic constriction; HFD, high-fat diet; WD, western diet. Data presented as mean ± SEM.

4.3.3. Metabolic protein expression

PPARα protein expression did not change in any of the control groups, whereas its expression was substantially reduced in both AC-SD and AC-WD hearts compared to their respective controls (figure 4.7). However, this decrease in PPARα expression between AC and Con groups was not observed in hearts from AC animals fed the HFD (figure 4.7).

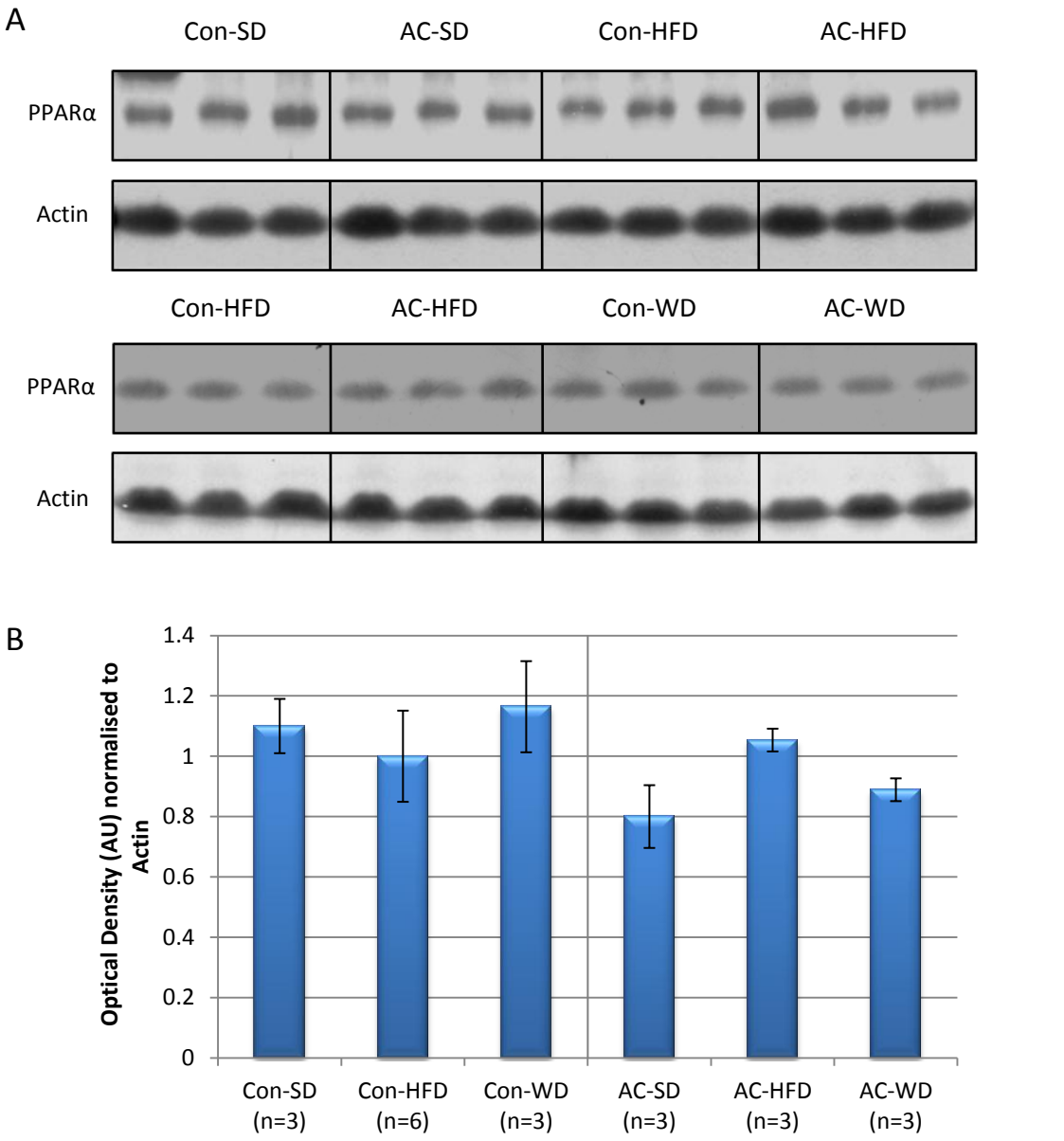

Figure 4.7 Western blot analysis of PPARα expression in from control and aortic constricted hearts exposed to different dietary regimens for 9 weeks. A) representative blots; B) densitometirc analysis normalised to actin expression and relative to Con-HFD. Con-HFD groups are duplicated due to the SD groups being a later additional experimental group. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. Data presented as mean ± SEM.

CD36 expression did not differ between any of the control groups (figure 4.8). However, there was an apparent increase in CD36 expression in Con-HFD relative to its control group, and the WD diet further enhanced CD36 expression (figure 4.7).

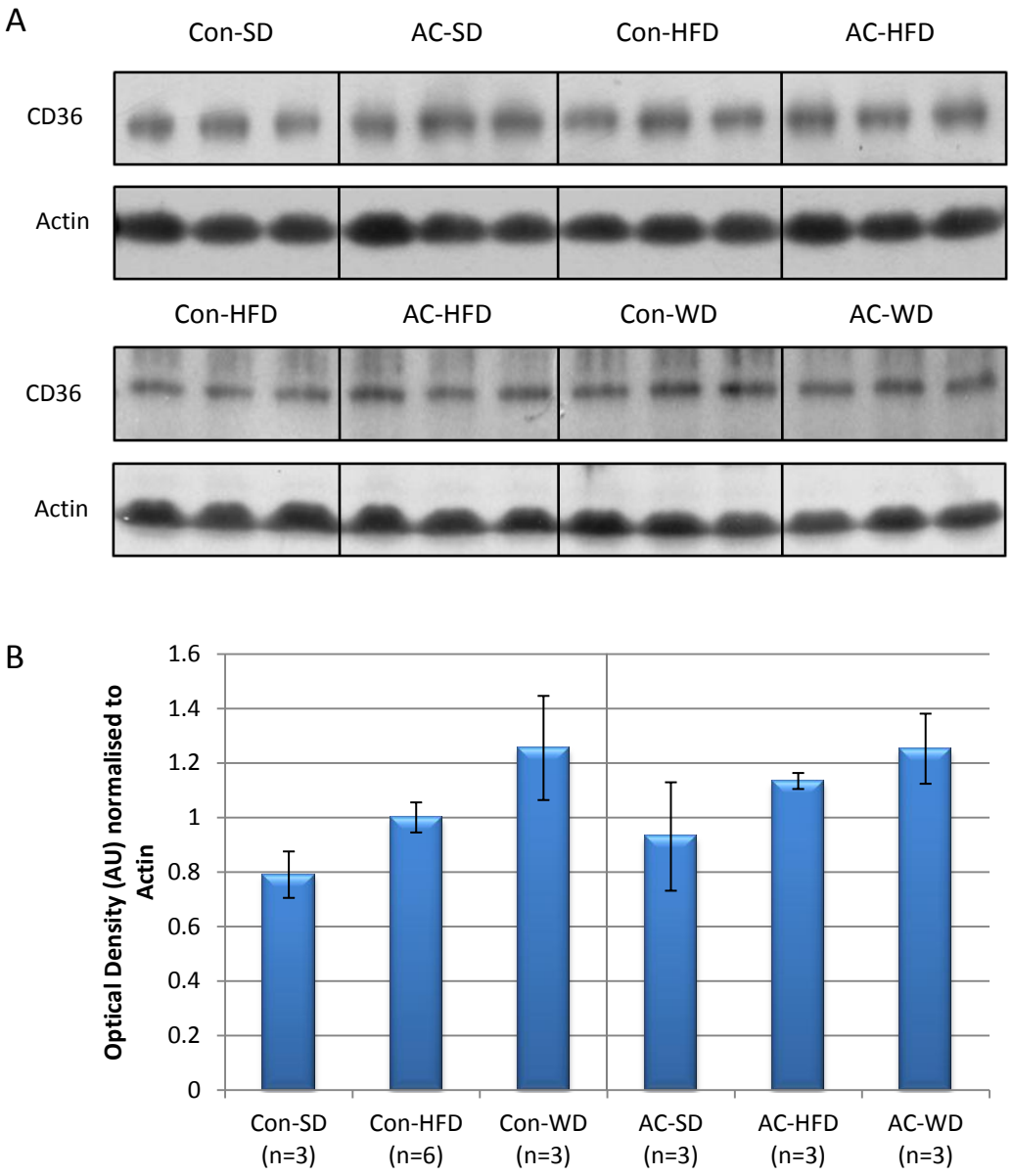

Figure 4.8 Western blot analysis of CD36 from control and aortic constricted hearts exposed to different dietary regimens for 9 weeks. A) representative blots; B) densitometirc analysis normalised to actin expression and relative to Con-HFD. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. Data presented as mean ± SEM.

A similar increase in CD36 expression was also observed in the AC group, with a progressive increase in CD36 expression in the HFD and WD hearts.

GLUT4 expression was determined in sarcolemmal (SR) and intracellular membranes (ICMs) to determine if there was a compensatory increase in carbohydrate uptake. Hearts from the Con-

WD group exhibited enhanced SR and ICM GLUT4 in comparison to the Con-HFD group. Hearts from the AC-HFD group also demonstrated an 88% increase in the SR content of GLUT4 compared to the control group (figure 4.9), whereas hearts from WD animals exhibited over 50% increase in GLUT4 expression in both SR and ICM fractions (figure 4.9). Both AC-HFD and AC-WD groups showed an increased expression of SR GLUT4 compared to the ICM fraction. Therefore, total GLUT4 was increased following WD feeding. Na+/K+ ATPAse was used as a marker for the SR fraction and was not detected in the ICM fraction.

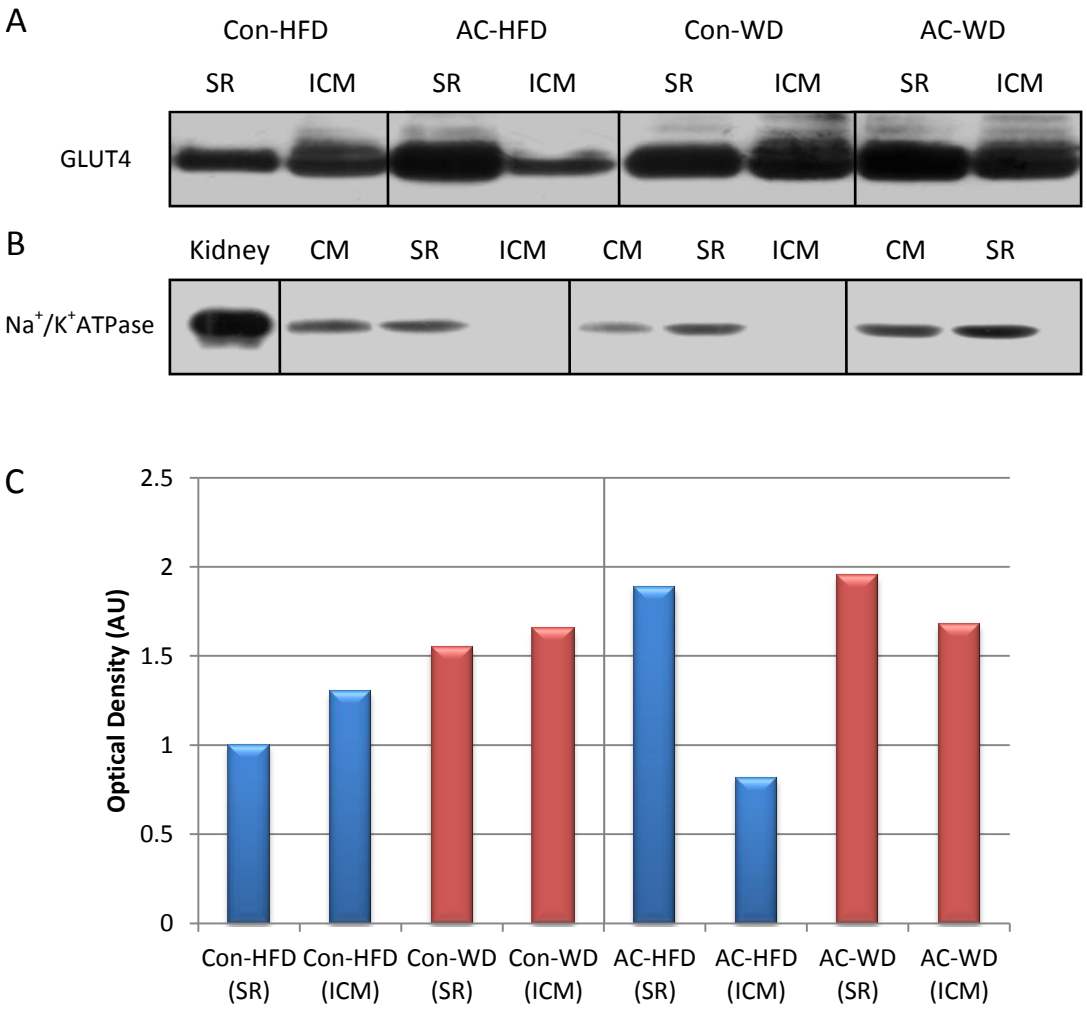

Figure 4.9 Subcellular isolation and expression of GLUT4 from control and aortic constricted hearts exposed to different dietary regimens for 9 weeks. A) representative blots of GLUT4 expression in the SR or ICM; B) characterisation of membranes using Na^+/K^+ATPase; C) densiometric analysis. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet; CM, crude membrane fraction; SR, sarcoplasmic reticulum fraction; ICM, intracellular membrane fraction. Each experimental group represents 12 pooled hearts.

4.3.4 Mitochondrial enzyme activity

Con-HFD hearts exhibited a significant decrease in percentage PDHa/PDHt relative to SD counterparts (figure 4.10) as would be expected due to higher dietary fat content. Hearts from the Con-WD group demonstrated a percentage PDHa/PDHt ratio that was significantly higher than the HFD group (figure 4.10) suggesting that enhancing dietary sugar can modulate the PDH response to increasing fat utilisation.

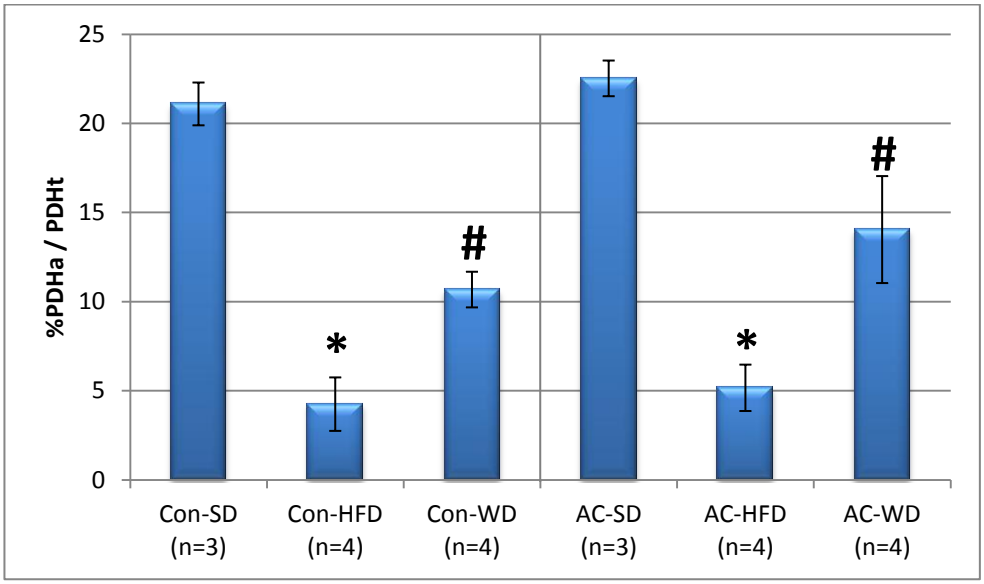

Figure 4.10 Percentage active pyruvate dehydrogenase activity in control and aortic constricted hearts exposed to different dietary regimens for 9 weeks. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet.* $P < 0.05$ vs. SD counterpart; # $P < 0.05$. vs. SD and HFD counterpart. Data presented as mean ± SEM.

In the AC groups the percentage of PDH in the active form relative to total PDH exhibited a similar pattern to the control groups (figure 4.10). Both AC-HFD and AC-WD groups showed a marked reduction in the PDHa/PDHt ratio compared to SD counterparts. Again, the AC-HFD group exhibited the lowest PDHa/PDHt ratio (figure 4.10). Neither dietary intervention nor AC had any impact upon PDHt content.

CS activity was significantly enhanced in the HFD group compared to SD counterparts, reflecting an increase in mitochondrial density. WD feeding produced a further increase in CS activity. A similar pattern was observed for all AC groups, with AC-WD hearts exhibiting the

greatest CS activity (table 4.2). Overall, these results indicate that mitochondrial content may be influenced by dietary manipulation – irrespective of the presence of hypertrophy.

