THE EFFECT OF MELATONIN ON MYOCARDIAL SUSCEPTIBILITY TO ISCHAEMIA AND REPERFUSION DAMAGE IN THE RAT MODEL OF HIGH-FAT DIET-INDUCED OBESITY

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Thesis presented in complete fulfilment of the requirements for the degree of Master of Science in Medical Sciences.

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December 2015

Declaration

I, Rafee'ah Kaskar, hereby declare that the work contained in this thesis is my original work and that I have not previously submitted it, in its entirety or in part, at any other university for a degree.

Date: 01 September 2015

ABSTRACT

Introduction:

Obesity has reached epidemic proportions worldwide and is currently a serious health problem. It is associated with metabolic abnormalities, oxidative stress, hypertension, insulin resistance and an increased disposition for the development of cardiovascular disease.

Elucidation of the pathophysiological mechanisms underlying obesity and its relationship with metabolic and cardiovascular diseases is essential for prevention and management of these disorders. Melatonin, the pineal gland hormone, is a powerful antioxidant and has been shown to protect the myocardium against ischaemia/reperfusion (I/R) injury. Long- as well as short-term melatonin treatment also reversed several of the harmful effects of obesity in an animal model of hyperphagia-induced obesity (DIO). However, its effects on myocardial I/R injury and intracellular signalling in obesity induced by a high fat diet (HFD) are still unknown.

Aims of study:

(i) To evaluate the ability of a high fat diet (HFD) to induce obesity in rats. Apart from evaluating its effects on the biometric parameters and resistance to ischaemia/reperfusion injury (as indicated by infarct size in regional ischaemia and functional recovery after global ischaemia), special attention will be given on the interplay between adiponectin, AMPK, leptin, and FFA in this model.

(ii) To evaluate the effect of daily oral administration of melatonin to rats on the HFD as well as their littermate controls, on the parameters listed above as well as on the development of obesity. In this study melatonin will be administered from the onset of the feeding of the high fat diet.

Methods:

Male Wistar rats were divided into 4 groups: (i) control rats (receiving normal rat chow) (C); (ii) control rats receiving melatonin (CM); (iii) obese rats (receiving HFD) (HFD); (iv) obese rats receiving melatonin (HM). Animals were kept on the diet for 16 weeks and melatonin treatment (10mg/kg/day, added to the drinking water) started at the onset of the feeding.

Following feeding and treatment, the animals were grouped into fasted/ non-fasted of which biometric parameters were recorded and blood collected at the time of sacrifice for metabolic and biochemical assays. Hearts were perfused in the working mode for evaluation of myocardial function and infarct size determination after exposure to 35min regional ischaemia/60min reperfusion. For study of intracellular signaling, hearts were perfused in the working mode, subjected to 20min global ischaemia/10min reperfusion and freeze-clamped for

Western blotting. Plasma leptin, adiponectin, free fatty acid, triglycerides, total cholesterol, phospholipids, conjugated dienes and thiobarbituric reactive substances (TBARS) levels were determined. Several kinases were investigated including, the RISK (reperfusion injury salvage kinase) (PKB/Akt and ERK p44/42) and SAFE (survivor activating factor enhancement) (STAT-3) pathways, AMPK and JNK under baseline conditions or following 10 min reperfusion. In addition, expression of UCP-3 and PGC1-α was determined.

Results:

Significant increases in body weight, visceral fat, blood glucose, insulin, HOMA index and leptin and a reduction in adiponectin levels were observed in the fasted high fat diet (HFD) group when compared with controls (C). Significant increases in free fatty acid and triglyceride levels were also noted the HFD group while other serum lipid parameters, including TBARS, remained unchanged. No differences in functional recovery during reperfusion or infarct size after exposure to 35 min regional ischaemia, as well as functional recovery during reperfusion after 20 min global ischaemia were observed between the control and HFD groups. Baseline and 10 min reperfusion data were similar for the RISK and SAFE pathway kinases for the control vs HFD groups. The HFD also had no effect on the expression and phosphorylation of myocardial AMPK and JNK, as well as on the expression of UCP-3 and PGC1-α, when compared to the controls. Treatment with melatonin significantly reduced body weight, visceral fat, blood glucose, HOMA index and serum leptin levels in HFD treated groups, while having no effect on the lipid profile. Although melatonin significantly reduced infarct size in both control [% of area at risk: 20.59 ± 2.29 (CM) vs 38.08 ± 2.77 (C)] and high-fat diet groups [% of area at risk: 11.43 ± 2.94 (HM) vs 38.06 ± 3.59 (H)], it was without effect on myocardial functional recovery during reperfusion. Melatonin had no effect on the intracellular signaling pathways studied.

Conclusions:

The HFD proved to be a useful model of diet-induced obesity with a more pronounced impact on biometric and metabolic changes compared to the DIO model. Long-term melatonin treatment successfully prevented the development of metabolic abnormalities associated with the high fat diet and obesity as well as significantly reduced myocardial infarct size. The mechanisms involved in melatonin-induced cardioprotection in obesity have not been fully elucidated in this study and require further investigation. However, the anti-obesogenic and cardioprotective properties of melatonin were very significant indeed and support the suggestion of this hormone as a potential tool in the treatment of obesity and associated cardiovascular complications.

ABSTRAK

Inleiding: Vetsug (obesiteit) het wêreldwyd epidemiese afmetings aangeneem en word tans as ‘n ‘n ernstige gesondheidsprobleem beskou. Vetsug word geassosieer met metaboliese afwykings, oksidatiewe stres, hipertensie, insulienweerstandigheid en is‘n belangrike risikofaktor vir die ontwikkeling van kardiovaskulêre siekte. Ten spyte hiervan, het onlangse studies ‘n gunstige effek van vetsug op die uitkomste van miokardiale infarksie in pasiënte gerapporteer, die sg obesiteitsparadoks. Kennis van die patofisiologiese meganismes onderliggend aan vetsug en die ontstaan van metaboliese afwykinge en hartsiekte is noodsaaklik vir die voorkoming en behandeling van hierdie toestande. Melatonien, die hormoon afgeskei deur die pineaalklier, is ‘n kragtige antioksidant en vry radikaal opruimer. Dit is voorheen aangetoon dat dit die hart teen iskemie/herperfusie (I/H) besering kan beskerm en sommige van die skadelike gevolge van vetsug in diermodelle kan omkeer. Die effek van melatonien op miokardiale I/H besering en intrasellulêre seintransduksie prosesse in vetsug geïduseer deur ‘n hoë vet dieet is egter nog onbekend.

Doelstellings:

(i) Die ontwikkeling en karakterisering van ‘n nuwe model van vetsug en insulien-weerstandigheid geïnduseer deur 'n hoë vet dieet (HVD) en die evaluering van die effek daarvan op miokardiale I/H besering en die gepaardgaande intrasellulêre seintransduksieprosesse;

(ii) Bepaling van die effek van daaglikse toediening van melatonien aan rotte op die HVD sowel as aan kontroles op ‘n standard dieet, op die ontwikkeling van dieet-geïnduseerde metaboliese veranderinge, miokardiale infarktgrootte en funksionele herstel na koronêre arterie afbinding, sowel as intrasellulêre seintransduksie.

Metodiek: Vier groepe van manlike Wistar rotte is bestudeer: (i) kontrole rotte (op‘n standaard dieet) (K); (ii) kontrole rotte op ‘n standard dieet plus melatonien (KM); (iii) dieetrotte (op‘n HVD); (iv) HVD rotte wat melatonien ontvang (HM). Die HVD en melatonien (10mg/kg/dag in die drinkwater) is vir 16 weke toegedien. Na die periode van behandeling, is die diere in vastende en nie-vastende groepe verdeel, die biometriese parameters genoteer en bloedmonsters vir metaboliese en biochemiese bepalings versamel, tydens verwydering van die harte. Harte is geperfuseer volgens die werkhartmodel vir bepaling van miokardiale funksie en infarktgrootte na blootstelling aan 35min streeksiskemie. Vir evaluering van intrasellulêre seintransduksie, is geperfuseerde werkende rotharte blootgestel aan 15min globale iskemie/10 min herperfusie en gevriesklamp vir latere analises volgens die Western kladtegniek.hart.

Serum leptien, adiponektien, vryvetsure, trigliseried, totale cholesterol, fosfolipiede, gekonjugeerde diene en tiobarbituursuur reaktiewe stowwe (TBARS) is bepaal. Met gebruik van Western kladtegniek, is die aktivering en/of uitdrukking van die RISK (PKB/ Akt en ERK p44/42) en SAFE (STAT-3) seintransduksiepaaie, AMPK, JNK, UCP-3 en PGC1-α, onder basislyn toestande of na 10 min herperfusie bestudeer.

Resultate: ‘n Beduidende toename in liggaamsgewig, visserale vet, die HOMA indeks, insulien en leptien vlakke is in die HVD groep waargeneem vergeleke met die kontrole (K) rotte. Adiponektien vlakke was laer in die HVD groep. Die HVD groep is ook gekenmerk deur ‘n beduidende styging in serum vryvetsuur en trigliseried vlakke, terwyl die ander lipied parameters, insluitende die TBARS vlakke, onveranderd was. Infarktgrootte en funksionele herstel tydens herperfusie na blootstelling aan 35 min streeksiskemie, asook funksionele herstel tydens herperfusie na 20 min globale iskemie het nie verskil tussen harte van die kontrole en HVD rotte nie. Aktivering van PKB/Akt, ERK p44/p42, STAT3, AMPK en JNK by basislyn en na 10 min herperfusie was soortgelyk in die kontrole en HFD groepe. Die HVD het ook geen effek op die uitdrukking van UCP-3 en PGC1-α in vergelyking met die kontrole gehad nie. Behandeling met melatonien het die liggaamsgewig, visserale vet, bloedglukose, HOMA indeks en serum leptien vlakke in die HVD groepe statisties beduidend verlaag, terwyl dit geen invloed op die lipiedprofiel gehad het nie. Melatonien behandeling het die miokardiale infarktgrootte beduidend en tot dieselfde mate verminder in beide kontrole [20.59 ± 2.29 (KM) vs 38.08 ± 2.77% (K)] en HVD groepe [11.43 ± 2.94 (HM) vs 38.06 ± 3.59% (HVD)]. Geen verskille is egter tussen die funksionele herstel gedurende herperfusie van die behandelde en onbehandelde kontrole en HVD groepe waargeneem nie. Melatonien het ook geen uitwerking op die intrasellulêre seintransduksiepaaie gehad nie.

Gevolgtrekkings: Die resultate het getoon dat die HFD 'n goeie model van dieet-geïnduseerde vetsug en insulien weerstandigheid ontlok, met 'n meer uitgesproke impak op biometriese en metaboliese veranderinge as die voorheen gebruikte hoë-sukrose dieet. Langtermyn melatonien- behandeling het die ontwikkeling van metaboliese abnormaliteite geassosieer met die HVD, voorkom, asook miokardiale infarktgrootte na koronêre afbinding beduidend verminder. Die meganismes betrokke in melatonien-geïnduseerde miokardiale beskerming moet egter in meer detail ondersoek word. Die resultate verkry steun die voorstel dat melatonientoediening voordelig sal wees in die behandeling van vetsug en sy kardiovaskulêre komplikasies.

ACKNOWLEDGEMENTS

I would like to extend my heartfelt thanks towards Prof. Amanda Lochner and Prof. Barbara Huisamen for excellent guidance, assistance and inspiration without whose knowledge and expertise this thesis would be incomplete.

I wish to express my gratitude to Sonia Genade for assisting with the heart perfusions and Dr Dee Blackhurst (Lipidology Division, University of Cape Town) for the lipid profile analysis as well as Prof. Stefan du Plessis, Prof. Hans Strijdom and my colleagues from the Divison of Medical Physiology (University of Stellenbosch) for encouragement and support.

The financial assistance of the Medical Research Council (MRC) and the Division of Medical Physiology (University of Stellenbosch) is hereby acknowledged.

I would like to thank my family for prayers, support and motivation throughout the pursuit of this degree.

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LIST OF ABBREVIATIONS

Units of measurement

cm: centimetre
g: gram
Hg: mercury
IU: international unit
kg: kilogram
kj: kilojoule
L: litre
m: molar
mg: milligram
min: minute
mM: millimolar
mm: millimetre
mmol: millimol
ºC: degree Celsius
v: volume
µ: micro
µl: microliter
µm: micrometer
µmol: micromol

Abbreviations

AA-NAT: Arylalkylamine N- acetyltranferase
AFMK: N- Acetyl- N- formyl- 5 methoxykynuramine
AMI (MI): Acute myocardial infarction
AMK: N1- Acetyl- 5- methoxy- kynurenine
AMP: Adenosine monophosphate
AMPK: Adenosine monophosphate kinase
AO: Aortic output
ATP: Adenosine triphosphate
BAT: Brown adipose tissue
BW: Body weight
Ca2+: Calcium
CAL: Coronary artery ligation
cAMP: Cyclic adenosine monophosphate
cGMP: Cyclic guanosine monophosphate
CO: Cardiac output
IRS: Insulin receptor substrate
JNK: c-Jun N-terminal kinase
LDH: Lactate dehydrogenase
LDL: Low density lipoprotein
LV: Left ventricle
MAPK: Mitogen activated protein kinase
MDA: Malondialdehyde
MPO: Myeloperoxidase
MPTP: Mitochondrial permeability transition pore
MetS: Metabolic syndrome
MT1/MT2: Melatonin receptor 1 and 2
NO: Nitric oxide
ONOO⁻: Peroxynitrite
P13-K: Phosphatidylinositol 3 kinase

CO_2: Carbon dioxide

COX- 2: Cyclooxygenase- 2

CREB: cAMP- response element binding protein

CVD: Cardiovascular disease

DAG: Diacylglycerol

DIO: Diet induced obesity

DP: Diastolic blood pressure

ERK 42/ 44: Extracellular signal regulated kinase p42/ p44

ETC: Electron transport chain

FFA: Free fatty acid

GLUT- 4: Glucose transporter 4

GPCR: G- protein coupled receptor

GPx: Glutathione peroxidase

GSH: Reduced glutathione

GSK-3: Glycogen synthase kinase- 3

H2O2: Hydrogen peroxide

HDL: High density lipoprotein

HFD: High fat diet

HOCL: Hyperchlorous acid

HOMA: Homeostasis model assessment

HR: Heart rate

HW: Heart weight

I/R I: Ischaemia and reperfusion injury

IFS: Infarct size

IP3: Inositol 1,4,5- triphosphate

IR: Insulin receptor

PGC1- α: Peroxisome proliferator-activated receptor Coactivator 1 alpha

PKA: Protein kinase A

PKB/ Akt: Protein kinase B

PKC: Protein kinase C

PPARγ: Peroxisome proliferator-activated receptor gamma

PSP: Peak systolic pressure

QUICK: Quantitative insulin sensitivity check

RAS/RAAS: Renin angiotensin/aldosterone system

RIA: radioimmunoassay

RISK: Reperfusion injury salvage kinase

RNS: Reactive nitrogen species

ROOH/ LOOH: Lipid hydroperoxide

ROS: Reactive oxygen species

SAFE: Survivor activating factor enhancement

SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM: Standard error of the mean

SIRT1: Sirtuin 1

SOCS3: Suppressor of cytokine signalling 3

SOD: Superoxide dismutase

STAT-3: Signal transducer and activator of transcription 3

TBARS: Thiobarbituric acid reaction substrate

TNF- α: Tumour necrosis factor α

TRIG: Triglyceride/ triacylglycerol

TZD: Thiazolidinediones

UCP- 3: Uncoupling protein 3

VLDL: Very low density lipoprotein

WAT: White adipose tissue

WHO: World Health Organisation

Wtot: Work total or total work

CHAPTER 1

LITERATURE REVIEW

1. OBESITY AND CARDIOVASCULAR DISEASE

Introduction

In the past two decades, obesity has reached epidemic proportions worldwide (for a review see Cao, 2014). Defined as having a body mass index (BMI) of or greater than 30 kg/m^2 (Jahangir *et al*., 2014), obesity is a condition characterized by an imbalance between food intake and energy expenditure resulting in adipose tissue expansion (Yingzhong *et al*. 2006) and increased health risk (Etelson *et al*., 2003).

