THE IMPACT OF OBESITY AND CHRONIC PPAR ALPHA AGONIST TREATMENT ON CARDIAC FUNCTION, METABOLISM AND ISCHAEMIC TOLERANCE
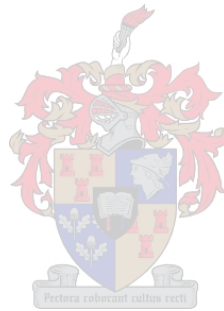
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Dissertation presented for the Degree of Doctor of Philosophy (Medical Physiology) in the Faculty of Health Sciences at the University of Stellenbosch

Promoters: Prof E.F. du Toit   **March 2012**

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DECLARATION

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ABSTRACT

Background: Myocardial oxidative fuel supply is increased in obese conditions. How this metabolic environment and altered cardiometabolic phenotype associated with pre-diabetic obesity impacts on cardiac function and tolerance to ischaemia/reperfusion injury remains uncertain. While obese individuals are likely to be treated with PPARα agonists, controversy exists as to how activation of the PPARα receptor influences cardiovascular function and post-ischaemic recovery. **Aims:** To determine in a model of hyperphagia-induced obesity 1) whether protracted obesity is associated with left ventricular (LV) mechanical dysfunction; 2) the responsiveness of these hearts to insulin stimulation; 3) whether insulin can afford cardioprotection against ischaemia/reperfusion damage; and 4) how obesity and chronic PPARα agonist (K-111) treatment influences myocardial function, substrate metabolism, mitochondrial function and post-ischaemic outcomes.

Methods: Male Wistar rats were fed standard rat chow or a high caloric diet. 1) *In vivo* LV mechanical function was assessed echocardiographically in 32 week fed animals. *Ex vivo* LV function was measured in the presence of glucose, insulin and/or fatty acid (FA); 2) *Ex vivo* myocardial insulin sensitivity was assessed by measuring insulin stimulated glycolytic flux in 16 week fed rats. Insulin was also administered prior to and during regional ischaemia to determine its effect on post-ischaemic function and infarct size; 3) K-111 was added to the drinking water during the last 10 weeks of feeding (feeding period of 18 weeks); a) Ventricular mitochondrial function was determined polarographically in the presence of either glutamate or palmitoyl-L-carnitine as substrates; b) Myocardial carbohydrate and lipid metabolism, and in a separate series of

perfusions, myocardial infarct size were determined in the presence of physiological or high insulin (30 or 50µIU/ml) and FA (0.7 or 1.5mM) concentrations.

Results: 1) Obese animals maintained normal *in vivo* LV mechanical function. Glucose perfused hearts from obese animals had depressed aortic outputs compared to the control group (32.58 ± 1.2 vs. 46.17 ± 0.91 ml/min; $p < 0.001$) which was abolished by the presence of FA; 2) Hearts from obese animals had reduced insulin stimulated glycolytic flux rates (1.54 ± 0.42 vs. 2.16 ± 0.57 µmol/g ww/min, $p < 0.01$). Although insulin reduced infarct size in the obese group (20.94 ± 1.60 vs. 41.67 ± 2.09 %, $p < 0.001$), its cardioprotective effect was attenuated in the presence of FA; 3) By simulating the *in vivo* metabolic environment of control and obese animals in *ex vivo* perfusions, elevated insulin and FA levels associated with obesity increased infarct sizes in the obese group compared to the control group (47.44 ± 3.13 vs. 37.17 ± 2.63 %, $p < 0.05$); 4) While chronic K-111 treatment reversed systemic metabolic abnormalities associated with obesity, neither obesity nor the drug influenced myocardial and mitochondrial function or post-ischaemic outcomes. K-111 was able to reduce palmitate oxidation in the obese group.

Conclusion: Elevated levels of circulating FFA may be important in maintaining normal LV mechanical function in the obese condition. While obesity had no impact on myocardial mitochondrial function and post-ischaemic outcomes during comparable perfusion conditions, the specific metabolic environment associated with obesity may augment post-ischaemic injury. K-111 is effective in reducing obesity related metabolic abnormalities, but has no effects on myocardial function, mitochondrial function or ischaemic tolerance.

OPSOMMING

Agtergrond: Miokardiale oksidatiewe substraat voorsiening is verhoog in vetsug. Hoe hierdie metaboliese omgewing en veranderde miokardiale metaboliese fenotipe in pre-diabetiese vetsug miokardiale funksie en iskemie/herperfusie skade beïnvloed, is onseker. Alhoewel vetsugtige individue met PPARα agoniste behandel kan word, is die resultate verkry van hierdie reseptor aktivering op miokardiale funksie en iskemiese skade teenstrydig.

Doelwitte: Om te bepaal of 1) verlengde vetsug linker ventrikulêre (LV) funksie beïnvloed; 2) hierdie harte sensitief vir insulien stimulasie is; 3) insulien die hart teen iskemie/herperfusie beskadiging beskerm; en of 4) vetsug en chroniese K-111 behandeling miokardiale funksie, substraat metabolisme, mitochondriale funksie en post-iskemiese herstel in vetsugtige, insulienweerstandige rotte beïnvloed.

Metodes: Manlike Wistar rotte is met gewone rotkos, of 'n hoé kalorie dieet gevoer. 1) *In vivo* LV funksie in 32 week gevoerde rotte is met behulp van eggokardiografie bepaal. *Ex vivo* LV funksie is met of sonder insulien en/of vetsure in die perfusaat bepaal; 2) Die *ex vivo* insuliensensitiwiteit is in 16 weke gevoerde rotte bepaal deur miokardiale glikolise te meet. Insulien is ook voor en tydens streeksiskemie toegedien, ten einde sy effek op miokardiale beskerming te bepaal; 3) K-111 is in die drink water van rotte toegedien vir die laaste 10 weke van hul dieet (voedingsperiode van 18 weke); a) Ventrikulêre mitochondriale funksie is polarografies bepaal in die aanwesigheid van glutamaat of palmitiel-L-karnitien; b) Miokardiale koolhidraat- en lipied metabolisme, en in 'n aparte groep rotte, infarktgrootte, is bepaal in die teenwoordigheid van fisiologiese of hoë insulien- (30 of 50μIU/ml) en vetsuurvlakke (0.7 of 1.5mM).

Resultate: 1) Vetsugtige rotte het normale *in vivo* LV funksie gehandhaaf. Glukose geperfuseerde harte van vet rotte se LV funksie was laer as die van kontroles (Aorta omset: 32.58±1.2 vs. 46.17±0.91 ml/min; p<0.001), maar dit het verbeter in teenwoordigheid van vetsure; 2) Harte van vetsugtige rotte het verlaagde insulien-gestimuleerde glikolise getoon (1.54±0.42 vs. 2.16±0.57 μmol/g ww/min, p<0.01). Alhoewel insulien infarktgrootte in die vetsugtige groep verlaag het (20.94±1.60 vs. 41.67±2.09 %, p<0.001), is sy beskermende effekte in die teenwoordigheid van vetsure verlaag; 3) deur die *in vivo* metaboliese omgewing van kontrole en vetsugtige rotte in die perfusaat van die harte *ex vivo* te simuleer, is dit aangetoon dat die verhoogde vlakke van insulien en vetsure, geassosieer met vetsugtigheid, infarktgroottes in die vetsugtige groep teenoor die kontrole groep verhoog het (47.44±3.13 vs 37.17±2.63 %, p<0.05); 4) Hoewel chroniese gebruik van K-111 die metaboliese abnormaliteite gepaardgaande met vetsug normaliseer het, het beide vetsug en die middel geen invloed op miokardiale of mitochondriale funksie of vatbaarheid vir iskemiese beskadiging gehad nie. K-111 het miokardiale palmitaatoksidasie in die vetsugtige behandelde groep verlaag.

Gevolgtrekking: Verhoogde bloed vetsuurvlakke in vetsug mag n belangrike rol in die handhawing van sistoliese funksie speel. Dit blyk dat die spesifieke *in vivo* omgewing geassosieer met vetsug wel tot verhoogte vatbaarheid vir iskemie/herperfusie skade mag lei. K-111 is effektief om die sistemiese metaboliese abnormaliteite gepaard met vetsugtigheid te verbeter, maar het geen effek op miokardiale funksie, mitochondriale funksie of vatbaarheid vir iskemie gehad nie.

ACKNOWLEDGEMENTS

TO GOD BE THE GLORY

I would like to offer my sincerest thanks to the following, without whom my PhD would not be possible:

Prof's Joss du Toit and Amanda Lochner

To my supervisors, thank you for your guidance and motivation over the last "few" years. Your support has meant more to me than you will know.

Candice Smith

To my wife, I would have never been able to complete this without you. Thank you for your understanding and support especially when the PhD had to take preference over many things.

My parents

Thank you for all the sacrifices you have made to get me to this point. Thank you for your continuous love and support.

Prof's Barbara Huisamen and Stephan du Plessis

Thank you for the motivation and advice during the PhD.

Friends from Medical Physiology (Tygerberg)

Derick van Vuuren, Ingrid Webster, James Fan, Corli Westcott, Erna Marais, Suzel Hatting:
Your friendship has meant a lot over the years. Thanks for always being willing to help, listen and advise.

Sonia Genade

Thank you for encouraging me and always being willing to help.

Colleagues at the Hypertension in Africa Research team
(North West University)

Thank you for giving me the time to complete the writing of my PhD during my first year with you.

I would like to thank the following for their contributions to the work in this PhD:

Prof Gavin Norton: Who performed the echocardiography and Langendorff perfusions during the 32 week study.

Prof Joss du Toit: Who performed the initial working heart perfusions in the presence of glucose. Prof du Toit also performed the determination of myocardial glycolytic flux rates in the 16 week fed rats.

Dr Dee Blackhurst: Who performed the analysis of myocardial intramyocardial triglyceride content.

Sonia Genade: Who performed the metabolic perfusions used to determine the rate of myocardial glycolysis and glucose oxidation.

Corli Westcot: Who assisted me with Western blotting.

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AO	Aortic output
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ATP	Adenosine triphosphate
bpm	Beats per minute
BMI	Body mass index
°C	Degree Celsius
CAL	Coronary artery ligation
CIRKO	Cardiomyocyte specific insulin receptor deletion
CoA	Co-enzyme A
CPT	carnitine palmitoyltransferase
CAT	carnitine:acylcarnitine translocase
dH$_2$O	Distilled water
dP/dt	Delta pressure over delta time
DF	Dilution factor
dw	Dry weight
FA	Fatty acid
FADH	Flavin adenine dinucleotide
FFA	Free fatty acid
FSend	Endocardial fractional shortening
FSmid	Mid-wall fractional shortening

GLUT	Glucose transporter
HCD	High caloric diet
HDL-C	High density lipoprotein cholesterol
HOMA-IR	Homeostasis model of assessment - insulin resistance
IR	Insulin receptor
IRS	Insulin receptor substrate
KE	Potassium chloride/ ethylenediaminetetraacetic acid solution
LAD	Left anterior descending
LCFA	Long chain fatty acid
LPL	Lipoprotein lipase
LV	Left ventricular
LVEDD	Left ventricular end diastolic diameter
LV Ees	Left ventricular end-systolic elastance
LVESD	Left ventricular end systolic diameter
m	milli
M	Molar
mRNA	Messenger ribonucleic acid
n	Nano
NADH$_2$	nicotinamide adenine dinucleotide
PCR	Phosphocreatine
PDH	pyruvate dehydrogenase
PDHK	pyruvate dehydrogenase kinase
PI3K	phosphoinositide 3-kinase
PKB/AKT	Protein kinase B
PKC	Protein kinase C

PFK	phosphofructokinase
PWT	Posterior wall thickness
RCI	Respiratory control index
RPP	Rate pressure product
SA	Specific activity
SRC	Standard rat chow
STAT3	Signal Transducer and Activator of Transcription 3
TBS-T	Tris-buffered saline mixed with Tween-20
Trig	Triglyceride
µ	Micro
vw	Ventricular weight
ww	Wet weight

CHAPTER 1:

INTRODUCTION

The prevalence of obesity is increasing world-wide. Not only are developed countries being affected, but also developing countries which are already burdened with various other disease epidemics (Pi-Sunyer, 2002; World Health Organization, 2002; Prentice, 2006). South Africa is not exempt from the obesity epidemic with 29.2% of men and 56.6 % of the women considered to be overweight or obese in 1998 (Puoane *et al.* 2002). Global statistics from 2005, suggested that 396 million adults were estimated to be obese and an additional 937 million were thought to be overweight (Kelly *et al*. 2008). The predicted number of overweight and obese individuals is expected to escalate to 1.35 billion and 573 million people respectively by 2030 (Kelly *et al*. 2008). The reality of these statistics is that obesity will have an immense impact on global health-care systems considering the various non-communicable diseases associated with the condition. In the United States of America, all-cause mortality resulting from obesity accounted for 280 184 deaths annually (Allison *et al*.1999) whereas 1 in 13 deaths in European countries have been attributed to obesity (Banegas *et al*. 2003) which is indicative of the detrimental nature of the epidemic. One of the major health concerns regarding obesity is its diverse and adverse effects on the cardiovascular system.

Obesity often occurs concurrently with various co-morbidities (Stamler *et al.* 1978; Kannel *et al.* 1979; Van Itallie *et al*. 1985; Colditz *et al*. 1995; Abbasi *et al.* 2002), each of which is a risk factor for cardiovascular disease (Kannel and McGee, 1979; Barbir *et al*. 1988; O'Donnell *et al.* 1997; Abassi *et al.* 2002; Rewers et al. 2004). Due to the cluster of cardiovascular risk factors associated with obesity, a number of

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factors may impact on the interaction between obesity and cardiac function and substrate metabolism (Lopaschuk *et al*. 2007). Consequently these factors may also influence other aspects of obesity related cardiovascular disease. Despite this, obesity has been shown to be a risk factor for the development of heart failure, independent of these traditional cardiovascular risk factors (Kenchaiah *et al*. 2002).

The impact of protracted obesity on cardiac function is controversial. Although it is widely acknowledged that obesity is associated with left ventricular (LV) diastolic dysfunction, the influence of obesity on LV systolic function is less clear. While many authors report normal or augmented systolic function (Berkalp *et al*. 1995; Iacobellis *et al*. 2002, 2004; Pascual *et al*. 2003; Otto *et al*. 2004; Dorbala *et al*. 2006) in response to obesity, it has been postulated that over time, severe obesity may lead to LV systolic dysfunction, with the length of morbid obesity being a strong indicator for the development of congestive heart failure (Alpert *et al*. 1995; 1997). More concerning are the findings of sub-clinical systolic dysfunction in obese and overweight individuals (Ferraro *et al*. 1996; Peterson *et al*. 2004b; Wong *et al*. 2004; Di Bello *et al*. 2006; Tumuklu *et al*. 2007, Kosmala *et al*. 2008a). Data from animal studies are also unclear as to the impact of obesity on LV mechanical function (Du Toit *et al*. 2005; Carroll *et al*. 2006, Wilson *et al*. 2007; Ouwens *et al*. 2007; Wilson *et al*. 2007; Aasum *et al*. 2008; Yan *et al*. 2009).

Both obesity and insulin resistance are risk factors for the development of type 2 diabetes. Indeed it is evident that a specific diabetic cardiomyopathy may develop in the diabetic population. Nevertheless, many of the myocardial abnormalities associated with type 2 diabetes such as excess intramyocardial lipid accumulation, altered substrate metabolism, reduced cardiac efficiency and mitochondrial

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dysfunction are already present in the obese pre-diabetic state (Szczepaniak *et al*. 2003; Mazumder *et al*. 2004; Buchanan *et al*. 2005; Kankaanpää *et al*. 2006; Wilson *et al*. 2007; Aasum *et al*. 2008; Niemann *et al*. 2011). Since both obesity and type 2 diabetes are risk factors for coronary artery disease and heart failure (Manson *et al*. 1991; Eckel and Krauss, 1998; Aronow and Ahn, 1999; Kenchaiah *et al*, 2002; Boudina and Abel, 2007), it is important to understand to what degree these factors (intramyocardial lipid accumulation, altered substrate metabolism, reduced cardiac efficiency and mitochondrial dysfunction), when present, are detrimental to cardiovascular functioning in the obese state.

Obesity is characterized by an overabundant supply of oxidative fuels and is often associated with abnormal myocardial substrate metabolism (Mazumder *et al*. 2004; Buchanan *et al*. 2005; Wilson *et al*. 2007; Aasum *et al*. 2008; Zhang *et al*. 2011) characteristic of an insulin resistant myocardium. Considering the various adaptations made by the myocardium in the obese state, hearts from obese individuals may indeed be more vulnerable to damage caused by ischaemic events. While it is clear that obesity (waist to hip ratio) is associated with a higher risk of developing myocardial infarction (Yusuf *et al*. 2005), the outcomes following myocardial infarction are more controversial. *In vivo* post-ischaemic outcomes in obese animals also provide no clear answer (Thim *et al*. 2006; Clark *et al*. 2010). An investigation promoting our understanding of the factors that could potentially contribute to adverse post-ischaemic outcomes in obesity in the absence of diabetes is warranted.

Peroxisome proliferator activated receptor alpha (PPARα) agonists are a family of pharmacological agents that show impressive results in improving whole body insulin

sensitivity and lipid profiles (Guerre-Millo *et al*. 2000; Robins *et al*. 2001). Due to the localization of the PPARα subtype in hepatocytes, cardiomyocytes and skeletal myocytes, PPARα agonists in particular represent an exciting option for the treatment of the cardiometabolic syndrome associated with obesity. Although the lipid lowering and insulin sensitizing capabilities of PPARα agonists are well known, the impact of manipulating this particular receptor in the heart in the context of ischaemia/reperfusion injury is controversial (Yue *et al*. 2003; Sambandam *et al*. 2006; Bulhak *et al*. 2009; Hafstad *et al*. 2009). Given that obese/diabetic patients are the most likely candidates to receive such interventions, notwithstanding their already high risk for acute myocardial infarction (AMI), it is imperative that more investigations are performed to determine the impact of chronic PPARα agonist treatment on the myocardial susceptibility to ischaemia/reperfusion injury, especially when new promising PPARα agonists emerge. Furthermore, as PPARα is a transcription factor involved in the up-regulation of many proteins involved in fatty acid metabolism (oxidation), the investigation of the impact of chronic PPARα agonism on the integrity of mitochondrial respiration is warranted.

CHAPTER 2

LITERATURE REVIEW

Obesity results from a constant imbalance between caloric intake (oxidative fuel supply) and utilization, genetic factors and reduced physical activity. Body mass index (BMI) represents a universal means of quantifying general adiposity. Normal body weight, pre-obesity/overweight and obesity are assigned BMI values of 18.5 - ≤ 24.9, 25.0 – 29.9 and ≥ 30 respectively (NHLBI Panel on the Identification, Evaluation and Treatment of Overweight and Obesity in Adults, 1998). However other measures such as waist circumference and waist to hip ratio's may also be used. The impact of obesity on the risk of developing cardiovascular disease is of great concern. BMI has been found to correlate positively with various risk factors (inverse correlation with HDL-C) implicated in the development of coronary heart disease (Lamon-Fava *et al*. 1996) and is itself a risk factor for coronary heart disease development (Manson *et al*. 1990; Eckel and Krauss, 1998). Obesity has further been shown to be an independent risk factor for congestive heart failure (Kenchaiah *et al*. 2002), and a strong predictor of increased left ventricular wall thickness (Peterson *et al*. 2004b), an indicator of cardiac hypertrophy. Of greater concern is that being overweight also confers risk for cardiovascular disease (Wilson *et al*. 2002).

Obesity seldom exits in isolation and is usually associated with various systemic and metabolic abnormalities. Obesity and particularly visceral obesity is associated with various co-morbidities including dyslipidaemia, insulin resistance, hyperinsulinaemia, diabetes and hypertension (Stamler *et al*. 1978; Kannel *et al*. 1979; Van Itallie *et al*. 1985; Colditz *et al*. 1995; Abbasi *et al*. 2002), all of which are considered strong risk factors for cardiovascular disease (Kannel and McGee, 1979; Barbir *et al*. 1988;

O'Donnell *et al*. 1997; Abassi *et al*. 2002; Rewers *et al*. 2004). In addition, the co-occurrence of these disorders within an individual constitutes the metabolic syndrome, a condition originally described by Reaven *et al*. (1988). Obesity together with changes in adipose tissue secretory function, insulin resistance and various other independent factors are thought to contribute greatly to the development of the metabolic syndrome (Grundy *et al*. 2004). Recently a collective definition of the metabolic syndrome was compiled to aid in the diagnosis thereof. To be diagnosed with the metabolic syndrome any 3 of the following conditions must be present: raised waist circumference (cut-off is population specific), elevated triglycerides (Trig's) levels ($\geq$ 150 mg/dl), reduced HDL-C levels (< 40.0 mg/dL in males; < 50 mg/dL in females), raised fasting glucose levels ($\geq$ 100 mg/dL) and elevated blood pressure (systolic $\geq$ 130 and/or diastolic $\geq$ 85 mmHg) (Alberti *et al*. 2009). The metabolic syndrome is associated with an increased risk of cardiovascular events, developing coronary heart disease and cardiovascular related and overall mortality (Lakka *et al*. 2002; Malik *et al*. 2004; Butler *et al.* 2006). Furthermore, the risk of developing cardiovascular disease may increase as the number of individual components of metabolic syndrome present within an individual increases (Klein *et al*. 2002). Due to the plethora of co-morbidities associated with obesity, it is evident and should be emphasized that it is exceptionally difficult to ascertain the direct effects of isolated obesity on cardiac function, structure and ischaemic complications since these endpoints may be influenced by the various co-morbidities present in metabolic syndrome patients. Data available on the cardiac effects of obesity should thus be carefully assessed in order to determine which co-morbidities are present. When comparing data generated from animal models of obesity, additional caution in the interpretation of data is required depending on whether genetic or diet induced models of obesity have been used.

2.1 Comparing the cardiovascular disease risk indexed by BMI or body fat distribution

Accumulating evidence suggests that the localization of excess adipose tissue in the viscera, at times occurring in the absence of general obesity, plays an important role in various metabolic complications associated with obesity. Although obesity is a risk factor for coronary heart disease (Manson *et al*. 1990; Eckel and Krauss, 1998), it has been shown that visceral/central obesity (sub-scapular skin-fold thickness) is a strong predictor of coronary heart disease independent of BMI (Donahue *et al*. 1987). Convincing evidence supporting the premise of the importance of body fat distribution in the development of coronary artery disease has been demonstrated in non-obese subjects (Nakamura *et al*. 1994; Kobayashi *et al*. 2001). Here it is thought that the presence of visceral fat may lead to coronary artery disease through the development of insulin resistance and altered lipid/lipoprotein profiles (Kobayashi *et al*. 2001). It may be that the metabolic profile of non-obese individuals that are viscerally obese may be similar to those with general obesity displaying signs of metabolic syndrome. Consequently, even in the absence of general obesity (as assessed from BMI), the presence of visceral obesity alone may be key to the development of insulin resistance, type-2 diabetes, metabolic syndrome and cardiovascular disease.

2.2 The impact of obesity on cardiac remodelling

Besides its associated vascular complications, obesity is associated with cardiac remodelling. Early studies characterizing cardiac remodelling in normotensive obesity indicated that obesity is generally associated with eccentric cardiac remodelling coupled with altered functional (increased cardiac output, stroke volume) and haemodynamic (increased central blood volume, plasma and total blood volume and reduced total peripheral resistance) parameters (Messerli *et al*. 1983a; Messerli *et al*. 1983b). Although a positive association between body mass, LV mass and LV wall thickness has been clearly demonstrated (Lauer *et al.* 1991; Crisostomo *et al.* 2001; Powell *et al.* 2006), data from various groups have argued that increased LV mass in the obese population is mediated by the degree of insulin resistance and/or hyperinsulinaemia (Sasson *et al.* 1993; Iacobellis *et al*. 2003), possibly independent of obesity and blood pressure (Sasson *et al*. 1993). This may be related to insulin's growth promoting effects (Samuelsson *et al*. 2006). Regardless of the aetiology of obesity related cardiac remodelling, augmented LV mass has been shown to be associated with a higher incidence of clinical cardiovascular events (Levy *et al*. 1990).

2.2.1 Mechanism of obesity induced cardiac remodelling

Obesity or excessive weight gain in humans is associated with an increase in both lean body mass and fat mass (Forbes and Welle, 1983), which implies that additional vascularization of this tissue would be required. BMI further correlates positively with whole body oxygen consumption (Bray *et al*. 1970) probably due to the increased metabolic demands, especially tissue oxygen requirements, associated with obesity. To meet the increased demand for oxygen, cardiac output is increased, which is

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thought to primarily be due to augmented stroke volume resulting from an obesity associated elevation in blood volume, as studies show that heart rate in obese individuals remains normal compared to lean individuals (Messerli *et al*. 1982). The increase in stroke volume may be explained by the increased blood volume (preload) associated with obesity as LV filling pressure and end diastolic volume subsequently increase, which in turn leads to a direct increase in stroke volume and consequently cardiac output. Over an extended time, the volume overload associated with obesity together with the increased cardiac output induces adaptational structural remodelling, which is evident by an increase in LV mass. Increased LV filling additionally induces alterations in LV cavity dimensions (eccentric left ventricular remodelling). Ultimately adaptive left ventricular hypertrophy and dilatation occur (Lauer *et al*. 1991; Paulson and Tahiliani, 1992; Kopelman *et al*. 2000).

Besides the preload-induced changes in cardiac structure associated with obesity, the co-occurrence of systemic hypertension (elevated afterload) may further impact on cardiac structure. Indeed the combination of the two conditions (increased preload and afterload) has a more pronounced effect on left ventricular remodelling and structure and has been proposed to enhance the long-term risk of congestive heart failure (Messerli *et al*. 1983a; Schmieder and Messerli, 1993).

Although for many decades the impact of obesity on LV structural remodelling has been well documented, the last few years have highlighted the importance of an altered obesity related cardio-metabolic phenotype in relation to parameters of cardiovascular functioning. Many of these myocardial metabolic alterations are similar in nature to those evident in type 2 diabetes. They include excessive intramyocardial lipid accumulation, altered myocardial substrate metabolism, reduced

cardiac efficiency and myocardial mitochondrial dysfunction (Szczepaniak *et al*. 2003; Mazumder *et al*. 2004; Buchanan *et al*. 2005; Kankaanpää *et al*. 2006; Wilson *et al*. 2007; Aasum *et al*. 2008; Niemann *et al*. 2011). These abnormalities may predispose the heart to future contractile complications especially when combined with the additive pre-existing co-morbidities associated with obesity. Furthermore, while contractile dysfunction may take decades to manifest, it is feasible that the altered cardiometabolic phenotype associated with obesity could itself leave the heart vulnerable to worse post-ischaemic outcomes. In order to better understand the altered metabolic phenotype associated with obesity, a brief overview of myocardial substrate metabolism in the healthy heart is warranted.

2.3 Metabolism

2.3.1 Substrate metabolism in the healthy heart

The heart is a dynamic organ, constantly requiring energy in the form of adenosine triphosphate (ATP) to meet its various metabolic and contractile demands. This is achieved through a constant supply of oxidizable substrates via the circulation. The most important substrates utilized by the heart include fatty acids (FA's), glucose and lactate. Although the adult heart is capable of oxidizing a variety of substrates, the majority of ATP (60-70%) generated by the heart originates from the oxidation of FA's (Zierler, 1976; Opie, 1998). However, in the presence of elevated glucose and insulin levels as occurs following a meal, 60-70% of ATP may be derived from glucose metabolism (Bertrand *et al*. 2008).

Circulating FA's are taken up by the heart either in the free form (free fatty acids (FFAs')) which are usually bound to albumin, or alternatively they can be released

from the Trig component of chylomicrons or very-low-density-lipoproteins (Van der Vusse *et al*. 2000). The concentration of FA's present in the blood greatly dictates their uptake and metabolism by the heart (Scott *et al*. 1962). Under normal physiological conditions, long chain FA's (LCFA's) are the principal FA oxidized by the heart (Coort *et al*. 2007). The entry of LCFA's across the sarcolemma into the cytoplasm of the cardiomyocyte occurs through passive diffusion or membrane protein mediated transport, the latter accounting for the majority of FA translocation into the cytosol (Figure 2.1) (Luiken *et al*, 1997). This membrane protein mediated transport is facilitated by fatty acid translocase (FAT)/CD36, plasma membrane fatty acid binding protein (FBPpm) or fatty acid transport protein (Luiken *et al*, 1997). Once inside the cell, it is thought that non-esterified LCFA's are transported via cytoplasmic heart-type fatty acid binding proteins through the cytoplasm to the location where they will be utilized (Fournier *et al*. 1978; Vork *et al*. 1993; Schaap *et al*. 1999). Hereafter LCFA are activated or esterified by acyl-CoA synthetase forming long chain fatty acyl-CoA's (LCFA-CoA) (Luiken *et al*. 2004). At this point, LCFA-CoA's can either be stored in intracellular lipid pools where they can be converted to additional lipid intermediates, or are transported to mitochondria where they undergo β-oxidation.

The transport of LCFA-CoA's across the mitochondrial membrane is facilitated by three enzymes (carnitine palmitoyltransferase 1 (CPT-1), carnitine:acylcarnitine translocase (CAT) and carnitine palmitoyltransferase 2 (CPT-2)) of which carnitine forms a major component (reviewed by McGarry and Brown, 1997). CPT-1, located on the surface of the outer mitochondrial membrane, converts the fatty acyl-CoA to fatty acylcarnitine. This conversion is vital in allowing the fatty acyl-CoA to transverse the space between the outer and inner mitochondrial membranes. CAT translocates

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the fatty acylcarnitine across the inner mitochondrial membrane space and once inside the mitochondrial matrix, the fatty acylcarnitine is converted back to a fatty acyl-CoA by CPT-2. The LCFA-CoA's are then able to undergo β-oxidation, a process whereby two carbons are subsequently cleaved from the LCFA-CoA carbon chain producing acetyl-CoA, nicotinamide adenine dinucleotide ($NADH_2$) and flavin adenine dinucleotide (FADH) (as summarized by Stanley *et al*. 2005).

Due to CPT-1's role in fatty acyl-CoA transport into the mitochondria, it follows that this enzyme may have a vital role in regulating the rate of FA β-oxidation. Indeed CPT-1 activity/expression is highly regulated at various levels. CPT-1 activity can be allosterically inhibited by malonyl-CoA, the activity of which is also tightly regulated. The cellular concentration of malonyl-CoA is related to its synthesis via acetyl-CoA carboxylase and its degradation by malonyl-CoA decarboxylase. Increased or reduced cardiac levels of malonyl-CoA correspond with reduced or increased levels of FA β-oxidation respectively (Paulson *et al*. 1984; Awan and Saggerson, 1993; Saddik *et al*. 1993; Kudo *et al*. 1995; Dyck *et al*. 1998; Onay-Besikci *et al*. 2006). Furthermore, enhanced glucose oxidation is also thought to suppress LCFA oxidation through the inhibition of CPT-1, resulting from an elevation in malonyl-CoA levels (Saddick *et al*. 1993). An additional regulator of CPT-1 activity is adenosine monophosphate (AMP)-activated protein kinase (AMPK) which inhibits acetyl-CoA carboxylase (Kudo *et al.* 1996). This mode of regulation is however more evident during stress related conditions where cytosolic AMP levels rise. Finally, LCFA themselves can indirectly influence the expression of CPT-1. LCFA are ligands for peroxisome proliferator activated receptor α (PPARα). PPARα, an orphan nuclear receptor transcription factor, is able to up-regulate genes transcribing proteins involved in FA metabolism such as CPT-1 (Mascaró *et al*. 1998). Murine models of

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cardiac specific over-expressed PPARα are further characterized by elevated myocardial CPT-1 expression and increased myocardial FA β-oxidation rates (Finck *et al*. 2002).

Effect of insulin or enhanced glucose oxidation indicated by (+) or (−)

A + inside the symbol indicates a stimulatory effect whereas a – indicates and inhibitory effect

FA

FATP/ CD36 (+)

FACS

Ceramide Synthesis in ER

FA-CoA

FACS

(−)

CPT1

CPT2

Glycerol lipid synthetic pathway in ER (+)

FA-CoA

ATP

β-Oxidation

ER-DAG

FA

TCA → ETC

Trig

MG

Enlarged view of Mitochondria

DAG
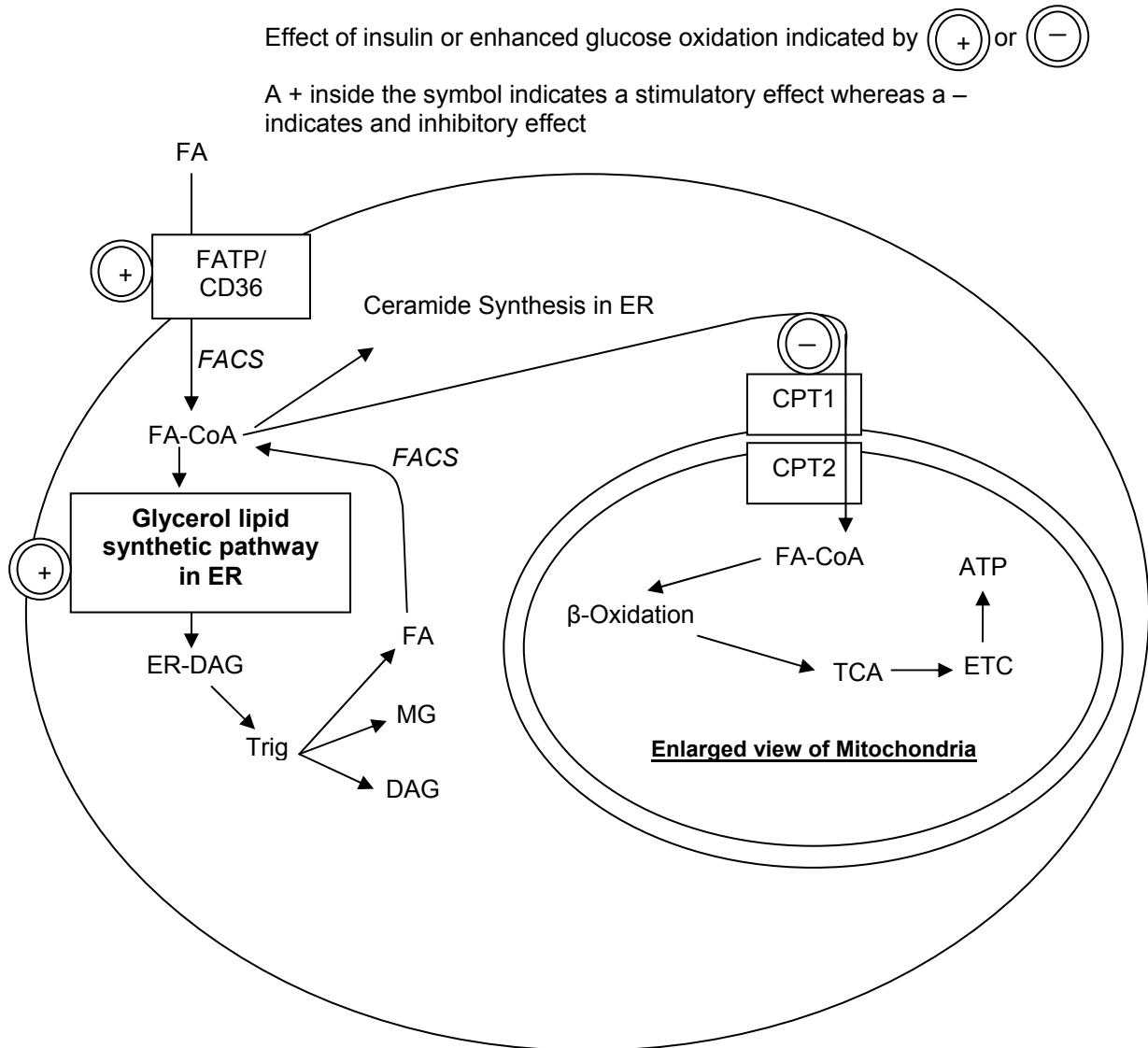
Figure 2.1: The pathways involved in LCFA uptake, storage and metabolism within the cardiomyocyte. *ATP* – Adenosine triphosphate; *CPT* – carnitine palmitoyltransferase; *DAG* – diacylglycerol; *ER* – endoplasmic reticulum; *ETC* – electron transport chain; *FA* – fatty acid; *FA-CoA* – fatty acyl-CoA; *FACS* – fatty acyl-CoA synthase; *FATP* – fatty acid transport protein; *MG* – monoacylglycerol; *Trig* – triacylglycerol. Modified from Brindley *et al*. 2010.

Another important substrate readily utilized by the heart as an oxidative fuel is glucose, which may enter the cardiomyocyte through either the basal uptake glucose transporter, GLUT 1, or via the insulin dependent glucose transporter, GLUT 4

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(Figure 2.2) (Kraegen *et al*. 1993). GLUT 4 is stored within cytoplasmic vesicles within the cardiomyocyte. These vesicles are recruited to the sarcolemma either upon insulin stimulation or cardiac contraction. Glucose itself can induce GLUT 4 translocation. Subsequently these events greatly determine the glucose flux into the cardiomyocyte (Zaninetti *et al*. 1988). Inside the cell the enzyme hexokinase, converts glucose to glucose-6-phosphate. Cytoplasmic glucose 6-phosphate can either be stored in the form of glycogen (conversion via glycogen synthase) or undergo glycolysis (an oxygen independent process) which eventually yields pyruvate and ATP. Another important enzyme of the glycolytic pathway involved in the eventual formation of pyruvate is phosphofructokinase 1 (PFK-1) which is responsible for the conversion of fructose-6-phosphate to fructose-1,6- bisphosphate. Although it will not be discussed in this review it is worth mentioning that glucose-6-phosphate may also be processed via the pentose phosphate and the hexosamine biosynthetic pathway. Excess glucose flux through these pathways is associated with insulin resistance and the production of toxic intermediates (Gupte *et al*. 2006; Fülöp *et al*. 2007).

During surplus oxygen supply, pyruvate is transported into mitochondria via a mitochondrial monocarboxylate transporter (Halestrap and Price, 1999) and subsequently oxidized by pyruvate dehydrogenase (PDH) producing acetyl-CoA (reviewed by Stanley *et al*. 1997). During anaerobic conditions such as during myocardial ischaemia, pyruvate may however be converted to lactate.

It is important to mention that elevated FA β-oxidation rates can reciprocally inhibit glucose oxidation. The activity of hexokinase, PFK and PDH are all inhibited by various metabolic products produced during FA metabolism (discussed by Hue and

Taegtmeyer, 2009). Thus there is a delicate interplay between the different myocardial substrates utilized, which in turn is dependent on their relative circulating levels.
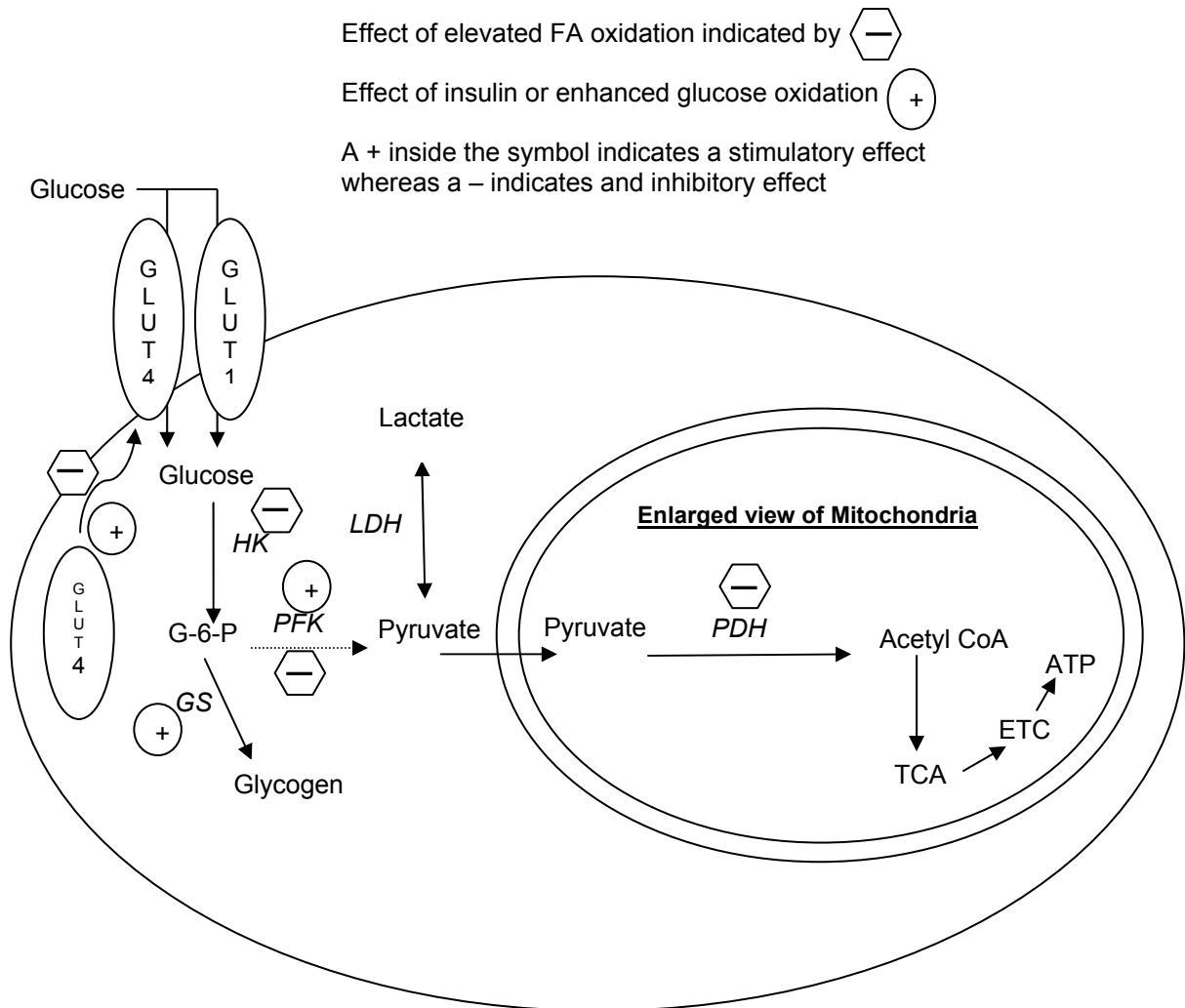
Figure 2.2: The pathways involved in glucose uptake and metabolism. *ATP* – Adinosine triphosphate; *ETC* – electron transport chain; *GLUT* – glucose transporter; *G-6-P* – glucose-6-phosphate; *GS* – glycogen synthase; *HK* – hexokinase; *LDH* – Lactate dehydrogenase; *PFK* – phosphofructokinase; *PDH* – pyruvate dehydrogenase complex; *TCA* – tricarboxylic acid cycle. See the text for mechanisms.

The common endpoint of glucose and FA oxidation is the production of acetyl-CoA. Acetyl-CoA derived from both fuel sources (glucose and FA) enters the tricarboxylic acid/Krebs/citrate cycle producing the reducing equivalents NADH and $FADH_2$.

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Additional NADH and $FADH_2$ are generated during the breakdown of FA and glucose (FA β-oxidation, glycolysis and the PDH reaction). These reducing equivalents play a central role in driving mitochondrial oxidative phosphorylation. Oxidative phosphorylation occurs in the electron transport chain, located in the inner mitochondrial membrane. In this process, NADH and $FADH_2$ are oxidized, yielding NAD^+, FAD, protons and electrons (Opie, 2004; Kim *et al*. 2008).

The electron transport chain consists of a series of complexes through which the electrons are transferred. The electrons ultimately bind to O_2 forming H_2O while the protons are pumped into the inter-mitochondrial membrane space by complexes I, III, IV of the electron transport chain. ATP production is greatly dependent on the fate of the protons as the formation of a proton gradient across the inner mitochondrial membrane essentially drives the F_1F_0 ATPase which itself enables the phosphorylation of ADP into ATP. Upon its production, ATP is subsequently transported to the cytosol where it is utilized for various energy dependent cellular functions. Lastly, the effectiveness of the proton gradient in the intermitochondrial membrane space is greatly determined by the amount of uncoupling proteins present in the membrane. Reducing the proton gradient via "proton leakage" through these uncoupling proteins would lead to the generation of heat instead of ATP, resulting in uncoupling of the respiratory chain (Opie, 2004; Kim *et al*. 2008). This essentially results in oxygen wastage which could compromise the cells' integrity during ischaemia where oxygen wastage should be avoided.

2.3.2 Insulin signaling in the regulation of myocardial substrate metabolism

The hormone insulin is an important regulator of myocardial substrate metabolism. Activation of the insulin receptor (IR) by insulin binding invokes a cascade of events which ultimately elicits enhanced glucose uptake and metabolism by the insulin sensitive tissue (Figure 2.3). Binding of insulin to its receptor results in autophosphorylation and activation of the IR's intrinsic tyrosine kinases which upon phosphorylation, further phosphorylates proteins such as the insulin receptor substrate (IRS) proteins. Phosphorylated IRS1 subsequently associates with phosphoinositide 3-kinase (PI3K) via its p85 subunit (Myers *et al*. 1992; Yonezawa *et al*. 1992), an event vital for initiating insulin's effects on glucose metabolism (reviewed by White and Kahn *et al*. 1994; Shepherd *et al*. 1998; Chang *et al*. 2004). Activated PI3K consequently induces (via various signaling mechanisms) insulin's major effector molecule, protein kinase B (PKB/AKT) (Alessi *et al*. 1996). PKB/AKT is a major signaling molecule which plays a pivotal role in glucose metabolism, regulating the translocation of the cytosolic glucose transporter, GLUT 4, to the sarcolemma (Alessi and Cohen, 1998; Foran *et al*. 1999). Due to the vital role played by PI3K and PKB/AKT in glucose metabolism, inhibiting the activity of these proteins greatly attenuates sarcolemmal translocation of GLUT 4, effectively reducing insulin stimulated glucose uptake (Clarke *et al*. 1994; Summers *et al*. 1998).

PI3K contains a catalytic (p110) and regulatory subunit (p85 (α and β isoforms)). The p85 subunit is important in insulin signaling as it enables the interaction of the PI3K catalytic subunit with IRS-1 (Yonezawa *et al*. 1992). Although PI3K activity is normally assessed as an indicator of the extent of insulin stimulation, phosphorylation of the p85 subunit (Tyr[458]) has been shown to increase with insulin stimulation, while in the presence of oleic acid and insulin, its phosphorylation is attenuated (Kim *et al*.

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2009). Significantly, PI3K p85 (Tyr$^{458/199}$)/p55 phosphorylation increases with cardiomyocyte glucose uptake upon insulin stimulation (Florian *et al*. 2010).

Besides facilitating glucose uptake via GLUT 4, insulin stimulation greatly increases glycolytic flux. This is achieved through activation of PFK-2 which in turn enhances the production of fructose-2,6-bisphosphate from fructose-6-phosphate (Rider and Hue, 1984; Hue and Rider, 1987; Hue *et al*. 2002). Fructose-2,6-bisphosphate stimulates PFK-1 activity, which will aid in enhancing glycolysis (summarized by Hue *et al*. 2002).

Elevated insulin levels further suppress tissue FA β-oxidation rates (Figure 1). This is most likely due to the impact of by-products of elevated glucose oxidation on malonyl-CoA levels (Saddik *et al*. 1993). In slight contrast, insulin facilitates the transport of LCFA into the cardiomyocyte by increasing the sarcolemmal distribution of FAT/CD36 (Luiken *et al*. 2002). Regardless of this, the augmented LCFA uptake resulting from insulin stimulation does not translate into enhanced FA β-oxidation, but rather increased esterification (Luiken *et al*. 2002). Insulin thus has a significant impact on both glucose and lipid metabolism.
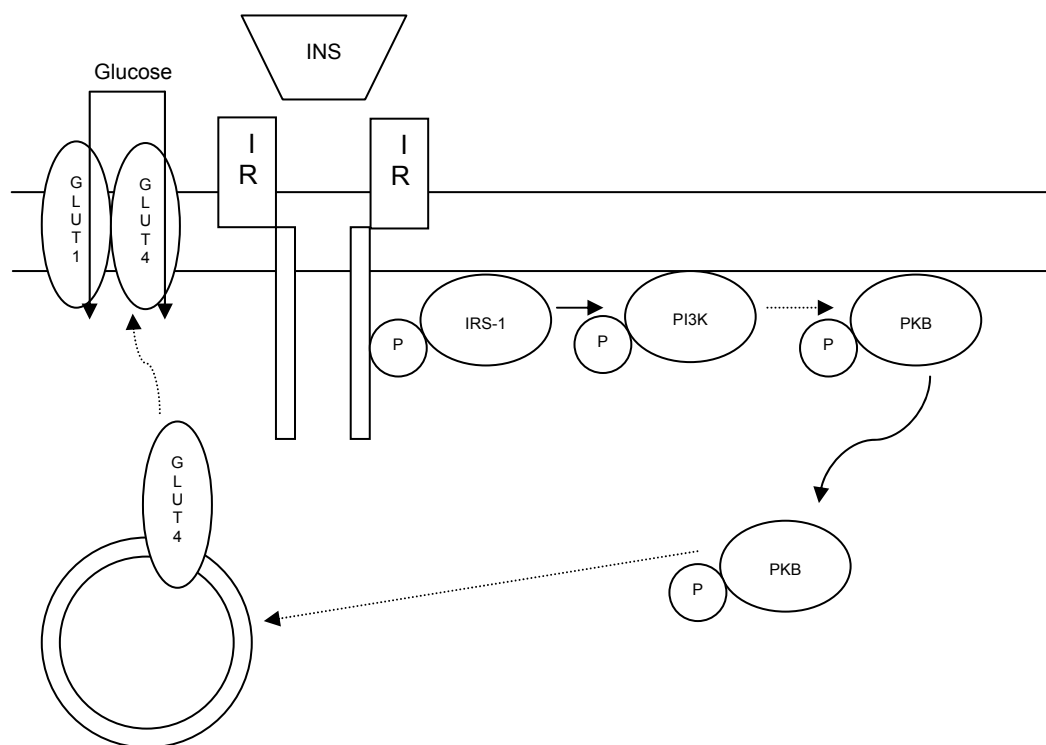
Figure 2.3: The mechanism of insulin stimulated glucose uptake. *GLUT* – glucose transporter; *IR* – insulin receptor; *IRS* – insulin receptor substrate; *P* – phosphorylated substrate. Adapted from Bertrand *et al.* (2008) and Feuvray, (2004).

2.3.3 The impact of obesity on myocardial insulin sensitivity and myocardial substrate metabolism

Insulin resistance ensues when normal physiological concentrations of insulin are no longer able to induce effective uptake of glucose by insulin sensitive tissue. Myocardial insulin resistance is demonstrated by reduced glucose oxidation rates and compromised intracellular insulin signaling pathways in experimental animal models of obesity (Zhang *et al.* 2010). As a compensatory mechanism aiming to maintain euglycaemia, pancreatic insulin secretion increases leading to a state of hyperinsulinaemia. The degree of glucose intolerance in insulin resistant individuals is thus dependent on the extent of the loss of the *in vivo* function of insulin, and the ability of the pancreas to adjust for this by secreting more insulin (Reaven, 1988; Reaven, 1995). Once elevated circulating levels of insulin are no longer able to maintain euglycaemia, a diabetic state ensues.

In most instances, obesity occurs concurrently with some degree of systemic insulin resistance. Many cardiovascular risk factors are speculated to be derived from the combination of obesity and insulin resistance (Leichman *et al*. 2006). Both insulin resistance and the probable ensuing type II diabetes are suggested to be prevented by lifestyle modification in the form of increased physical activity and dietary intervention (Seidell, 2000).

Obesity is mostly associated with an insulin resistant myocardium similar to that seen in the diabetic heart. Myocardial FA oxidation rates are normal or elevated, while more importantly, glucose oxidation rates are reduced with or without insulin stimulation (*ex vivo* experiments) (Mazumder *et al*. 2004; Buchanan *et al*. 2005; Wilson *et al*. 2007; Aasum *et al*. 2008; Zhang *et al*. 2010). Even in certain instances of obesity and insulin resistance where myocardial FA and glucose oxidation rates are similar to those reported in lean control animals, insulin stimulated myocardial glycolytic flux rates remain suppressed (Lopaschuk and Russell, 1991; Atkinson *et al*. 2003). In humans similar pronounced features of obesity related myocardial insulin resistance are not as common. Nevertheless, while myocardial glucose uptake and utilization and FA uptake, utilization and oxidation rates are not significantly different between obese and non-obese subjects, it has been shown that the degree of systemic insulin resistance in women relates to the degree of myocardial FA uptake, utilization and oxidation (Peterson *et al*. 2004a). It also seems that the myocardial metabolic response to obesity differs between men and women as it was shown that the female gender independently predicted lower myocardial glucose uptake, utilization and utilization/plasma insulin (Peterson *et al*. 2008).

Interestingly, two studies have reported data on diabetic patients with or without ischaemic heart disease displaying systemic insulin resistance while presenting similar rates of myocardial glucose uptake compared to control subjects in response to insulin infusion (Utriainen *et al*. 1998; Jagasia *et al*. 2001). Thus, although it is generally believed that obesity and diabetes are associated with altered myocardial FA metabolism in conjunction with reduced glucose metabolism, the previously mentioned studies allude to the possibility that systemic insulin resistance does not always lead to myocardial insulin resistance in humans. Unfortunately myocardial glucose oxidation rates were not determined in the two previously mentioned studies.

The mechanisms whereby obesity contributes to reduced muscle insulin sensitivity have been extensively investigated. In particular, the downstream impact of altered adipocyte function characteristic of the obese state is implicated in the development of insulin resistance. These alterations include 1) increased release of FFA (Randle *et al*. 1963; Roden *et al*. 1996), 2) reduced secretion of the insulin sensitizing adipokine, adiponectin, coupled with lower adiponectin receptor densities (Kadowaki *et al*. 2006) and, 3) increased secretion of pro-inflammatory cytokines such as TNFα from macrophages (Rydén and Arner, 2007). Lastly, as the use of antioxidants abolishes the ensuing insulin resistance in animal models of obesity, oxidative stress may also contribute to the development of insulin resistance (Nduhirabandi *et al*. 2011). In this review, only the role of FFA's in the development of muscle insulin resistance will be covered.

Probably the most influential determinant of systemic insulin sensitivity is increased levels of circulating FFA (Kahn *et al*. 2006). As over-nutrition is the primary cause of obesity, it is not surprising that the abundant availability of circulating oxidative fuels

is implicated in the development of systemic insulin resistance. The concentration of circulating FFA is largely governed by the rate of lipolysis. In obesity, the rate of lipolysis in response to β-adrenergic stimulation may be elevated in certain fat depots (Lönnqvist *et al*. 1995). Due to the oversupply of FFA, obesity and particularly diabetes are further characterized by the storage of excess lipids in non-adipose tissue such as skeletal muscle (Manco *et al*. 2000) and in certain instances, cardiac muscle (Szczepaniak *et al*. 2003; Kankaanpää *et al*. 2006; McGavock *et al*. 2007; Rijzewijk *et al*. 2008). Besides its association with myocardial insulin resistance, excess intramyocardial lipid accumulation is further implicated in cardiac dysfunction (see section 2.4.3).

The notion that elevated levels of circulating FFA play an important contributory role in the development of cellular insulin resistance has been repetitively demonstrated. As most of the mechanistic experiments focusing on the development of insulin resistance have been performed in skeletal muscle, the interaction between circulating FFA, intracellular lipid accumulation and insulin resistance in both skeletal and cardiac muscle will be discussed.

2.3.4 The interaction between circulating free fatty acids, intracellular lipid accumulation and insulin resistance

2.3.4.1 Skeletal muscle

An inverse correlation exists between intramuscular lipid content and insulin sensitivity. Measurements of insulin sensitivity (120 min euglycaemic hyperinsulinaemic clamp) in skeletal muscle from healthy humans demonstrated that subjects with higher levels of intramuscular lipid content had lower whole body insulin stimulated glucose uptake. These individuals also had higher circulating FFA's during hyperinsulinaemia, reduced tyrosine phosphorylation of the IR and lower IRS-1 mediated PI3K activation than subjects with low intramuscular lipid content (Virkamäki *et al*. 2001). Comparisons between lean and obese individuals have made similar observations (Manco *et al.* 2000; Sinha *et al*. 2002). Boden *et al.* (2001) identified strong associations between serum FFA levels, intramuscular lipid content and insulin resistance. Elevated circulating FFA levels, achieved by lipid injection, were shown to be associated with a gradual increase in intramuscular lipid content, an observation which correlated with a 40% increase in insulin resistance. These findings are supported by Kelley *et al.* (1993) who similarly observed reduced skeletal muscle glucose uptake in response to elevated levels of FFA in humans.