The activity of medium-chain acyl-CoA dehydrogenase (MCAD) did not differ significantly between groups, irrespective of diet or AC (table 4.2). When MCAD activity was normalised to CS. the MCAD:CS ratio (a putative indicator of β-oxidation) (Chess et al., 2007) was significantly decreased in the Con-WD group when compared to SD and HFD counterparts. AC groups showed a similar pattern to the control groups, with the AC-WD group showing the greatest reduction the MCAD:CS ratio (table 4.2)

Table 4.2 Cardiac mitochondrial enzyme activity

	Con-SD (n=6)	Con-HFD (n=-17)	Con-WD (n=12)	AC-SD (n=6)	AC-HFD (n=20)	AC-WD (n=12)
CS (µM/min/g)	55.2 ±2.9	71.1 ±2.8#	102 ±2.1*	46.1 ±1.8	66.7 ±2.0#	92.6 ±3.3*
MCAD (µM/min/g)	12 ±1.0	13.8 ±0.5	14.2 ±0.2	11 ±1.0	12.0 ±0.5	12.1 ±0.3
MCAD:CS	0.22 ±0.01	0.20±0.01	0.14±0.01*	0.23 ±0.00	0.18 ±0.01	0.13 ±0.01*

Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet.* $P < 0.05$ vs. SD and HFD counterpart, # $P < 0.05$ vs. SD counterpart. Data presented as mean ± SEM.

4.3.5 Myocardial glycogen and triglyceride content

Glycogen progressively accumulated with increasing dietary sugar (table 4.3), with Con-WD exhibiting the greatest degree of glycogen accumulation. AC hearts from HFD and WD rats also showed increased glycogen accumulation. However, WD feeding in the AC group did not lead to further increases in glycogen (table 4.3).

Table 4.3 Endogenous storage substrates

	Con-SD	Con-HFD	Con-WD	AC-SD	AC-HFD	AC-WD
Glycogen ($x10^3$ µmol glucose equivalents/g)	2.7 ±0.2 (n=3)	3.8 ±0.3* (n=6)	5.0 ±0.1# (n=6)	2.6 ±0.4 (n=6)	3.6 ±0.3* (n=6)	3.8 ±0.4* (n=6)
Triglyceride (µmol/g)	1.6 ±0.1 (n=3)	1.5 ±0.1 (n=17)	2.7 ±0.1‡ (n=12)	1.7 ±0.2 (n=4)	1.5 ±0.2 (n=20)	2.3 ±0.1‡ (n=12)

Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. * $P < 0.05$ vs. respective SD counterpart. # $P < 0.05$ vs. SD HFD counterpart. ‡ $P < 0.01$ vs. SD and HFD counterparts. Data presented as mean ± SEM.

TG concentrations were unaffected by SD or HFD alone (table 4.3). However, WD feeding produced a significant increase in myocardial TG accumulation in control hearts (table 4.3). Similarly, AC hearts from both SD and HFD groups demonstrated no significant difference in TG concentration (table 4.3), whereas AC-WD hearts exhibited a significantly greater concentration of TG in comparison (table 4.3).

4.3.6 Determination of contractile and basal oxygen consumption

Based upon the earlier findings of enhanced function in the AC-HFD and AC-WD groups (table 4.1), the oxygen cost of contractile function and basal metabolism were determined in HFD and WD hyperkalaemia (20 mM) protocol. This protocol allowed determination of total MVO_2 (reflecting the combined cost of contractile function and metabolic demand during normoxia), and the oxygen requirement of basal metabolism (calculated during the period of arrest following hyperkalaemia).

Following K^+ arrest, all experimental groups demonstrated a significant decrease in RPP (figure 4.11). Consequently, this led to significant decrease in the oxygen requirement in all groups giving a measure of the metabolic cost (figure 4.12). Importantly, the decrease in MVO_2 observed following K^+ arrest was unaffected by diet or AC, with basal metabolism accounting for 67-73% of the total MVO_2 (figure 4.12).

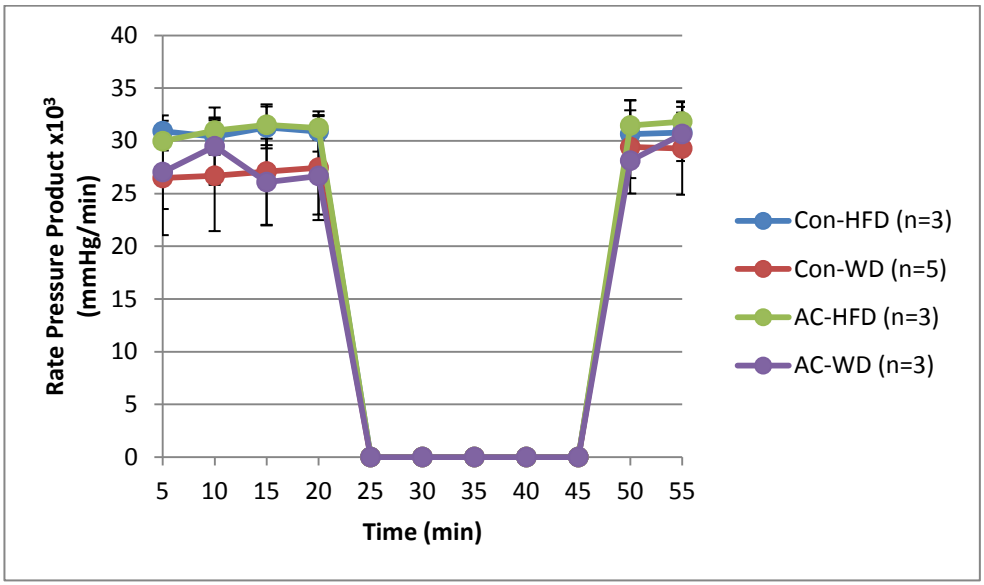

Figure 4.11 Rate pressure product in control and aortic constricted hearts exposed to either a HFD or WD for 9 weeks. Perfusate containing a high potassium concentration was infused between 20 and 45 minutes. Con, control; AC, aortic constriction; HFD, high-fat diet; WD, western diet. Data presented as mean ± SEM.

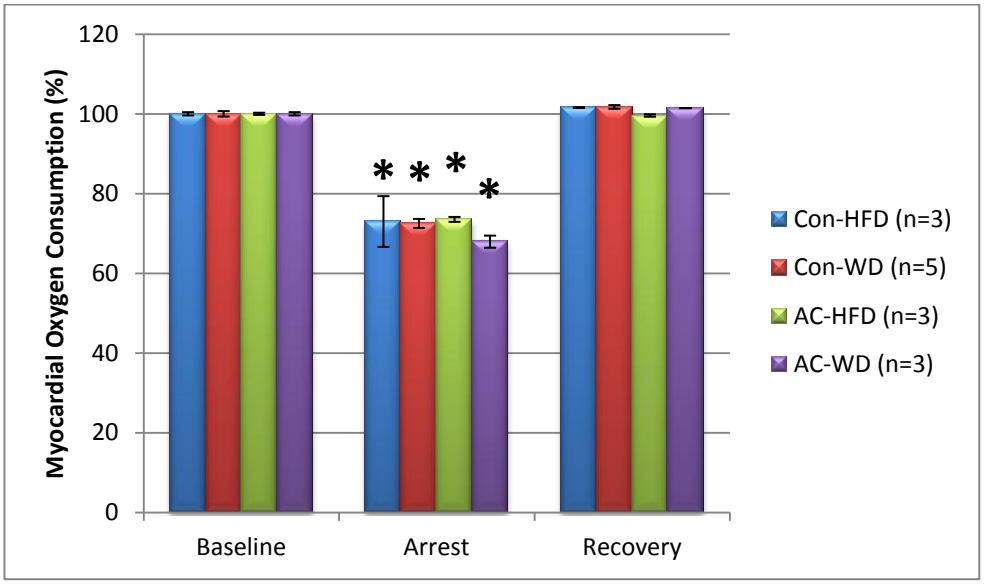

Figure 4.12 Mean myocardial oxygen consumption during contraction and arrest in hearts isolated from control or aortic constricted animals after 9 weeks of dietary intervention. Con, control; AC, aortic constriction; HFD, high-fat diet; WD, western diet. * $P < 0.05$ vs. respective baseline and recovery values. Data presented as mean ± SEM relative to baseline values.

4.4 Discussion

The present study identified that dietary manipulation had a profound effect upon cardiac metabolism. A specific role of the combination of high dietary lipid and sucrose (WD) promoted a reduction in FAO, increased oxidation of carbohydrate and accumulation of glycogen and TG. Importantly, these metabolic changes were not associated with alterations in *in vitro* cardiac function despite a significant decrease in MVO_2, and were not apparent in the SD or HFD groups. Conversely, hypertrophied hearts exposed to a HFD or WD exhibited enhanced contractility when compared to controls. This together with the decrease in MVO_2 and increased efficiency indicates a compensatory phase of cardiac hypertrophy.

In parallel to the hyperfunction observed in AC-HFD and -WD hearts, substantial cardiac metabolic remodelling was evident which was more pronounced in WD-fed animals, characterised by decreased cardiac palmitate oxidation and a compensatory increase in carbohydrate usage. Furthermore, this change in the oxidative profile was associated with increased TG accumulation. These data suggest that by 9 weeks, diets high in lipid and sucrose have no deleterious impact upon cardiac function but lead to substantial metabolic remodelling.

4.4.1 *In vitro* functional response to dietary intervention

In vitro cardiac function was unaffected by dietary intervention (table 4.1, figures 4.3 and 4.5). Indeed, this observation is consistent with both rodent (Okere et al., 2006a, Wilson et al., 2007) and human studies (Holloway et al., 2011). Indeed, Holloway et al. (2011) demonstrated no change in stroke volume or ejection fraction following 14 days 75% fat diet despite a significant reduction in the cardiac PCr/ATP ratio. Conversely, Sprague-Dawley rats fed a HFD for 9 weeks showed reduced RPP and elevated MVO_2, subsequently reducing efficiency (Akki and Seymour, 2009). However, the cause of this reduction was related to a decrease in HR following a HFD, as there was no difference in LVDP (Akki and Seymour, 2009). An increase in MVO_2 was also observed by Wilson et al. (2007) with a reduced CE. As hearts were paced at 300 bpm in the present study, this may explain the observed preservation of cardiac function following a HFD.

Wilson et al. (2007) showed that 48 weeks of a WD reduced cardiac power, speculated to be due to inadequate activation of genes associated with FAO (Wilson et al., 2007). Indeed, this blunted FAO gene response and reduced FAO may support the current observation of reduced MVO_2 following WD feeding (figure 4.4). In addition to the effect of enhanced fat consumption, rats fed a diet supplemented with fructose-sweetened drinking water showed signs of reduced cardiac function and a reduction in stroke volume and ejection fraction (Deng et al., 2007). Although the metabolic properties of fructose differ to other sugars, similar results have also been observed with high sucrose diets (Duda et al., 2008), suggesting increased dietary sugar per se may be an important determinant of cardiac function.

Previous studies have suggested a potential role of dietary lipid as a regulator of cardiac hypertrophy (Morgan et al., 2006b, Okere et al., 2006b, Chess et al., 2008). Interestingly, the present study identified significant hypertrophy in all AC groups (chapter 3, section 3.3.2), yet hyperfunction was only observed in the HFD and WD group, reflected by an augmented LVDP and RPP (table 4.1, figures 4.3 and 4.5). Thus, these observations would suggest that a HFD can maintain cardiac function during pressure-overload hypertrophy. Indeed, Morgan et al. (2006b) demonstrated when mice with infarct-induced HF were fed a diet rich in lipid, cardiac function was not worsened in comparison to low-fat fed mice, suggesting a positive effect of dietary lipid on maintaining cardiac function. Similarly, 16 weeks of a HFD in a model of TAC-induced HF did not adversely affect ejection fraction compared to low-fat fed controls (Chess et al., 2008). Furthermore, the deterioration in function associated with cardiac hypertrophy derived from chronic hypertension in DSS rats was substantially attenuated by consumption of a HFD, with a reduction in both diastolic and systolic volumes, as well as enhanced ejection fraction (Okere et al., 2006b). However, this observation is not uniform and other groups have demonstrated that HFDs cause dysfunction in the hypertrophied heart over short- and long-term feeding regimes (Raher et al., 2008, Akki and Seymour, 2009). This discrepancy between studies may in part relate to the FA composition of the experimental diets, and their ability to regulate cardiac gene expression (Bordoni et al., 2007) and cardiomyocyte apoptosis (Aguila et al., 2005).

Increasing consumption of dietary carbohydrate at the expense of lipid can be more detrimental to cardiac function than diets high in fat (Okere et al., 2006b). Consumption of standard rodent chow (high in carbohydrate) increased functional remodelling associated with hypertension and worsened function in comparison to a HFD (Okere et al., 2006b).

Furthermore, mortality was significantly greater in a hypertensive group of rats fed a high carbohydrate diet, and those in which oxfenicine was used to inhibit FAO (Okere et al., 2006b). Oxfenicine is a potent CPT1 inhibitor, and therefore suggests that maintaining the ability of mitochondria to oxidise LCFA may be important in not only supplying sufficient energy, but potentially reducing intracellular TG accumulation as a result of depressed oxidation. When hypertensive DSS rats were fed a diet rich in fructose for 8 weeks systolic dysfunction became evident, with reduced ejection fraction (Sharma et al., 2008). Similarly, in TAC mice, high-starch diets maintained EDD, ESD, and ejection fraction at levels similar to control mice, however, high-fructose feeding led to depressed systolic performance (Chess et al., 2007). Data also support the concept that sucrose impactes negatively upon cardiac function, with a significant decrease in ejection fraction (Chess et al., 2007). The functional consequences following HFD and WD feeding may therefore be derived from a combination of increased lipid, heightened bioavailability of carbohydrate, and – similar to HFDs – the systemic effects the diet promotes. Combined, these 3 parameters may account for the disparity in results between different studies.

Basal MVO_2 was determined during a period of arrest and was found to be unaffected by a HFD or WD, or AC (figure 4.12). Previous studies by How et al. (2005, 2006) demonstrated that increasing the supply of FAs to the myocardium reduced CE by enhancing the oxygen cost of the unloaded heart, but did not reduce cardiac function. Furthermore, in the db/db heart the increase in MVO_2 may be due to futile metabolic cycling (How et al., 2006). This concept was demonstrated by Wilson et al., showing increased uncoupling protein (UCP) 3, cytosolic thioesterase 1 (CTE1), and mitochondrial thioesterase 1 (MTE1) mRNA expression in hearts exposed to a HFD or WD (Wilson et al. 2007), and by Cole et al. (2011). WDs have been shown partially to activate futile cycling elements, and that the full activation observed following a HFD may prevent harmful oxidation of lipids and cardiac dysfunction (Wilson et al., 2007). Elevated expression of UCP3 would be expected to dissipate the inner mitochondrial membrane proton gradient and thereby increase oxygen consumption, as well as facilitating removal of FA species from the mitochondrial matrix (Schrauwen et al., 2001). Furthermore, others have shown that in the ob/ob mouse contractile function is impaired with an elevation in MVO_2, causing a reduction in CE (Mazumder et al., 2004, Dong et al., 2006). Indeed, this mechanism for reducing CE has been observed in response to a HFD (Akki and Seymour, 2009). Although difficult to reconcile, the reduction in MVO_2 and preserved cardiac function observed in WD hearts may relate to changes in cardiac metabolism, with a shift away from the

oxidation of FAs towards carbohydrate. Taken together, this would suggest that changes in cardiac energy provision precede the development of contractile dysfunction.

4.4.2 Metabolic protein expression

PPARα expression was slightly lower in hypertrophied hearts compared to non-hypertrophied controls (figure 4.7). However, this decrease in protein expression was only observed in hypertrophied hearts from rats fed either a SD or WD. As such, this may suggest that in the AC-HFD group, dietary lipid is sufficient to maintain PPARα protein expression at a level similar to Con-HFD hearts. Indeed, the reduced protein expression of PPARα in the AC-WD group may be a reflection of greater remodelling.

Both PPARα mRNA and protein expression are decreased in experimental models of LVH and in hypertensive and failing human hearts (Sack et al., 1996, Barger et al., 2000), consistent with the findings here. Similarly, the co-activators of PPARα (RXR and PGC-1α) are also reduced (Osorio et al., 2002, Garnier et al., 2003, Morgan et al., 2006a, Ventura-Clapier et al., 2008). Attenuating the decline in PPARα expression in the hypertrophied heart has frequently produced conflicting data, with reports of no change in or an improvement (Chess et al., 2008, Morgan et al., 2006b, Okere et al., 2006b), or a substantial decrease (Raher et al., 2008, Akki and Seymour, 2009) in cardiac function. Thus, the interpretation of the reactivation of PPARα in the hypertrophied heart remains controversial. However the disparity between the studies described above may be due to the source of dietary lipid. Indeed, the fat used by Raher et al (2008) was derived from lard whereas that of Okere et al. (2006b) and Chess et al. (2008) was derived from cocoa butter, and may therefore expose the heart to a very different systemic environment. Raher et al. (2006) reported that a HFD derived from lard increased plasma insulin concentrations, whereas the HFD derived from cocoa butter did not (Chess et al., 2008).

The disparity between the effect of high-fat diets upon the expression of PPARα extends to the use of dietary carbohydrate. Duda et al. (2008) found no effect of either starch or sucrose upon PPARα mRNA expression in hypertrophied hearts. Conversely, Sharma et al. (2007) showed that high-fructose feeding led to a significant decrease in PPARα mRNA expression compared to high-fat or high-starch feeding in DSS rats. Furthermore, standard rodent chow (containing 55% energy from starch and 5% from sucrose) caused a significant reduction in PPARα protein in the hypertrophied heart (Akki et al., 2008), and would support the

observation in the AC-SD group. Indeed, this is most likely due to reduced ligand-activation, explaining the observed normalisation of PPARα protein in the AC-HFD group (figure 4.7). The present data also indicate that increasing sucrose consumption in the face of high dietary fat is insufficient to maintain PPARα protein expression in the AC-WD group. Thus, it may be inferred that the combination of high dietary fat and sugar leads to the incomplete activation of PPARα which may be reflected in alterations in downstream target proteins associated with FA metabolism.