Obesity poses a risk for several disorders such as, hypertension, insulin resistance and fatty liver disease, with an increased predisposition for the development of cardiovascular disease, type 2 diabetes mellitus (Emanuela *et al*., 2012; for a review see Cao, 2014) and cancer (Haslam *et al*., 2006). Mounting evidence have also linked obesity to obstructive sleep apnoea, osteoarthritis (Haslam & James, 2005) and chronic kidney disease (Ruan & Guan, 2009).

Although obesity has long been associated with serious adverse health conditions, in recent years, along with body weight management, it is rapidly becoming the center of attention for public health experts (Kopelman, 2000; James, 2008). According to the World Health Organization (WHO), globally 600 million adults (18years and older) are obese and 42 million children are overweight/obese, with escalating obesity rates in both adults and children (Wang *et al*., 2008).

Elucidation of the pathophysiological mechanisms underlying obesity and its relationship with metabolic and cardiovascular diseases are essential for prevention and management of these disorders (Ohashi *et al*. 2014).

Obesity is a multifactorial disease and the mechanisms underpinning the development thereof have been associated with dysfunction of adipocytokine pathways (Vazquez-Vela *et al*., 2008) while its comorbidities, such as increased fat accumulation, lipotoxicity, increased oxidative stress and insulin resistance, have been suggested as possible mechanisms linking it to increased cardiovascular risk (For a review see Bluher, 2012).

In particular, obesity is a major risk factor for the development of cardiovascular disease and heart failure, causing a reduction in life expectancy and quality of life (see for example Diercks et al., 2006; Mahamat et al., 2003; Chase et al., 2014). Obese patients with cardiovascular disease are also more likely to develop acute coronary syndromes, likely because of concomitant comorbidities that tend to cluster with obesity, which include hypertension, diabetes mellitus and hyperlipidemia, as mentioned above (Wolk et al., 2003). Thus, elucidation of the pathophysiological mechanisms underlying obesity and its relationship with metabolic and cardiovascular diseases is essential for prevention and management of these disorders (Ohashi *et al*. 2014).

In view of the above, the first sections of the literature survey will focus on the physiological role of adipose tissue, the changes induced by obesity as well as the possible underlying mechanisms in cardiovascular disease and associated metabolic abnormalities.

1.1 Physiological role of adipose tissue and adipocytes

It is well-established that adipose tissue (AT), being the most widespread tissue in humans, plays a dynamic role in physiological processes occurring throughout the body. Adipose tissue (subcutaneous and visceral fat) is known to cushion and insulate the body, but more importantly to store energy, specifically in white adipose tissue (WAT) depots (Golan *et al*., 2012; Haas *et al*., 2012).

Its main constituent, adipocytes, is considered to be a corner stone in the homeostatic control of whole body metabolism (Ali *et al*. 2013). Adipocytes store triglycerides (Ntambi & Young-Cheul, 2000) derived from the diet and accounts for the majority of fat stored in the body. Lipolysis of adipose tissue triglycerides is responsible for the release of free fatty acids (FFA), the principal lipid fuel present in the circulation. Insulin is the main regulator of adipocyte fat content, enhancing FFA uptake and triglyceride synthesis (for a review see Hajer *et al*. 2008).

In contrast to WAT, brown adipose tissue (BAT) is specialized in generating heat in response to cold or diet (nonshivering thermogenesis) rather than oxidizing fatty acids (Jimenez-Aranda *et al*. 2013).

Besides the classical function of storing fat, numerous biologically active substances (adipocytokines/adipokines) are secreted by adipocytes (Greenberg & Obin, 2006; Jensen *et al*., 2006).

Thus fat is considered to be a major endocrine organ with profound effects on the regulation of normal body processes (Ali *et al*. 2013) and is increasingly being recognized as an important role player in the development of obesity (Kershaw & Flier, 2004; Fonseca-Alaniz *et al*., 2006; for a review see Khan & Joseph *et al*., 2014), which will be discussed below.

1.2 Adipose tissue-derived secretions: adipokines

Adipose tissue plays a central role in metabolic alterations (insulin resistance, type 2 diabetes, amongst others) triggered by lipid over-accumulation, leading to disordered adipocyte function (For a review see Schuster, 2010; El Akoum *et al*., 2011).

Given the importance of adipocytes and adipose tissue in obesity and obesity-related diseases (Rosen & Spiegelman, 2006), adipokines have been studied extensively as they are known to regulate pivotal aspects of metabolism (Pajvani *et al*., 2003; Gunawardana, 2014), reflect adipose tissue function and have noteworthy effects on the brain, muscle, liver and pancreas (For a review see Bluher, 2012; Trujillo & Scherer, 2006).

It has been well reviewed that increased adiposity leads to adipose tissue dysfunction mirrored by impaired adipokine secretion and activation of pro-inflammatory signaling (Bastard *et al*., 2006; Wellen & Hotamisligil, 2005), suggesting a potential link for obesity and inflammation, insulin resistance and adverse cardiovascular outcomes (Flehmig *et al*., 2014; Dunmore & Brown, 2013; Wellen & Hotamisligil, 2005).

The adipose tissue-derived hormone, leptin, surfaced as a 'central player' in the cloning of genes involved in body weight control (Zhang *et al*., 1994; for a review see Yingzhong *et al*., 2006), which further stimulated the discovery of new adipose tissue derived signals (Haluzik *et al*., 2004; Gomez *et al*.,2009). Among the many putative molecules, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) have been identified to play a role in the inflammatory response. While adipokines such as omentin, vaspin, chemerin, resistin, and fatty acid binding protein 4 have been associated with insulin resistance (For reviews see Bluher, 2012; Haas *et al*. 2012; Haluzik *et al*. 2004), adiponectin has been shown to positively correlate with insulin sensitivity and supplementation thereof attenuates insulin resistance (Yamauchi *et al*., 2002).

Research has focused on the clinical relevance of adipokines as markers or predictors of obesity related diseases and as potential therapeutic tools or targets thereof (For review see Bakhai, 2008; Bluher, 2012). The importance of adipokines in regulating normal body processes, such as energy metabolism, appetite control (For a review see Fisman & Tenenbaum, 2014), insulin sensitivity (For a review see Bluher, 2012), inflammation and cardiac function (Flehmig *et al*., 2014) have been highlighted. Conversely, a role for adipokines in the pathophysiology of several metabolic diseases has been established (Bluher, 2009; for a review see Fisman & Tenenbaum, 2014). The respective roles of leptin and adiponectin in health and obesity are summarized below.

1.3 Leptin

Leptin, a 16kDa hormone, was first identified by Zhang and coworkers in a mouse model of obesity (Zhuang *et al*., 2009). It is abundantly expressed in adipose tissue, specifically adipocytes, and is a key factor in the regulation of energy homeostasis (Jung & Choi, 2014).

It is encoded by the obese (ob) gene and is known to inhibit appetite/food intake and stimulate energy expenditure (Fortuno *et al*., 2003) via three neurons in the hypothalamus, neuropeptide Y (NPY), γ-aminobutyric acid (GABA), and pro-opiomelanocortin (POMC) (Morton *et al*., 2005).

Although leptin's actions are predominantly exerted via the hypothalamus, it also directly affects peripheral tissues (Schwartz *et al*., 2000). In vivo studies have shown that leptin improves insulin sensitivity (Wang *et al*., 1999; Kahn & Flier, 2000), possibly by preventing

fat deposition in the liver and skeletal muscle and directly enhances muscle fatty acid oxidation (in vitro) by activating AMP-activated protein kinase (AMPK) (Muoio, 1997).

It is well-established that circulating leptin levels and its mRNA expression are elevated in obese subjects (Kim-Motoyama *et al*., 1997). A phenomenon known as leptin resistance occurs as a result of excessive food intake, impairing leptin's actions (Lin *et al*., 2000) in leptin-responsive areas in the brain (Jequier, 2002). Strong associations between leptin resistance and endothelial dysfunction, heart-failure, atherosclerosis and hypertension, among many other related metabolic disorders, have been made (for a review see Gomez *et al*., 2009). The anti-diabetic effect of leptin is mediated by activation of the (PI3K)/Akt pathway that stimulates insulin sensitivity in peripheral tissues (Morton, *et al*. 2005).

Growing evidence suggests that leptin may contribute to the development of cardiac dysfunction (for reviews see Karbowska & Kochan, 2012; Hou & Luo, 2011). In a large prospective study leptin has been shown to an independent risk factor for coronary heart disease. In animal studies chronic leptin infusion increased heart rate and blood pressure and it has been demonstrated that circulating leptin levels are elevated in patients with heart failure. Moreover, a role for this hormone in mediating myocardial hypertrophy has been suggested. Findings thus far have suggested that leptin acting directly of through the sympathetic nervous system may have adverse effects on cardiac structure and function and that chronic hyperleptinemia may greatly increase the risk of cardiac disorders.

1.4 Adiponectin

Adiponectin or adipocyte-complement related protein of 30 kDa (Acrp30) is present in high levels in human plasma relative to other hormones (for a review see Diez & Iglesias, 2003), with concentrations ranging from 5 to 30mg/L, accounting for more or less 0.01% of total plasma protein (Arita *et al*., 1999). Due to the remarkable effects of adiponectin on various systems in the body, much attention has been given to the receptors (AdipoR1 and AdipoR2) through which it acts (Yamauchi *et al*., 2003).

Adiponectin differs from other adipokines in that it is secreted solely by adipose tissue (Maeda *et al*., 1996), and found at reduced levels in the presence of obesity (Arita *et al*., 1999), type 2 diabetes and heart disease (For a review see Lee & Kwak, 2014). It is well established that plasma adiponectin levels are negatively associated with the accumulation of body fat, particularly visceral fat (Matsuzawa *et al*., 2004).

Adiponectin has diverse functions ranging from aiding in lipid and glucose metabolism to immune cell function. It exerts direct actions on the liver, skeletal muscle (Berg *et al*., 2001) and cardiovascular system (Ouchi *et al*., 2006) and may possess both protective and ameliorating effects in many metabolic diseases, with its insulin sensitizing effects being one of its key properties (For reviews see Berg *et al*., 2002; Trujillo & Scherer, 2006).

Being the most abundantly secreted adipocyte derivative, adiponectin has been implicated in the interrelationship between obesity, insulin resistance and inflammation (Silva *et al*., 2014; for a review see Fisman & Tenenbaum, 2014).

Clinical studies indicate a close association of low adiponectin levels (hypoadiponectinemia) with endothelial dysfunction and insulin resistance (Kadowaki *et al*., 2007; Matsuzawa *et al*., 2004; Gomez *et al*., 2009). Hypoadiponectinemia also positively correlates with elevated levels of the pro-inflammatory cytokine, tumor necrosis factor alpha (TNFα) in obesity (Hector *et al*., 2007). Long-term administration of globular adiponectin to rats on a high-fat diet enhanced

insulin signaling and lipid storage in adipose tissue, resulting in improvements of the metabolic profile (Matafome *et al*., 2014). Increased fatty acid oxidation and inhibition of hepatic glucose production have been reported as the key mechanisms through which adiponectin enhance insulin sensitivity (Kadowaki *et al*., 2007; Silva *et al*., 2014).

Several studies investigated the role of adiponectin in cardiac diseases associated with obesity (Okamoto, 2011). It is well known that cardiovascular risk factors such as obesity, dyslipidemia and increased blood pressure are associated with low levels of adiponectin, suggesting a link between hypoadiponectinemia and cardiovascular disease (Arita *et al*., 1999; Matsuzawa *et al*., 2004). Associations of hypoadiponectinemia have further been made with cardiac hypertrophy (Okamoto, 2011) and coronary artery disease (Ouchi *et al*., 1999; Hotta *et al*., 2001).

The significance of adiponectin in the response of the heart to ischaemia/reperfusion was illustrated in a study by Shibata *et al*. (2005) who showed increases in myocardial infarct size and apoptosis, as well as elevated expression of TNF-α, in adiponectin knock-out mice. Pretreatments with adiponectin of these knock-out mice reduced the infarct size and apoptosis after ischemia/reperfusion, suggesting a cardioprotective role for adiponectin in cardiac myocytes (Shibata *et al*., 2005).

1.5 DEVELOPMENT OF INSULIN RESISTANCE AND OBESITY

Obese individuals tend to be both insulin resistant and at increased risk to develop cardiovascular disease. However, a large epidemiological study showed although 36% of individuals in the most insulin-resistant group were obese, not all overweight/obese individuals are insulin resistant and not all insulin resistant individuals are overweight/obese (McLaughlin et al., 2004; Reaven 2011).

1.5.1 Pathophysiology of insulin resistance

Insulin is a pleiotropic hormone produced in the pancreatic β-cells. It has effects on muscle, liver and adipose tissue thereby influencing metabolism and various cellular processes throughout the body (Jellinger, 2007). Insulin orchestrates the regulation of glucose homeostasis through promoting glucose uptake in peripheral tissue, such as muscle (skeletal and heart), fat (brown and white), and in suppressing hepatic glucose production (Benito, 2011).

Apart from insulin's effect on nutrient metabolism, it is also involved in protein synthesis, growth, generation of new mitochondria (biogenesis), autophagy, proliferation and differentiation (For a review see Bunner *et al*., 2014).

Insulin resistance is a generalized metabolic disorder characterized by inefficient insulin function or decreased cellular sensitivity to insulin in skeletal muscle, liver and adipocytes (Haag & Dipenaar, 2005). Growing evidence support the notion that increased circulating FFA levels play an important role in the development of insulin resistance and deterioration of β-cell function due to hyperglycemia (Shulman, 1999; Evans *et al*., 2003). Insulin resistance hinders the normal actions of insulin such as increased muscle and cellular glucose uptake,

glycogen synthesis and reduction in hepatic glucose production (Petersen & Shulman, 2002; Haag & Dipenaar, 2005).

Insulin resistance manifests differently in different tissues. For example, it can originate in adipose tissue, where it results in increased lipolysis, thereby releasing glycerol and fatty acids (FFA) into the circulation. In the liver, insulin resistance is manifested by impairing the ability of insulin to suppress glucose production thereby raising blood glucose levels (Kahn & Flier, 2000; Manrique *et al*., 2014; Muniyappa *et al*., 2008).

Insulin resistance can be measured by a number of approaches, including the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) (Matthews *et al*., 1985), the quantitative insulin sensitivity check (QUICK) index (Katz *et al*., 2000), and the gold standard, hyperinsulinemic-euglycemic clamp test (DeFronzo *et al*., 1979).

The nuclear receptor, peroxisome proliferator-activated receptor λ, important in lipid metabolism, has been identified as an important target of insulin sensitizing drugs, such as the thiazolidinediones (TZDs), to counteract insulin resistance and type 2 diabetes (For a review see Bunner *et al*., 2014). TZDs promote differentiation of preadipocytes into white adipocytes and are assumed to redirect FFA away from skeletal muscle towards adipose tissue (the so-called lipid steal hypothesis). The TZDs are also implicated in positively influencing insulin resistance by decreasing the expression of TNFα, IL-1 and resistin while increasing the adiponectin production (Haas *et al*., 2012).

1.5.2 Mechanisms of obesity-induced insulin resistance

1.5.2.1 Insulin signaling in insulin sensitive and resistant tissue

Binding of insulin to its receptor (IR) triggers its phosphorylation and activation via an intrinsic kinase activity, leading to tyrosine phosphorylation of the insulin receptor substrate (IRS) proteins 1 to 4 (Saltiel & Kahn, 2001).

Normal insulin signaling following stimulation of the insulin receptor (IR) is mediated by two different pathways: the phosphatidylinositol-3-OH kinase pathway (PI3K pathway) which is predominant in metabolic tissue and a second, growth factor-like promitotic pathway namely mitogen-activated protein kinase (MAPK) (Muniyappa & Sowers, 2013; Manrique *et al*., 2014).

Tyrosine phosphorylation of insulin receptors (1 to 4) initiates the PI3K pathway to produce phosphatidylinositol-3,4,5-triphosphate (PIP_3), and its increased production results in activation of PKB/Akt via phosphoinositide-dependent kinase 1 (PDK1) activation (Shulman *et al*., 1999) which in turn activates PKC (ζ and λ) (Bertrand *et al*., 2008). Other downstream effectors which mediate insulin action include translocation of the glucose transporter (GLUT1 or 4) to the plasma membrane (Shulman *et al*., 2000) and PKB/Akt mediated inhibition of GSK-3 (Cheng *et al*., 2010).