2.3.4.2 Cardiac muscle

Similar associations between excess intramyocellular lipid accumulation and reduced insulin sensitivity as noted in skeletal muscle, have been observed in cardiac tissue from obese insulin resistant animals (Young *et al*. 2002; Atkinson *et al*. 2003; Yan *et al*. 2009). Indeed, in obese insulin resistant JCR:LA-cp rats, the increased supply of circulating FFA was associated with a 50% increase in myocardial Trig content, which corresponded with a 50% reduction in myocardial glycolytic flux rates (Atkinson

et al. 2003). Significantly in humans, plasma FFA levels correlate with intramyocardial Trig levels (Kankaanpää *et al*. 2006), which are also evident in obese (Szczepaniak *et al*. 2003; Kankaanpää *et al*. 2006), obese glucose intolerant (McGavock et al. 2007) and diabetic (McGavock *et al*. 2007; Rijzewijk *et al*. 2008) individuals. Excessive intramyocardial Trig accumulation thus precedes the development of type II diabetes. Furthermore intramyocardial Trig levels tend to increase linearly with the degree of systemic insulin resistance (McGavock *et al*. 2007). Unfortunately no human data exist that quantify and correlate intramyocardial Trig content and the degree of myocardial insulin resistance. It should be emphasized that although in many cases intracellular Trig accumulation is associated with skeletal and cardiac muscle insulin resistance, the accumulation of more specific lipid intermediates such as ceramide and diacylglycerol may play a more causal role in the development of tissue specific insulin resistance (Turinsky *et al*. 1990; Ussher *et al*. 2010; Zhang *et al*. 2010), since not all cases of myocardial insulin resistance are associated with elevated intramyocardial Trig levels (Aasum *et al*. 2008).

Excess intracellular lipids accumulating within the myocardium are believed to stem from either 1) increased FA supply to the heart which is in excess of its oxidative capacity, or 2) suppressed myocardial FA oxidation rates (Lopaschuk *et al*. 2010).

While the majority of studies indicate that myocardial FA β-oxidation rates are elevated in obesity (Mazumder *et al*. 2004; Buchanan *et al*. 2005; Wilson *et al*. 2007; Aasum *et al*. 2008; Zhang *et al*. 2010), a limited number of studies suggest that reduced myocardial FA β-oxidation rates are responsible for intramyocardial lipid accumulation (Young *et al*. 2002). While non-fasted obese Zucker rats have comparable myocardial FA oxidation rates and intramyocardial lipid content

compared to lean control rats, an increased supply of FFA's, induced by fasting, was shown to significantly increase myocardial FA oxidation rates and myocardial function and decrease intramyocardial lipid content in control animals. In strong contrast to this data, hearts from obese Zucker rats were unable to augment FA oxidation rates or myocardial function, while the intramyocardial lipid content rose significantly in response to the fasting induced increased myocardial FFA supply (Young *et al*. 2002). Lopaschuk *et al*. (2010) has cautioned the implications of these findings as the authors did not consider the contributory effect of the excess pool of intramyocardial lipids on the overall rate of myocardial FA oxidation. Nevertheless, while the hypothesis that reduced FA oxidation rates may contribute to intracellular lipid accumulation is an attractive one, the majority of the available data indicate that myocardial FA oxidation rates in obesity are seldom lower than in lean controls (Mazumder *et al*. 2004; Buchanan *et al*. 2005; Wilson *et al*. 2007; Aasum *et al*. 2008; Zhang *et al*. 2010).

2.3.5 The cellular mechanisms of lipid induced insulin resistance

As previously mentioned, an increase in the supply of one oxidative substrate (FA or glucose) will result in that particular substrate being favoured with regards to its utilization. Enhanced FA metabolism has been shown to inhibit glucose metabolism at multiple levels. Firstly, elevated levels of $NADH/NAD^+$ and acetyl-CoA/CoA, produced during FA β-oxidation inhibits PDH activity possibly via PDH kinase 4 (PDHK4) activation (an inhibitor of PDH) (Denton *et al*. 1975; Hansford and Cohen, 1978; Holness and Sugden, 2003). Additionally, augmented FA β-oxidation causes a rise in citrate levels, an enzyme capable of directly inhibiting phosphofructokinase (Garland *et al*. 1963) which in turn inhibits hexokinase activity through the accumulation of glucose-6-phosphate (Regen *et al*. 1964). Secondly, FA's may exert

direct inhibitory effects on components of the insulin signaling pathway. The presence of palmitate in the perfusate of glucose and insulin perfused hearts induced an inhibitory effect on PKB/AKT activity and phosphorylation which coincided with reduced myocardial glucose uptake, glycolysis and glucose oxidation rates (Soltys *et al*. 2002). In rat skeletal muscle cells, palmitate was shown to inhibit IR gene expression, possibly via protein kinase C (PKC) ε, thus disrupting the insulin signaling pathway (Dey *et al*. 2005). These inhibitory actions of FA's become more relevant following chronic exposure to an elevated FFA supply and could contribute to decreased systemic and cellular insulin sensitivity.

In conditions of over-nutrition, not all FA's entering the cell are utilized for oxidative purposes. Non-oxidative pathways exist whereby the intracellular pool of long chain fatty acyl-CoA's are increased. Consequently long chain acyl-CoA's can enter lipid pathways for the formation of diacylglycerol, Trig and ceramide (Figure 2.1). Experimental evidence suggests that the intracellular formation and accumulation of diacylglycerol and ceramide in particular, adversely affect insulin signaling pathways and consequently carbohydrate metabolism (Figure 2.4) (Turinsky *et al*. 1990; Ussher *et al*. 2010; Zhang *et al*. 2010).

In rat skeletal muscle, experimentally induced elevated circulating FFA were shown to increase intracellular acyl-CoA and diacylglycerol (a known activator of PKC) levels coupled with increased membrane bound and/or active protein kinase C (PKC) theta. These changes were accompanied by increased IRS-1 serine phosphorylation, reduced IRS-1 tyrosine phosphorylation and reduced IRS-1 associated PI3K activity (Griffin *et al*. 1999; Yu *et al*. 2002). Phosphorylation of IRS-1 at serine[307] is sufficient to hinder IRS-1's interaction with PI3K. Similarly, the development of myocardial

insulin resistance in obese rats coincided with elevated cardiac diacylglycerol levels and was accompanied by increased membrane translocation of PKCα, increased p70s6k activity and IRS-1 phosphorylation at Ser$^{332/336}$ (Zhang *et al*. 2010). Both PKCα and p70s6k are capable of phosphorylating IRS-1 at serine/threonine residues thereby reducing IRS1 association with PI3K. It is subsequently speculated that myocardial diacylglycerol accumulation together with PKCα membrane translocation and phosphorylated p70s6k play a central role in specifically high fat diet (HFD) induced myocardial insulin resistance (Zhang *et al*. 2010). Although various PKC isoforms are implicated in the aetiology of insulin resistance, these experimental observations are in agreement with the general view that PKC can exert inhibitory effects on IRS-1 activation (Hegarty *et al*. 2003). The net result is disrupted insulin signaling which prevents IRS-1's association with insulin's mediating molecule PI3K. Due to reduced IRS-1 associated PI3K activity following insulin stimulation, glucose transport into the cell may be reduced (Dresner *et al*. 1999).

Another lipid intermediate implicated in the pathogenesis of insulin resistance is ceramide (Summers, 2006). Elevated cardiac and skeletal muscle ceramide levels have been observed in rodent models of obesity/diabetes and insulin resistance (Zhou *et al*. 2000; Ussher *et al*. 2010) and are thought to inhibit components of the insulin signaling cascade. While ceramide levels are increased in skeletal muscle of obese insulin resistant rats, it was further shown to be the only lipid intermediate to correlate significantly with glucose intolerance (Ussher *et al*. 2010). In the same study, myriocin was shown to abolish the development of insulin resistance which coincided with reduced skeletal muscle ceramide (but not diacylglycerol) content. The signaling pathway through which ceramide may induce insulin resistance is

poorly understood, but may involve the inhibition of PKB/AKT phosphorylation (Summers *et al*. 1998).

The current data regarding the mechanism of lipid induced insulin resistance is in agreement with the hypothetical model previously proposed by Coort *et al*. (2007), in which an obesity/diabetes related permanent translocation of CD36 exists on the cardiomyocyte sarcolemma. This is associated with increased LCFA uptake with a subsequent rise in intracellular Trig's. The intracellular pool of Trig intermediates such as acyl-CoA's, ceramide and diacylglycerol are consequently increased which in turn impairs insulin signaling, possibly mediated via protein kinase C, resulting in decreased insulin-stimulated GLUT 4 translocation to the sarcolemma.
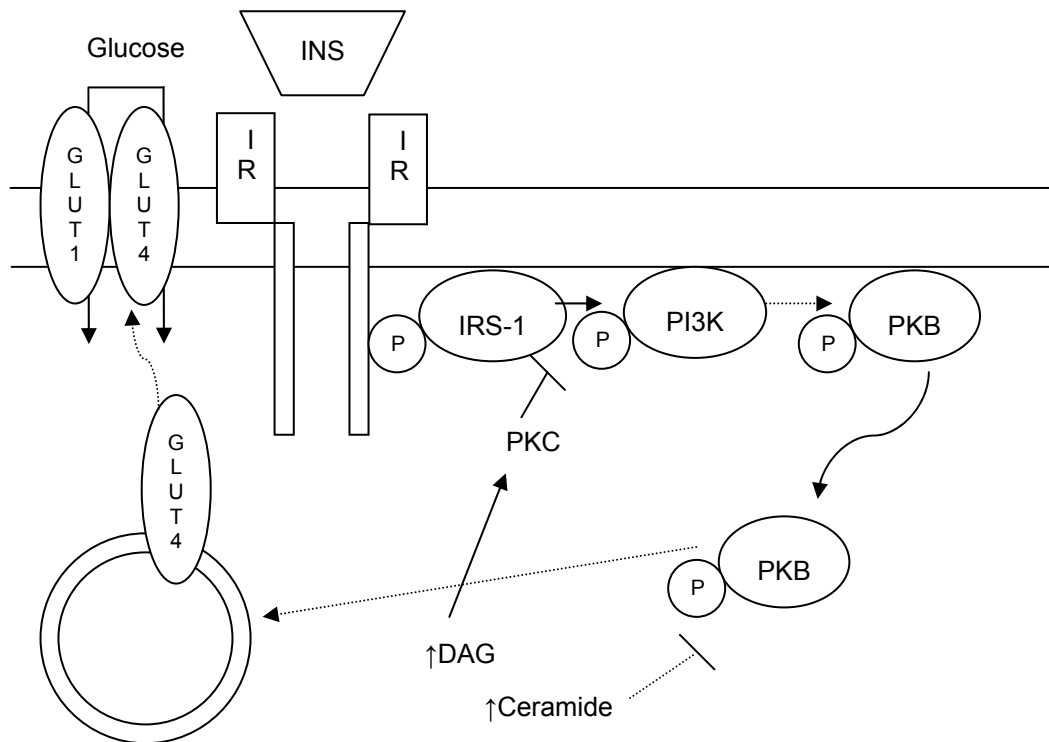
Figure 2.4: The proposed signaling involved in lipid induced insulin resistance. *DAG* – diacylglycerol; *GLUT* – glucose transporter; *IR* – insulin receptor; *IRS* – insulin receptor substrate; *P* – phosphorylated substrate. See text for references

2.4 Cardiac function

2.4.1 Cardiac function in obese humans

Many studies have demonstrated that obesity is associated with normal or increased LV mechanical function in the presence or absence of cardiac hypertrophy (Berkalp *et al*. 1995; Iacobellis *et al*. 2002, 2004; Pascual *et al*. 2003; Otto *et al*. 2004; Dorbala *et al*. 2006). There is however a general consensus that protracted obesity is associated with some form of diastolic dysfunction (Berkalp *et al*. 1995; Iacobellis *et al*. 2002; Pascual *et al*. 2003; Otto *et al*. 2004). While obesity remains an independent predictor of heart failure (Kenchaiah *et al*. 2002), it is uncertain whether clinically significant LV mechanical dysfunction occurs as a result of protracted obesity.

Alpert *et al.* (1995; 1997) has suggested that longstanding morbid obesity together with the associated LV hypertrophy could lead to cardiac dysfunction. A study conducted by the previously mentioned research group determined an association between the duration of morbid obesity, LV mass and reduced LV systolic and diastolic function. The authors further reported that weight reduction correlated with decreased LV mass and concomitant improvements in cardiac systolic and diastolic function (Alpert *et al*. 1995). In addition, morbid obese individuals with increased LV mass demonstrate reduced LV systolic function in response to exercise (Alpert *et al*. 1989). In line with these findings, it is speculated that compensatory hypertrophy may over decades become maladaptive leaving a vulnerable ventricle that is simply unable to adapt to the volume overload, consequently leading to the manifestation of systolic dysfunction. Obesity associated with LV diastolic dysfunction, together with possible systolic dysfunction has been proposed to progress to heart failure

(Kopelman, 2000), a notion agreeing with the observation that obesity is an independent risk factor for heart failure (Kenchaiah *et al*. 2002).

Although systolic dysfunction may be feasible in morbid obese individuals, the recent observation that general obesity may indeed be accompanied by sub-clinical systolic dysfunction is intriguing (Ferraro *et al*. 1996; Peterson *et al*. 2004b; Wong *et al*. 2004; Di Bello *et al*. 2006; Tumuklu *et al*. 2007, Kosmala *et al*. 2008a). Sub-clinical alterations in both LV structure and systolic function have even been reported in overweight individuals (Wong *et al*. 2004). Additionally, analysis of 295 apparently healthy overweight subjects revealed that BMI, fasting insulin and age were independent predictors of sub-clinical LV systolic dysfunction (strain, peak strain rate), while it was almost absent in the absence of hyperinsulinaemia (Kosmala *et al*. 2008b). Wong and Marwick, (2007) have commented that preserved or augmented LV systolic function observed in obese individuals in many studies may be confounded by the insensitivity and load-dependency of the standard echocardiographical techniques used (fractional shortening and ejection fraction), while newer techniques investigating myocardial tissue velocity and strain index are sensitive enough to identify sub-clinical LV systolic alterations. Whether sub-clinical systolic dysfunction may progress to clinically relevant systolic dysfunction is uncertain.

Due to the lack of longitudinal studies investigating obesity related cardiac dysfunction, it is not possible to establish whether obesity indisputably causes myocardial dysfunction (Abel *et al*. 2008). Indeed, the notion of the existence of an obesity cardiomyopathy has been questioned (Owan and Litwin, 2007).

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2.4.2 Cardiac function in obese insulin resistant animals

Valuable insights regarding the myocardial functional response to obesity have been gained from animal models of obesity, however as with the human data, it seems that animal models of obesity also yield inconclusive observations.

Genetic animal models of pre-diabetic obesity display cardiac dysfunction (Young *et al*. 2002). Although genetic models of obesity do not accurately resemble the phenotype of human obesity, the recent development of a number of diet-induced models of obesity have contributed to a better understanding of the impact of obesity on myocardial function. Animal models of diet-induced obesity have reported normal (Carroll *et al*. 2006, Wilson *et al*. 2007; Yan *et al*. 2009) or reduced (Du Toit *et al*. 2005; Ouwens *et al*. 2007; Wilson *et al*. 2007; Aasum *et al*. 2008) cardiac function. It is often difficult to identify the precise mechanism contributing to cardiac dysfunction as various animal models of obesity displaying both normal (Carroll *et al*. 2006) and abnormal (Yan *et al*. 2009) serum insulin and glucose levels have been associated with normal cardiac function. The possibility further exists that studies employing models of diet-induced obesity are too short in duration for cardiac dysfunction to develop. Wilson *et al*. (2007) only noted cardiac dysfunction in obese insulin resistant rodents following 32-48 weeks of Western diet feeding. Cardiac dysfunction may thus only be evident with protracted obesity during which the heart would be subjected to excess oxidative fuel supply and possibly extensive cardiac remodelling over a prolonged period of time. However adverse cardiac remodelling (indexed by increased ventricular weight) was absent in the study of Wilson *et al*. (2007).

2.4.3 Evidence supporting reduced cardiac function due to lipotoxicity

The accumulation of excess myocardial metabolic byproducts derived from lipid metabolism and the hydrolysis of myocardial Trig's are implicated in what has been termed cardiac lipotoxicity (Unger and Orci, 2002; McGavock et al. 2006). In particular the generation of ceramide is strongly implicated in cellular dysfunction and apoptotic cell death (lipo-apoptosis) (Zhou *et al*. 2000; Unger and Orci, 2002). Although excess intracellular ceramide accumulation may better explain lipotoxicity in a mechanistic manner, the majority of research has been aimed at determining the relationship between myocardial function and intramyocardial Trig content. This may not be the most appropriate approach as it has been proposed that the incorporation of excess FA into the pool of intracellular Trig's prevents the formation of toxic lipid intermediates suggesting that these lipid stores may actually protect against lipotoxicity (Listenberger *et al*. 2003).

In humans, associations exit between excess intramyocardial lipid accumulation and LV function. While reductions in intramyocardial lipid (Trig) content are associated with improved LV ejection fraction following exercise training in obese subjects (Schrauwen-Hinderling *et al*. 2010), an inverse relationship exists between septal lipid (Trig) content and LV systolic function (septal thickening) (Szczepaniak *et al*. 2003). However, no correlation exists between septal Trig content and ejection fraction (Szczepaniak *et al*. 2003). Despite these associations in humans, causality between intramyocardial lipid accumulation and cardiac dysfunction remains to be shown.

The use of animal models of obesity and/or diabetes allows for a more careful investigation into the relationship between intramyocardial lipid content and cardiac

dysfunction. The evidence available thus far suggests a close relationship between these two events. While increasing myocardial FA supply by lipid infusion is followed by myocardial lipid droplet accumulation and diminished *in vivo* cardiac function in rabbits (Hexeberg *et al*. 1995), the co-occurrence of excess intramyocardial lipid accumulation (intracellular phospholipid and Trig) and cardiac dysfunction is evident in certain obese insulin resistant animals (Ouwens *et al*. 2007). Further insight into this relationship is gained by studies using genetic manipulations aimed at enhancing myocardial FA metabolism. Mice over-expressing cardiac specific long chain acyl-CoA synthase display high intramyocardial levels of Trig's and ceramide, accompanied by increased DNA fragmentation and cytochrome c release, indicative of apoptosis. These mice develop early cardiac hypertrophy, followed by LV dysfunction and eventually die prematurely (Chiu et al. 2001). Furthermore, mice over-expressing cardiac specific human lipoprotein lipase, develop excess intramyocardial lipid accumulation which is associated with myocyte hypertrophy, dilated hearts and reduced LV systolic function (Yagyu *et al*. 2003). In a separate study, fasting-induced elevations in circulating FFA levels were associated with increased myocardial FA β-oxidation rates, normal intramyocardial lipid content and augmented cardiac output in lean Zucker rat hearts. Obese Zucker rat hearts however failed to increase myocardial FA β-oxidation rates, displaying elevated intramyocardial lipid levels and were further unable to elevate their cardiac output relative to the values obtain by the lean animals, indicative of cardiac dysfunction (Young *et al*. 2002). The authors ascribe such cardiac dysfunction to the heart's inability to increase FA β-oxidation in response to elevated FA availability. This finding coincided with a reduced expression of genes regulating myocardial FA metabolism with a concomitant rise in intracellular Trig content. Lastly, one of the most significant findings in this regard was the observation that by reducing cardiac

Trig and ceramide levels pharmacologically, myocardial lipoapoptosis was prevented while cardiac dysfunction was reduced in obese diabetic animals (Zhou *et al*. 2000).

Despite these convincing reports, Wilson *et al*. (2008) reported *ex vivo* cardiac dysfunction in rodents following long term consumption of a western diet in the absence of elevated intramyocardial lipid (Trig) content. These results either argue against a causal role of intramyocardial lipid accumulation in the pathogenesis of cardiac dysfunction, or more specifically allude to the importance of determining the levels of lipid intermediates such as ceramide. Indeed, of the various lipid metabolites, ceramide may be the most detrimental in inducing myocardial lipo-apoptosis (Zhou *et al*. 2000), while diacylglycerol accumulation may be more involved in the pathogenesis of myocardial insulin resistance (Zang *et al*. 2010). Alternatively other mechanisms may exist whereby excess calories may induce cardiac dysfunction.

2.4.3.1 Possible mechanisms of lipid induced cardiotoxicity

Models of cardiac lipotoxicity have demonstrated the co-occurrence of elevated myocardial ceramide levels together with increased indices of apoptosis (Zhou *et al*. 2000; Chiu *et al.* 2001). Similarly neonatal cardiomyocytes incubated with physiological concentrations of palmitate have increased intracellular Trigs and ceramide levels coupled with increased levels of apoptosis (Hickson-Bick *et al*. 2000). A mechanism implicated in ceramide-induced apoptosis has been proposed (Unger and Orci, 2002) and although the pathway is poorly delineated, it may involve activation of nuclear factor-kappa β (Machleidt *et al*. 1994), which in turn up-regulates the expression of the inducible isoform of nitric oxide synthase (iNOS). The resulting increase in nitric oxide production (Shimabukuro *et al*. 1997) may cause a

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subsequent rise in the formation of peroxynitrite, which has been shown to induce mitochondrial cytochrome c release (Ghafourifar *et al*. 1999) and subsequent apoptosis (see Figure 2.5).
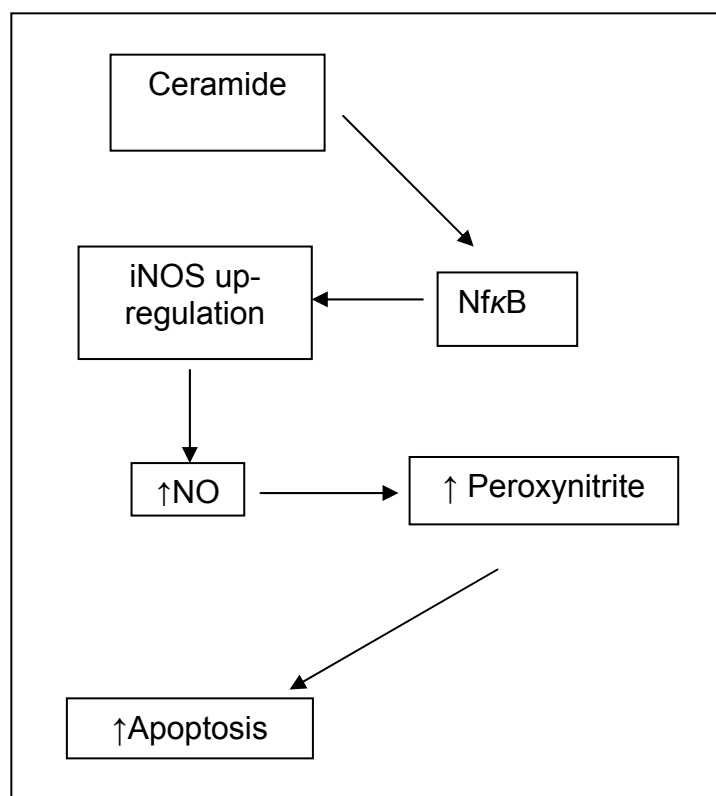
Figure 2.5: Proposed mechanism of ceramide induced apoptosis. Although this is the recognized pathway in pancreatic β cells, it could be similar in other tissues including the heart. *iNOS* – inducible form of nitric oxide synthase; *NfkB* - nuclear factor kappa B; *NO* – nitric oxide; *PKB/AKT* – protein kinase B. Modified from Unger, (2005).

2.4.4 Other potential causes of cardiac dysfunction in obesity/diabetes not highlighted in detail

Although we have discussed lipotoxicity as a means of inducing cardiac dysfunction in obesity, it is important to mention that other mechanisms such as activation of the renin-angiotensin system, increased reactive oxygen species (ROS) production, abnormal calcium handling and toxicity caused by glucose intermediates (Clark *et al*. 2003; Hu *et al*. 2005; Minhas *et al*. 2005; Cesario *et al*. 2006; Schwanke *et al*. 2006;

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Boudina and Abel, 2007; Ballal *et al*. 2010) may additionally contribute to cardiac dysfunction in diabetes and at times in obesity.

Most of these functional abnormalities described in the previous paragraph including lipotoxicity may be related to an oversupply of oxidative fuels or the source of the excess calories supplied to the heart. A series of impressive studies from the Taegtmeyer research group have yielded exciting evidence identifying novel mechanisms of obesity related cardiac dysfunction. Wilson *et al*. (2007) postulated that the source of the excess calories (i.e. derived from fat or carbohydrate) may be important in the aetiology of obesity related cardiovascular disease. In a well designed study Wilson *et al*. (2007) fed rats a normal diet (10% fat, 70% carbohydrate), high fat diet (60% fat, 20% carbohydrate) or Western diet (high fat and high carbohydrate) (45% fat, 35% carbohydrate) for an acute (1 day to 1 week), short (4-8 weeks), intermediate (16-24 weeks) or long (32-48 weeks) period. Interestingly, cardiac dysfunction was only evident in rats fed the Western diet and only after 32-48 weeks of feeding. This corresponded with an inability of myocardial FA response genes to be sufficiently activated. While both the high fat and Western diets were able to increase mRNA transcripts of proteins involved in uncoupling, the former was able to do this to a greater degree than the Western diet group. Further analysis implicated reduced anaplerotic flux of the Krebs cycle and oxidative stress (reduced anti-oxidant enzyme transcription and evidence of glucolipotoxicity) related injury as mechanisms contributing to cardiac dysfunction in the model. In addition, hearts from Western diet fed rats displayed increased apoptosis with reduced expression of anti-apoptotic Bcl2 and increased mitochondrial biogenesis (indicative of mitochondrial damage), all of which are implicated in the mechanism of cardiac dysfunction induced by long term Western-diet consumption (Ballal *et al*. 2010).

Many of these detrimental changes associated with Western diet feeding were absent in the HFD fed group after the same period of feeding.

As a cautionary note to this section it should be emphasized that the FA composition of the diet may also play an important part in the mechanism of cardiac dysfunction. It is known that lipotoxicity is often seen when cardiomyocytes are treated with saturated FA's (Hickson-Bick *et al*. 2000). While cells cultured with different doses of long chain saturated FA's undergo cell death, it is intriguing to find that culturing cells with similar concentrations of unsaturated LCFA does not induce cell death (Listenberger *et al*. 2003). Furthermore cells co-cultured with saturated and unsaturated FA's do not develop lipotoxicity (Listenberger *et al*. 2003). Indeed it has been suggested that a diet high in unsaturated fatty acids and low in refined carbohydrates may afford lipoprotection (Taegtmeyer and Stanley, 2011). We again emphasize that the relationship between FA metabolism, lipid accumulation and cardiomyopathy remains unclear in humans and causality remains to be shown (Lopaschuk *et al*. 2007).

2.4.5 Myocardial substrate flexibility and cardiac function in hearts from healthy and obese animals

While the majority of ATP produced from the heart is derived from the oxidation of FA's (Zierler, 1976; Opie, 1998), experimental studies have shown that the healthy heart is metabolically flexible, able to achieve normal LV mechanical function even when substrate supply is shifted away from FA metabolism (Yan *et al*. 2009). This is further reflected by conditions where isolated hearts from healthy lean animals are able to achieve comparable indices of LV mechanical function regardless of whether being perfused with glucose alone or glucose in combination with FA's (Lopaschuk *et*

al. 1999; Liu *et al*. 2002; Boudina *et al*. 2005). It has however been shown that acute elevations of the FA concentrations in the perfusate of isolated hearts from healthy mice fail to impact on LV mechanical function (How *et al*. 2005). With respect to hearts from obese animals, one study has shown that isolated hearts from ob/ob mice, when retrogradely perfused, are able to achieve comparable LV function compared to hearts from lean mice in the presence of glucose alone or glucose in combination with palmitate in the perfusate (Boudina *et al*. 2005). This is however in contrast to studies reporting reduced LV mechanical function in isolated glucose perfused working hearts from obese rats (Du Toit *et al*. 2005; Nduhirabandi *et al*. 2011). The discrepancy between these findings may be related to the use of different perfusion models (non-working Langendorff perfused heart vs. working heart) used to assess LV function.

In response to increased workload elicited by β-adrenergic receptor stimulation or the addition of calcium to the perfusion medium, the healthy heart reacts by increasing glucose oxidation rates, while FA oxidation rates have been reported to become slightly elevated, remain constant or even become reduced (Collins-Nakai *et al*. 1994; Wilson *et al*. 2007; Yan *et al*. 2009). During this increased LV workload, the contribution of glucose to myocardial ATP production increases greatly (Collins-Nakai *et al*. 1994). Even then healthy hearts from lean animals are still able to achieve comparable peak LV function whether being perfused with glucose as the sole energy source or in combination with FA (Boudina *et al*. 2005). This is in contrast to hearts from obese ob/ob mice which fail to achieve comparable LV function to hearts from lean mice when perfused with glucose alone or in combination with FA in response to increased workload (Boudina *et al*. 2005). This study however failed to investigate the contributory effect of insulin on LV functional reserve, which is

important, given the hyperinsulinaemic state associated with obesity. Recently, LV functional reserve in response to epinephrine or calcium supplementation of the perfusate has been shown to be normal in isolated hearts from diet induced obesity rodents when perfused with glucose, insulin and FA. (Wilson *et al*. 2007; Yan *et al*. 2009). Hearts from obese animals thus appear to have sufficient metabolic reserve in response to elevated workloads.

2.5 Mitochondrial abnormalities related to obesity, insulin resistance and type II diabetes

Normal mitochondrial function is vital to ensure adequate cellular energetics. Among various other factors, reduced mitochondrial function is thought to contribute to the LV dysfunction associated with the diabetic cardiomyopathy (Ren *et al*. 2010; Bugger and Abel, 2010) and as such any alteration in mitochondrial function even during pre-diabetic obesity could induce risk for the development of LV dysfunction should disease progression advance. Mitochondrial dysfunction may culminate in reduced ATP production which could eventually contribute to LV dysfunction. Indeed mitochondrial dysfunction (reduced state 3 respiration rates) (Sharov *et al*. 2000) and decreased phosphocreatine, ATP and phosphocreatine/ATP (PCR/APT) ratios (Beer *et al*. 2002) are evident in patients with heart failure.

2.5.1 Mitochondrial dysfunction in obese and diabetic humans

Data regarding myocardial mitochondrial function in humans are limited and when available, relate to mitochondria isolated from atrial cardiomyocytes. While evidence of cardiomyocyte mitochondrial dysfunction is present in type II diabetic patients (Anderson *et al*. 2009), it is interesting to note that mitochondrial dysfunction is also

evident in young obese insulin resistant individuals as right atrial cardiomyocyte mitochondria isolated from young obese pre-diabetic (HbA1c < 6) humans display impaired respiratory capacity and a reduced biogenic response compared to age-matched non-obese subjects. Furthermore these mitochondria displayed signs of enhanced oxidative stress and elevated mRNA levels of pro-apoptotic proteins (Niemann *et al*. 2011).

A review by Lesnefsky *et al*. (2001) noted that although a reduction in electron transport chain enzymatic activity may lead to a reduced rate of ATP synthesis, respiratory chain enzymes are generally present in excess of the oxidative phosphorylation potential of the cell. Consequently only a 30-50% reduction in ETC enzymatic activity is thought to be adequate to attenuate mitochondrial ATP production (Lesnefsky *et al*. 2001). It however remains to be seen whether the reduction in myocardial mitochondrial respiratory chain capacity noted in obese pre-diabetic individuals (Niemann *et al*. 2011) is sufficient to alter mitochondrial ATP production. In addition a study investigating the relationship between these mitochondrial abnormalities and load independent indices of LV systolic function is warranted.

2.5.2 Mitochondrial dysfunction in obese and diabetic animals

Animal models of obesity, insulin resistance and type 2 diabetes also reveal signs of mitochondrial dysfunction and inadequate mitochondrial biogenesis. While ventricular mitochondrial state 3 respiration rates appear to be normal in genetic (*ob/ob*) (Boudina *et al*. 2005) and diet-induced (Essop *et al.* 2009; Cole *et al*. 2011) obese insulin resistant pre-diabetic animals, protein levels of components of the respiratory chain appear to be reduced in these *ob/ob* non-diabetic mice (Boudina *et al*. 2005) as

is seen in diabetic db/db mice (Boudina *et al*. 2007). Despite the normal respiration rates described in these models, myocardial mitochondrial ATP/O ratios and in certain cases the amount of ATP synthesized/ADP phosphorylated were reduced in response to obesity (Boudina *et al*. 2005; Essop *et al*. 2009; Cole *et al*. 2011).

2.5.3 Cardiac efficiency and mitochondrial uncoupling

Cardiac mechanical efficiency is the term used to describe the relationship between external cardiac power and LV energy consumption (energy consumed to produce LV mechanical work) and can be indirectly determined by expressing the work performed by the heart as a ratio of its oxygen consumption (Bing and Michal, 1959; How *et al*. 2006; Lopaschuk *et al*. 2010). In other words, should there be an increase in myocardial oxygen consumption, without an equivalent increase in LV mechanical function, cardiac efficiency is said to be reduced. Reduced cardiac efficiency is evident in obese insulin resistant and diabetic animals as well as healthy hearts exposed to elevated FFA supply (Mazumder *et al*. 2004; Buchanan *et al*. 2005; How *et al*. 2005; How *et al*. 2006; Cole *et al*. 2011). Reduced cardiac efficiency is further noted prior to the development of hyperglycemia and LV dysfunction (Buchanan *et al*. 2005). Similarly Peterson *et al*. (2004a) recently reported that BMI predicted increased myocardial oxygen consumption and reduced cardiac efficiency (due to increased myocardial oxygen consumption) in young obese insulin resistant women. Although the oxidation of 1 molecule of palmitate utilizes 46 atoms of oxygen and produces 105 ATP molecules, only 12 atoms of oxygen are required to oxidize 1 molecule of glucose producing 32 ATP molecules. It therefore follows that the enhanced FA utilization, as is the case in obesity, will translate into augmented myocardial oxygen consumption. Despite this, the increase in work-independent myocardial oxygen consumption seen in obesity/diabetes is often more that what can

be explained by the elevated consumption of FA as a substrate (How *et al*. 2006). Consequently other factors such as increased uncoupling of mitochondrial respiration may further contribute to the enhanced myocardial oxygen consumption evident obesity/diabetes (Boudina *et al*. 2005).

Reduced ATP production and ATP/O ratios could result from mitochondrial uncoupling, i.e. disruption of the mitochondrial respiratory chain proton gradient. Experimental evidence suggests that FA utilization augments mitochondrial uncoupling (via increased UCP3 expression or activity) in both obese insulin resistant (Cole *et al.* 2011) and diabetic animals (Boudina *et al*. 2007) leading to greater myocardial oxygen consumption at the expense of ATP production. FA's are thought to induce mitochondrial uncoupling through enhanced mitochondrial ROS production (Boudina *et al*. 2007; Bugger and Abel *et al.* 2010). Bugger and Abel (2010) have further proposed that LV dysfunction evident in type 2 diabetics may in part result from enhanced mitochondrial uncoupling related decreases in myocardial high-energy reserves. Importantly enhanced mitochondrial uncoupling is evident in heart failure (Murray *et al*. 2004).

2.5.4 Mitochondrial biogenesis

In the cell, should the need arise to increase substrate oxidation, compensatory mitochondrial biogenesis occurs to meet the oxidative requirements induced by the increased fuel supply. Recent data however suggest that while mitochondria from obese insulin resistant and diabetic animals show signs of dysfunction, the biogenic response may additionally be impaired. Myocardial mitochondrial numbers, DNA copy and volume density are reported to be elevated in obesity related insulin resistance (Duncan *et al*. 2007) and diabetes (*db/db* mice) (Boudina *et al*. 2007).

Despite this mitochondrial biogenic response, ADP stimulated respiration and ATP synthesis rates are reported to be reduced in these models (Boudina *et al*. 2007; Duncan *et al*. 2007).

2.5.5 High energy phosphate metabolism

Insulin resistant or diabetic patients demonstrate abnormal myocardial high-energy phosphate ratios. Reduced myocardial PCr/ATP ratios have been observed in overweight, obese insulin resistant (pre-diabetic) and diabetic individuals (Diamant *et al*. 2003; Scheuermann-Freestone *et al*. 2003; Perseghin *et al*. 2007) and correlate with the degree of insulin insensitivity (Perseghin et al. 2007). These alterations in energy status precede diabetes related myocardial systolic dysfunction and correlate with diastolic dysfunction. Indeed reduced myocardial PCr/ATP ratios have been described as a significant predictor of cardiovascular mortality in diabetic patients presenting with dilated cardiomyopathy (Neubauer *et al*. 1997). Reduced PCr/ATP ratios are thought to develop due to the altered substrate metabolism profile associated with insulin resistance (Scheuermann-Freestone *et al*. 2003). Although a reduced PCr/ATP ratio may predict mortality in diabetic patients with dilated cardiomyopathy, the observations that an altered myocardial energetic status is present prior to the development of type II diabetes is a point of concern.

2.5.6 Mitochondrial function and insulin sensitivity

Mitochondrial respiration rates may be influenced by the degree of insulin sensitivity and the extent of visceral obesity and general obesity. Measuring mitochondrial respiration in permeablized skeletal muscle fibers, Chanseaume *et al*. (2010) observed that viscerally obese individuals (waist circumference: 88-93cm and 94-101cm, BMI<30kg/m^2) had higher state 2 and 3 respiration rates than lean (waist

circumference ≤87cm), and obese insulin resistant individuals (waist circumference ≥102cm, BMI≥30kg/m^2). Despite the higher mitochondrial respiration rates, the 88-93cm waist circumference group had a similar ATP/O ratio compared to the lean group, while the 94-101cm waist circumference group's ATP/O ratio was significantly reduced compared to the lean group. In strong contrast, mitochondrial state 2 and 3 respiration rates and ATP/O ratios obtained in the obese insulin resistant group (waist circumference ≥102cm, BMI≥30kg/m^2) were significantly lower compared to the lean group. These functional observations were independent of changes in oxidative enzyme activity. Thus it appears that skeletal muscle mitochondrial function and efficiency (ATP/O ratio) are dependent on the degree of insulin sensitivity, visceral adiposity and general adiposity. It further seems as if a clear adaptational phase in mitochondrial respiratory function occurs, which is followed by signs of mitochondrial dysfunction as obesity and insulin resistance progress.

Using mice with cardiomyocyte specific insulin receptor deletion (CIRKO), Boudina *et al*. (2009) recently reported enhanced mitochondrial oxidative stress and uncoupling accompanied by reduced Krebs cycle and FA oxidative capacity and reduced ATP production. These mice further display reduced LV mechanical function and increased mortality following *in vivo* myocardial infarction (Sena *et al*. 2009). These findings together with those of Chanseaume *et al*. (2010) suggest an important role for impaired insulin signaling in the development of mitochondrial dysfunction.

2.5.7 Mitochondrial function and anoxia/ischaemia

While reduced respiratory capacity, reduced cardiac efficiency and increased mitochondrial uncoupling may not pose an immediate threat to LV mechanical function in obese insulin resistant pre-diabetic individuals, these factors could well leave the myocardium vulnerable to ischaemic events as myocardial ischaemia per

se further impairs mitochondrial respiratory capacity. This mitochondrial dysfunction would further impact negatively on myocardial ischaemia/reperfusion injury (Lesnefsky *et al*. 2001). As noted by Sena *et al*. (2009), impaired post-ischaemic myocardial insulin signaling can augment post-ischaemic damage. Essop *et al*. (2009) reported reduced post-anoxia state 3 respiration rates in isolated ventricular mitochondria from obese insulin resistant rats in the presence of a FA derived oxidizable substrate. The authors proposed that the underlying reduced myocardial mitochondrial bio-energetic capacity in these obese insulin resistant rats may account for the enhanced post-ischaemic damage (increased myocardial infarct sizes) identified in isolated heart perfusions (Essop *et al*. 2009).

2.6 Peroxisome proliferator activated receptor alpha

Peroxisomes are organelles that are present in most eukaryotic cells and are capable of metabolizing FA through β-oxidation, although to a much lesser degree than mitochondria. Initial studies performed on rodent hepatic tissue documented peroxisomal proliferation in response to various substances termed peroxisome proliferators (Issemann and Green, 1990; Lock *et al*. 1989). In 1990 the receptor to which peroxisome proliferators bind was isolated and characterized and became known as the peroxisome proliferator-activated receptor (PPAR) (Issemann and Green, 1990). Interestingly, although the peroxisomal proliferative response to peroxisome proliferators was only induced with the alpha subtype in rodents, the proliferative response is absent in humans and certain other species (Lock *et al*. 1989; Kliewer *et al*. 2001).

PPARs are a family of ligand activated receptors belonging to the orphan nuclear receptor super-family. These receptors act as transcription factors regulating gene expression in various cell types. PPARs have naturally occurring and synthetic ligands, which upon entering the cell bind and activate PPARs residing in the cytoplasm. This initial activation leads to subsequent binding of PPAR to another transcription factor, the retinoid X receptor/retinoic acid receptor (RXR/RAR) (Gearing *et al*. 1993). The formation of this heterodimeric complex is essential for PPAR function and enables 1) translocation of the complex into the nucleus from the cytoplasm and 2) binding of the complex to a peroxisome proliferator response element in the promoter region of the target gene within the nucleus (Gearing *et al*. 1993; Kieć-Wilk *et al*. 2005). Through these processes PPAR's are able to regulate genes involved in carbohydrate and lipid metabolism.

Presently three distinct PPAR isoforms have been identified namely α, β/δ and γ. These isoforms are differentially distributed throughout the body and are encoded by different genes (Kliewer *et al*. 2001). PPARα has traditionally been ascribed a role in lipid metabolism, specifically FA oxidation (Kieć-Wilk *et al*. 2005). It has also become evident that PPARα additionally exerts anti-inflammatory actions and plays a role in gluconeogenesis (Colville-Nash *et al*. 2005; Grau *et al*. 2006). PPARγ activation enables lipid storage into adipocytes, adipogenesis and may also perform an anti-inflammatory role (Berger and Moller, 2002). Lastly, agonists of PPARβ/δ demonstrate lipid lowering and insulin sensitizing effects (Oliver *et al*. 2001) while the receptor has also been implicated in cancer and placental development (Jaeckel *et al*. 2001; Nadra *et al*. 2006). Although PPARγ and PPARβ/δ have important physiological roles, reviewing their function is beyond the scope of this dissertation

and accordingly, information relevant to PPARα and the cardiovascular system will be discussed.

As PPARα plays a fundamental role in lipid metabolism, its activity and ability to influence gene expression is tightly regulated at multiple levels, many of which are poorly understood. PPARα can be activated by a host of natural and synthetic ligands including LCFA's, certain eicosanoids and hypolipidaemic drugs (e.g. ciprofibrate, bezafibrate, fenofibrate and clofibrate) (Forman *et al*. 1997; Yu *et al*. 1995; Brown *et al*. 1999).

The activity of PPARs is dependent on their interaction with co-activators or co-repressors (reviewed by Yu and Reddy, 2007). PPARγ co-activator 1α (PGC1α) is a transcriptional co-factor capable of interacting with a wide variety of transcription factors including PPARα. PGC1α is an important co-activator of PPARα and as such, its presence and consequent binding to PPARα greatly enhances the potential of PPARα regulated genes being transcribed (Vega *et al*. 2000). Adipocyte derived factors may further influence the level of PPARα activity or expression. Adiponectin is thought to stimulate PPARα gene expression via its interaction with PGC1α (You *et al*. 2005), while PPARα expression may be down-regulated by TNFα (Beier *et al*. 1997). In addition to the presence of co-activators and co-repressors, Shalev *et al*. (1996) demonstrated that insulin can phosphorylate PPARα leading to an increase in its transcriptional activity (Shalev *et al*. 1996). Unfortunately little is known about PPARα function as a phospho-protein. Intriguingly, glucose was recently shown to bind PPARα with high affinity inducing a conformational change in PPARα's secondary structure (Hostetler *et al*. 2008). This study made use of COS-7 cells and it was shown that glucose alone reduced the interaction of PPARα with its co-

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activator steroid receptor co-activator-1 (SRC-1). In the presence of the PPARα ligands arachidonic acid and clofibric acid, even low concentrations of glucose increased PPARα binding to SRC-1. Glucose further enhanced PPARα-RXRα binding to DNA, while attenuating LCFA-CoA binding to PPARα (there was no such inhibitory effect on LCFA's), which paradoxically led to augmented transcription of genes favouring FA metabolism.

2.6.1 PPARα expression and gene targets of PPARα

In rats, PPARα is co-expressed in various cell types. In particular, PPARα has been identified in hepatocytes, cardiomyocytes, skeletal muscle, enterocytes, the proximal tubule cells of the kidney, and endothelial cells (Braissant *et al*. 1996; Su *et al*. 1998; Marx *et al*. 1999).

In the heart, varying reports exist regarding the expression of PPARα in the context of obesity and type 2 diabetes. Hearts from obese insulin resistant animals have been reported to have elevated myocardial PPARα protein expression in the initial stages of high caloric feeding (8 weeks) (N Bezuidenhout, MSc dissertation, 2011), while longer periods of feeding (16 weeks) are shown to produce comparable levels of PPARα mRNA (Aasum *et al.* 2008) and protein expression compared to hearts from lean control animals (N Bezuidenhout, MSc dissertation, 2011). In similar fashion, obese Zucker rat hearts have comparable levels of PPARα mRNA expression in the fed state, while fasting attenuates PPARα mRNA expression in these animals when compared to lean controls (Young *et al*. 2002). In the previously mentioned study (Young *et al*. 2002), fasting resulted in increased mRNA expression in certain (but not all) PPARα target genes in these obese animals. The expression levels of myocardial PPARα mRNA however seems to be inconsistent in obese

Zucker rats as elevated PPARα mRNA expression levels have been reported elsewhere (Pagano *et al*. 2008). The expression of myocardial PPARα content in the context of obesity may further be dependent on dietary influence. While an obesity inducing Western diet failed to influence myocardial PPARα mRNA expression during a 32-48 week feeding period, an obesity inducing high fat diet resulted in reduced myocardial PPARα mRNA expression after 4-8 weeks and 16-24 weeks of feeding relative to the control and western diet fed group (Wilson *et al*. 2007).

In hearts from diabetic animals, PPARα mRNA expression levels appear to be elevated relative to lean controls (Boudina *et al*. 2007).

Activation of PPARα in a particular tissue may induce the expression of various PPARα target genes which encode proteins and enzymes predominantly involved in FA metabolism. These PPARα gene products were summarized in a review by Barger and Kelly, (2000) and include FA transport protein (FATP) (Motojima *et al*. 1998), FA translocase, (CD36/FAT)) (Motojima *et al*. 1998), FA binding protein (FABP) (van Bilsen *et al*. 1997; Motojima, 2000), fatty acyl-CoA synthase (Van der Lee *et al*. 2000), CPT-1 (Mascaró *et al*. 1998; Djouadi *et al*. 1999), malonyl-CoA decarboxylase (Campbell *et al*. 2002) medium, long and very long chain acyl-CoA dehydrogenase (Gulick *et al*. 1994; Aoyama *et al*; 1998; Djouadi *et al*. 1999; Van der Lee *et al*. 2000), short chain 3-hydroxyacyl-CoA dehydrogenase (Aoyama *et al*. 1998); short chain specific 3-ketoacyl-CoA thiolase (Aoyama *et al.* 1998); long chain acyl-CoA synthetase (Aoyama *et al*. 1998), malic enzyme (Aoyama *et al*. 1998) and acyl-CoA oxidase (Djouadi *et al*. 1999). Activation of PPARα can thus be seen to increase the expression of proteins implicated in the transport of FA into the cell, through the cytosol and into the mitochondria. Additionally proteins involved in peroxisomal and mitochondrial β-oxidation are also expressed. In rodent hearts,

PPARα further regulates the expression of uncoupling protein 3 (Young *et al*. 2001). It is noteworthy that activation of myocardial PPARα resulting from diet induced obesity does not always correspond with the up-regulation of every PPARα related gene/protein product, but rather a specific cassette of PPARα related genes (Wilson *et al*. 2007). These observations indicate that additional unknown factors may exist which further regulate PPARα's transcriptional effects.

2.6.2 The impact of PPARα activation

Examining the phenotypic characteristics of PPARα null mice and mice with cardiac specific PPARα over-expression has provided valuable insight regarding the physiological role of PPARα. PPARα null mice display lower levels of hepatic FA metabolism related enzymes which correspond with reduced hepatic FA β-oxidation activity (Aoyama *et al*. 1998). Furthermore, after 72 hours of fasting, PPARα null mice display excessive hepatic lipid accumulation (Lee *et al*. 2004). These mice (fed) also have elevated total serum cholesterol, HDL-C, and apolipoprotein A-I levels (Peters *et al*. 1997) and do not develop insulin resistance when fed a high fat diet (Guerre-Millo *et al*. 2001). Data on PPARα null mice further support an anti-inflammatory role of PPARα activation by fibrates (Delerive *et al*. 1999). In addition PPARα null mice demonstrate myocardial substrate switching, reflected by a higher reliance on glucose oxidation as opposed to FA β-oxidation compared to wild type mice (Luptak *et al*. 2005, Hafstad *et al*. 2009). This metabolic switching is associated with reduced LV performance during normal perfusion conditions and in response to elevated workload (Luptak *et al*. 2005; Loichot *et al*. 2006). Distinct from their metabolic characteristics, PPARα null mice display increased vascular lesion formation (Howroyd *et al*. 2004). Lastly, the lack of PPARα does not seem to influence heart rate or systemic blood pressure (Loichot *et al*. 2006).

Conversely, hearts from mice with cardiac specific PPARα over-expression are insulin resistant and exhibit increased expression of genes involved in FA transport and oxidation, while the expression of genes related to glucose uptake and transport are attenuated (Finck *et al*. 2002). These genetic modifications corroborate with attenuated insulin signaling and elevated FA β-oxidation and reduced glucose uptake and oxidation rates in the heart (Park *et al*. 2005; Sambandam *et al*. 2006), thus representing a phenotype similar to the diabetic heart (Finck *et al*. 2002). Hearts from these mice are also hypertrophied (Finck *et al*. 2002), exhibit excess myocardial lipid accumulation (Finck *et al*. 2002; Yang *et al*. 2007) and reduced *in vivo* myocardial systolic function (Finck *et al*. 2002; Yang *et al*. 2007), while being more susceptible to ischaemia/reperfusion induced injury (Sambandam *et al*. 2006).

2.6.3 The effect of PPARα agonists/activation on lipid metabolism

In humans, fibrate treatment has been shown to lower circulating Trig's and elevate HDL-C levels (Robins *et al*. 2001). Certain authors have also reported reductions in circulating LDL-C levels following PPARα agonist treatment (Malmendier and Delcroix, 1985). The mechanisms whereby PPARα agonists (fenofibrate) improve the lipid profile may involve increasing the synthesis of both apolipoprotein A-I (Malmendier and Delcroix, 1985) and A-II (Vu-Dac *et al*. 1995) thereby assisting in elevating the levels of HDL-C, while reducing the amount of apolipoprotein B, the major lipoprotein constituent of LDL-C. PPARα agonists further increase transcription of the hepatic LPL gene (Schoonjans *et al*. 1996), while reducing ApoC-III (ApoC-III may inhibit LPL mediated Trig hydrolysis) levels (Minnich *et al*. 2001). This results in increased Trig hydrolysis which aids in reducing circulating Trig levels. Elevated levels of hepatic mitochondrial β-oxidation in response to PPARα agonism (Minnich

et al. 2001) further reduce the level of FFA, an essential component of VLDL and LDL-C formation.

2.6.4 The effect of PPARα agonists on systemic insulin sensitivity

PPARα agonists have repeatedly been demonstrated to have both systemic and tissue specific insulin sensitizing effects (Guerre-Millo *et al*. 2000; Ide *et al*. 2004; Bergeron *et al*. 2006; Tsunoda *et al*. 2008) and consequently have tremendous potential in the treatment of diabetes.

The mechanisms whereby PPARα agonists achieve enhanced insulin sensitivity have been widely investigated. Administration of the selective PPARα agonist Wy-14,643 significantly increases hepatic and skeletal muscle IR and IRS-tyrosine phosphorylation, while increasing IRS-associated PI3K activity in ob/ob mice (Ide *et al*. 2004). These beneficial effects of Wy-14,643 on the insulin signaling pathway were accompanied by reduced hepatic and skeletal muscle Trig and fatty acyl-CoA levels. In the previously mentioned study it was interesting to note that these beneficial effects afforded by PPARα agonism were absent in the lean treated group. Nevertheless, similar reductions in myocardial Trig levels in response to PPARα agonism have been reported elsewhere (Forcheron *et al*. 2009). Due to the association between intramuscular lipid accumulation and insulin resistance (Boden *et al.* 2001), PPARα agonists may alleviate insulin resistance secondary to their systemic and tissue specific lipid lowering effects.

2.6.5 Additional cardiovascular effects of PPARα activation

The impact of PPARα activation on myocardial susceptibility to ischemia/reperfusion injury is controversial. Administration of the highly selective and potent PPARα agonist GW-7647 to healthy mice, 3 days prior to *in vivo* CAL induced a dose dependent decrease in myocardial infarct size, accompanied by a significant improvement in LV contractile function. These cardioprotective effects of GW-7647 were absent in PPARα null mice (Yue *et al*. 2003). The cardioprotection afforded by GW-7647 was associated with reduced circulating FFA's, increased expression and activity of cardiac FA oxidation enzymes and increased *in vivo* FA oxidation relative to vehicle treated controls after 24 hours reperfusion (Yue *et al*. 2003). Additional studies describing the cardioprotective effects of PPARα agonism following ischaemia/reperfusion have been reported in healthy (Bulhak *et al*. 2006; Tabernero *et al*. 2002, Wayman *et al*. 2002, Yeh *et al*. 2006), obese and diabetic (Aasum *et al*. 2008; Bulhak *et al*. 2009) animals. These studies have highlighted metabolic and anti-inflammatory effects (Yue *et al*. 2003), inhibiting various apoptotic pathways (Yeh *et al*. 2006) and stimulating signaling via the PI3K/Akt pathway coupled with the production of nitric oxide (Bulhak *et al*. 2006; Bulhak *et al*. 2009) as possible mechanisms contributing to the protection elicited by PPARα agonism/activation following ischaemia/reperfusion episodes. Moreover, PPARα agonists have also been reported to reduce myocardial fibrosis in deoxycorticosterone acetate salt hypertensive rats and Zucker diabetic rats (Ogata *et al*. 2004; Forcheron *et al*. 2009).

In contrast to the reported cardioprotective effects ascribed to PPARα agonist treatment, certain studies have observed no effect (Aasum *et al*. 2003b; Xu *et al*. 2006) or have shown PPARα activation to be detrimental to the ischaemia/reperfused heart (Hafstad *et al*. 2009). The later report (Hafstad *et al*. 2009) concurs with post-

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ischaemic observations made in cardiac specific PPARα over-expressed mouse hearts, while hearts from PPARα null mice recover to a similar degree as wild-type mice after ischaemia/reperfusion induced injury (Sambandam *et al*. 2006). Remarkably, PPARα agonist treatment (tetradecylthioacetic acid) has been shown to increase the rate of myocardial necrosis (Pruimboom-Brees *et al*. 2006). Clearly the issue of whether PPARα agonists are detrimental or beneficial during ischaemia/reperfusion needs further investigation.

2.6.6 The need to develop new lipid lowering and insulin sensitizing drugs

Dislipidaemic patients at risk for cardiovascular disease are generally placed on statin therapy (Hydroxymethyl-glutamyl-coenzyme A reductase inhibitors). Although statins significantly reduce cardiovascular disease risk (Baigent *et al*. 2005), primarily accomplished by a reduction in LDL-cholesterol (Baigent *et al*. 2005), a degree of atherogenic/cardiovascular disease risk still remains in high risk patients due to elevated circulating Trigs and lowered HDL-C levels (Cziraky *et al*. 2008; Alagona, 2009). As niacin and fibrates positively influence Trig and HDL-C levels, combination therapy involving niacin/statin or fibrate/statin therapy, is advised for the treatment of cardiovascular disease (Cziraky *et al*. 2008; Alagona, 2009).