Additional evidence regarding the consequences of PPARα activation comes from the use of specific synthetic PPARα ligands. Indeed, fenofibrate administration reduced HW in AC rats (Rose et al., 2007), endothelin (ET)-1-induced cardiac hypertrophy (Irukayama-Tomobe et al., 2004), aldosterone-induced LVH (LeBrasseur et al., 2007), and in DSS rats (Ichihara et al., 2006). Linz et al. (2009) showed that the PPARα agonist, AVE8134, improved cardiac output in hearts subject to MI, and increased life expectancy in aged spontaneously hypertensive rats. These studies further support the concept that activation of PPARα can attenuate the progression of hypertrophy, supporting the present observations. The mechanisms by which fibrates modulate the antihypertrophic effect, however, remain largely unknown, but may relate to their effects on lipid metabolism (Staels et al., 1998). Indeed, fibrates increase lipolysis of TG-rich lipoproteins (TRLs) and lead to enhanced hepatic uptake of FAs via increased expression of FA transport protein (FATP) and lowering of apolipoprotein C-III (apoC-III) gene expression (Motojima et al., 1998, Minnich et al., 2001). Furthermore, it was shown that ureido-fibrate-5 increased mitochondrial palmitate oxidation in both liver and skeletal muscle, and induced CPT1 expression via a PPARα-dependent mechanism (Minnich et al., 2001). Therefore, in the heart, fibrates may stimulate PPARα and the associated genes involved with FA transport and oxidation. It may be speculated dysregulation of FA uptake and oxidation may be the cause of dysfunction in some models of LVH.

CD36 protein expression, although not markedly different in any of the AC groups, showed a progressive increase in expression in AC-HFD and AC-WD hearts (figure 4.8). Previously, it has been shown that the increase in CD36 mRNA associated with high-fat feeding is blunted by the development of hypertrophy (Okere et al., 2006b), suggesting that LVH is associated with a decreased ability of LCFA transport. Interestingly, other studies showed that high-fat feeding led to increased expression of CD36 protein, irrespective of LVH (Akki and Seymour, 2009). Thus, decreased CD36 protein expression may be protective in the hypertrophied heart by

limiting the entry of LCFA into the cardiomyocyte when the capacity for FAO is reduced. Indeed, a recent study has suggested that CD36 KO mice subjected to TAC showed reduced cardiac function but were protected from dysfunction when they were faced with a WD (Steinbusch et al., 2011). Consequently, heightened expression of CD36 in the AC-WD group and reduced PPARα expression suggests dysregulation of FA metabolism and uptake.

In addition to changes in CD36 protein expression, differences were observed in the subcellular distribution of GLUT4 protein in the hearts of AC-HFD and AC-WD animals, (figure 4.9). Total GLUT4 protein was increased in the WD group suggesting responsiveness to circulating insulin, but increased reliance on carbohydrate as a source of energy. Hypertrophied hearts exhibit a reduced GLUT4/GLUT1 ratio suggesting an impaired ability to increase GLUT4 protein expression in response to insulin (Paternostro et al., 1999), therefore suggesting specific cardiac IR in the hypertrophied heart. Furthermore, it has recently been demonstrated that both GLUT1 and GLUT4 expression are inversely related to CD36 expression in ventricular biopsies from patients diagnosed with aortic stenosis (Heather et al., 2011). Specifically, it was observed that as cardiac mass increases, CD36 expression decreased with a proposed compensatory elevation in GLUT1 and GLUT4 expression (Heather et al., 2011). Although different to the present data, this may be partly explained by the high dietary fat and sugar content, which promotes both CD36 and GLUT4 expression. However, the increased content of GLUT4 within the ICM fraction of the AC-WD group may imply impaired GLUT4 vesicle trafficking, in comparison to HFD counterparts (Zierath et al., 1997, Wright et al., 2009). Importantly, mice with cardiac-specific GLUT4 deletion develop a more pronounced hypertrophy when subjected to AC (Domenighetti et al., 2010). Thus, the potentially impaired GLUT4 trafficking in WD hearts may represent a critical stage in the remodelling process associated with the progression of LVH. Determining the expression of GLUT1 would further add to the understanding of metabolism in the present model.

4.4.3 Modification of mitochondrial metabolism in LVH

AC-HFD and AC-WD exhibited lower rates of palmitate oxidation than their respective control groups (figure 4.6), but a parallel increase in the oxidation of lactate, suggesting a reversion to a foetal metabolic phenotype (Kolwicz Jr and Tian, 2009). Importantly, this increased reliance upon carbohydrate was more pronounced in the WD group.

Similar to the control groups, AC-HFD and AC-WD groups demonstrated a significant increase in CS activity when compared to the AC-SD group, with the highest activity observed in the WD group (table 4.2). Consequently, this would suggest a direct role of dietary lipid and sugar in regulating mitochondrial density (Okere et al., 2006a). However, CS activity was consistently lower in AC hearts than controls (table 4.2). In the failing heart, CS activity is reduced indicating impaired energy provision from the TCA cycle (Kalsi et al., 1999, Lei et al., 2004), or reduced mitochondrial content. Furthermore, when dog hearts subjected to chronic tachycardia-induced HF recovered, CS activity remained reduced, suggesting persistent derangement (Qanud et al., 2008). Therefore, preventing the decline in CS activity (and thereby mitochondrial number) in the hypertrophied heart may be advantageous in maintaining energy provision.

Morgan et al. (2006b) have shown no additive effect of increased dietary lipid or PPARα agonism with fenofibrate upon the activity of CS in infarcted hearts or hypertrophied ventricles (Chess et al., 2008, Chicco et al., 2008, Chess et al., 2009). Rather, these studies demonstrate that high-fat feeding prevents the decline in CS activity associated with LVH (Okere et al., 2006b), suggesting a positive effect of a HFD in maintaining mitochondrial number. Additionally, diets high in fructose (61%) or-sucrose (61%) fail to maintain CS activity in DSS rats (Sharma et al., 2008), further supporting the concept that increased lipid consumption is an important determinant of mitochondrial content. Indeed, the improvement in cardiac function observed following certain HFDs (Okere et al., 2006b, Chess et al., 2009) may be the result of preserved mitochondrial content. However, it has been shown that a WD induces oxidative stress and reduced expression of anti-apoptotic protein Bcl2 and an increased ratio of Bcl2/BAD, alongside increased mitochondrial biogenesis (Ballal et al., 2010). In this regard, the increased biogenesis may reflect a compensatory mechanism to normalise overall mitochondrial function whilst Bcl2 expression is low (Ballal et al., 2010), and thus mitochondrial biogenesis in the HFD and WD may be a compensatory effect due to defective mitochondrial function. Determining mitochondrial function in the HFD and WD groups would be an important addition to this work.

In contrast, the activity of MCAD was not significantly affected by diet but was consistently lower in AC hearts when compared to their respective controls (table 4.2). This decrease in the activity of MCAD following the development of hypertrophy has been well documented (Sack et al., 1996, Depre et al., 1998, Barger et al., 2000). Previously, HFDs maintained the activity of

MCAD at a similar level to non-hypertrophied ventricles (Okere et al., 2006b, Akki and Seymour, 2009), or increased MCAD activity in comparison to hypertrophied hearts isolated from animals fed a low-fat diet (Chess et al., 2008). In the present study, when MCAD activity was normalised to CS activity, there was a significant decrease observed in AC-WD hearts indicating that despite an increase in mitochondrial number, β-oxidation is selectively decreased (table 4.2). Furthermore, this decrease in the MCAD:CS ratio was not observed in other AC groups, and this is further supported by the observation that PPARα expression was reduced in AC-WD hearts but not in the AC-HFD group. The observation that a HFD maintains the MCAD:CS ratio is in agreement with previous studies in the hypertrophied heart (Okere et al., 2006b, Chess et al., 2008). However, the increased supply of carbohydrate in the WD group may negatively regulate the expression of PPARα-governed genes, or facilitate the enhanced reliance on carbohydrate oxidation, which is energetically more efficient than FAO (figure 4.6).

Increased reliance on carbohydrate is reflected in the activity of PDH. Indeed, the percentage of PDHa was greatest in hypertrophied hearts from rats fed either a SD, or WD (figure 4.10), consistent with previous studies (Akki et al., 2008). Increased expression of PDK4 mRNA has been observed in hypertrophied hearts from high-fat fed rats (Chess et al., 2008, Akki and Seymour, 2009). Stimulation of PPARα would therefore, be anticipated to increase transcription of PDK4 and thus lead to inhibition of PDH (chapter 1, section 1.1.4). Indeed, this is supported by the observation that when PPARα was reduced (as in the AC-WD group, figure 4.7), the percentage of PDHa was increased (figure 4.10). Although this would account for the increased PDHa activity observed in the AC-WD group, PPARα protein expression remained unchanged in the control group (figure 4.7), suggesting that both experimental groups exhibit disruption to the PPARα regulation mechanism of cardiac metabolism.

4.4.4 Changes in endogenous substrate in the hypertrophied heart

Cardiac glycogen content was significantly increased in AC-HFD and AC-WD hearts (table 4.3). However the mechanism by which dietary intervention led to glycogen accumulation remains unclear. Previous studies have suggested that diets high in fat lead to reduced glycogen synthesis via impaired insulin sensitivity and attenuated glycogen synthase activity (Chalkley et al., 1998). However, Miller et al. (2005a) demonstrated that elevated AMPK activity enhanced UDP-glucose pyrophosphorylase and subsequent glycogen accumulation. Stimulation of AMPK has been documented in response to increases in FAs (Watt et al., 2006), but does not increase glycogen accumulation following a HFD. In AC-WD hearts, the absence of a further increase in

glycogen (as observed in the respective control group, table 4.3) may reflect AMPK desensitivity to endogenous metabolites or diversion of glucose into other pathways, including the pentose phosphate or hexosamine monophosphate pathways (Chess and Stanley, 2008, Chatham and Marchase, 2010).

4.4.5 Triglyceride accumulation in WD hearts

Myocardial TG accumulation was evident in both Con and AC hearts from the WD group, consistent with reduced expression of PPARα, increased CD36, and attenuated β oxidation. Importantly, the degree of lipid accumulation and the magnitude of the reduction in β-oxidation were similar irrespective of LVH. The mechanism for the accumulation of cardiac TG in the Con- and AC-WD groups may therefore be different. In the control group, the increased expression of PPARα protein may not be sufficient to maintain mitochondrial β-oxidation in the face of heightened FA uptake, therefore leading to TG accumulation. Indeed, this would be similar to models of PPARα overexpression (Finck et al., 2003). However, reduced PPARα protein expression in the AC group may ultimately lead to a decrease in β-oxidation, which when combined with unaltered CD36 expression, leads to TG accumulation. However, crucially, at this stage in the metabolic remodelling process, function was not compromised.

Data regarding the impact of TG accumulation in the hypertrophied heart are conflicting. Akki and Seymour (2009) showed that 9 weeks of high-fat feeding led to cardiac TG accumulation and depressed cardiac function in AC hearts. Conversely, others have shown that a HFD led to TG and neutral lipid accumulation with no deterioration in function in the hearts of hypertensive DSS rats (Okere et al., 2006b). However, Chess et al. (2009) showed that high-fat feeding did not increase cardiac TG in TAC mice, a more severe model of hypertrophy. The consequence of decreased FAO alongside preserved expression of CD36, may manifest as significant TG accumulation in hearts of the WD group, and reinforces the observed disturbances to the regulation of metabolism.

Increasing dietary sugar consumption in combination with a HFD led to lipid accumulation in the absence of cardiac dysfunction (Axelsen et al., 2010), supporting the observations in the present study. Interestingly, there was no evidence of neutral lipid accumulation in AC-WD hearts. This could potentially be explained by incorporation of lipids into the phospholipid membrane (Christoffersen et al., 2003) before droplets became visible through microscopy.

Indeed, incorporation of endogenous FAs into the phospholipid membrane has been shown to have differential effects upon membrane structure and subsequent enhanced susceptibility to peroxidation (Lemieux et al., 2011). The decrease in FAO observed in AC-WD hearts yet increased CD36 protein expression, may lead to a situation where uptake of FAs exceeds their rate of oxidation, hence promoting storage. The mechanisms behind the dysfunction observed in some experimental models following HFDs may ultimately relate to the fate of non-oxidised FA species.

4.4.6 Summary

In the present model, 9 weeks of high-fat or WD-feeding did not lead to changes in cardiac function. When combined with AC, both the HFD and WD improved cardiac function when compared to SD counterparts. WD hearts exhibited substantial metabolic changes, characterised by a reduction in FAO despite an increase in mitochondrial density, and a significant increase in TG content. Although function was not compromised, these metabolic changes may underpin the transition to HF via lipotoxicity.

Chapter 5 Cardiac lipotoxicity and ceramide content

5.1 Introduction

5.1.1 Cardiac lipotoxicity

Long-chain fatty acids remain the predominant source of ATP in the healthy adult heart; however pathology can result from a mismatch between the uptake and oxidation of FAs, increasing acyl-CoA species (figure 5.1) (Unger and Orci, 2002). The deleterious effects of increased lipid supply and accumulation has been described as lipotoxicity. Lipotoxicity in cardiac tissue has been observed in models of PPARα overexpression (Finck et al., 2002, Finck et al., 2003), disrupted FAO and starvation (Bakermans et al., 2011), short-term lipid infusion (Hexeberg et al., 1995), increased lipid uptake (Yagyu et al., 2003, Park et al., 2008), and HF (Sharma et al., 2004). Thus, dietary constituents may hold a crucial role upon the development of the lipotoxic phenotype.

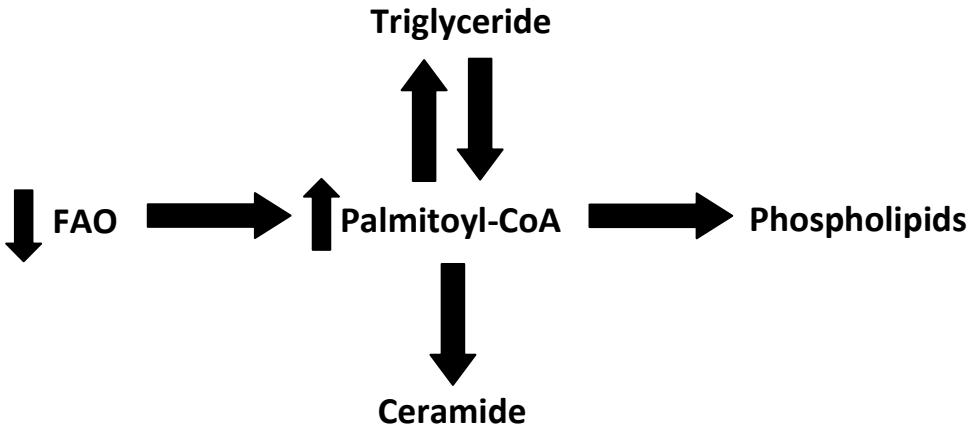

Figure 5.1 Consequences of increased cellular acyl-CoA. A decrease in the ability to oxidise FAs leads to the accumulation of acyl-CoA species and potential shuttling into triglyceride formation, phospholipid, or ceramide synthesis. Adapted from Unger (2005).

There have been widely documented reports of numerous FAs species behaving as inducers of cellular stress and apoptosis, including palmitate (Kong and Rabkin, 2000, Ostrander et al., 2001, Fauconnier et al., 2007), and stearate (Listenberger and Schaffer, 2002, Rabkin and Lodha, 2009). Importantly however, apoptosis associated with SFAs does not occur with oleate (de Vries et al., 1997). Indeed, it has been suggested that MUFAs can attenuate apoptosis associated with SFAs (de Vries et al., 1997, Van Bilsen et al., 1997, Miller et al., 2005b). Furthermore, apoptosis induced by palmitate can be attenuated by the addition of carnitine (Miller et al., 2005b), suggesting that reduced mitochondrial oxidation precipitates increased

apoptosis. This concept is further supported by the observation that despite similar levels of TG, high PUFA feeding reduces the number of apoptotic events associated with SFAs in hearts of Wistar rats (Okere et al., 2006a).

5.1.2 Palmitate as a mediator of cellular dysfunction

Cell death induced by palmitate has been regarded as an apoptotic process, evidenced by cytochrome c (cyt c) release into the cytosol, caspase activation and DNA laddering (Kong and Rabkin, 2000), although it has been demonstrated that palmitate may also induce necrotic cell death (Kong and Rabkin, 2003). Importantly, mitochondria appear to be central to cell death associated with palmitate in cardiomyocytes (Kong and Rabkin, 2000). Characteristically, palmitate induces mitochondrial dysfunction via disruption of membrane potential, induction of mitochondrial swelling, and release of cyt c (Sparagna et al., 2000, Ostrander et al., 2001). Cytosolic cyt c leads to activation of caspases and subsequent stimulation of apoptosis. Importantly, palmitate may potentially stimulate mitochondrial dysfunction via generation of ceramide, increased ROS production, and decreased cardiolipin synthesis (Kong and Rabkin, 2000, Ostrander et al., 2001, Fauconnier et al., 2007), although it is arguable as to whether ceramide formation is required for palmitate-induced cell death (Listenberger et al., 2001).

5.1.3 Ceramide synthesis

Sphingolipid (SL) synthesis and catabolism is a complex process involving several distinct but often overlapping metabolic pathways, ultimately producing a wide array of metabolic intermediates (figure 5.2). Importantly ceramides lie at the centre of this network due to their requirement as a precursor for the synthesis of more complex SLs.