The growth and mitogenic actions of insulin involve the MAPK-dependent pathway, which is initiated by activation of Shc, Grb-2, Ras and Sos (Carel *et al*., 1996). Following Ras activation, Raf is recruited to the cell membrane which stimulates a signalling cascade by phosphorylation of MAPK which in turn phosphorylates and activates downstream proteins such as extracellular regulated kinase (ERK1/2) (Sasaoka *et al*., 1994).

Contrary to normal insulin signaling, conditions of insulin resistance lead to inhibition

of the metabolic pathway (PI3K) and overstimulation of the growth-factor like pathway (MAPK), resulting in a decrease in glucose uptake and possibly affecting normal metabolic function (Van Gaal *et al*, 2006).

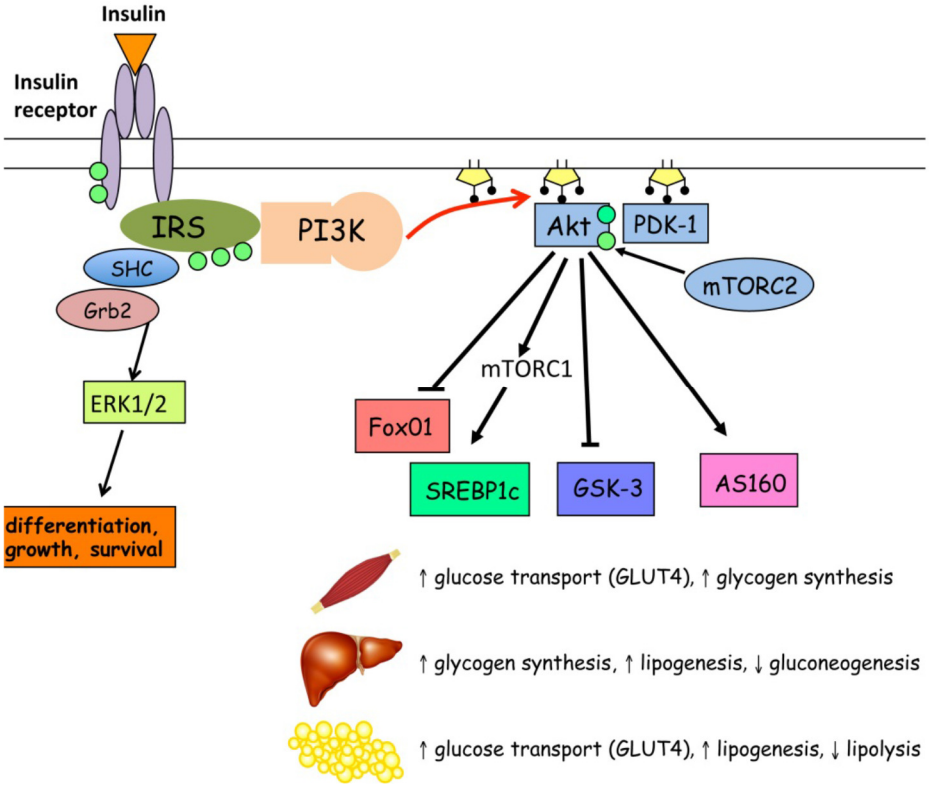

Figure 1.1 Insulin signaling pathway. Binding of insulin to the insulin receptor initiates a signaling cascade that involves multiple phosphorylation events (green circles) and leads to alterations in glucose and lipid metabolism (Turner, 2013).

IRS, insulin receptor substrate; SHC, Src Homology 2 domain; GRB2, growth factor receptor-bound protein 2; ERK, extracellular- signal-regulated kinases or classical MAP kinases; PI3K Phosphoinositide 3-kinase; PDK1, phosphoinositidedependent protein kinase 1; mTORC mammalian target of rapamycin complex; FoxO1 Forkhead box protein O1; SREBP1c sterol regulatory element binding protein 1c; GSK-3, glycogen synthase kinase 3; AS160, 160 kDa Akt substrate.

The molecular mechanisms of insulin resistance in type 2 diabetes have not been fully characterized, although many important biochemical, metabolic, and genetic features have been identified. Accumulated findings have highlighted several pathways in insulin resistance,

including lipid accumulation, oxidative stress, and inflammation (For a review see Bunner *et al*., 2014).

An important common feature of these mechanisms is the activation of stress-sensitive kinases including protein kinase C ζ (PKCζ). PKCζ depends on lipids for activation (such as diacylglycerol/DAG and fatty acyl-CoA). When activated, PKCζ has the ability to enhance the inhibition of nuclear factor kappa-B (IKK-β) while both c-Jun N-terminal kinase 1 (JNK1) and PKCζ will phosphorylate IRS-1 on serine residues, thereby hindering tyrosine phosphorylation. Diminished tyrosine phosphorylation of IRS-1 reduces signaling through the PI3-kinase/Akt pathway interfering with binding of IRS1/2 to the insulin receptor, ultimately affecting insulin signalling (Schmitz-Peiffer & Biden, 2008; Morino *et al*., 2006).

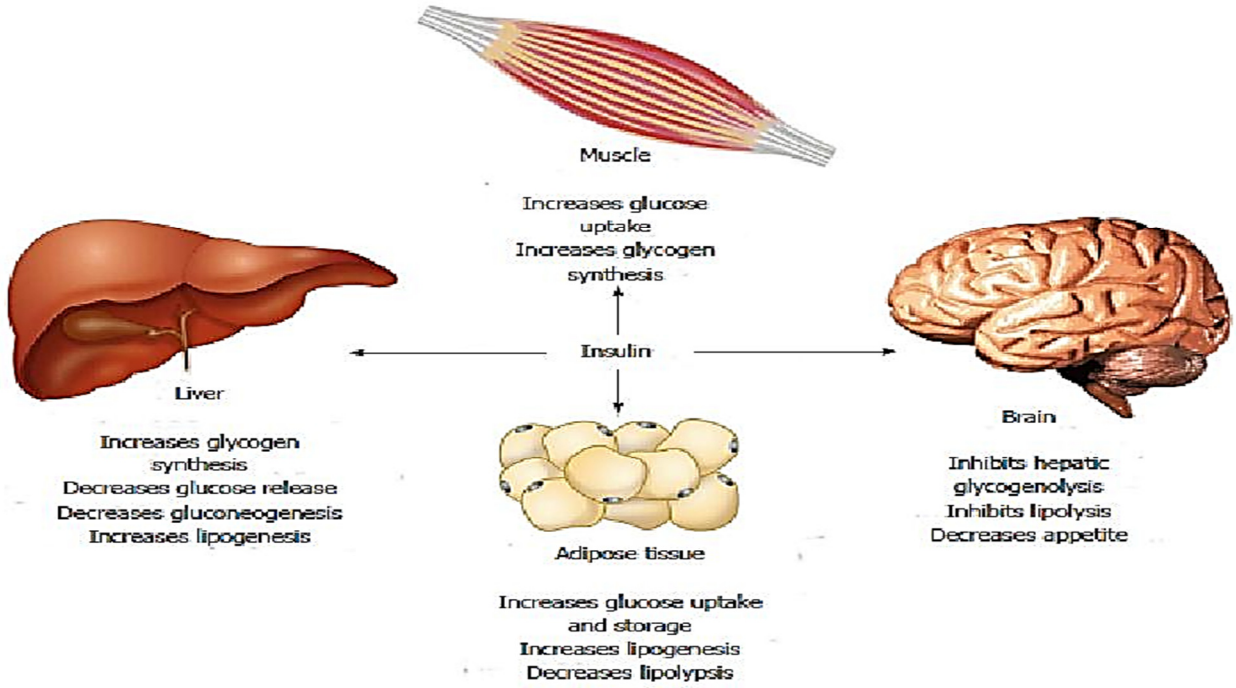

Figure 1.2 Insulin actions in insulin-sensitive tissues (Bunner A *et al*., 2014).

The different actions of insulin in insulin-sensitive tissues. In muscle, insulin increases glucose uptake and glycogen synthesis. In liver, it promotes glycogen synthesis and lipogenesis and decreases gluconeogenesis and the release of stored glucose. In adipose tissue, it increases

glucose uptake and lipogenesis and decreases lipolysis. In the brain, insulin inhibits hepatic glycogenolysis and lipolysis and decreases appetite.

1.5.3 The role of adipose tissue-derived hormones (adipokines) in insulin resistance

Several possible mechanisms linking insulin resistance to obesity have been postulated. Qatanani and Lazar (2007) very elegantly stated that the link between obesity and insulin resistance is possibly a 'cause and effect' relationship as changes in body weight (loss/gain) are closely correlated to insulin sensitivity/resistance, respectively.

Many of the adipose tissue secretions have been found to contribute to insulin resistance, metabolic disturbances and cardiovascular risk (Van Gaal *et al*., 2006).

1.5.3.1 TNFα

It is well known that in obesity, circulating levels of TNFα are elevated which may be attributed to macrophage infiltration in the expanded adipose tissue (Qatanani & Lazar, 2007). TNFα increases the release of FFA (Sethi & Hotamisligil, 1999), which in turn leads to increased accumulation of triglycerides, LDL-c and VLDL-c, while HDL-c levels are decreased. TNFα can also directly interfere with insulin signaling in adipocytes by down-regulating several steps in the insulin signalling cascade. It achieves this by increasing serine phosphorylation of insulin receptor substrate-1 (IRS-1), inhibiting insulin receptor tyrosine kinase activity, thus blocking the downstream events of insulin signalling, including the insulin-sensitive expression of the glucose transporter GLUT4 as well as the translocation of GLUT4 to the plasma membrane, eventually reducing insulin-induced glucose uptake. TNFα also stimulates the expression of leptin, plasminogen activator inhibitor-1 (PAI-1) and IL-6 (For review see Bakhai, 2008; Grattagliano *et al*., 2008).

1.5.3.2 Leptin and insulin resistance

The physiological role of leptin in both obese and lean subjects has received much attention (For reviews see Yingzhong *et al*., 2006; Jung & Choi, 2014). Initial studies performed on leptin demonstrated that it controls body weight and appetite via the hypothalamus. Interestingly, inflammation induced by metabolic stress negatively regulates leptin signaling in a manner similar to insulin receptor signalling as both of these signalling pathways in the hypothalamus are integrated through the PI3K pathway, among others. Also, loss of leptin and/or insulin signalling in the hypothalamus can promote obesity and type 2 diabetes (Morton *et al*., 2005; Zhang *et al*., 2008; for a review see Cao, 2014). Leptin signalling includes the JNK and the activators of transcription (JAK-STAT) pathways (Yingzhong *et al*., 2006; Xu *et al*. 2013).

Recent evidence now indicates that the brain processes information from adiposity signals such as insulin and leptin, which circulate in proportion to body fat mass, and integrates this input with signals from nutrients such as fatty acids (Qatanani & Lazar, 2007). Apart from the action of leptin to modify metabolism via the brain, leptin may have important effects through direct action on peripheral target cells, including β cells, liver, muscle, and fat (Kahn and Flier, 2000). In addition, hyperinsulinemia and insulin resistance are associated with elevated serum leptin levels (Kim-Motoyama *et al*., 1997) through dysregulation of the adipose tissue-hypothalamic axis (Almanza-Perez *et al*., 2008). Morrison *et al*. (2009) reported that leptin can either modulate insulin sensitivity or be induced by insulin.

1.5.3.3 Adiponectin and insulin resistance

Expression of adiponectin in adipose tissue is lower in subjects with obesity and insulin resistance than in lean subjects where it is associated with higher degrees of insulin sensitivity and lower adipose TNF-α expression (For a review see Jung & Choi, 2014).

Researchers employing rhesus monkeys to study obesity and type 2 diabetes found reduced adiponectin levels in the advancement of insulin resistance and type 2 diabetes (Hotta *et al*., 2001). In addition, several groups have reported that obese mice lacking adiponectin develop insulin resistance as a result of TNFα overproduction in adipose tissue causing defects in insulin signalling and reduced responsiveness to PPARγ (Maeda *et al*., 2001; For a review see Cao, 2014).

The adiponectin receptors (AdipoR1 and AdipoR2) are important in adiponectin signaling and are vital components of metabolic processes mediating increased AMP kinase (AMPK) activity, fatty acid oxidation (in peripheral tissue) (Tomas *et al*., 2002) glucose uptake and PPARγ ligand activity in the liver thereby promoting insulin sensitivity (Kadowaki *et al*., 2007; Pajvani *et al*., 2003).

1.5.4 Insulin resistance and oxidative stress

Oxidative stress

It has now been firmly established that obesity is associated with chronic, low-grade inflammation due to changes in function of adipocytes and macrophages (for a review see Hajer *et al.* 2008) and oxidative stress.

Under normal conditions, tissue oxidant and antioxidant activity is balanced. This is achieved by antioxidants, including enzymes such as cytosolic or mitochondrial superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase as well as non-enzymatic compounds such as vitamins E and C. However, a 'pro-oxidative' condition arises when the balance between oxidants and antioxidants is disturbed, resulting in oxidative tissue damage or oxidative stress (Bondia-Pons *et al*., 2012).

Thus, oxidative stress is defined as an impaired balance between production of reactive oxygen species (ROS) and/ or reactive nitrogen species (RNS) and an insufficient antioxidant capacity to overcome the production (Eriksson, 2007), resulting in accumulation of oxidative products. Major ROS variants include hydrogen peroxide (H2O2), hyperchlorous acid (HOCL), superoxide ($\bullet O2^-$), hydroxyl (•OH), peroxyl ($\bullet RO2^-$) and hydroperoxyl ($\bullet HO2^-$) radicals while RNS consist of nitric oxide (NO) and peroxynitrite anions ($ONOO^-$) (Evans *et al*., 2005). It is well-known that oxidative stress plays an important role in many pathological conditions and human disease states, as well as with the onset, and all components of cardiovascular complications (For a review see Wellen & Hotamisligil, 2005; Furukawa *et al*., 2004). Malondiadehyde (MDA), lipid hydroperoxides and thiobarbituric reactive acid substrates (TBARS) are established markers for determining the levels of lipid peroxidation in tissue damaged by oxidative stress (Siddiqui *et al*. 2014; Van Gaal *et al*., 2006).

1.5.4.1 Oxidative stress and insulin resistance

Oxidative stress as a causal factor in the development of insulin resistance has been supported by several studies and many findings suggest that the reversal of oxidative stress by the use of antioxidants may improve insulin resistance (Qatanani & Lazar, 2007).

Since oxidative stress is considered to be the common factor underlying insulin resistance, type 2 diabetes and cardiovascular disease, it may explain the presence of inflammation in all of these conditions (Van Gaal, 2006). In response to converting excessive amounts of FFA and other nutrients into fat, adipocytes can develop signs of oxidative and endoplasmic reticulum (ER) stress (Van Gaal, 2006; Boden, 2011).

A decrease in mitochondrial function associated with obesity and insulin resistance has been highlighted by Evans and coworkers (2002). Although paradoxical, the increases in ROS production induced by FFA, requires functional mitochondria, suggesting that FFA oxidation may occur before mitochondrial dysfunction and establishment of insulin resistance (Petersen & Schulman, 2006)

The peroxisome proliferator-activated receptor coactivator 1 (PGC-1α) is highly expressed in tissue containing numerous mitochondria such as brown adipose tissue (BAT) and skeletal muscle, with lower expression in white adipose tissue (WAT) and the liver (Puigserver *et al*., 1998).

PGC-1α, an important regulator of mitochondrial biogenesis, is activated by increased nutrient supply as seen in obesity (Lin *et al*., 2005) and has been associated with improved mitochondrial function as well as increased insulin sensitivity, which supports the idea that insulin resistance might arise from defects in mitochondrial function. This in turn leads to increases in FFA metabolites, such as fatty acyl-CoA and DAG that disrupt insulin signaling in muscle and liver (Qatanani & Lazar, 2007).

1.6 The metabolic syndrome (MetS)

The metabolic syndrome (MetS) is a cluster of cardiovascular risk factors including hypertension, hyperglycemia, hypertriglyceridemia, decreased high-density lipoprotein-cholesterol, and central obesity. MetS was reported to be associated with increased risks of type 2 diabetes mellitus, cardiovascular and cerebrovascular events, and chronic kidney disease (Isomaa et al., 2001; Meigs et al., 2007).