Although most fibrates are classified as "safe drugs", the high dosages required (Berger and Moller, 2002) and some of the reported side effects (reviewed by Rubenstrunk *et al*. 2007), warrant the development of new, more potent and specific PPARα agonists.

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For the purposes of this dissertation, information will be provided on K-111, a novel PPARα agonist which has shown potential in alleviating the metabolic manifestations associated with obesity and type II diabetes.

2.6.7 K-111/BM 17.0744
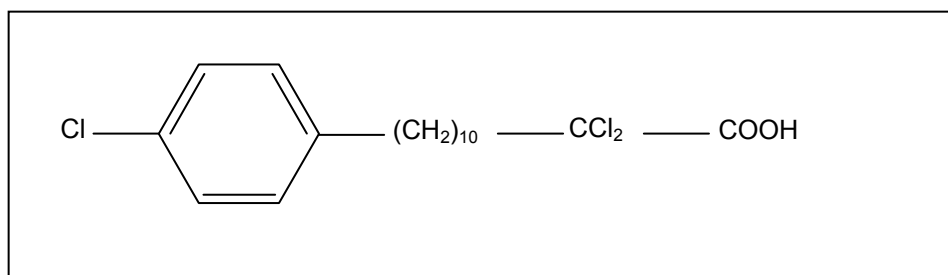
Figure 2.6: The chemical structure of K-111

K-111 ((2,2-dichloro-12-(p-chlorophenyl)-dodecanoic acid)), formally known as BM 17.0744, is a ω-substituted alkyl carboxylic acid developed by Kozva Co Ltd, under the licensing of Roche Holding AG, as a oral PPARα agonist. K-111 has a high specificity for PPARα, stronger than other fibrates such as Wy-14.643, while it does not interact with PPARγ (Meyer *et al*. 1999). K-111 was reported to be undergoing phase II clinical trials (Drew and Calkin, 2007). To date, only a handful of scientific papers in accredited journals have been published regarding K-111. The reports published concur that K-111 has potent insulin sensitizing and lipid lowering properties attributed to its specific interaction with PPARα. K-111 treatment has also been shown to exert various biometric effects such as reducing body weight gain, and increasing liver and kidney weights (an effect common in rodents exposed to PPARα agonists) (Meyer *et al*. 1999).

2.6.7.1 Murine and rodent studies

The insulin sensitizing capabilities of K-111 have been well documented. For example, in cultured 3T3-L1 adipocytes, the use of K-111 enhanced insulin stimulated glucose uptake (Pill and Kühnle, 1999). K-111 administration was further shown to improve systemic insulin sensitivity in 4 different rodent models of insulin resistance (Yellow KK mice, ob/ob mice, db/db mice and obese *fa/fa* Zucker rat) (Pill and Kühnle, 1999). The insulin sensitizing/glucose lowering effects of K-111 have subsequently been confirmed in diabetic *db/db* mice by various other authors (Aasum *et al*. 2002, 2005; Bratkovsky *et al*. 2006).

Similarly to the fibrate class of PPARα agonists, K-111 has a potent effect on lipid metabolism, reducing circulating Trigs and total cholesterol (Meyer *et al*. 1999) in healthy rats. K-111 has also been shown to reduce circulating Trig and FFA levels in diabetic mice (Aasum *et al*, 2002).

In dose response studies, male Sprague-Dawley rats were administered a range of K-111 doses (0, 1.5, 3, 6 and 12.5 mg/kg/day) in an attempt to detect quantifiable serum concentrations of the drug. On day 22 of drug administration, K-111 was detectable in the serum, but only at the highest investigated dosage (12.5mg/kg/day) yielding the following pharmokinetics: c_{maxss} 0.44mg/l, t_{maxss} 1hr, $t_{1/2ss}$ 2hrs and $AUC_{0>24ss}$ 0.6mg/hr/l (Meyer *et al*. 1999). Although no further rodent data are currently available on K-111's effects at dosages between 6 and 12.5mg/kg/day, it could be speculated that K-111 doses below 6mg/kg/day, would mainly elicit hepatic effects as these doses did not result in detectable drug levels in the blood (Meyer *et al*. 1999). Indeed, at the K-111 doses investigated a significant dose dependent increase in hepatic FA oxidation enzymes was found (Meyer *et al*. 1999). In rodents,

cardiac PPARα regulated target genes may theoretically only be up-regulated in response to chronic K-111 treatment at dosages of 12.5mg/kg/day and above. Unfortunately no other studies have investigated K-111's *in vivo* transcriptional effects on cardiac target genes in rats. However, while cultured rodent cardiomyocytes are responsive to K-111 treatment, hearts from db/db mice receiving K-111 at 24.5-37.9mg/kg/day for 4-5 (Aasum *et al*. 2002) or 8-9 weeks (Aasum *et al*. 2005) displayed no myocardial transcriptional response to PPARα agonism. A similar cardiac unresponsiveness to K-111 was seen in normal mouse hearts at similar doses (Aasum *et al*. 2005). These data suggests that K-111 at sufficiently high concentrations is either unable to up-regulate cardiac PPARα target gene expression *in vivo*, or that the myocardial responsiveness to K-111 may differ between mice and other species.

2.6.7.2 Studies in non-human primates

Besides smaller mammals, K-111 administration has also yielded beneficial effects in non-human primates. Obese hypertriglyceridaemic hyperinsulinaemic pre-diabetic rhesus monkeys treated with K-111 at various doses displayed a marked improvement in systemic insulin sensitivity, while body weight and circulating Trig's were reduced in a dose dependent manner. K-111 also improved HDL-C levels in these animals, without influencing blood pressure, plasma fibrinogen or total cholesterol levels. An insignificant effect on liver weight, adipogenesis, blood chemistry and hematological profile was observed following chronic K-111 treatment (Bodkin *et al*. 2003; Schäfer *et al*. 2004). K-111 further elicited augmented skeletal muscle glycogen synthase activity in these animals, which has been proposed as a contributory mechanism of K-111's insulin sensitizing capabilities (Ortmeyer *et al*. 2005).

2.6.7.3 Species differences

The effectiveness of K-111 may be species dependent (Meyer *et al*. 1999). While rats develop enlarged livers and kidneys together with significant increases in hepatic enzyme activity ((carnitine acetyl transferase activity (CAT): 200 fold increase; acetyl CoA oxidase (ACO): 20 fold increase) in response to K-111 treatment, treated dogs have normal kidney and liver weights. They also have more modest increases in hepatic enzyme activity (CAT: 6-fold; ACO: 2-fold) of PPARα related target genes. Furthermore, although doses of up to 12.5mg/kg/day K-111 are well tolerated in rats, 4 and 6mg/kg/day K-111 appear to be toxic in dogs. In strong contrast to dogs, K-111 at a dose of 12.5mg/kg/day has no influence on serum Trig's or total cholesterol in guinea-pigs, while hepatic enzyme activity of CAT and ACO are elevated and liver and kidney weights remain unchanged (Meyer *et al*. 1999). Thus clear species differences exist in their response to K-111 treatment. Rodents may display a more exaggerated response following PPARα treatment due to the high density of hepatic PPARα receptors in comparison to other species (Tugwood *et al*. 1998).

2.6.7.4 Myocardial substrate metabolism

K-111 has a profound and paradoxical effect on cardiac substrate metabolism. In response to chronic K-111 treatment, db/db mice displayed increased *ex vivo* myocardial glycolysis and glucose oxidation rates while FA oxidation rates were reduced (Aasum *et al*. 2002, 2005), despite increased FA oxidation rates being observed in cardiomyocytes cultured with K-111 (Aasum *et al*. 2005). The inability of K-111 to increase myocardial FA oxidation *in vivo* may be due to an inhibitory effect of K-111 on cardiac heparin-releasable LPL activity (Carroll and Severson, 2001; Aasum *et al*. 2005). Reduced cardiac LPL activity could result in the *in vivo* myocardium being transcriptionally conditioned to a lower myocardial FFA supply.

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Indeed Aasum *et al*. (2005) have proposed that the decrease in FA oxidation rates in response to K-111 treatment may reflect an *in vivo* adaptation to reduced myocardial FFA supply resulting from increased hepatic FFA clearance.

2.6.7.5 Additional effects of K-111

Similar to other PPARα agonists, K-111 was shown to elicit an anti-inflammatory effect. K-111 added to adipocytes (3T3-L1 cells) co-cultured with a lipopolysaccharide stimulated macrophage cell line (RAW 263.7 cells) indirectly, suppressed adipocyte TNFα secretion by attenuating macrophage interleukin-6 release (Murakami *et al*. 2007). K-111 could thus also improve systemic insulin sensitivity by reducing the systemic inflammatory response evident in obesity/diabetes.

Collectively, these beneficial effects of K-111 agonism allude to its potential use in the management of obesity and diabetes. The strong affinity of K-111 for PPARα coupled with the low doses required to effectively attenuate lipid levels and improve insulin sensitivity make K-111 an even more attractive drug. By reducing body weight gain, exerting anti-inflammatory actions, reducing Trig levels and increasing HDL-C levels, K-111 can afford added anti-atherogenic benefits. Although elevated liver and kidney weights are observed in so-called "high responders" (rodents), the minimal effect on liver and kidney mass in non-human primates underscores the potential for its future use in humans.

Although potent new insulin sensitizing and lipid lowering drugs are required, the importance of investigating these substances outside the scope of their normal insulin sensitizing and lipid lowering abilities is critical. As these drugs are

administered to patients at risk for developing various cardiovascular diseases, their ability to not antagonize endpoints of normal cardiovascular functioning or cardiovascular disease is vital. Thus far K-111 has shown promise in the alleviation of insulin resistance and dyslipidaemia, but more information is required on its specific cardiac effects, and the impact on ischaemia/reperfusion injury.

2.7 Myocardial ischaemia/reperfusion injury

Ischaemia is derived from the Greek words "*isch*" meaning restriction and "*haema*" meaning blood and thus refers to a condition where a tissue or an organ is partially or completely deprived of blood flow. Depending on the severity of the myocardial coronary flow restriction, ischaemia may be classified as either moderate or severe. Ischaemia may also ensue when insufficient blood reaches the heart muscle in response to a stimulus requiring increased myocardial contractility (demand-induced ischaemia) (reviewed by Stanley *et al*. 1997).

Depending on the severity of the ischaemic insult, various metabolic alterations occur within the cardiomyocyte, and in the case of severe ischaemia, should reperfusion not be instituted with immediate effect, necrosis and the development of infarction occurs. Although reperfusion therapy is absolutely essential to salvage the ischaemic myocardium and prevent the progression to irreversible ischaemic damage, reperfusion itself brings about a certain degree of damage or cell death (as reviewed by Kharbanda, 2010). The extent of the myocardial damage (cell death, arrhythmia, contractile dysfunction and microvascular dysfunction) incurred is largely dependent on the duration and severity of ischaemia, effective reperfusion and the myocardium's intrinsic signaling response to ischaemia and reperfusion (as reviewed

by Kharbanda, 2010). Myocardial ischaemia/reperfusion injury is a relevant concern in clinical practice and is evident in various interventions (thrombolysis, angioplasty and coronary bypass surgery) aimed at restoring blood flow to an ischaemic area. Ischaemia/reperfusion injury may also be evident during cardioplegic arrest and aortic cross clamping. As such, interventions aimed at reducing the extent of ischaemia/reperfusion injury are invaluable.

2.7.1 Metabolic and structural consequences of ischaemia and reperfusion

Various metabolic and ultrastructural changes occur during ischaemia. These include ATP and PCr depletion. Structurally, sarcoplasmic reticulum swelling, myofibril relaxation and mitochondrial damage are also evident (Puri *et al*. 1975; Jennings and Ganote, 1976). The extent of these abnormalities are however dependent on the length of the ischaemic episode, which subsequently influences the recovery of the metabolic parameters upon reperfusion (Puri *et al*. 1975). Other metabolic abnormalities ensue as ATP dependent ion pumps are unable to function, culminating in the cytosolic accumulation of sodium, hydrogen and calcium ions and intracellular acidosis development (Tani and Neely, 1989).

A fundamental feature of myocardial ischaemia is reduced oxygen availability and consequently, lower mitochondrial oxidative phosphorylation rates which disturbs the tightly coupled ATP breakdown and re-synthesis equilibrium existing during normoxia. Consequently cellular ATP levels become depleted, the extent of which is again dependent on the duration and severity of ischaemia (Puri *et al*. 1975). Whereas oxidative metabolism is reduced following an ischaemic event, experimental work has shown that after coronary artery ligation, there is an initial increase in glycolytic flux which quickly declines to normal levels (Opie, 1976).

Although during aerobic conditions, glycolysis accounts for only a small amount of the total ATP production, glycolytically produced ATP during mild myocardial ischaemia becomes invaluable in maintaining intracellular ion pump activity (Opie, 2004). However, glycolytically produced ATP is often insufficient to maintain normal contractile function (Opie, 2004). While glycolytically generated ATP may aid in maintaining ion homeostasis, in the virtual absence of glucose supply to the ischaemic portion of the heart, glycogen stores undergoing glycolysis contributes greatly to cytosolic proton accumulation and consequently to the decline in intracellular pH (Garlick *et al*. 1979). Despite this, elevated glycogen levels at the onset of myocardial ischaemia have been associated with improved functional recovery during reperfusion of the ischaemic myocardium (Van Rooyen *et al*. 2002). The beneficial effects of glycogen in the ischaemic myocardium may however be dependent on the severity of the ischaemic insult.

In humans, AMI is associated with elevated catecholamine release, which in turn increases circulating FFA levels, reduces pancreatic insulin release and induces hyperglycemia (Vetter *et al*. 1974; Opie, 2008). Oliver *et al*. (1968) noted that high levels of FFA were evident for hours following AMI and that these elevated FFA levels were associated with an increased incidence of arrhythmias. In line with this, systemic glucose intolerance (Allison *et al*. 1967) and delayed insulin responsiveness ensue (Vetter *et al*. 1974) in the hours following the onset of AMI. These clinical observations coincide with our current understanding of the fatty acid-glucose cycle and it follows that a state of myocardial insulin resistance is possible after catecholamine induced mobilization and elevations of FFA during and following an AMI.

2.7.2 Obesity and post-ischaemic outcomes

As discussed previously, obesity is associated with a higher risk of developing cardiovascular disease. In a case-control study consisting of 27098 participants, obesity defined by waist-to-hip ratio's was shown to be associated with a higher risk of developing myocardial infarction (Yusuf *et al*. 2005). Healthy obese pre-diabetic individuals may also be at risk for developing ischaemic events. During increased myocardial workload, obese normotensive pre-diabetic individuals without cardiovascular disease present defects in myocardial perfusion (silent ischaemia) which are predominantly observed in subjects with insulin resistance and visceral adiposity (Nasr and Sliem, 2010).

Despite their high risk for developing cardiovascular disease, the prognosis of obese patients seems far better than that of lean patients following an AMI and/or heart failure. This phenomenon is known as the obesity paradox (reviewed by Morse *et al*. 2010). Although the obesity paradox is well documented, conflicting post-ischaemic outcomes associated with obesity are reported in the literature. Certain clinical studies have reported an increased risk of coronary events, or death related to obesity following an AMI (Rea *et al*. 2001; Rana *et al*. 2004), while others have reported reduced or unaltered risk for overall mortality following myocardial infarction (Lopez-Jimenez *et al*. 2004; Nikolsky *et al*. 2006; Eisenstein *et al*. 2005; Shiraishi *et al.* 2007). Nigam *et al*. (2006) demonstrated that obese and overweight individuals hospitalized following AMI had significantly lower risk of mortality within the first 6 months following AMI, while long-term mortality remained similar between the groups. Despite this, the risk of recurrent AMI and cardiac-related death remained higher in the overweight and obese group (Nigam *et al*. 2006). Using magnetic resonance imaging, obese patients (extreme obese patients were excluded) have

been reported to have smaller infarct sizes compared to lean patients (Pingitore *et al*. 2007) (This finding must be interpreted with caution as the sample size of the obese group was only 14 compared to the 75 patients in the non-obese group). Similarly, lean patients presenting with coronary artery disease have significantly higher risk of cardiac mortality and one-year mortality following percutaneous coronary intervention when compared with obese patients (Gruberg *et al*. 2002). Considering this, the possibility exists that in the obese pre-diabetic hyperinsulinaemic state, elevated insulin levels may afford a cardioprotective effect following coronary events which is lost in the diabetic state (myocardial unresponsiveness to insulin). Notwithstanding this paradox, non-diabetic obese patients with the metabolic syndrome have been reported to have worse outcomes following myocardial infarction (Clavijo et al. 2006). Nevertheless, the post-ischaemic outcomes associated with obesity remain controversial.

Although the existence of the obesity paradox requires further elucidation, Ozeke *et al*. (2011) have hypothesized that obese individuals with obstructive sleep apnea may be protected from future cardiovascular events due to chronic intermittent hypoxic episodes associated with obstructive sleep apnea. Obstructive sleep apnea occurs predominantly in the obese population and these hypoxic episodes are thought to elicit a cardioprotective preconditioning stimulus thought to be responsible for the improved cardiovascular outcomes seen in these patients.

Myocardial susceptibility to ischaemia/reperfusion injury has been investigated in many animal models of obesity/insulin resistance and type II diabetes to try and ascertain at what point, if any, in the aetiology of obesity, the myocardium becomes more susceptible to ischaemia/reperfusion induced injury. The use of animal models

allows for the control of confounding factors such as age from influencing post-ischaemic outcomes. In response to experimentally induced ischaemia/reperfusion, certain studies have reported that diabetic animals have greater infarct sizes and poorer LV systolic functional recovery compared to non-diabetic animals (Marfella *et al*. 2002) while other studies have observed smaller infarct sizes but reduced LV systolic functional recovery (Kristiansen *et al*. 2004). Available data generated from animal models of obesity and insulin resistance without overt type II diabetes, demonstrate that myocardial ischaemia/reperfusion injury is either increased (Sidell *et al*. 2002; Du Toit *et al*. 2005; Clark *et al*. 2010; Huisamen *et al*. 2011; Nduhirabandi *et al*. 2011) or comparable (Thim *et al* 2006; Aasum *et al.* 2008; Dixon *et al*. 2009) to that observed in lean control groups. Obesity has additionally been shown to increase myocardial vulnerability to repetitive ischaemic episodes (Thakker *et al*. 2008), while the offspring of obese animals are also more susceptible to ischaemia/reperfusion injury (Calvert *et al*. 2009). Animal models of obesity are further associated with adverse LV remodelling (LV dilation and cardiac hypertrophy) after a myocardial infarction (Thakker *et al*. 2006). Obesity may also increase the resistance of the myocardium to known cardioprotective interventions such as ischaemic preconditioning (Kristiansen *et al*. 2004; Katakam *et al*. 2007) and post-conditioning (Bouhidel *et al*. 2008). The majority of the available animal data collectively suggests that obesity either increases or has no effect on the myocardial susceptibility to ischaemia/reperfusion injury, while abolishing the effectiveness of cardioprotective interventions.

2.7.3 Oxidative fuel supply and ischaemia/reperfusion injury

It is probable that the combination of the increased supply of oxidative fuels and abnormal myocardial substrate metabolism characteristic of the obese state may influence the myocardiums' tolerance to ischaemia/reperfusion injury. Indeed in obese diabetic mice, post-ischaemic LV mechanical function is positively associated with pre-ischaemic myocardial glucose oxidation rates and negatively with pre-ischaemic myocardial palmitate oxidation rates (Hafstad et al. 2007). In addition, a detrimental role for FA on post-ischaemic functional outcomes has been shown in hearts from healthy animals (Lopaschuk *et al*. 1988, 1990; Liu *et al*. 2002; Gambert *et al*. 2006).

Early studies in dogs indicated that experimentally induced elevations in circulating FFA during coronary artery ligation (CAL) were associated with increased left ventricular dilatation, thus implicating elevated FFA levels with depressed pump function during ischaemia (Kjeksjus and Mjøs, 1972). More recent studies have focused on the impact of FA's on cardiac function during reperfusion. While the rates of myocardial glucose oxidation during reperfusion are often similar to the pre-ischaemic period, FA's once again constitute the major oxidative fuel predominantly oxidized during the reperfusion of the previously ischaemic myocardium, as rates of FA β-oxidation remain unchanged or become slightly elevated during this period (Lopaschuk *et al*. 1990; Aasum *et al*. 2003a).

Hearts from healthy animals perfused with a combination of 1.2mM FA and 11mM glucose have significantly poorer myocardial functional recoveries compared to hearts perfused with glucose alone (Lopaschuk *et al*. 1990). Other studies have similarly documented depressed cardiac function during myocardial reperfusion in response to elevated FA levels in hearts from healthy animals (in most instances

compared to conditions where glucose was the only substrate) (Lopaschuk *et al*. 1988; Liu *et al*. 2002; Folmes *et al*. 2006; Gambert *et al*. 2006). It is thought that the myocardium becomes stunned during the initiation of reperfusion in the presence of FA's due to their inhibitory effect on myocardial glucose oxidation rates (Lopaschuk *et al*. 1990). Furthermore, during reperfusion elevated FA's appear to have a more potent inhibitory effect on myocardial glucose oxidation rates while glycolytic flux rates are minimally affected (Lopaschuk *et al*. 1993). This dissociation between myocardial glycolysis and glucose oxidation upon reperfusion is associated with enhanced proton production, a decline in the recovery of intracellular pH and LV dysfunction (Liu *et al*. 2002; Folmes *et al*. 2006). Of great importance, is the observation that stimulation of glucose oxidation and not glycolysis, or reducing FA metabolism during reperfusion, is associated with improved myocardial functional recovery (Lopaschuk *et al*. 1990; Lopaschuk *et al*. 1993; Dyck *et al*. 2004; Gambert *et al*. 2006). Besides proton accumulation, other authors have linked the use of FA to excess ROS production which contributes to post-ischaemic cardiac dysfunction (Gambert *et al*. 2006). It is uncertain whether the post-ischaemic implications of elevated FFA supply on myocardial infarct size have been investigated.

Despite the general consensus that the presence of elevated FFA concentrations negatively impact on myocardial function in the healthy heart following ischaemia, a few studies have reported either no or beneficial effects associated with elevated FFA supply after ischaemia. This is evident in studies where supplementation of the perfusion medium of isolated perfused hearts with palmitate fails to impact on myocardial functional parameters before or after hypoxia/ischaemia (Gmeiner *et al*. 1975; King *et al*. 2001). Similarly, increasing the supply of FFA to the hearts of conscious dogs during reperfusion after only 10 minutes regional ischaemia was

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associated with improved reperfusion function, an effect abolished by oxfenicine pretreatment (CPT-1 inhibitor) (Van de Velde *et al*. 1996). Additionally, isolated rodent hearts perfused with 5.5mM glucose and 1.2mM palmitate exhibit improved mechanical function when compared to hearts perfused with glucose alone following 20 minutes global ischaemia (Broderick and Glick, 2004). Strong evidence supporting a beneficial role for FFA following ischaemia in diseased animals exists as hearts from streptozotocin-induced type I diabetic mice achieved a poorer rate pressure product recovery compared to hearts from control animals when perfused with glucose as the only substrate. The addition of palmitate in conjunction with glucose to the perfusate however enabled hearts from these diabetic animals to achieve comparable functional recoveries to the control group (King *et al*. 2001).

Considering the conflicting data regarding the impact of FA on post ischaemic myocardial function, Van der Vusse and van Bilsen, (2006) have cautioned authors against extrapolating experimental findings to the clinical setting as experimental data can be influenced by a wide range of confounding factors. Significantly the length and severity of the ischaemic episode may impact strongly of the findings reported. Nevertheless, considering all data currently available, one may speculate that obesity could be associated with adverse post-ischaemic effects due to excess availability of circulating FFA.

2.7.4 The cardioprotective effects of insulin administration

Insulin is a hormone produced by the β cells of the islets of Langerhans in the pancreas. Upon binding to insulin receptors on insulin sensitive tissues, insulin stimulates increased glucose uptake and glycogen formation, while FA utilization by the cells is reduced. As previously described, there is considerable literature

documenting the detrimental consequences of elevated levels of FA β-oxidation specifically during reperfusion on the myocardial functional recovery following an ischaemic episode (Lopaschuk *et al*. 1988, 1990; Liu *et al*. 2002; Folmes *et al*. 2006; Gambert *et al*. 2006). By virtue of its enhancement of myocardial glucose utilization by the heart, before, during and after the ischaemic episode, insulin may confer cardioprotection.

The cardioprotective effects of insulin or glucose-insulin-potassium in the setting of ischaemia/reperfusion injury have been extensively documented in the literature (Baines *et al*. 1999; Jonassen *et al*. 2000a, b; Jonassen *et al*. 2001; Nawata *et al*. 2002; Van Rooyen *et al*. 2002; LaDisa *et al*. 2004; Hafstad *et al*. 2007; Fuglesteg *et al*. 2008; Yu *et al*. 2008; Oates *et al*. 2009; Xing *et al*. 2009; Ji *et al*. 2010; Wong *et al*. 2011). While there is consensus that insulin is cardioprotective in experimental physiology, the majority of insulin's cardioprotective effects have been reported to occur when administered 5 minutes prior to reperfusion of the ischaemic myocardium (LaDisa *et al*. 2004; Jonassen *et al*. 2000a; Jonassen *et al*. 2001; Fuglesteg *et al*. 2008; Yu *et al*. 2008; Ji *et al*. 2010; Wong *et al*. 2011), with a few articles documenting cardioprotective effects when it was administered before, during and after ischaemia (Jonassen *et al*. 2000a; Nawata *et al*. 2002; Van Rooyen *et al*. 2002; Oates *et al*. 2009; Xing *et al*. 2009). Despite this, LaDisa *et al*. (2004) and Hafstad *et al*. (2007) could not demonstrate a cardioprotective effect of insulin in healthy hearts when present throughout the experimental period. Insulin further seems unable to evoke cardioprotection when administered prior to and during the ischaemic period only (Jonassen *et al*. 2001). However pre-treating isolated hearts with insulin as a pre-conditioning stimulus (Fuglesteg *et al*. 2009), or merely administering insulin for 5 minutes followed by a 10 minute washout period prior to regional ischaemia (Baines

et al. 1999) significantly reduced myocardial infarct size in response to simulated AMI *ex vivo*. This elaborate body of evidence indicates that insulin exerts its cardioprotective effects predominantly during reperfusion. Jonassen *et al*. (2001) further determined that the initial 15 minute reperfusion phase is a pivotal period during which insulin exerts its cardioprotective effects.

Some of insulin's cardioprotective mechanisms have been unraveled and include early activation of PKB/AKT and p70s6 kinase during reperfusion (Jonassen *et al*. 2001), activation of the K_{ATP} channel (established with a non-selective K_{ATP} antagonist) (LaDisa *et al*. 2004), improved re-synthesis of myocardial high energy phosphates upon reperfusion (Nawata *et al*. 2002), exerting anti-adrenergic effects in response to β-adrenergic stimulation (Yu *et al*. 2008), inhibiting peroxynitrate formation (Ji *et al*. 2010) and activation of Signal Transducer and Activator of Transcription 3 (STAT3) (Fuglesteg *et al*. 2008). Insulin has been shown to reduce neonatal cardiomyocyte apoptosis during hypoxia by attenuating caspase-3 cleavage in the absence of glucose (Morisco *et al*. 2007). Although improved glucose uptake and glycolytic flux rates can induce cardioprotection (Malhotra and Brosius, 1999), additional signaling mechanisms seem to be important in the overall cardioprotective effect. As far as we know, only Nawata *et al*. (2002) (streptozotocin induced type I diabetes) and Hafstad *et al*. (2007) (in diabetic db/db mice) have demonstrated a cardioprotective role for insulin in diseased animal models. A cardioprotective role for insulin has yet to be demonstrated in diet-induced obese insulin resistant animals. As such additional studies are warranted investigating the cardioprotective role of insulin in diseased animal models.

2.8 Hypotheses and objectives

In view of the literature and the various controversies regarding the cardiovascular effects of obesity that were described in the previous sections, a few research questions were identified requiring further investigation.

The general aim of the current dissertation is to determine the impact of obesity on cardiac function, myocardial sensitivity to insulin administration, substrate metabolism, mitochondrial function and susceptibility to ischaemia/reperfusion injury. The impact of an insulin sensitizing/lipid lowering PPARα agonist (K-111) on these parameters will also be investigated.

For this study, we have developed the following hypotheses:

1) Protracted obesity is associated with compromised *in vivo* and *ex vivo* cardiac function.

2) Isolated hearts from obese animals are less responsive to insulin administration than hearts from lean control animals.

3) Insulin present at normal physiological concentrations confers cardio-protection from myocardial ischaemia/reperfusion injury in healthy animals, while its protective efficacy is reduced in obese animals.

4) Under comparable perfusion/substrate conditions, hearts from obese animals exhibit altered myocardial substrate metabolism, mitochondrial respiration and increased susceptibility to ischaemia/reperfusion injury.

5) Under comparable perfusion/substrate conditions, hearts from animals subjected to chronic PPARα agonist treatment are able to improve myocardial substrate metabolism and mitochondrial respiration which is associated with reduced myocardial susceptibility to ischaemia/reperfusion injury.

To address these hypotheses, we made use of a pre-diabetic rodent model of diet-induced obesity which is characterized by general and visceral obesity, systemic insulin resistance and dyslipidaemia.

Our objectives were to determine:

1) The impact of protracted obesity on *in vivo* and *ex vivo* cardiac mechanical function.

2) The responsiveness of hearts isolated from control and obese insulin resistant animals to insulin administration and the impact thereof on the myocardial susceptibility to ischaemia/reperfusion injury and the role of the substrate present.

3) The impact of obesity and chronic PPARα agonist treatment on *ex vivo* cardiac function, substrate metabolism, mitochondrial function and susceptibility to ischaemia/reperfusion injury.

CHAPTER 3

GENERAL MATERIALS AND METHODS USED FOR STUDIES 1, 2 and 3

In this chapter details are given regarding the animals and their diet as well as the techniques routinely used throughout this study. Further details regarding the various study designs, protocols, experimental procedures and results are given in the subsequent chapters.

3.1.1 Animals

All studies were performed according to the guidelines set by the Principles of Laboratory Animal Care of the South African Medical Research Council and the Guide for the Care and use of Laboratory Animals of the National Academy of Sciences (NIH publication no 80-23. revised 1985) and were approved by the Committee for Experimental Animal Research of the Faculty of Health Sciences of the University of Stellenbosch (P06/10/016).

Male Wistar rats were used in all three studies and housed in the Central Research Facility (AAALAC accredited), Faculty of Health Sciences, University of Stellenbosch. The holding rooms had a 12-hour artificial day-night cycle, while temperature and humidity were constantly maintained at 22°C and 40% respectively. The animals were weaned at 4 weeks of age and allowed free access to food and water.

3.1.2 The experimental diet

A central component to all the studies performed in this thesis was the high caloric diet (HCD), which has been reported to facilitate hyperphagia-induced obesity in rats (Pickavance *et al*. 1999). The macronutrient composition of the HCD was reported to

consist of 65% carbohydrates, 19% protein and 16% fat, while the standard rat chow (SRC) contained 60% carbohydrate, 30% protein and 10% fat (Pickavance *et al*. 1999). The HCD consisted of 33% SRC, 33% condensed milk (clover), 7% sucrose and 27% water (Pickavance *et al*. 1999). As the HCD promoted hyperphagia, these rats had a higher average daily energy consumption compared to the SRC group (Du Toit *et al*. 2005).

	SRC	HCD
Food consumption:	29 g/day	57 g/day
Energy content	13.1 kJ/g	10.1 kJ/g
Energy consumption per day	**379.9 kJ**	**575.7 kJ**

(Food consumption x energy content)

Due to the condensed milk present in the HCD, the experimental group (later referred to as the obese or the HCD fed group) received fresh food daily to avoid fermentation of the food.

The experimental group was placed on the HCD when age matched rats weighed between 180-200g. Details regarding the length of feeding will be provided in the upcoming chapters.

3.1.3 Isolated heart perfusions

Isolated rat heart perfusions formed a fundamental component of all the studies presented in this thesis. In all cases, hearts were rapidly excised from anaesthetized animals (sodium pentobarbitone 60 mg/kg) and immediately placed in ice-cold Krebs-

Henseleit buffer. Within 60 seconds of excision, the hearts were mounted on the appropriate perfusion apparatus for the respective interventions.

3.1.3.1 Perfusion buffer

All perfusions were performed with a standard Krebs-Henseleit buffer (118.46mM NaCl, 25mM $NaHCO_3$, 4.75mM KCl, 1.19mM KH_2PO_4, 1.19mM $MgSO_4.7H_2O$, 1.25mM $CaCl_2.2H_2O$ and 10mM glucose (Merck Pty.Ltd., Darmstadt, Germany)) which was at times supplemented with insulin (Eli Lilly and Company, Indianapolis, IN 46285, USA) and/or fatty acid (FA) (palmitic acid (Sigma-Aldrich, St.Louis, MO)). The perfusion protocols employed used different concentrations of insulin and/or FA to simulate the specific *in vivo* plasma insulin and FA content of each group of animals. Based on the *in vivo* insulin and FA concentration obtained in control and obese rats, the following perfusion buffer modifications were utilized (unless otherwise stated) – Control animals: 30µIU/ml insulin, 0.7mM FA; Obese animals: 50µIU/ml insulin, 1.5mM FA (See Table 5.2). Fresh perfusion buffer was prepared daily and gassed with a mixture of 95% O_2 and 5% CO_2 before being filtered through a 0.45µm mixed cellulose ester pore filter (Advantec MFS Inc, Pleasanton USA). The perfusate was further oxygenated with the same gas mixture within the perfusion apparatus for the duration of the experimental protocol.

3.1.3.2 Preparation of the FA perfusion buffer

The preparation of the FA perfusion buffer was performed according to the guidelines set out by Lopaschuk and Barr (1997).

Due to the abundance of palmitic acid in the serum of HCD fed rats (Huisamen *et al*. unpublished data), it was decided to used palmitic acid as the FA in the perfusion

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buffer. Palmitic acid (which contributed 0.4mM or 1.2mM to the total FA concentration) was brought into solution with 0.5mM or 1.5mM sodium carbonate respectively, 95% ethanol and heat. After the majority of the ethanol had boiled away (as determined by carefully smelling the mixture for the presence of ethanol) the palmitate solution was carefully added to a warm solution of Krebs-Henseleit buffer containing 3% (final concentration) bovine serum albumin (Bovine serum albumin, Fraction V, Roche, Germany). Prior determination with gas chromatography showed that the bovine serum albumin contributed an additional 3mM to the total FA concentration of the perfusion mixture. The concentrated FA perfusion buffer was subsequently dialyzed (Spectra/Por dialysis tubing (MW cut-off 6000-8000) Thomas Scientific, USA) overnight in 9.5 volumes of glucose free Krebs-Henseleit buffer to saturate the calcium binding sites of albumin and to allow excess ethanol to diffuse into the Krebs buffer (Lopaschuk and Barr, 1997). The following day the dialyzed FA solution was diluted with Krebs-Henseleit buffer to the final working volume. At this point 10mM glucose and insulin were added to the perfusion buffer after which the solution was filtered through Whatman #1 (Whatman Ltd, England) filter paper. The final FA perfusion buffer therefore consisted of Krebs-Henseleit buffer and 0.7mM or 1.5mM FA and 30 or 50µIU/ml insulin.

3.1.3.3 Motivation for the concentrations of insulin and glucose used

Both the insulin (30 or 50µIU/ml) and the FA (0.7mM or 1.5mM) concentrations used in the various perfusion buffers were determined to be relevant to the *in vivo* conditions of the animals used (Table 5.2). In the absence of insulin, many studies use 0.8mM or 1.2mM FA in combination with 10/11mM glucose in the perfusion buffer. However, when insulin is used together with glucose, the glucose concentration is normally reduced to 5mM. It is however worth mentioning that when

hearts are perfused with 5mM glucose, insulin and FA, the insulin concentrations are at times higher (3-fold higher in certain cases) (Wilson *et al*. 2007; Zhang *et al*. 2010) than those used in the current study. Despite this a number of authors have determined cardiac substrate metabolism in normal and insulin resistant animals in the presence of 11mM glucose, insulin (140µIU/ml or 2000 µIU/ml) and FA (0.4mM or 1mM) (Lopaschuk *et al*. 1991; Buchanan *et al*. 2005). Consequently we decided to continue with 10mM glucose in the presence of different concentrations of insulin (30 or 50µIU/ml) and FA (0.7 or 1.5mM) in our experiments as: 1) the insulin concentrations used in our study were lower than those used by the previously listed authors and 2) to limit the amount of variation between our perfusion protocols (Krebs-Henseleit perfusion vs. Krebs-Henseleit + FA + insulin perfusion).

3.1.4 Comparisons between the isolated working rat heart perfusion apparatus and the Langendorff perfusion apparatus (balloon model)

Throughout this thesis, reference will be made to both the isolated working rat heart perfusion apparatus and the Langendorff perfusion apparatus. Here the fundamental differences between these two perfusion models are highlighted.
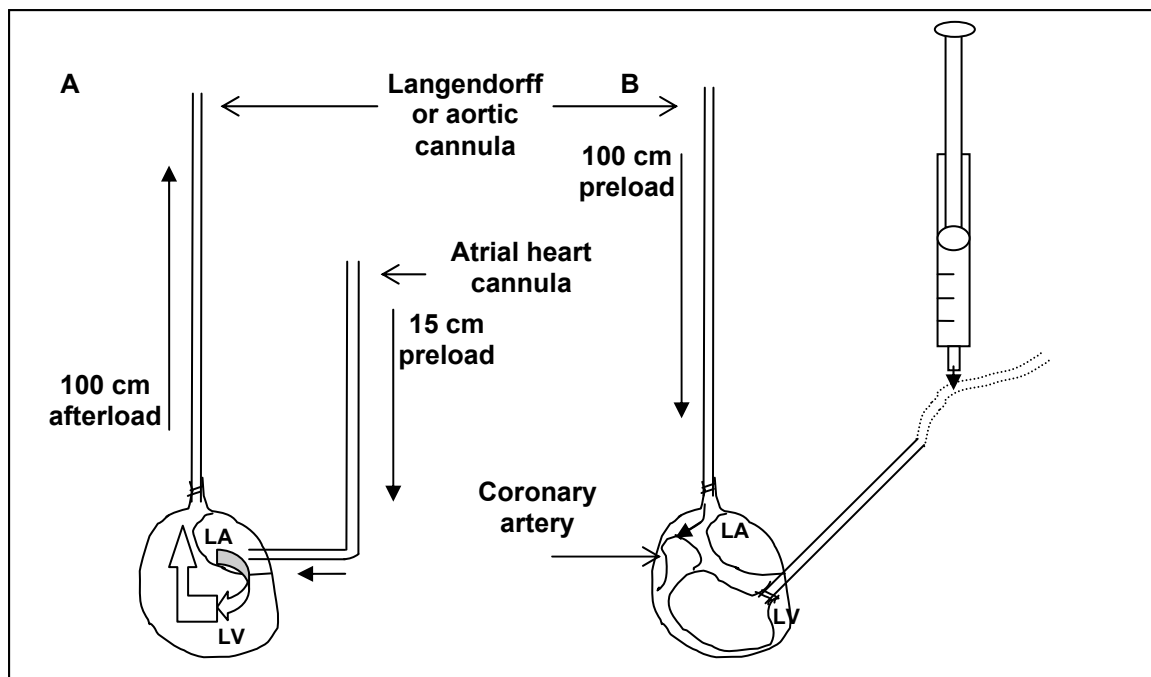
Figure 3.1: Schematic representation of the direction of perfusion buffer flow in the isolated working rat heart perfusion apparatus (a), and the isolated Langendorff perfusion apparatus (balloon model) (b).

LA – left atrium; *LV* – left ventricle

The isolated working rat heart perfusion apparatus used in our experiments was based on the model originally described by Neely *et al*. (1967) and modified by Opie *et al*. (1971). It allows for perfusion of the isolated heart in a working mode (Figure 3.1a). The aorta of the heart is mounted on the aortic cannula where it immediately receives retrograde perfusion in the Langendorff mode. Excess tissue is trimmed off from the heart. The left atrium is subsequently cannulated through one of the pulmonary veins via the working heart left atrial cannula. Using a set of stopcocks, the perfusion of the heart can be switched between retrograde/Langendorff and working heart perfusion/mode at the appropriate times. During the working mode of heart perfusion, buffer enters the left atrium via the left atrial cannula of the working heart perfusion apparatus and is subsequently pumped out by the left ventricle through the aorta. The preload placed on the heart during working heart mode is determined by the height of the overflow of the working heart bubble trap supplying

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the left atrium. For rat hearts, the preload is normally fixed at 15cm H_2O, but may be adjusted if required. Increasing the preload would be an attempt to simulate clinical situations such as volume overload (as seen in obesity), or increased venous return (as evident in exercise).

The isolated Langendorff perfusion apparatus was originally described by Oskar Langendorff (Langendorff *et al*. 1895). Isolated hearts are mounted onto the aortic cannula of the Langendorff perfusion apparatus (Figure 3.1b) and retrogradely perfused. Once the excess tissue has been removed, a water filled balloon (latex or plastic where appropriate) tipped cannula is inserted via the atrium directly into the left ventricle via the pulmonary veins. Once positioned correctly, the balloon is filled with fluid via a syringe attached to the line connecting the cannula to the pressure transducer. The force of contraction and relaxation exerted by the left ventricle on the balloon generates a pressure that is converted to an electrical signal by the pressure transducer.

An important fundamental difference exists regarding the myocardial workload being performed during working heart perfusion and Langendorff mode perfusion of the isolated heart. During working heart mode perfusion, perfusate enters the left atrium, is expelled by the atrium into the left ventricle, which once it contracts pumps the perfusate out via the aorta. In contrast, when the heart is retrogradely perfused, perfusate never enters the left atrium or ventricle as the aortic valve remains closed. The perfusate merely enters the coronary circulation due to retrograde flow of buffer down the aorta thus supplying the heart with essential nutrients and oxygen. The workload and the metabolic requirements placed on the heart are therefore greater during working heart mode perfusion than during Langendorff mode.

Both the isolated working heart and Langendorff perfusion apparatus are well suited to provide information regarding LV mechanical function. During the working heart mode (isolated working heart apparatus), buffer is expelled through the aorta as previously described. The expelled buffer represents the aortic output of the heart. The buffer that is ejected against the 100cm H_2O afterload is collected in a graded measuring cylinder over a period of time allowing the aortic output of the heart to be measured in ml/min. Aortic output closely reflects the LV mechanical function of the *in vivo* heart. Coronary flow is also manually measured, and as the name indicates, is the volume of fluid that is drained from the coronary circulation. On the working heart perfusion system, a side arm from the aortic cannula is connected to a pressure transducer, which in turn is connected to a computer. From this device, measurements for peak systolic/diastolic aortic pressure, heart rate, work total, and aortic $dP/dt_{max/min}$ are monitored and documented.

When using the Langendorff perfusion apparatus a water filled balloon tipped cannula is inserted into the left ventricle. The cannula is connected to a pressure transducer which converts the pressure changes to signals that are then displayed on the computer monitor, thus allowing myocardial isovolumic contractile function to be monitored. In this way, both heart rate and the LV developed pressure can be determined, the product of which is termed the rate pressure product, which represents the major functional parameter assessed on the Langendorff perfusion apparatus. The coronary flow is also measured as described previously.

3.1.4.1 Myocardial temperature control during the isolated heart perfusions

Once the heart is mounted and heart perfusion initiated, a small incision is made into the coronary sinus through which a temperature probe can be inserted. A water bath (Grant instruments, Cambridge, England) maintains the temperature of the perfusate by re-circulating warm water through the water-jacketed glassware of the perfusion system. A separate water bath (Grant instruments, Cambridge, England) is used to maintain the temperature of the water-jacket surrounding the perfused heart.

During the experimental procedures of the current study, the following myocardial temperatures were maintained for the various stages of the perfusion protocols: 1) Working heart apparatus: Langendorff mode: 34.5°C-35.6°C; Working heart mode: 36.0°C-37.0°C and regional ischaemia 35.8-36.3°C; 2) Langendorff perfusion apparatus: Langendorff mode: 36.5°C-37.0°C; global ischaemia 36.0°C-36.5°C.

Circulation of the buffer through the perfusion system was maintained by a peristaltic pump (Watson Marlow Ltd, UK).

CHAPTER 4

METHODS AND RESULTS FOR STUDY 1

AN *IN VIVO* AND *EX VIVO* INVESTIGATION ON THE IMPACT OF PROTRACTED OBESITY ON MYOCARDIAL MECHANICAL FUNCTION

4.1 Aim of the study

The aim of this sub-study was to determine the impact of protracted obesity on *in vivo* and *ex vivo* myocardial function in control and obese animals. The contributory role of insulin and FA to LV mechanical function was also investigated.

4.2 Methods

4.2.1 Study design

To determine the consequences of obesity on *in vivo* and *ex vivo* myocardial LV mechanical function, the feeding regime of our existing model of diet induced obesity (Du Toit *et al*. 2005; Clark *et al*. 2011) was extended from 16/18 weeks to 32 weeks of feeding to exacerbate any potential myocardial functional consequences related to obesity. The feeding period was of a similar duration to the "long term" feeding period utilized by Wilson *et al*. (2007) who documented *ex vivo* myocardial dysfunction in isolated hearts from rats fed a Western diet for 32-48 weeks.

Age and weight matched rats weighing 190±10g, were randomly assigned to control or experimental groups and fed either a SRC or a HCD respectively for 32 weeks. Upon completion of the feeding period, myocardial mechanical function was determined using either standard echocardiography techniques (*in vivo*) or isolated

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heart perfusions (*ex vivo*). A separate series of animals was used for each determination (echocardiography, isolated Langendorff perfusions and isolated working heart perfusions). The importance of glucose and simulated *in vivo* concentrations of insulin and FA on myocardial LV mechanical function was assessed using isolated heart perfusions. Different animals were used for each different perfusion condition. Random measurements of body weight, retroperitoneal and gonadal fat mass and tibia lengths were measured and following the completion of the experimental protocol, heart weights (wet weight) were determined. To determine the influence of long term HCD consumption on the blood lipid profile and glucose and insulin concentrations following the 32 week feeding period, a separate series of animals was fasted overnight (duration of fast not documented) and blood was collected.

4.2.2 *In vivo* assessment of LV mechanical function

4.2.2.1 Echocardiography

In vivo LV systolic function was determined by two-dimensional targeted M-mode echocardiography using a 7.5 MHz transducer and a Hewlett Packard Sonos 2500 sector scanner according to the guidelines set by the American Society of Echocardiography convention and as previously described by Norton *et al.* (2002). Rats were anaesthetized with a combination of ketamine and xylazine (Norton *et al.* 2002) and echocardiography was performed blinded, and in a random order by a single observer. Two images were obtained from each rat and mean values for LV end-diastolic diameter (LVEDD), end-systolic diameter (LVESD) and posterior wall thickness (PWT) were determined. These variables were measured using the leading edge technique (Norton *et al.* 2002). To assess LV functional reserve of the animals, 0.01 mg/kg isoproterenol HCl (Sigma-Aldrich, St.Louis, MO) was injected

intraperitoneally and LV functional measurements were documented at regular intervals for 30 minutes.

LV systolic chamber function was determined from the LV endocardial fractional shortening (LV FSend) and LV systolic myocardial function from the LV midwall fractional shortening (LV FSmid). These parameters were calculated as previously described by Norton *et al*. (2002).
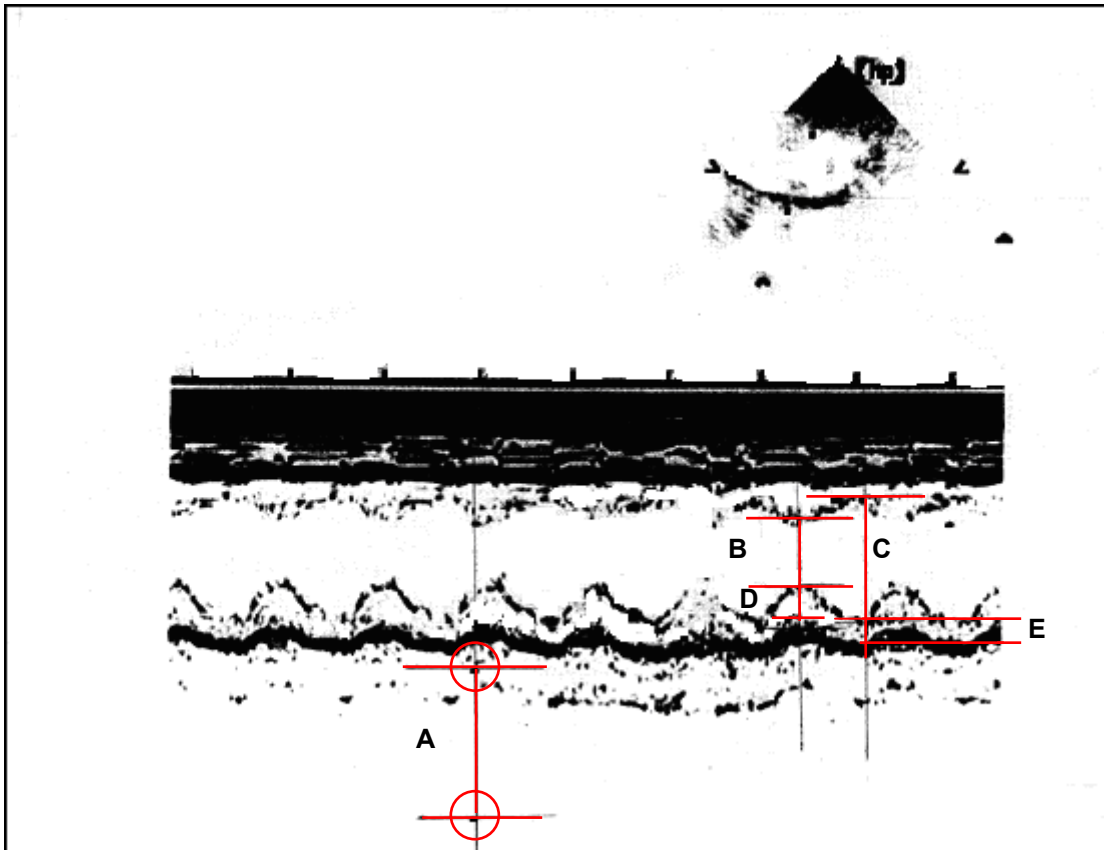
Figure 4.1: A typical echocardiograph indicating the dimensions of the rat heart that were measured

- A: The scale given on the original print-out was 3 cm between the markings (circles). However since the image had been enlarged, the scale had to be adjusted accordingly.

- B: indicates the ESD

- C: indicates the EDD

- D: indicates the PWT_{syst}

- E: indicates the PWT_{diast}

The various echocardiographic parameters were calculated as follows:

- Endocardial fractional shortening (FSend) was calculated from:

    ((EDD - ESD) / EDD)) x 100.

- Midwall fractional shortening (FSmid) was calculated from:

    $$(((EDD + PWT_{diast}) – (ESD + PWT_{syst})) / (EDD + PWT_{diast})) \times 100$$

- Posterior wall thickening was calculated from:

    $$((PWT_{syst} - PWT_{diast}) / (PWT_{diast})) \times 100$$

4.2.3 *Ex vivo* assessment of LV mechanical function

To assess the importance of simulated *in vivo* concentrations of insulin and FA, on myocardial mechanical function, LV function was assessed *ex vivo* on both the isolated Langendorff perfusion apparatus and the isolated working rat heart perfusion apparatus. The Langendorff, isovolumetric, constant coronary flow model allowed for the measurement of LV intrinsic myocardial mechanical function independent of different loading conditions and possible alterations in obesity related coronary flow rate. In contrast, the isolated working heart model was used to assess LV mechanical function on a working heart which has a coronary flow rate which is dictated by intrinsic vascular tone of the coronary arteries.

4.2.3.1 Isolated Langendorff perfusions (Balloon model)

Hearts were perfused with standard Krebs-Henseleit perfusion buffer containing glucose (10mM) as the only substrate. To determine the impact of insulin on LV mechanical performance, a separate series of hearts was used and the perfusate supplemented with the relevant *in vivo* concentrations of insulin (Control: 30µIU/ml;

Obese: 50µIU/ml). Only the effect of insulin on LV mechanical function was assessed in this model.

Once hearts were mounted, the coronary flow rate was determined volumetrically and adjusted to achieve a constant flow rate of 12 ml/min/g heart weight. The hearts were paced at 300bpm with platinum wire electrodes attached to the left atrium and the apex of the heart with the voltage set at 10% above threshold. A water-filled balloon-tipped cannula coupled to a pressure transducer was inserted into the LV cavity and after an initial 10 minute stabilization period LV developed pressure was determined. A thin-walled latex balloon with a zero pressure filling volume beyond maximum LV lumen capacities was selected for this study to avoid the stiffness of the balloon wall contributing to LV pressure at higher filling volumes. The volume of the balloon was assessed with a water-displacement technique, and the same balloon was used for each heart. A micromanipulator was used to incrementally increase LV filling volumes to values that resulted in no further change in LV developed pressure. LV pressures were determined at as many multiple small increments in volume as were practically possible to improve the accuracy of curve fitting during later analysis. A range of LV pressures and volumes were determined in hearts perfused with glucose, or glucose together with the relevant *in vivo* concentration of insulin.

Peak LV systolic function was determined in isolated hearts from a separate series of animals under similar perfusion conditions as described above. This was achieved by the addition of isoproterenol HCL (10^{-8}mM) (Sigma-Aldrich, St.Louis, MO) to the perfusate. The load-independent LV systolic chamber performance was then determined from the slope of the peak LV systolic pressure-volume curve generated, (LV end-systolic elastance (LV Ees)).

4.2.3.2 Isolated working heart perfusions

To further assess the impact of physiological concentrations of insulin and FA on LV mechanical function, hearts were either perfused with a standard Krebs-Henseleit perfusion buffer (see 3.1.3) containing glucose (10mM) or supplemented with different combinations of insulin and or FA. The simulated *in vivo* concentrations of insulin and FA used for the different groups were as follows: Control - 30µIU/ml insulin, 0.7mM FA; Obese: 50µIU/ml insulin, 1.5mM FA (see 3.1.3). When FA's were present in the perfusion buffer, the perfusate (200ml) was continuously re-circulated. It was not expected that re-circulating the buffer would influence myocardial function as hearts perfused under these conditions were able to maintain normal working heart function for up to one hour (preliminary investigations). Furthermore, it was thought that the 200ml re-circulating volume was sufficient to buffer possible contributory effects of substrate metabolic by-products on cardiac function.

Once mounted on the perfusion apparatus, hearts were retrogradely perfused for an initial 10 minute stabilization period. The perfusion was then changed to working heart mode with the preload set at 15cm H_2O. A further 10 minute stabilization period was initiated under these conditions. To assess myocardial functional reserve under the various perfusion conditions, the preload was incrementally increased from 15cm to 17.5cm and then to 20cm H_2O at 5 minute intervals. Aortic output was documented at the end of each perfusion period of a particular filling pressure. Thus, aortic output was measured at various preloads, with different combinations of insulin and FA in the perfusate.

4.2.4 Biometric measurements

4.2.4.1 Determination of retroperitoneal and gonadal fat content

After the heart was removed, abdominal incisions were made to access the abdominal fat depots. Both the retroperitoneal and gonadal fat depots were removed and weighted.

4.2.4.2 Indices of cardiac hypertrophy

4.2.4.2.1 Ventricular weight to tibia length ratio

Yin *et al*. (1982) demonstrated that the ventricular weight to tibia length ratio was a far more accurate index to reliably identifying cardiac hypertrophy than the ventricular weight to body weight ratio in models where weight fluctuations (such as due to aging) were prevalent.

Measurements of ventricular weight to tibia length ratio were collected at random. The tibia was cleaned off and the length measured with a caliper and expressed as a ratio to the heart weight.

4.2.5 Biochemical analysis

4.2.5.1 Blood sample collection

Unless otherwise stated, blood samples were collected from the thoracic cavity of rats following the excision of their hearts. Blood was transferred directly into a specific BD Vaccutainer (Lasec SA, Cape Town, South Africa). For whole blood analysis, BD Vaccutainer's containing anticoagulant was used. When serum was required for biochemical determinations, BD Vaccutainer's specific for serum separation were used. In these cases the tubes were centrifuged at 3000rpm at 4ºC for 10 minutes according to the manufacturer's instructions. The separated serum was aliquoted into tubes and stored at -80 ºC until required for specific assays.