Ceramide can be generated either through *de novo* synthesis requiring serine, palmitoyl-CoA and another fatty acyl-CoA species, or via recycling of more complex SL species, principally sphingomyelin (SM) (figure 5.2). *De novo* synthesis of ceramide begins with the condensation of palmitoyl-CoA with serine, catalysed by the rate limiting enzyme, serine palmitoyltransferase (SPT). The important intermediate of this pathway is dihydroceramide, formed via (dihydro)ceramide synthase. Dihydroceramide does not possesses any of the apoptotic properties associated with ceramide and can even hinder its activity (Siskind et al., 2006).

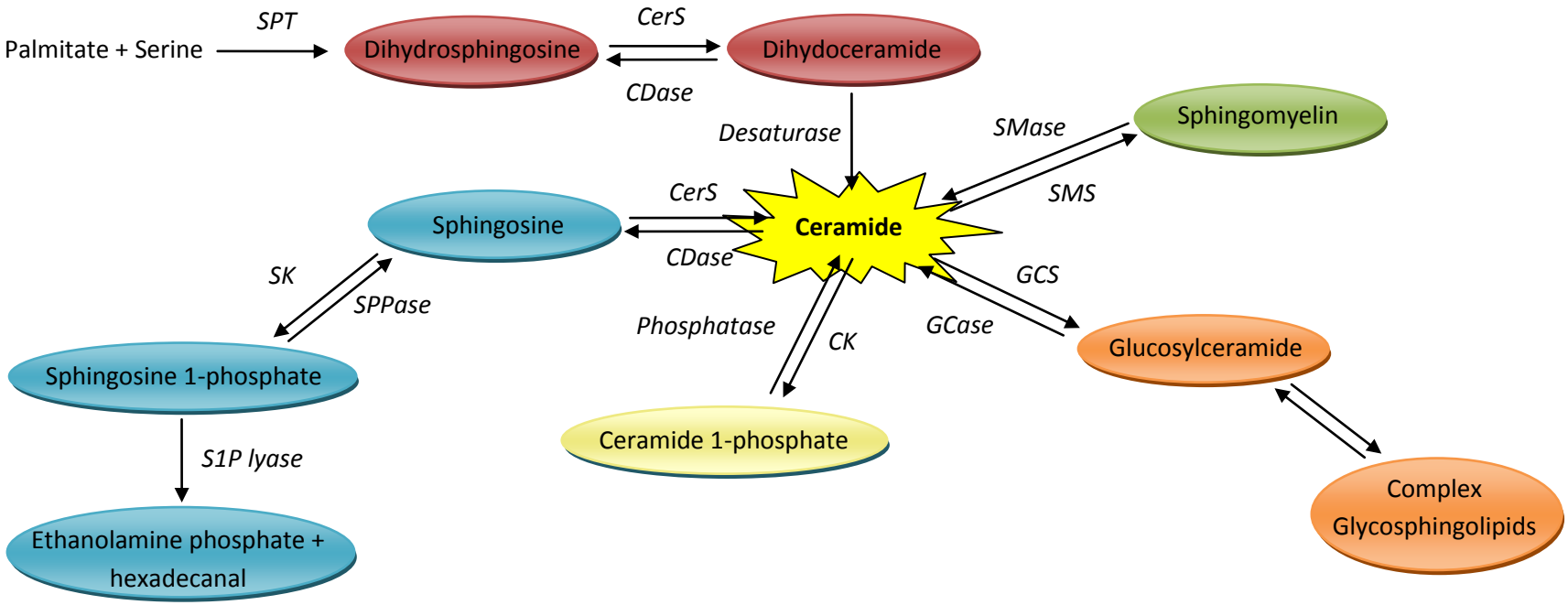

Figure 5.2 Principle pathways of sphingolipid synthesis and catabolism. Numerous pathways converge upon the formation of ceramide. Although ceramide can be converted to several different metabolites, the only exit from the sphingolipid series of reactions is via sphingosine-1-phosphate (S1P) lyase. CDase, ceramidase; CK, ceramide kinase; DAG, diacylglycerol; GCase, glucosyl ceramidase; GCS, glucosylceramide synthase; PC, phosphatidylcholine; SK, sphingosine kinase; SMase, sphingomyelinase; SMS, sphingomyelin synthase; SPPase, sphingosine phosphate phosphatase; SPT, serine palmitoyl transferase. Adapted from (Hannun and Obeid, 2008).

Ceramide is produced from dihydroceramide by desaturation via desaturase (figure 5.2). Additional sources of intracellular ceramide are hydrolysis of membrane SM via sphingomyelinases (SMases), or more complex SLs such as glucosylceramide and galactosylceramide, and from sphingosine. The only exit for ceramide is via the formation of sphingosine-1-phosphate (S1P) from sphingosine (SP) and subsequent cleavage via sphingosine-1-phosphate lyase (S1P lyase). Indeed, the balance between ceramide, sphingosine, and S1P may hold an important role in determining cell fate (Olivier, 2002).

5.1.4 Location of sphingolipid generation

The different pathways of ceramide synthesis and degradation have been shown to occur in different subcellular locations. Synthesis of ceramide via the *de novo* pathway occurs predominantly on the surface of the endoplasmic reticulum (ER) (Stiban et al., 2008), and on the surface of other membranes associated with the mitochondria, termed mitochondrial-associated membranes (MAM), whereas synthesis of the more complex SLs such as SM and GluCer occurs within the Golgi (Halter et al., 2007). ER-generated ceramide can potentially translocate to several different cellular locations, indicating the complexity of interpreting alterations in ceramide content.

5.1.5 Ceramide and cardiomyocyte apoptosis

Studies have suggested that ceramide accumulation in cardiomyocytes may be positively associated with apoptosis in *in vitro* (Bielawska et al., 1997, Dyntar et al., 2001) and i*n vivo* (Bielawska et al., 1997, Zhou et al., 2000, Zhang et al., 2001, Okere et al., 2006a) studies. Indeed, several key studies by Siskind et al. (2000, 2002) have demonstrated that ceramide can induce pore formation in mitochondrial membranes and mitochondrial fission (Parra et al., 2008) thus allowing intramembraneous cyt c release, and activation of the apoptotic cascade (Wang et al., 2000).

5.1.6 Objectives

The objective of this study was to determine the impact of pressure-overload hypertrophy and increased dietary lipid and sucrose upon cardiac long-chain ceramide formation. The most robust method for measuring individual ceramide species is mass spectrometry. However, an additional aim was to develop and optimise high-performance liquid chromatography (HPLC) to separate cardiac ceramide species for subsequent coupling with time of flight (TOF) mass spectrometry (MS).

5.2 Materials and methods

5.2.1 Identification of ceramide species

Stock solutions of ceramide standards were prepared in HPLC-grade ethanol at 5 µg/ml. Ceramides of longer acyl-chain length (C20 and C24) required warming to solubilise at this concentration. Experiments to optimise MS acquisition parameters were performed using C16, C17, and C18 ceramides. Solutions of 50 ng/µl were prepared and injected into the mass spectrometer (Applied Biosystems API QSTAR Pulsar i) using a Hamilton-Microsyringe, an infusion pump and a TurboIonSpray electrospray ionisation (ESI) source at 10 µl/min. Spectra were acquired in the positive ion (+) mode. Optimised MS parameters for maximal peak detection were as follows: ion source (IS) voltage: 5000 V; temperature: 400°C; gas source 1 and 2 (GS1 and GS2): 30 and 50, respectively; curtain gas (CUR) 20.0; declustering potential (DP): 45 V; focussing potential (FP): 145 V; DP2: 15 V; collision gas (CAD): 3.0; ion release delay (IRD): 6.0; ion release width (IRW): 5.0; pulse duration was set at 8µs. The mass spectrometer was set to record ions with a mass-to-charge ratio (m/z) from 400 to 600 (based upon the ceramide standard molecular weights).

Initial experiments revealed two major ions (figure 5.3), the higher m/z corresponding to the singly charged molecular ion $(M+H)^+$ and the lower m/z corresponding to a fragmentation ion due to the loss of water $((M-H_2O)+H)^+$. Two other minor ions, 1 mass unit higher than the major ions, were also observed. These correspond to ions containing 1 atom of ^{13}C (due to the natural isotopic abundance of ^{13}C).

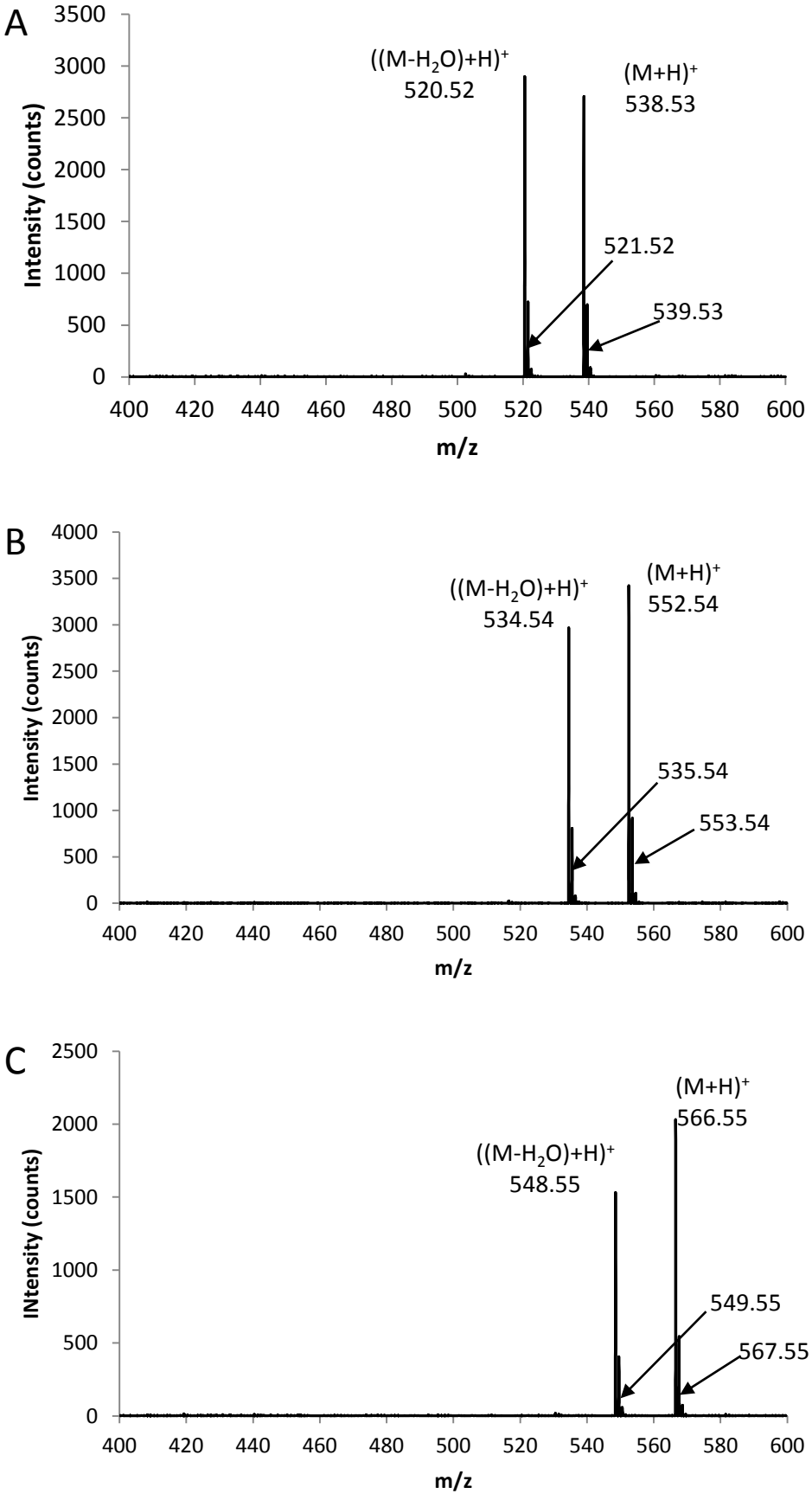

Figure 5.3 Positive ESI-MS spectra of ceramide species. A, C16 ceramide; B, C17 ceramide; C, C18 ceramide.

5.2.2 HPLC separation, LC-MS and LC-MS/MS of ceramide species

HPLC experiments were performed using an Agilent 1100 system fitted with a C_{18} 5 µm 150 x 3.2 mm column (Phenomenex, UK) at 40°C in a column oven. The initial HPLC mobile phase mixtures were based on those described by Merrill et al. (2005) and comprised of solvents A [containing methanol, water, and formic acid (74:25:1 v/v/v)] and solvent B [containing methanol and formic acid (99:1 v/v)]. Both solvent A and B contained ammonium formate (5mM). Ceramide standards were prepared in 0.5 ml A:B (20:80 v/v) in glass autosampler vials (Agilent, UK). 5 µl samples were used for preliminary studies, starting with a composition of 20:80 (v/v) A:B. Solvent B was increased to 100% over 8 min and maintained at this level for 2 min, after which was returned to 80% with a flow rate of 0.5 ml/min throughout.

The HPLC was coupled to a QSTAR tandem mass spectrometer using the TurboIonSpray source, set at 5400 V. MS settings were optimised for HPLC separation; DP and FP were reduced to 30 V and 130 V, respectively with an acquisition time of 15 minutes. For MS/MS experiments, quadrupole 2 (Q2) was set to fragment selected ions from quadrupole 1 (Q1). This collision-induced dissociation (CID) was performed to aid molecular identification of ceramide species and their fragmentation patterns. It is known that the ions m/z 262.5 and 264.5 can be generated from SLs, corresponding to major ion fragments from sphingadienine and sphingosine, respectively (Colsch et al., 2004). Only the m/z 264.5 ion was of interest in this study due to its association with ceramide. GS2 was increased to 80 and CUR to 30. Collision gas was increased to 6 and collision energy was set at 25 V (expt2) and 40 V (expt3). The mass spectrometer was set to record ions with an m/z from 100 to 600 to capture smaller fragmentation ions.

Results using the Phenomenex column with separate injections of C16, C17, and C18 ceramide indicated good separation between components (figure 5.4). C16 eluted at 8.36 min, C17 at 9.63 min and C18 at 10.93 min. Furthermore, these standards gave identical ESI-MS spectra to those previously obtained (figure 5.3).

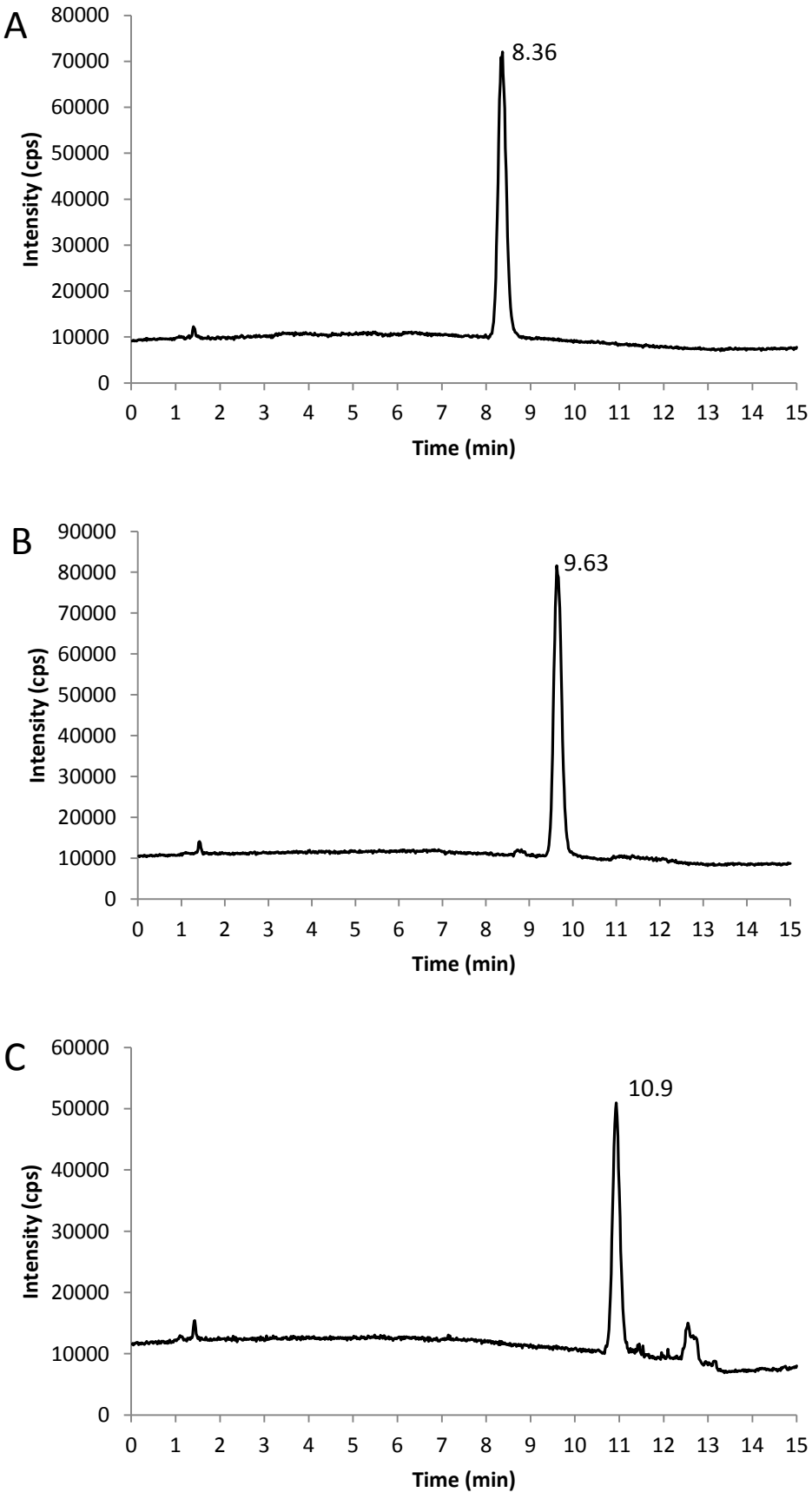

Figure 5.4 LC-MS TIC for C16, C17, and C18 ceramide species. A) C16 ceramide; B) C17 ceramide; C) C18 ceramide.