The pathophysiological mechanisms through which overweight/obesity progress to the metabolic syndrome, remain unclear. However, it is known that obesity-induced inflammation plays an important role in the development of insulin resistance that triggers the associated comorbidities of metabolic syndrome, as mentioned above. In addition to an increased risk for type 2 diabetes and cardiovascular disease, the comorbidities of MetS also include, obstructive sleep apnoea syndrome, fatty liver disease as well as sleep/wake disturbances (Wilcox *et al*., 1998; For a reviews see Bakhai, 2008 and Nduhirabandi *et al*., 2012).

Obesity is associated with a clustering of atherogenic risk factors, and when three or more are present, it generally signifies insulin resistance or metabolic syndrome (Reavan, 2004). This is promoted by weight gain and visceral adiposity (Veronica & Esther, 2012). The metabolic syndrome is further characterized by high levels of fasting glucose and triglycerides, low levels of high-density lipoprotein (HDL) and hypertension (Slanovic-Kuzmanovic *et al*., 2013).

Accumulating evidence suggest that visceral obesity is a central component of this syndrome and insulin resistance a triggering factor, as well as genetics, ethnicity, hormonal changes and ageing, which may also be involved in the development of this syndrome. However, the precise underlying cause is yet to be established (Hausman *et al*., 2001).

Matsuzawa *et al.* (2004) performed clinical studies on obese individuals to investigate the associated morbidities and showed that the distribution of body fat, rather than the extent of fat accumulation, is the more important determinant of the morbidity of obesity-related disorders. The accumulation of visceral adipose tissue is therefore an important factor in the developing of diabetes mellitus, hypertension, hyperlipidemia, atherosclerosis, or their coexistence, as seen in the metabolic syndrome (Hausman *et al*., 2001).

In addition to adiposity, a subtle interplay for adipokines in the development of MetS has been suggested (for a review see Cao, 2014). This occurs by dysregulation of adipose tissue (adipocytes) caused by visceral fat accumulation leading to oversecretion of leptin and TNFα and diminished secretion of adiponectin, resulting in metabolic disorders, specifically dyslipidemia, characterized by small dense low-density lipoprotein (LDL) (Grattagliano *et al*., 2008).

A potential role for oxidative stress is increasingly being recognized as a common factor in MetS (Furukawa *et al*., 2004) as well as its involvement in the pathogenesis of vascular alterations such as atherosclerosis (Palmieri *et al*., 2006), obesity and diabetes associated with the metabolic syndrome (Grattagliano *et al*., 2008).

1.7 MYOCARDIAL ISCHAEMIA-REPERFUSION: AN OVERVIEW

Ischaemia refers to an imbalance between the demand for and supply of coronary blood to tissue, causing an inadequate supply of oxygen and nutrients to maintain cellular metabolism (Hearse, 1977).

Myocardial ischaemia or ischemic heart disease (IHD) is the leading cause of death worldwide (Hausenloy & Yellon, 2008). Several risk factors are involved in the development thereof

(Ferdinandy *et al*., 2007) and even though this condition has mixed environmental and genetic etiologies, it occurs mainly in the context of severe and prolonged myocardial ischemic events or in clinical situations where untreated atherosclerotic plaques in coronary arteries, can progress to myocardial infarction (MI) or cell death. (Garcia-Dorado *et al*., 2014; Opie, 2004)

The changes in the myocardium induced by ischaemia following acute coronary occlusion (20 or more minutes) include: a block in oxidative metabolism, mitochondrial swelling, rapid ATP depletion (Braasch *et al*, 1968), lactate accumulation, tissue acidosis (Lemasters *et al*, 1996) associated with changes in intracellular sodium and calcium ions (Turer & Hill, 2010).The subsequent loss of structural integrity is believed to be due to sarcolemma (SL) disruption as well as swelling of mitochondria (for a review see Jennings, 2013). In addition, the damage caused by myocardial ischaemia can be classified into reversible and irreversible, depending on the severity and length of the reduction in coronary flow as well as the location of the ischemic insult (Skyschally *et al*., 2008).

Early reperfusion is essential for maintaining cell viability and protecting the heart against infarction. The process of restoring coronary blood flow is associated with the washout of ischaemic metabolites along with the re-admission of oxygen and metabolic substrates, causing changes in structure, function and biochemistry (Edoute *et al*., 1983), and may ultimately determine the fate of the cell (survival or death). Paradoxically, myocardial reperfusion per se can also induce injury and cell death thereby reducing its beneficial effects (Frolich *et al*., 2013), the so-called phenomenon of ischaemia/reperfusion injury. The following section will briefly highlight the myocardial alterations and damage incurred by ischaemia/reperfusion.

1.7.1 ISCHAEMIA-REPERFUSION INJURY

The concept of ischaemia/reperfusion (I/R) injury usually refers to a combination of the injury induced by sustained ischaemia and reperfusion. Experimentally it is difficult to discern

whether a damaged piece of myocardium was injured by ischaemia or reperfusion, or a blend of both (Skyschally *et al*., 2008).

At the onset of ischaemia, rapid ultrastructural changes occur in the myocardium (Edoute 1983, Ferdinandy *et al*., 2007). The duration of the ischemic episode and the application of reperfusion will determine whether the injury to the myocardium is reversible or irreversible (Yellon & Hausenloy, 2007). Three types of myocardial injury have been identified i.e. arrhythmias, myocardial stunning and irreversible/lethal reperfusion injury.

Arrhythmias occur as a result of electrical changes in ion channels or disrupted ion homeostasis and are usually associated with a short period of ischaemia (±5 minutes) and a reduction in blood flow and contractility, but can be reversed by reperfusion (Roden *et al*., 2002).
Myocardial stunning (or 'mechanical dysfunction') refers to a transient period of depression in myocardial function occurring during reperfusion after a usually longer period of ischaemia and represents a non-lethal form of reperfusion injury (Yellon & Hausenloy, 2007; Frolich *et al*., 2013; Kloner *et al*., 1989). Although there is still some controversy regarding the exact causal factors, oxidative stress (as occurs during early reperfusion) and intracellular calcium overload are important role players in this phenomenon (Bolli & Marban, 1999).

The third type of injury is associated with ischaemic periods lasting longer than 20 minutes, resulting in myocardial infarction (Skyschally *et al*. 2008; Opie *et al*. 2004). In a study using a canine heart model, Jennings (1957) demonstrated that increased amounts of necrosis became evident after a prolonged ischemic episode of 60 minutes. Failure to recover upon reperfusion, indicated irreversible injury. Histological features of the irreversibly damaged canine myocardium included cardiomyocyte swelling, contraction bands (myofibrils), disrupted sarcolemma and the presence of intramitochondrial calcium phosphate granules (for a review see Jennings, 2013).

Irreversible injury can further be defined as death/loss of cardiomyocytes (through apoptosis and necrosis) which were viable during ischaemia and irreversibly damaged upon reperfusion (Kloner *et al*., 1989; Skyschally *et al*., 2008).

In view of this, several studies focused on the ability of interventions induced at the beginning of reperfusion to reduce myocardial infarct size. Reductions in myocardial infarct size of 40-50% have been reported, making it an important target for cardioprotection (For reviews see Yellon & Hausenloy, 2007; Frolich *et al*., 2013).

1.7.2 Pathophysiology of ischaemia-reperfusion injury

Restoration of myocardial blood flow to reversibly injured myocytes leads to recovery of mitochondrial respiration, ATP synthesis, normalization of hydrogen, sodium and calcium concentrations, marked ultrastructural improvement and generation of creatine phosphate, despite the presence of ischaemia-reperfusion injury (Edoute *et al*., 1983). The deleterious effects of reperfusion during the first few minutes include macrophage activation, ROS overproduction and oxidative stress (Tapuria *et al*., 2008).

After prolonged ischaemia, reperfusion can no longer reverse the continued damage causing intracellular calcium overload and ATP depletion resulting in the eventual opening of mitochondrial permeability transition pore (MPTP) and cell death (Dominguez-Rodriguez *et al*., 2014).

Other factors implicated in reperfusion injury include, extracellular and intracellular edema contributing to changes in the mechanical function of the myocardium, platelet accumulation, (Garcia-Dorado *et al*., 2014), renin-angiotensin system (RAAS) (angiotensin-II increases

calcium and induces necrosis) and complement system activation (directly induces cell injury by increasing cell permeability and releasing histamine) (Dominguez-Rodriguez *et al*., 2014). Innovative treatment strategies aimed at protecting the myocardium against the detrimental effects of ischaemia/reperfusion injury are needed to improve the prognosis of CHD patients. In order to do so, knowledge of events occurring at an intracellular level is a prerequisite.

1.7.3 The mitochondrial permeability transition pore (MPTP)

In the heart, mitochondria serve to provide ATP for contraction and maintenance of ionic homeostasis. The inner membrane of mitochondria (in mammals) contains a mitochondrial permeability transition pore (MPTP) (for a review see Halestrap & Richardson, 2014), which allows communication between the cytoplasm and mitochondrial matrix (Hunter *et al*., 1976). Opening of the MPTP during reperfusion injury has indeed been associated with increased Ca^{2+} levels, apoptosis, cytochrome c (Cyt C) release and elevated ROS levels (Littlejohns *et al*., 2014; for reviews see Perrelli *et al*., 2011; Halestrap & Richardson, 2014).

Furthermore, MPTP opening is also involved in determining the extent of injury to the myocardium during reperfusion, after a prolonged ischaemic episode (Hausenloy *et al*., 2009; for a review see Halestrap & Richardson, 2014).

Interestingly, in a study using a rabbit model, MPTP inhibitors (cinnamic anilides) were shown to be cardioprotective against acute myocardial infarction (AMI), (Fancelli *et al*., 2014) which, along with other established cardioprotective regimes, mediate their effects via inhibition of MPTP opening (for a review see Halestrap & Richardson *et al*., 2014). Other inhibitors of MPTP opening include cyclosporine A (CsA) (Hausenloy *et al*., 2003) and melatonin (Petrosillo *et al*., 2009).

1.7.4 Concepts of cell death: Necrosis, Apoptosis and Autophagy

According to Tian *et al*. (2013), cell death (progressive or acute) denotes an irreparable damage of plasma membrane integrity and can be classified into necrosis, apoptosis and autophagy, each with different morphological manifestations.

Cell death is a physiological process that serves to regulate tissue homeostasis and is considered as a 'hallmark characteristic' of cardiovascular diseases, including acute myocardial infarction (Whelan *et al*., 2010).

Apoptosis is a tightly regulated, energy-dependent process that occurs under both physiological and pathological conditions. It is responsible for normal cell turnover and immune system function in addition to its ability to act as a defence mechanism when cells are damaged (Elmore *et al*., 2007). It consists of two pathways namely, the death receptor pathway (stimulated by TNF-α) and the mitochondrial pathway which is activated under conditions of ischaemia with increased Ca^{2+}, ROS and acidosis, playing a significant role in myocardial infarction (Kim & Kang, 2010).

Necrosis or 'uncontrolled cell-death' refers to the changes that occur in cells and tissue after the cells have died (Kanduc *et al*., 2002). Unlike apoptosis, necrosis is associated with energy depletion, swelling, inflammation and disruption of sarcolemma and mitochondria. With regard to the myocardium, it has been reported to occur after prolonged ischaemia and is associated with irreversible injury (for a review see Jennings, 2013).

Lastly, autophagy is a cell survival mechanism involving degradation and recycling of cytoplasmic components and restoration of homeostasis (for reviews see Booth *et al*., 2014; Tian *et al*., 2013). However, if stresses persist and autophagy can no longer support cell survival, cells respond to apoptosis for elimination. This process also occurs without

inflammation, suggesting a cross-talk between apoptosis and autophagy (for a review see Booth *et al*., 2014).

In addition, autophagy is regulated by several signalling pathways including nutrient, insulin/growth factor and energy sensing pathways and is also involved in ischemic heart disease and heart failure (Tian *et al*., 2013).

1.7.5 Protection of the ischemic heart: ischemic preconditioning (IPC) and postischemic conditioning (IPOC)

The heart has a powerful endogenous protective mechanism at its disposal against ischaemia/reperfusion damage. Several forms have been identified namely ischemic preconditioning (IPC), remote preconditioning and ischemic postconditioning (IPOC) (for a review see Bousselmi *et al*., 2013). A detailed description of these phenomena falls beyond the scope of this thesis.

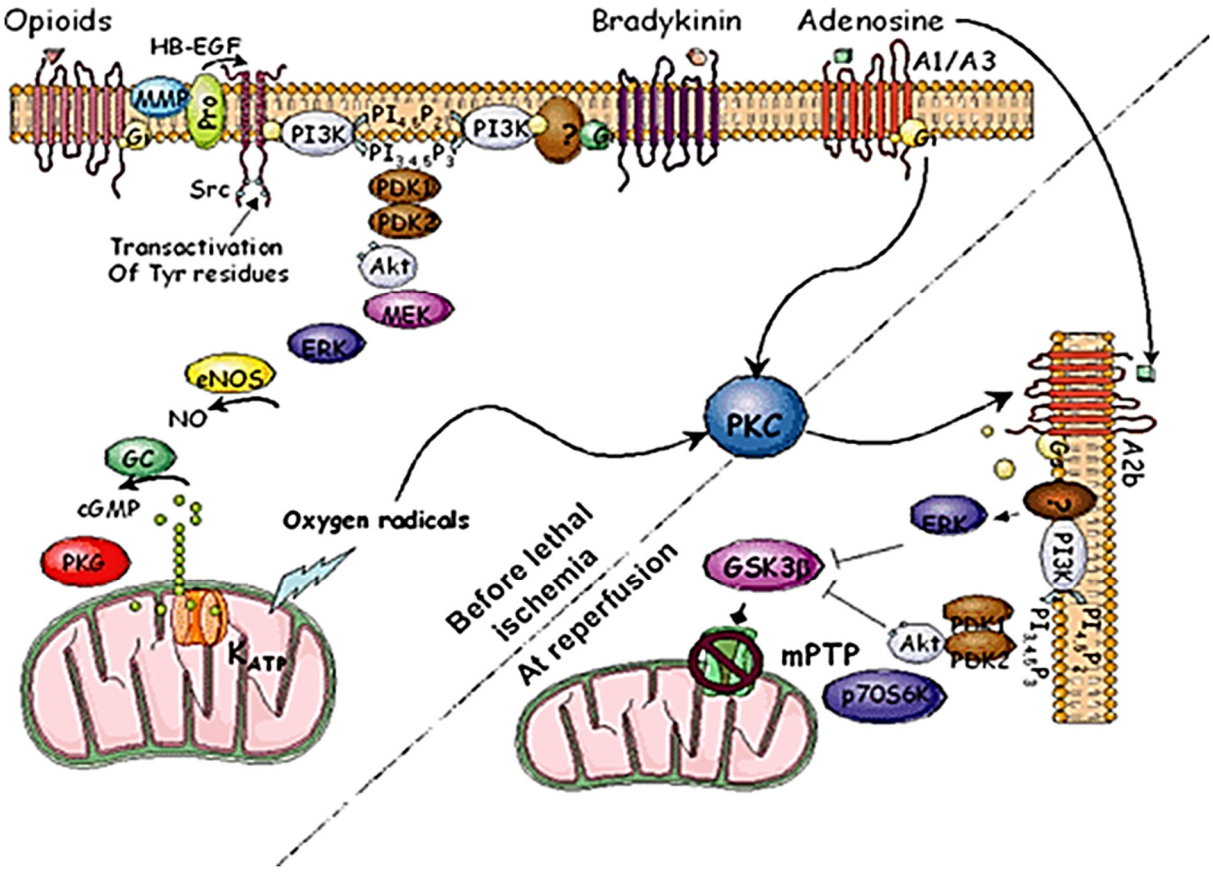

Figure 1.3 Mapping preconditioning's signaling pathways (Downey *et al*., 2008).

During ischaemic preconditioning, adenosine, opioids, and bradykinin are released from the heart. Opioid and bradykinin receptors causes activation of Akt via phosphatidylinositol 3-kinase (PI3-kinase) activation. PI3-kinase causes extracellular signal regulated kinase (ERK)-dependent activation of endothelial nitric oxide synthase (eNOS), which activates guanylyl cyclase resulting in cyclic GMP-dependent protein kinase (PKG) activation through production of cyclic guanosine monophosphate (cGMP). This activation causes the opening of ATP-sensitive potassium channels on the inner membrane of the mitochondria, resulting in generation of free radicals during reperfusion when oxygen is reintroduced. These radicals then activate protein kinase C (PKC) and put the heart into the protected phenotype that persists for one to two hours. Although adenosine receptors activate PI3-kinase, they also have a second direct coupling to PKC and thus bypass the mitochondrial pathway.