4.2.5.2 Blood determinations

4.2.5.2.1 Blood glucose

Fasting blood glucose levels provide valuable information regarding steady state glucose levels. Raised blood glucose levels during post-prandial conditions allude to either a failure of normal or elevated levels of insulin to maintain normal blood glucose levels, or insufficient insulin being produced. Elevated blood glucose levels are further a characteristic of type II diabetes.

Fasting glucose values were determined by placing a drop of blood onto the provided absorbent film of a slip/cassette that was inserted into a glucometer (Gluco Plus^{TM}; distributed by Cipla Dibcare, Bellville, South Africa).

4.2.5.2.2 HbA1c

The measurement of HbA1c is a clinical endpoint used to determine the efficiency of blood glucose management of the body over a 3 month period. HbA1c is a specific subtype of haemoglobin A. Once the rat (non-fasted) had been anaesthetized an incision was made into the hind limb exposing the femoral vein which was then heparinised. A few minutes later, blood was collected and taken to the Department of Chemical Pathology of Tygerberg Hospital, for further analysis.

Principle of Determination

Glucose binds (or glycosylates) haemoglobin in the erythrocyte to form the A1c subtype, over a period of time. The decomposition of HbA1c occurs very slowly and therefore strongly correlates with the average blood glucose levels over the previous 3 months. When the percentage glycosylated haemoglobin is under 7 %, one may

correctly assume that the animal's blood glucose levels were normal over the previous 3 months.

4.2.5.2.3 Blood lipids

Similarly to blood glucose determinations, a drop of blood from fasted animals was placed onto the absorbent film of a PTS strip/cassette which was inserted into a CardioCheck instrument (CardioCheck™, Polymer Technology Systems Inc, Indianapolis, USA) which quantified total cholesterol, triglyceride and HDL-C levels.

4.2.5.3 Serum determinations
4.2.5.3.1 Insulin levels

Serum samples from fasting animals were stored at -80 ºC and were used to determine insulin levels by means of competitive radioimmunoassay (Coat-A-Count ® Insulin, Diagnostic Products Corporation, LA, USA).

Kit Principle

A set amount of radio-labelled (^{125}I) insulin competes with the insulin present in the serum sample for antibody binding sites specific for insulin. As the walls of the provided polypropylene tubes are coated with antibody, decanting the tubes following the incubation period isolates the antibody bound fraction containing radio-labelled and sample insulin.

Once the assay was completed, the amount of radioactivity was measured with a gamma scintillation counter (Cobra II Auto Gamma, A.D.P, South Africa) and the specific insulin concentrations determined using a standard curve obtained from

known insulin concentrations. Calculations were performed using the appropriate computer software.

4.2.6 Statistical analysis

All data are presented as means ± the standard error of the mean. Means were considered to be statistically significant from each other when the p-value was smaller or equal to 0.05. Statistical comparisons between control and obese animals were determined with an unpaired Students t-test. Comparisons of *in vivo* functional data were assessed with a one-way ANOVA followed by the Bonferroni post-test. For multiple comparisons between various groups a two-way ANOVA (repeated measures where appropriate) was applied followed by the Bonferroni post-test (except for the retrograde perfusions where a Tukey post-test was performed). Statistical Analysis was performed on Graph Pad Prism 4 and 5 software.

RESULTS: STUDY 1

4.3 Results

4.3.1 Biometric data

Table 4.1: Biometric data of control and obese animals

	Control	Obese
Body weights (g)	550±16 (n=15)	673±19 * (n=15)
retroperitoneal and gonadal fat weight (g)	27.20±0.89 (n=12)	54.6±2.50 * (n=15)
VW weights (g)	1.10±0.01 (n=13)	1.27±0.02 * (n= 13)
VW/TL (mg/mm)	24.90±0.18 (n=13)	28.95±0.50 * (n=13)

VW – ventricle weight; TL – tibia length

*p<0.05 control vs. obese group

Consumption of a HCD for 32 weeks induced a 22% increase in body weight compared to rats fed a SRC diet (Table 4.1) (p<0.05). Similarly the HCD fed group had more retroperitoneal and gonadal fat content. In addition HCD fed rats had greater VW's while their VW/TL ratio's were also significantly greater than the corresponding values observed in the SRC fed group (p<0.05).

4.3.2 Metabolic data

Table 4.2: Metabolic data of control and obese animals that were fasted overnight. HbA1c levels were determined from non-fasted animals

	Control	Obese
Glucose (mmol/l)	7.13±0.37 (n=10)	7.61±0.42 (n= 13)
Insulin (μIU/ml)	33.50±6.07 (n=7)	54.40±7.60 * (n=10)
HbA1c (%)	4.13±0.16 (n=6)	4.10±0.02 (n=6)
Triglycerides (mmol/l)	0.62±0.08 (n=6)	1.25±0.09 * (n=6)
Total cholesterol (mmol/l)	1.60±0.26 (n=6)	1.83±0.19 (n=6)
HDL-C (mmol/l)	0.40±0.06 (n=6)	0.50±0.07 (n= 6)

HbA1c **– glycosylated haemoglobin;** *HDL-C* **– high density lipoprotein cholesterol.**

*$p<0.05$ vs. control

After 32 weeks of feeding, blood glucose levels were comparable between the control and obese groups, however obese animals had significantly higher serum insulin levels compared to the control group ($p<0.05$) (Table 4.2). As glucose and insulin determinations were not obtained in the same animals in all cases the homeostasis model assessment index for insulin resistance (HOMA-IR) could not be calculated, but by studying the means of these groups and comparing them to similar data presented in table 5.2 and 6.2, obese animals in this study could be regarded as being insulin resistant. Additionally, as the HbA1c values of the two groups were comparable, it can be concluded that 32 weeks of HCD feeding was sufficient to induce systemic insulin resistance but not type II diabetes. Furthermore, blood Trig levels were significantly elevated in obese animals while there was no difference in total cholesterol and HDL-C between the groups.

4.3.3 *In vivo* and *ex vivo* myocardial functional data

4.3.3.1 *In vivo* myocardial LV dimensions and mechanical function

Table 4.3: LV dimensions determined echocardiographically in control and obese animals

	Control (n=5)	Obese (n=5)
LVEDD (cm)	0.80±0.02	0.81±0.03
LVESD (cm)	0.52±0.03	0.48±0.03
LV PWT (cm)	0.67±0.09	0.73±0.07

LVEDD **– left ventricular end diastolic diameter;** *LVESD* **– left ventricular end systolic diameter;** *LVPWT* **– left ventricular posterior wall thickness.**

Echocardiograph measurements of the various LV dimensions were comparable between control and obese animals (Table 4.3). There was no evidence of LV remodelling present in the obese group (LV PWT, $p > 0.05$) despite a greater VW/TL ratio, indicative of enhanced cardiac mass, in a similar obese group (Table 4.1). The absence of significant differences in LV PWT may be due to the small sample size used for the *in vivo* determinations.
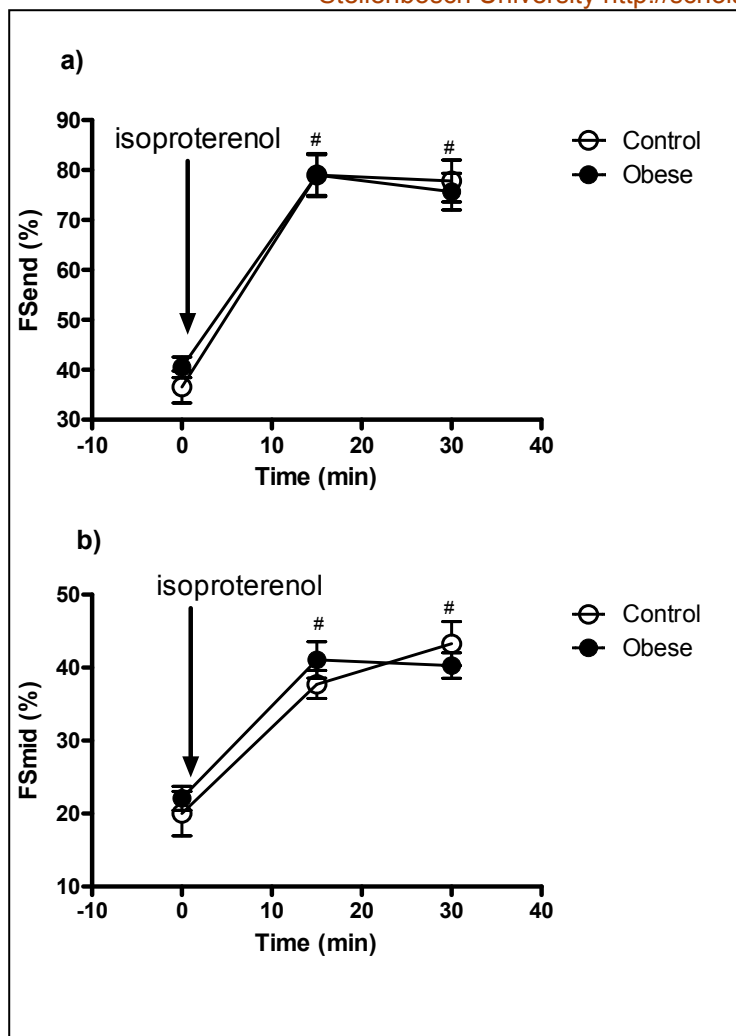
Figure 4.2: The effect of isoproterenol administration on *in vivo* LV chamber (a) and intrinsic myocardial (b) function in hearts from control and obese animals. *FSend* – endocardial fractional shortening; *FSmid* – midwall fractional shortening.

[#]p<0.001 vs. Baseline for both groups

n = 5 rats per group

Echocardiography assessments of control and obese animals during resting conditions revealed similar LV chamber (Figure 4.2a) and intrinsic myocardial (Figure 4.2b) function between these groups. In response to β-adrenergic receptor stimulation (0.01 mg/kg isoproterenol-HCl intraperitoneal injection) both the control and obese animals were able to significantly increase their FSend and FSmid to the same degree. Under resting and increased myocardial workload conditions, obese animals were thus able to functionally (LV chamber and intrinsic myocardial

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functional measurements) match their control counterparts suggesting the absence of any form of myocardial dysfunction in the obese animals after 32 weeks HCD feeding.

4.3.3.2 *Ex vivo* functional determinations

4.3.3.2.1 Isolated Langendorff perfusions
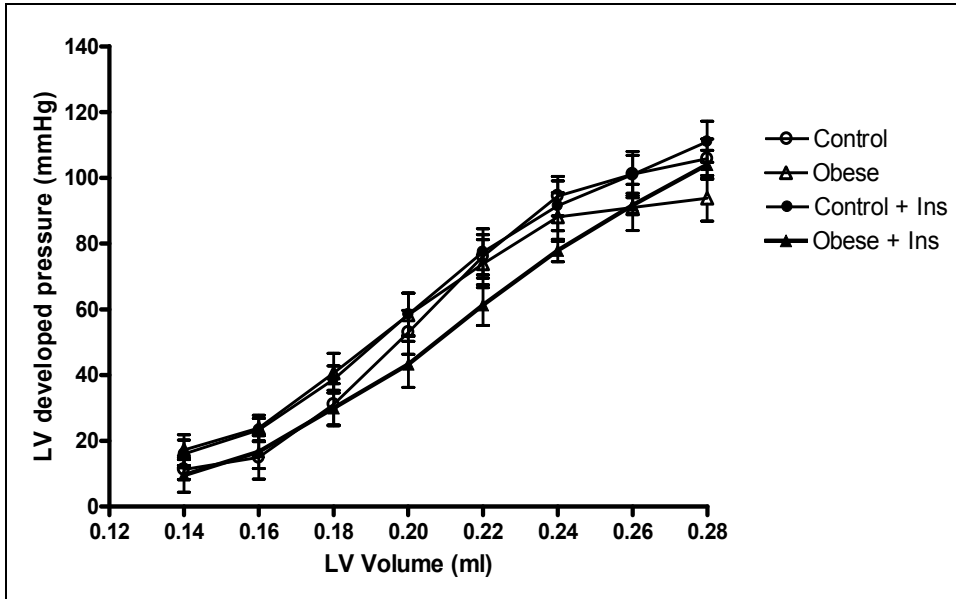
Figure 4.3a: The impact of simulated *in vivo* concentrations of insulin (Control: 30µlU/ml; Obese: 50µlU/ml) on LV systolic chamber function assessed at different LV preloads in retrogradely perfused hearts isolated from control and obese animals. *Ins* – insulin.

n = 8 hearts per group
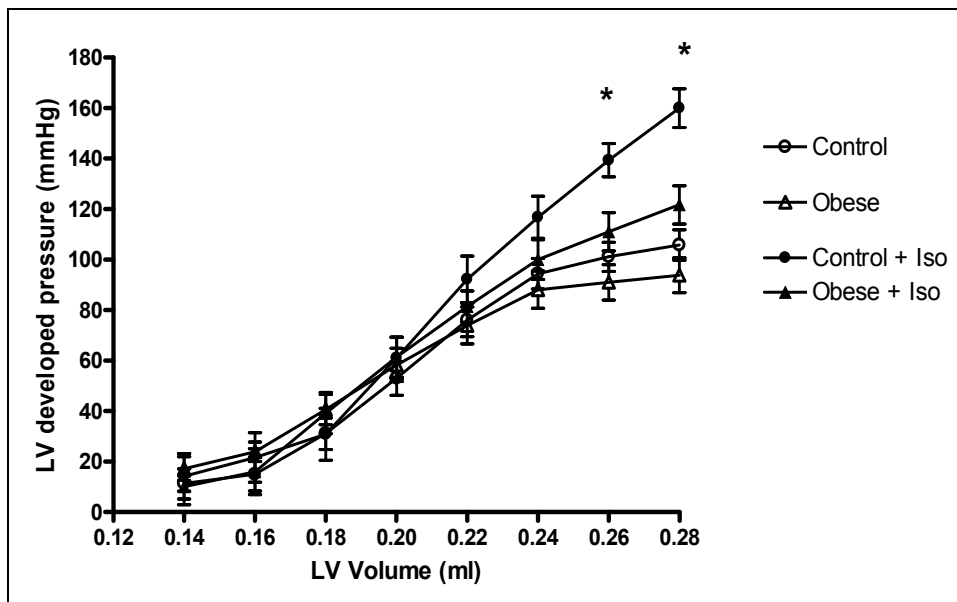
Figure 4.3b: The impact of β-adrenergic receptor stimulation on LV systolic chamber function assessed at different LV preloads in retrogradely perfused hearts isolated from control and obese animals. *Iso* – isoproterenol.

*p<0.05 control vs. control + Iso

n = 8 hearts per group
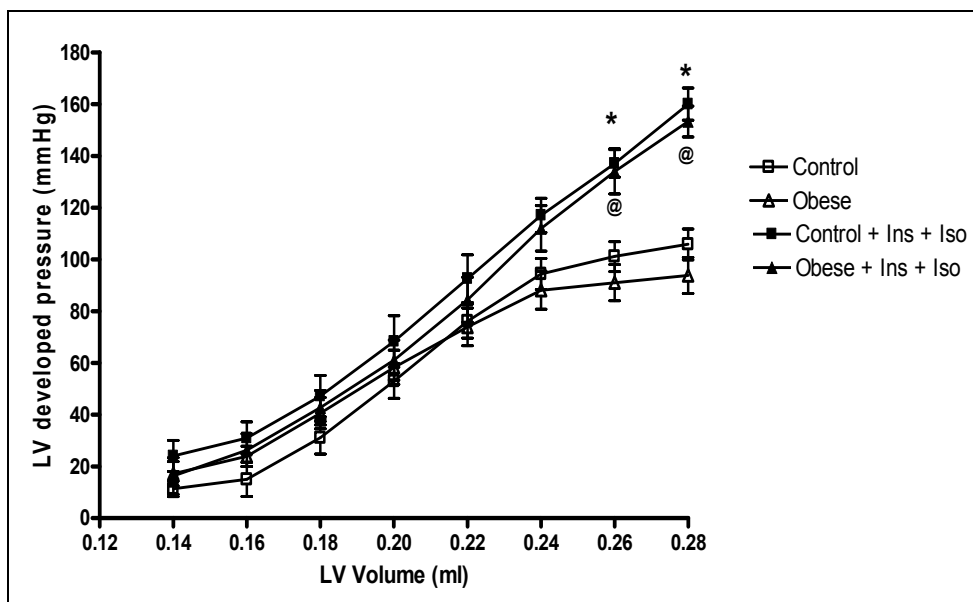
Figure 4.3c: The impact of β-adrenergic receptor stimulation and simulated *in vivo* concentrations of insulin (Control: 30µIU/ml; Obese: 50µIU/ml) on LV systolic chamber function assessed at different LV preloads in retrogradely perfused hearts isolated from control and obese animals. *Ins* – insulin; *Iso* – isoproterenol.

*p<0.05 control vs. control + Ins +Iso

@p<0.05 obese vs. obese + Ins + Iso

n = 8 hearts per group
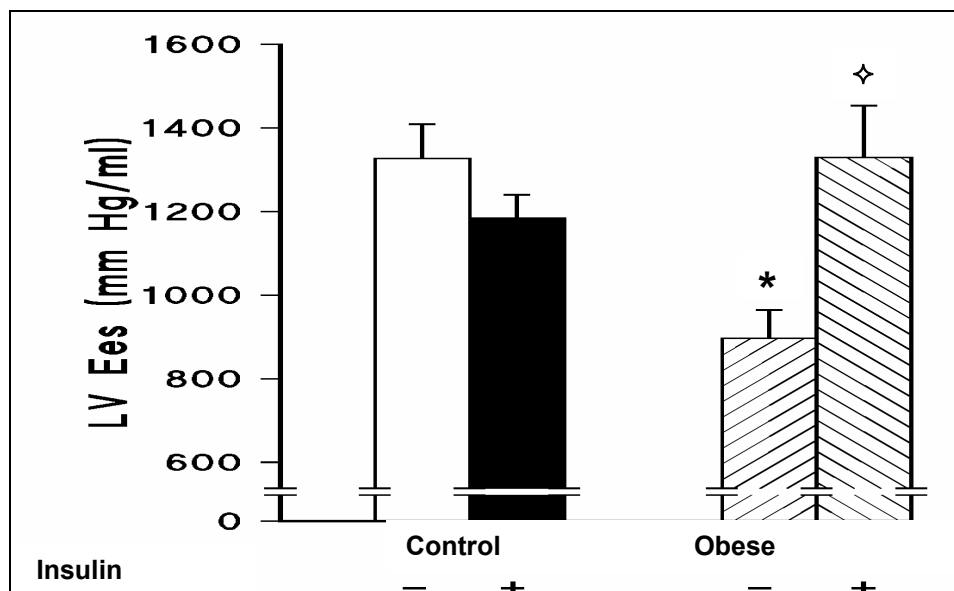
Figure 4.3d: The impact of β-adrenergic receptor stimulation and simulated *in vivo* concentrations of insulin (Control: 30μIU/ml; Obese: 50μIU/ml) on the slope of the pressure volume relations (LV Ees) generated in Figure 4.3c in hearts isolated from control and obese rats.

*p<0.05 vs. Control; ⬧p<0.05 vs. Obese – Insulin

n = 8 hearts per group

Isolated retrograde perfused hearts from control and obese animals generated comparable pressure volume curves indicative of normal LV systolic chamber function in these groups. This finding was comparable to that observed *in vivo* (Figure 4.3a (resting conditions)). Significantly, perfusing hearts with their respective *in vivo* concentrations of insulin did not alter the pressure volume curves generated indicating that insulin did not elicit a positive inotropic response in retrogradely perfused hearts (Figure 4.3a). Interestingly, when the myocardial metabolic demand was increased through the addition of isoproterenol (β-adrenergic receptor stimulation) to the perfusate, hearts from the control group were able to significantly increase their LV systolic chamber function at the higher filling volumes (p<0.05) compared to untreated controls hearts perfused in the absence of isoproterenol. These findings mimicked what was observed *in vivo* following the isoproterenol

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injection. In strong contrast to the control group, in the presence of isoproterenol, isolated hearts from obese animals were unable to significantly increase their LV developed pressures at the higher LV filling volumes beyond that generated in the absence of isoproterenol (Figure 4.3b). However, when the respective *in vivo* concentrations of insulin were present in the perfusion buffer together with isoproterenol, hearts from the obese group were able to generate significantly greater (yet similar to controls) LV developed pressure at the higher LV filling volumes compared to hearts from obese animals perfused in the absence of isoproterenol and insulin (Figure 4.3c). This is evident when the slope of the pressure volume relations (LV Ees) generated in figure 4.3c was assessed (Figure 4.3d). The current finding alludes to an important contributory role of elevated *in vivo* concentrations of insulin in the maintenance of normal myocardial LV functional reserve in obese animals.

4.3.3.2.2 Isolated working heart perfusions

The effects of 10mM glucose and different combinations of simulated *in vivo* concentrations of insulin and FA on LV mechanical function.
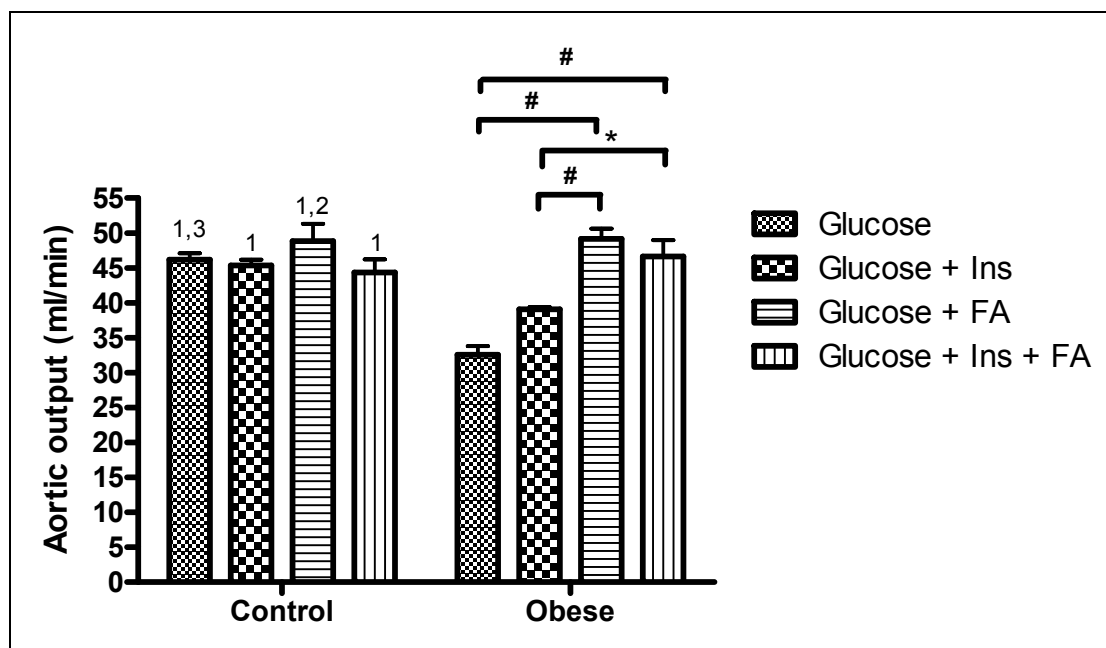
Figure 4.4: The impact of obesity and simulated *in vivo* concentrations of insulin (Control: 30µlU/ml; Obese: 50µlU/ml) and FA (Control: 0.7mM; Obese: 1.5mM) on LV mechanical function determined in isolated working heart perfusions with a 15cm H_2O preload. *Ins* – insulin; *FA* – fatty acid
*p<0.05; #p<0.001
[1]p<0.001 vs. obese glucose
[2]p<0.01 vs. obese glucose + Ins; [3]p<0.05 vs. obese glucose + Ins
n = 6-8 hearts per group
(Figure 4.4 was analyzed using a repeated measures two-way ANOVA where data from control and obese animals at all the preloads were included)

Simulated *in vivo* concentrations of insulin, FA or the combination thereof, when present in the perfusate, had no effect on the aortic output generated by the control group in the presence of glucose alone (p<0.05) (Figure 4.4). This indicates effective myocardial flexibility in substrate use by the control group in maintaining optimal LV mechanical function. In strong contrast, glucose perfused hearts from obese animals

achieved significantly poorer aortic outputs compared to the control group (32.58±1.2 vs. 46.17±0.91 ml/min; p<0.001) under comparable perfusion conditions. Although the addition of various factors in the perfusate had no functional impact on hearts from the control animals, hearts from obese animals reacted more favourably to simulated *in vivo* concentrations of insulin, FA or the combination thereof.

Hearts isolated from the obese group were unable to significantly augment their aortic output in response to *in vivo* concentrations of insulin compared to obese hearts perfused with glucose alone (p>0.05). Of great interest was that the addition of FA (p<0.001) or FA and insulin (p<0.01) to the perfusion buffer enabled hearts in the obese group to significantly increase their aortic output above that achieved by obese rat hearts perfused in the presence of glucose alone or glucose and insulin. More importantly, only in the presence of FA or FA and insulin were hearts from the obese group able to achieve similar aortic outputs compared the control groups at all the different perfusion conditions assessed. Insulin produced no additive effect on LV mechanical function in the FA perfused obese group indicating that the presence of simulated *in vivo* concentrations of FA in combination with 10mM glucose, were by themselves sufficient to increase LV mechanical function maximally at the normal preload. These data demonstrate myocardial dependence (in the obese animal) on simulated *in vivo* concentrations of FA to maintain normal LV mechanical function during control perfusion conditions.

The LV functional response to increments in preload when perfused with 10mM glucose and different combinations of simulated *in vivo* concentrations of insulin and FA.
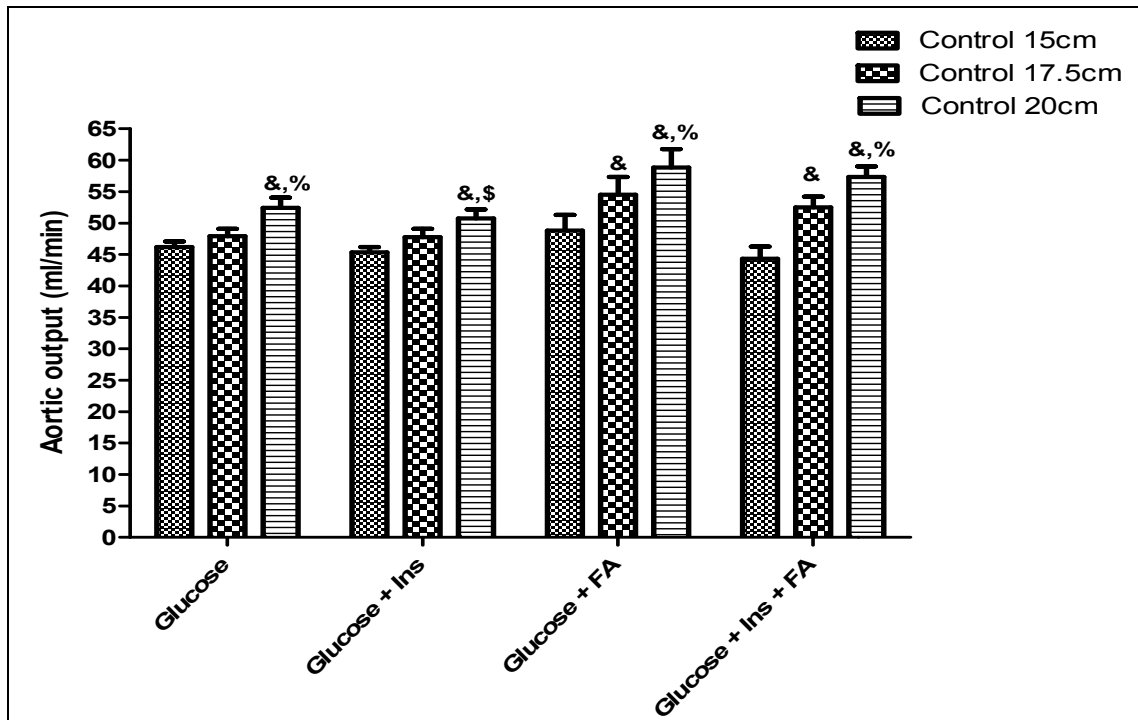
Figure 4.5a: The aortic output generated by isolated working hearts from control animals following incremental increases in preload when perfused with different combinations of 10mM glucose and simulated *in vivo* concentrations of insulin (30µIU/ml) and FA (0.7mM). *Ins* – insulin; *FA* – fatty acid.
[&]p<0.001 vs. 15cm with particular substrate; [%]p<0.001 vs. 17.5cm with particular substrate; [$]p<0.05 vs. 17.5cm with particular substrate.
n = 6 hearts per group
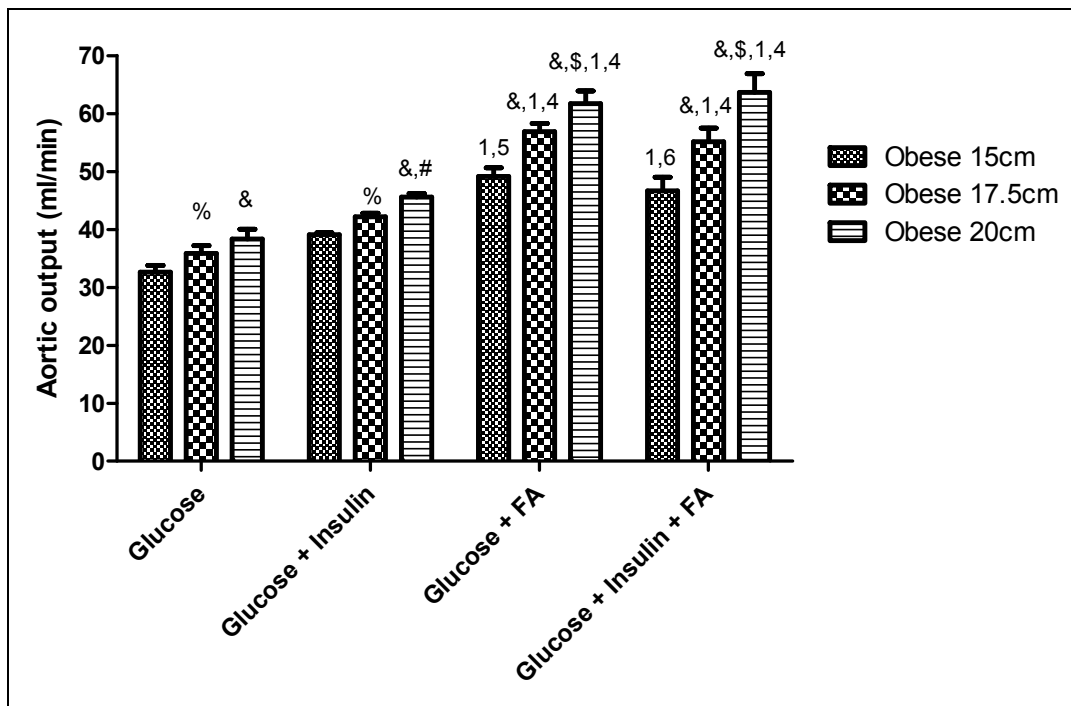
Figure 4.5b: The aortic output generated by isolated working hearts from obese animals following incremental increases in preload when perfused with 10mM glucose and different combinations of simulated *in vivo* concentrations of insulin (50μIU/ml), and FA (1.5mM). *Ins* – insulin; *FA* – fatty acid.
[&]p<0.001 vs. 15cm; [%]p<0.05 vs. 15cm; [$]p<0.001 vs. 17.5cm; [#]p<0.01 vs. 17.5cm within the particular substrate group.
[1]p<0.001 vs. glucose at the particular preload; [2]p<0.01 vs. glucose at the particular preload; [3]p<0.05 vs. glucose at the particular preload.
[4]p<0.001 vs. glucose + insulin at the particular preload; [5]p<0.01 vs. glucose + insulin at the particular preload; [6]p<0.05 vs. glucose + insulin at the particular preload.
n = 6-8 hearts per group.

Working hearts from both control and obese animals were able to significantly augment aortic output in response to incremental increases in preload with all combinations of glucose and simulated *in vivo* concentrations of insulin and FA (Figure 4.5a, b). Hearts from the control animals were able to achieve peak aortic outputs (20cm preload) in the presence of all perfusion conditions.

In contrast to that seen in the control group, hearts from obese animals were only able to achieve peak aortic output at the 20cm preload when FA's were included in the perfusate (in a separately conducted repeated measures two-way ANOVA, only the FA perfused obese groups had comparable function to the FA perfused control groups). This was further reflected by the significant differences in aortic output achieved at the 20cm preload between hearts from obese animals perfused in the presence of glucose + FA/FA +Ins compared with those hearts perfused with glucose or glucose + insulin.

Technical note

For the isolated working heart experiments conducted in this study, hearts in the glucose and glucose + insulin groups were paced at 280bpm. Due to an unforeseen problem with the pacer, subsequent perfusions in the presence of glucose + FA or glucose + FA + insulin were performed without pacing. The average heart rates during the 3 different preloads used for these groups are provided below (Table 4.4). According to the Guide for the care and use of experimental animals released by the Canadian Council on Animal Care (Olfert *et al*. 1993), the heart rate of a rat ranges between 250-450 beats per minute. In our laboratory, using the perfused working heart system, heart rates varied between 220 and 300bpm.

Table 4.4: Average heart rate (bpm) achieved during the functional experiments in un-paced hearts from control and obese animals in the presence 10mM glucose and different combinations of simulated *in vivo* concentrations of insulin and FA. Heart rates were averaged over the three different workloads

Control		Obese	
Glucose + FA	Glucose + FA + insulin	Glucose + FA	Glucose + FA + insulin
282.78±11.13	269.39±6.54	249.44±13.35	241.58±11.31

n = 6-8 hearts per group

As functional differences between the groups were assessed with a 2-Way Analysis of variance, we performed similar statistical analysis on the heart rate data. The heart rate of the groups in Table 4.4 did not differ statistically from one another although diet played a role in predicting lower heart rates (p=0.01). Despite this, we do not believe that changes in heart rate would contribute significantly to the aortic outputs observed as the working heart compensates for changes in heart rate by adjusting its stroke volume.

4.4 Summary of the findings

LV mechanical function assessed echocardiographically was similar between control and obese rats at baseline and following β-adrenergic receptor stimulation indicating the absence of myocardial dysfunction in the obese group *in vivo* after long term (32 weeks) of HCD feeding. The normal *in vivo* LV mechanical function and functional reserve observed in these obese animals occurred in the presence of elevated circulating levels of insulin and FFA (FFA levels are already elevated at 16 weeks (Table 5.2)). Hearts from the control animals were able to maintain normal LV mechanical function in the presence of various combinations of glucose, and simulated *in vivo* concentrations of insulin and FA in the perfusate. In contrast, *ex vivo* assessment of LV mechanical function in the obese group revealed compromised LV function when perfused with glucose alone. While addition of insulin was able to augment myocardial function in the obese group, under conditions of greater myocardial workload only the presence of simulated *in vivo* concentrations of FA were able to normalize baseline function and functional reserve in hearts from these animals. This data suggests that in the obese insulin resistant pre-diabetic state, the heart may rely more heavily on an elevated FFA levels as seen *in vivo,* to maintain effective LV mechanical function particularly when workload is increased.

CHAPTER 5

METHODS AND RESULTS FOR STUDY 2

THE IMPACT OF INSULIN ON GLYCOLYLTIC FLUX RATES AND INDICES OF

ISCHAEMIA/REPERFUSION INJURY IN EX VIVO PERFUSED HEARTS

5.1 Aim of the study

The aim of this sub-study was to determine the responsiveness of hearts isolated from control and obese insulin resistant animals to insulin administration and the impact thereof on the myocardial susceptibility to ischaemia/reperfusion injury.

5.2 Methods

5.2.1 Study design

Age matched rats weighing 190±10g were randomly fed either SRC or a HCD for 16 weeks. After 16 weeks the rats were weighed and used accordingly. To determine the extent of insulin's metabolic impact on obese and control rat hearts, isolated Langendorff perfused hearts were perfused with D-[5- ^{3}H (N)]- glucose together with different combinations of glucose and insulin to determine the rate of myocardial glycolytic flux. The impact of obesity on myocardial susceptibility to ischaemia/reperfusion induced injury was assessed in isolated glucose perfused working hearts as working heart functional parameters could be monitored in addition to performing a standard myocardial infarction induction protocol. To determine whether insulin could afford cardioprotection in a model of obesity, a separate series of glucose perfused hearts received insulin supplementation prior to and during regional ischaemia. In yet another series of experiments, the glucose and insulin

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perfusate was supplemented with FA to determine whether insulin's post-ischaemic outcomes could be attenuated by the use of FA as a substrate. To assess changes in the blood profile induced by the HCD following 16 weeks of feeding, blood was collected from fasted rats (duration of fast not documented) for glucose, insulin, HbA1c, Trig, total cholesterol, HDL-C and FFA analyses. To assess the impact of obesity on cardiac morphology echocardiography analysis was employed. Lastly, retroperitoneal and gonadal fat was removed from animals to determine the impact of the HCD on retroperitoneal and gonadal fat mass.

5.2.2 Determination of myocardial glycolytic flux rate

Hearts were mounted on the Langendorff perfusion apparatus to determine the effect of obesity and insulin (30 or 50µIU/ml) on myocardial glycolytic flux rates. After a 10 minute stabilization period, hearts were perfused with D-[5-[^{3}H(N)]-glucose (Amersham) in the presence of insulin, and the myocardial glycolytic flux rate was determined by measuring the amount of ^{3}H$_2$O released into the coronary effluent following the metabolism of the D-[5-[^{3}H(N)]-glucose. The specific buffer (100ml) containing D-[5-[^{3}H(N)]-glucose was re-circulated. Coronary effluent samples were collected every two minutes over an eight minute period.

To separate the ^{3}H$_2$O from the 5-[^{3}H] glucose in the coronary effluent, collected samples were passed through ion exchange resin columns (200-400 mesh Dowex 1x4 (Sigma-Aldrich, St.Louis, MO)) pre-treated with Na$_2$B$_4$O$_7$. Samples (0.1ml) were loaded onto the column and eluted with 0.8ml H$_2$O into scintillation vials for determination of radioactivity using a Beckman liquid scintillation counter (Beckman Instruments, USA) (for a more complete description of the methodology see section

6.2.4). The glycolytic flux rates (µmol/g ww/min) determined during the eight minute perfusion period was averaged to obtain an average glycolytic flux rate.

5.2.3 Isolated working rat heart perfusions

Perfusion Protocol

Isolated hearts were perfused with a standard glucose (10mM) containing Krebs-Henseleit perfusion buffer as described previously (Section 3.1.3). Figure 5.1 demonstrates the standard perfusion protocol used to simulate an acute myocardial infarction which aimed to assess ischaemia/reperfusion injury following a regional ischaemic episode. Briefly, hearts were retrogradely perfused in Langendorff mode for an initial 10 minute stabilization period after which the perfusion mode was changed to working heart mode for 20 minutes. During working heart mode, hearts would work at a preload of 15cm H_2O against an afterload of 100cm H_2O. At the end of the 20 minute working heart period functional measurements were taken. The perfusion was then switched to the Langendorff mode for 10 minutes prior to the induction of regional ischaemia. The total stabilization time prior to ischaemia was therefore 40 minutes. To induce an infarct, myocardial regional ischaemia was applied by ligating the left anterior descending (LAD) coronary artery, approximately 3mm from the coronary sinus, with a Ethicon silk suture (black braided silk non-absorbable suture, 3/0, 26mm ½c Taper, Johnson&Johnson Medical (PTY) LTD, South Africa). Occlusion of the LAD coronary artery with the suture was maintained for a period of 40min. After 40 minutes, the suture was released in order to reperfuse the ischaemic myocardium. The reperfusion phase consisted of a 10 minute retrograde perfusion followed by a 20 minute working heart perfusion after which functional parameters were again measured. At the end of the perfusion protocol the LAD coronary artery was re-occluded by tying the suture (i.e. 30 minutes of

reperfusion). The heart was stained with 0.6ml Evans blue (0.5%) injected through the aortic cannula, which enabled the visualization of the viable myocardium (now stained blue) from the area at risk and the necrotic area. Stained hearts were placed in a sealed plastic envelope and stored at -20°C for further analysis. The determination of myocardial infarct size was performed on stored hearts within a week of experimentation. While many laboratories utilize 2 hours of reperfusion for determining myocardial infarct size, in our laboratory, both 30 minutes and 2 hours reperfusion have yielded similar infarct sizes in control animals (Fan *et al*. 2009). We therefore used a 30 minute reperfusion period.

To determine whether insulin would afford cardioprotection, the perfusion solution of glucose perfused hearts was changed to a glucose and insulin supplemented Krebs-Henseleit perfusion buffer (30µIU/m or 50µIU/ml) 10 minutes prior to the onset of regional ischaemia. Insulin perfusion continued for the duration of the ischaemic episode and was not present during the reperfusion period (See Figure 5.1). When assessing the impact of FA's on insulin's post-ischaemic outcomes, hearts were perfused with a glucose, insulin and FA supplemented Krebs-Henseleit perfusion buffer (Control: 30µIU/ml insulin and 0.7mM FA; Obese: 50µIU/ml insulin and 1.5mM FA) (See details for the FA perfusion buffer in section 3.1.3) for the same duration as described for insulin administration. At the end of the ischaemic period, the suture was loosened and within 5 seconds, the perfusion solution was changed to the standard glucose containing Krebs-Henseleit perfusion buffer for the duration of the reperfusion period. Although we used a model of coronary occlusion, a small volume (up to 6% of pre-ischaemic coronary flow rate) of the perfusion buffer could be expected to reach the occluded zone via collateral flow (Maxwell *et al*. 1987).

During the period of insulin and FA perfusion, a 200ml glucose, insulin and FA supplemented Krebs-Henseleit buffer was re-circulated in the heart perfusion system.
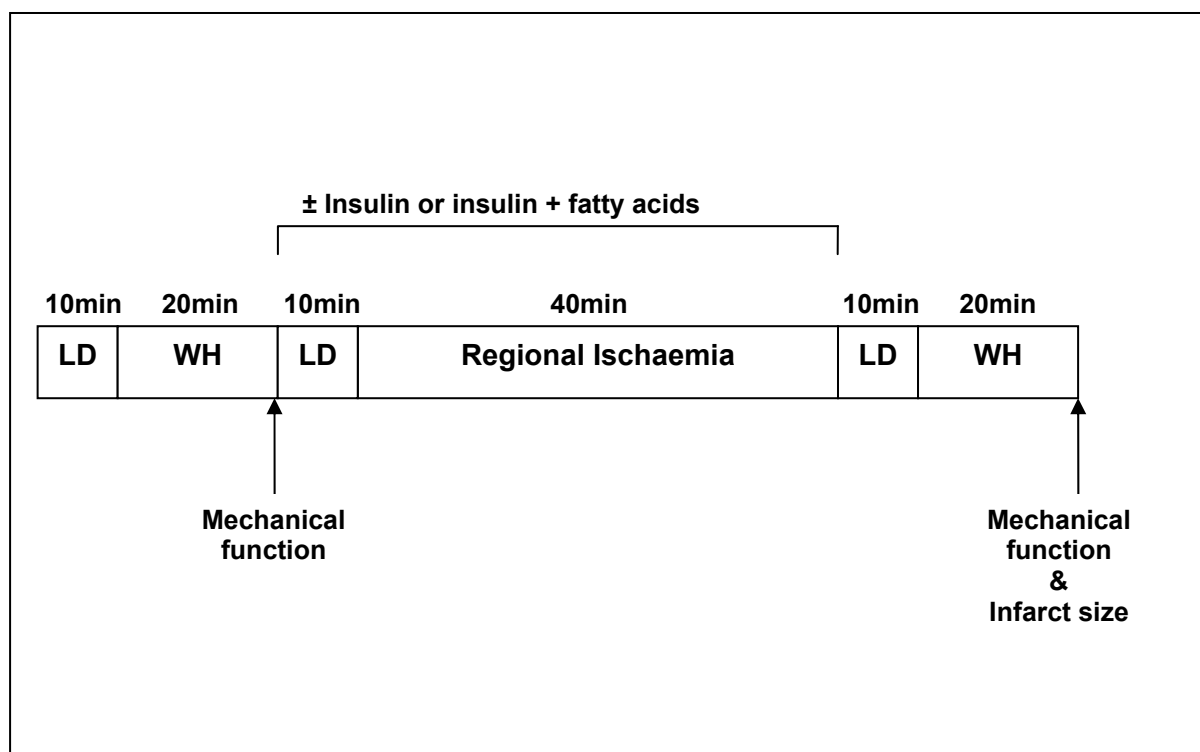
Figure 5.1: Diagrammatic representation of the perfusion protocol followed to determine myocardial infarct size and recovery of function following a period of coronary artery ligation in hearts from control and obese animals. *LD*, **Langendorff perfusion;** *WH,* **working heart perfusion.**

5.2.4 Indices of myocardial ischaemia/reperfusion injury

5.2.4.1 Myocardial infarct size

Frozen hearts were cut into 6-7 slices, 2-3mm thick. The slices were stained with a 1% 2,3,5 triphenyltetrazolium chloride phosphate buffer (20mM $NaH_2PO_4.2H_2O$ and 80mM Na_2HPO_4) (pH7.4) for 20min at 37ºC. Slices were then left to stand in 34% formaldehyde for two hours. The heart slices were blindly traced onto a transparency paper. Afterwards, an unbiased researcher not involved with the study blindly assessed the 3 identified areas on the traces. The transparency was scanned into a

computer and the area of the viable tissue, area at risk and infarct size was delineated and quantified using computerized planimetry (UTHCSA Image Tool program, University of Texas Health Science Center at San Antonio, TX, USA). The ventricular area at risk did not differ between experimental groups. Infarct size was expressed as a percentage of the area at risk to account for differences in heart size between the various groups.

5.2.4.2 Myocardial functional recovery

To monitor myocardial mechanical function, aortic output was documented during the working heart periods before and after regional ischaemia. Myocardial functional recovery, an indirect index of ischaemic injury, was then calculated as a percentage as follows:

$$\text{Myocardial functional recovery} = \frac{\text{Pre-ischaemic AO}}{\text{Post-ischaemic AO}} \times 100$$

It is important to mention that while both infarct size and functional recovery are well recognized indices used to assess ischaemia/reperfusion injury, functional recovery is not regarded as an accurate indicator of ischaemic damage in a model of regional ischaemia, due to concomitant stunning. In our laboratory larger infarct sizes do not always translate in poorer functional recoveries following regional ischaemia. Greater significance will therefore be given to infarct size than to functional recovery in determining the extent of post-ischaemic damage. Indeed myocardial infarct size is regarded to be a better predictor of future clinical outcomes than post-ischaemic LV functional performance (Wu *et al*. 2008).

5.2.5 Biometric measurements

A description of the methodology used to quantify retroperitoneal and gonadal fat content has been reported in section 4.2.4.1.

5.2.6 Biochemical analysis

Blood glucose (4.2.5.2.1), HbA1c (non-fasting) (4.2.5.2.2), Trigs (4.2.5.2.3), total cholesterol (4.2.5.2.3), HDL-C (4.2.5.2.3) and serum insulin (4.2.5.3.1) levels were determined from fasted animals as previously described. In this series of experiments, glucose levels were determined from blood collected from the tail vein. The fasting glucose and serum insulin levels presented in this section were taken from the MSc of Mr. W Smith to save on the cost of additional biochemical determinations. These measurements were performed at a similar time to the infarct size experiments presented in this sub-study.

5.2.6.1 Serum determinations

5.2.6.1.1 Non-esterified free fatty acids

FFA levels were determined in stored serum samples (-80 ºC), collected from fasting animals, by means of a Free Fatty Acid Half-micro Test (Roche Diagnostics, Penzberg, Germany).

The test follows an enzymatic colorimetric approach and relies on the addition of enzymes to the FFA containing sample yielding a particular breakdown product. A catalyst (peroxidase) is added which reacts with the breakdown product and a colour reaction is visible which can be measured at 546nm. The concentration of the FFA is subsequently determined using a standard curve plotting the absorbancies of known amounts of FFA.

5.2.6.1.2 Determination of systemic insulin sensitivity

Systemic insulin sensitivity was determined by the homeostasis model assessment (HOMA-IR) index. The HOMA-IR index was determined from the product of blood glucose (mM) and the serum insulin (µIU/ml) concentrations determined in the fasting state. This value was then divided by 22.5.

5.2.7 Statistical analysis

The data were presented as described in section 4.2.6. Comparisons between 2 groups were made with an unpaired Student's t-test. When different values within a particular group were compared a one-way ANOVA was used followed by the Bonferroni post-test. Multiple comparisons between various groups were made by using a two-way ANOVA followed by the Bonferroni post-test.

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RESULTS: STUDY 2

5.3 Results

5.3.1 Biometric Data

Table 5.1: Biometric data of control and obese animals

	Control	Obese
Body Weight (g)	463.90±8.38 (n=28)	537.30± 9.91 $^{\$}$ (n=30)
Retroperitoneal and gonadal fat weights (g)	24.21± 2.31 (n=10)	40.70± 2.64 $^{\#}$ (n=12)

$^{\$}$**p<0.001; $^{\#}$p=0.002 vs. control**

Compared to the SRC fed rats those that received the HCD for 16 weeks developed obesity characterised by both general (p<0.001) and visceral adiposity (p=0.002) (Table 5.1).

Although not shown along with the biometric data, 16 week HCD fed rats had greater heart weights and larger myocyte size compared to the control group. This data was previously reported in the MSc dissertation of Mr W Smith (2005).

5.3.2 Metabolic data

Table 5.2: Metabolic data of control and obese animals that were fasted overnight

	Control	Obese
Glucose (mmol/l)	4.82±0.18 (6)	5.28±0.15 (6)
Insulin (µlU/ml)	31.40±2.80 (11)	49.50±6.20 * (11)
HOMA-IR	6.20±0.70 (6)	12.50±2.00 * (6)
HbA1c (%) (non fasting)	3.97±0.18 (13)	4.36±0.13 (16)
Trig (mmol/l)	0.72±0.07 (13)	1.91±0.18 * (13)
Total cholesterol (mmol/l)	1.43±0.05 (13)	1.29±0.06 (13)
HDL-C (mmol/l)	0.87±0.03 (13)	0.56±0.03 * (13)
FFA (mmol/l)	0.79±0.10 (8)	1.70±0.31 [#] (8)

FFA – free fatty acids; HOMA-IR – homeostasis model assessment for insulin resistance; HbA1c – glycosylated haemoglobin; HDL-C – high density lipoprotein cholesterol. The group size is indicated by the number is brackets. [#]**p<0.02; *p<0.05 vs. control**

Obese rats had similar glucose levels compared to the control rats, however their insulin levels were significantly elevated (p<0.05) and consequently the degree of systemic insulin sensitivity was reduced (HOMA-IR index; p<0.05) (Table 5.2). Obese rats were however not diabetic as reflected by the normal HbA1c values. In comparison to control rats, obese rats additionally displayed an unfavourable blood lipid profile evident by reduced HDL-C (p<0.05) and elevated Trig (p<0.05) and FFA (p<0.02) levels.

5.3.3 Myocardial glycolytic flux rates
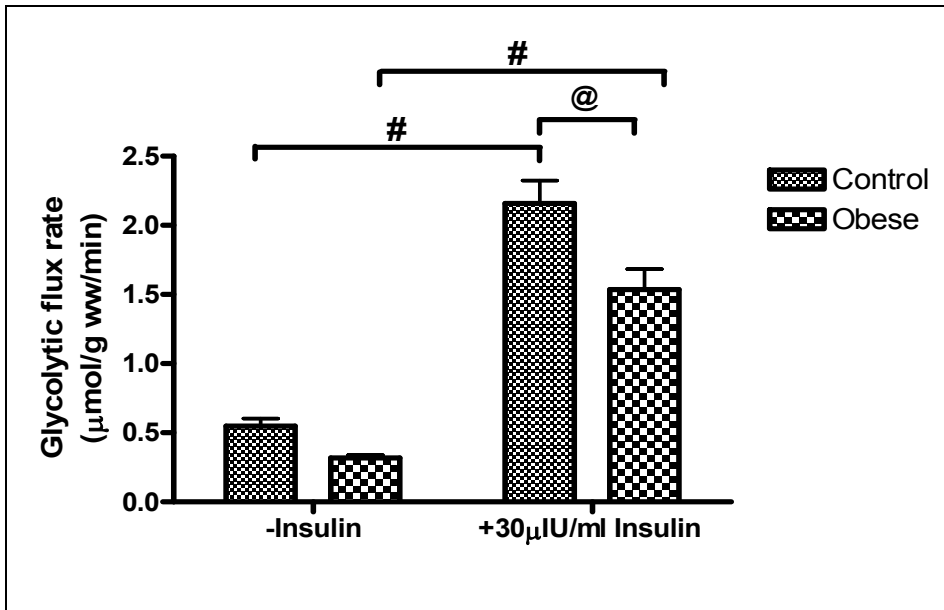
Figure 5.2a: Average myocardial glycolytic flux rates obtained from Langendorff perfused rat hearts from control and obese rats during normoxic conditions. Hearts were perfused in the presence or absence of 30µIU/ml insulin.

[@]p<0.01; [#]p<0.001

n = 6-12 hearts per group
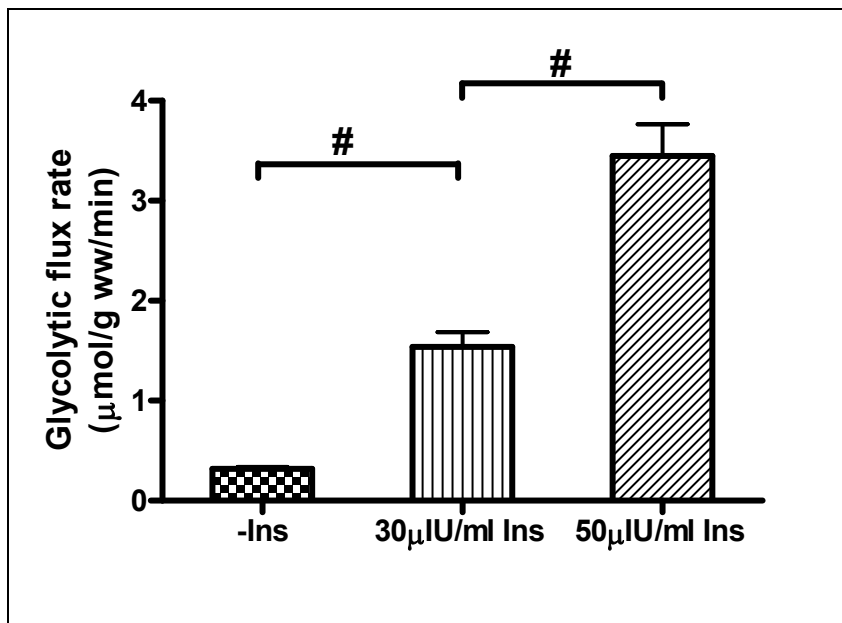
Figure 5.2b: Average myocardial glycolytic flux rates obtained from isolated Langendorff perfused obese rat hearts during normoxic conditions. Hearts were perfused in the presence or absence of insulin (30 or 50µIU/ml).
#p<0.001
n = 6-8 hearts per group

In the absence of insulin, myocardial glycolytic flux rates did not differ between the control and obese groups. In response to insulin present in the perfusate, hearts isolated from both the control and obese animals, were able to significantly increase their glycolytic flux rates (Control: 2.16±0.57 vs. 0.55±0.14 µmol/g ww/min, p<0.001; Obese: 1.54±0.42 vs. 0.32±0.05 µmol/g ww/min, p<0.001) (Figure 5.2a). Despite this increased response in glycolytic flux following insulin stimulation (30µIU/ml), hearts from obese animals had lower rates of glycolysis compared to the control group (1.54±0.42 vs. 2.16±0.57 µmol/g ww/min, p<0.01). Hearts from obese animals were however able to incrementally increase their glycolytic flux rate in response to different insulin concentrations (Figure 5.2b). This may suggest a mild form of insulin resistance in these hearts.