All ceramide species analysed by LC-MS/MS produced a characteristic fragment with an m/z ratio of 264 (figure 5.5) with complete absence of the $(M+H)^+$ ion. During MS/MS scans, precursor ions ($(M+H)^+$) were selected in Q1 and fragmented in Q2 using the collision gas. These fragments were transferred into the TOF analyser and recorded. In expt3, the greater collision energy in Q2 reduced the appearance of the $((M-H_2O)+H)^+$ and increased the intensity of the 264 fragment (data not shown).

To confirm that the separation on HPLC could separate compounds in cardiac tissue, a ceramide mixture was prepared containing equal amounts (30 ng/µl) of C8, C16, C17 and C18 (figure 5.6). Analysis of the TIC showed clear separation of the four ceramide standards (figure 5.6), with C8 ceramide eluting at 2.66 min. All other ceramide standards were detected at similar times to those described in figure 5.6.

Larger ceramide species (C20 and C24) were also analysed within the same 15 minutes. The initial step in achieving this was replacement of the Phenomenex column with an ACE C_{18} column (Advanced Chromatography Technologies, 5 µm, 100 x 3 mm). Employing a modification of the Merrill gradient combined with the ACE column, increased the speed at which the ceramide standards eluted from the column without affecting peak separation, and hence allowed a greater window of analysis post C18 ceramide. Specifically, C8 eluted at 1.86 min, C16 at 4.20 min, C17 at 4.81 min, and C18 at 5.72 min.

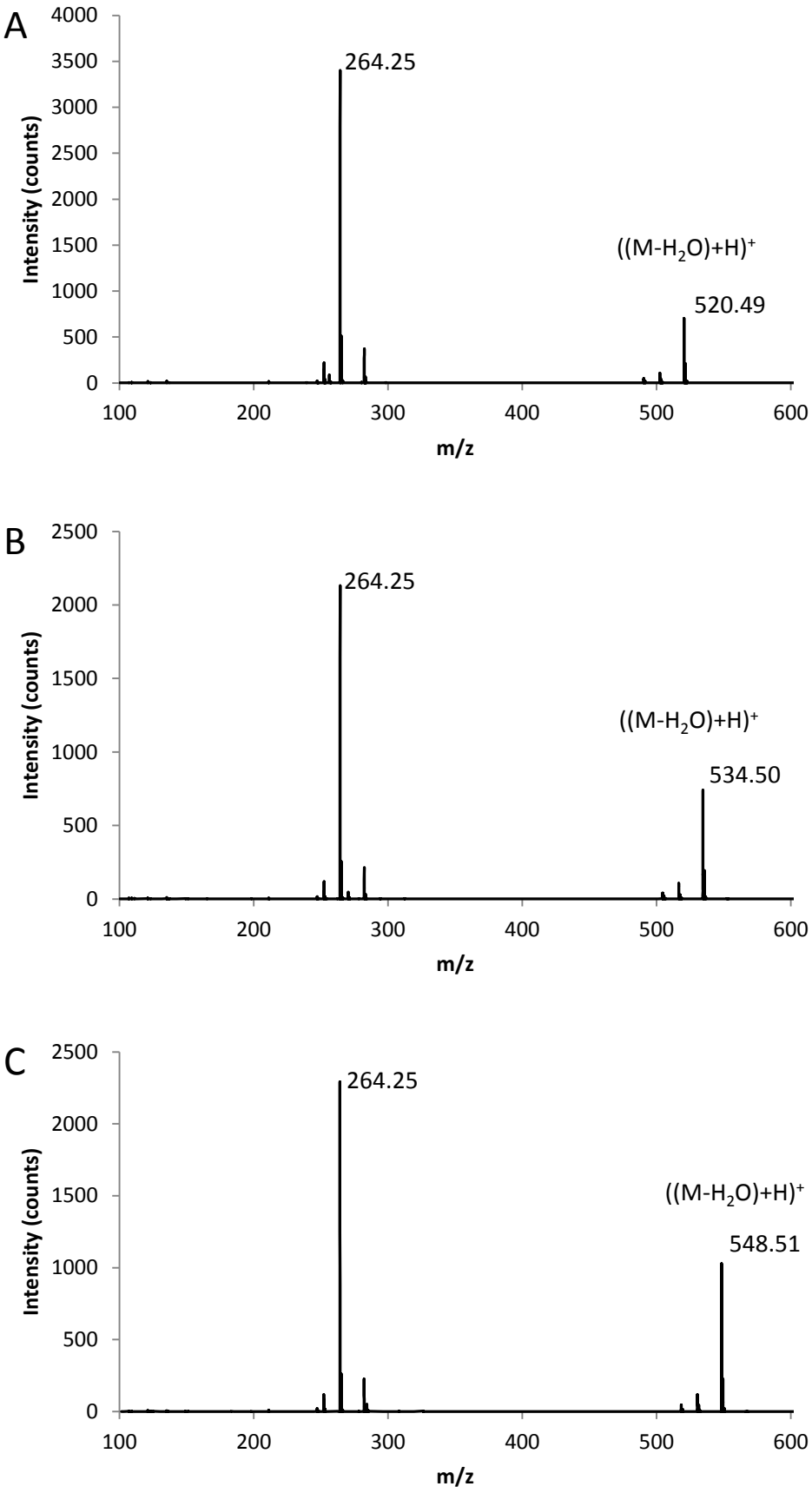

Figure 5.5 Product ion scan of C16, C17 and C18 ceramide species. A) C16 ceramide (precursor ion m/z 538.53); B) C17 ceramide (precursor ion m/z 552.54); C) C18 ceramide (precursor ion m/z 566.55).

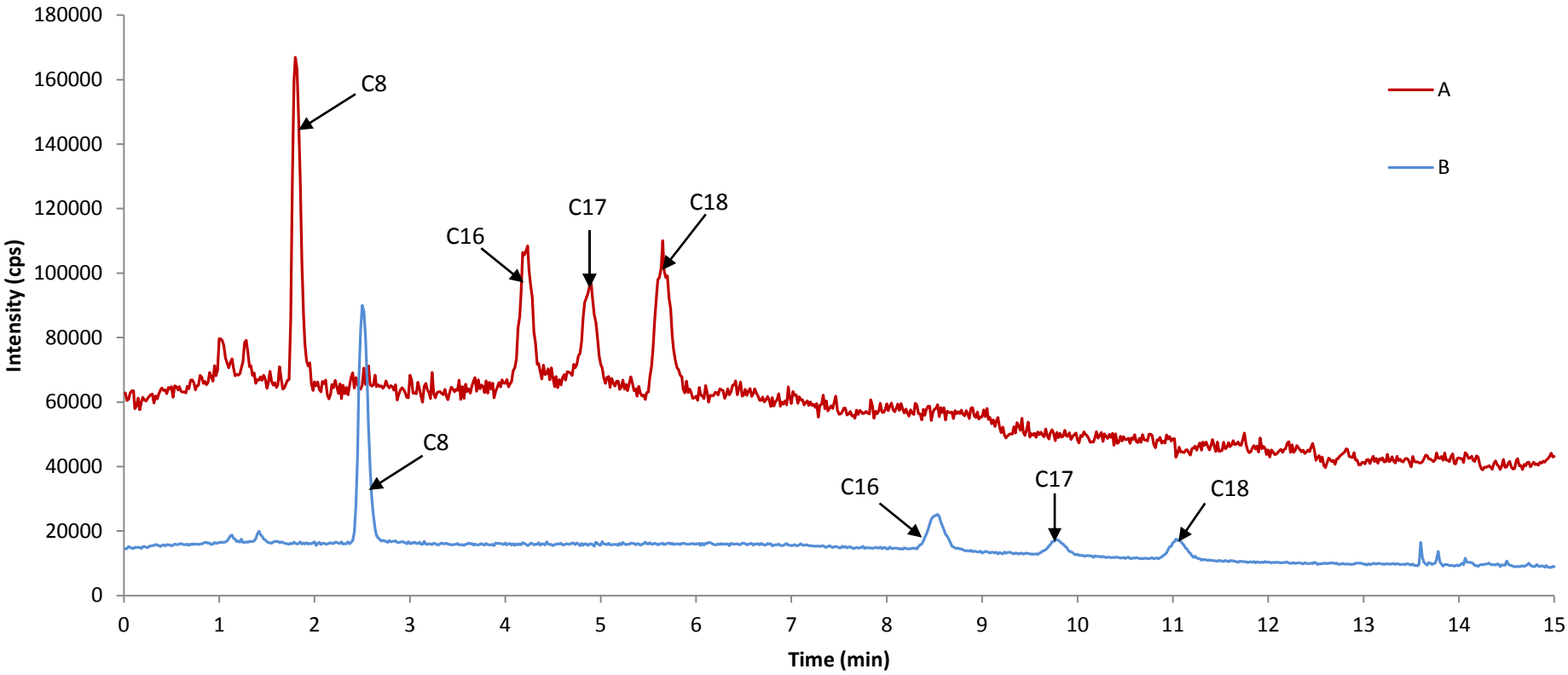

Figure 5.6 LC-MS TIC of ceramide standard mixtures separated on different HPLC columns. A: modified gradient (as shown in figure 5.2) with ACE C_{18} 5 µm 100 x 3 mm (Advanced Chromatography Technologies, UK) ; B: modified gradient with C_{18} 5 µm 150 x 3.2 mm column (Phenomenex, UK).

Following optimisation of the HPLC gradient system, C20 and C24 ceramide were initially analysed individually. Both ceramide species were readily observable (figure 5.7, A-B) and produced the characteristic two-ion pattern ($(M+H)^+$ and $(M-H2O)+H)^+$) exhibited by all other ceramide species analysed (figure 5.7, C-D). Fragmentation of C20 and C24 ceramide also yielded the characteristic m/z 264 fragment (figure 5.8).

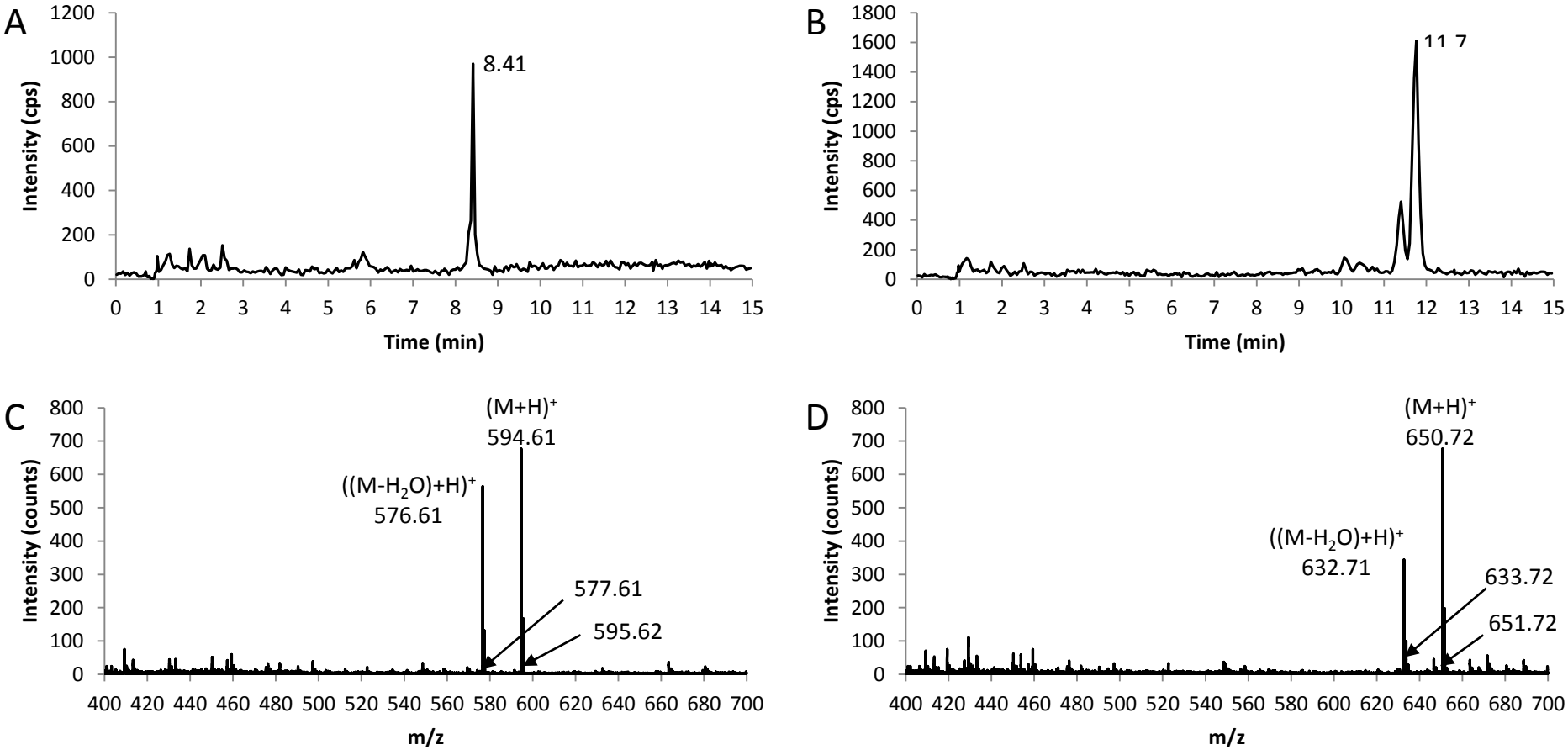

Figure 5.7 LC-MS spectra for C20 and C24 ceramide. A) TIC for C20; B) TIC for C24. C) LC-ESI-MS spectra for C20 ceramide; D) LC-ESI-MS spectra for C24 ceramide.

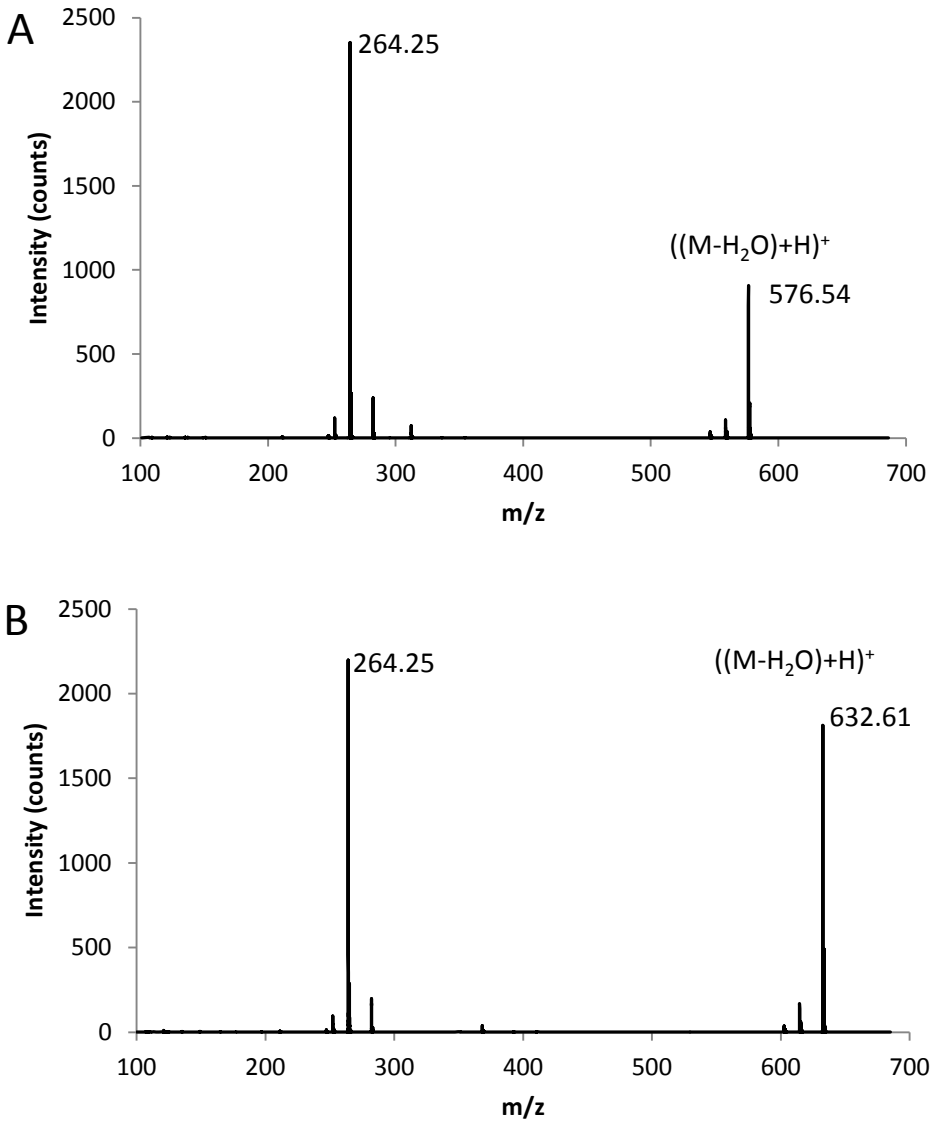

Figure 5.8 Product ion scan of C20 and C24. A) C20 ceramide (precursor ion m/z 594.61); B) C24 ceramide (precursor ion m/z 650.72).