1.7.6 Cardioprotection associated with the RISK and SAFE pathways

Early coronary artery reperfusion is still known as the most effective means of controlling infarct size (Hausenloy & Yellon, 2007). Interventions such as IPC and several pharmacological agents (opiods, bradykinins, growth factors, etc.) have been associated with the activation of important cardioprotective pathways during early reperfusion. The Reperfusion Injury Salvage Kinase (RISK) pathway which includes the pro-survival kinases, PKB/Akt and ERK1/2, which when activated at the time of reperfusion, is associated with powerful protection by preventing lethal reperfusion injury (Fig 1.2). It can also be perceived as a mediator of 'programmed cell survival' involving inhibition of MPTP opening, normalizing Ca^{2+} levels as well as recruitment of antiapoptotic pathways (for a review see Yellon & Hausenloy, 2007).

Protection can also be conferred by The Survivor Activating Factor Enhancement (SAFE) pathway consisting of signal activator and transducer 3 (STAT-3) and tumor necrosis factor-α (TNF-α). Activation of the SAFE pathway, induced by low doses of TNF-α, has been shown to protect against experimental lethal reperfusion injury (Lecour, 2009).

In addition, inhibition of the mitochondrial permeability transition pore (MPTP) and ATP-dependent mitochondrial potassium channel (mKATP) as well as protein kinase C have all shown to be involved in cardioprotection (for reviews see Hausenloy & Yellon, 2008 and Yellon & Hausenloy, 2007).

1.7.7 Effects of obesity on myocardial ischaemia/reperfusion injury

As stated in the introduction of this chapter, obesity is considered to be a serious risk factor in the development of cardiovascular disorders and is traditionally regarded to impact negatively

on the outcome of myocardial ischaemia. However, despite the evidence for a higher prevalence of obesity in myocardial ischaemia patients, a number of recent publications suggested that obesity in humans with ischaemic heart disease is associated with reduced morbidity and mortality, the so-called "obesity paradox" (Chase et al., 2014; Clark et al., 2014; Diercks et al., 2006). The mechanism underlying this paradox is complex and remains unclear.

To elucidate the pathophysiological mechanisms underlying the relationship between obesity and cardiovascular disease (CVD), extensive use has been made of experimental animals fed high calorie or high fat diets. Unfortunately experimental data on the impact of diabetes, obesity and insulin resistance on myocardial ischaemia/ reperfusion injury (IRI) are very controversial (for reviews see references Miki et al., 2012; Whittington et al., 2012). In contrast to the vast amount of research done to assess the susceptibility of the heart to ischaemia/reperfusion damage in types I and II diabetes (Miki et al., 2012; Whittington et al., 2012; Balakumar et al., 2009), not much is known about the effects of obesity per se in this regard. Experimental studies have reported a decreased myocardial tolerance to ischaemia-reperfusion damage in *in vivo* (Clark et al., 2011; Wensley et al., 2013) and *ex vivo* (Du Toit et al., 2008; Nduhirabandi et al., 2011) studies using hyperphagia-induced obese insulin resistant male rats as well as in other animal models (Sidell et al., 2002; Thakker et al., 2008; Bouhidel et al., 2008). Together, these studies have demonstrated an association between increased infarct size and poor functional recovery in hearts from obese animals compared to the controls. However, in a hyperphagia-induced obese rat model, Donner and coworkers have reported smaller infarcts and improved functional recovery during reperfusion (Donner et al., 2013). Similar beneficial effects of obesity have been observed in our own laboratory (Salie et al., 2014), suggesting that the variation in susceptibility to ischaemia/reperfusion damage may be due to differences in the age of the animals and the duration of obesity.

It is now generally accepted that activation of the reperfusion injury salvage kinase pathway (RISK) is characteristic of a reduction in injury during reperfusion (Hausenloy et al., 2005, Hausenly & Yellon, 2007), which is further associated with activation of the prosurvival/anti-apoptotic kinase PKB/Akt, a key enzyme in the insulin signalling pathway (Huisamen, 2003). However, conclusions about the RISK pathway's involvement in models of obesity and insulin resistance are hampered by the facts that not only do the models of diabetes differ, but tissue samples are taken at different times during the experimental protocol. For example, samples were collected at the end of the stabilization time (Tsang et al., 2004), during reperfusion after sustained ischaemia (Xu et al., 2004) or under baseline conditions (Donner et al., 2013; Strniskova et al., 2003). Baseline activation of ERK was found to be increased in streptozotocin-induced diabetes (Strniskova et al., 2003) while both ERKp44/p42 and PKB/Akt phosphorylation were reported to be lower in rats on a high fat diet (Wensley et al.,2013). ERK activation during reperfusion of streptozotocin-induced diabetic hearts is dependent on the duration of hyperglycaemia: an increment is seen after 4 weeks followed by a significant reduction after 20 weeks (Xu et al., 2004), while PKB/Akt activation showed a similar early stimulation followed by a reduction (Ma et al., 2006). Clearly more experimental work is required to elucidate these discrepancies.

1.8 EFFECTS OF MELATONIN ON OBESITY AND THE HEART

An overview

Several physiological processes are influenced by the 12 hour day/night circadian cycle.

An important function of this cycle is the adjustment of the master circadian clock, the hypothalamus, including sleep/activity regulation and appetite control (for reviews see Froy 2010, 2011; Lima *et al*., 2013).

Melatonin (*N*-acetyl-5-methoxytryptamine) is an evolutionary conserved, pineal gland hormone that is found in animals, humans (Reiter, 1999; Hardeland & Poeggeler, 2012) and plants (Reiter *et al*., 2007).

Melatonin is undoubtedly under the control of the master circadian clock and is synthesized and released in a rhythmic fashion such that its production is circumscribed to the night (Reiter, 1991) with circulating levels of 43-400pM at night and 10-60pM during the day (Barrenetxe *et al*., 2004).

Melatonin was initially identified by Lerner and coworkers (1958) and subsequently demonstrated in a variety of organisms, from protozoa, bacteria, invertebrates to vertebrates (Hardeland & Fuhrberg, 1996). Its biosynthesis has been described by Pandi-Perumal *et al*. (2006), Reiter *et al*. (2007), Tan *et al*. (2002) and Zawilska *et al*. (2009) and involves two key enzymes: serotonin (arylalkylamine)-N-acetyltransferase (AA-NAT) and hydroxyindole-O-methyl transferase (HIOMT) ,which are responsible for the conversion of serotonin to melatonin.

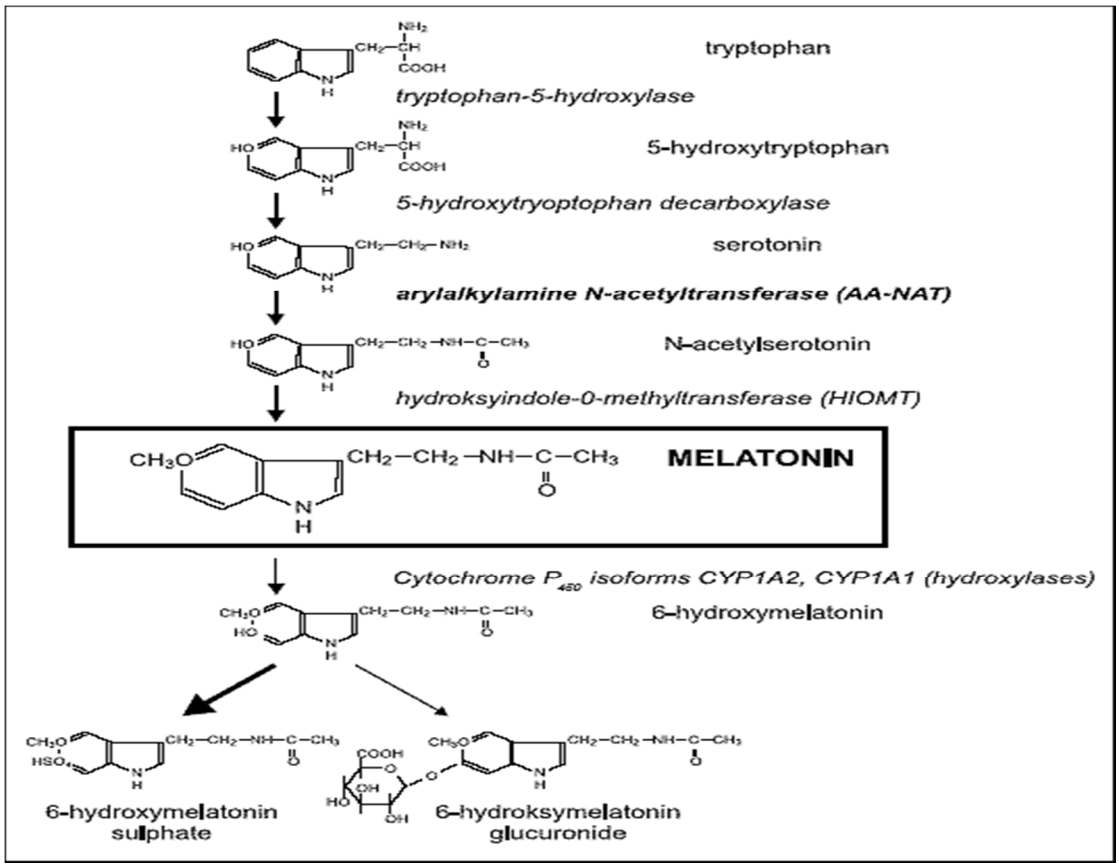

Figure 1.4 Melatonin biosynthesis and metabolism (Karasek & Winczyk, 2006).

The synthetic pathway and metabolism of melatonin from tryptophan to 6-hydroxymelatonin sulphate and 6-hydroxymelatonin glucuronide.

Due to its amphiphilic nature, melatonin can enter body fluids as well as all cellular and sub-cellular compartments (Vural *et al*., 2001), displaying a wide range of physiological functions such as regulation of circadian rhythms acting as a chronobiotic or 'internal synchronizer'. It possesses powerful antioxidant, anti-inflammatory (for review see Tengattini 2008; Hardeland *et al*., 2006), anti-adrenergic actions (Lochner *et al*., 2013), as well as body weight and energy metabolism regulation (Korkmaz *et al*., 2009).

In view of this, several studies have sought to investigate melatonin's actions at a cellular/molecular level to better understand its pleiotropic effects (Luchetti *et al*., 2010) which

occur mainly through the activation of membrane or nuclear receptors in various tissues, allowing them to respond to the circadian melatonin message (Reiter *et al*., 2008).

1.8.1 Melatonin signaling: role of melatonin receptors

Melatonin possess all characteristics of a classical hormone, being centrally produced in an endocrine gland, having the ability to circulate in a free and albumin-linked form, and acting through specific G-protein-coupled membrane receptors namely, MT1 and MT2 (for a review see Cipolla-Neto *et al*., 2014). These receptors have been identified in several organs including the liver, lungs, heart and the central nervous system (CNS) (Naji *et al*., 2004), however, its signaling in the heart has not been studied in sufficient detail.

It is well established that coupling of melatonin to its receptors (MT1 and MT2) can activate various signal transduction cascades, eliciting a number of cellular responses (Pandi-Perumal *et al*., 2008), including the regulation of antioxidant enzymes (Rodriguez *et al*., 2004).

Melatonin's intracellular signaling is mediated by the G inhibitory protein, which upon binding of melatonin to its receptors (MT1 and MT2), leads to increases in cytosolic Ca^{2+} due to stimulation of phospholipase C and phosphorylation of protein kinase C (PKC) (for a review see Luchetti *et al*., 2010), ultimately leading to activation of CREB (cAMP responsive element binding protein) and the regulation of antioxidant enzymes, accounting for the indirect antioxidant actions of melatonin (Tengattini *et al*., 2008; for a review see Luchetti *et al*., 2010). On the other hand, the direct free radical scavenging actions of melatonin account for its receptor-independent actions (Cipolla-Neto *et al*., 2014; Nduhiranadi *et al*., 2012).

1.8.2 Melatonin, a powerful antioxidant

The antioxidant properties of melatonin have been widely reviewed in view of the fact that several pathophysiological conditions are associated with increased oxidative stress (e.g. cardiovascular disease, type 2 diabetes, Alzheimer's disease, amongst many others) (Allegra *et al*., 2003; Reiter *et al*., 2009; Lochner *et al*., 2013;).

The discovery of the high efficacy of melatonin as a free radical scavenger in 1993 (Tan *et al*., 1993) stimulated several studies. It was found that melatonin does not require the formation of a complex with a receptor or molecule to be able to act as a free radical scavenger. Thus its receptor-independent actions only require that melatonin be in the vicinity of ROS/RNS when it is generated, for scavenging of the free radicals (Reiter *et al*., 2008)

Melatonin has been documented as a potent scavenger of the hydroxyl radical (·OH) since excess ·OH generation due to ionizing radiation was found to be reduced in its presence (Zhou *et al*., 2006; for a review see Reiter *et al*., 2008). Moreover, the molecule that is formed when melatonin scavenges two ·OH radicals or when it interacts with the peroxynitrite anion ($ONOO^-$), 3-hydroxymelatonin (3-OHMEL), also possesses potent free radical scavenging properties and in doing so, N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) is formed (Tan *et al*., 2000). In turn when AFMK encounters ROS/RNS, this leads to the formation of N1-acetyl-5-methoxykynuramine (AMK), and its interaction with ($ONOO^-$) also produces ROS/RNS scavengers. Other free radicals that melatonin can scavenge include, hydrogen peroxide, superoxide and nitric oxide, amongst others.

It was also shown that melatonin can reduce mitochondrial free radical formation, and inhibit the opening of the mitochondrial permeability transition pore (MPTP), suggesting a cardioprotective role of melatonin (Petrosillo *et al*., 2009). It has further been determined that

a single melatonin molecule (via AFMK) has the ability to scavenge up to 10 free radical species (ROS/RNS) (Tan *et al*., 2007).

This process whereby melatonin along with its metabolites neutralizes toxic reactants is known as the antioxidative cascade (for reviews see Hardeland, 2005; Tan *et al*., 2007; Reiter *et al*., 2008). Importantly, studies employing melatonin (up to 1 g daily), far above its physiological range, showed no toxicity (Bonnefont-Rousselot & Colin, 2010; Tan *et al*., 2011).

It is also known that melatonin has the ability to stimulate antioxidant enzymes, such as glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GRd) and glucose-6-phosphate dehydrogenase (G6PD) (for reviews see Reiter *et al*., 2000, 2008). In addition, exogenous melatonin treatment reduces the activity of the pro-oxidant enzymes (nitric oxide synthases, myeloperoxidases) and downstream inflammatory cascades (Hardeland, 2005; Galijsevic *et al*., 2008). Positive correlations with the level of antioxidants and serum melatonin have also been made, suggesting a role for melatonin as a modulator of oxidative stress via these antioxidant actions (Bonnefont-Rousselot & Collin, 2010).

1.8.3 Melatonin, metabolic regulation and obesity

1.8.3.1 Body weight regulation

The ability to balance energy intake, storage and expenditure is a prerequisite of life and ultimately determines the final body weight. The state of being overweight or obese results from an inbalance between energy intake and expenditure.

The circadian clock is known to govern all peripheral clocks and as a result, behavioural and/or physiological changes in many species (Panda et al., 2002; Reppert & Weaver, 2002). It is reportedly known to regulate metabolism and energy homeostasis in several organs, including the liver and peripheral tissue. Growing evidence suggests that metabolism, energy intake and timed meals are linked to circadian rhythms. Furthermore, metabolic disorders have been associated with disruption of circadian rhythms (for a review see Froy, 2010).

Melatonin is well-known for its chronobiotic effects and circadian entrainment (Reiter *et al*., 2012). Bartness & Wade (1985) reported on its role in body weight regulation by demonstration of body weight gain in pinealectomized rats with low circulating melatonin levels. On the other hand, intraperitoneal (ip) melatonin supplementation of 30mg/kg/day for 3 weeks to pinealectomized rats, reversed the body weight gain (Prunet-Marcassus *et al*., 2003).