5.3.4 The impact of obesity and insulin treatment prior to and during ischaemia on myocardial infarct size and functional recovery
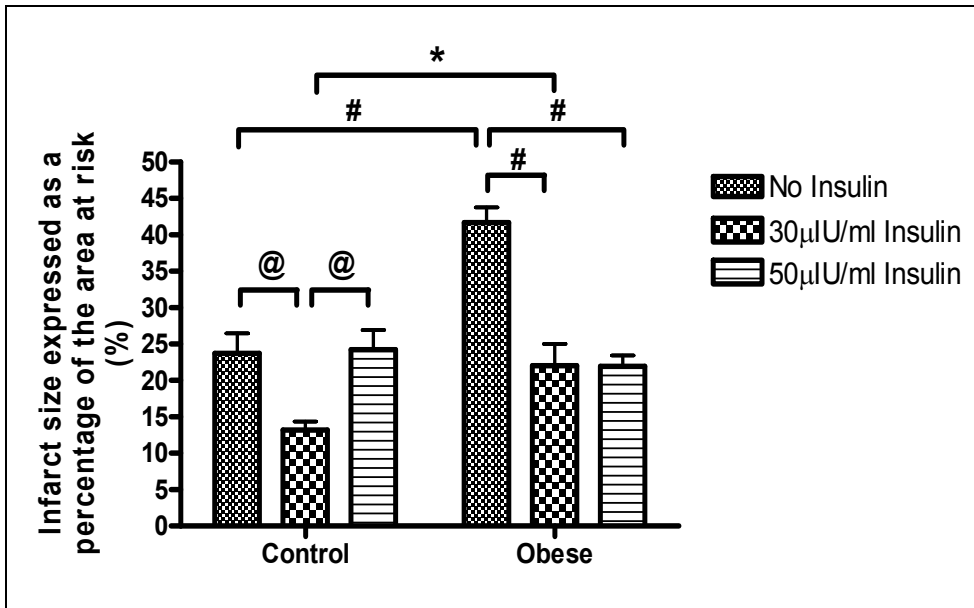
Figure 5.3a: Myocardial infarct size expressed as a percentage of the area at risk obtained in isolated rat hearts from control and obese animals perfused in the presence or absence of insulin (30 or 50µIU/ml). Hearts were subjected to 40 minutes regional ischaemia followed by 30 minutes reperfusion. Insulin administration, when present, occurred 10 minutes prior to the onset of regional ischaemia and ended upon the initiation of reperfusion.
[#]p<0.001;[@]p<0.01; *p<0.05
n = 6-8 hearts per group
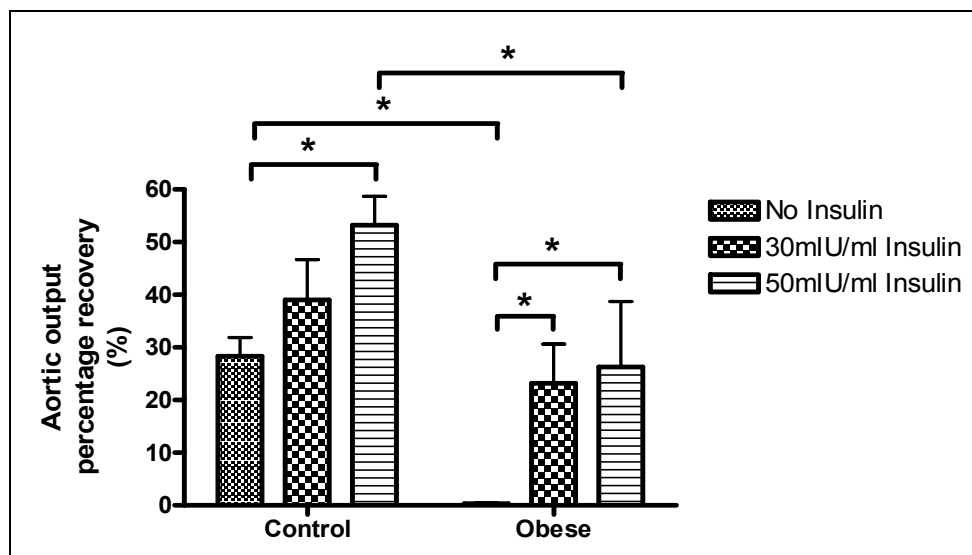
Figure 5.3b: The percentage aortic output recovery obtained from isolated rat hearts from control and obese animals perfused in the presence or absence of insulin (30µIU/ml or 50µIU/ml). Hearts were subjected to 40 minutes regional ischaemia followed by 30 minutes reperfusion. When insulin was present, insulin administration occurred 10 minutes prior to the onset of regional ischaemia and ended upon the initiation of reperfusion.

*p<0.05

n = 6-8 hearts per group

Glucose perfused hearts from obese animals were more susceptible to ischaemia/reperfusion induced injury. This was reflected by the larger infarct sizes (41.67±2.09 vs. 22.74±2.72 %; p<0.001) and poorer post-ischaemic functional recoveries (0.00±0.00 vs. 28.30±3.56 %, p<0.05) in comparison to the control group (Figure 5.3a). Insulin, when present in the perfusate, had a cardioprotective effect by attenuating infarct size in all cases (Control with 30µIU/ml insulin: 13.85±1.23 vs. 22.74±2.72 %, p<0.01; Obese with 30µIU/ml insulin: 21.75±2.64 vs. 41.67±2.09 %, p<0.001; Obese with 50µIU/ml insulin: 20.94±1.60 vs. 41.67±2.09 %, p<0.001) except for the control 50µIU/ml insulin group. Insulin, irrespective of the concentration used, further improved myocardial functional recovery in the obese groups (26.26±12.48 and 23.16±7.49 vs. 0.00±0.00 %, p<0.05), while in the control group,

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only the 50µIU/ml concentration of insulin was able to improve LV functional recoveries (53.24±5.45 vs. 28.30±3.56 %, p<0.05) (Figure 5.3b). A dissociation between infarct size (no change) and functional recovery (improved recovery) was observed in the control + 50µIU/ml insulin which is in agreement with previous findings from our laboratory that indicate that functional recovery does not always follow the same pattern as infarct size.

5.3.5 The impact of obesity and insulin or insulin and FA, administered prior to and during ischaemia, on myocardial infarct size and functional recovery
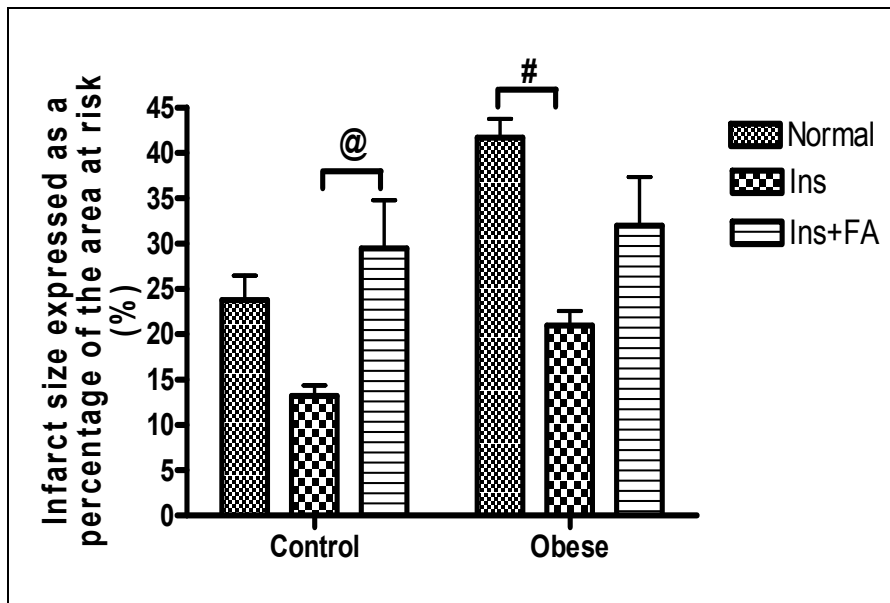
Figure 5.4: Myocardial infarct size expressed as a percentage of the area at risk obtained from isolated hearts perfused in the presence or absence of simulated *in vivo* concentrations of insulin (Control: 30µIU/ml; Obese: 50µIU/ml) or insulin+FA (Control: 30µIU/ml insulin + 0.7mM FA; Obese: 50µIU /ml insulin + 1.5mM FA). Hearts were subjected to 40 minutes regional ischaemia followed by 30 minutes reperfusion. When insulin or insulin and FA were present in the perfusate, administration occurred 10 minutes prior to and during regional ischaemia and ended upon the initiation of reperfusion.
[#]p<0.001;[@]p<0.01;
n = 6-8 hearts per group

To determine whether insulin's post-ischaemic effects would be abolished by FA's, myocardial ischaemic tolerance was investigated in the presence of glucose and simulated *in vivo* concentrations of insulin and FA (Figure 5.4). In the control groups, the reduction in infarct size afforded by 30µIU/ml insulin was lost when the FA perfused hearts were included in the statistical model. Despite this, the presence of FA abolished insulin's infarct lowering effect as the insulin+FA group had significantly

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larger infarct sizes compared to the insulin treated group (29.45±5.33 vs. 13.17±1.17 %, p<0.01). Nevertheless, infarct sizes between the glucose perfused hearts and the insulin + FA perfused hearts were similar.

In the obese group, insulin's infarct sparing effect remained when compared to the glucose perfused obese hearts (20.94±1.60 vs. 41.67±2.09 %, p<0.001). Myocardial infarct sizes did not differ between the insulin and the insulin + FA group. The data suggest that in the obese groups, the protection afforded by insulin was attenuated by the combination of insulin + FA in the perfusate as infarct sizes observed between the glucose perfused and the glucose, insulin and FA perfused hearts were similar.
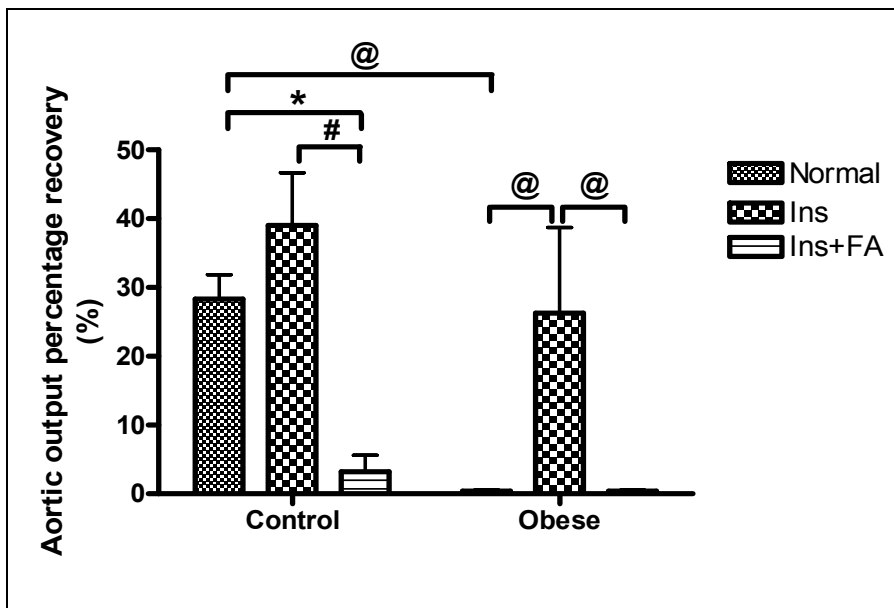
Figure 5.5: The percentage aortic output recovery obtained from isolated rat hearts perfused in the absence or presence of simulated *in vivo* concentrations of insulin (Control: 30µIU/ml; Obese: 50µIU/ml) or insulin+FA (Control: 30µIU/ml insulin + 0.7mM FA; Obese: 50µIU/ml insulin + 1.5mM FA). Hearts were subjected to 40 minutes regional ischaemia followed by 30 minutes reperfusion. In the presence of insulin, administration occurred 10 minutes prior to and during regional ischaemia.
[#]p<0.001; [@]p<0.01; *p<0.05
n = 6-8 hearts per group

As previously shown, simulated *in vivo* concentrations of insulin (30µIU/ml) did not improve myocardial functional recovery in the control group. However both the normal control and the control + insulin group had significantly greater functional recoveries compared to the control + insulin + FA group (28.30±3.56 and 39.01±7.68 vs. 3.22±2.40 %, p<0.05 and p<0.001 respectively) (Figure 5.5).

In the obese groups, hearts perfused with simulated *in vivo* concentrations of insulin had significantly improved post-ischaemic functional recoveries compared to the hearts perfused in the absence of insulin (26.26±12.48 vs. 0.00±0.00 %, p<0.01) (Figure 5.4) and those perfused with FA in combination with insulin and glucose

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(26.26±12.48 vs. 0.00±0.00 %, p<0.01). Glucose perfused hearts had similar functional recoveries compared to hearts perfused with glucose, insulin and FA present in the perfusate prior to and during regional ischaemia.

5.4 Summary of the findings

Hearts from obese animals responded favourably to insulin administration in a dose dependent manner as reflected by significant changes in glycolytic flux rates during normoxic perfusion. Nevertheless, compared to the control group, hearts from obese animals achieved lower rates of glycolysis when perfused with comparable insulin concentrations indicating that these hearts were insulin resistant.

Glucose perfused obese rat hearts were more susceptible to ischaemia/reperfusion injury (greater infarct sizes and poorer aortic output recoveries) compared to control hearts. Apart from its impact on glycolysis, simulated *in vivo* concentrations of insulin, when included in the perfusate 10 minutes prior to and during regional ischaemia, reduced infarct size in both the control and obese groups. This cardioprotective effect of insulin was attenuated when hearts were additionally perfused with simulated *in vivo* concentrations of FA's. This suggests that insulin's cardioprotective effects may in part be related to its metabolic actions.

CHAPTER 6

METHODS AND RESULTS FOR STUDY 3:

THE IMPACT OF OBESITY AND CHRONIC K-111 TREATMENT ON MYOCARDIAL FUNCTION, SUBSTRATE METABOLISM AND SUSCEPTIBILITY TO ISCHAEMIA/REPERFUSION INDUCED INJURY

6.1 Aim of the study

The aim of this sub-study was to determine the impact of obesity and chronic PPARα agonist treatment on *ex vivo* cardiac function, substrate metabolism, mitochondrial function and susceptibility to ischaemia/reperfusion injury.

6.2 Methods

6.2.1 Study design

To determine the effects of obesity and chronic PPARα agonist treatment on various cardiac and circulating blood parameters, age and weight matched male Wistar rats, upon reaching 200 ± 20g, were randomly placed on either a SRC diet or the HCD. After 8 weeks on their respective diets the animals were weighed. The SRC group was randomly sub-divided and one sub-group received the PPARα agonist K-111 (10mg/kg/day) administered via their drinking water. The same random sub-division was performed with the HCD fed group, with one half of the rats receiving both the HCD and K-111 (10mg/kg/day) in their drinking water and the other half remaining on the HCD without treatment (Figure 6.1). The rats were maintained on their respective feeding and drug administration protocols for an additional 8 to 10 weeks extending the experimental period to a total of 16-18 weeks (This group will be referred to as the 18 week fed group). To determine the impact of obesity and chronic PPARα

agonist treatment on myocardial substrate metabolism, hearts were mounted on a modified Langendorff perfusion apparatus. Myocardial glycolytic flux, glucose oxidation and palmitate oxidation rates were determined during baseline normoxic perfusion and during reperfusion following a period of total global normothermic ischaemia. To determine the effects of obesity and chronic PPARα agonist treatment on the myocardial susceptibility to ischaemia and reperfusion induced injury, hearts from the various groups were perfused in the working rat heart mode and subjected to 40 minutes CAL and 1 hour reperfusion. Myocardial function measurements were documented at regular intervals before and after ischaemia. Upon completion of the perfusion protocol, hearts were stained for the determination of myocardial infarct size.

To determine the influence of obesity and chronic PPARα agonist treatment on myocardial mitochondrial respiratory capacity, isolated ventricular mitochondrial respiration was assessed 1) in the presence of oxidizable substrates (glutamate and malate or palmitoyl–L–carnitine) and 2) in the combined presence of these substrates and ADP. Mitochondrial recovery of respiration in the presence of the oxidizable substrates and ADP was also assessed following 25 minutes of anoxia.

The impact of obesity and chronic PPARα agonist treatment on the expression of various myocardial proteins was investigated using standard Western blotting techniques with the appropriate antibodies. These proteins included CPT-1, a target of PPARα transcriptional upregulation, and phosphorylated and total PKB and the p85 subunit of PI3K as indicators of the insulin signaling pathway.

To determine the influence of obesity and chronic PPARα agonist treatment on various blood parameters, a separate series of animals were fasted overnight, and blood samples were collected for glucose, insulin, Trig, total cholesterol, HDL-C and FFA determinations. Blood from non-fasted animals was also collected for HbA1c analysis. To determine whether chronic K-111 treatment influenced adipogenesis, retroperitoneal and gonadal fat content was quantified. Lastly to determine whether obesity and chronic PPARα treatment influenced myocardial intracellular Trig levels, hearts isolated, freeze clamped and stored for subsequent analysis of myocardial intracellular Trig content.
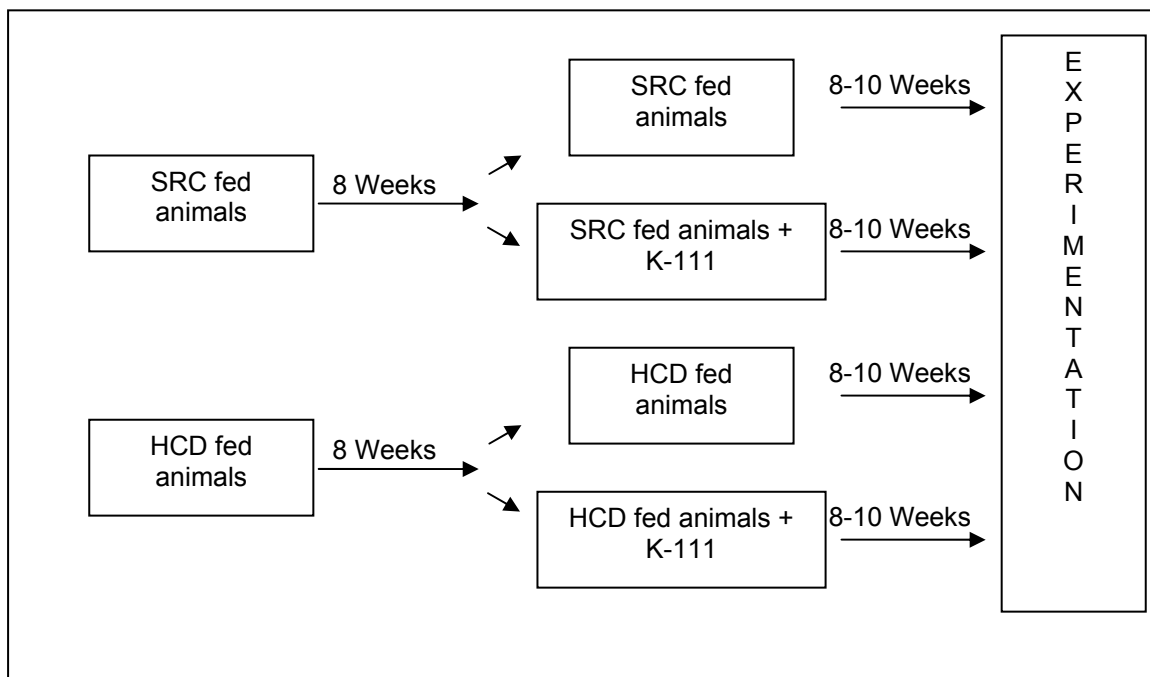
Figure 6.1: Flow diagram depicting the feeding and treatment protocol followed for control and obese animals

6.2.2 The PPARα agonist K-111

The PPARα agonist used in this study is a highly selective compound displaying a high affinity for PPARα (Phill and Kühnle, 1999; Meyer *et al*. 1999). The compound was previously named BM 17.0744 but is currently referred to as K-111. K-111 was

kindly supplied by KOWA Company, Ltd (Fugi Research Laboratories). At the commencement of the study, K-111 was reported to be undergoing phase II clinical trials. We were advised by KOWA not to exceed a dosage of 10mg/kg/day per animal. In rodents a dosage of 12mg/kg/day was however shown not to be toxic (Meyer *et al*. 1999).

6.2.2.1 Maintenance of the required K-111 dosage

Rats were administered K-111 in their drinking water at an approximate dosage of 10mg/kg/day. Five to six rats were housed per cage. One week prior to the commencement of K-111 treatment, the average daily water consumption per rat was calculated for a particular cage. At the beginning of week 8 before K-111 treatment started, the groups that would receive K-111 were weighed. The average water consumption per rat and average body weight per rat for a specific cage was then used to prepare a K-111 containing solution that would provide an approximate dosage of 10mg/kg/day for the specific cage. During the week, the water consumption for each cage was monitored along with the new average body weight per rat and used to calculate the K-111 dosage to be prepared for the following week. Thus, each week a fresh stock of K-111 was prepared based on the previous weeks average water consumption per rat and the average body weight per rat. Water consumption between concurrent weeks did not differ markedly. Water bottles were refilled with fresh drug containing water every second day.

6.2.3 Isolated heart perfusions for the determination of myocardial substrate metabolism

To determine the effect of obesity and chronic PPARα agonist treatment on myocardial substrate metabolism, hearts were perfused with either D-[5-[^{3}H(N)]-glucose (Perkin Elmer, USA) and D-[^{14}C(U)]-glucose (Perkin Elmer, USA) or 9,10-[^{3}H]-palmitic acid (Amersham) to determine the respective rates of myocardial glycolytic flux, glucose oxidation and palmitate oxidation before and after 15 minutes of global ischaemia.

Initially metabolic determinations were to be performed on isolated working heart preparations. Since the metabolism of D-[^{14}C(U)]-glucose yields $^{14}CO_2$, the perfusion apparatus was sealed air tight to prevent the escape of $^{14}CO_2$ from the perfusion system. Trial perfusions using a sealed isolated working rat heart perfusion apparatus were however not satisfactory as we were not able to maintain a stable myocardial systolic pressure during a one hour working heart perfusion. Consequently the aortic output was not stable and at times dropped dramatically. The same perfusion apparatus was then modified and the working heart component of the perfusion system was sealed and excluded, thus leaving only the Langendorff component operational. The Langendorff component was then used for the metabolism experiments (a satisfactory rate pressure product was obtained following 1hr perfusion using the LV balloon to measure pressure). The apparatus was designed to allow perfusate to re-circulate through the system once it had passed through the heart. A small hole was drilled into the perspex dome of the chamber in which the heart was contained to allow a temperature probe access to the heart. Temperature could therefore be monitored throughout the experiment. Once the

temperature probe had been placed into the coronary sinus of the heart, the hole was sealed.

When only 9,10-[^{3}H]-palmitate was incorporated into the perfusate, the perfusion system was not required to be sealed. During these conditions a fluid filled balloon tipped cannula was inserted into the LV for ventricular function determinations during the protocol (as previously described in section 3.1.4).

6.2.3.1 Preparation of the radio-labelled fatty acid perfusion buffer

The perfusion buffer containing FA was prepared as previously described in section 3.2.3 (Lopaschuk and Bar, 1997). The various tracers were added at different steps during the preparation of the buffer (to be discussed below).

After the FA perfusion buffer was prepared, the final working solution was supplemented with D-[^{14}C(U)]-glucose (25µCi per 100ml working solution; specific activity 250-360 mCi/mmol) or D-[5-[^{3}H(N)]-glucose (25µCi per 100ml working solution; specific activity 10-20 Ci/mmol) and stirred for 20 minutes. When using 9,10-[^{3}H]-palmitate (20µCi per 100ml working solution; specific activity 30-60 mCi/mmol), the tracer was added to the boiling palmitate sodium carbonate solution prior to the mixture being added to the concentrated bovine serum albumin solution. As two of the radio-labelled substrates had the same isotope (^{3}H) one series of hearts was perfused with D-[^{14}C(U)]-glucose and D-[5-[^{3}H(N)]-glucose and the other with 9,10-[^{3}H]-palmitate.

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6.2.3.2 Perfusion protocol followed when determining myocardial substrate metabolism

Before experimentation, samples were taken from the radio-labelled perfusion buffer prior to being placed into the perfusion system to determine the specific activity of the appropriately labelled isotope. The radio-labelled buffer (130ml) was re-circulated throughout all the perfusion lines of the perfusion system for 5 minutes prior to experimentation. After re-circulating the radio-labelled FA buffer, buffer samples, which would later be used as blanks, were taken in duplicate from the various injection ports in the system.

Isolated rat hearts were mounted on the perfusion apparatus via the aortic cannula and retrogradely perfused with Krebs-Henseleit perfusion buffer entering the system via a separate perfusion line (preload 100cm H_2O). Once all the excess tissue had been trimmed and the blood washed out (5 minute Krebs perfusion) the system was sealed and the perfusion medium immediately changed to the radio-labelled FA buffer. Two perfusion protocols were used differing only in the levels of insulin and FA used. Insulin and FA concentrations simulating the *in vivo* conditions of the control animals will be referred to as normal insulin + FA (30μIU/ml insulin and 0.7mM FA), whereas insulin and FA concentrations simulating the *in vivo* conditions of the obese animal will be referred to as high insulin + FA (50μIU/ml insulin and 1.5mM FA). In each perfusion protocol, 10mM glucose was also present.

Perfusate samples (containing both the tracer and its metabolic by-products) were collected from the perfusion system after 30 and 40 minutes of aerobic perfusion. Thereafter, hearts were subjected to 15 minutes of normothermic total global ischaemia followed by 30 minutes aerobic reperfusion. Additional buffer samples

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were collected 20 and 30 minutes after the onset of reperfusion in order to determine the metabolic rate of the hearts during the reperfusion phase of the protocol.

At the end of the perfusion period, the hearts were carefully removed from the perfusion system and the connective tissue and atria were trimmed off. The ventricles were cut into three slices, placed in an oven overnight and weighed to obtain dry weights.

6.2.3.3 Collection and processing of radio-labelled metabolic end products

Myocardial glycolytic flux, glucose oxidation, and palmitate oxidation rates from the various groups were determined by collecting the various metabolic end products produced from the metabolism of D-[5-[^{3}H(N)]-glucose, D-[^{14}C(U)]-glucose and 9,10-[^{3}H]-palmitate. ^{14}CO$_2$ released by the metabolism of [^{14}C]-glucose was present in both the gaseous and liquid phases in our experimental conditions. Collection of the gaseous ^{14}CO$_2$ was achieved by bubbling gas from the perfusion system directly into 20N NaOH (20ml). ^{14}CO$_2$ binds to the NaOH effectively trapping the ^{14}C in the NaOH solution. It was noted that the 20ml NaOH did not become completely saturated with ^{14}CO$_2$. An injection port with a plastic tube leading directly into the NaOH solution was used to withdraw samples at the appropriate time intervals. The alternative solution to trapping ^{14}CO$_2$ is hyamine hydroxide, but due to the exorbitant cost thereof, we chose to use NaOH. NaOH has previously been used in our laboratory to successfully trap ^{14}CO$_2$ (McCarthy *et al*. 2011). Both the liquid phase ^{14}CO$_2$, generated from the metabolism of D-[^{14}C(U)]-glucose and ^{3}H$_2$O, produced from the metabolism of both D-[5-[^{3}H(N)]-glucose and 9,10-[^{3}H]-palmitate were collected in a similar manner. Briefly, perfusate samples (2.5ml) were directly withdrawn with a

syringe through a 3-way stop cock that was inserted into the line between the water jacket surrounding the heart and the main reservoir containing the radio-labelled FA buffer. Glass scintillation vials were prepared with 3ml mineral oil. Perfusate samples required for the analysis of liquid phase $^{14}CO_2$ were injected directly under the layer of mineral oil to prevent the release of $^{14}CO_2$ from the buffer.

6.2.4 Methodology used to determine substrate metabolism

6.2.4.1 Myocardial glycolytic flux rate (Saddik and Lopaschuk, 1991)

Principle

The amount of ^3H_2O released from the metabolism of D-[5-[^{3}H(N)]-glucose was determined by passing samples through Dowex$^®$ 1-4X anion exchange columns (Sigma-Aldrich, St.Louis, MO). As glucose has a net negative charge, all glucose molecules would adhere to the column, while the positively charged ^3H_2O were eluted through the column and collected.

Procedure

The Dowex resin was conditioned with 2 washes of 1M NaOH, 4 washes of dH_2O, 2 washes of 0.9M boric acid, 2 washes of $Na_2B_4O_2$ and finally another 4 washes of dH_2O.The conditioned dowex slurry was stored at 4ºC.

The Dowex slurry was added to a 5ml syringe to form a column. We aimed to achieve a consistent 1ml Dowex column for all analyzed samples. Collection tubes were placed below each syringe. 200µl samples were slowly added to their respective columns and left to stand for 30 minutes. Hereafter, samples were eluted with 800µl dH_2O. From the volume of eluted ^3H_2O, 900µl was added to scintillation vials containing 5ml scintillation fluid and counted in a β scintillation counter for 2

minutes (Beckman LS 6500 Multi Purpose Scintillation Counter, Beckman Instruments, USA).

Calculation of the myocardial glycolytic rate

The following formula was used to determine the amount of glucose metabolized via glycolysis at a particular time point:

(Sample counts – blank) x $\underline{\text{total perfusion volume}}$ x $\underline{1}$ x $\underline{1}$ x DF
Sample volume (0.2ml) SA dw

where SA refers to the specific activity, dw to myocardial dry weight and DF to the dilution factor. The SA was determined by counting the 200µl buffer sample and dividing the counts by the amount of glucose (counts/µmol) present in the 200µl sample.

The final glycolytic flux rate was then calculated by dividing the amount of glucose metabolized via glycolysis (µmol/g dw) by the particular time interval at which the sample was taken (e.g. 30 minutes) (µmol/g dry weight/min). The glycolytic flux rates for the two samples taken during the pre-ischaemic perfusion period were averaged to determine the average basal glycolytic flux rate. A similar calculation was then used to determine the reperfusion glycolytic flux rate. However to account for possible metabolic occurrences during the global ischaemic period, the amount of glucose metabolized via glycolysis after 40 minutes perfusion (the sample directly before global ischaemia) was subtracted from the 75 and 85 minute sample (i.e. was used as the new blank).

6.2.4.2 Myocardial glucose oxidation rate (Saddik and Lopaschuk, 1991)

As mentioned previously, $^{14}CO_2$ was generated by the metabolism of D-[^{14}C(U)]-glucose and was present in both the gaseous and liquid phase. Collected gaseous phase samples trapped in NaOH were directly placed into scintillation vials containing 5ml scintillation fluid and were counted using a β scintillation counter. Liquid phase $^{14}CO_2$ was quantified using sealed metabolic flasks.

Principle

Liquid phase $^{14}CO_2$ present in perfusate samples were placed into sealed metabolic flasks containing 9N H_2SO_4. The metabolic flask consisted of an Erlenmeyer flask fitted with a rubber stopper. The rubber stopper contained an injection port, for the injection of the sample, and a fitting into which a glass tube could be securely inserted. The glass tube contained Whatman #1 filter paper which was laced with 3N NaOH. H_2SO_4 would react with the [^{14}C] bicarbonate ions in the perfusate sample liberating $^{14}CO_2$. The $^{14}CO_2$ passes into the glass tube and is trapped on the filter paper.

Procedure

1ml perfusate samples were injected into the metabolic flasks containing 9N H_2SO_4, and placed on a shaker. After 1 hour, the filter paper was removed, placed in scintillation vials containing 5ml scintillation fluid and counted in a β scintillation counter.

Calculation of the myocardial glucose oxidation rate

The same formulas and calculations were used to determine the average basal and reperfusion glucose oxidation rates as were described for glycolytic flux rate determinations. The only difference was that the amount of glucose oxidized in the gas and liquid phase samples were combined to determine the overall myocardial glucose oxidation rate. In addition, a dilution factor was not required in the calculation.
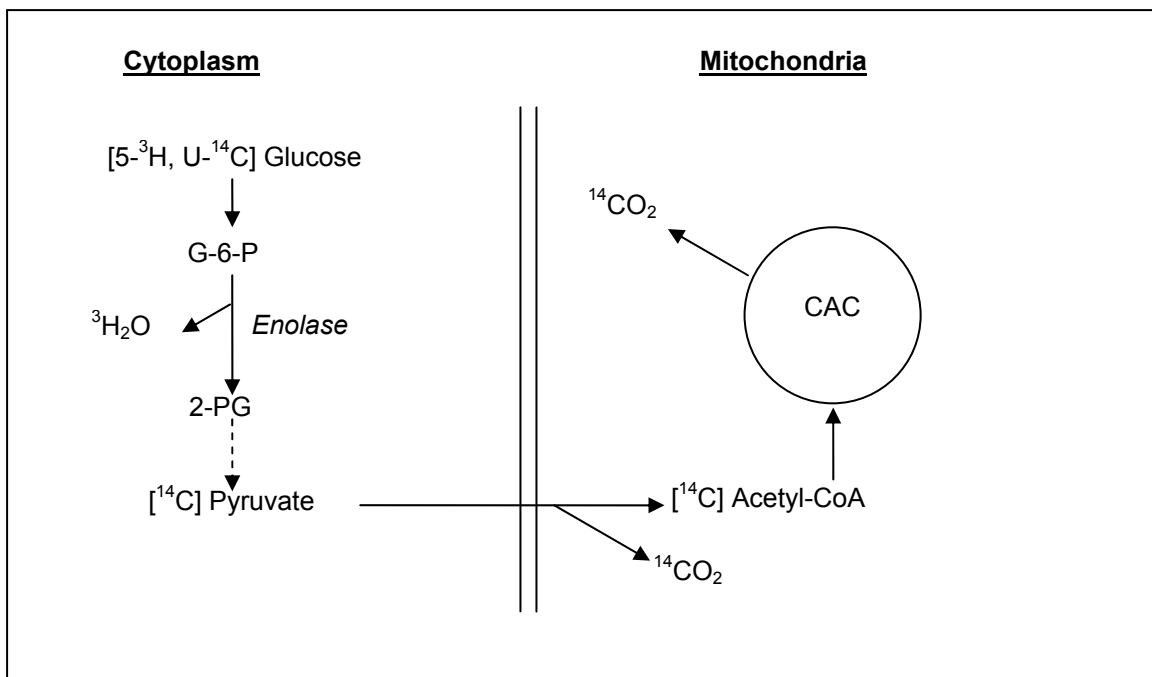
Figure 6.2: Diagram depicting the fate of ^{3}H and ^{14}C labelled glucose in the heart. (Taken from Lopaschuk and Barr, 1997)
CAC – **citric acid cycle;** *G-6-P* – **glucose -6- phosphate;** *2-PG* – **2-phosphoglycerate**

6.2.4.3 Myocardial palmitate oxidation rate (Personal communication between A Lochner and GD Lopaschuk)

Principle

Myocardial palmitate oxidation rates were calculated by measuring the amount of ^3H_2O released from the metabolism of 9,10-[^3H]-palmitate. The perfusate samples collected during the perfusion protocol contained both ^3H_2O and 9,10-[^3H]-palmitate. The evaporation method allowed the separation and collection of evaporated ^3H_2O from the perfusate sample. The volume of evaporated ^3H_2O collected from the perfusion was then counted and the final amount of ^3H_2O present in the specific sample was determined by factoring in the ratio of the processed and unprocessed internal standard (to be discussed below).

When using the evaporation method, the assumption is made that all the ^3H_2O from the perfusate sample, present within the microfuge tube will evaporate and be transferred into the scintillation vial during the evaporation process. To account for any possible transfer of ^3H originating from the un-metabolized 9,10-[^3H]-palmitate into the scintillation vial together with metabolized ^3H_2O following the evaporation procedure, two precisely identical internal standards were prepared in duplicate (four standards in total). The internal standards consisted of a known amount of 9,10-[^3H]-palmitate (1mCi/ml) added to 1ml Krebs-Henseleit buffer. Of these standards, one internal standard (in duplicate) was processed via the evaporation method (processed internal standard). The other internal standard (in duplicate) (unprocessed internal standard) was merely counted with the rest of the samples at the end of the procedure. This enabled us to determine the percentage transfer of 9,10-[^3H]-palmitate during a particular experimental run and to compensate for it when calculating the final amount of ^3H_2O present in each sample. The amount of 9,10-[^3H]-palmitate carry over varied form 1-4%.

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Procedure

200µl aliquots of the blank, sample and internal standard (to become the processed standard) were pipetted in duplicate into capless microfuge tubes (tube volume: 250µl). The microfuge tubes were carefully placed in a 7ml scintillation vial containing 500µl dH$_2$O. Capped scintillation vials were kept in an incubator (50ºC) for 24 hours. Subsequently, all vials were transferred to the cold room (4ºC) overnight. Microfuge tubes were then carefully removed from the scintillation vials by dragging them against the side of the vial to remove any ^{3}H$_2$O. The microfuge tubes were discarded and the scintillation vials were all filled with 5ml scintillation fluid. The unprocessed internal standard (the standard that did not go through the evaporation method) was also added to a scintillation vial and filled with scintillation fluid.

The un-metabolized perfusion solution (200µl taken before the addition of perfusate to the perfusion system) was placed into a separate scintillation vial and 5ml scintillation fluid was added. This vial served to determine the specific activity of the tracer used in the perfusate. All the samples were counted in a β-scintillation counter.

Calculation of palmitate oxidation rate

The following formula was used to determine the amount of palmitate oxidized by the heart:

$$(\text{Sample counts} - \text{blank}) \times \frac{\text{volume of buffer in perfusion system}}{\text{Sample volume (0.2ml)}} \times \frac{1}{\text{SA}} \times \frac{1}{\text{dw}}$$

where SA refers to the specific activity and dw to myocardial dry weight. The specific activity was determined by counting the 200µl SA sample and dividing it by the amount (nmol) of palmitate present.

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The final rate of palmitate oxidation rate was expressed as nmol/g dry weight/min. The palmitate oxidation rates for the two samples taken during the pre-ischaemic perfusion period were averaged to determine the basal palmitate oxidation rate. A similar calculation was used to determine the reperfusion palmitate oxidation rate. However to account for possible metabolic occurrences during the 15 minute global ischaemic period, the amount of palmitate oxidized at 40 minutes (the sample directly before global ischaemia) was subtracted from the 75 and 85 minute (post-ischaemic) sample (i.e. the 40 minute sample acted as the blank for the 75 and 85 minute samples).
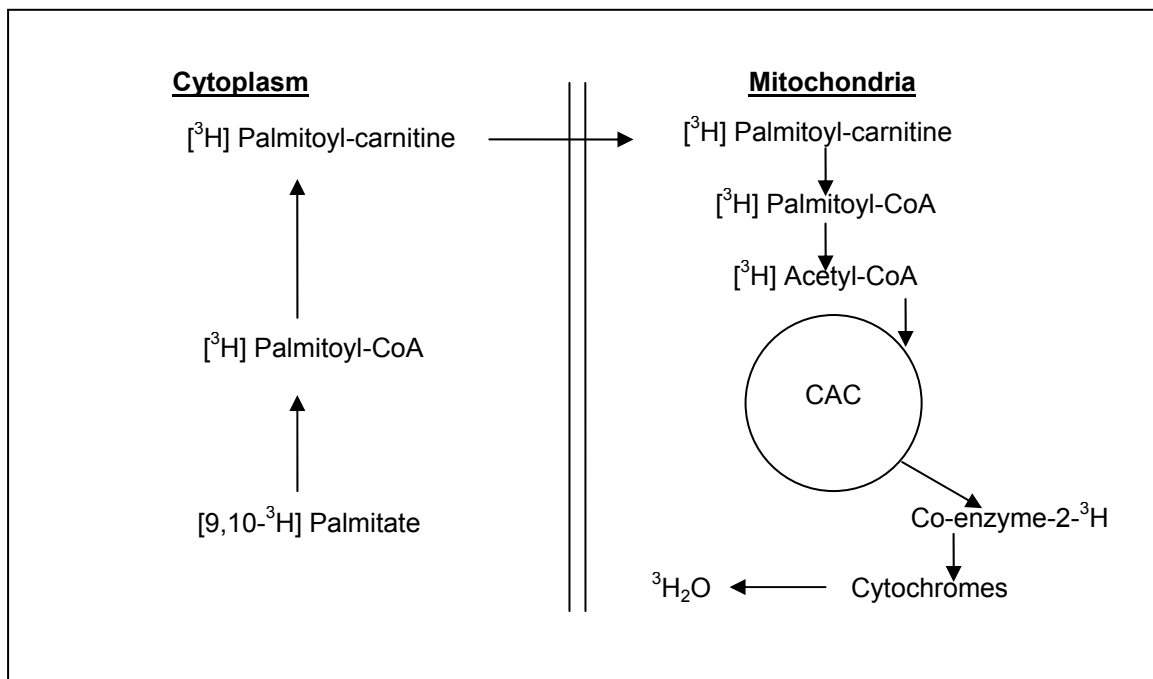
Figure 6.3: Diagram depicting the fate of ^{3}H and ^{14}C labelled palmitate in the heart. (Taken from Lopaschuk and Bar, 1997)

6.2.5 Perfusion protocol used to assess the myocardial susceptibility to ischaemia/reperfusion injury

The impact of obesity and chronic PPARα agonist (K-111) treatment on the myocardial susceptibility to ischaemia/reperfusion injury was assessed in isolated working rat hearts as it allowed for working heart function to be determined. As with the metabolic perfusions, two different perfusion protocols were used differing only in the concentration of insulin and FA used. As described in section 6.2.3.2 these two different perfusion solutions will again be referred to as the "normal" or "high" insulin + FA concentrations. This approach further enabled us to elucidate the impact of altered substrate and insulin supply on post-ischaemic outcomes.

Hearts from each group were subjected to a standard myocardial infarction perfusion protocol (Figure 6.4). While being mounted on the perfusion apparatus, all hearts were initially perfused with Krebs-Henseleit perfusion buffer for 5 minutes to ensure that all the blood was washed out from the hearts. After 5 minutes the perfusion buffer was switched to the appropriate glucose containing Krebs-Henseleit perfusion buffer supplemented with insulin and FA. This solution (200 ml) was re-circulated for the duration of the perfusion protocol. The perfusion protocol consisted of an initial 10 minute retrograde perfusion which served as a stabilization period. The perfusion was then changed to the working heart mode for 20 minutes after which the hearts were again retrogradely perfused and the left anterior descending coronary artery ligated with a silk suture. Occlusion of the coronary artery induced regional ischaemia which was maintained for a period of 40 minutes, and was followed by 60 minutes of reperfusion after the suture had been loosened. The reperfusion period consisted of 10 minutes Langendorff perfusion, 20 minutes working heart perfusion and a further 30 minutes of Langendorff perfusion. During the working heart perfusion periods,

myocardial LV mechanical function was monitored and documented (See section 3.1.4 for the list of functional measurements documented in isolated working hearts).

Hearts that were unable to pump adequately during the working heart reperfusion period were perfused in an assisted working heart mode (a working heart mode with the Langendorff perfusion tap slightly opened). This ensured that an adequate perfusion pressure was maintained during the working heart period. These hearts could therefore not generate data for reperfusion function. The number of hearts that were perfused in an assisted mode can be determined by the difference between the sample size of the pre-ischaemic function and the sample size of the post-ischaemic function. In instances where the reperfused working heart was pumping adequately and developing pressure but not able to generate aortic output, the hearts were allowed to continue in the working heart mode alone, and a *dp/dt* value was measured and an aortic output value of 0 ml/min recorded.
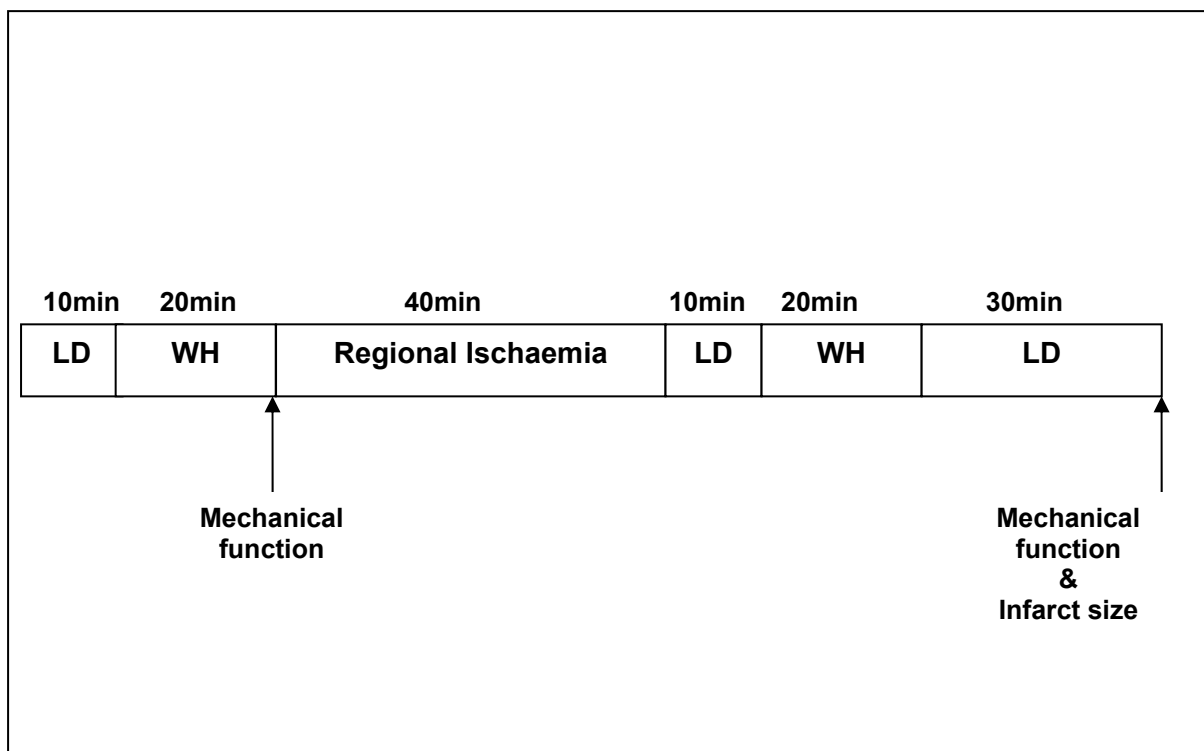
Figure 6.4: Perfusion protocol followed to document the impact of obesity and chronic K-111 treatment on the myocardial susceptibility to ischaemia and reperfusion injury.
LD **– Langendorff mode;** *WH* **– Working heart mode.**

6.2.6 Indices used to assess myocardial ischaemia/reperfusion induced injury

6.2.6.1 Myocardial infarct size

Myocardial infarct size was assessed as a direct measure of ischaemia/reperfusion injury. The protocol for the determination of myocardial infarct size has previously been described (Section 5.2.3.1).

6.1.6.2 Myocardial functional recovery

The aortic output recovery was used as an index of the severity of ischaemia/reperfusion damage and has already been described (section 5.2.3.2). The total work and cardiac output (aortic output + coronary flow) recoveries were calculated using the formula previously described (Section 5.2.4.2).

As previously mentioned, while both infarct size and functional recovery are well recognized indices of the severity of ischaemia/reperfusion injury, functional recovery is not regarded as an accurate indicator of ischaemic damage in a model of regional ischaemia.

6.2.7 Isolated mitochondria experiments

To determine the influence of obesity and chronic K-111 treatment on mitochondrial respiratory capacity, ventricular mitochondria from the various groups were isolated and mitochondrial respiration measured in the presence of different oxidizable substrates (glutamate or palmitoyl – L – carnitine). The choice of glutamate and palmitoyl – L – carnitine as substrate was merely to assess respiratory capacity and not the effect of the substrate per se. The ability of these mitochondria to regain their normal respiratory capacity was also assessed following a 25 minute period of anoxia. Mitochondrial oxygen consumption (nAtoms O_2/ml/min) was monitored by means of Clarke electrode (Oxygraph, Hansatech Instruments Bannan, UK). For the purpose of this study we only wished to determine the impact of obesity and K-111 agonist treatment on total mitochondrial respiratory capacity. Consequently we did not perform complex analysis of the electron transport chain.

6.2.7.1 Preparation of ventricular mitochondria

After rats were anaesthetized, their hearts were removed and all excess tissue and atria removed. The ventricles were placed in a Sorvall tube containing ice cold potassium chloride ethylenediaminetetraacetic acid (KE) (0.18M KCl, 0.01M ethylenediaminetetraacetic acid, pH set at 7.4 with 2M Tris) isolation medium and cut into small fine pieces with a scissor. The pieces of ventricle were washed 4-5 times in ice cold KE isolation medium to remove all traces of blood. Mitochondria were

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prepared according to the method of Sordahl *et al.* (1971). Ventricles were homogenized (on ice) with a Polytron (PT10) homogenizer for 2 cycles of 4 seconds at setting 4. The homogenate was centrifuged at 2500 rpm (755 x g) for 10 minutes in a Sorvall centrifuge (rotor SS34). The supernatant was then transferred into a clean Sorvall tube and centrifuged again at 12500 rpm (18 800 x g) for 10 minutes. The supernatant was discarded and the remaining pellet, which represented the ventricular mitochondria population (subsarcolemmal fraction), was re-suspended in 0.5ml KE isolation medium with the help of a pre-cooled glass Teflon Potter Elvehjem homogenizer.

6.2.7.2 Determination of mitochondrial protein content

From the 0.5ml re-suspended pellet containing the mitochondria, 50µl was placed into a tube containing 1ml trichloroacetic acid (TCA) (10%) overnight to precipitate the mitochondrial protein. The mitochondrial protein content was assessed using the Lowry method (Lowry *et al.* 1951). Briefly, the precipitated protein was isolated by centrifuging the mitochondrial TCA suspension at 2500 rpm for 15 minutes. The supernatant was discarded and the pellet re-suspended in 500µl 1N NaOH. These samples were subsequently placed in a water-bath at 70°C for 10 minutes. Once the samples were clear (or after the 10 minute incubation) an additional 500µl dH$_2$O was added to each tube and the samples mixed with a vortex.

For the assay, a 0.5N NaOH solution was used as the blank, while 3 different concentrations of an albumin standard were used to generate a standard curve. 50µl of mitochondrial samples, standards or blank were aliquoted in triplicate into lucham-tubes. A 1ml solution of 2% NaK-Tartrate, 1% CuSO$_4$.5H$_2$O, 2% Na$_2$CO$_3$ was added to each tube at 10 second intervals and vortexed. After 10 minutes, 100µl Folin

Ciocalteu's (33%) was added to each tube, at 10 second intervals and vortexed. A colour reaction developed and after 30 minutes the optical density was measured at 750nm in a spectrophotometer. The absorbancies of the 3 different known concentrations of albumin were plotted to generate a standard curve, which was used for the determination of the protein content of the unknown samples.

6.2.7.3 Determination of ventricular mitochondrial respiration and the post-anoxic recovery of respiration

Mitochondrial oxidative phosphorylation was determined using an oxygraph (Oxygraph, Hansatech Instruments Bannan, UK). During all calibrations and experimental conditions a small magnet was placed in the chamber and set to stir to ensure that a homogeneous solution was maintained at all times. At the start of each experimental day, the oxygraph was calibrated as follows: Incubation medium (see the next paragraph for details) was placed in the chamber. Upon reaching the experimental temperature of 26°C (previous authors have used temperatures of either 25°C (Boudina *et al*. 2005; Duncan *et al*. 2007) or 37° (Khalid *et al*. 2011)), the oxygen concentration was set at 100%. Sodium dithionite, which acts as an oxygen scavenger, was added and the 0% oxygen concentration was set once the decline in oxygen concentration reached a steady state. The chamber surrounding the electrode containing the incubation medium and sodium dithionite was then sealed for 20 minutes to determine whether the chamber's o-rings sealed air tight (in the event of a leak, the oxygen concentration in the chamber would rise within 20 minutes). Hereafter the chamber was repeatedly washed to remove all traces of sodium dithionite.

The incubation solution used for the experiments consisted of 250mM sucrose, 10mM Tris HCL, 8.5mM K_2HPO_4, 2mM malate and either 5mM glutamate or 0.45mM palmitoyl-L-Carnitine as oxidative substrates. The pH of the incubation solution was set at 7.4. All experiments were performed at 26ºC. Isolated mitochondria were kept on ice during the entire procedure until they were ready to be placed into the chamber.

At the start of each experiment, incubation solution containing either glutamate or palmitoyl-L-Carnitine as oxidizable substrates was added to the oxygraph chamber. The solution was allowed to reach 26ºC before experimentation commenced. Once a stable oxygen concentration trace was maintained in the chamber, the recording was started. Isolated mitochondria were then added to the chamber and state 2 respiration was recorded (See figure 6.5). Thereafter, 50μl of a 7.48mM ADP (in total 374nmol ADP) solution was added and the chamber sealed. This event induced mitochondrial state 3 respiration during which oxygen was consumed at a rapid rate due to the production of ATP from ADP. Once all the ADP had been phosphorylated, state 4 respiration ensued. After a measurable period of state 4 respiration, the seal of the chamber was removed and an oversupply of ADP (100μl of a 10X solution) was added to the mitochondrial suspension. The chamber was then resealed. This "oversupply" of ADP allowed the mitochondria to utilize all oxygen present in the chamber, effectively inducing anoxia in the chamber with excess ADP remaining in the medium. Following 25 minutes of anoxia, the seal was removed and the chamber re-oxygenated using a plastic Pasteur pipette, restoring the oxygen concentration too approximately half of that present in the chamber prior to the addition of the mitochondria. A re-oxygenation state 3 respiration rate ensued due to the presence of excess ADP previously added. Mitochondrial susceptibility to anoxia re-

oxygenation was then calculated by expressing the post-anoxia state 3 respiration as a percentage of the pre-anoxia state 3 respiration rate.

6.2.7.4 Mitochondrial parameters measured and investigated

The various measurements obtained during the experimental protocol are depicted in figure 6.5. In this figure (A) represents state 2 respiration (the slope of the curve), representing mitochondrial respiration in the presence of the specific substrates in the incubation solution in the absence of ADP. (B) Represents state 3 respiration (the slope of the curve), which corresponds with the rate at which the substrates were oxidized in the presence of ADP. During this period oxidative phosphorylation occurs where ADP is converted to ATP. State 4 respiration (the slope of the curve), represented by (C), illustrates mitochondrial respiration in the absence of ADP (all the ADP was converted to ATP). The value of the state 4 respiration is often similar to that of state 2 respiration. The value of state 4 respiration may vary slightly due to the presence of various breakdown products in the incubation solution following oxidative phosphorylation.