5.2.3 Quantitation of ceramide species

A mixture of ceramide standards ranging from C16-C24 was prepared to determine adequate peak separation within a 15 minute period (figure 5.9). Mixtures of C16, C18, C20, and C24 ceramide standards were prepared from original stock solutions at concentrations ranging from 1ng/µl to 30 ng/µl. C17 ceramide was included as an internal standard (IS) at a concentration of 25 ng/µl.

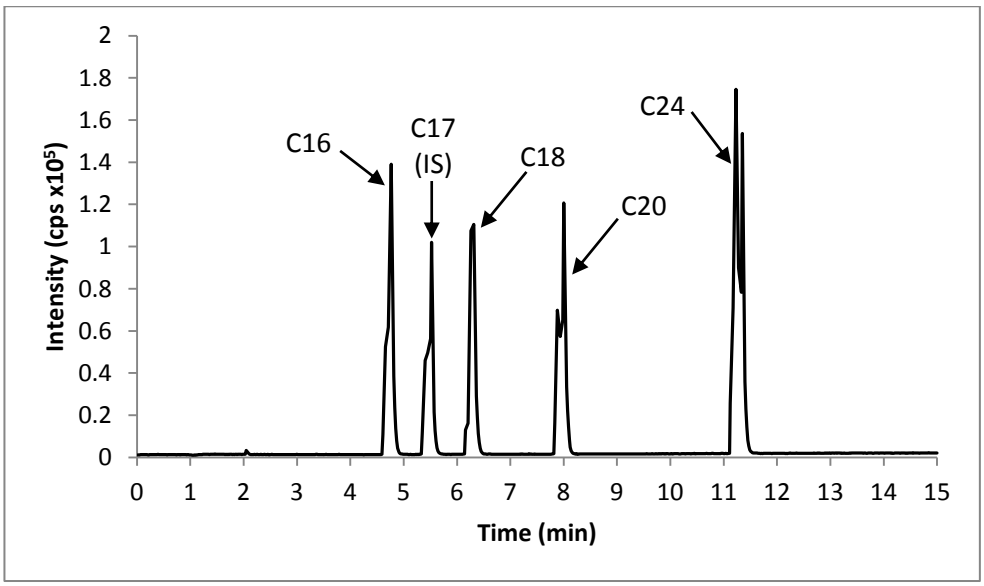

Figure 5.9 Example of LC-MS TIC mixture of ceramide standards. Separation was performed using the gradient profile described in figure 5.2. Naturally occurring ceramides, C16, C18, C20 & C24, at 30 ng/µl; internal standard, C17, at 25 ng/µl.

For quantitation purposes, the peak area of the analyte was normalised to the peak area of the IS. Both peak areas were obtained by integration, with manual adjustment, using the quantitation routine provided within Analyst QS (version 1.1). Subsequently, the ratio of the analyte peak area to IS peak area (APA:ISPA) was used to generate a series of standard curves (figure 5.10).

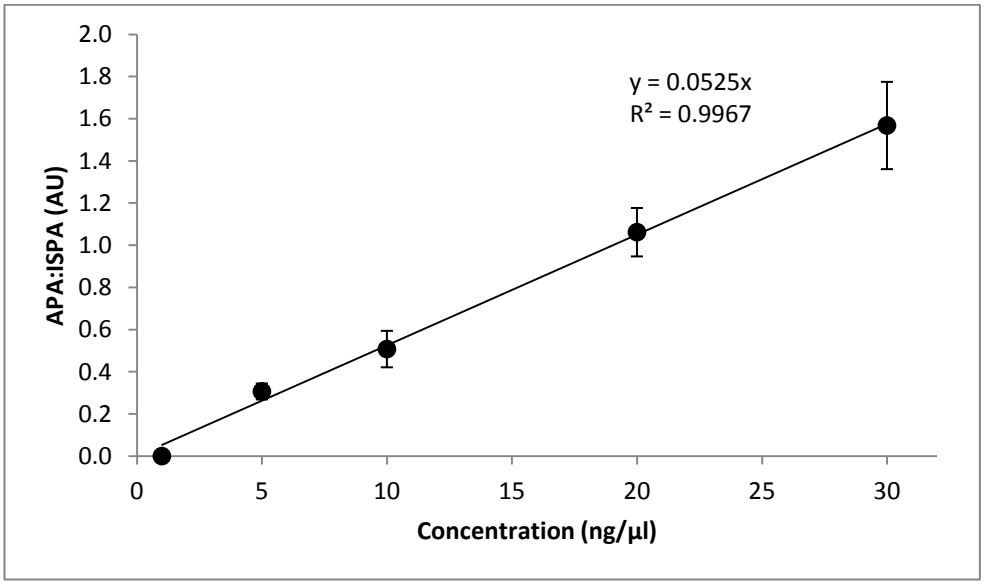

Figure 5.10 Example standard curve for C16 ceramide. Analyte peak area (APA) was normalised to the internal standard peak area (ISPA). Each point is the average of 4 readings.

5.2.4 Experimental groups

48 hours post-surgery, animals were assigned to dietary regimens as described in section 2.2.3:

- Control + standard chow (Con-SD)
- Control + high-fat diet (Con-HFD)
- Control + western diet (Con-WD)
- Aortic Constriction + standard chow (AC-SD)
- Aortic Constriction + high-fat diet (AC-HFD)
- Aortic Constriction + western diet (AC-WD).

Separate experiments were performed using an established model of HF induced using adriamycin (ADR) (Lu et al., 2009). Animals were maintained on a WD for 9 weeks but were dosed with 6 i/p injections of ADR from weeks 7 to 9 (2.5 mg/kg for a cumulative dose of 15 mg/kg) (Lu et al., 2009). Control animals were dosed with equal volumes of sterile isotonic saline

After 9 weeks, animals were anaesthetised, hearts were excised, rinsed, freeze-clamped and processed as described in 2.2.10.

5.2.5 Sample preparation for analysis of ceramides by LC-MS

300 mg of powdered cardiac tissue was homogenised in 1ml of PBS (pH 7.4) for 10 x 30 s using an Ultra Turrax homogeniser at maximum speed at 4°C. The homogenate was sonicated using a probe-type sonicator for 2 x 5 s. From this, 0.1 ml was transferred to a clean 10-SV screw-topped glass vial (13 mm x 100 mm, Chromacol, UK) and prepared as described by Merrill et al. (2005). Briefly, 0.75 ml of chloroform:methanol (2:1 v/v) was added and the sample mixed thoroughly. 10 µl C17 internal standard (500 ng/µl) was added to each tube and samples heated at 48°C for 14 h. 75 µl of 1M KOH in methanol was added to each vial, sonicated for 15 min, and incubated at 37°C in a waterbath for 120 min. Samples were centrifuged at 760 x g for 10 min at room temperature, the supernatant transferred to glass autosampler vials (Agilent, UK), and dried using a SC210A SpeedVac Plus concentrator (Thermo-Scientific, UK). Samples were resuspended in 0.5 ml 50% methanol.

5.3 Results

All morphological characteristics of the experimental groups were similar to those previously observed in chapter 3.

5.3.1 Cardiac ceramide content

No significant difference in any ceramide species analysed was observed in control groups fed a SD or HFD (figure 5.11). Following WD intervention, levels of the 4 ceramide species assayed were significantly increased when compared to SD and HFD counterparts (figure 5.11). No significant difference was observed in any ceramide species analysed between AC-SD and AC-HFD groups (figure 5.11). However, the combination of AC and WD led to a significant increase in all ceramide (figure 5.11). No intra-group differences were observed.

A common ceramide profile was observed in all experimental groups, with C18 and C24 ceramides being the most predominant species observed (figure 5.11). WD feeding had the most pronounced effect upon C18 ceramide (figure 5.11).

5.3.2 Cardiac ceramide content in adriamycin-administered hearts

A significant increase in cardiac C16 and C18 ceramide content was observed following ADR administration but no change in C20 and C24 ceramide (figure 5.12).

5.3.3 Total cardiac ceramide content

Total cardiac ceramide content was not affected following HFD intervention, or AC alone (figure 5.13). WD feeding augmented total ceramide content in both Con and AC hearts, potentially due to increased C18 and C24 (figure 5.13). Total ceramide content was significantly elevated in hearts of WD animals compared to other dietary groups, in part due to increased C16 and C18 ceramide content (figure 5.13).

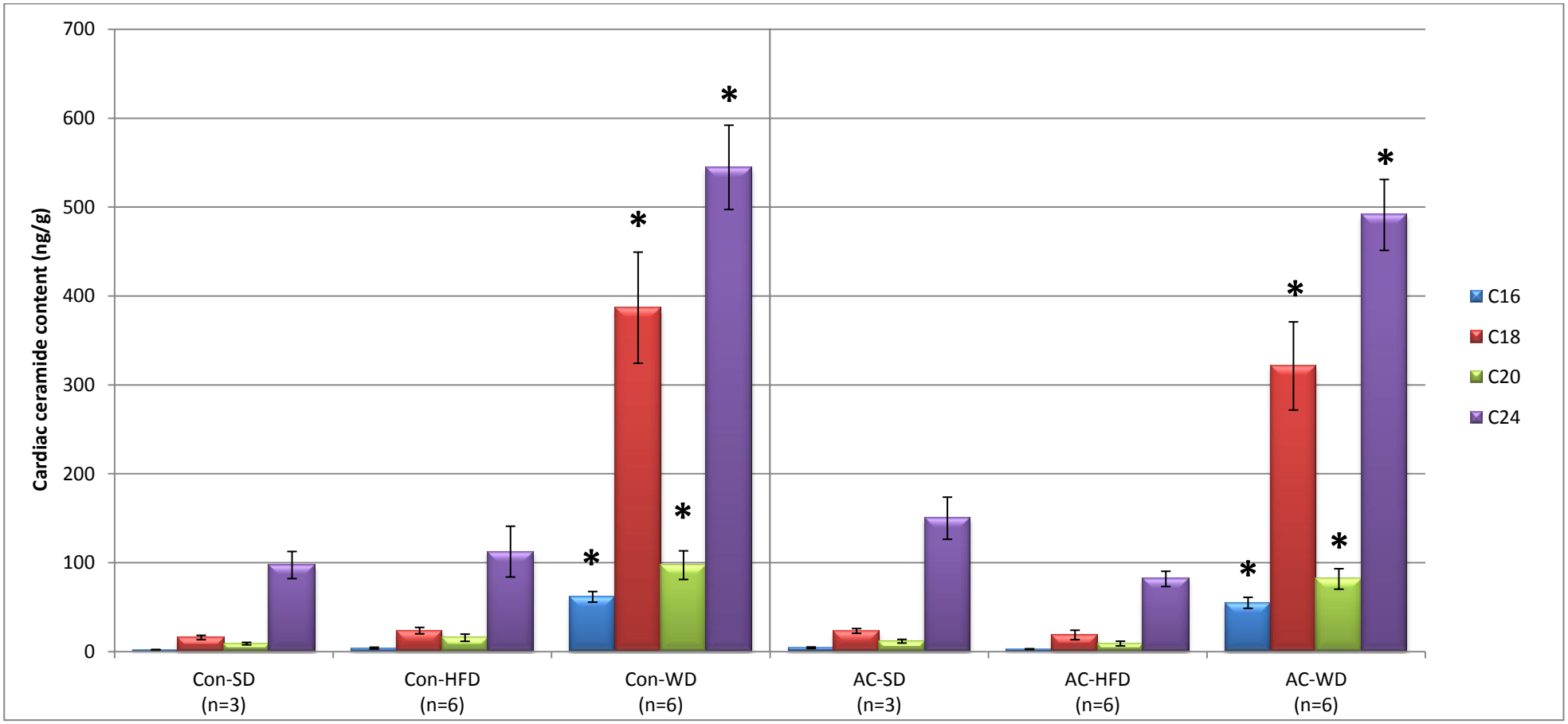

Figure 5.11 Alterations to cardiac ceramide content following 9 weeks dietary intervention or aortic constriction. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD, western diet. *$P < 0.001$ vs. SD and HFD counterparts. Data show mean ± SEM.

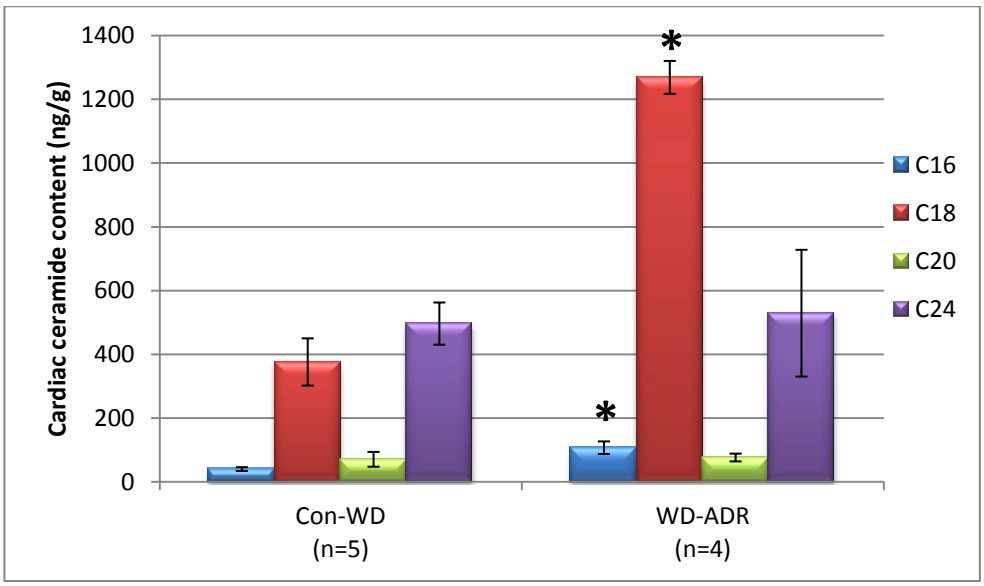

Figure 5.12 Changes in the cardiac ceramide profile following dietary intervention and adriamycin administration. Con-WD, control western diet; WD-ADR, western diet + ADR administration. *P < 0.01 vs. same ceramide species in Con-WD group. Data show mean ± SEM.

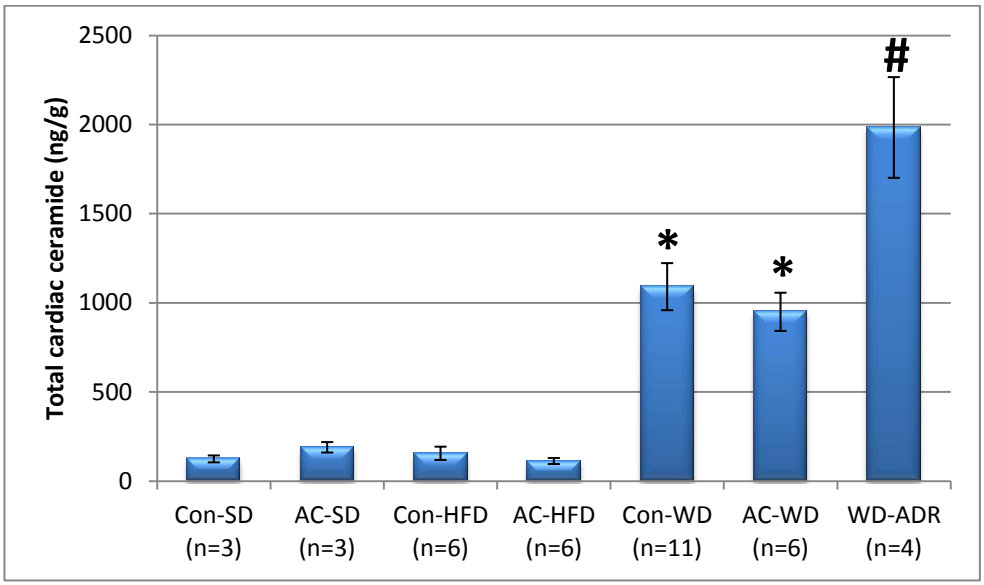

Figure 5.13 Total cardiac ceramide (C16-C24) content following dietary intervention, aortic constriction, and adriamycin administration. Con, control; AC, aortic constriction; SD, standard diet; HFD, high-fat diet; WD; WD-ADR, western diet; ADR, adriamycin.*P < 0.001 vs. SD and HFD counterparts, # P < 0.05 vs. SD, HFD, Con-WD, and AC-WD. Data show mean ± SEM.

5.3.4 Physiological data of adriamycin-administered animals

By the end of the dosing protocol, administration of ADR to rats led to a deterioration in health including lethargy, poor coat condition, and porphyrin staining around the nose and eyes.

HW:BW and HW:TL were significantly reduced following ADR treatment (table 5.1). No difference was observed in the degree of lung fluid content, EAT or RPAT, or kidney weights between the Con and ADR-administered group (table 5.1).