The role of melatonin in body weight regulation is further underscored by the demonstration that, in a rat model of diet-induced obesity, a daily melatonin supplementation was shown to significantly reduce plasma glucose, leptin, triglyceride (TG) as well as total cholesterol levels, along with reductions in body weight gain, independent of food consumption (Rasmussen *et al*., 1999; Wolden-Hanson *et al*., 2000; for a review see Nduhirabandi *et al*., 2012).

In a 12 week high-fat diet study carried out by Hussein *et al*. (2007), the diet significantly increased body weight gain and melatonin treatment reversed the body weight gain as well as lowered blood glucose, cholesterol and triglyceride levels, consistent with previous studies by Bartness *et al*. (2002) and Shieh *et al*. (2009)

Although a wealth of data has accumulated on melatonin's involvement in energy expenditure and body fat mass regulation (Prunet-Marcassus *et al*., 2003; Rasmussen *et al*.,1999; Alonso-Vale *et al*., 2008; for reviews see Barrett & Bolborea, 2012; Nduhirabandi *et al*., 2012, Cipolla-Neto *et al*., 2014), the hypothesized anti-obesogenic effect of melatonin may in part be due to

its regulatory role on energy balance through activation of brown adipose tissue (BAT) (see below), thereby aiding in body weight regulation (for a review see Cipolla-Neto *et al*., 2014), however, the underlying mechanism of obesity and its related pathologies are complex and needs to be further investigated.

1.8.3.2 Melatonin and brown adipose tissue (BAT)

It is well established that obesity can be reduced by promoting energy expenditure. (Jimenez-Aranda *et al*., 2013) While white adipose tissue (WAT) functions to store excess triglycerides (Ntambi & Young-Cheul, 2000) and positively correlates with increased fat mass, brown adipose tissue (BAT) has the ability to dissipate energy (ATP) by producing heat (thermogenesis) (Ginter & Simko, 2012; Jimenez-Aranda *et al*., 2013; Haas *et al*., 2012) and is negatively correlated with body mass index (Benito, 2011).

This physiological process is mediated by an important mitochondrial transporter protein, the uncoupling protein 1 (UCP1) (Zhou *et al*., 2014) and its upstream regulator, the peroxisome proliferator activated receptor gamma coactivator 1 (PGC1-α) (Zhou *et al*., 2014; Austin & St-Pierre, 2012).

Interest in white adipose tissue (WAT) led to the discovery of brown-like (‘beige’) adipocytes in WAT of animals and humans and it has been shown that browning of WAT can be induced with cold stimuli in animals (Jimenez-Aranda *et al*., 2013).

Several investigators have shown that BAT activity can be increased with melatonin treatment (Holtorf *et al*., 1985; Jimenez-Aranda *et al*., 2013). In a recent study on the effects of melatonin on obesity, it was found that melatonin-treated obese rats displayed reductions in body weight mass with raised core body temperature, due to the direct regulatory actions of melatonin on

energy expenditure through its metabolism-activating effects in BAT and in the browning of WAT (Heldmaier *et al*., 1981; Tan *et al*., 2011; for a review see Cipolla-Neto *et al*., 2014).

PGC1-α expression is reduced in insulin-resistant and diabetic humans, and PGC1-α-null mice has been shown to have skeletal and cardiac muscle contractility defects, possibly due to insulin resistant humans having fewer mitochondria in their muscles (for a review see Kim *et al*., 2008). In a study by Jimenez-Aranda *et al*., 2013, it was shown that increased expression of PGC1-α was found in beige depots of lean melatonin-treated animals compared to the untreated obese animals (Jimenez-Aranda *et al*., 2013), suggesting that melatonin increased the activity of the 'master regulator' of thermogenesis, PGC1-α (Puigserver *et al*., 1998).

With regard to the uncoupling proteins, UCP1 expression is unique to brown adipose tissue, UCP2 is ubiquitously expressed and UCP3 in skeletal and heart muscle. UCPs play a vital role in mitochondrial function by regulating heat and ROS generation (for a review see Kim *et al*., 2008). In the same above-mentioned study, melatonin increased UCP1 expression in beige depots in diabetic rats where it was previously absent (Jimenez-Aranda *et al*., 2013; for a review see Cipollo-Neto *et al*., 2014).

Melatonin treatment at 10 mg/kg/day also induced browning of WAT in both Zucker diabetic fatty (ZDF) and Zucker lean (ZL) rats. These effects of melatonin may be a part of the mechanisms associated with its anti-obesity and antidiabetic properties (Jimenez-Aranda *et al*., 2013).

1.8.3.3 Melatonin and insulin resistance

Given the importance of melatonin in a range of physiological processes in the body and the widespread distribution of its receptors including in the pancreatic β-cells (McMullan *et al*.,

2013), melatonin has been implicated in the regulation of insulin secretion and glucose metabolism (Nishida 2005; Peschke 2008). In this regard, in vivo studies have shown that melatonin administration leads to a significant reduction in insulin secretion (Rasmussen *et al*., 1999; Wolden-Hanson *et al*., 2000) with similar findings in vitro (Picinato *et al*., 2002; Peschke, 2008).

Frese and co-workers (2009) showed reduced endogenous melatonin synthesis in type 2 diabetic (Goto-Kakizaki) rats while chronic melatonin administration of 2.5 mg/kg, daily enhanced plasma melatonin and reduced insulin levels in these animals (Peschke *et al*., 2010), similar to the above-mentioned in vitro and in vivo findings.

In a high-fat/high-sucrose animal diet study, improvements of not only metabolic profile and oxidative stress status, but also increased insulin sensitivity and decreased blood glucose levels were evident using melatonin and its agonist, NEU-p11 (She *et al*., 2009). Interestingly, a 2 week melatonin treatment of rats fed a high-fructose diet for 6 weeks showed no changes in body weight, however it ameliorated insulin resistance (Kitagawa *et al*., 2012; for a review see Nduhirabandi *et al*., 2012).

In addition, melatonin supplementation in type 2 diabetic rats significantly reduced insulin levels and improved insulin sensitivity (McMullan *et al*., 2013). Furthermore, both short- and long-term melatonin consumption improved insulin sensitivity and glucose intolerance in type 2 diabetic rats as well as in high-fat diet induced obesity rats (for a review see Nduhirabandi *et al*., 2012).

Several studies have also employed pinealectomized rats for better insight into melatonin's role in insulin resistance (Picinato *et al*., 2002; McMullan *et al*., 2013). For example, it has been reported that such rats exhibited glucose intolerance (impaired GLUT 4 expression), reduced

muscle and liver glycogenolysis, high blood glucose levels (Picinato *et al*., 2002) and decreased adipose tissue responsiveness to insulin (McMullan *et al*., 2013). On the contrary, hypoglycemia, restored GLUT-4 content and increases in glucose tolerance were noted after infusion of pineal extracts in rats (Picinato *et al*., 2002; Zanquetta *et al*., 2003). Experimental evidence also exist for melatonin and the autonomic nervous system output as facilitators of the master clock in regulation of circadian plasma glucose and insulin levels (Cailotto *et al*., 2005, for a review see Cipolla-Neto *et al*., 2014).

The mechanisms involved in the effect of melatonin on insulin resistance, are not yet fully understood. The insulin resistance cascade is associated with the coupling of insulin to the insulin receptor (IR), phosphorylation of the substrate proteins (IRS-1/2) and PI3-K, PKB/Akt and PKC activation. Melatonin treatment, on the other hand, is associated with stimulation of receptor-mediated signal transduction pathways, exerted through G_i protein-coupled receptors, MT1 and MT2 (Peschke *et al*., 2008). It has been reported that in rat pancreatic beta cells, melatonin exerts its effects via three intracellular pathways, namely the G_i-α dependent cyclic adenosine monophosphate (cAMP) pathway, cyclic guanosine monophosphate (cGMP) and G_i-α- Inositol 1,4,5-triphosphate (IP_3) independent pathway (for reviews see Peschke *et al*., 2008; Zanuto *et al*., 2013).

Melatonin may interact with the insulin signalling cascade by activating the insulin receptor-mediated PI3-K, PKB/Akt and the mitogen-activated extracellular signal-regulated kinase (MEK/ERK) pathway by tyrosine phosphorylation of IRS1 and 2 (Picinato *et al*., 2008).
Melatonin also stimulates glycogen synthesis and increased phosphorylation of hepatic glycogen synthase kinase 3 β (GSK3-β) (Shieh *et al*., 2009; for a review see Nduhirabandi *et al*., 2012)

Even more so, the use of melatonin antagonist, luzindole, blocked the above-mentioned beneficial effects of melatonin, implying involvement of melatonin receptors in its metabolic actions (Ha *et al*., 2006).

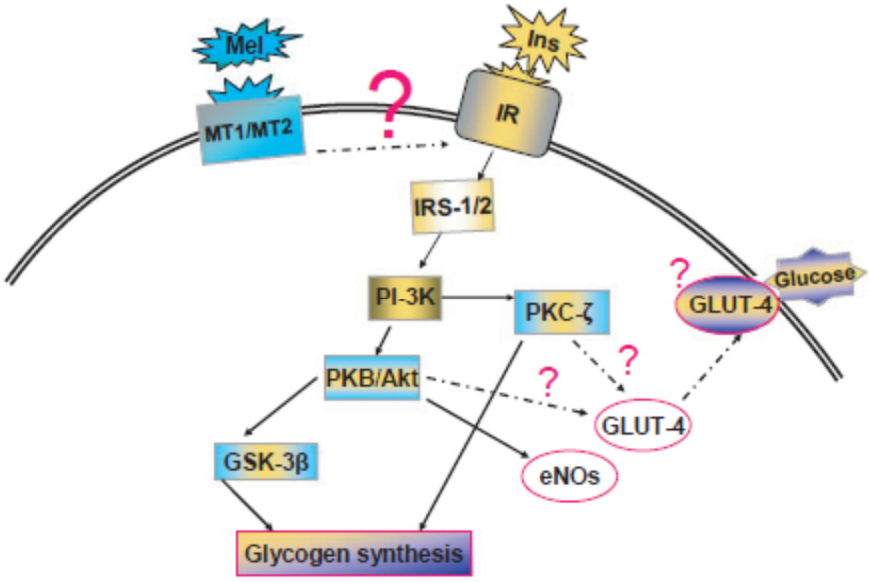

Figure 1.5 Effect of melatonin administration on hepatic glycogen synthesis and skeletal muscle glucose homeostasis (for a review see Nduhirabandi *et al*., 2012).

Melatonin administration was associated with significant increases in hepatic glycogen synthesis and improved skeletal muscle glucose homeostasis by eNOs (endothelial nitric oxide synthase) activation. The effect of melatonin on IRS phosphorylation and glucose uptake has not been established. The mitogen activated protein kinase (MAPK) pathway was not included in the figure; *IR, insulin receptor; IRS-1, insulin receptor substrate 1; PI-3K, phosphatidylinositol-3-kinase; PKC-ζ, protein kinase C-zeta; GSK-3b, glycogen synthase kinase-3 beta; GLUT-4, glucose transporter-4; Mel, melatonin; MT1/2, melatonin receptor 1, 2; Ins, insulin.*

1.8.4 Melatonin and the heart

It is well-known that melatonin regulates many physiological processes in a circadian manner (Dominguez-Rodriguez et al., 2010). In addition, studies have reported that melatonin influences multiple factors of cardiovascular function (Tengattini et al., 2008). Moreover, receptors for melatonin have been identified in the cardiovascular system (Ekmekcioglu *et al*., 2003).

Under physiological conditions, heart function is unaffected by melatonin treatment (Patel *et al*., 2010; Nduhirabandi *et al*., 2011). However, profound effects on the heart were seen with chronic melatonin consumption including, a reduction in absolute and relative heart weight (Bojkova et al., 2008) and increased glycogen content (Kassayova et al., 2006). Additionally, chronic melatonin treatment counteracted the heightened susceptibility of MPTP opening due to calcium overload (Petrosillo et al., 2009), plus improvements in myocardial hypertrophy were noted (Reiter *et al*., 2010).

Further associations between melatonin and the heart were found in patients with coronary heart disease (CHD) or acute myocardial infarction (AMI) who exhibited low circulating levels of melatonin (Brugger *et al*., 1995).

Another interesting association is the increased risk for stroke and myocardial infarction in the morning with coinciding low plasma circulating melatonin levels (Macchi & Bruce, 2004).

Melatonin was also shown to influence blood pressure (Kitajima *et al*. 2001) and heart function (Abete *et al*.1997) directly and/or indirectly by influencing risk factors associated with heart disease such as increased visceral fat accumulation and dyslipidaemia (Agil *et al*. 2011, Kozirog *et al*. 2011, Nduhirabandi *et al*. 2011).

1.8.4.1 Melatonin and myocardial ischaemia/reperfusion injury

The cardioprotective actions of melatonin on the ischaemic/reperfused heart are well-established (for review see Nduhirabandi *et al*., 2012; Lochner *et al*., 2013; Reiter *et al*., 2003; Tengatini *et al*., 2008).

It has been reported that melatonin has significant protective actions against the events that occur during ischaemia/reperfusion (I/R). In addition, pharmacological doses of melatonin both before ischaemia and/or after reperfusion reduced ventricular fibrillation and premature ventricular contractions associated with reperfusion and decreased arrhythmias (Tan *et al*., 1998), possibly owing to melatonin's free-radical scavenging actions (Lee *et al*., 2002; Sahna *et al*., 2002; for a review see Reiter & Tan, 2003).

Using confocal microscopy of live cardiomyocytes, Salie and coworkers (2001) showed that melatonin protected these cells against I/R damage by inhibition of ROS generation and intracellular Ca^{2+} accumulation. Melatonin has also been shown to reduce myocardial infarct size (Sahna *et al*., 2002; Dobsak *et al*., 2003; Lochner *et al*., 2006) and improve functional recovery during reperfusion after ischaemia (Tengattini et al., 2008; for a review see Reiter & Tan, 2003).

1.8.4.2 Melatonin-induced cardioprotection: RISK and SAFE pathways

As mentioned previously, activation of prosurvival signalling pathways, at the time of reperfusion, is associated with limitation of reperfusion injury (Lacerda *et al*., 2009; Hausenloy & Yellon, 2008).

These cardioprotective pathways include, the Reperfusion injury Salvage Kinase (RISK) pathway, (PKB/Akt and ERK 1/2) and the Survivor Activating Factor Enhancement (SAFE)

pathway (Genade *et al*., 2008; Lamont *et al*. 2011), which involves the Janus Kinase (JAK) activation and signal transducer and activation of transcription 3 (STAT-3) (Lecour, 2009).

Genade et al. (2008) assessed the involvement of these prosurvival pathways in the presence and absence of melatonin, and found that under conditions of ischaemia, followed by 10 minutes reperfusion and subsequent western blotting, PKB/Akt was phosphorylated/activated and the opposite was seen for the pro-apoptotic kinase p38 MAPK. Activation of this prosurvival pathways was seen regardless of whether melatonin was administered before or after ischaemia. Lamont et al (2011) reported that melatonin treatment induced activation of STAT3 prior to induction of ischaemia. Furthermore, melatonin elicited no protection against I/R injury in TNF-α receptor 2 and myocardial STAT-3 knockout mice, suggesting that activation of the SAFE pathway *before* the onset of ischaemia is important in cardioprotection. In both these studies activation of the survival pathways was associated with a significant reduction in infarct size.

Interestingly, the dosage of melatonin administered may not appear to be important in cardioprotection in this scenario. For example, Lamont *et al*. (2011) showed that hearts pretreated with melatonin in the picomolar range, exhibited a reduction in infarct size during reperfusion, However, most workers made use of higher concentrations of melatonin, for example Lochner et al. (2006) and Genade et al. (2008) used melatonin at a concentration of 50μM.

Apart from this, cardioprotection associated with melatonin has been linked to melatonin receptors (MT1/2) that has been identified in the heart (Ekmekcioglu *et al*., 2003; Lochner *et al*., 2006). In addition, in a study on rats by Sallinen and coworkers (2007) on the effect of myocardial infarction and melatonin receptor expression, it was shown that post-myocardial

infarction, the animals had increased pineal melatonin synthesis, associated with raised plasma melatonin levels along with a consequent enhanced melatonin receptor expression. These results suggest an important role of endogenous melatonin plus its receptors after myocardial infarction (Sallinen *et al*., 2007). In support of this, Lochner *et al*. (2006), using the melatonin receptor antagonist luzindole, found that the cardioprotective effects of melatonin were abolished.