The slope of the curves for A, B and C, was determined by software provided with the oxygraph to effectively quantify State 2, 3 and 4 respiration ($nmolO_2$/min/ml). The final respiration rate was expressed as $natomsO_2$/min/mg protein. Mitochondrial protein content of the various groups was determined using the Lowry method for protein determination (Lowry *et al*. 1951) as previously described (6.2.7.2).
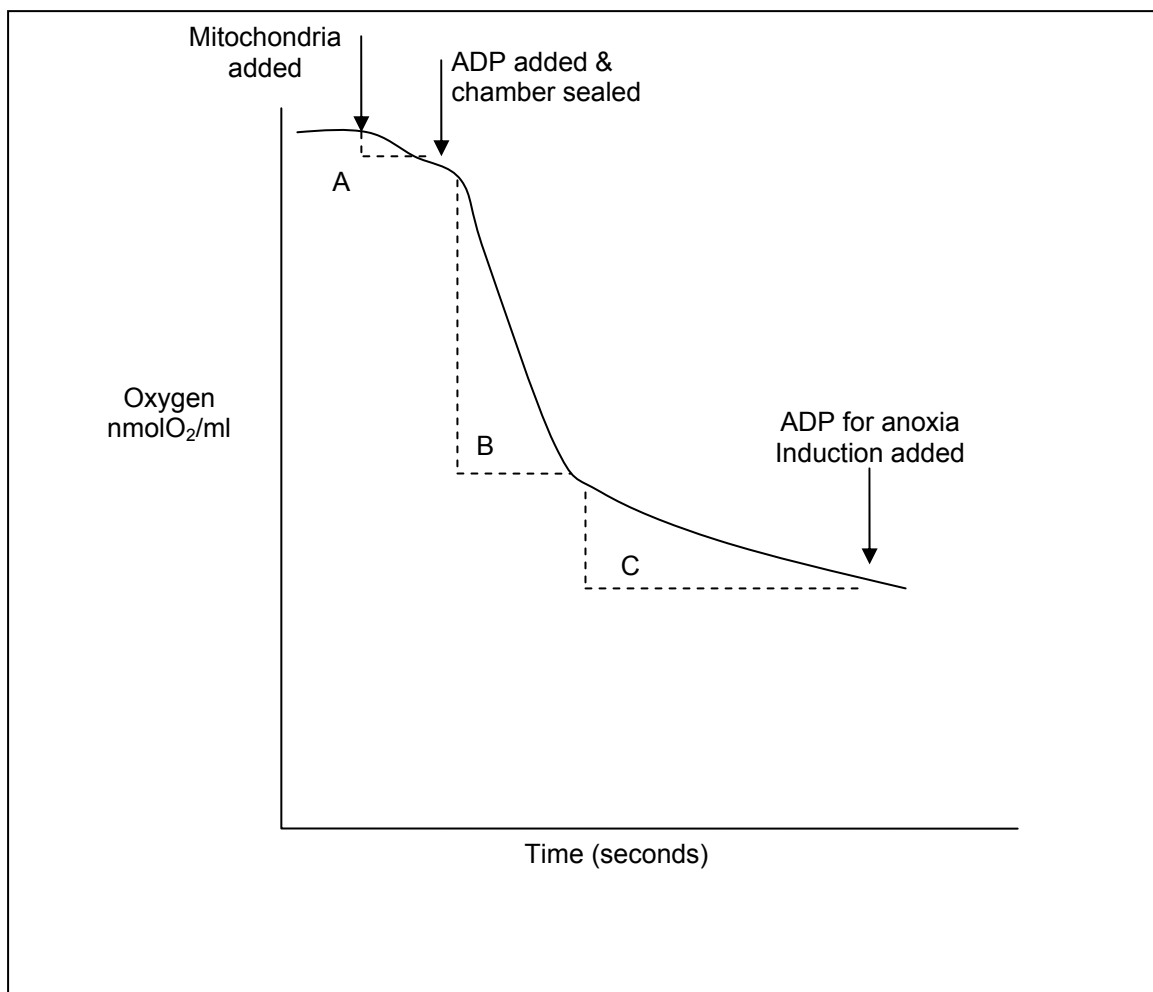
Figure 6.5: Diagrammatic representation of the respiration curves obtained on the oxygraph. A, represents state 2 respiration; B, represents state 3 respiration; and C, represents state 4 respiration

From the graph presented in Figure 6.5, both the ADP/O ratio and the respiratory control index (RCI) index could be calculated. The ADP/O ratio represents the relationship between ATP synthesis and oxygen consumption. In the presence of glutamate as a substrate the theoretical ADP/O ratio is calculated to be 3, whereas in the presence of palmitoyl-L-carnitine the theoretical value should be 2.6. The ADP/O ratio is determined by dividing the total amount of ADP added by the amount of oxygen consumed during state 3 respiration. For all experiments, aliquots of a stock solution of ADP were made and the absorbance of the stock solution was measured at 259nm with a spectrophotometer. Using the absorbance of the ADP solution, and

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the molar extinction coefficient of ADP at 259 nm (15.4) the exact ADP concentration used could be determined. Once the ADP/O ratio had been calculated, the oxidative phosphorylation rate (nmol ADP/min/mg protein) was calculated from the product of the ADP/O ratio (nmol ADP/nAtom O_2) and state 3 respiration (nAtoms O_2/min/mg protein).

The RCI is the ratio of state 3:state 4 respiration and is an indication of the tightness of the coupling between respiration and phosphorylation.

6.2.8 Biometric measurements

Retroperitoneal and gonadal fat mass was determined as previously described (4.2.4.1).

6.2.8.1 Determination of pericardial fat mass

Hearts were carefully removed from anaesthetized rats. Once mounted on a perfusion apparatus, the fat surrounding the heart was removed and weighed.

6.2.9 Biochemical analysis

6.2.9.1 Determination of intramyocardial triglyceride content

Hearts isolated from animals were immediately placed in ice cold Krebs-Henseleit buffer. The atria and connective tissue were removed and the heart cut in half. The two halves were placed in a new container of ice cold Krebs-Henseleit buffer to wash away the remaining blood from the ventricles. These pieces of ventricular tissue were then freeze clamped with pre-cooled Wollenberger tongs and placed into liquid nitrogen, where they were stored until required for further analysis.

Analysis of the intramyocardial Trig content was performed by the Lipid Clinic at the Cape Heart Center, University of Cape Town (South Africa). Briefly, lipids were extracted using the Folch extraction (Folch *et al*. 1957) and the quantity of Trig present in each sample determined spectrophotometrically using the KAT enzymatic, spectrophotometric kit (KAT Laboratory and Medical (PTY) Ltd , Gauteng, South Africa, catalogue number KAT801T). The protein concentration of each sample was determined by the Markwell spectrophotometric method (a modification of the Lowry protein determination method) (Markwell *et al*. 1978). The amount of Trig present in each sample was then expressed in terms of the protein concentration of the sample.

6.2.9.2 Blood and serum determinations

Blood glucose (4.2.5.2.1), lipid (Trig's, total cholesterol and HDL-C) (4.2.5.2.3), and serum insulin (4.2.5.3.1), FFA (5.2.6.1.1) and HbA1c (4.2.5.2.2) levels were determined in non-fasting animals as previously described. Lastly, systemic insulin sensitivity was determined from the HOMA-IR index as previously described in section 5.2.6.1.2.

6.2.10 Western blot analysis

To determine whether obesity and chronic K-111 treatment influenced the expression of various proteins involved in carbohydrate and FA metabolism, Western blot analysis was performed. Hearts were isolated and stored as described in section 6.2.9.1.

6.2.10.1 Preparation of Western blot lysates

Approximately 200mg of frozen ventricular tissue was crushed using a mortar and pestle which had been pre-cooled in liquid nitrogen. The crushed tissue was quickly placed into Sorvall tubes containing 900µl lysis buffer (20mM Tris-HCl pH 7.4, 1mM EGTA, 25mM NaCl, 1mM Na_3VO_2, 10mM NaF, 1% (v/v) triton X-100, 10µg/ml leupeptin, 10µg/ml aprotinin, 1mM benzamidine. 1mM Phenylmethyl-sulphonyl fluoride was only added before usage) for the extraction of the myocardial proteins. The same lysis buffer was used to prepare lysates for all the proteins investigated in this study.

The tubes containing the lysis buffer and ventricular tissue were homogenized with a Polytron (PT10) (2 cycles of 5 seconds each at setting 5). Following the homogenization step, the tubes were kept on ice for 30 minutes to ensure adequate protein extraction. The samples were then centrifuged at 14000rpm for 14 minutes to remove particulate matter. From each sample, an aliquot was used to determine the protein concentration of the supernatant using the Bradford protein determination technique (Bradford, 1976). Once the exact amount of protein present in each sample was known, aliquots of each sample were made and diluted with Laemmli sample buffer (3:1). The aliquots were boiled for 5 minutes and stored at -20ºC. Western blot analysis was performed within 2 weeks of lysate preparation.

6.2.10.2 The Bradford protein determination method

Principle

A standard curve is generated plotting the absorbancies and concentrations of different known amounts of albumin. The absorption of each of the unknown protein samples can then be determined at 595nm and their concentration determined by using the linear equation of the standard curve.

Procedure

The Bradford assay was preformed in duplicate. A standard curve was prepared by performing serial dilutions from a stock of albumin to create a standard curve with protein concentrations ranging from 1-20µg protein. Protein samples from the experimental group were diluted 1:10 with dH_2O. From this dilution 5µl was aliquoted into a new tube and further diluted with 95µl dH_2O. These dilutions ensured that the protein content of the diluted sample would be less than 20µg thus allowing the unknown samples to fit on the standard curve. 0.9ml Bradford reagent (Coomassie Brilliant Blue G-250 0.01% (w/v), ethanol 4.7% (v/v), and phosphoric acid 8.5% (v/v)) was added to each tube (standards and unknown proteins) and vortexed. Between 15 and 30 minutes following the addition of the Bradford reagent, the absorption of each sample was spectrophometrically determined at 595nm. The unknown protein concentrations could then be determined from the standard curve as was described above.

6.2.10.3 Separation of the proteins

Prior to use, each lysate was boiled for 5 minutes followed by centrifugation at 14000rpm for 5 minutes. The proteins from each sample were then separated on a SDS-polyacrylamide gel using the Bio-Rad Mini-PROTEAN III system (Bio-Rad, Life

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Science Group, USA) (140V, 140mA for 80 minutes). The samples were loaded onto a 4% stacking gel and electrophoretically separated onto a 10% running gel. After this, the separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Immobilon Transfer Membranes, USA) by electro-blotting. Thereafter, the membranes were washed with Tris-buffered saline mixed with 0.1% Tween-20 (TBS-T). The wash step was repeated for 2 hours with 5% fat-free milk powder in TBS-T solution in order to block all non-specific binding sites on the membrane.

Following the TBS-T milk powder incubation, membranes were washed (TBS-T) and incubated on a shaker with the appropriate primary antibody overnight at 4ºC (see specific dilution details below). Following the incubation procedure, membranes were once again washed (TBS-T) and incubated with a horseradish peroxidase-labelled secondary antibody (1:4000 in 5% fat-free milk powder in TBS-T, unless otherwise stated) for 1 hour at room temperature. The membranes were again washed (TBS-T) after which they were ready to be developed. Membranes were subsequently incubated with electro-chemiluminescence detection agents (ECL Solution Detection Reagents 1 and 2, GE Healthcare, UK) for 1 minute. The membranes were then briefly exposed to high performance chemiluminescence film (Hyperfilm ECL) allowing for a light emission to portray the protein band on the film. The protein bands were then quantified by laser scanning densitometry and analyzed with the relevant software (UN-SCAN-IT, Silkscience, USA).

Relevant information relating to each protein analyzed will be discussed below.

PI3-kinase p85 subunit

60µg protein fractions were loaded onto a 10% SDS-polyacrylamide gel. Membranes were incubated with total PI3K p85 or the phospho-PI3K p85 (Tyr 458) (Cell Signalling Technology, Inc) primary antibody overnight. The primary antibodies were diluted 1:1000 in TBS-T.

PKB

30µg protein fractions were loaded onto a 10% SDS-polyacrylamide gel. Membranes were incubated with total PKB or the phospho-PKB (Ser 473) (Cell Signalling Technology, Inc) primary antibody overnight. The primary antibodies were diluted 1:1000 in TBS-T.

CPT-1

60µg protein fractions were loaded onto a 10% SDS-polyacrylamide gel. Membranes were incubated with CPT-1 – M(H-120): sc-20670 (Santa Cruz Biotechnology, Inc) primary antibody overnight. The primary antibodies were diluted 1:1000 in TBS-T.

β-Tubulin

A membrane that had been used for PI3K determination was stripped (3 x dH_2O, 1 x 1N NaOH, 3 x dH_2O) and probed for B-tubulin. Membranes were incubated with B-Tubulin (Cell Signalling Technology, Inc) primary antibody overnight. The primary antibodies were diluted 1:1000 in TBS-T.

6.1.10.4 Calculation of the amount of protein in each sample

A total of 6 hearts were used per group. On each gel only 3 samples were loaded per group as only 12 sample wells were available per gel. To enable comparisons between 2 different gels for a specific protein investigated, an internal standard was loaded onto each gel. The internal standard used was proteins from a heart isolated from a healthy 250g rat. After completing the densitometry of each protein band, the number of pixels for each band was expressed as a ratio of the internal standard. Using this approach the amount of pixels obtained for a specific protein in a specific group spread over two gels could simply be combined.

6.2.11 Statistical analysis

Statistical comparisons were made as previously described in section 5.2.7. In addition, the comparison between pre- and post-ischaemic rates of myocardial substrate metabolism within a particular group were assessed by means of a paired Students t-test.

RESULTS: STUDY 3

6.3 Results

6.3.1 Biometric and metabolic data after 8 weeks of feeding

Table 6.1: Body weight of control and obese animals after 8 weeks of feeding prior to receiving the K-111 treatment.

	Control	Obese
Body Weight (g)	333.50±5.57 (n=58)	375.60±5.35* (n=59)

*p<0.001

After 8 weeks of feeding and prior to rats receiving chronic K-111 treatment, HCD fed rats were already significantly heavier than the control group (Table 6.1). Although we did not determine any metabolic parameters after 8 weeks of HCD feeding (to reduce the number of animals required for this study), it is important to mention our research group has previously shown that such rats have increased retroperitoneal and gonadal fat mass, are euglycaemic and have elevated fasting insulin levels compared to control animals (Huisamen *et al.* unpublished data). Thus the 8 week HCD fed rats in this study may be considered to be obese (both general and visceral) and have systemic insulin resistance when compared to the lean control animals.

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6.3.2 Biometric and biochemical data after 18 weeks

Table 6.2: Biometric and biochemical data of non-fasted control and obese animals with and without chronic K-111 treatment.

	Control	Obese	Control + K-111	Obese + K-111
BW (g)	428.50±17.23	506.14±10.61[@]	403.13±17.08	410.42±11.78*
Retroperitoneal and gonadal fat weights (g)	15.15±1.18	30.45±1.99[#]	12.29±0.86	17.53±1.70[%,$]
Retroperitoneal and gonadal fat as % BW (%)	3.53±0.19	5.99±0.30[#]	3.06±0.20	4.27±0.38[&,^]
Pericardial fat (g)	0.11±0.02	0.28±0.06[@]	0.13±0.01	0.14±0.02*
Intramyocardial Trig (μg/mg protein)	852.81±69.85	895.36±108.62	1017.09±121.41	847.37±63.83
Glucose (mmol/l)	12.08±0.70	12.78±0.83	9.75±0.28*	10.29±0.64*
Insulin (μIU/ml)	30.24±4.55	60.18±7.25[#]	21.70±2.56	37.99±3.76*
HOMA-IR (AU)	16.19±2.85	35.66±6.26[@]	9.86±1.25	17.31±2.32[^]
HbA1c (%)	2.62±0.07	2.78±0.04	2.78±0.05[^]	2.77±0.05
Trig (mmol/l)	0.97±0.19	2.53±0.24[#]	0.57±0.00	0.57±0.00[$]
HDL-C (mmol/l)	0.98±0.12	0.85±0.08	0.56±0.14*	0.48±0.07*
FFA (mmol/l)	0.26±0.02	0.47±0.05[#]	0.23±0.02	0.23±0.02[$]

BW – body weight; *HbA1c* – glycosylated haemoglobin; *HDL-C* – high density lipoprotein cholesterol; *HOMA-IR* – homeostasis model assessment for insulin resistance; *FFA* – free fatty acid; *Trig* – Triglyceride.
[#]p<0.001, [@]p<0.01 vs. control; [&]p<0.001, [%]p<0.05 vs. control treated; [$]p<0.001, [^]p<0.01 *p<0.05 vs. corresponding untreated group
n = 6-8 rats per group; intramyocardial triglyceride content: n = 3-5 hearts per group

After 18 weeks, HCD fed rats were significantly heavier and had more retroperitoneal and gonadal fat (absolute mass and expressed as a percentage of body weight) and pericardial fat compared to their control littermates (Table 6.2). Chronic K-111 treatment had a beneficial effect on adiposity and prevented the increase in body weight, retroperitoneal and gonadal fat and pericardial fat mass associated with HCD feeding. HCD fed K-111 treated rats did however have more retroperitoneal and gonadal fat in comparison to control K-111 treated animals. Furthermore, although chronic K-111 treatment did not influence the body weight, retroperitoneal and gonadal fat or pericardial fat mass in this specific group of control rats (compared to the untreated controls), certain series of K-111 treated control animals did indeed have lower body weights compared to their untreated littermates (data not shown). Similar observations were made between the HCD K-111 treated and control K-111 treated groups where in certain series of animals, the body weight of the HCD K-111 treated group was greater than the control K-111 treated group (yet still less than the HCD untreated group).

Neither the HCD nor chronic K-111 treatment influenced the intramyocardial triglyceride content, suggesting the absence of lipotoxicity in the model after 18 weeks of feeding.

After the 18 week feeding period, HCD fed rats developed hyperinsulinaemia, while normoglycaemia was maintained and consequently these animals displayed systemic insulin resistance (reflected by a raised HOMA-IR index) (Table 6.2). This was accompanied by raised blood Trig's and FFA levels compared to the control group. The HCD did not change the percentage glycosylated haemoglobin or HDL-C levels.

Chronic K-111 treatment attenuated many of the metabolic abnormalities associated with obesity. This included reducing blood glucose, insulin, Trig and FFA levels, while whole body insulin sensitivity was markedly improved (treated vs. untreated HCD fed rats). The metabolic influence of K-111 was less pronounced in the control group where only a reduction in blood glucose was noted. Although the control K-111 treated group had raised glycosylated haemoglobin levels compared to the control untreated group, these values remained within the normal range. Surprisingly chronic K-111 treatment reduced HDL-C levels within both dietary groups. Lastly, no standard error of the means were included for blood Trig levels in the K-111 treated groups as the Trig levels obtained were lower than the detectable limit of the Cardio-check instrument. Thus the value reported was the lower detection limit of the Cardio-check instrument.

Although the HCD K-111 treated animals in Table 6.2 were not heavier than the control or control treated animals, they will also be referred to as the obese or HCD fed K-111 treated group as at the time of K-111 administration, these animals were significantly heavier compared to their control littermates (Table 6.1).

6.3.3 *Ex vivo* myocardial function and substrate metabolism obtained on the Langendorff perfusion apparatus

6.3.3.1 Normal insulin and FA concentrations in the perfusate
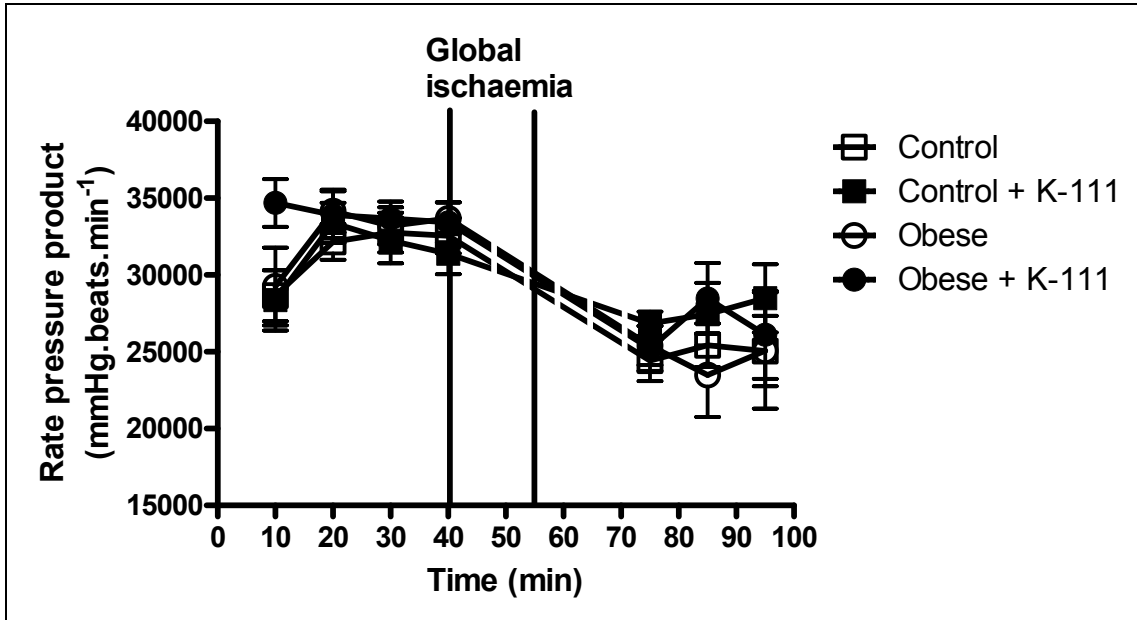
Figure 6.6: Rate pressure product obtained from isolated rat hearts perfused with glucose (10mM) and normal insulin (30μIU/ml) and FA (0.7mM) concentrations. Hearts were subjected to 15 minutes normothermic total global ischaemia followed by 30 minutes reperfusion.

n=4-8 hearts per group

Neither obesity nor chronic PPARα agonist treatment influenced myocardial RPP before and during reperfusion (Figure 6.6). Similarly, there was no difference in the RPP percentage recovery between the four groups (data not shown).

Table 6.3: Average normoxic baseline and reperfusion myocardial substrate metabolism obtained from isolated rat hearts perfused with glucose (10mM) and normal insulin (30µIU/ml) and FA (0.7mM) concentrations. Hearts were subjected to 15 minutes normothermic total global ischaemia. Baseline samples were collected after 30 and 40 minutes of normoxic perfusion. Reperfusion samples were collected after 20 and 30 minutes post-ischaemic perfusion.

	Baseline			
	Control	**Obese**	**Control + K-111**	**Obese + K-111**
Glycolysis (µmol/g dw/min)	0.62±0.11	0.80±0.18	0.79±0.20	1.24±0.26
Glucose oxidation (µmol/g dw/min)	0.11±0.03	0.07±0.02	0.13±0.03	0.09±0.03
Palmitate oxidation (nmol/g dw/min)	5.69±1.84	6.32±1.70	5.64±1.80	2.37±0.69
	Reperfusion			
	Control	**Obese**	**Control + K-111**	**Obese + K-111**
Glycolysis (µmol/g dw/min)	$1.48±0.24^b$	$1.27±0.12^a$	$1.84±0.21^c$	$1.65±0.21^a$
Glucose oxidation (µmol/g dw/min)	0.15±0.05	0.16±0.05	0.18±0.04	0.28±0.10
Palmitate oxidation (nmol/g dw/min)	7.95±2.15	$12.71±3.78^a$	9.32±1.55	$8.25±1.51^a$

dw **– dry weight**
[a]**p<0.05;** [b]**p<0.01;** [c]**p<0.001 vs. baseline value**
Glycolytic flux rate: n = 5-7 hearts per group
Glucose Oxidation: n = 4-6 hearts per group
Palmitate oxidation n = 4-8 hearts per group

In the presence of normal levels of insulin and FA neither obesity nor chronic K-111 treatment influenced myocardial glycolytic flux, glucose oxidation or palmitate oxidation rates (Table 6.3) prior to and following global ischaemia. Notably, glycolytic

flux rates of all the groups and palmitate oxidation rates of the obese and the obese

treated groups were elevated from baseline during the reperfusion phase.

6.3.3.2 High insulin and FA concentrations in the perfusate
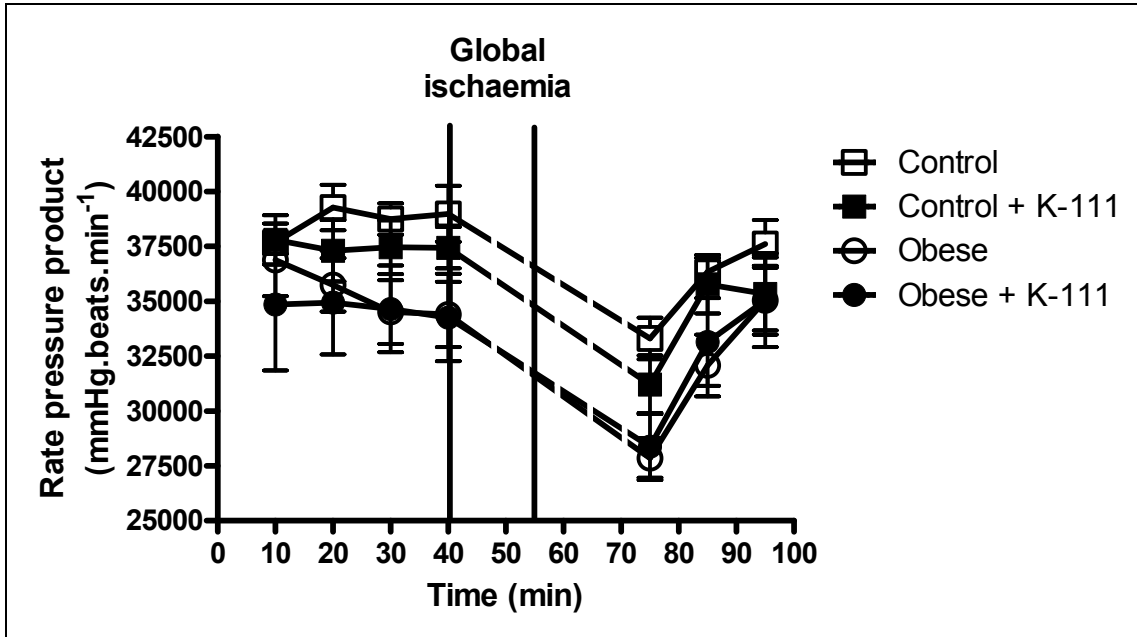
Figure 6.7: Rate pressure product obtained from isolated rat hearts perfused with glucose (10mM) and high insulin (50µIU/ml) and FA (1.5mM) concentrations. Hearts were subjected to 15 minutes global ischaemia followed by 30 minutes reperfusion.
N= 4-8 hearts per group

Neither obesity nor chronic PPARα agonist treatment influenced the myocardial rate pressure product during baseline and reperfusion (Figure 6.7). In addition there was no difference in the percentage RPP recovery between the four groups (data not shown).

Table 6.4: Average normoxic baseline and reperfusion myocardial substrate metabolism obtained from isolated rat hearts perfused with glucose (10mM) and high insulin (50µIU/ml) and FA (1.5mM) concentrations. Hearts were subjected to 15 minutes normothermic total global ischaemia. Baseline samples were collected after 30 and 40 minutes of normoxic perfusion. Reperfusion samples were collected after 20 and 30 minutes post-ischaemic perfusion.

	Baseline			
	Control	**Obese**	**Control + K-111**	**Obese + K-111**
Glycolysis (µmol/g dw/min)	0.29±0.04	0.38±0.06	0.32±0.06	0.56±0.11
Glucose oxidation (µmol/g dw/min)	0.07±0.01	0.05±0.01	0.07±0.01	0.10±0.02
Palmitate oxidation (nmol/g dw/min)	25.01±5.31	35.49±2.92	38.01±6.41	20.07±2.90*[#]
	Reperfusion			
	Control	**Obese**	**Control + K-111**	**Obese + K-111**
Glycolysis (µmol/g dw/min)	0.86±0.14[a]	0.91±0.13[a]	1.90±0.34*[b]	1.38±0.20[b]
Glucose oxidation (µmol/g dw/min)	0.15±0.05	0.12±0.03[a]	0.09±0.02	0.15±0.04
Palmitate oxidation (nmol/g dw/min)	104.32±12.96[b]	81.59±18.86[d]	100.09±10.43[c]	83.33±4.03[c]

dw – dry weight; *HCD* – high caloric diet
*p<0.05 vs. similar dietary untreated group; [#]p<0.05 vs. control treated group;
[a]p<0.05; [b]p<0.01; [c]p<0.001; [d]p =0.05 vs. baseline value
Glycolytic flux rate: n = 6- 8 hearts per group
Glucose Oxidation: n = 5 hearts per group
Palmitate oxidation n = 4-7 hearts per group

The HCD had no impact on the rate of myocardial substrate metabolism. This was reflected by similar myocardial glycolytic flux, glucose oxidation and palmitate oxidation rates compared to the control group when hearts were perfused with elevated levels of insulin and FA (Table 6.4). The impact of K-111 treatment on myocardial substrate metabolism was dependent on the presence or absence of HCD feeding. While HCD fed K-111 treated rats had lower pre-ischaemic palmitate oxidation rates compared to the HCD fed untreated animals, similar differences were found when being compared to the control K-111 treated group. During reperfusion, comparable rates of substrate metabolism were observed between the various groups. The only exception was with the control K-111 treated group that achieved significantly higher glycolytic flux rates compared to the untreated control group.

Reperfusion had a marked effect on the pattern of substrate metabolism with the rates of glycolytic flux, and palmitate oxidation for the various groups being higher during reperfusion compared to baseline values. Similarly glucose oxidation rates were higher during reperfusion compared to baseline values in the obese group, while a similar non-significant trend was observed in the other experimental groups.

6.3.3.3 Comparisons of myocardial function and substrate metabolism between control and obese animals perfused with both 10mM glucose and normal or high concentrations of insulin and FA
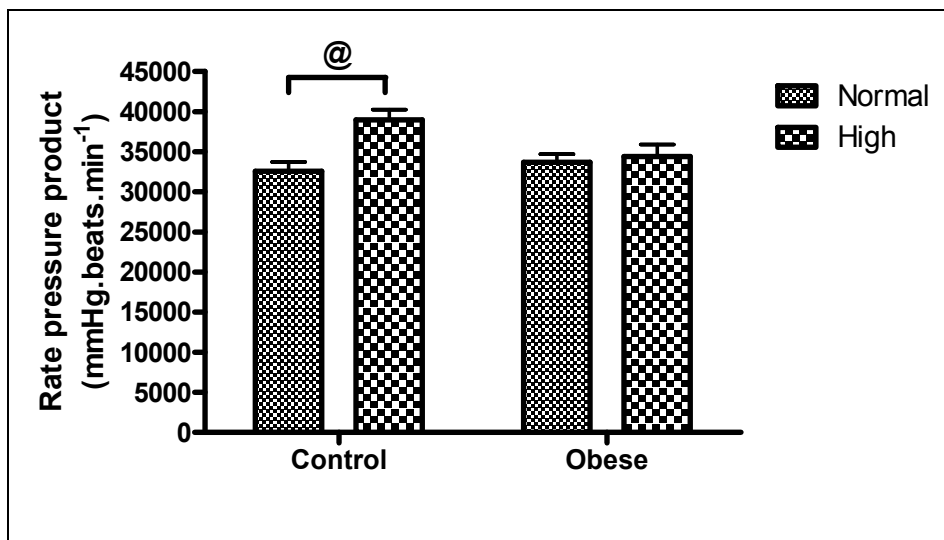
Figure 6.8: Pre-ischaemic rate pressure product obtained from isolated rat hearts perfused with glucose (10mM) and normal (30µIU/ml insulin and 0.7mM FA) or high (50µIU/ml insulin and 1.5mmol/l FA) insulin and FA concentrations. Measurements were taken after 40 minutes normoxic perfusion.
@p<0.01
n = 4-8

Hearts from control animals perfused with elevated insulin and FA concentrations had significantly greater baseline RPP's compared to hearts from control animals perfused with the low insulin and FA concentrations (38984.25±1281.63 vs. 32558.50±1162.01 mmHg.beats.min^{-1}, p<0.01) (Figure 6.8). In contrast, the presence of elevated insulin and FA in the perfusate did not cause the baseline RRP values to differ from those obtained when these hearts were perfused with normal insulin and FA concentrations.

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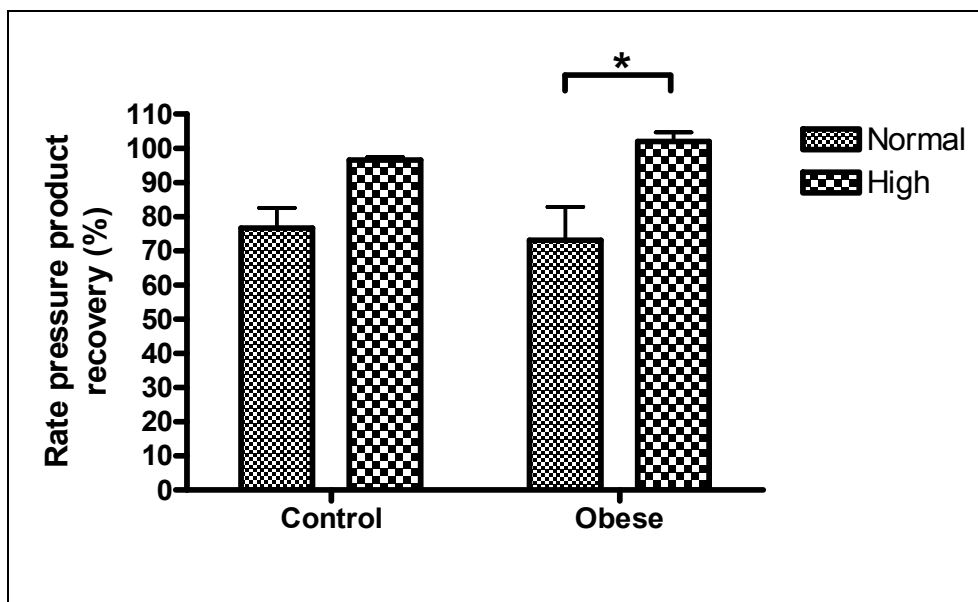
Figure 6.9a: Post-ischaemic rate pressure product percentage recovery obtained from isolated rat hearts perfused with glucose (10mM) and normal (30µIU/ml insulin and 0.7mM FA) or high (50µIU/ml insulin and 1.5mmol/l FA) insulin and FA concentrations. Hearts were subjected to 15 minutes global ischaemia followed by 30 minutes reperfusion. Functional values were taken at the end of the pre- and post-ischaemic periods.

*p<0.01

n = 4-8
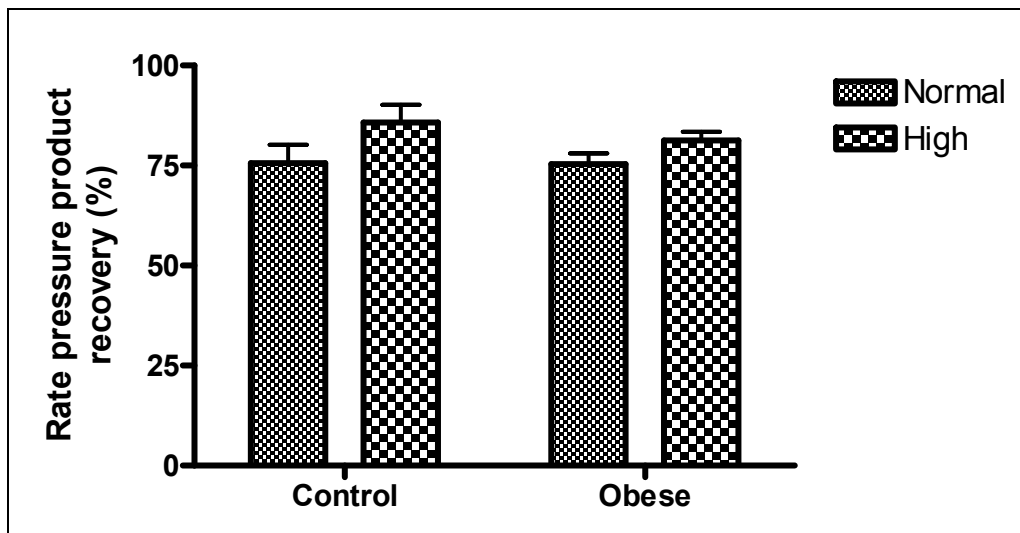
Figure 6.9b: Post-ischaemic rate pressure product percentage recovery obtained from isolated rat hearts perfused with glucose (10mM) and normal (30µIU/ml insulin and 0.7mM FA) or high (50µIU/ml insulin and 1.5mmol/l FA) insulin and FA concentrations after 20 minutes reperfusion. Hearts were subjected to 15 minutes global ischaemia followed by a reperfusion period. Functional values were taken at the end of the pre-ischaemic and after 15 minutes of reperfusion.

n = 4-8

In the control hearts the recovery in RPP was not influenced by the concentration of insulin and FA (Figure 6.9a) present in the perfusate. However in the obese group, high insulin and FA levels enabled these hearts to achieve a significantly better RPP percentage recovery compared to hearts from the same group perfused with low insulin and FA concentrations (102.00±2.64 vs. 73.12±9.71 %, p<0.05). While this may suggest that hearts from obese animals respond better to ischaemia in the presence of elevated insulin and FA concentrations, the improvement in functional recovery observed in the obese group has to be interpreted with caution. The raw RPP percentage recovery data for the low insulin + FA obese group was as follows: 58.46, 85.02, 31.87, 52.01, 98.01, 89.33, 97.16. Three of these hearts obtained significantly lower RPP's recoveries primarily due to unstable sinus rhythm

(ventricular arrhythmia's) during the last 10 minutes of reperfusion which contributed to a lower RPP values. Figure 6.9b demonstrates that after 20 minutes reperfusion, *prior to the development of these rhythm disturbances in some of the hearts*, the RPP recovery was comparable between the obese groups.
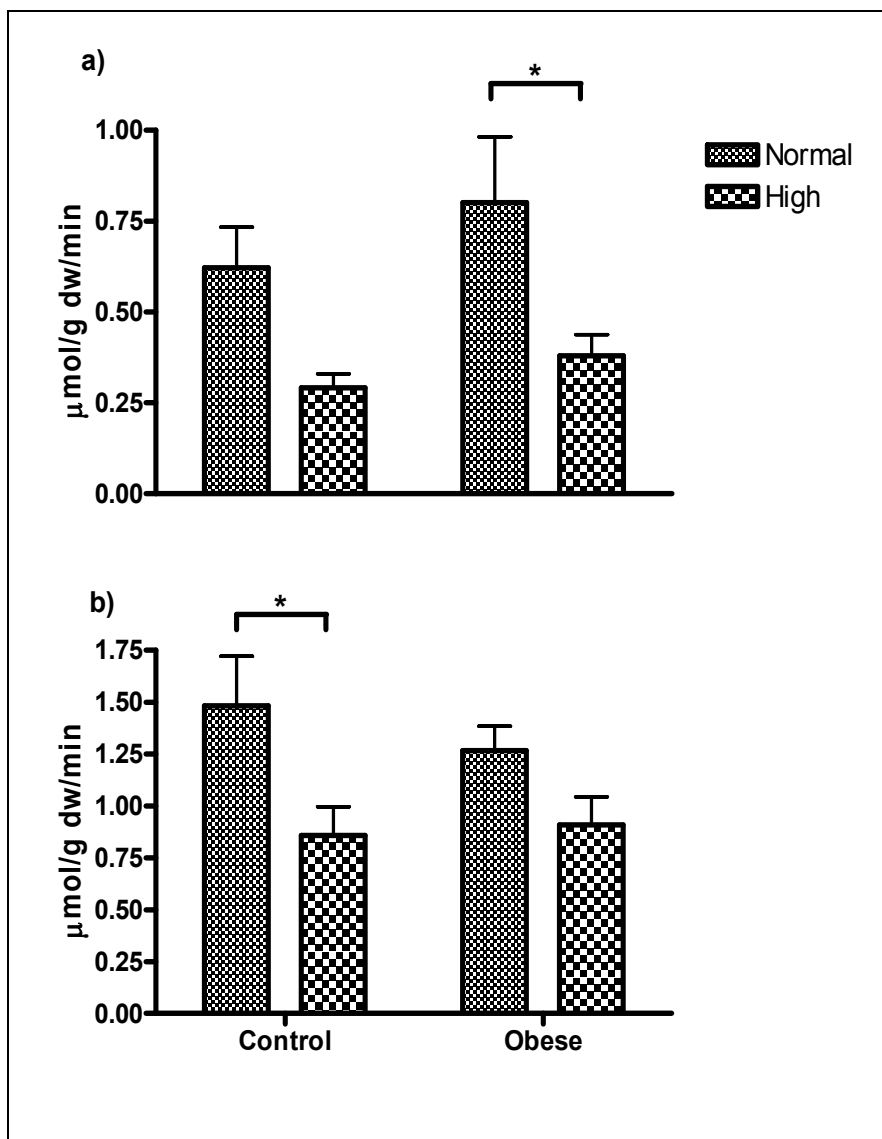
Figure 6.10: Average a) baseline and b) reperfusion myocardial glycolytic flux rates obtained from isolated rat hearts perfused with glucose (10mM) and either normal (30µIU/ml insulin and 0.7mM FA) or high (50µIU/ml insulin and 1.5mmol/l FA) insulin and FA concentrations. Hearts were subjected to 15 minutes global ischaemia followed by 30 minutes reperfusion. Baseline samples were collected after 30 and 40 minutes of normoxic perfusion. Reperfusion samples were collected after 20 and 30 minutes post-ischaemic perfusion.

*p<0.05

n = 5-7 hearts per group

Although there was a tendency for baseline glycolytic flux rates in the control groups to be influenced by the different insulin and FA concentrations present in the

perfusate, this proved to be insignificant (Figure 6.10a). However during the reperfusion phase, elevated insulin and FA present in the perfusate resulted in these hearts generating lower glycolytic flux rates compared to similar hearts perfused in the presence of normal insulin and FA concentrations (0.86 ± 0.14 vs. 1.48 ± 0.24 µmol/g dw/min; $p < 0.05$) (Figure 6.10b).

In the presence of high insulin and FA levels, hearts from the obese group had significantly lower baseline myocardial glycolytic flux rates compared to those hearts perfused with normal insulin and FA concentrations (0.38 ± 0.06 vs. 0.80 ± 0.18 µmol/g dw/min, $p < 0.05$) (Figure 6.10a). During reperfusion, the presence of high insulin and FA levels in the perfusate did not significantly affect myocardial glycolytic flux rates in either of the obese groups (Figure 6.10b).

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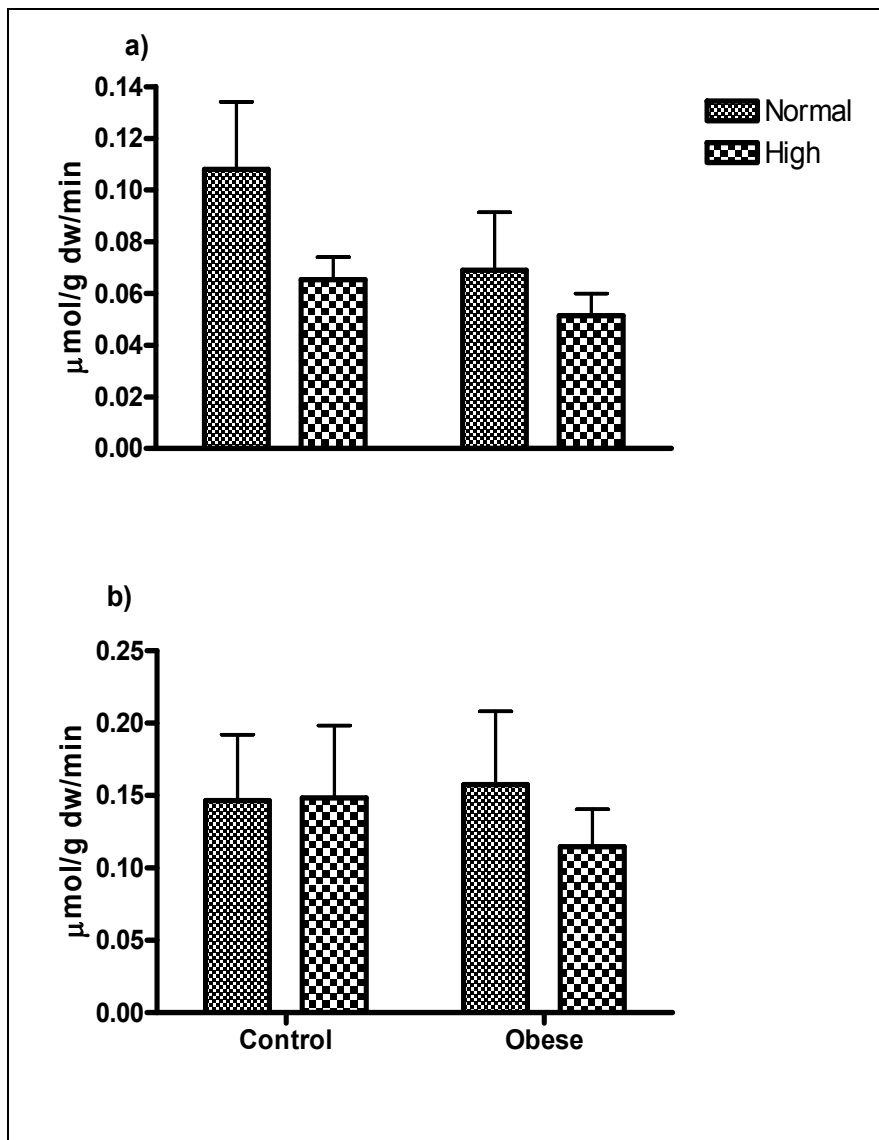
Figure 6.11: Average a) baseline and b) reperfusion myocardial glucose oxidation rates obtained from isolated rat hearts perfused with glucose (10mM) and either normal (30µIU/ml insulin and 0.7mM FA) or high (50µIU/ml insulin and 1.5mmol/l FA) insulin and FA concentrations. Hearts were subjected to 15 minutes global ischaemia followed by 30 minutes reperfusion. Baseline samples were collected after 30 and 40 minutes of normoxic perfusion. Reperfusion samples were collected after 20 and 30 minutes post-ischaemic perfusion.

n = 4-5 hearts per group

Increasing the concentration of insulin and FA in the perfusate of hearts from control and obese animals did not influence their glucose oxidation rates either prior to (Figure 6.11a) or during reperfusion following global ischaemia (Figure 6.11b).
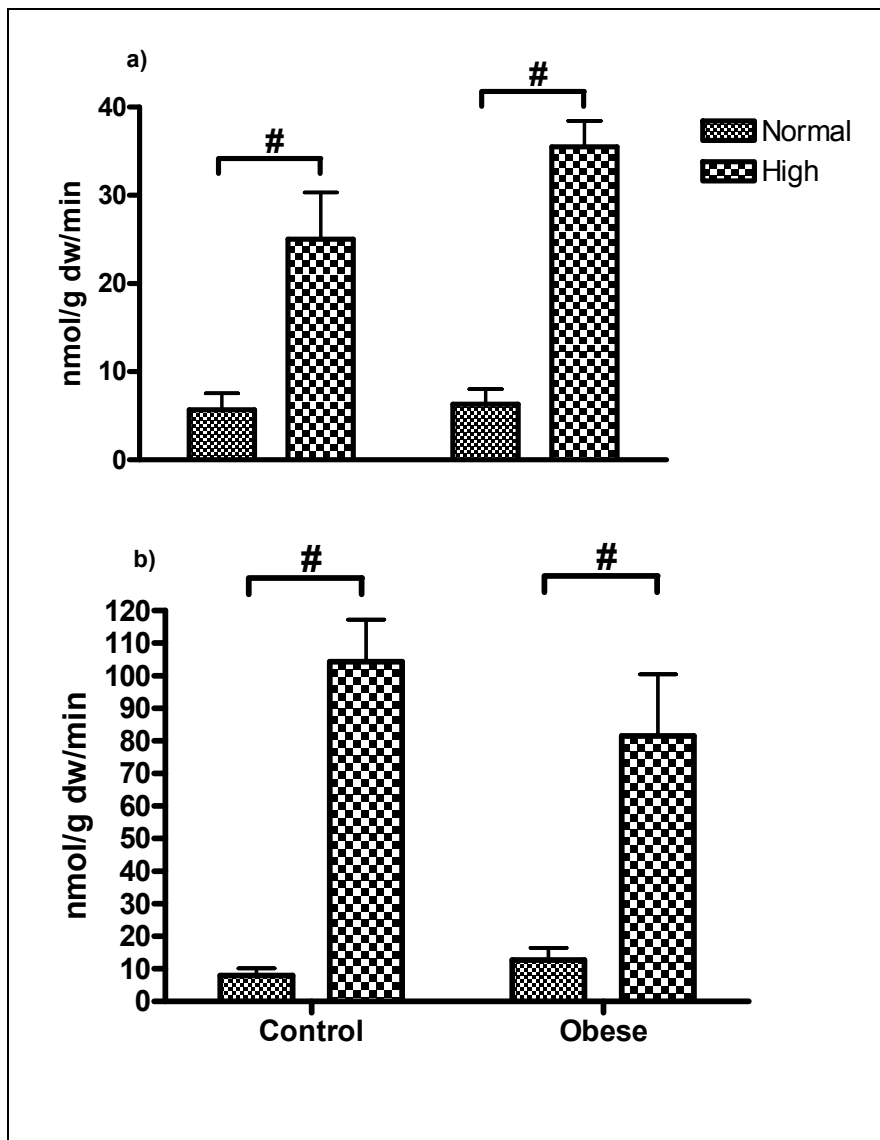
Figure 6.12: Average a) baseline and b) reperfusion myocardial palmitate oxidation rates obtained from isolated rat hearts perfused with glucose (10mM) and either normal (30µIU/ml insulin and 0.7mM FA) or high (50µIU/ml insulin and 1.5mmol/l FA) insulin and FA concentrations. Hearts were subjected to 15 minutes global ischaemia followed by 30 minutes reperfusion. Baseline samples were collected after 30 and 40 minutes of normoxic perfusion. Reperfusion samples were collected after 20 and 30 minutes post-ischaemic perfusion.

[#]p<0.001

n = 4-8

High insulin and FA levels present in the perfusate had an substantial impact on

myocardial palmitate oxidation rates by significantly increasing these values during

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baseline and reperfusion in both the control (Baseline: 3.25±1.01 vs. 20.01±4.24 nmol/g dw/min, $p<0.0001$; Reperfusion: 4.55±1.23 vs. 83.46±10.37 nmol/g dw/min, $p<0.0001$) and obese groups (Baseline: 3.61±0.97 vs. 28.39±2.34 nmol/g dw/min, $p<0.001$; Reperfusion: 7.26±2.16 vs. 65.28±15.09 nmol/g dw/min, $p<0.001$) (Figure 6.12a, b).

6.3.4 E*x vivo* pre- and post-ischaemic myocardial functional parameters

6.3.4.1 Normal insulin and FA concentrations in the perfusate

Table 6.5: The effect of obesity and chronic K-111 treatment on myocardial functional parameters assessed prior to and following 40 minutes of regional ischaemia when perfused with glucose (10mM) and normal insulin (30µIU/ml) and FA (0.7mM) concentrations

	Baseline data			
	Control **n = 11**	**Obese** **n = 11**	**Control** **K-111** **n= 11**	**Obese** **K-111** **n = 11**
Aortic output (ml/min)	49.3±1.3	46.4±1.8	48.9±1.0	43.8±2.0
Coronary flow (ml/min)	19.9±1.0	19.3±1.4	17.6±0.7	17.7±0.4
Cardiac output (ml/min)	69.1±1.3	65.7±2.7	66.6±1.3	61.5±2.3
SP (mmHg)	99.1±1.0	96.0±1.0	98.4±1.0	95.4±1.4
DP (mmHg)	61.4±1.4	61.6±1.8	59.6±1.2	62.2±1.1
Heart rate (bpm)	282±5	295±6	275±6	287±5
Work total (W)	15.3±0.3	14.1±0.6	14.6±0.3	13.1±0.6
dP/dt_{max} (mmHg/s)	28.4±1.9	26.8±1.8	25.0±0.9	27.1±1.4
dP/dt_{min} (mmHg/s)	-21.1±1.0	-24.30±0.8*	-22.5±1.0	-21.2±0.9[&]
	Reperfusion data			
	Control **n = 7/8**	**Obese** **n = 10**	**Control** **K-111** **n = 10**	**Obese** **K-111** **n = 9**
Aortic output (ml/min)	3.4±1.6	0.0±0.0	4.9±2.0	1.6±1.3
Coronary flow (ml/min)	19.7±2.0	19.7±3.2	23.1±2.2	19.0±2.2
Cardiac output (ml/min)	23.1±3.0	19.7±3.2	28.0±3.6	20.6±2.6
SP (mmHg)	76.3±5.5	68.1±4.1	82.2±2.4	73.7±4.2
DP (mmHg)	56.4±5.7	53.9±3.8	64.8±2.3	58.2±3.4
Heart rate (bpm)	294±16	285±13	299±12	296±18
Work total (W)	3.9±0.8	3.8±1.0	5.2±0.7	3.6±0.6
dP/dt_{max} (mmHg/s)	13.1±2.0	9.3±0.8	15.3±1.0	11.1±0.9[$]
dP/dt_{min} (mmHg/s)	-7.7±0.9	-8.3±0.5	-9.6±0.8	-8.0±0.7

DP – aortic diastolic pressure; *dP/dt* – delta aortic pressure over delta time; *SP* – aortic systolic pressure

*$p<0.05$ vs. control; [$]$p<0.05$ vs. control + K-111; [&]$p<0.05$ vs. obese

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Neither the HCD nor chronic K-111 treatment had any effect on either of the functional parameters assessed prior to and following myocardial regional ischaemia, when isolated working hearts were perfused with normal insulin and FA concentrations (Table 6.5). The exception was aortic dP/d$t_{max/min}$. As the aortic outputs between all these groups were comparable, this finding has no immediate functional implication.

6.3.4.2 High insulin and FA concentrations in the perfusate

Table 6.6: The effect of obesity and chronic K-111 treatment on myocardial functional parameters assessed prior to and following 40 minutes of regional ischaemia when perfused with glucose (10mM) and high insulin (50µIU/ml) and FA (1.5mM) concentrations

Baseline data				
	Control **n = 10**	**Obese** **n = 12**	**Control K-111** **n = 11**	**Obese K-111** **n = 10**
Aortic output (ml/min)	48.3±1.9	45.7±2.3	47.0±1.5	47.5±1.3
Coronary flow (ml/min)	19.3±1.1	20.3±1.1	18.9±0.7	20.1±1.0
Cardiac output (ml/min)	67.6±2.6	66.0±3.1	65.9±1.8	67.6±2.0
SP (mmHg)	99.7±1.4	99.8±1.0	97.8±1.3	98.8±1.2
DP (mmHg)	61.8±0.9	60.1±1.2	60.7±1.4	59.9±1.7
Heart rate (bpm)	279±6	269±8	284±7	283±8
Work total (W)	15.1±0.6	14.8±0.8	14.4±0.5	14.9±0.5
dP/dt_{max} (mmHg/s)	30.6±1.5	30.5±1.8	28.6±1.7	26.5±1.1
dP/dt_{min} (mmHg/s)	-18.3±1.0	-22.0±0.95*	-22.6±0.9*	-22.8±1.3
Reperfusion data				
	Control **n = 8**	**Obese** **n = 11**	**Control K-111** **n = 9**	**Obese K-111** **n = 9**
Aortic output (ml/min)	10.7±3.8	8.0±1.9	7.2±1.8	9.9±3.3
Coronary flow (ml/min)	21.8±1.1	24.5±2.0	21.8±2.7	22.8±3.0
Cardiac output (ml/min)	32.4±4.5	32.5±3.4	29.0±3.9	32.7±5.9
SP (mmHg)	89.0±1.5	88.8±0.8	81.4±6.7	81.6±4.6
DP (mmHg)	67.4±1.8	65.7±1.4	59.9±5.3	60.8±4.2
Heart rate (bpm)	284±10	282±10	272±20	279±18
Work total (W)	6.5±0.9	6.5±0.7	5.7±0.9	6.2±1.2
dP/dt_{max} (mmHg/s)	18.3±1.7	16.6±1.7	16.6±2.0	15.9±1.7
dP/dt_{min} (mmHg/s)	-9.2±0.6	-11.3±1.1	-9.8±0.9	-9.6±1.4

DP – aortic diastolic pressure; *dP/dt* – delta aortic pressure over delta time; *SP* – aortic systolic pressure

*p<0.05 vs. control

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When hearts were perfused with elevated levels of insulin and FA, a similar myocardial functional profile was noted amongst the various groups as was previously observed in the presence of normal insulin and FA levels. Again no differences between any of the functional parameters existed among the various groups, with the exception of the aortic dP/dt_{min} values (Table 6.6). Although differences in aortic dP/dt_{min} exist, these findings have no immediate functional implications.