Table 5.1 Physiological characteristics of adriamycin-administered animals

	Con-WD (n=5)	WD-ADR (n=4)
Terminal Body weight (g)	552 ±14	544 ±12
Tibia length (cm)	4.3 ±0.06	4.6 ±0.09
Heart weight (g)	2.05 ±0.09	1.87 ±0.15
HW:BW (g/kg)	3.73 ±0.21	3.45 ±0.11
HW:TL (g/cm)	0.47 ±0.03	0.40 ±0.02*
Lung wet wgt:dry wgt (%)	75 ±2	78 ±3
EAT (g)	11.5 ±0.6	12.4 ±0.9
RPAT (g)	16.5 ±0.8	14.0 ±1.7
L K:R K	1.0 ±0.0	1.0 ±0.0

Con-WD, control western diet; WD-ADR, western diet + adriamycin treatment, HW:BW, heart weight-to-body weight; heart weight-to-tibia length, HW:TL; EAT, epididymal adipose tissue; RPAT, retroperitoneal + perirenal adipose tissue; L K:R K, left kidney:right kidney ratio. * $P < 0.05$ vs. Con-WD. Data show mean ± SEM.

5.4 Discussion

The present study indicates that dietary manipulation has a significant impact upon cardiac ceramide generation. Furthermore, this remodelling is most apparent in hearts exposed to a WD, with an increase in all ceramide species measured. Importantly, the dietary effect of ceramide accumulation was not exacerbated by AC, suggesting that, at this stage of LVH, WD *per se* exerts greater influence upon the cardiac ceramide pool. Importantly, WD animals also exhibited the most pronounced metabolic remodelling, characterised by reduced FAO and increased TG accumulation (chapter 4, sections 4.3.4 and 4.3.5). Interestingly, no significant impact was observed in the HFD group when compared to the SD-fed animals (figure 5.12). The mechanisms underlying the increase in ceramide content following oversupply of FAs may involve increased activation of PPARα via FA ligands, or disruption to β-oxidation in the face of unaltered uptake. This latter element is particularly important in organs with a high turnover of FAs, such as the heart (Schaffer, 2003).

5.4.1 Cardiac ceramide changes following dietary intervention

9 weeks of WD-feeding led to a significant increase in all ceramide species measured and elevated total cardiac ceramide content when compared to other dietary groups (figures 5.12 and 5.13). In support of the deleterious impact of WD, Steinbusch et al. (2011) have shown that 16 weeks of WD-feeding in mice led to the accumulation of lipid and ceramide. Importantly, this accumulation of ceramide was attenuated with deletion of CD36 (Steinbusch et al., 2011), suggesting that ceramide accumulation may be a product of enhanced FA uptake in the face of altered FA metabolism. However, others have shown that longer WD-feeding protocols (up to 48 weeks) do not affect cardiac ceramide content (Harmancey et al., 2010). Consequently, the mechanisms by which diet is able to regulate ceramide formation require further investigation.

Ceramide metabolism has been more widely studied in response to HFDs. In mice with cardiac-specific overexpression of PPARα, 8 weeks of a HFD increased cardiac TG accumulation and enhanced ceramide content despite upregulation of β-oxidation (Finck et al., 2003). This study found no evidence of apoptosis despite significant ventricular dysfunction. Similarly, others have suggested that heightened levels of ceramide are associated with increased DNA laddering, apoptosis, and a decline in cardiac function (Zhou et al., 2000, Sharma et al., 2004); findings which are in contrast to the impact of a WD upon function in the current study

(chapter 4, section 4.3.1). Thus, modulating lipid metabolism may be an important way in which ceramide synthesis is regulated.

Further support for the importance of PPARα in regulating ceramide synthesis is offered by Baranowski et al. (2007). Pharmacological activation of PPARα with WY-14643 (a synthetic PPARα ligand) or a high-PUFA diet increased all long chain ceramide species assayed (C14-C24). Stimulation of PPARα combined with the high-fat feeding increased SPT activity, but not in animals not receiving WY-14643. However, a potential explanation for the increased ceramide content from exogenous PPARα stimulation and high PUFA feeding is suggested by the observation that the high PUFA diet reduced nCDase, aCDase, and alCDase expression, thereby attenuating the clearance of ceramide (figure 5.2) (Baranowski et al., 2007). Consequently, elevated dietary lipid may lead to ceramide formation, but may also alter the rate of its clearance from the cell. Indeed, this concept is interesting as the HFD and WD used in the present study contained the same percentage of fat, suggesting that the cardiac metabolic profile promoted by WD-feeding may be the sole driver of ceramide accumulation.

Further complicating the relationship between intracellular TG and ceramide are studies which suggest dietary FAs can differentially influence ceramide content independently of TG concentration (Okere et al., 2006a). Specifically, a reduction in C16 ceramide content was observed with a 60% MUFA/PUFA diet (composed of oleic and linoleic), with no change in TG content. Conversely, 60% SFA-feeding raised TG and ceramide content compared to the MUFA/PUFA diet group with a concomitant increase in the number of apoptotic events (Okere et al., 2006a). Additionally, high-fat feeding in mice for 12 weeks induced a near 2-fold increase in ceramide without increasing cardiac TG (Sung et al., 2011). Interestingly, the accumulation of ceramide did not impair cardiac function, consistent with the present observations (chapter 4, section 4.3.1).

Increasing SFA may facilitate elevated flux through SPT and ceramide generation via the de novo pathway. In support of this, high PUFA-feeding alone did not increase SPT activity in rabbit hearts (Baranowski et al., 2007). Indeed, in mice with cardiac-specific overexpression of LPL, deletion of the LCB1 subunit of SPT prolongs survival and improves systolic function (Park et al., 2008). In contrast, wildtype mice are not protected from increased de novo ceramide formation, and the excess FAs generated via the overexpressed LPL are shuttled into ceramide

formation (Park et al., 2008). Using rapeseed oil feeding for 20 weeks Dewailly et al. (1977) demonstrated a two-fold increase in cardiac SM content, through enhanced incorporation of euricic acid (+10.5%). Similarly, Riediger et al. (2008) noted substantial changes in phospholipid composition, including changes to SM following feeding with *n*-3 PUFAs. Consequently, susceptibility of the membrane to diet-induced changes may lead to the potential for generation of ceramide via the action of SMases (figure 5.2).

The present study showed that of the ceramide species measured, C24 ceramide was the most abundant (figure 5.12), consistent with previously observations in interfibrillar and subsarcolemmal mitochondrial membranes, and cardiac tissue homogenates from standard chow-fed rats (Monette et al., 2010). In contrast, Baranowski et al. (2007) observed that C16 and C18 ceramide were the most predominant species, irrespective of diet. However, their study also demonstrated that ceramide remodelling extended far beyond the traditionally studied C16 ceramide (Okere et al., 2006a), supporting the concept that dietary influences can affect many different ceramide species. Ceramides of varying chain length can also be generated via the activities of specific (dihydro)CerS isoforms, and by utilisation of dihydrosphingosine derived from recycling of endogenous SM and varying fatty acyl-CoAs, ranging from C14-, C16-, C18-, C20-, and C24-acyl-CoA (Mizutani et al., 2005). The contribution of SM derived ceramide precursors remains to be determined, but nSMase activity was elevated in hearts of rats subjected to MI, alongside a decrease in Bcl-2 protein content and increased cleavage of caspase 3 indicative of apoptosis (Adamy et al., 2007). Mitochondrial hydrolysis of SM by targeted expression of bacterial SMase led to increased cyt c release and subsequent apoptosis (Birbes et al., 2001). In addition, the presence of ceramide rafts in the plasma membrane (Silva et al., 2007) also raised questions regarding the specific location of ceramide synthesis/accumulation. Importantly, ceramide formation must be a tightly regulated process so as not to induce apoptosis. Cell death associated with ceramide accumulation may therefore be influenced by the rate of ceramide degradation to an inactive form, or by the metabolism to S1P, itself an important anti-apoptotic molecule (Maceyka et al., 2002).

5.4.2 Cardiac ceramide changes following dietary intervention and AC

AC did not affect the ceramide profile irrespective of the dietary intervention compared to controls (figure 5.11). However, AC-WD hearts exhibited a significant increase in the levels of all ceramide species measured (figure 5.11), although this was not different to their respective

controls. Furthermore, total ceramide was increased in AC-WD hearts, similar to the Con-WD group, indicating that at this stage in disease progression, WD may be more detrimental to cardiac health than LVH (figure 5.11).

There have been few studies recording the changes in ceramide content in the hypertrophied heart when challenged with increasing dietary fat consumption. However, it has previously been shown that increased activity of SPT could lead to cardiac hypertrophy, ventricular dilation, and depressed function in LPL transgenic hearts (Park et al., 2008). Indeed, this would suggest that oversupply of lipid to the heart could induce systolic dysfunction and ceramide generation. However, following 45% fat feeding, ceramide content is not significantly different to control animals, despite a significant increase in tissue TG (Morgan et al., 2006b). Moreover, C16 ceramide content is elevated in the hearts of low-fat/high salt fed rats treated with the CPT1 inhibitor, oxfenicine (Okere et al., 2006b), suggesting that a decrease in mitochondrial FAO might lead to the diversion of acyl-CoA species from oxidation into the formation of sphingolipids. Indeed, increasing SFA supply (60% of daily calories) to rats with HF doubles C16 ceramide content and increases TG levels, but had no impact upon function (Rennison et al., 2009), supporting the observations in the present study. Recent studies, examining the impact of WD feeding upon cardiac ceramide levels, support the current data. 16 weeks of WD feeding in mice subjected to TAC produces a significant increase in cardiac ceramide (Steinbusch et al., 2011). Importantly, this study also demonstrated that both sham and TAC-operated animals showed similar levels of ceramide levels, recapitulating the present data (Steinbusch et al., 2011). Thus, similar to the control group, WD-feeding infers a greater degree of SL remodelling, whether imposed upon AC or not.

5.4.3 Cardiac ceramide changes following adriamycin administration

The present study demonstrated that ADR treatment in combination with WD led to significant alterations in the cardiac ceramide profile (figure 5.12). Specifically, this was characterised by an increase in C16 and C18 ceramide, with other ceramide species assayed remaining unchanged (figure 5.12). Consequently, ADR-administered animals had the greatest degree of ceramide accumulation of all experimental groups (figure 5.13).

The elevation in C18 ceramide has been recently been shown to be a novel serum marker of tumour response to ADR and Gemcitabine treatment (Saddoughi et al., 2011). In particular,

serum C18 ceramide correlates positively with tumour response, whereas no relationship is observed with C16 ceramide (Saddoughi et al., 2011). Consequently, elevated levels of C18 ceramide in the hearts of WD-ADR rats may lead to increased vacuolisation and apoptosis (Lu et al., 2009). In addition, increased lipid consumption sensitises the heart to ADR-induced toxicity via increased oxidative stress and impaired ATP synthesis (Mitra et al., 2008)

The mechanisms underlying the generation of intracellular ceramide in response to ADR treatment are conflicting. ADR administration amplified the activity of aSMase with a subsequent decrease in SM and increase in ceramide in adult rat cardiomyocytes (Andrieu-Abadie et al., 1999). This suggested that enhanced liberation of SM was in part responsible for the increased ceramide formation in the hearts of ADR-administered rats. Conversely, ceramide could also be generated via the de novo pathway in response to ADR treatment (Rath et al., 2009). C18 ceramide is preferentially elevated by ADR treatment via specific increased expression of human longevity assurance gene 1 (LASS1) mRNA [encoding (dihydro)CerS which generates C18 ceramide] (Senkal et al., 2007). Furthermore, when de novo synthesis of ceramide is blocked by the addition of fumonisin B1 [FB1, an inhibitor of (dihydro)CerS], ceramide levels are not enhanced following ADR treatment (Rath et al., 2009), suggesting ceramide might also be increased via hydrolysis of SM. Importantly, these authors noted that neither nSMase and aSMase were affected by ADR treatment, confirming the importance of the de novo pathway (Rath et al., 2009), in contrast to the study of Andrieu-Abadie et al. (1999). Thus, depending upon the model used, ADR treatment may lead to differential activation of ceramide synthesis pathways.

5.4.4 Mechanisms for ceramide-induced cellular dysfunction

Ceramide has a wide range of cardiac effects, including interaction with the respiratory chain of mitochondria (Gudz et al., 1997, Di Paola et al., 2000), membrane permeabilisation (Siskind and Colombini, 2000, Siskind et al., 2002, Siskind et al., 2006), involvement in mediating the effects of ROS production (Hernandez et al., 2000), and mitogenic signalling via regulating the phosphorylation state of Akt and glycogen synthase kinase 3 beta (GSK-3β) (Park et al., 2008). In addition, isolated cardiomyocytes incubated with S1P develop hypertrophy characterised by an increase in cell size, enhanced incorporation of phenylalanine, and expression of brain natriurietic peptide (BNP) (Robert et al., 2001). Furthermore, inhibition of the S1P receptor indicates that the S1P hypertrophic response requires G_i coupled signalling pathways, with activation of MAPKs, Akt, and p70S6K (Robert et al., 2001).

Ceramide activates cardiac serine/threonine protein phosphatases (PP1 and PP2A) (Puthanveetil et al., 2011). Interestingly, the finding that increased dietary lipid manifests heightened PP2A expression (Puthanveetil et al., 2011) is not seen in cardiac tissue from rats fed a high-sucrose diet (Vasanji et al., 2006) suggesting that ceramide-activated protein phosphatases (CAPPS [PP1 and PP2A]) activity may be related to dietary lipid content.

Cardiac c-Jun N-terminal kinases (JNKs) are activated by ceramides following hypoxia-reoxygenation; a response which can be blocked by pre-treatment of cells with antioxidants suggesting that oxidative stress is important in regulating ceramide synthesis and stimulation of JNK (Hernandez et al., 2000). Furthermore, targeted cardiac expression of dominant negative JNK reduces c-Jun kinase activity and increases hypertrophic remodelling following TAC (Liang et al., 2003). Similarly, inhibition of JNK during ischaemia reduces phosphorylation of c-Jun, attenuates infarct size and significantly reduces the number of TUNEL-positive cells, suggesting a reduction in apoptotic cell-loss (Ferrandi et al., 2004). As such, increased ceramide-JNK signalling may promote apoptosis, and lead to organ dysfunction as described in studies examining the impact of high fat- and WD-feeding (Fang et al., 2008, Ballal et al., 2010). Importantly, however, no such dysfunction was observed following HFD- or WD-feeding in the present study. Thus, ceramide signalling cascades in the hypertrophied heart faced with increased dietary lipid require further investigation.

Sung et al. (2011) have shown that elevated ceramide levels in the mouse heart following a HFD are associated with reduced phosphorylation of AMPK. Furthermore, ceramide treatment of AC16 cardiomyocytes leads to decreased phosphorylation of Akt and GSK-3β (Park et al., 2008), suggesting a potential role for ceramide in regulating cardiac growth. Indeed, activation of protein kinase C (PKC) ζ via ceramide has also been shown to inhibit Akt translocation (Powell et al., 2003), potentially dependent upon PP2A (Guenther et al., 2008). Additionally, augmented ceramide production reduces pro-insulin gene expression in pancreatic β-cells via decreased activity of the ERK pathway due to increased PP2A (Guo et al., 2010). Thus, ceramide accumulation may directly regulate cardiomyocyte metabolism and morphology. Furthermore, impaired Akt signalling as a result of ceramide accumulation may result in reduced inhibition of the pro-apoptotic Bcl-2 family members Bad, Bax, Bid, and Bim, and hence increase apoptosis (Datta et al., 1997). Indeed, a well-studied mechanism of ceramide mediated cellular dysfunction involves disruption to mitochondrial function and activation of the intrinsic death pathway (Siskind, 2005), although there is a paucity of cardiac-specific data.

However, long-term WD feeding reduced the expression of Bcl-2 proteins in the heart, an observation not seen with HFDs (Ballal et al., 2010). Furthermore, hearts from WD-fed animals exhibit increased caspase-3 cleavage and show a greater number of TUNEL-positive cells, indicating enhanced apoptosis (Ballal et al., 2010).

5.4.5 Summary

In the current model, 9 weeks of a HFD did not lead to changes in cardiac long-chain ceramide content. In comparison, WD-feeding led to a significant elevation of C16-C24 ceramide. Furthermore, AC did not further augment the increase in ceramide content. When animals fed a WD were subjected to ADR-induced HF, a specific increase in C16 and C18 ceramide content was observed, with no change in C20 and C24 ceramide. These data suggest that HF induces a specific change in the ceramide profile of the heart, strongly suggesting an important role for ceramide in the development of HF following lipid accumulation.

Chapter 6 Discussion and future directions

6.1 Discussion

Surgical induction of pressure-overload hypertrophy resulted in significant LVH in all experimental groups. Furthermore, the hypertrophic response was found to differ following dietary intervention, with diets high in carbohydrate (SD and WD) producing the greatest increase in LVH compared to a diet high in fat (HFD). At 9 weeks, cardiac function was not compromised following AC or dietary intervention, despite substantial changes in cardiac metabolism. Consequently, this suggests that consumption of a WD – although not initially detrimental to function – may in the long term lead to deterioration in function secondary to metabolic remodelling. Indeed, increased TG may lead to the accumulation of ceramide observed in the hearts of animals consuming a WD (figure 6.1). These data suggest that metabolic remodelling precedes the development of contractile dysfunction in the hypertrophied heart. Furthermore, the metabolic phenotype generated by the WD may offer a speculative mechanism by which this dietary pattern enhances the risk of developing HF. A summary

The HFD attenuated the development of LVH and prevented the increase in HW associated with pressure-overload, a process which was exaggerated by increasing carbohydrate inclusion in diet. It is interesting that the beneficial effect of the HFD was lost with WD feeding, despite a similar FA profile. This suggests that the favourable effect of lipid is lost with heightened sugar consumption and implicates systemic changes in the ventricular remodelling response. However, besides attenuating the increase in HW associated with AC combined with SD and WD feeding, the effect of the HFD occurred despite significant increases in adiposity and leptin, challenging the view that increasing fat mass is detrimental to cardiac performance. Although these observations are in line with previous studies (Chess et al., 2009) and to high-salt feeding (Okere et al., 2005, Okere et al., 2006b), and exacerbated by high levels of carbohydrate (Chess et al., 2007, Sharma et al., 2008), extending the duration of the experimental protocol may elicit greater ventricular remodelling in response to both diet and AC. Indeed, a more pathological model would complement the current experimental design, and allow changes in cardiac function and metabolism to be assessed as the disease progresses.