As previously mentioned, the opening of the MPTP during myocardial reperfusion injury is associated with cell death. It was shown that melatonin inhibited this opening via prevention of mitochondrial cardiolipin peroxidation, thus associated with cardioprotection (Petrosillo *et al*., 2009; for a review see Lochner *et al*., 2013).

Melatonin's antioxidant, anti-inflammatory, anti-adrenergic activities as well as its ability to enhance antioxidant enzyme activities, have all been associated with its conferred cardioprotection (Genade *et al*., 2008; Dominguez-Rodriguez *et al*., 2010).

Another interesting observation was made by administration of exogenous melatonin to pinealectomized animals which not only raised the level of circulating melatonin, but also maintained a high level up to 3 weeks after treatment (Djeridane *et al*., 2000), suggesting that the beneficial effects of melatonin treatment could last for days after treatment (Lochner et al., 2006). These long-term effects of melatonin may be due upregulation of the melatonin receptors, since pretreatment of rats with a combination of melatonin and the melatonin receptor blocker, luzindole, abolished cardioprotection (Lochner & Genade, unpublished observations). However, further investigations are required for elucidation of these effects.

1.5 MOTIVATION FOR STUDY

As stated before, obesity is currently considered as a global major health issue and risk factor for type 2 diabetes and cardiovascular disease, especially ischaemic heart disease (Eckel *et al*., 2005; Emanuela *et al*., 2012).

There is increasing evidence for the role of adipose tissue dysfunction, dysregulated adipocytokines and elevated free fatty acid release in promoting oxidative stress in obesity (Furukawa *et al*., 2004; for a review see Hajer *et al*., 2008). Furthermore, it is well-established that oxidative stress is associated with several pathological states, including heart disease and particularly damage to the ischaemic-reperfused myocardium (Opie, 2004; Yellon & Hausenloy, 2007).

Melatonin is considered by many researchers as the ideal free radical scavenger and antioxidant (Reiter 1995; Tan *et al*., 2002; Hardeland *et al*., 2005). It has been shown to have marked beneficial effects in animal models of obesity (Prunet-Marcassus *et al*., 2003; Hussein *et al*., 2007; Shieh *et al*., 2009) and to protect the heart against ischaemia/reperfusion injury (Salie *et al*., 2001; Tengattini *et al*., 2008; Petrosillo *et al*., 2009; Nduhirabandi *et al*., 2011) suggesting a potential role for melatonin in both obesity management and cardioprotection.

Nduhirabandi and coworkers (2011 & 2014) previously showed that melatonin treatment reduced infarct size in both control and obese rats. However, in view of the obesity paradox, it is not known how melatonin treatment will affect cardioprotection. Interestingly, melatonin abrogates protection induced by prior preconditioning, due to its free radical scavenging properties (Genade et al., 2006). In this context melatonin may affect diet-induced resistance to ischaemia/reperfusion injury and thus exploration of melatonin effects in models of obesity remains important.

A previous study in our laboratory made use of a hyperphagia-induced rat model of obesity, achieved by adding excess sucrose to the diet. However, the changes induced by this diet were relatively small and the animals, despite becoming insulin-resistant, did not develop type 2 diabetes. It was therefore decided to adjust the diet by adding cooking fat to the sucrose diet in the hope that this will induce more marked changes in the myocardium, leading to, amongst others, myocardial hypertrophy and type 2 diabetes.

We hypothesize that melatonin, in view of its free radical scavenging properties, may alter the response of the heart to obesity-induced cardioprotection. This, of course, is based on the assumption that the high fat diet will elicit the obesity paradox.

The aims of this study were therefore:

(i) To evaluate the ability of a high fat diet (HFD) to induce obesity in rats. Apart from evaluating its effects on the biometric parameters and resistance to ischaemia/reperfusion injury (as indicated by infarct size in regional ischaemia and functional recovery after global ischaemia), special attention will be given on the interplay between adiponectin, AMPK, leptin, and FFA in this model.

(ii) To evaluate the effect of daily oral administration of melatonin to rats on the HFD as well as their littermate controls, on the parameters listed above as well as on the development of obesity. In this study melatonin will be administered from the onset of the feeding of the high fat diet.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMAL CARE AND ETHICS

96 Male Wistar rats (weighing ±200g) were obtained from the University of Stellenbosch Central Research Facility. The milieu in which the animals were maintained included controlled conditions of a 12-h dark/light setting with lights on from 6:00 am until 6:00 pm, with constant temperature (22°C) and humidity (40%). Ethical clearance was obtained from the Committee for Ethical Research: Animal Care and Use, University of Stellenbosch (SU-ACUM13-00035). Animals were treated according to the guidelines of the South African National Standard for the Care and Use of Animals for Scientific Purpose (SANS 10386:2008).

2.2 EXPERIMENTAL DESIGN

The study consisted of three parts. The first part included grouping, feeding and treatment. For the second part, biometric measurements (body weight, visceral fat etc.), blood collection (for analyses of blood glucose, plasma insulin, leptin, adiponectin, free fatty acids , triglycerides, cholesterol, phospholipids, conjugated dienes and thiobarbituric acid reactive substances (TBARS) as well as heart tissue excision and perfusions were performed. Lastly, biochemical determinations of blood and heart tissue collections were carried out.

2.2.1 Feeding and treatment

Male Wistar rats (200±20 g) were divided into four groups (24 each) consisting of: group C (control) which received standard rat chow and drinking water; group C+M (control + melatonin) received a standard rat chow with melatonin in the drinking water; group HFD, received a high-fat diet (HFD) with normal drinking water and lastly, group HFD+M received a high-fat diet (HFD) with melatonin in the drinking water. The feeding and treatment were initiated simultaneously and lasted for 16 weeks with fresh food/water given daily to all of the above mentioned groups. The high fat diet was prepared as follows: normal rat chow (2.4Kg); sugar (520g); condensed milk (full cream) 8 cans; Holsum cooking fat (550g); water (2L).

The compostion of the control rat chow and the HFD is summarized in Table 1.

Table 1. Composition of the diets

	Control rat chow	HFD
Total fat (g/100g)	4.8	11.5
Saturated fat (g/100g)	0.9	7.6
Cholesterol (mg/100g)	2.2	13
%Protein	17.1	8.3
%Carbohydrate	34.6	42
Sucrose	5.3	20.4

Analysis of the diets was done by Microsept, Cape Town

2.2.2 Melatonin administration

Melatonin (Sigma Aldrich, St Louis, MO, USA), was first dissolved in 1 ml of absolute ethanol and added to 1L drinking water (ethanol concentration 0.1% (v/v)). The final concentration of melatonin in the drinking water was 75µg/ml or 50µg/ml for the HFD and control rats respectively. These amounts were based on the daily fluid intakes of the HFD and control animals to obtain a dosage of 10mg/kg/day. The animals were weighed on a regular basis (once

a month) and the amounts of melatonin added to the drinking water adjusted so that the dosage of 10mg/kg/day was maintained throughout the experimental period. Since melatonin is light sensitive, the bottles containing the melatonin water were covered with aluminium foil. Water, melatonin and food were available ad libitum. Drinking water with or without melatonin was replaced every day one hour before light off as previously indicated (Wolden-Hanson *et al*., 2000; Prunet-Marcassus *et al*., 2003). The melatonin solution was prepared freshly every 2 days and stored in a refrigerator at 4ºC until used.

2.3 EXPERIMENTAL PROTOCOL

Following the 16 week period of feeding and treatment, the animals were grouped into non-fasted and overnight fasted (for baseline measurements). They were then anaesthetized by intraperitoneal injection of 30mg/kg pentobarbital, body weights were determined, blood glucose levels measured (from tail tip) and then sacrificed. The excision of the heart was followed by blood collection from the thoracic cavity as well as collection and weighing of visceral fat. Blood samples were centrifuged at 3000rpm (4^{o} C), the plasma collected and kept frozen at -80^{o} C until analyses were done. The hearts were either perfused (non-fasted) or freeze-clamped (fasted). For each rat the adiposity index was calculated (ratio of visceral fat mass to body weight multiplied by 100).

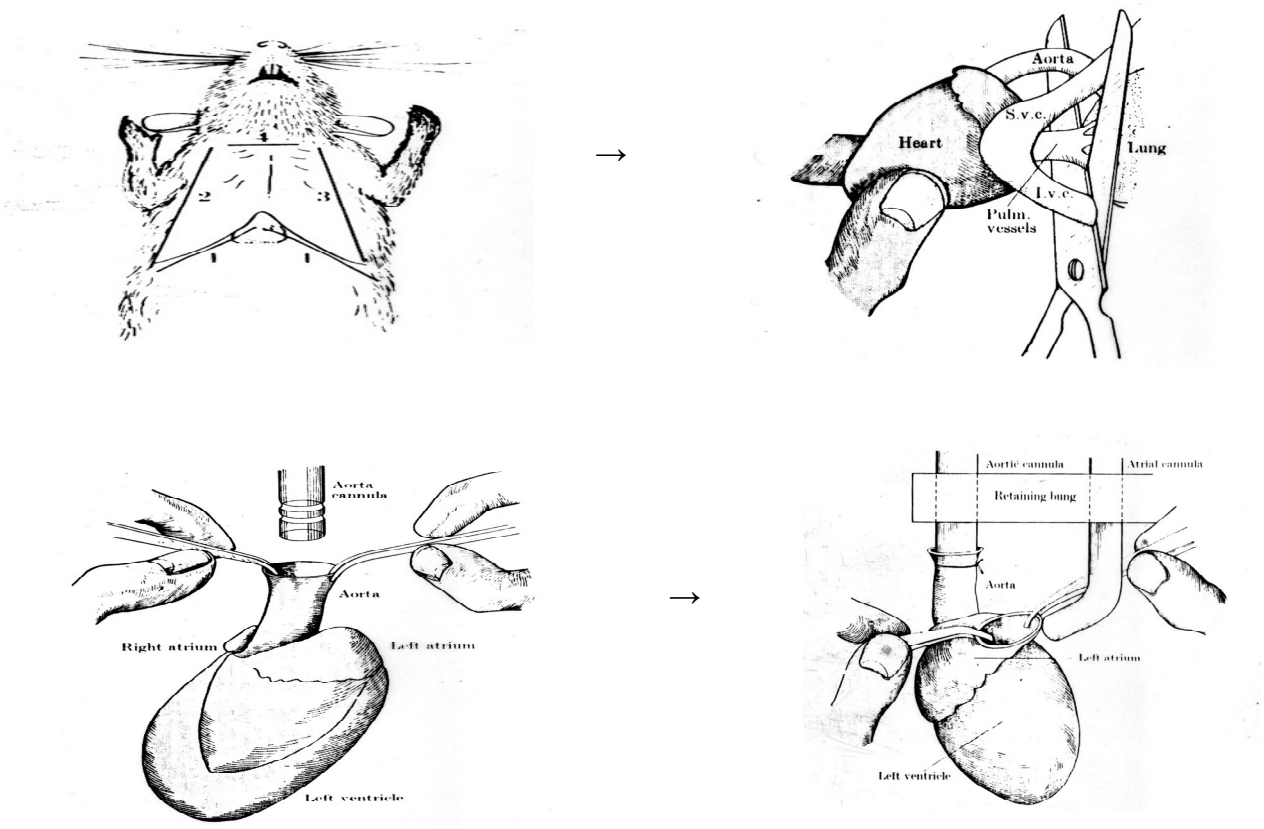

Figure 2.1 After excision, the heart was mounted onto the aortic cannula, followed by insertion of the atrial cannula into the pulmonary vein (Lochner A, BHons lectures, 2013).

2.3.1 Perfusion technique

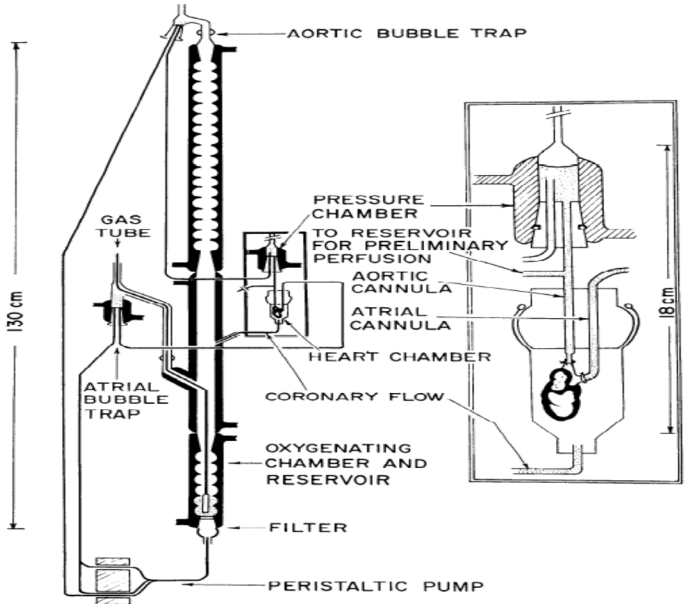

Figure 2.2 Perfusion apparatus for working rat hearts (Neely *et al*., 1967).

Following removal of the rat heart, it was placed into ice-cold Krebs-Henseleit solution for arrest and then mounted *via* the aorta onto the aortic cannula and tied with a silk suture as demonstrated in fig 2.1. The hearts were perfused with Krebs-Henseleit buffer containing (in mM): NaCl 119, $NaHCO_3$ 24.9, KCl 4.7, KH_2PO_4 1.2, $MgSO_4.7H_20$ 0.59, Na_2SO_4 0.59, $CaCl_2.H_2O$1.25 and glucose 10. The buffer was gassed with 95%O_2/5%CO_2 to obtain a pH of 7.4. For stabilization, the hearts were retrogradely perfused (Langendorff mode), in a non-recirculating manner (at 100 cm H2O) for 15 minutes, followed by 15 minutes of atrial perfusion, in the working heart mode, at a preload of 15cm H_2O and afterload of 100cm H_2O (see fig 2.1).

The hearts were then subjected to either (i) global ischaemia or (ii) regional ischaemia induced by ligation of the left descending coronary artery (see fig 2.3.2 (i/ii)). The temperature of the hearts was monitored by inserting a temperature probe into the pulmonary artery and kept at constant temperatures of 37°C and 36.5°C, during perfusion and ischaemia, respectively. Manual measurements of coronary flow (CF) and aortic output (AO) were made before and after ischaemia while the heart was perfused in the working mode, while a Viggo-Spectramed pressure transducer inserted into the aortic cannula, coupled to a computer system, was used to measure the aortic pressure (systolic/diastolic (mmHg)) and heart rate (HR in beats per minute, bpm).

Total work performance (pressure power) was calculated according to the formula of Kannengieser *et al*. (1975):

$0.002222(P_{AO} -11.25).(CO)$

Where, P_{AO} = aortic pressure

CO =cardiac output (aortic output +coronary flow rate)

For each heart, the recovery of function during reperfusion was calculated for each parameter by expressing function during reperfusion as a percentage of function before induction of ischaemia.