6.3.5 Myocardial susceptibility to ischaemia/reperfusion injury

6.3.5.1 Normal insulin and FA concentrations in the perfusate
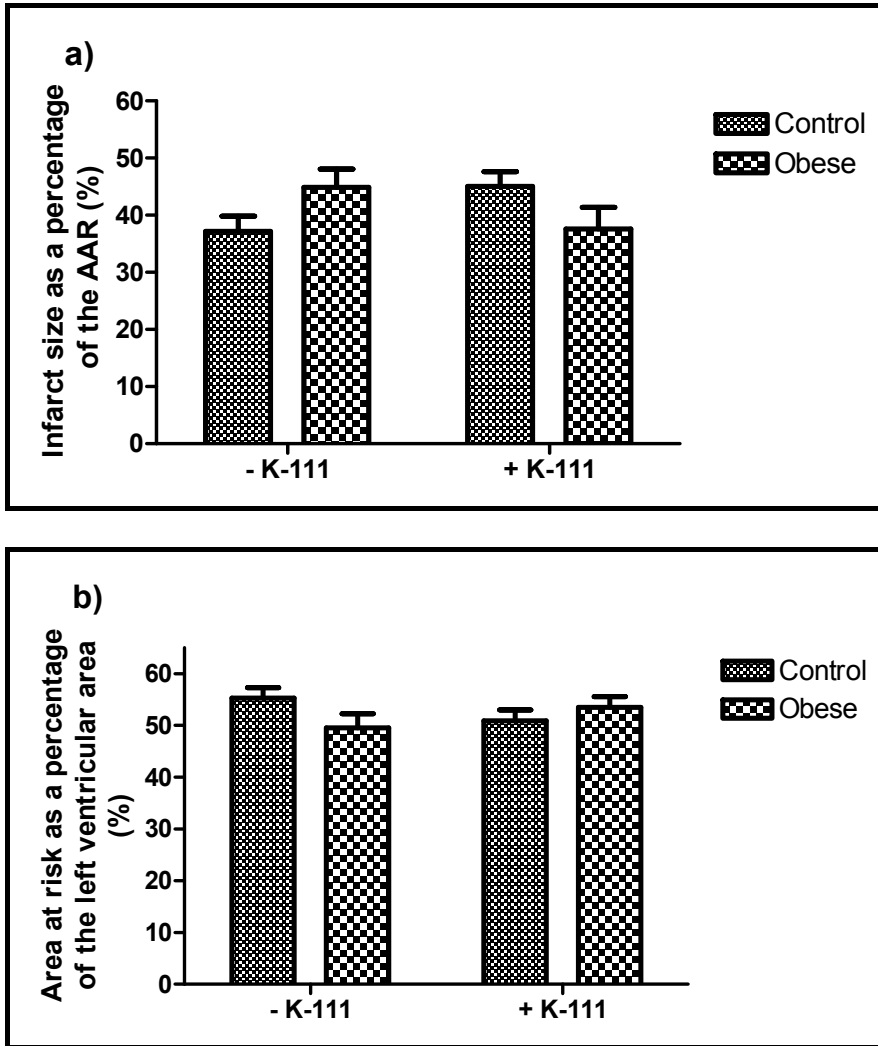
Figure 6.13: a) Myocardial infarct size expressed as a percentage of the area at risk and b) the area at risk expressed as a percentage of the LV area. Hearts perfused with glucose (10mM) and normal insulin (30µIU/ml) and FA (0.7mM) concentrations were subjected to 40 minutes regional ischaemia followed by 60 minutes reperfusion.

n = 11 hearts per group

Table 6.7: Post-ischaemic functional recovery assessed in working hearts in the presence of 10mM glucose and normal insulin and FA concentrations

	Control n = 8	Obese n = 10	Control + K-111 n = 10	Obese + K-111 n = 9
AO (%)	7.23±3.39	0.00±0.00	9.64±3.85	3.30±2.80
CO (%)	34.07±4.64	27.00±4.35	41.66±4.54	33.05±3.60
Total work (%)	25.97±5.06	26.88±6.65	35.48±4.24	26.76±3.78

CO – cardiac output; *AO* – Aortic output

Obesity and chronic K-111 treatment had no impact of the measured indices of ischaemia/reperfusion injury when hearts from these groups were perfused with normal insulin and FA concentrations. This was reflected by comparable infarct sizes (with similar at risk areas) (Figure 6.13 a, b) and similar functional recoveries (aortic output, cardiac output, total work) (Table 6.7) between the various groups.

6.3.5.2 High insulin and FA concentrations in the perfusate
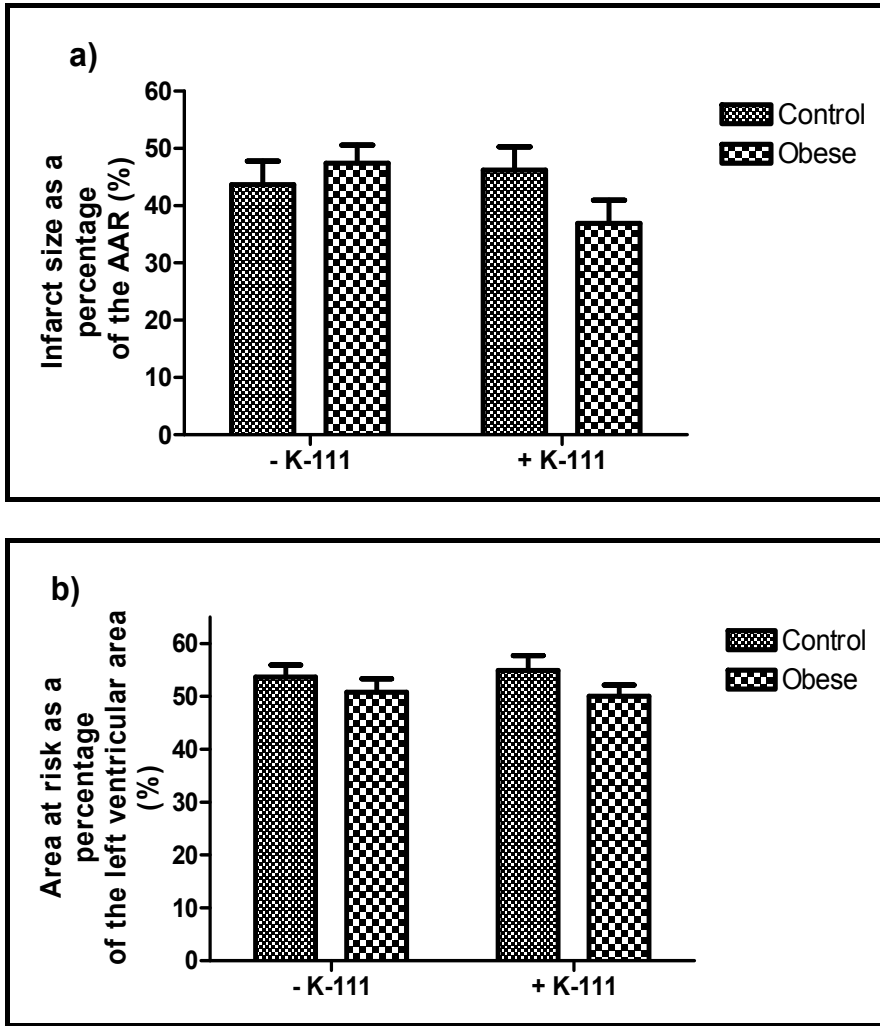
Figure 6.14: a) Myocardial infarct size expressed as a percentage of the area at risk and b) The area at risk expressed as a percentage of left ventricular area. Hearts perfused with glucose (10mM) and high insulin (50μIU/ml) and FA (1.5mM) concentrations were subjected to 40 minutes regional ischaemia followed by 60 minutes reperfusion.

n = 10-12 hearts per group

Table 6.8: Post-ischaemic functional recovery assessed in working hearts in the presence of 10mM glucose and high insulin and FA concentrations

	Control **n = 8**	**Obese** **n = 11**	**Control** **+ K-111** **n = 9**	**Obese** **+ K-111** **n = 9**
AO (%)	21.47±6.92	17.28±4.12	15.89±3.80	19.91±5.93
CO (%)	47.17±5.51	37.43±2.35	37.18±7.53	47.40±7.26
Total work (%)	42.52±5.30	43.99±3.92	37.53±6.29	41.05±6.93

CO – cardiac output; *AO* – Aortic output

Neither obesity nor chronic K-111 treatment influenced post-ischaemic outcomes when hearts from each group were perfused with elevated insulin and FA levels. Both myocardial infarct size, the area at risk (Figure 6.14a,b) and functional recoveries (Aortic output, cardiac output and total work) (Table 6.8) were comparable between the various groups.

6.3.5.3 Comparisons of indices of ischaemia/reperfusion injury in the control and obese groups perfused with both 10mM glucose and normal and high insulin and FA concentrations
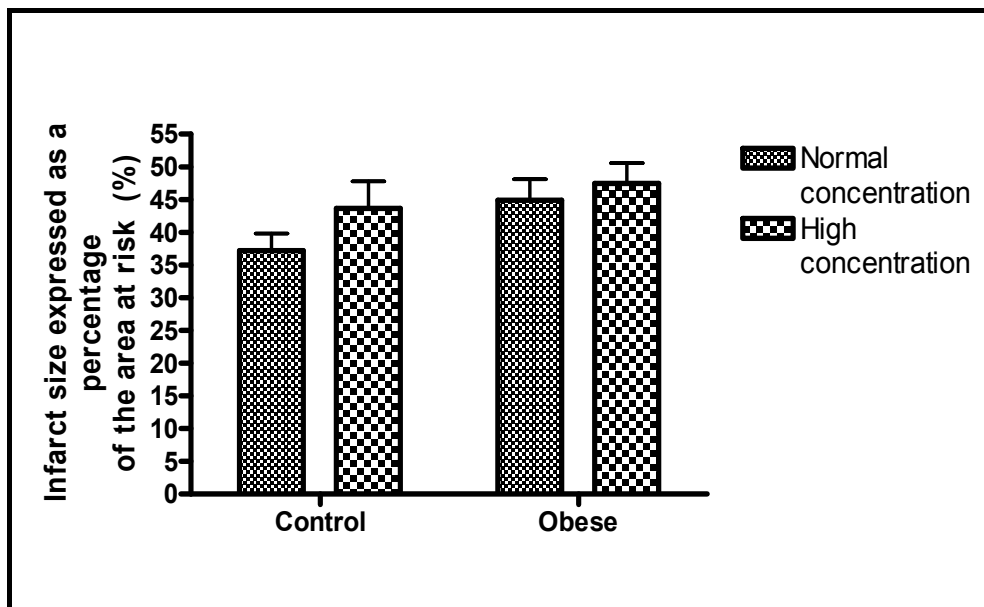
Figure 6.15: Myocardial infarct size expressed as a percentage of the area at risk. Hearts perfused with either glucose and normal (30µIU/ml insulin and 0.7mM FA) or high (50µIU/ml insulin and 1.5mmol/l FA) insulin and FA concentrations were subjected to 40 minutes regional ischaemia followed by 60 minutes reperfusion.

n = 10-12 hearts per group

The size of the myocardial infarction did not differ within groups when comparisons were made between control and obese hearts perfused with normal and high insulin and FA concentrations (Figure 6.15).

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Table 6.9: Comparison of the post-ischaemic percentage functional recovery attained in the presence of 10mM glucose and normal and high insulin and FA concentrations, assessed in working hearts from control and obese animals

	Control		Obese	
	Normal **n = 8**	**High** **n = 10**	**Normal** **n = 8**	**High** **n = 11**
AO (%)	7.23±3.39	21.47±6.92	0.00±0.00	17.28±4.12[@]
CO (%)	34.07±4.64	47.17±5.51	27.00±4.35	37.43±2.35
Total work (%)	25.97±5.06	42.52±5.30	26.88±6.65	43.99±3.92*

CO – cardiac output; *AO* – aortic output

[@]p<0.01, *p<0.05 vs. obese normal

When comparing the effect of the different perfusion conditions on the myocardial functional recovery, it was intriguing to observe how well hearts from obese animals responded to elevated concentrations of insulin and FA in the perfusion medium. In the presence of elevated insulin and FA concentrations, hearts from obese animals achieved significantly greater aortic output and total work recoveries compared to similar hearts perfused with normal insulin and FA concentrations (Table 6.9). Although a similar trend was seen in the control groups, these effects were not significant.

6.3.6 Comparisons of myocardial substrate metabolism and infarct size between hearts from control and obese animals when simulating the group specific relevant *in vivo* conditions

Table 6.10: Comparison of myocardial glycolytic flux, glucose oxidation and palmitate oxidation rates in isolated hearts from control and obese animals in the presence of 10mM glucose and simulated *in vivo* concentrations of insulin and FA

	Baseline	
	Control	**Obese**
Glycolysis (µmol glucose/g dw/min)	0.62±0.11	0.38±0.06
Glucose oxidation (µmol glucose/g dw/min)	0.11±0.03	0.05±0.01*
Palmitate oxidation (nmol palmitate/g dw/min)	3.25±1.05	28.39±2.34[#]
	Reperfusion	
	Control	**Obese**
Glycolysis (µmol glucose/g dw/min)	1.48±0.24	0.91±0.13
Glucose oxidation (µmol glucose/g dw/min)	0.15±0.05	0.12±0.03
Palmitate oxidation (nmol palmitate/g dw/min)	4.55±1.23	65.28±15.09[#]

dw – dry weight

[#]$p<0.001$, *$p=0.057$

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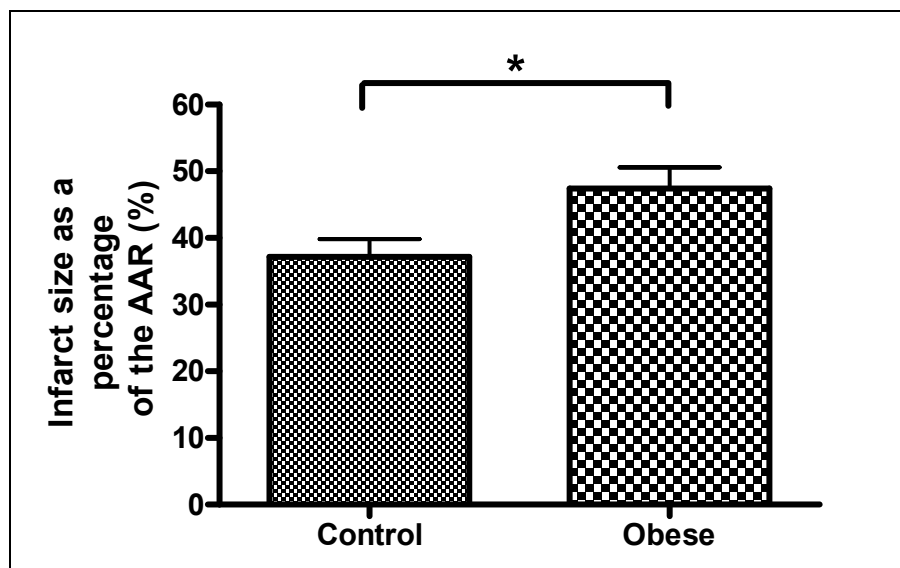
Figure 6.16: Myocardial infarct size expressed as a percentage of the area at risk obtained from hearts isolated from control rats perfused with glucose (10mM) and normal insulin and FA concentrations (insulin (30µIU/ml) and FA (0.7mM)) and hearts from obese animals perfused with glucose (10mM) and high insulin and FA concentrations (insulin (50µIU/ml) and FA (1.5mmol/l)). Hearts were subjected to 40 minutes regional ischaemia followed by 60 minutes reperfusion. Administration of substrate and insulin was done throughout the perfusion protocol
*p<0.05
n = 10-12 hearts per group

When comparisons between the control and obese groups were made simulating the specific *in vivo* environment that the heart would be expected to be exposed to, significant observations were made. In the presence of simulated *in vivo* insulin and FA concentrations, baseline myocardial glucose oxidation rates were reduced (0.05±0.01 vs. 0.11±0.03 µmol/g dw/min, p<0.057) whereas palmitate oxidation rates were increased (28.39±2.34 vs. 3.25±1.01 nmol/g dw/min, p<0.001) in the obese group compared to the control group (Table 6.10). Baseline glycolytic flux rates were comparable between the two groups. While no significant differences existed between the myocardial reperfusion glycolytic flux and glucose oxidation rates

between the control and obese groups, reperfusion palmitate oxidation rates were significantly elevated in the obese group (65.28±15.09 vs. 4.55±1.23   nmol/g dw/min) relative to the control group.

Of particular interest was the increase in the size of infarction observed in the obese group compared to the control group (47.44±3.13 vs. 37.17±2.63 %, p<0.05) (Figure 6.16). It is worth mentioning that comparisons of myocardial infarct size specifically assessed with the unpaired Students t-test were not significant between the control and obese group when perfused with comparable perfusion conditions (i.e. unpaired Students t-test performed on the control and obese groups in Figures 6.13 a and 6.14 a).

6.3.7 Measurements of mitochondrial function

6.3.7.1 Substrate: glutamate

Table 6.11: The effect of obesity and chronic K-111 treatment on measurements of mitochondrial respiration and susceptibility to anoxia induced mitochondrial dysfunction in the presence of glutamate. Following state 3 and 4 respiration, mitochondria were made anoxic for 25 minutes and reoxygenated for the measurement of state 3 respiration.

	Control n = 11	Obese n = 9	Control + K-111 n = 10	Obese + K-111 n = 12
ADP/O (nmol ADP/nAtoms oxygen)	2.65±0.19	2.67±0.17	2.56±0.12	2.80±0.15
RCI (State3/State4)	7.17±0.73	7.39±0.43	6.55±0.65	6.88±0.44
State 2 Resp. (nAtoms oxygen/min/mg)	21.50±1.94	17.91±0.96	19.67±1.28	19.31±1.48
State 3 Resp. (nAtoms oxygen/min/mg)	145.10±11.22	146.67±9.087	138.27±12.65	147.39±11.46
State 4 Resp. (nAtoms oxygen/min/mg)	21.41±2.16	19.93±0.86	21.98±1.95	21.93±1.68
Oxphos rate (nmol ADP/min/mg)	390.87±42.62	388.78±32.42	362.39±46.55	410.34±44.85
State 3 % recovery during reoxygenation after anoxia (%)	54.52±5.87	53.62±5.35	47.24±5.77	53.79±4.13

ADP/O **– adenosine diphosphate phosphorylation/oxygen uptake;** *Oxphos* **– oxidative phosphorylation rate;** *Resp* **– respiration**

In the presence of glutamate as substrate, indices of mitochondrial respiration in control and obese untreated and treated animals were similar (Table 6.11). Neither obesity nor chronic K-111 treatment impacted on the amount of ADP utilized per atom of oxygen, or the coupling of the mitochondria during oxidative respiration. Lastly, neither obesity nor chronic K-111 treatment affected ventricular mitochondrial susceptibility to anoxia/reoxygenation.

6.3.7.2 Palmitoyl – L – carnitine as the oxidative substrate

Table 6.12. The effect of obesity and chronic K-111 treatment on measurements of mitochondrial respiration and susceptibility to anoxia induced mitochondrial dysfunction in the presence of palmitoyl – L – carnitine. Following baseline state 3 and 4 respiration mitochondria were made anoxic for 25 minutes and reoxygenated for the measurement of state 3 respiration.

	Control n = 8	Obese n = 10	Control + K-111 n = 9	Obese + K-111 n = 11
ADP/O (nmol ADP/nAtoms oxygen)	2.44±0.22	2.49±0.20	2.30±0.14	2.61±0.93
RCI (State3/State4)	5.67±0.32	6.27±0.27	5.75±0.51	6.68±0.46
State 2 Resp. (nAtoms oxygen/min/mg)	30.94±3.90	29.56±2.38	28.84±3.24	28.33±3.76
State 3 Resp. (nAtoms oxygen/min/mg)	204.12±17.95	211.30±13.37	201.07±25.89	204.69±18.01
State 4 Resp. (nAtoms oxygen/min/mg)	36.50±3.48	33.91±2.05	35.15±2.70	30.99±2.16
Oxphos rate (nmol ADP/min/mg)	491.34±57.43	523.67±50.52	464.17±67.59	551.06±79.04
State 3 % recovery during reoxygenation after anoxia (%)	21.69±6.41	25.52±4.05	26.49±5.67	27.97±4.74

ADP/O **– adenosine diphosphate phosphorylation/oxygen uptake;** *Oxphos* **– oxidative phosphorylation rate;** *Reox* **– reoxygenation;** *Resp* **– respiration.**
@p<0.01 vs. control+K-111

Ventricular mitochondrial respiration parameters were unchanged by the HCD and chronic K-111 treatment in the presence of palmitoyl – L – carnitine as substrate (Table 6.12). Mitochondrial ADP/O ratios, RCI values and susceptibility to anoxia/reoxygenation were comparable between the groups.

6.3.7.3 Comparisons between glutamate and palmitoyl – L – carnitine on mitochondrial respiration and anoxic injury susceptibility

Table 6.13: Comparison of the effects of glutamate and palmitoyl – L – carnitine on measurements of respiration and anoxia injury susceptibility in ventricular mitochondria isolated from control and obese animals. Following baseline state 3 and 4 respiration mitochondria were made anoxic for 25 minutes and reoxygenated for the measurement of state 3 respiration

	Glutamate		Palmitoyl-L-carnitine	
	Control n = 11	Obese n = 9	Control n = 8	Obese n = 10
ADP/O (nmol ADP/nAtoms oxygen)	2.65±0.19	2.67±0.17	2.44±0.22	2.49±0.20
RCI (State3/State4)	7.17±0.73	7.39±0.42	5.67±0.32	6.27±0.27
State 2 Resp. (nAtoms oxygen/min/mg)	21.50±1.94	17.91±0.96	30.94±3.90*	29.56±2.38[@]
State 3 Resp. (nAtoms oxygen/min/mg)	145.10±11.22	146.67±9.09	214.12±17.95[@]	211.30±13.37[@]
State 4 Resp. (nAtoms oxygen/min/mg)	21.41±2.16	19.93±0.86	36.50±3.48[#]	33.91±2.05[#]
Oxphos rate (nmol ADP/min/mg)	390.87±42.62	388.78±32.42	491.34±57.43	523.67±50.52
State 3 % recovery during reoxygenation after anoxia (%)	54.52±5.88	53.62±5.35	21.69±6.41[#]	25.52±4.05[@]

ADP/O – adenosine diphosphate phosphorylation/oxygen uptake; *Oxphos* – oxidative phosphorylation rate; *Reox* – reoxygenation; *Resp* – respiration. *p<0.05, [@]p<0.01, [#]p<0.001 vs. glutamate.

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Interesting observations were made when comparing the use of glutamate and palmitoyl – L – carnitine as oxidizable substrates (Table 6.13). Although the ADP/O (Control: 2.72±0.26 vs. 2.42±0.26; Obese: 2.75±0.17 vs. 2.47±0.21) and RCI (Control: 7.43±0.81 vs. 5.77±0.35; Obese: 7.57±0.43 vs. 6.35±0.29) values within the control and obese groups were similar irrespective of the substrate present, state 2, 3 and 4 respiration values were higher in both these groups when palmitoyl – L – carnitine was being oxidized. Furthermore, the presence of palmitoyl – L – carnitine significantly reduced the mitochondria's ability to function effectively during reoxygenation following a period of anoxia when compared to experiments where only glutamate was used.

6.3.8 Western blot analysis
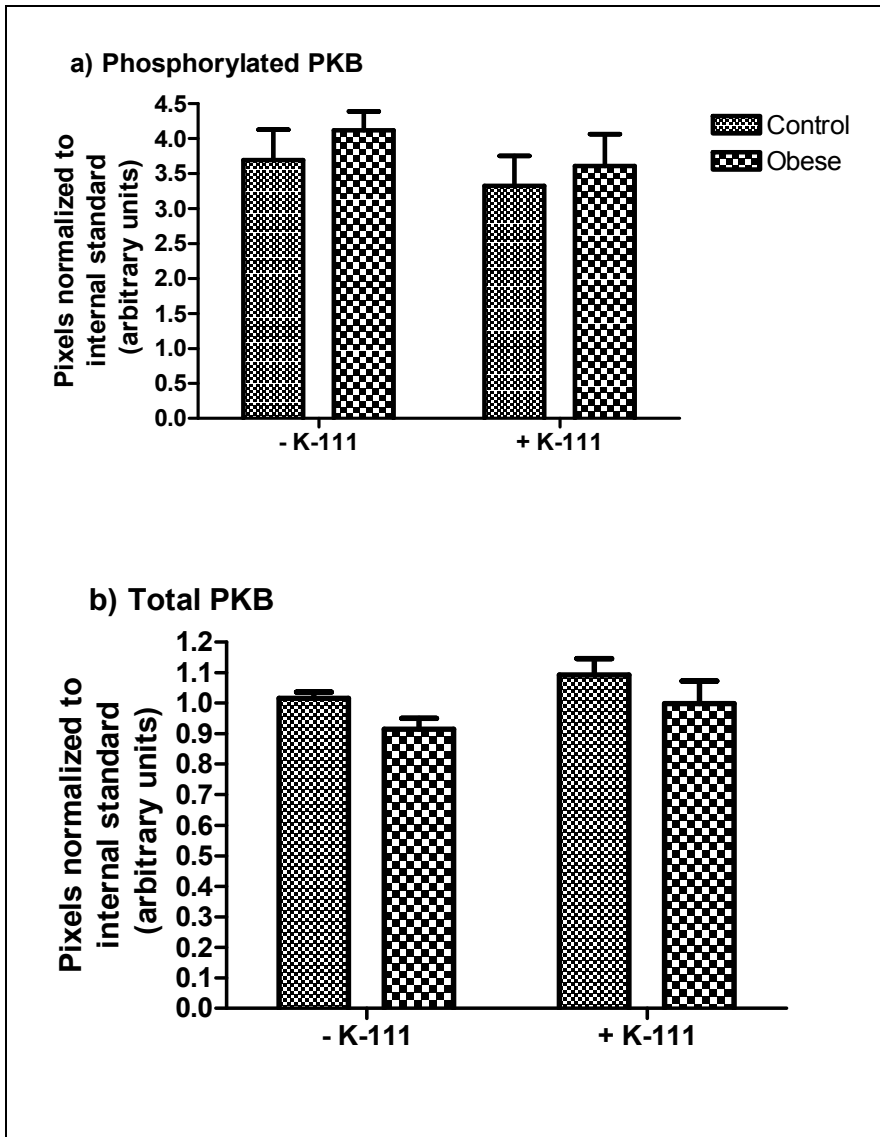
Figure 6.17: The impact of obesity and chronic K-111 treatment on phosphorylated PKB (a) and total PKB (b) expression in ventricular tissue. Hearts were freeze clamped after being removed from the animals.
n = 6 hearts per group

Phosphorylated PKB (Figure 6.17a) and total PKB (Figure 6.17b) expression were similar in hearts from control and obese animals. In addition, K-111 treatment did not influence total PKB expression or phosphorylation in the ventricular tissue.
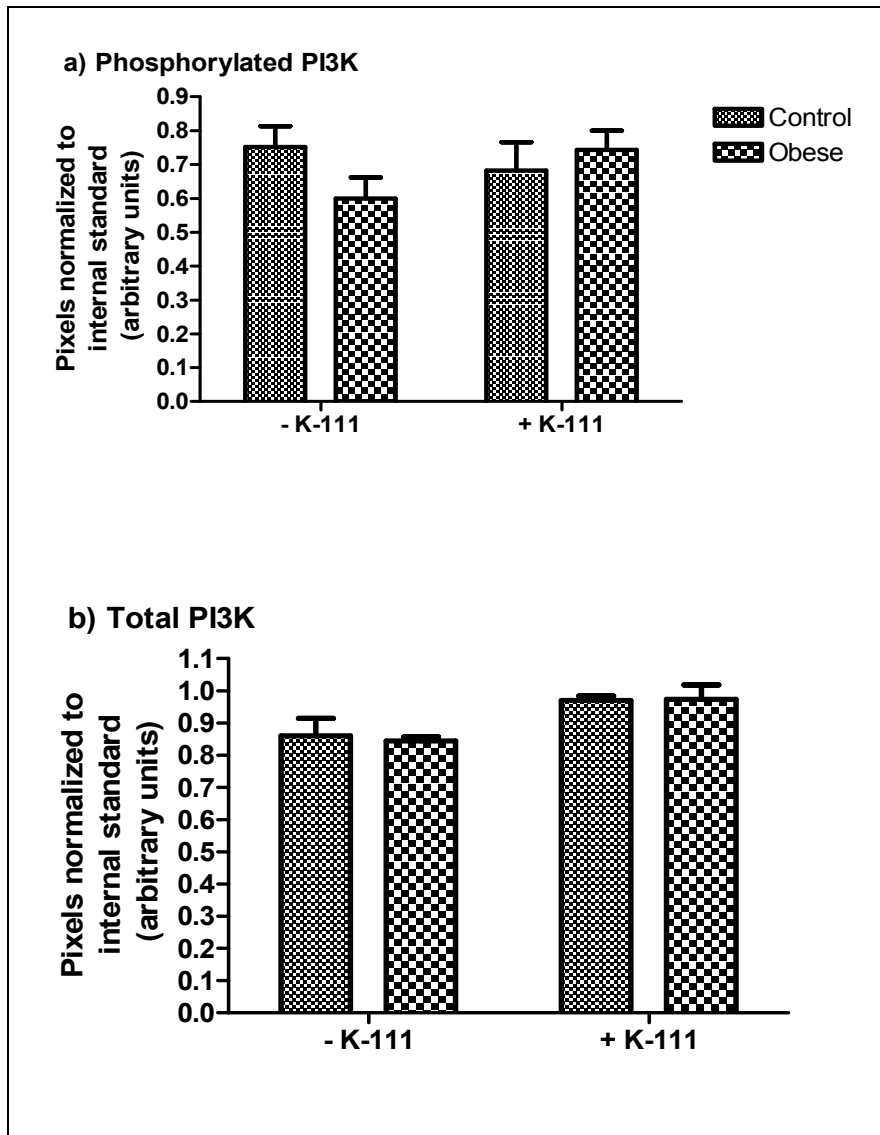
Figure 6.18: The impact of obesity and chronic K-111 treatment on phosphorylated p85 subunit of PI3K (a) and total PI3K (b) expression in left ventricular tissue. Hearts were freeze clamped after being removed from the animals.

n = 6 hearts per group

The expression of both the phosphorylated p85 subunit of PI3K (Figure 6.18a) and total PI3K (Figure 6.18b) was unaltered between the groups, with neither obesity nor K-111 treatment affecting the levels of protein expression.
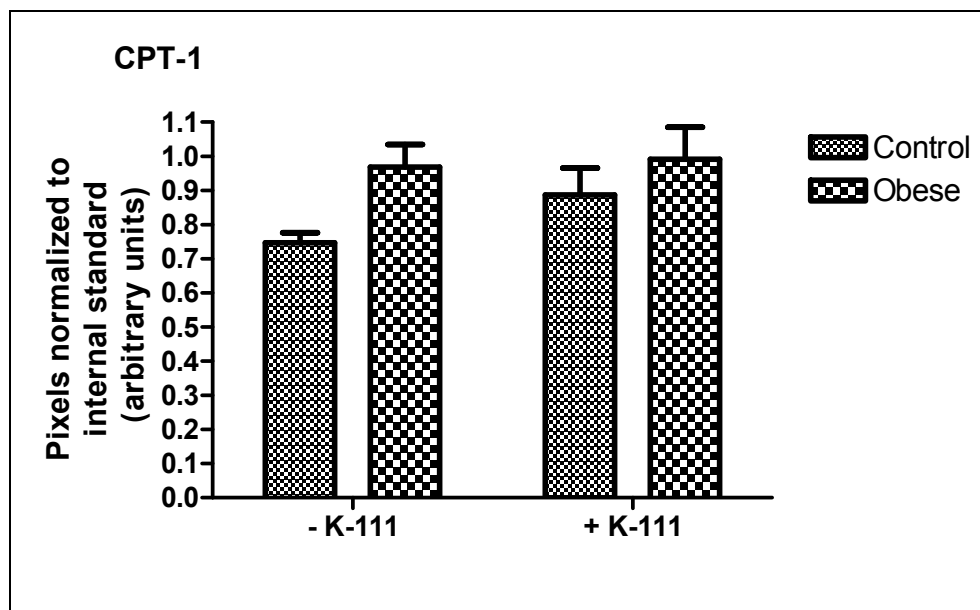
Figure 6.19: The impact of obesity and chronic K-111 treatment on CPT-1 expression in left ventricular tissue. Hearts were freeze clamped after being removed from the animals.

n = 6 hearts per group

Although the 2-way analysis of variance determined that the HCD contributed significantly to the variance seen in CPT-1 expression (p=0.03), post test analysis indicated no significant differences between the four groups (Figure 6.19). (When assessed with an unpaired Students t-test, the expression of CPT-1 was greater in the obese group compared to the control group). K-111 treatment did not influence myocardial CPT-1 expression in the LV.
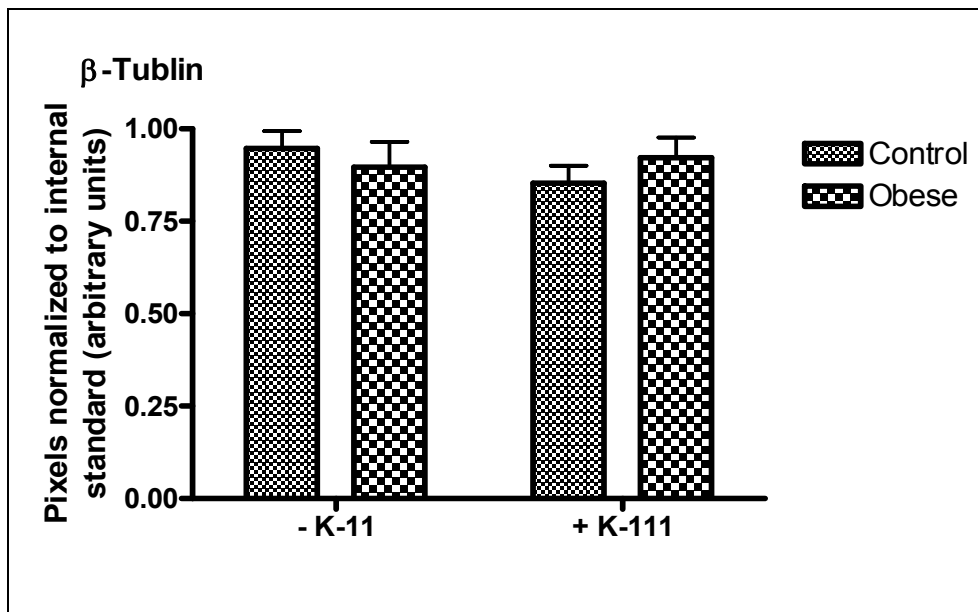
Figure 6.20: The impact of obesity and chronic K-111 treatment on β-Tubulin expression in left ventricular tissue. Hearts were freeze clamped after being removed from the animals.

n = 6 hearts per group

There was no difference in β-tubulin between the groups (Figure 6.20) indicating that equal amounts of protein from the various groups were loaded onto the membranes prior to being separated by electrophoresis.

6.4 Summary of findings

When perfused with glucose and comparable concentrations of insulin and FA, hearts from obese animals had comparable LV mechanical function, rates of substrate metabolism and tolerance to ischaemia/reperfusion injury relative to the control hearts. The infarct sizes obtained in the hearts from control and obese animals perfused in the presence of their respective simulated *in vivo* insulin and FA condition were compared and it emerged the *in vivo* environment associated with obesity contributed significantly to increased infarct sizes in hearts from obese rats. These perfusion conditions were associated with the most dramatic shifts in myocardial substrate metabolism between the control and obese untreated groups.

K-111 treatment attenuated many of the systemic metabolic abnormalities associated with obesity, while it did not impact on LV or mitochondrial oxidative phosphorylation function. K-111 treated obese hearts maintained lower palmitate oxidation rates compared to the untreated obese group (when perfused in the presence of high insulin and FA concentrations in the perfusate), however this occurred without any significant change in the expression of the PPARα regulated enzyme CPT-1. Despite K-111's beneficial systemic and metabolic effects, it did not induce cardioprotection following an episode of ischaemia/reperfusion. Nevertheless, considering some of the adverse effects associated with myocardial PPARα activation, the fact that K-111 did not detrimentally affect post-ischaemic outcomes in our study is of great clinical importance regarding the usefulness of PPARα agonists in the treatment of patients at high risk for developing myocardial ischaemic disorders.

The rates of myocardial substrate metabolism should however be interpreted with caution (See Discussion section 7.3).

CHAPTER 7

DISCUSSION

Using a pre-diabetic rodent model of diet induced obesity and insulin resistance, the aims of the present studies were to determine the impact of protracted obesity on myocardial mechanical function, the responsiveness of isolated hearts from obese insulin resistant animals to insulin treatment prior to and during a simulated acute myocardial infarction. The effects of obesity on myocardial substrate metabolism, ventricular mitochondrial function and susceptibility to ischaemia/reperfusion injury were also investigated. The same rodent model of obesity was further used to investigate the impact of chronic PPARα agonist treatment (K-111) on myocardial mechanical function, substrate metabolism, ventricular mitochondrial function and susceptibility to ischaemia/reperfusion injury.

The main findings of the present study were as follows: 1) Protracted obesity, in the present model, was associated with normal *in vivo* myocardial LV mechanical function and functional reserve. The study unmasked an inability of hearts from obese animals to utilize glucose effectively as a fuel source (myocardial substrate inflexibility) in the maintenance of normal LV mechanical function *ex vivo*. The importance of simulated *in vivo* concentrations of FFA's in the maintenance of both normal *in vivo* LV mechanical function and functional reserve in the obese state was further highlighted; 2) While glucose perfused hearts from obese animals were metabolically responsive to insulin present in the perfusate, this response was not to the same degree as observed in hearts isolated from the control animals, indicating some degree of myocardial insulin resistance in these animals. Isolated hearts from obese insulin resistant animals further showed increased susceptibility to

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ischaemia/reperfusion injury when perfused with glucose alone, while insulin administration prior to and during ischaemia conferred significant cardioprotection in the obese group and to a lesser extent in the control group; 3) In the presence of similar concentrations of glucose, insulin and FA in the perfusate, hearts from obese animals had similar *ex vivo* myocardial mechanical function, substrate metabolism and post-ischaemic outcomes compared to the control group. In addition, mitochondrial function and tolerance to anoxia were also comparable between the obese and control groups in the presence of glutamate or palmitoyl-L-carnitine as substrate; 4) The current data further suggest that the specific metabolic environment associated with obesity (elevated circulating insulin and FA levels) may contribute to detrimental post-ischaemic outcomes in the obese state; 5) Lastly, although chronic K-111 treatment improved the systemic metabolic profile associated with obesity, it had a limited impact on cardiac substrate metabolism (reduced palmitate oxidation rates in the presence of high insulin and FA levels in the perfusate), while it did not influence myocardial function or myocardial ischaemic tolerance irrespective of the different perfusion conditions implemented. Similarly chronic K-111 treatment did not influence ventricular mitochondrial function or their tolerance to anoxia in the presence of either glutamate or palmitoyl-L-carnitine as substrates in either the obese or control treated groups.

7.1 The model of diet induced obesity

Obesity seldom exists in isolation from various other co-morbidities such as dyslipidaemia, hyperinsulinaemia, insulin resistance, diabetes and hypertension, each of which constitutes a risk for cardiovascular disease (Stamler *et al*. 1978; Kannel *et al*. 1979; Kannel and McGee, 1979; Van Itallie *et al*. 1985; Barbir *et al*.

1988; Colditz *et al.* 1995; O'Donnell *et al.* 1997; Abassi *et al.* 2002; Rewers *et al.* 2004). Not surprisingly, it was found that 16, 18 and 32 weeks of HCD feeding significantly increased rodent body weight and visceral (retroperitoneal and gonadal) fat content (Table 4.1, 5.1, 6.2). Obese rats further displayed a metabolic profile similar to pre-diabetic metabolic syndrome seen in humans, evident by the co-occurrence of normoglycaemia, hyperinsulinaemia, insulin resistance (HOMA-IR), and hypertriglyceridaemia, while in certain groups of animals HDL-C levels were significantly reduced compared to the lean animals (Table 4.2, 5.2, 6.2). Additional biochemical determinations only performed after 16 or 18 weeks of feeding revealed an increase in circulating FFA levels in response to HCD feeding (Table 5.2, 6.2). Despite this increased supply of FFA, intramyocardial Trig levels of control and obese animals did not differ (Table 6.2). Normal levels of glycosylated haemoglobin further confirmed the absence of diabetes and effective blood glucose management in our model of obesity (Table 5.2, 6.2).

Comparisons between 32 week and 16 week control and HCD fed animals (data not shown) revealed that 32 week HCD fed rats had a 25% and 34% increase in body weight and visceral fat mass relative to the 16 week fed rats (p<0.001). Similarly, the body weight of the 32 week fed control rats was 19% more than the 16 weeks control group (p<0.001), while no differences in visceral fat mass were seen (comparisons not shown). Comparisons of metabolic parameters between 32 and 16 week rats were not possible due to differences in the overnight fasting periods between these two groups.

Other rodent models of diet induced obesity likewise displayed similar biometric and metabolic profiles to that observed in the current study (Wilson *et al.* 2007), while in certain models, raised glucose levels may also develop following high caloric feeding

(Ouwens *et al*. 2007; Ussher *et al*. 2010). Although the caloric composition of the HCD utilized in the present study differed slightly from that used by other groups (Ouwens *et al*. 2007; Wilson *et al*. 2007; Ussher *et al*. 2010), it was beyond the scope of this study to determine whether alterations in the macronutrient content of the diet influenced the various cardiovascular outcomes associated with obesity. Such a study has previously been conducted by Wilson *et al.* (2007) who documented differences in LV mechanical function in response to altered dietary macronutrient content.

In the present study, the impact of HCD feeding on myocardial ventricular remodelling appeared to be mild at most. Our research group has previously shown greater echocardiographically determined LV posterior wall thickening with significant cardiomyocyte hypertrophy (W Smith, MSc. Dissertation, 2005) and greater heart weight to body weight ratios (indicative of ventricular remodelling) in 16 week HCD fed animals (Du Toit *et al*. 2005). Although 16 week HCD fed rats had greater LV posterior wall thickening than control rats, these remodelling differences were absent after 32 weeks of HCD feeding (Table 4.3). This finding could however be due to the small sample size used for these echocardiography determinations in the 32 week HCD fed group. Indeed, while a non-significant tendency was evident for greater LV posterior wall thickening to be present in these animals, a larger group of 32 week HCD fed rats was shown to have significantly greater ventricular weight to tibia length ratios (indicative of ventricular remodelling) (Table 4.1). These discrepant findings could suggest that the ventricular remodelling observed in our model of diet induced obesity may not be of a pathological nature. In support of this, mice fed a HCD similar to that used in the present study had normal heart weights and heart to body weight ratio's (Aasum *et al*. 2008).

Format of the discussion

Although this thesis consists of 3 interrelated studies, the overlapping nature of the work justifies the discussion to follow the major overlapping outcomes investigated, i.e. cardiac function, substrate metabolism, mitochondrial function and susceptibility to ischaemia/reperfusion injury. The impact of chronic K-111 treatment on these parameters will be discussed separately.

7.2 The impact of obesity on cardiac mechanical function

Obesity has traditionally been associated with normal or augmented cardiac systolic function whereas diastolic function is usually compromised (Berkalp *et al*. 1995; Iacobellis *et al*. 2002, 2004; Pascual *et al*. 2003; Otto *et al*. 2004; Dorbala *et al.* 2006). Recent data have however identified the presence of sub-clinical myocardial systolic dysfunction in obese (Ferraro *et al*. 1996; Peterson *et al*. 2004b; Wong *et al*. 2004; Di Bello *et al*. 2006; Tumuklu *et al*. 2007, Kosmala *et al*. 2008a) and overweight (Wong *et al*. 2004) individuals occurring at times independently of ventricular loading conditions. As obesity is an independent risk factor for the development of heart failure (Kenchaiah *et al*. 2002), it is plausible that protracted obesity may be associated with sub-clinical alterations in cardiac function.

A few studies have documented the impact of altered *ex vivo* myocardial substrate supply (glucose alone or glucose + FA in the perfusate) on LV function both during baseline perfusion conditions and in response to augmented workload (Lopaschuk *et al*. 1999; Liu *et al*. 2002; Boudina *et al*. 2005) in isolated hearts from healthy animals.

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In addition, only Boudina *et al*. (2005) has reported on the effects of glucose alone or in combination with FA present in the perfusate on LV mechanical function and functional reserve in hearts from obese animals. Neither of these studies has investigated the contributory role of insulin on LV mechanical function (Lopaschuk *et al*. 1999; Liu *et al*. 2002; Boudina *et al*. 2005). This is important as obesity is normally associated with a state of hyperinsulinaemia. Furthermore, Boudina *et al*. (2005) made use of retrogradely perfused hearts from leptin deficient ob/ob mice. While this model is a well recognized animal model of obesity, it is important to note that while leptin may not always influence LV mechanical function in normal healthy hearts, it still impacts on myocardial FA metabolism (Atkinson *et al*. 2002). Furthermore, the contractile dysfunction identified in isolated cardiomyocytes from obese ob/ob mice, has been show to be abolished by the presence of leptin administered *in vitro* (Dong *et al*. 2006). Interestingly, the previous study found that these contractile abnormalities were not present in cardiomyocytes isolated from diet-induced obese mice. These findings suggest that *ex vivo* LV functional data generated from hearts from *ob/ob* mice may have to be interpreted with caution due to the influence of leptin on cardiac metabolism and the impact of *in vitro* leptin administration on cardiomyocyte contractile performance. Although these effects of leptin on the various cardiovascular parameters were documented upon *ex vivo* administration, it is possible that hearts not being conditioned by leptin *in vivo* may respond differently in *ex vivo* preparations. Lastly, although certain aspects regarding the impact of altered substrate supply on LV mechanical function at baseline and in response to increased workload have been investigated, the present study is the first to combine data from both *in vivo* and *ex vivo* (retrograde perfused heart to assess LV contractility and working heart to assess LV function in a working heart) models to

develop a sound conclusion regarding the impact of altered cardiac substrate and insulin supply on normal and peak LV function in obese animals.

7.2.1 *In vivo* data

In the present study, pre-diabetic obese insulin resistant animals following 32 weeks of HCD feeding displayed comparable *in vivo* mechanical function (FSend, FSmid) relative to control animals. Moreover, myocardial functional reserve (FSend, FSmid), in response to β-adrenergic receptor stimulation, was also similar between the experimental groups (Figure 4.2a, b). The data indicate that protracted obesity associated with euglycaemia, hyperinsulinaemia and elevated circulating FFA levels, in the absence of overt type II diabetes and hypertension (blood pressure data not shown), is associated with normal *in vivo* cardiac function under both basal and augmented workloads. Unfortunately the *in vivo* measurements of LV function used in the present study were not independent of LV loading conditions. However, LV end-systolic elastance (a load independent measure of LV systolic function) was comparable between the control and obese groups in the presence of isoproterenol, glucose and insulin (Figure 4.3d), suggesting that the normal LV function observed *in vivo* could be independent of LV loading conditions. Nevertheless, our *in vivo* observations are in agreement with other models of diet induced obesity which similarly report normal *in vivo* LV myocardial function (Carroll *et al*. 2006; Yan *et al*. 2009; Zhang *et al*. 2010). As Wilson *et al*. (2007) documented obesity related *ex vivo* cardiac dysfunction only after 32-48 weeks of western diet feeding, it is unclear whether the absence of cardiac dysfunction in these previously mentioned studies (Carroll *et al*. 2006, Yan *et al*. 2009, Zhang *et al*. 2010) was due to the shorter feeding periods employed (12, 20 and 10 weeks respectively). Our data however indicate that diet induced obesity (32 weeks of feeding) that developed over a similar

feeding period as used by Wilson *et al*. (2007), was not associated with *in vivo* cardiac dysfunction. Although not investigated in the current study, it is plausible that subtle changes in the fat and carbohydrate composition of the diets used may impact on these findings. Whereas a diet consisting of 60% of the calories derived from fat was unable to elicit cardiac dysfunction after 32-48 weeks of feeding (Wilson *et al*. 2007), a HFD in which 50.4% of the calories derived from fat led to cardiac dysfunction after only 8 weeks of feeding (Owens *et al.* 2007), thus illustrating the possible impact of dietary composition on various cardiovascular outcomes. In the previously mentioned study cardiac dysfunction ensued after a relatively short period of feeding in the absence of obesity, but with evidence of advanced ventricular remodelling (Owens *et al*. 2007), which was absent in the present study and those of others (Carroll *et al*. 2006; Yan *et al*. 2009; Zhang *et al*. 2010). Thus results from the previously mentioned studies emphasize the importance of strict dietary control in studies assessing the cardiovascular outcomes associated with diet induced obesity.

7.2.2 *Ex vivo* data

While the majority of cardiac energy is derived from the oxidation of circulating FFA (Zierler, 1976; Opie, 1998), healthy hearts are thought to be capable of adapting to an altered substrate supply enabling them to maintain normal LV mechanical function (Yan *et al*. 2009). In agreement with previous studies (Lopaschuk *et al*. 1999; Liu *et al*. 2002; Boudina *et al*. 2005), the present study using isolated working and retrogradely perfused hearts from control animals found that these hearts exhibited effective metabolic substrate flexibility in the presence of different combinations of simulated *in vivo* substrate and hormone concentrations enabling them to achieve normal LV mechanical function irrespective of the substrate/hormone supply perfusion conditions (Figures 4.3a, 4.4). Despite obese animals having comparable

in vivo LV mechanical function relative to the control group, *ex vivo* function differed markedly from that observed *in vivo*. Isolated working hearts from obese animals were unable to achieve similar LV mechanical function compared to the control groups in the presence of glucose and at times in the presence of glucose + insulin (Figure 4.4). The reduced LV end-systolic elastance (a load-independent measure of LV systolic chamber performance) observed in paced retrograde glucose perfused hearts from obese animals (Figure 4.3d) suggests that the *ex vivo* cardiac dysfunction seen in working hearts in the presence of glucose was related to intrinsic contractile abnormalities independent of changes in LV loading conditions. Significantly, when working hearts from obese animals were perfused with simulated *in vivo* concentrations of glucose + FA or glucose + insulin + FA that were physiologically relevant to the obese animal, LV myocardial mechanical function was normalized relative to the control group (Figure 4.4). Our data suggest that hearts from obese animals depend on elevated (physiologically relevant to the obese state) FA concentrations for the maintenance of normal LV function, while exhibiting an inability to maintain normal LV function when provided with carbohydrate as its sole substrate supply. Supporting this notion is the observation that the reduced LV mechanical function (aortic output and cardiac output) generated by obese insulin resistant *ob/ob* mouse hearts when perfused with glucose, 0.4mM palmitate and 1nmol/l insulin, was reversed when the palmitate concentration in the perfusate was elevated to 1.2mM (Mazumder *et al*. 2004). Although it appears that the heart from the obese animal has a preference for an elevated FA supply to maintain normal LV function, this adaptation may come at the expense of increased oxygen utilization (How *et al*. 2005; 2006). Furthermore this substrate preference could render these hearts vulnerable to enhanced injury during an ischaemic event (To be discussed in Section 7.6).

Despite working hearts from obese animals displaying *ex vivo* LV mechanical dysfunction in the presence of glucose or glucose + insulin, this finding was contradicted by the normal (relative to the control group) pressure-volume relations generated by retrogradely perfused hearts from the obese group using a similar perfusate (Figure 4.4 vs. 4.3a). Boudina *et al.* (2005) similarly reported comparable LV function between control and ob/ob retrogradely perfused mouse hearts in the presence of glucose alone. The discrepancy between the data may be explained by the different workloads placed on the heart by the two different perfusion modes (i.e. working vs. non-working retrograde) used. Consequently we believe that the higher myocardial workload, and therefore energy demand, placed on the working heart had unmasked functional abnormalities between the control and obese groups not seen in the retrogradely perfused hearts. This argument is supported by the presence of functional differences between retrogradely perfused hearts from control and obese animals when perfused in the presence of glucose and the β-adrenergic receptor agonist, isoproterenol (Figure 4.3 b), which would impose an increase in workload. A second reason may relate to the balloon inserted into the left ventricle in the retrogradely perfused heart. Whereas the balloon may assist diastolic relaxation by the elastic recoil of the compressed water inside the balloon, this assistance is absent in the working heart, which itself is dependent on normal diastolic relaxation to maintain aortic output.

7.2.3 The effect of substrate on peak LV function

In response to increased β-adrenergic receptor stimulation (increasing myocardial workload), the healthy heart reacts by increasing glucose oxidation rates, while FA oxidation rates have been reported to become slightly elevated, remain constant or

even become reduced (Collins-Nakai *et al*. 1994; Wilson *et al*. 2007; Yan *et al*. 2009). During these events the contribution of glucose to myocardial ATP production increases greatly (Collins-Nakai *et al*. 1994). In the present study, it was demonstrated that control and obese animals were able to increase their LV mechanical function equally in response to increased workload (β adrenergic stimulation) *in vivo* (Figure 4.2). Assessment of *ex vivo* myocardial functional reserve showed that both groups were able to augment LV mechanical function in response to increments in preload regardless of the substrate and hormonal conditions employed (Figure 4.5a, b). Whereas hearts from the lean group were able to achieve peak aortic output with all substrate and insulin perfusion conditions, it emerged that hearts from obese animals were only able to achieve peak aortic output values (as defined by the aortic output values achieved by the lean FA perfused groups at the 20cm preload) in the presence of simulated *in vivo* concentrations of FA (FA alone or together with insulin). These *ex* vivo observations concur with the *ex vivo* observations of Wilson *et al*. (2007) and Yan *et al*. (2009) who similarly found comparable functional reserve between control and obese animals when glucose, insulin and FA were present in the perfusate. In contrast hearts from obese ob/ob mice were not able to achieve comparable LV function at high workloads compared to hearts from the lean group in the presence of glucose and palmitate (Boudina *et al*. 2005). The reason for this discrepancy may be due to a more advanced form of mitochondrial dysfunction in these hearts (Boudina *et al*. 2005).

Although the healthy heart's response to increased workload is to increase glucose oxidation rates, while FA oxidation rates are slightly elevated, maintained or even reduced (Collins-Nakai *et al*.1994; Wilson *et al*. 2007; Yan *et al*. 2009), Pagliaro *et al*. (2002) noted that retrogradely glucose perfused hearts from normal animals rely

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on energy derived from the oxidation of endogenous myocardial lipids to maintain effective LV contractile function in response to incremental increases in LV filling (Frank-Starling mechanism). Although our study did not assess the functional impact of endogenous lipid stores, we concede that endogenous lipids would have contributed to the substrate supply in glucose perfused hearts from the current study. Although not different between the control and obese groups, these endogenous Trig's may have aided LV mechanical function. Nevertheless, the present study demonstrated that with the use of the more physiological working heart model, glucose metabolism in addition to the contribution of endogenous lipid stores was not sufficient to induce effective LV mechanical function in response to incremental increases in LV preload and hence filling conditions in the obese state (Figure 4.5a, b). Consequently a physiologically relevant exogenous supply of FA is essential to achieve normal Frank-Starling responses in the obese state.

Although not investigated, it can be speculated why working hearts from obese animals did not achieve normal LV mechanical function in the presence of glucose or glucose + insulin. Firstly, it is well known that cardiomyocyte glucose transport is regulated by the glucose transporters, GLUT 1 and GLUT4, with the sarcolemmal translocation of the latter being regulated by insulin (Zaninetti *et al*. 1988; Kraegen *et al*. 1993). Wright *et al.* (2009) reported on HFD fed mice having reduced myocardial glucose uptake, glycolysis and glucose oxidation rates coupled with reduced myocardial GLUT 4 content and attenuated insulin stimulated GLUT4 sarcolemmal translocation after short term feeding. Obese animals in the present study similarly display reduced insulin stimulated myocardial rates of glycolytic flux (Figure 5.2a), while our research group has previously identified attenuated rates of insulin stimulated glucose uptake in cardiomyocytes isolated from these obese animals

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(Huisamen, unpublished data). Secondly pre-diabetic obesity is associated with reduced myocardial PDH activity (reduced activity of the active fraction of PDH) (Boudina *et al*. 2005). It is well known that elevated myocardial FA supply may inhibit PDH activity through the up-regulation of the PDH inhibitor, PDK4 (Denton *et al*. 1975; Hansford and Cohen, 1978; Huang *et al*. 2002; Holness and Sugden, 2003). Consequently high rates of FA supply in the context of high caloric feeding or obesity could reduce myocardial glucose oxidation by reducing the activity of PDH. As circulating FFA are elevated in our model of obesity after 16 weeks of feeding, it is plausible that following long-term obesity (32 weeks of HCD feeding), myocardial PDH activity would be reduced in these obese rats. Although the current study made use of isolated perfused hearts it is possible that the inhibition of the PDH complex by PDK4 may persist in these hearts when perfused. Taken together, abnormalities in myocardial glucose transporter content, insulin stimulated GLUT4 translocation and reduced PDH activity associated with obesity would aid to diminish myocardial glucose uptake and utilization, thus compromising myocardial energetics in the absence of FA as a fuel source which would in turn result in cardiac dysfunction in the glucose or glucose + insulin perfused heart. Consequently energy derived from FA metabolism may be vital for the maintenance of effective contractile function in the obese insulin resistant state.

In the current study, the improvement in *ex vivo* LV mechanical function attributed to the presence of simulated *in vivo* FA concentrations in the obese group help explain our previous published findings and those of others. We have previously reported reduced *ex vivo* cardiac mechanical function in hearts from 16 week HCD fed animals perfused with glucose alone (Du Toit *et al*. 2005; Nduhirabandi *et al*. 2011), while cultured cardiomyocytes isolated from non-obese insulin resistant rats

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demonstrate functional abnormalities in media containing glucose as the only oxidative substrate (Schwanke *et al*. 2006). These findings could therefore be influenced by the lack of FA as a fuel source.