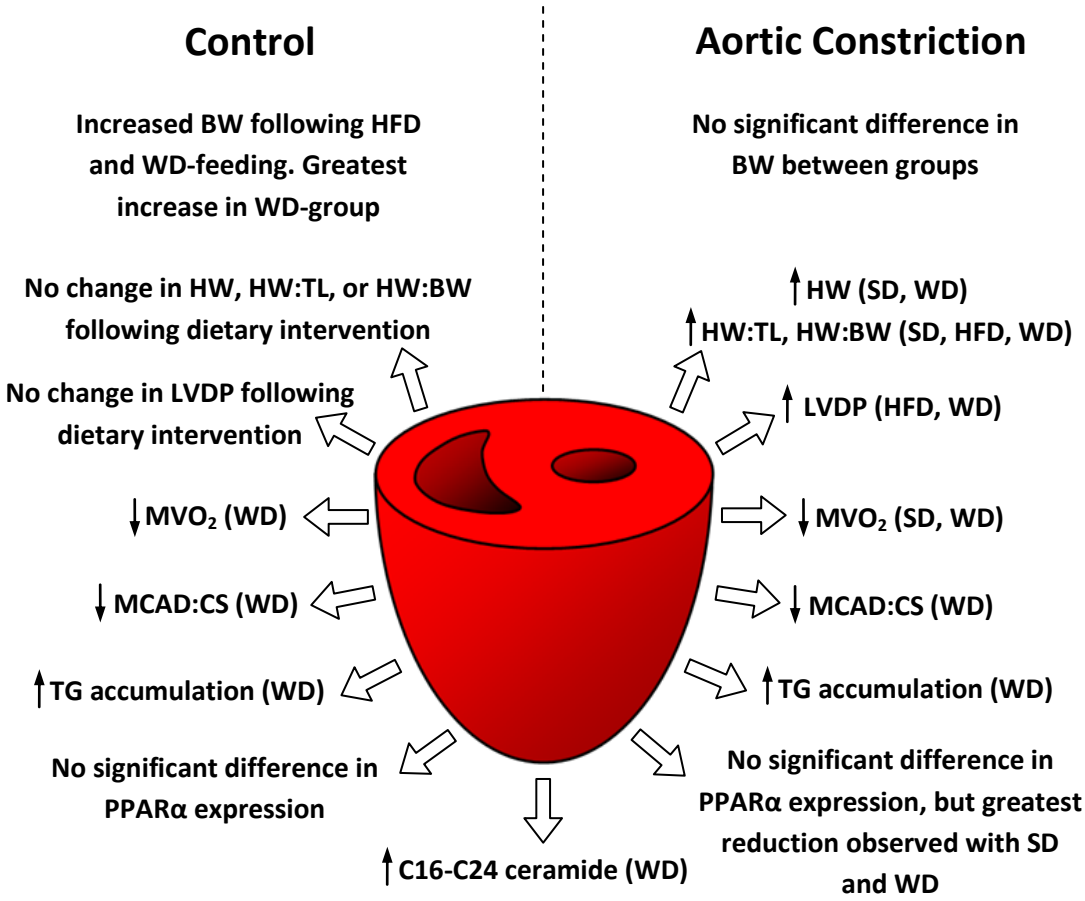

Figure 6.1 Summary of main observations following dietary intervention or the combined effect of dietary manipulation and cardiac hypertrophy. BW, body weight; HW, heart weight; HW:TL, heart weight-to-tibia length; HW:BW, heart weight-to-body weight; LVDP, left ventricular developed pressure; MVO_2, myocardial oxygen consumption; MCAD:CS, medium-chain acyl-CoA dehydrogenase:citrate synthase; TG, triglyceride; PPARα, Peroxisome proliferator-activated receptor; SD, standard diet; HFD, high-fat diet; WD, western diet

Decreased FAO (Planavila et al., 2005, Akki et al., 2008) and increased reliance upon glycolytic substrates (Allard et al., 1994b, Akki et al., 2008, Leong et al., 2002) has been observed in hypertrophied hearts, and also in HF (Sack et al., 1996, Depre et al., 1998, Barger et al., 2000), and are in agreement with this study. However, the subtle difference between control and experimental animals in the present study likely reflects the severity of the procedure, as rats subjected to a more severe experimental intervention show greater remodelling (Chess et al., 2009).

Facilitating FAO in HF has been shown to improve cardiac function and reduce mortality. Increased carnitine supplementation, and thereby greater mitochondrial uptake of LCFAs, improved mortality in HF patients (Ioannis, 2000), and elevated consumption of SFAs led to improved survival in hamsters with genetic cardiomyopathy (Galvao et al., 2012). Furthermore, rats fed a HFD showed an improved cardiac response to a dobutamine stress test (Berthiaume et al., 2010). Blocking LCFA uptake via inhibition of CPT1 with oxifenicine did not improve cardiac function in the hypertrophied DSS rat heart (Okere et al., 2006b) or manifest cardiac hypertrophy in Wistar rats fed a HFD (Okere et al., 2007). However, in HF of ischaemic origin, the use of Trimetazidine (TMZ) to inhibit long-chain 3-ketoacyl thiolase – and therefore FAO – improved cardiac function (Fragasso et al., 2006, Fragasso et al., 2011), and has been shown to increase the fractional oxidation of glucose in a model of pressure-overload hypertrophy following ischaemia, without affecting palmitate oxidation (Saeedi et al., 2005). However, this effect was not observed prior to ischaemia. It would therefore be interesting to study cardiac function following ischaemia in the HFD and WD models to determine if increased oxidation of FA under basal conditions predisposes the heart to enhanced dysfunction. Thus, regulating cardiac FAO in the hypertrophied or failing heart via dietary intervention may be an important additional therapy for the treatment of HF.

A HFD did not lead to significant changes in the metabolic markers studied other than reducing PDHa, as anticipated with increasing FA supply and oxidation (Randle, 1998), suggesting that a HFD may prevent the decline in FAO associated with LVH. Increased dietary carbohydrate augmented the left ventricular remodelling process in response to hypertension in the DSS rat (Okere et al., 2006b), supporting the observation in the present study. Additionally, the potential activation of PDH in response to such a small increase in dietary sucrose requires further investigation. Although spectrophotometric determination of enzyme activity is useful, for an enzyme which is as highly regulated as PDH the measurement of pyruvate flux through the enzyme *in vivo* would be more insightful. This has been achieved by using hyperpolarised 1-^{13}C pyruvate in the hearts of diabetic rats (Schroeder et al., 2008).

Additionally, it has been shown that pressure-overload hypertrophy is associated with reduced activity of malonyl-CoA dehydrogenase (MCD) (Young et al., 2001). The blunted PPARα response observed in AC-WD hearts may in part lead to the combined effect of attenuated FAO via decreased transcription of enzymes associated with β-oxidation, and mitochondrial FA transport, ultimately resulting in TG accumulation. Additional support for this notion is

provided by studies using transgenic mouse models of increased glucose uptake, which show a decrease in PPARα and elevated ACC mRNA (Yan et al., 2009). Thus, the provision of a substrate for endogenous TG synthesis when FAO is reduced would facilitate increased TG accumulation, as observed in WD hearts. Determination of MCD and ACC protein expression, in addition to CPT1 activity in the different dietary groups would allow the dissociation of altered FAO and mitochondrial FA uptake to be examined fully, thus allowing identification of the cause of TG accumulation following the WD protocol.

Chronic AC did not impact upon the measured ceramide species when combined with SD and HFD feeding. In contrast to this, WD feeding led to a significant increase in cardiac ceramide content. Furthermore, when animals fed a WD are treated with ADR to induce HF (Lu et al., 2009), total ceramide was further increased with the specific accumulation of C16 and C18 ceramide. Lipotoxicity is a recognised mechanism for the deterioration in function in HF patients, in particular in association with pathologies which increase FA delivery to the myocardium (Park et al., 2008) such as high-fat feeding (Zhou et al., 2000), obesity and diabetes (Sharma et al., 2004).

Although no difference was observed in the measured saturated ceramide content in AC hearts following HFD intervention it is possible that other ceramide species undergo remodelling. However, the present data are supported by a previous study examining the effect of diet alone (Baranowski et al., 2007). WD-feeding led to a significant increase in ceramides containing SFA species, irrespective of AC. The mechanisms behind this increase are unclear, especially due to the diet containing the same FA profile to that of the HFD, but may relate to increased SPT activity or reduced removal of ceramide by ceramidases (Baranowski et al., 2007). Indeed, inhibition of SPT via myriocin reduced ceramide accumulation in hearts overexpressing LPL and rescued the deterioration in function associated with this phenotype (Park et al., 2008). Furthermore, obese failing human hearts showed reduced PPARα, CPT1, and MCAD mRNA which can be restored to non-failing levels by obesity, however the consequence of this is increased lipid accumulation (Sharma et al., 2004). In contrast to this, we observed hyperfunction in AC hearts fed a WD, and normal function in respective controls despite TG and ceramide accumulation, supported by a previous study (Okere et al., 2006a). It may be anticipated that by maintaining the activity of enzymes associated with FAO prevented the accumulation of TG when challenged with a heightened supply of FAs. This may be due to all dietary regimens commencing at the same time of the induction of hypertrophy. Therefore,

for the HFD, hypertrophy develops alongside a heightened supply of FAs. This may attenuate the development of hypertrophic metabolism (decreased FAO and increased carbohydrate usage). Indeed, the ability of HFDs to upregulate the full cassette of FA responsive genes may be crucially important for attenuating the increase in intracellular ceramide, and highlights metabolic regulators of FAO as important targets for modulating cardiac metabolism in disease.

6.2 Strengths and limitations

The Langendorff method of heart perfusion has been widely used for the study of cardiac function in response to numerous interventions, including diabetes (Nawata et al., 2002, Aasum et al., 2003, Belke et al., 2004), ischaemia/ischaemia reperfusion injury (Nawata et al., 2002, Wei and Vander Heide, 2010), measurement of infarct size (Ferrera et al., 2009), hypertrophy (Eberli et al., 1998, Akki et al., 2008, Dupont et al., 2012), and for measuring electrical activity (Yanagi et al., 2001), although as reviewed by Skrzypiec-Spring et al. (2007), the rat heart suffers from a short action potential duration which therefore limits its use in arrrhythmogenic studies. However, the development of sophisticated computer programs combined with the use of numerous isotopic substrate labels has allowed determination of myocardial metabolism *in vitro* (Malloy et al., 1990).

The isolated Langendorff heart perfusion has several important advantages, including a large degree of reproducibility and a low level of technicality in comparison to the working-heart preparation. Similar to the working heart preparation, excision of the heart from the thorax removes all associations with the autonomic and central nervous system, and endogenous neurohormonal control mechanisms, thereby allowing the direct study of intrinsic cardiac function and metabolism. Although this is highly desirable, there are limitations associated with this approach. For metabolic studies, isolation of the heart from the body removes the potential for plasma substrate usage. Indeed, this may become increasingly important when analysing cardiac function from animals which exhibit substantial serum changes, such as following dietary intervention. However, the opportunity to include a wider range of physiological substrates within the perfusate is an strength of the current method, in comparison to those which have been perfused solely with carbohydrate substrates (Konior et al., 2011). Additionally, the presence of albumin in the crystalloid buffer permits the use of FAs

and reduces the well-known osmotic effects of such perfusates (Bell et al., 2011). However, a limitation of such a buffer is its low oxygen-carrying capacity in comparison to whole-blood (Bell et al., 2011) and the necessity for a relatively high CFR which may increase endothelial sheer stress.

Although the composition of the perfusate medium was designed to mimic *in vivo* conditions, there is potential that the increased function observed in AC hearts from HFD and WD groups could be due to substrate unloading, as previous studies have shown a significant contribution of endogenous lipoproteins to cardiac metabolism (Niu et al., 2004, Niu and Evans, 2008). Furthermore, the endogenous source of FAs derived from *in vivo* hydrolysis of lipoproteins may in part account for an elevated supply of FAs and increased TG content in WD hearts. Indeed, the absence of lipoproteins from the perfusate may be important in the current model and may indirectly modulate myocardial metabolism *ex vivo*, accounting for the high lactate consumption in hearts perfused with ^{13}C substrates.

The oxygen consumption experiments described in chapter 4 (section 4.3.6) demonstrate an approximately 30% reduction in MVO_2 following arrest, suggesting that in the present perfusion method, cardiac contraction would account for approximately 30% of the oxygen cost. In comparison, values of 80% are observed with the working heart preparation (Pepe and McLennan, 2002). Additionally, it has been shown that *in* vivo, work is greater than in experimentally perfused hearts (Chatham et al., 1999), with this factor potentially altering substrate metabolism. An additional limitation is the difficulty in extrapolating *in vitro* observations to *in vivo* conditions and to human disease. In particular, the additional metabolic regulation mechanisms such as variations in heart rate, oxygen content, and hormonal influences are absent in the present experimental model (Lydell et al., 2002), and are a recognised limitation of the isolated heart preparation.

6.3 Future work

By 9 weeks of pressure-overload, all groups exhibited signs of LVH with preserved or mild hyperfunction. However, although cardiac function is evidently not compromised at this stage, subclinical alterations may be evident in addition to the metabolic changes observed in the present study. A longer period of study intervention would allow a more severe cardiac

phenotype to develop, which in WD hearts may be due to excessive TG accumulation and ceramide generation. In addition to the cardiac phenotype, long-term studies would allow more systemic abnormalities to develop following different dietary patterns, including hyperglycaemia, hyperinsulinaemia, and diabetes. Indeed, the observed accumulation of hepatic lipid in the present study suggests disturbances to the actions of insulin at this experimental stage (Bugianesi et al., 2005), which may actually be detrimental to the hypertrophied heart in terms of excessive insulin-stimulated protein synthesis. It would be interesting to observe the effect of improving insulin sensitivity upon cardiac metabolism in hypertrophied hearts from the WD group.

The pathways for ceramide generation - although well studied in numerous cancers – remain largely unstudied in the heart, although a recent study has attempted to examine this (Baranowski et al., 2007). The intimate association between elevated ceramide levels and apoptosis (Okere et al., 2006a) necessitates further studies to quantify this in the present model. Indeed, apoptosis may be evident and increased by WD feeding yet at this experimental stage does not significantly influence function, similar to the finding of Okere et al. (2006a). Furthermore, it would be insightful to measure the activity of SPT in control and hypertrophied hearts in the presence of myriocin, thus allowing the contribution of de novo ceramide synthesis to be determined. Additionally, the activities of ceramidases and SMases could be examined, further enhancing the understanding of the mechanisms for altered ceramide biology in the experimental model.

In the present model of LVH, subtle metabolic remodelling was observable at 9 weeks and showed susceptibility to dietary influence. Consistent with previous work from the laboratory, PPARα protein expression was lower in hypertrophied hearts fed a standard rodent diet (Akki et al., 2008). Following high-fat feeding, PPARα protein expression was not reduced in the hypertrophied heart, yet was diminished following WD intervention. The PPARα response may in part also be regulated by PGC-1α (Puigserver and Spiegelman, 2003). In particular, as well as a cofactor for PPARα, PGC-1α is involved in regulating mitochondrial biogenesis (Ventura-Clapier et al., 2008). CS activity was used as a marker of mitochondrial density and was enhanced following increased fat and sucrose consumption; potentially secondary to heightened mitochondrial damage (Ballal et al., 2010). Thus, determining PGC-1α expression in the hypertrophied hearts in the current model may lead to a greater understanding of the

dietary impact upon mitochondrial function, as excessive mitochondrial biogenesis has been shown to manifest cardiomyopathy (Russell et al., 2004).

Publications

Papers

BUTLER, T. J., SEYMOUR, A-M. L., and ASHFORD, D. 2012 Long-chain ceramide accumulation in the hypertrophied heart – impact of western diet (in preparation).

Presented Abstracts

BUTLER, T.J., and SEYMOUR, A-M. L. Impact of obesity on left ventricular hypertrophy. Presented at Society for Heart and Vascular Metabolism, Padova, Italy (2009).

BUTLER, T. J., SEYMOUR, A-M. L., and ASHFORD, D. Impact of dietary fat upon myocardial ceramide species in cardiac hypertrophy. Presented at Society for Heart and Vascular Metabolism, Kananaskis, Canada (2010).

BUTLER, T. J., SEYMOUR, A-M. L., and ASHFORD, D. Western diet and the hypertrophied heart: the sugary path to failure? Presented at Society for Heart and Vascular Metabolism, Brussels, Belgium (2011) and The Northern Cardiac Meeting, Hull, UK (2011).

BUTLER, T. J., SEYMOUR, A-M. L., and ASHFORD, D. Alterations in the myocardial ceramide pool following dietary intervention in an experimental model of cardiac hypertrophy. To be presented at the Frontiers in Cardiovascular Biology, London, 2012.

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