7.2.4 FA supply and cardiac function

The relationship between altered cardiac substrate supply, metabolism and contractile function is complex, delicate and not fully understood, and has received much attention over recent years where the focus has been on the metabolic effects associated with chronically increased myocardial substrate delivery as seen in obese, insulin resistant and diabetic conditions.

Defective or insufficient FA oxidative metabolism in response to high FA supply coupled with the activation of alternative glucose metabolic pathways resulting from an enhanced myocardial oxidative fuel supply (both glucose and FA) may explain the relationship between altered cardiac substrate supply, metabolism and LV dysfunction associated with models of obesity (Young *et al*. 2002; Wilson *et al*. 2007; Yan *et al*. 2009; Ballal *et al*. 2010). This form of cardiac dysfunction is thought to stem from myocardial FA overload (due to reduced FA metabolism) and in certain cases a combination of FA and glucose overload (lipo- and glucotoxicity) leading to the activation of lipotoxic pathways or the generation of reactive oxygen species, which could induce cardiomyocyte apoptosis and attenuate cardiac function (Zhou *et al*. 2000; Young *et al*. 2002; Yan *et al*. 2009; Ballal *et al*. 2010).

The present study provides additional insight into the complex interplay between substrate supply and cardiac function. Although enhanced FA supply to the heart plays a major role in the development of myocardial insulin resistance, this oversupply of oxidative fuel is inevitable in the progression of obesity. Consequently

the heart adapts metabolically to an elevated FA supply possibly by up-regulating PPARα response genes which culminates in enhanced FA metabolism (outlined by Barger and Kelly, 2000). It may be that during the initial developmental phases of obesity related myocardial insulin resistance, elevated levels of FA become more important in the maintenance of normal LV mechanical function and functional reserve, especially in light of attenuated glucose metabolism. However, unless body weight/caloric intake is controlled, the enhanced supply of FA and/or carbohydrates coupled with a reduced expression of PPARα response gene products (Wilson *et al*. 2007), may eventually become a burden (or become disrupted) to the point where the heart is unable to effectively utilize the elevated substrate supply and metabolic and mechanical cardiac dysfunction ensue (Young *et al.* 2002; Wilson *et al*. 2007; Yan *et al*. 2009).

7.2.5 Study limitations

A limitation to this study was the failure to determine fasting FFA levels present in the 32 week animals. This was due to insufficient serum being collected from fasted animals. Consequently fasting FFA levels determined from 16 week control and HCD fed rats (Table 5.2) were used as a reference for the FFA levels in the present study. Although these values may not be a true reflection of 32 week fed condition, it did ensure that the FFA levels used in the *ex vivo* perfusions were in the range that hearts from these animals may have been exposed to. A further possible limitation to this study was that we did not determine whether normal insulin and FA levels (as seen in the control animal) present in the perfusate, would be sufficient to sustain cardiac function after 32 weeks of HCD feeding. Although 16 week HCD fed animals have reduced LV mechanical function in the presence of glucose alone (Du Toit *et al*. 2005), it is interesting to note that obese animals, following 18 weeks of HCD feeding

in the present study have normal LV mechanical function when perfused with concentrations of insulin and FA seen in the control animals (Table 6.5). Although this may suggest myocardial dependency on FA's independent of their concentration, it is uncertain whether this observation will remain following 32 weeks of HCD feeding where the adverse myocardial effects of obesity may be more advanced.

The data of the present study suggest that protracted obesity in the absence of overt type II diabetes is not associated with cardiac dysfunction in a rodent model characterised by normoglycaemia, hyperinsulinaemia, hypertriglyceridaemia, raised FFA levels and normotension (blood pressure data not shown). Our study proposes that during the initial adaptational phase of obesity related myocardial insulin resistance, energy derived from FFA metabolism plays a much more important role in maintaining normal LV mechanical function and functional reserve than in the healthy heart. This is possibly due to the heart's loss of flexibility in utilizing glucose effectively as an energy source for the maintenance of normal LV mechanical function.

7.2.6 Future directions

Future work investigating the relationship between FA levels and LV mechanical function in obese insulin resistant rats should do so using different doses of CPT-1 inhibitors, such as oxfenicine, in either the perfusate or administered chronically. In this way, the extent to which obese insulin resistant animals rely on FA as a fuel source for the maintenance of LV mechanical function can further be investigated. It was recently shown 8 weeks of oxfenicine treatment in rats fed either a high saturated, high unsaturated or medium chain FA diet had no effect on *in vivo* cardiac mechanical function. It should however be noted that this feeding period was not

associated with significant changes in body weight (Okere *et al*. 2007). Consequently the myocardial response to the elevated FA load may not have been long enough to determine the significance of CPT-1 inhibition on LV mechanical function in the obese insulin resistant condition.

7.3 The impact of obesity on myocardial substrate metabolism

Insulin resistant hearts are characterized by an insensitivity to insulin administration, either in terms of glucose uptake, glycolysis or glucose oxidation, (Atkinson *et al*. 2003; Mazumder *et al*. 2004; Ouwens *et al*. 2007; Zhang *et al*. 2010).

In the present study, hearts from 16 week HCD fed obese insulin resistant animals responded surprisingly well to insulin treatment by exhibiting a dose dependent increase in myocardial glycolytic flux rates (Figure 5.2b). In spite of this, myocardial glycolytic flux rates remained lower in the obese group compared to the control group in the presence of comparable insulin concentrations (Figure 5.2a), a finding which is indicative of an insulin resistant myocardium. This is in agreement with reduced myocardial glycolytic fluxes observed in other models of insulin resistance and diabetes (Atkinson *et al*. 2003). Hafstad *et al*. (2006) similarly noted that despite isolated hearts from diabetic *db/db* mice showing attenuated glucose metabolism, they responded remarkably well to insulin stimulation, since changes in substrate oxidation rates elicited by insulin stimulation seemed more pronounced in the db/db mouse hearts compared to the control group during certain experimental conditions. Thus, despite hearts from obese insulin resistant rats (present study) and diabetic mice (Hafstad *et al*. 2006) being insulin resistant, it appears that these hearts are indeed able to respond to insulin stimulation.

Systemic insulin resistance is a predictor of myocardial FA uptake, utilization and oxidation in obese insulin resistant humans (Peterson *et al*. 2004a). Following 18 weeks of HCD feeding it was aimed to obtain a more complete picture of the myocardial substrate metabolism profile of these obese animals. Surprisingly, under comparable perfusion conditions, no changes in myocardial glycolytic flux, glucose oxidation, or palmitate oxidation rates were observed between hearts from the control and obese animals, either before or after myocardial global ischaemia (Table 6.3, 6.4). This is in contrast to many studies describing marked alterations in myocardial substrate metabolism in diet induced obese and/or insulin resistant animals (Wilson *et al*. 2007; Aasum *et al*. 2008; Wright *et al*. 2009; Zhang *et al*. 2010). As expected (How *et al*. 2005; 2006), palmitate oxidation was significantly increased in hearts from both groups perfused with high levels of insulin and FA compared to hearts perfused with normal insulin and FA levels (Figure 6.12). The last mentioned perfusion conditions coincided with a non-significant tendency for glucose metabolism to be attenuated (Figure 6.10; 6.11). In addition, myocardial rates of palmitate oxidation were elevated from baseline values during the reperfusion phase (especially in the presence of high insulin and FA in the perfusate) (Table 6.3, 6.4) which is in accordance with previous findings in isolated working mouse hearts (Aasum *et al*. 2003a).

The absolute values obtained for the different metabolic parameters in the present study appeared rather low. An extensive search of the literature showed that most of the recent studies on myocardial substrate metabolism were done on isolated working mouse hearts (Muzumder *et al*. 2004, Kuang *et al*. 2004, Buchanan *et al*. 2005; Aasum *et al*. 2008; Wright *et al*. 2009; Zhang *et al*. 2010), which may

profoundly influence the values obtained. One study also using Langendorff perfused rat hearts yielded different FA oxidation values to those seen in the present study, but differences in the FA/albumin ratio used and the absence of insulin in the perfusion buffer (Heather *et al*. 2007) complicate any comparisons with our own work (Evans *et al*. 1963). Similarly, comparisons of the present findings with that of Shipp and co-workers (1961), using a similar model could not be done due to differences in substrate composition (5mM glucose, 0.4mM palmitate, 0.5% albumin vs. 10mM glucose, 30 or 50µIU/ml insulin and 0.4 or 1.2mM palmitate, 3% albumin (containing an additional 0.3mM FA)).

In the current study, Western blot analysis for the assessment of the expression of metabolically related proteins involved in insulin signalling did not provide additional insight regarding the insulin sensitivity of the myocardium from the obese animals as no differences in the total or phosphorylated PKB or the p85-subunit of PI3K were observed between the two groups (Figure 6.17, 6.18). This is in contrast to studies which show that reduced glucose uptake may be associated with attenuated PKB and p85-subunit of PI3K phosphorylation (Summer *et al*. 1998; Florian *et al*. 2010). However myocardial CPT-1 levels were significantly elevated in the obese group (Figure 6.19 (assessed with a t-test)), which together with elevated circulating levels of FFA suggest increased FFA flux into the mitochondria.

It is however important to consider methodological differences between the present study and a recent study published from our research group (Huisamen *et al*. 2011). While hearts in the current study were snap frozen upon removal from the body (i.e. without perfusion) for Western blotting analysis, Huisamen *et al*. (2011) perfused isolated hearts from 16 week HCD fed rats with insulin prior to being frozen. Under these conditions Huisamen *et al*. (2011) observed reduced insulin stimulated PKB

and eNOS (endothelial derived nitric oxide synthase) phosphorylation, reduced IRS-1 expression and increased ser[612] IRS-1 phosphorylation (an inhibitory action) in the hearts from obese animals compared to hearts from control animals. Our research group has further observed reduced insulin stimulated glucose uptake in cardiomyocytes isolated from obese rat hearts (16 weeks of HCD feeding) (Huisamen, unpublished data) while another research group using a similar HCD have reported reduced myocardial glucose oxidation and increased palmitate oxidation rates in isolated hearts from HCD fed mice (Aasum *et al*. 2008). Taken together these findings indicate that HCD feeding induces an insulin resistant myocardium, as was also indicated by the reduced effect of insulin on the myocardial glycolytic flux rate of obese animals (Figure 5.2a).

Studies have documented an association between intramyocardial Trig accumulation and myocardial insulin resistance (Atkinson *et al*. 2003; Yan *et al*. 2009). The present study has however shown myocardial insulin resistance in the absence of elevated intramyocardial Trig levels in the hearts from obese animals. This finding is corroborated by a few other studies (Wilson *et al*. 2007; Aasum *et al*. 2008). Together, these observations question the relevance of intramyocardial Trig accumulation as a contributor to myocardial insulin resistance. More specific lipid intermediates such as diacylglycerol may be a better mediator of myocardial insulin resistance (Zhang *et al*. 2010).

7.4 Ventricular mitochondrial function

Ventricular mitochondrial dysfunction (impaired respiratory capacity leading to reduced ATP production), evident in diabetic animals (Boudina *et al*. 2007; Khalid *et al*. 2011) is speculated to contribute to the LV functional abnormalities associated with the diabetic cardiomyopathy and metabolic syndrome (Ren *et al*. 2010; Bugger and Abel, 2010). While obesity is an independent risk factor for the development of heart failure (Kenchaiah *et al*. 2002), the observations of myocardial mitochondrial dysfunction in obese insulin resistant humans (Niemann *et al.* 2011) and animals (Boudina *et al*. 2005; Essop *et al*. 2009) are disconcerting. Indeed, it has been suggested that reduced mitochondrial bioenergetic capacity evident in obese insulin resistant animals may account for reduced LV mechanical function and the worsening of post-ischaemic outcomes reported in these animals (Essop *et al*. 2009).

7.4.1 The impact of obesity on ventricular mitochondrial function

In contrast to previous findings (Boudina *et al*. 2005; Essop *et al*. 2009), the present study found the oxidative phosphorylation function to be normal in mitochondria isolated from obese insulin resistant animals (Table 6.11, 6.12). This was evident from coupled oxidative phosphorylation (ADP/O and RCI ratio's), and comparable rates of oxygen consumption during ADP stimulated respiration (State 3 respiration) between the control and obese groups. Additionally states 2 and 4 respiration and oxidative phosphorylation rates were similar in the two groups. These respiration rates were similar, regardless of the oxidative substrates used.

Essop *et al.* (2009), using the same rodent model as that used in the present study, reported reduced ADP phosphorylation rates in mitochondria isolated from obese rat

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hearts in the presence of palmitoyl-L-carnitine. While this observation is in contrast to the findings of the present study, it is noteworthy that obese rats in the latter study had more visceral fat than obese rats in the present study (49.5g vs. 30.45g). While intra-abdominal mass has been shown to correlate with systemic insulin sensitivity (Park *et al*. 1991), which in turn correlates with the degree of mitochondrial morphological and functional abnormalities (Kelley *et al*. 2002), it is possible that the differences noted between the current study and those of Essop *et al*. (2009) and Boudina *et al*. (2005) may be due to differences in the degree of systemic insulin resistance present in the respective models of obesity. Based on the data obtained, this would suggest that during the early stages of obesity related insulin resistance, ventricular mitochondria have normal function in the presence of glutamate and palmitoyl – L – carnitine as substrates. The respiratory capacity of these mitochondria isolated from obese rats may however deteriorate as the degree of systemic insulin resistance increases. It is important to mention that while the current study measured mitochondrial respiration by challenging the tricarboxylic acid cycle, β-oxidation and the electron transport chain, it failed to investigate the impact of flux through PDH on mitochondrial respiratory capacity. It is however clear that mitochondrial respiratory capacity in response to pyruvate may be reduced in obese conditions (Boudina *et al*. 2005) given the reduced LV mechanical function observed in obese rat hearts when perfused with glucose alone.

Mitochondrial susceptibility to ischaemia/reperfusion was also evaluated by exposing isolated mitochondria to hypoxia followed by reoxygenation. The results of the present study showed that the recovery of state 3 respiration following anoxia was not influenced by obesity. This finding is also in contrast with the reduced post-anoxia recovery of state 3 respiration observed in isolated ventricular mitochondria from

obese insulin resistant rats reported by Essop *et al*. (2009). These differences may again be explained by the greater degree of systemic insulin resistance in the obese rats used in the Essop *et al*. (2009) study.

7.4.2 The impact of the oxidative substrate on ventricular mitochondrial function

Comparisons between the different substrates used revealed that ventricular mitochondria incubated in the presence of palmitoyl-L-carnitine as substrate had higher rates of state 2, 3 and 4 respiration, while the oxidative phosphorylation rate was similar to that obtained with glutamate as an oxidative substrate. Interestingly, the recovery of the mitochondrial state 3 respiration after exposure to hypoxia was lower in the palmitoyl-L-carnitine groups compared to the glutamate groups (Table 6.13). As the ADP/O and RCI ratio's were similar between mitochondria incubated with glutamate or palmitoyl-L-carnitine as substrate, the higher respiration rates noted in the presence of palmitoyl-L-carnitine may simply reflect an adaptive ability of mitochondria to use FA as a fuel source due to the greater reliance of cardiac tissue on FA. Compared to glutamate, the use of palmitoyl-L-carnitine as an oxidizable substrate results in the formation of more reducing equivalents (the use of FA leads to the formation of reducing equivalents produced during both FA β-oxidation and the Krebs cycle, whereas the use of glutamate only produces reducing equivalents via the Krebs cycle). Bugger and Abel, (2010) have further proposed that enhanced FA oxidation resulting from increased myocardial FA supply characteristic of type 2 diabetes leads to a greater production of reducing equivalents. This elevated supply of reducing equivalents to the respiratory chain may lead to increased ROS production which may induce uncoupling protein activation, with a concomitant increase in mitochondrial oxygen consumption. Although the present study failed to

demonstrate mitochondrial uncoupling in the presence of palmitoyl-L-carnitine (ADP/O and RCI ratio's), it is however still be possible that a slight build up of ROS during the normoxic period may contribute to the reduced recovery of state 3 respiration following the anoxic period. However, this remains to be further investigated.

7.4.3 Obesity and mitochondrial biogenesis

In certain instances insulin resistance may be associated with a defective mitochondrial biogenic response characterised by an increased mitochondrial content without a concomitant increase in total mitochondrial respiration rates, while ATP synthesis is reduced (Duncan *et al*. 2007). Although the present study did not assess mitochondrial quantity, our research group has previously shown that the ventricular mitochondrial content is reduced in HCD fed rats after 16 weeks (N Bezuidenhout, MSc dissertation, 2011). Since the mitochondrial oxygen consumption values were expressed in terms of mitochondrial protein, the present data suggest that despite the possible lower total myocardial mitochondrial content in the obese group, their mitochondria are still able to maintain normal respiration rates. This does however portray a delicately balanced system which may become maladaptive with time.

7.5 The impact of insulin administration on myocardial susceptibility to ischaemia/reperfusion injury

The cardioprotective effects of insulin in the setting of ischaemia/reperfusion injury are well documented (Baines *et al*. 1999; Jonassen *et al*. 2000a, b; Jonassen *et al*. 2001; Nawata *et al*. 2002; Van Rooyen *et al*. 2002; LaDisa *et al*. 2004; Hafstad *et al*. 2007; Fuglesteg *et al*. 2008; Yu *et al*. 2008; Oates *et al*. 2009; Xing *et al*. 2009; Ji *et*

al. 2010; Wong *et al*. 2011). Insulin (insulin alone or a glucose-insulin-potassium combination) has previously been shown to elicit cardio-protection when administered 5 minutes prior to reperfusion (LaDisa *et al*. 2004; Jonassen *et al*. 2000a; Jonassen *et al*. 2001; Fuglesteg *et al*. 2008; Yu *et al*. 2008; Ji *et al*. 2010; Wong *et al*. 2011) and in certain instances when present during the entire perfusion protocol (Jonassen *et al*. 2000a; Nawata *et al*. 2002; Van Rooyen *et al*. 2002; Oates *et al*. 2009; Xing *et al*. 2009). Insulin administered as a preconditioning stimulus also induces cardio-protection (Baines *et al*. 1999; Fuglesteg *et al*. 2009).

Despite the substantial amount of evidence supporting a cardioprotective role for insulin, few studies have sought to determine the extent of insulin's cardio-protective effects in insulin resistant animals. While Hafstad *et al*. (2007) have shown that insulin can improve post-ischaemic LV function (aortic output) in diabetic mouse hearts, the cardioprotective effects of insulin and especially insulin's impact on myocardial infarction remained to be documented in models of diet induced obesity and insulin resistance. In contrast to the findings obtained regarding insulin administration times in a previous study (Jonassen *et al*. 2001), the present study has demonstrated that insulin administration 10 minutes prior to and during regional ischaemia improved myocardial tolerance to ischaemia/reperfusion. This was reflected by reduced myocardial infarct size (except for the 50µIU/ml insulin perfusion in the control group) and improved post-ischaemic LV functional recovery (except for the 30µIU/ml insulin perfusion in the control group) in isolated hearts from control and obese animals after 16 weeks of feeding (Figure 5.3a, b). These observations are in agreement to a similar study from Baines *et al*. (1999) where insulin was used as a preconditioning agent. In the latter study reduced infarct sizes were reported

following 10 minutes insulin perfusion followed by a washout period prior to the induction of regional ischaemia in isolated hearts from healthy animals.

While it was demonstrated that all concentrations of insulin protected hearts from obese animals, only physiological concentrations of insulin were able to reduce myocardial infarct size in the control group (Figure 5.3a). The failure of elevated insulin concentrations (50µIU/ml) to induce cardioprotection (infarct size reduction) in the control group of the present study and those of others when administered 10 minutes prior to ischaemia (Jonassen *et al*. 2001), while being protective in hearts from obese animals in the current study may suggest possible metabolic thresholds of protection in normal and diseased animals. The fact that the higher dose was still effective in hearts from obese animals, may yet be another indicator of the reduced responsiveness to insulin in these hearts. The time of administration and the insulin dosage used, appear to be important determinants in the outcomes obtained. While elevated levels of insulin are cardioprotective when administered upon reperfusion, insulin administered prior to and during ischaemia at doses ranging from 0.3 – 5mU/ml have been shown to have no effect myocardial infarct size (Jonassen *et al*. 2001). In contrast when insulin is administered prior to global ischaemia at 40U/ml it has a detrimental effect on post-ischaemic outcomes (Schaefer and Ramasamy, 1997). These opposing outcomes emphasizes the importance of assessing cardioprotective interventions in both normal and diseased animal models at various concentrations as appropriate treatment doses may differ between these groups depending on their metabolic status.

Although the present study did not determine the mechanism of insulin (insulin alone or glucose-insulin-potassium combination) induced cardioprotection, protection

elicited by insulin has previously been shown be associated with or to occur via improved glucose metabolism (glycolytic flux rates) (van Rooyen *et al*. 2002), early activation of PKB/AKT and p70s6 kinase during reperfusion (Jonassen *et al*. 2001), activation of (sarcolemmal or mitochondrial) K_{ATP} channel activity (LaDisa *et al*. 2004), improved re-synthesis of myocardial high energy phosphates upon reperfusion (Nawata *et al*. 2002), anti-adrenergic effects during β-adrenergic stimulation (Yu *et al*. 2008), inhibition of peroxynitrate formation during reperfusion (Ji *et al*. 2010) and activation of Signal Transducer and Activator of Transcription 3 (Fuglesteg *et al*. 2008).

The study of Baines *et al*. (1999) reported that insulin's cardioprotective effects may be related to enhanced PI3K signalling and the results from the present study suggest that insulin's cardioprotective effects may in part relate to the metabolic side of PI3K signalling (increased glucose uptake). This is evident by the marked increase in glycolytic flux rates following insulin stimulation in hearts from both groups of animals (Figure 5.2a, b). Although a model of severe ischaemia (40 minutes coronary artery ligation) was used in the present study, the regional ischaemic zone of a rat heart may receive up to 6.1% collateral flow (coronary collateral flow as a percentage of the non-ischaemic coronary flow) during the ischaemic episode (Maxwell *et al*. 1987), suggesting that insulin, when administered 10 minutes prior to ischaemia, will continue to penetrate the ischaemic zone at a low rate during the ischaemic period and exert its beneficial effects. In addition, the inclusion of simulated *in vivo* concentrations of FA (control: 30µIU/ml insulin with 0.7mM FA; obese: 50µIU/ml insulin with 1.5mM FA) concentrations in the perfusate prior to and during regional ischaemia was shown to increase infarct size and reduce functional recovery to values comparable with the glucose perfused hearts (Figure 5.4, 5.5). This indicates

that the protection elicited by insulin in the current study may have been in part due to metabolic effects. For example, insulin may increase myocardial glycogen levels prior to ischaemia, an effect which has been proposed to be associated with beneficial post-ischaemic outcomes (Van Rooyen *et al.* 2002). The reversal of the beneficial effects of insulin by inclusion of FA's may be due to the profound effects that FA's have on myocardial glucose metabolism (Liu *et al*. 2002 Lopaschuk *et al*. 1993).

7.5.1 Future directions

It is recommend that future experiments should aim to administer super-physiological concentrations of insulin in combination with physiologically relevant concentrations of glucose, and FA when assessing insulin's cardio-protective effects. The timing of insulin administration is also important. Although the present study has demonstrated a cardioprotective role for insulin when administered prior to and during ischaemia in hearts isolated from obese insulin resistant rats, most interventions are applied upon reperfusion of the ischaemic myocardium and as such future studies aiming to investigate insulin's cardioprotective effects should do so.

7.6 The impact of obesity on myocardial susceptibility to ischaemia/reperfusion injury

Although obesity is associated with a higher risk of developing myocardial infarction (Yusuf *et al*. 2005), clinical outcomes post infarction are controversial (Rea *et al*. 2001; Rana *et al*. 2004; Lopez-Jimenez *et al*. 2004; Nikolsky *et al*. 2006; Eisenstein *et al*. 2005; Clavijo et al. 2006; Shiraishi *et al.* 2007). Animal studies are not always able to mimic human obesity and its associated cardiovascular disease entirely, and

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as such, extrapolation of animal data to the clinical setting is difficult. Nevertheless, the use of rodent studies allows for the investigation of obesity related cardiovascular disorders in the absence of confounding factors such as age and diet.

Obese diabetic animals (*db/db* mice) have been shown to be more susceptible to ischaemic injury (reduced LV pump function) and this is thought to result from altered myocardial substrate metabolism coupled with reduced cardiac efficiency (Hafstad *et al*. 2007). Indeed post-ischaemic LV mechanical function in these animals correlates positively with pre-ischaemic myocardial glucose oxidation rates and negatively with pre-ischaemic myocardial palmitate oxidation rates (Hafstad *et al*. 2007), implying that an insulin resistant myocardium associated with diabetes may predispose the heart to poorer post-ischaemic LV function. In contrast to the reduced LV pump function reported in diabetic mice, the insulin resistant myocardium from non-diabetic obese mice display normal post-ischaemic LV mechanical function (Aasum *et al*. 2008). Although post-ischaemic LV mechanical function is a valid index of ischaemia/reperfusion induced injury, myocardial infarct size, which is independent of LV stunning and loading conditions, is thought to be a better predictor of future clinical events (Wu *et al*. 2008). Few studies have assessed the impact of diet induced obesity coupled with insulin resistance on post-ischaemic infarct size, and even then these findings are inconclusive (Thim *et al*. 2006; Clark *et al*. 2011).

After 16 weeks of HCD feeding, hearts from glucose perfused obese animals developed larger infarct sizes and poorer functional recoveries following regional ischaemia compared to hearts from control animals (Figure 5.3a, b). These data are in agreement with previous studies indicating increased post-ischaemic damage in isolated glucose perfused hearts from obese insulin resistant rats (Du Toit *et al*.

2005; Huisamen *et al*. 2011; Nduhirabandi *et al*. 2011) and *in vivo* data from obese and non-obese insulin resistant rats (Morel *et al*. 2003; Clark *et al.* 2011). Interestingly, after 16 weeks of feeding, it was further demonstrated that in response to simulated *in vivo* levels of insulin and FA present in the perfusate prior to and during regional ischaemia (but not during reperfusion), hearts from control and obese animals had comparable infarct sizes (Figure 5.4). As the addition of FA's at various stages of the perfusion protocol may differentially influence indices of ischaemia/reperfusion injury (Lopaschuk *et al*. 1992), it is vital that insulin and FA be present in the perfusate during the entire perfusion protocol to make sound comparisons. Regardless of this, when comparable levels of insulin and FA were present through the perfusion protocol, obesity did not impact on either myocardial infarct size or functional recovery (18 week fed animals) (Figure 6.13a, 6.14a, Table 6.9). Furthermore post-ischaemic comparisons within the control and obese groups in the presence of normal and high levels of insulin and FA revealed that the concentration of these factors did not have a significant effect on myocardial infarct size within these individual groups (Figure 6.15) (18 week fed animals)).

Altering myocardial substrate and hormonal supply in present study allowed interesting observations to be made between the myocardial ischaemic remodelling response in hearts from control and obese animals. The observation of similar infarct sizes between the control and obese groups in the presence of comparable levels of glucose, insulin and FA in the perfusate was indeed interesting. Whereas insulin had an infarct sparing effect in the present study, the observation that differences in infarct size existed between the control and obese group when glucose was the sole substrate would suggest that the ischaemic remodelling response in hearts from healthy lean animals is more sensitive to the inclusion of FA in a glucose and insulin

containing buffer. While the negative effects of the inclusion of FA into a glucose and insulin supplemented perfusate on post-ischaemic LV function have previously been reported in isolated hearts from lean healthy animals (Folmes *et al*. 2006), one may speculate that hearts from obese animals have become accustomed to the elevated *in vivo* FFA supply and have developed an adaptive mechanism buffering the harmful effects of FA's during and following ischaemia. Indeed in obese rodents, increased mitochondrial uncoupling induced by HFD feeding has been proposed to be an adaptive response in preventing excess ROS formation and the subsequent development of cardiac dysfunction in these animals (Wilson *et al*. 2007). It is therefore feasible that in hearts from obese animals, during the early stages of myocardial insulin resistance a similar protective/adaptive mechanism exists, enabling these hearts to effectively utilize FA during the ischaemic and reperfusion periods.

When comparisons of myocardial infarct size were made between control and obese animals in the presence of normal and high insulin and FA concentrations (control normal and high Ins+FA and obese normal and high Ins+FA) using a one-way ANOVA followed by the Dunnetts post-test (data not shown) (The Dunnets post-test makes comparisons between all the groups relative to the control which in this case was the control heart perfused with normal insulin and FA levels), infarct sizes did not differ between the various groups. As obese animals in the current study had elevated levels of circulating insulin and FA's compared to their lean littermates, an attempt was made to determine what the possible impact of this altered substrate environment on myocardial infarct size *in vivo* would be. Consequently myocardial infarct size was compared between hearts from control animals perfused with glucose and normal insulin and FA levels and hearts from obese animals perfused

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with glucose and high insulin and FA levels (insulin and FA being present throughout the experimental protocol). These experiments revealed a modest, yet significant increase in myocardial infarct size in the obese group relative to the control group (Figure 6.16). Considering the results of the One-Way ANOVA, the effects of elevated insulin and FA levels on infarct size in the obese animals are small, and may thus only in part explain the increased susceptibility of the obese animal to ischaemia/reperfusion injury *in vivo*. It is likely that other factors present in the obese animal *in vivo*, may further impact on post-ischaemic outcomes. This may include elevated catecholamine and angiotensin II levels noted in these HCD fed obese rats (W. Smith, MSc dissertation, 2005), both of which have previously be shown to contribute to ischaemia/reperfusion injury (Waldenström *et al*. 1978; Schwartz *et al*. 1997). Nevertheless, our data suggests that should FFA levels be elevated in obese insulin resistant patients with AMI, that interventions be employed aimed at reducing myocardial FFA supply.

The observations noted in figure 6.16 are in agreement with recent findings from our laboratory where 18 week HCD fed rats developed greater infarct sizes compared to control animals after 45 minutes of regional ischaemia and 2 hours of reperfusion *in vivo* (Clark *et al*. 2011). Consequently the observations of the present study may help explain the contrasting post-ischaemic observations between our research group's *in vivo* study, and that of Thim *et al*. (2006) where the lipid profile of the obese animals differed markedly. In our research group's rodent model, obesity was associated with elevated circulating Trig and FFA levels (determined in the present study), while myocardial infarct size following *in vivo* regional ischaemia was greater compared to lean control animals. In contrast, obese rats in the study of Thim *et al*. (2006) had lower fasting circulating Trig levels and similar non-fasting FFA levels compared to

the lean control group, while infarct sizes following regional ischaemia (induced *in vivo*) were comparable between the two groups.

Although the adverse effects of an elevated supply of FA on post-ischaemic LV mechanical function are well documented (Lopaschuk *et al*. 1990; Liu *et al*. 2002; Gambert *et al*. 2006), to our knowledge, this is the first study to suggest a role for elevated levels of insulin and FA's as a contributor to increased post-ischaemic injury (specifically infarct size) in diet induced obese insulin resistant animals. This implies that while obesity is a risk factor for cardiovascular disease (Hubert *et al*. 1983) and specifically acute myocardial infarction in obese humans (Yusuf *et al*. 2005), the present data suggest that the unique metabolic milieu associated with obesity, characterised by elevated insulin and FA levels may itself contribute to exacerbate post-ischaemic outcomes. This notion agrees with many studies demonstrating improved post-ischaemic outcomes following the attenuation of FA metabolism or stimulation of glucose metabolism upon reperfusion (Lopaschuk *et al*. 1990; Liu *et al*. 2002). Further supporting our hypothesis is the observation that healthy hearts perfused with serum isolated from hypertriglyceridaemic hypertensive rats have reduced post-ischaemic LV functional recoveries (Carvajal and Baños, 2002).

Despite the role that elevated levels of FFA's may play in influencing post-ischaemic outcomes in obesity, the controversy regarding clinical outcomes following AMI between obese and lean patients cannot be ignored (Rea *et al*. 2001; Rana *et al*. 2004; Lopez-Jimenez *et al*. 2004; Nikolsky *et al*. 2006; Eisenstein *et al*. 2005; Clavijo et al. 2006; Shiraishi *et al.* 2007). It has however recently been proposed that sleep apnea, which is highly associated with obesity, may afford a cardioprotective role in these individuals (Ozeke *et al*. 2010). The presence of sleep apnea may contribute to

improving post-ischaemic events in the obese population by acting as a pre-conditioning stimulus, subsequently masking the detrimental effects of myocardial insulin resistance and elevated FFA supply on post-ischaemic outcomes.

7.7 The impact of chronic PPARα agonist treatment on biometric and metabolic outcomes in control and obese animals

PPARα agonists are well suited for treating the metabolic manifestations associated with the metabolic syndrome, primarily due to their lipid (Trig) lowering (Robins *et al*. 2001) and insulin sensitizing effects (Guerre-Millo *et al*. 2000; Ide *et al*. 2004; Bergeron *et al*. 2006; Tsunoda *et al*. 2008). Unfortunately, the fibrate class of PPARα agonists requires administration of high doses to achieve clinically beneficial effects and while they can effectively activate PPARα they may also activate PPARγ albeit to a much lesser degree (Willson *et al*. 2000; Shek and Ferrill, 2001). New PPARα agonists are consequently being developed to aid in the treatment of obesity, diabetes and the related metabolic syndrome.

Although various biometric and metabolic outcomes related to K-111 treatment have been investigated in normal animals, obese rhesus monkeys and genetic modified animals displaying obesity and diabetes (Meyer *et al*. 1999; Pill and Kühnle, 1999; Aasum *et al*. 2002; Bodkin *et al*. 2003; Schäfer *et al*. 2004; Aasum *et al*. 2005; Ortmeyer *et al*. 2005; Bratkovsky *et al*. 2006), the present study is the first to document the effects of chronic K-111 administration in an animal model of diet induced obesity and insulin resistance (8 week old rats were obese and insulin resistant before treatment commenced). Similar to the previously mentioned studies, it was demonstrated that chronic K-111 treatment (10mg/kg/day) was able to

significantly ameliorate biometric and metabolic perturbations associated with obesity and the metabolic syndrome (Table 6.2). This was reflected by the prevention of general and visceral fat weight gain and the lowering of non-fasting glucose, insulin (consequently improved HOMA-IR), Trigs and FFA levels associated with obesity in our model of HCD feeding. Interestingly while K-111 had a favourable effect on serum lipids in the obese treated animals, chronic K-111 treatment did not alter the intramyocardial triglyceride content. This is however in agreement with a previous study where obese mice treated chronically with fenofibrate developed an improved lipid profile, while there was no change in intramyocardial Trig content (Aasum *et al*. 2008).

A concern regarding insulin sensitizing drugs such as the Thiazolidinedione's is that of weight gain (body weight and fat mass) (Miyazaki *et al*. 2001; Miyazaki *et al*. 2002). However the current study demonstrated that K-111's insulin sensitizing effect in obese treated animals was rather associated with weight loss. The insulin sensitizing effects afforded by K-111 in obese animals (as indexed by reduced insulin levels and a lower HOMA-IR index) in the present study corroborate findings in obese insulin resistant monkeys and db/db mice treated with K-111 (Pill and Kühnle, 1999; Schäfer *et al*. 2004), while the Trig lowering effects have previously been reported in healthy rats and obese diabetic mice (Pill and Kühnle, 1999; Meyer *et al*. 1999).

The impact of chronic K-111 treatment on the insulin sensitivity (glucose and insulin levels and HOMA-IR index) and blood Trig levels of control animals in the present study was less significant. While K-111 reduced non-fasting blood glucose levels, there were tendencies for K-111 to reduce non-fasting insulin and Trig levels, but these were found to be non-significant. Whereas the lipid lowering (Trig's) effects

observed in K-111 treated animals have previously been reported in healthy animals (Meyer *et al*. 1999), failure to observe similar changes in our study could be explained by the reduced sensitivity of the CardioCheck machine for rodent use, as all measured Trig values in the K-111 treated groups fell below the detection limit of the apparatus. Lastly chronic K-111 treatment significantly attenuated HDL-C levels in both the control and obese treated groups, which is strongly contrasted by findings reported elsewhere in obese pre-diabetic rhesus monkeys (Bodkin *et al*. 2003). This finding may however be specific to rodents as it has been reported that fibrate treatment reduces the circulating levels of apolipoproteins A-I and A-II in rodents (Staels *et al*. 1992).

K-111 did however significantly increase the percentage glycosylated haemoglobin in the lean animals, however the values were maintained within the normal range of healthy animals, suggesting that blood glucose was well managed and controlled under these conditions. Indeed K-111 reduced non-fasting blood glucose levels in both the control and obese treated groups.

One concern regarding the applicability of the present studies' data to those in humans would be the higher hepatic PPARα receptor density known to be present in rodents compared with other species including humans (Palmer *et al*. 1998; Holden and Tugwood, 1999). As such, extrapolation of the beneficial effects of K-111 on body weight, lipid levels and insulin sensitivity reported in this study need to be done with caution. The novel assessment of K-111 in a model of diet induced obesity does however support the current body of evidence advocating the usefulness of K-111 in treating systemic metabolic complications associated with obesity and type 2

diabetes. It will be interesting to see whether the beneficial effects of K-111 noted in the present and other studies are mirrored in the clinical trials.

7.8 The impact of chronic K-111 treatment on cardiac function, substrate metabolism and susceptibility to ischaemia/reperfusion injury

Obesity, insulin resistance and diabetes are generally associated with an altered metabolic profile, which extends to the myocardium, where enhanced FA utilization and reduced glucose utilization predominates (Wilson *et al*. 2007; Hafstad *et al*. 2007; Aasum *et al*. 2008; Zhang *et al*. 2010). As previously discussed, these myocardial metabolic alterations associated with obesity and diabetes are thought to impact on myocardial mechanical function, mitochondrial function and tolerance to ischaemia/reperfusion induced injury. Metabolic modification/manipulation of carbohydrate and lipid utilization often done by genetic or pharmacological interventions has been shown to influence LV mechanical function (Aasum *et al*. 2008) and post-ischaemic outcomes (Vik-Mo *et al*. 1986, Lopaschuk *et al*. 1988; Hafstad *et al*. 2007; Aasum *et al*. 2008) in healthy, obese and diabetic animals. The application of metabolic modulators has also proved useful in treating diabetic patients with ischaemic cardiomyopathy resulting in the improvement of LV function, glucose metabolism and endothelial function (Fragasso *et al*. 2003). It is therefore vital to determine the impact of new metabolic manipulators on these endpoints. This is especially true with regards to PPARα agonists since genetic over-expression of PPARα in the heart has been shown to induce a metabolic phenotype characteristic of the diabetic heart coupled with cardiac dysfunction (Finck *et al*. 2002).

7.8.1 The impact of chronic K-111 treatment on basal cardiac function

In the present study, chronic K-111 treatment did not alter basal *ex vivo* LV mechanical function in either the control or obese treated animals in comparison to their untreated counterparts irrespective of the insulin and FA concentrations present in the perfusate (Table 6.5, 6.6). This finding concurs with other studies using K-111 that have previously reported a lack of LV functional effects in healthy and diabetic animals (Aasum *et al*. 2002; Aasum *et al*. 2005). In contrast, Aasum *et al*. (2008) demonstrated that obese mice treated chronically with the PPARα agonist fenofibrate, had improved myocardial LV function, whereas Zungu *et al*. (2009) demonstrated that healthy rats treated with the PPARα agonist Wy-14,643 for 14 days exhibited significantly attenuated LV functional parameters. The disparity in functional outcomes between the previously mentioned studies (including the present study) and that of Zungu *et al*. (2009) (i.e. no effect or protective vs. detrimental) may be due to the differences in PPARα agonists used (fenofibrate and K-111 vs. Wy-14,643). In addition, only Wy-14,643 was able to induce changes in myocardial PPARα regulated response genes (mRNA levels) in contrast to the present study (K-111 failed to induce alter myocardial CPT-1 expression) and those of others (Aasum *et al*. 2002; Aasum *et al*. 2005, Aasum *et al*. 2008). Although the majority of evidence suggests that chronic PPARα agonist treatment does not impact negatively on myocardial function, murine models of cardiac specific PPARα over-expression exhibit cardiac dysfunction (Finck *et al*. 2002). From the observations made by Zungu *et al*. (2009) the possibility therefore exists, that agonists of the PPARα family capable of augmenting myocardial PPARα response genes may predispose the myocardium to contractile dysfunction.

7.8.2 The impact of chronic K-111 treatment of myocardial substrate metabolism

Studies using cardiac specific PPARα over-expressed mice further suggest that myocardial PPARα activation increases myocardial FA oxidation rates (Finck *et al*. 2002; Park *et al*. 2005; Sambandam *et al*. 2006). In the present study, it was demonstrated that chronic K-111 administration had no impact on control treated, while limited outcomes were observed in the myocardial substrate metabolism profile of obese K-111 treated animals (Table 6.3, 6.4). Hearts from K-111 treated obese animals achieved reduced palmitate oxidation rates prior to global ischaemia in relation to the untreated obese and K-111 treated control rat hearts, but only in the presence of high insulin and FA levels in the perfusate. Although the reduction in myocardial palmitate oxidation rates in the K-111 treated obese group was unexpected, the present data agree with that of Aasum *et al*, (2002; 2005; 2008) who demonstrated similar myocardial metabolic changes in palmitate oxidation rates in K-111 treated db/db mice (Aasum *et al*, 2002; 2005) and fenofibrate treated obese insulin resistant mice (Aasum *et al*. 2008).

Interestingly Carroll and Severson (2001) and Aasum *et al*. (2005) reported an inhibitory effect of K-111 on cardiac lipoprotein lipase activity. The present study further noted that K-111 lowered circulating Trig's and FFA levels in the obese rat, which would imply that the K-111 treated obese rat heart would be accustomed to being exposed to lower circulating FFA supply *in vivo*. In accordance with what was proposed by Aasum *et al*. (2005), it is thought that the systemic effects of K-111, coupled with the absence of K-111 related myocardial effects (the absence of PPARα target gene up-regulation in the present study) may explain the reduced myocardial FA oxidation rates observed in the K-111 treated obese group compared to the

untreated obese group. A similar conclusion to that of Aasum *et al*. (2008) can be made, namely that the myocardial metabolic effects associated with chronic PPARα agonist treatment may result primarily from the drugs' systemic lipid lowering effects.

Contrary to what has been described in the previous paragraph, rodent cardiomyocytes cultured with K-111 have increased FA oxidation rates (Aasum *et al*. 2005). In the same study, when K-111 was administered *in vivo*, it was shown to induce changes in the gene expression of certain PPARα related gene targets in hepatic tissue but not cardiac tissue. The discrepancies observed between the lower FA oxidation rates observed in isolated perfused hearts (current study; Aasum *et al*, 2002; 2005) and higher FA oxidation rates reported in cultured cardiomyocytes (Aasum *et al*. 2005) in response to K-111 treatment, suggest that K-111 primarily acts on the liver and not the heart when administered *in vivo* (as described by Aasum *et al*. 2005).

Although obese rats in the present study maintained euglycaemia, primarily due to an elevated insulin secretion, glucose has been shown to potentiate the effects of PPARα agonists by increasing their binding to co-activators and DNA (Hostetler *et al*. 2008). The discrepancy regarding the myocardial metabolic effects of chronic K-111 treatment in control and HCD fed animals could possibly also be explained by the higher carbohydrate content of the experimental diet which could aid in potentiating PPARα's hepatic effects. This hypothesis however remains to be established.

7.8.3 The impact of chronic K-111 treatment on the expression of myocardial metabolic proteins

As previously mentioned, myocardial CPT-1 expression was not upregulated in both K-111 treated groups. It was further interesting to note that chronic K-111 treatment did no influence the expression or phosphorylation of PKB or the p85-subunit of PI3K. While phosphorylation of these two proteins may relate to the degree of tissue insulin sensitivity and more specifically the degree of glucose uptake (Summer *et al*. 1998; Florian *et al*. 2010), previous studies have documented the lack of induction of PDK4 (inhibits the PDH complex and consequently will influence myocardial glucose oxidation rates) mRNA expression by both fenofibrate and K-111 (Aasum *et al*. 2005; 2008). It therefore seems that certain groups of PPARα agonists have no effects on myocardial gene regulation while others do (Zungu *et al*. 2009). Alterations in *ex vivo* myocardial substrate metabolism seen with certain PPARα agonists may thus reflect the conditioned changes brought about by altered *in vivo* myocardial substrate supply (systemic effects) and not direct myocardial transcriptional effects.

Various authors have documented an inability of fenofibrate and K-111 to induce myocardial target gene expression (Aasum *et al*. 2002; Aasum *et al*. 2005, Aasum *et al*. 2008). Although K-111 was unable to upregulate myocardial CPT-1 levels in the present study, which is in agreement with the finding of the previously listed authors, the conclusion that K-111 does not upregulate the protein expression of myocardial PPARα taget genes in the present rodent model needs to be further evaluated by measuring additional PPARα regulated proteins following chronic K-111 treatment.

7.8.4 The impact of chronic K-111 treatment on isolated ventricular mitochondrial function

While synthetic ligands for PPARα are used to combat the metabolic abnormalities associated with obesity and type II diabetes, it is disputed whether these compounds have beneficial effects on mitochondrial function (Scatena *et al*. 2004). Indeed PPARα agonists administered *in vivo* and *in vitro* have been shown to attenuate ADP dependent oxidative phosphorylation in cardiomyocyte mitochondria (respiring in the presence of glutamate, pyruvate or palmitoyl-L-carnitine) isolated from healthy animals (Zungu *et al*. 2006; Khalid *et al*. 2011), while having no effect on cardiomyocyte mitochondria isolated from diabetic animals (Khalid *et al*. 2011).

Results obtained in the present study showed that lean and obese insulin resistant animals treated chronically with the PPARα agonist K-111 have normal ventricular mitochondrial function irrespective of the oxidative substrate used (Table 6.11, 6.12). Although no effects were observed on mitochondrial function, these findings are novel with respect to both the drug and model in which the drug was used.

The mitochondrial functional findings identified in a model of diet induced obesity could be clinically relevant. Both the PPARα agonists fenofibrate and Wy 14,643 when administered to isolated mitochondria from either skeletal muscle or cardiac ventricles have been reported to inhibit complex I respiratory activity (Brunmair *et al*. 2004; Zungu *et al*. 2006), coupled with reduced rates of oxidative phosphorylation *in vitro* (Zungu *et al*. 2006). While the possibility exists that K-111 may produce similar effects *in vitro*, the present study has demonstrated that chronic *in vivo* administration of K-111 to lean and obese animals does not adversely influence oxidative phosphorylation rates of mitochondria isolated from myocardial tissue. It may

however be necessary to investigate the impact of chronic K-111 treatment on the respiratory capacity of hepatic mitochondria since this tissue may be the primary site of action for K-111 (Aasum *et al* 2005). Nevertheless this data provides additional support for the clinical use of K-111.

7.8.4.1 Future directions

Although the aim was to measure mitochondrial respiratory capacity, the present study did not assess mitochondrial respiratory function in the presence of pyruvate. As mitochondrial respiration in the presence of this substrate may be compromised in obese conditions (Boudina *et al*. 2005), it would be interesting to see whether the improved systemic insulin sensitivity associated with K-111 treatment in obese treated animals would be associated with improved mitochondrial respiratory capacity in the presence of pyruvate.

Although the present study could not show a detrimental effect of chronic *in vivo* K-111 administration on mitochondrial oxidative phosphorylation rates, the fact that similar agonists inhibit complex I respiration and the rates of oxidative phosphorylation in mitochondria are concerning (Zungu *et al*. 2006). Since the primary site of action for K-111 may be the liver (Aasum *et al*. 2005), future studies should aim at investigating the impact of chronic PPARα agonism on hepatic mitochondrial respiration.

7.8.5 The impact of chronic K-111 treatment on myocardial susceptibility to ischaemia/reperfusion injury

Although K-111 had favourable systemic and at times metabolic (lowering palmitate oxidation rates in the presence of high insulin and FA perfusion conditions) effects in obese treated animals, chronic treatment with the drug did not afford cardioprotection (infarct size or post-ischaemic LV functional recovery) following coronary artery ligation and subsequent reperfusion in either the control or obese treated groups, independent of circulating insulin and FA levels present in the perfusate (Figure 6.13a, 6.14a, and Table 6.7, 6.8). This data is in agreement with the findings of Aasum *et al* (2003b; 2005) and together with their observations would suggest that attenuated myocardial FA oxidation prior to ischaemia is not always associated with cardioprotection during reperfusion.

Aasum *et al*. (2003b) previously noted that K-111, when administered at 0.24mg/ml for 3 weeks, was not able to protect isolated hearts from type 2 diabetic db/db mice against ischaemia/reperfusion injury (pump function). The authors speculated that protection from ischaemia/reperfusion injury may be limited to PPARγ activation in the heart. Despite this, PPARα agonists have subsequently been shown to induce cardioprotection in response to ischaemia/reperfusion induced injury in both healthy and diseased animal models (Tabernero *et al*. 2002; Wayman *et al*. 2002; Yue *et al*. 2003; Bulhak *et al*. 2006; Yeh *et al*. 2006; Aasum *et al*. 2008; Bulhak *et al*. 2009). In contrast, only a few studies have noted no or enhanced post-ischaemic injury following PPARα agonism/activation (Aasum *et al*. 2002; Xu *et al*. 2006; Hafstad *et al*. 2009). It may be that the lack of protection (LV pump function) afforded by K-111 in the study by Aasum *et al*. (2002) was influenced by the short duration of drug treatment (3 weeks). In addition, the myocardium of type 2 diabetic mouse may be

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more prone to irreversible ischaemic injury compared to that of the obese insulin resistant animal. However neither of these possibilities may be correct as db/db mice of a similar age as used in the study of Aasum *et al*. (2002), when treated with the pan-PPAR ligand Tetradecylthioacetic acid (which has a high affinity for PPARα) for eight days showed enhanced cardiac output recovery following low flow ischaemia (Khalid *et al*. 2011). In contrast to the present study, Aasum *et al*. (2008) using a similar HCD to that used in the current study, demonstrated increased post-ischaemic LV mechanical function in hearts from obese insulin resistant mice following chronic treatment with the PPARα agonist fenofibrate. Thus the absence of protection afforded by K-111 in a similar model of diet induced obesity may be related to the specific nature of the drug, which may differ subtlety from other classes of PPARα agonists demonstrating cardioprotection from ischaemia/reperfusion injury (Tabernero *et al*. 2002; Wayman *et al*. 2002; Yue *et al*. 2003; Bulhak *et al*. 2006; Yeh *et al*. 2006; Aasum *et al*. 2008; Bulhak *et al*. 2009). The difference in cardioprotection between the present study and that of Aasum *et al*. (2008) (using mice fed a similar HCD treated with fenofibrate) could also be explained by the different endpoints used (infarct size vs. functional recovery) or species differences (rat vs. mouse).

The current study is the first to investigate post-ischaemic outcomes as indicated by myocardial infarct size following chronic K-111 treatment in both normal and diet induced obese animals. Since adverse myocardial post-ischaemic outcomes have been reported in mice with enhanced PPARα activation (Sambandam *et al*. 2006), the current study is clinically significant given the high risk for developing coronary heart disease and myocardial infarction in the obese and diabetic population (Hubert *et al*. 1983; Grundy *et al*. 1999; Yusuf *et al*. 2005), a population that is likely to receive metabolic modulators such as PPARα agonists. Thus the present

observation that chronic K-111 treatment did not augment post-ischaemic injury is extremely important.

7.8.5.1 Future directions

Despite the absence of an infarct lowering effect afforded by chronic K-111 treatment in the present study, the outcomes of these experiments may be confounded by the *ex vivo* nature of the experiments as perfusions performed using similar FA and insulin levels did not simulate the specific *in vivo* environments of the untreated and treated obese animals. In line with the study's hypothesis that the altered metabolic environment associated with obesity may play a role in augmenting post-ischaemic outcomes, combined with the observation that K-111 treatment attenuated FFA and triglyceride levels while improving whole body insulin sensitivity, it is tempting to speculate that chronic K-111 treatment may reduce myocardial infarct size in obese animals *in vivo*. Although hearts from obese K-111 treated rats perfused with "normal" insulin and FA levels did not have smaller infarct sizes compared to the untreated obese group perfused with high levels of insulin and FA (comparison not shown), an *in vivo* study would better explain this relationship. Consequently such an *in vivo* study is warranted to further explore the cardioprotective role of chronic K-111 treatment in models of obesity. Furthermore due to the uncertainty of the post-ischaemic outcomes associated with PPARα agonist treatment, the clinical use of novel PPARα agonists in the treatment of the obesity/diabetes/metabolic syndrome, a population with a high risk for developing AMI, should proceed with caution prior to their post-ischaemic impact being fully investigated.

CONCLUSIONS

The present study made used of a rodent model where obesity was induced by hyperphagia. These obese animals had more retroperitoneal and gonadal fat mass, displayed an altered lipid profile and developed insulin resistance which did not progress to type 2 diabetes even after long term feeding (32 weeks).

While it is known that an oversupply of oxidative fuels characteristic of obesity impacts on cardiac energy metabolism and eventually may contribute to cardiac dysfunction, the present study suggests that prior to the development of cardiac dysfunction, hearts from obese insulin resistant animals (32 week fed rats) are more dependent on physiologically relevant concentrations of FFA to maintain LV mechanical function and functional reserve than hearts from lean animals. This may be due to the ineffective or reduced capability of the insulin resistant heart to utilize glucose as a fuel source.

The impact of obesity on post-ischaemic outcomes is controversial. When obesity was induced over shorter feeding periods myocardial mitochondrial respiration and susceptibility to anoxia were normal. Although hearts from obese animals had larger infarct sizes when perfused with glucose alone, obesity did not impact on myocardial post-ischaemic injury in the presence of similar substrate and hormonal supply in the perfusate. An *ex vivo* simulation, using *in vivo* predicted insulin and FA levels specifically relevant to the control and obese state however suggest that the specific metabolic environment found in the obese state may aid to reduce myocardial tolerance to an acute myocardial infarction. However it is feasible that other factors present *in vivo* in the obese condition may additionally contribute to these outcomes.

Thus on the one hand, obese insulin resistant hearts rely more readily on elevated FFA as a fuel source to maintain normal LV mechanical function and functional reserve, but at the same time, this metabolic environment may contribute to adverse post-ischaemic outcomes. As such metabolic strategies aimed at reducing myocardial supply of circulating FFA in obese patients at risk for AMI are essential. While these strategies may lower the risk for adverse post-ischaemic outcomes, they may additionally improve myocardial insulin sensitivity enabling more effective use of glucose as a fuel source in maintaining LV mechanical function and functional reserve. Data from the present study in conjunction with other studies additionally suggest that the insulin resistant heart in the setting of myocardial infarction may be responsive to and protected by exogenous insulin administration when attempting to revascularize the ischaemic myocardium.

The present study was the first to investigate the cardiovascular effects of the PPARα agonist K-111, in a model of diet induced obesity. While K-111 improved various biometric and systemic metabolic parameters (hyperlipidaemia and insulin resistance) associated with obesity, it did not improve any of the measured cardiovascular outcomes (besides attenuating myocardial palmitate oxidation in the obese group when perfused with high insulin and FA concentrations). Amid the controversy surrounding the impact of PPARα agonists on especially mitochondrial function and post-ischaemic outcomes, the present findings have significant clinical value as it appears that chronic administration of K-111 does not adversely impact on normal myocardial mitochondrial respiration or post-ischaemic infarct size following a simulated acute myocardial infarction. The absence of an infarct sparing effect afforded by K-111 may however be due to the *ex vivo* nature of the experiments as

mimicking the *in vivo* substrate environment of obese and obese K-111 treated animal was not possible.

In conclusion, hearts from obese animals in the present study show no signs of LV contractile dysfunction or mitochondrial dysfunction. The metabolic environment associated with obesity may however contribute to exacerbate post-ischaemic outcomes. *In vivo* studies investigating the effects of metabolic modulators on post-ischaemic outcomes in models of obesity and or diabetes are however warranted.

FINAL COMMENTS

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A final search in PubMed revealed no additional articles related to the use of K-111. An internet search however revealed that phase II clinical trials involving K-111 have been discontinued (http://diabesitydigest.com/targets.php?id=62). The reasons for the discontinuation of this trial are not available.

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