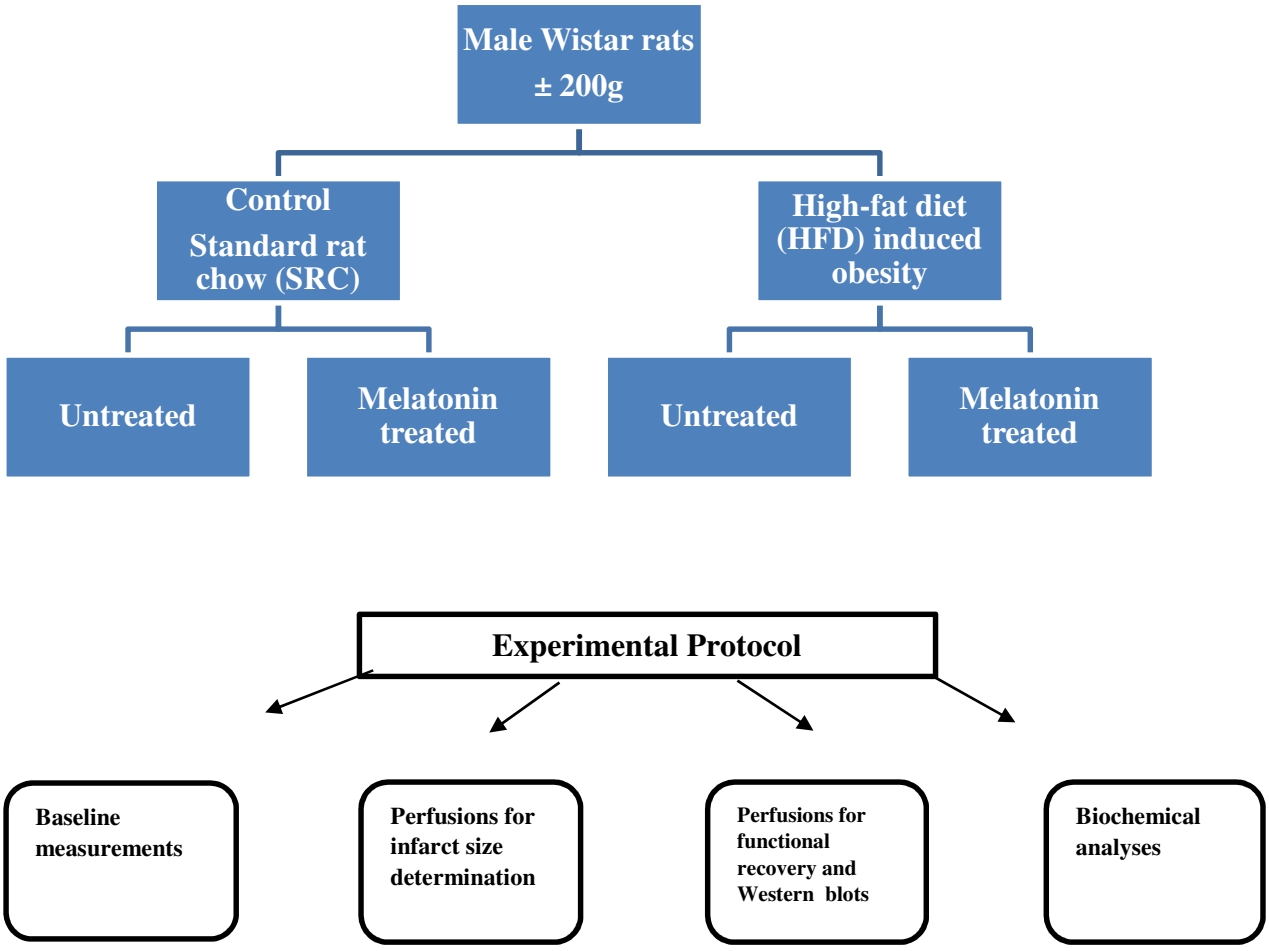

Figure 2.3.1 Experimental approach: Four groups of animals were studied. In each group biometric measurements were made and blood collected for biochemical analyses collected. Hearts were then removed for perfusion for measurement of functional performance, infarct size and collection of tissue for Western blotting.

2.3.1.1 Regional and global ischaemia

Following the stabilization period, regional ischaemia was induced by occlusion of the left anterior descending coronary artery (LAD) with a silk suture (inserted and tied below the LAD) for 35 minutes at 36.5°C. A reduction of 33% in coronary flow signified sufficient regional ischaemia. Once the 35 minutes had elapsed, the silk suture was removed and the hearts perfused for an additional 60 minutes (10 min retrograde, 20 min working mode, 30 min retrograde) after which infarct size was determined. Mechanical function was measured before and after induction of ischaemia during perfusion in the working mode. See Fig 2.3.2 (i).

For global ischaemia, the same stabilization period was used; however, thereafter, the aortic cannula was completely occluded resulting in no flow to the heart (no-flow ischaemia) for 20 minutes at 36.5°C. To reperfuse the aortic cannula was reopened and the hearts were perfused for 30 minutes (10 min retrograde; 20 min working mode) for determination of function. At the end of reperfusion, hearts were freeze-clamped and stored in liquid nitrogen for subsequent biochemical analysis. A similar intervention was performed for determination of kinase activity with freeze-clamping of the hearts after 10 minutes of reperfusion following global ischaemia (20 minutes). See Fig 2.3.2 (ii).

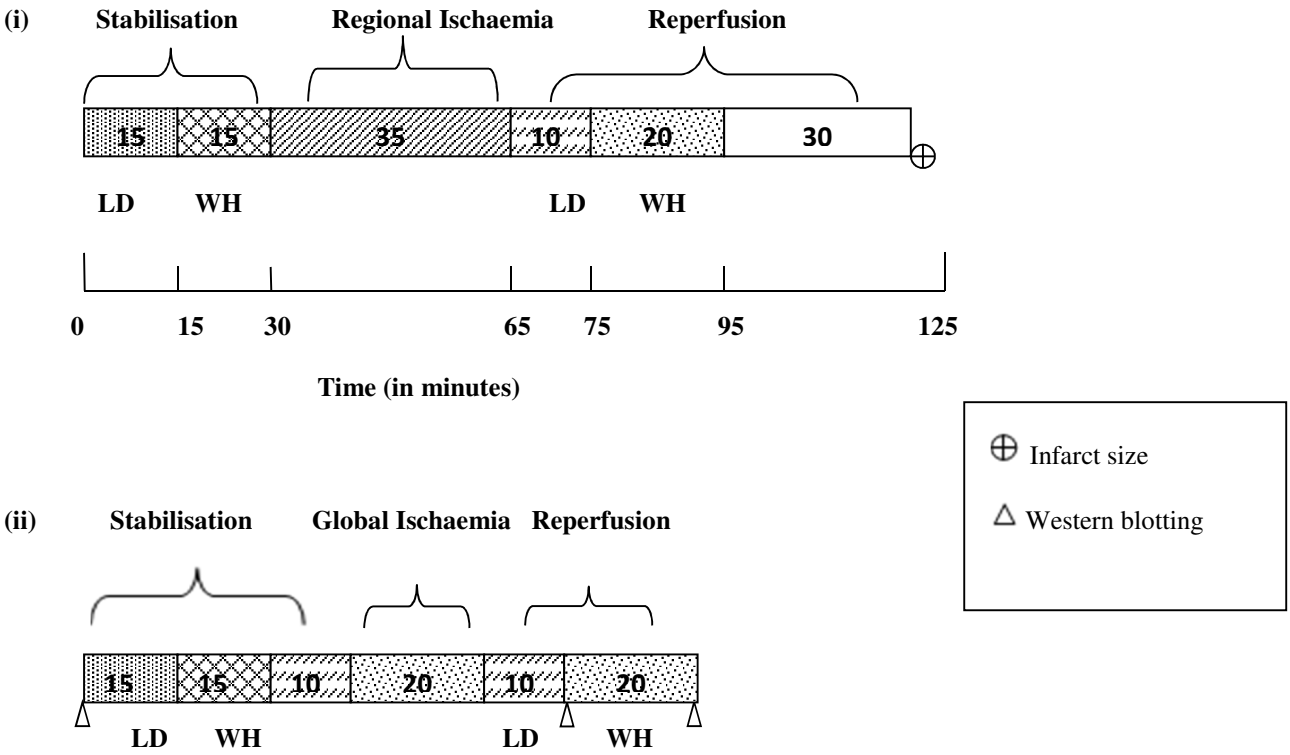

Figure 2.3.2 Perfusion protocol: (i) Regional ischaemia (ii) Global ischaemia with infarct size and mechanical function (recovery) respectively as endpoints.

2.3.1.2 Determination of area at risk and infarct size

At the end of the perfusion protocol, the coronary artery was tied (with the silk suture) and 1ml of 0.5% Evans blue injected via the aorta cannula. Following over-night freezing of the hearts, they were cut into 2mm slices, then stained for 15 minutes with 1% w/v triphenyl tetrazolium chloride (TTC) in phosphate buffer (pH 7.4) at 37°C. Thereafter, the slices were fixed in 10% v/v formaldehyde solution which enabled the distinction between viable tissue (indicated by the blue area), non-viable/infarcted tissue (the white (unstained) area) and the area at risk which constitutes the white area as well as the red area surrounding it. All slices were drawn on transparencies and then scanned to determine the area at risk (AR) and area of infarcted tissue,

using the UTHCSA (University of Texas Health Science Center at San Antonio, TX, USA) Image Tool software. Infarct size (IFS) was expressed as a percentage of the area at risk (IFS/AR %).

2.4 BIOCHEMICAL ANALYSES

2.4.1 Western Blotting

2.4.1.1. Tissue collection and lysates preparation

Hearts were freeze-clamped either without perfusion (baseline conditions) or after 10 or 30 min reperfusion (see protocol fig 2.3.1). The tissues were stored at -80^{0}C until preparation of lysates.

The lysis buffer consisted of (in mM): Tris-HCl 20, p-nitrophenylphosphate 20, EGTA 1, EDTA 1, NaCl 150, tetra-sodium-pyrophosphate 2.5, β-glycerophosphate 1, sodium orthovanadate 1, phenylmethyl sulphonyl fluoride 1, aprotinin and leupeptin both at 10µg/ml and Triton-X100 1%. The frozen tissue was pulverized and homogenized in 600-900 µl of lysis buffer using a Polytron homogenizer. Following centrifugation (1000xg for 10 minutes) of the samples, the supernatant was collected and diluted according to the lysates preparation protocol. The amount of protein in the lysate was determined using the Bradford technique (1976), using a standard curve of known BSA concentration to obtain the concentration of the unknowns. The lysis buffer and protein samples were then diluted in 3X Laemmli sample buffer to a final concentration of 50 µg of protein per 15 µl for loading into each well of the PAGE. The final lysates were then boiled for 5 min and stored at -20°C.

For STAT-3 lysates preparation, the same procedure was followed except that the frozen tissue was homogenized in lysis buffer without the addition of Triton-X100 1%. Following centrifugation at 14500 rpm for 10 minutes the supernatant was collected as the cytosolic fraction. The pellet was then resuspended in lysis buffer (600-900 µl) containing Triton-X100 (1%) of which its supernatant (after additional centrifugation) was used for the nuclear fraction. The same procedure was followed to prepare the final lysates.

2.4.1.2 Western blot technique

After boiling and centrifugation of the lysates (for 5 minutes each), the protein was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad® mini-Protean III system. Either 10% (for AMPK, JNK, STAT-3 and UCP) or 12% (for PKB/Akt, ERK1/2 and PGC1-α) resolving gels (matrix to contain and separate molecules) were prepared with a 4% stacking gel on top of the resolving gel. The samples were loaded into the wells of the gels along with a commercial molecular weight marker/ladder (used to approximate the size of the protein separated on the gels) and placed into running buffer containing Tris 25 mM, glycine 192 mM and 0.1% SDS). After the separation process, proteins were transferred to PVDF membranes using transfer buffer (Tris 25 mM, glycine 192 mM and methanol (20% v/v) and then washed in methanol and allowed to air-dry. Once dried, the membranes were stained with Ponceau red reversible stain for confirmation of adequate protein transfer. The membranes were scanned using a ChemiDoc MP imaging system for later normalization. Blocking of non-specific sites on the membrane with 5% fat-free milk powder TBS-T solution (for 1 to 2 hours) or 5% Bovine serum albumin (BSA) in TBS-T (for UCP3 and PGC1-α) required thorough washing of the membrane before and after with Tris-buffered saline plus 0.1% Tween 20 (TBS-T). The membranes were then incubated overnight in primary

antibody (5µl antibody in 5ml of TBS-T or 5% milk-TBS-T) at 4°C. Thereafter, the membranes were washed (at least 3 times for 5 minutes) with TBS-T and incubated in horse-radish peroxidase coupled secondary antibody (5µl antibody in 20ml TBS-T or 5% milk-TBS-T). The secondary antibody was then washed off using the same washing procedure as mentioned above. The details regarding the blotting technique and the preparation of the gels are summarized in tables 2 and 3.

2.4.1.3 Western Blot detection and validation

After washing, a detection reagent, Enhanced chemiluminescence (ECL), was used to detect the protein of interest. This was achieved using the ChemiDoc MP imaging system (Bio-Rad®). Normalization and analysis of data involved using the stored image of the Ponceau stained membrane and superimposing it on the image obtained from the antibody detection step. This results in a ratio of the signal from the protein-antibody reaction to the total protein in a specific lane.

2.4.2 Western blot analysis

Table 2- Western blot information table

Protein	Molecular weight (kDa)	Resolving gel %	Blocking time (hours)		1° Ab dilution	2° Ab dilution
			Total	Phosphorylated		
PKB/Akt	60	12	1	2	1:1000 TBS-T	All 1: 4000 TBS-T 1:4000 5% milk (p-AMPK)
ERK ½	42/44	12	1	1h30	1:1000 TBS-T	
JNK	43-47	10	1h30	2+	1:1000 TBS-T	
AMPK	62	10	1h30	2	1.5:1000 TBS-T	
PGC1-α	90	12	1	1	1:1000 TBS-T	
UCP	30-34	10	1	1	1:1000 TBS-T	
STAT-3	86-90	10	1h30	1h30	1:1000 TBS-T	

Table 3- Preparation of SDS-PAGE gels

Reagents	Stock	Resolving gels		Stack gel
		10%	12%	4%
Distilled H_2O		**4.9 ml**	**3.35 ml**	**3.05 ml**
Tris pH8.8(gel)/pH6.8(stack)	**1.5 M / 0.5 M**	**250µl (1.5M)**	**250µl (1.5M)**	**1.25 ml (0.5M)**
SDS	**10%**	**100 µl**	**100 µl**	**50 µl**
Acrylamide	**40%**	**2.5 ml**	**3 ml**	**0.5 ml**
APS (0.1g in 1ml dH_20)	**10%**	**50 µl**	**50 µl**	**50 µl**
TEMED	**99%**	**20 µl**	**20 µl**	**10 µl**

2.5. BLOOD ANALYSIS

2.5.1 Blood collection

After excision of the hearts, blood collected from the thoracic cavity was placed in K3-EDTA (Greiner bio-one™) tubes and kept on ice until centrifugation at 3000 rpm for 10 minutes at 4°C (within 30 minutes of collection). The plasma was stored in Eppendorf tubes at -80°C until required for analyses.

2.5.2 Blood glucose determination

Blood samples were collected from the tip of the rat's tail (before sacrifice) for measuring blood glucose levels using disposale test strips inserted into the GlucoPlus™ meter (CIPLA DIBCARE, Bellville, South Africa) afterwhich the reading was displayed in mmol/L.

2.5.3 Free Fatty Acid Quantitation assay

Plasma FFA levels were measured using the Free fatty acid quantitation kit MAK044 (Sigma-Aldrich®, St Louis, MO) in which the fatty acid concentration was determined by a coupled enzyme assay resulting in a colorimetric product proportional to the fatty acids present in the samples.

Palmitic acid standards containing 0 (blank), 2, 4, 6, 8, and 10 nmoles/well were placed in the 96 well plate provided. For the reaction wells, plasma samples (50µl) were pipetted directly into the wells. All standard and plasma samples were prepared in duplicate. Thereafter, 2 µl of acyl-CoA synthetase (ACS reagent) was added to all the wells and incubated (30 minutes at 37°C). Thereafter a master reaction mix containing a fatty acid probe was prepared and added to each well (50 µl/well). The plate was covered, shaken and incubated under the same conditions as above. The absorbance at 570nm was measured using a plate-reader (FLUOstar Omega, BMG Labtech. The blank reading was subtracted from all other values obtained after which a standard curve (for palmitic acid standard) was plotted and the fatty acid concentration of the samples (unknown) calculated from the curve.

2.5.4 Leptin assay

The leptin rat ELISA (enzyme-linked immunosorbent assay) kit (ab100773, abcam®) was used for measurement of plasma leptin levels. This assay employs an antibody specific for leptin coated on a 96 well plate. Standards (prepared according to the manufacturer's instructions) and samples were pipetted into the wells and the leptin present binds to the immobilized antibody on the wells. The wells were washed and biotinylated anti-rat leptin antibody added. After washing the unbound biotinylated antibody, HRP-conjugated streptavidin was added to the wells. The wells were again washed, a Tetramethylbenzidine (TMB) substrate solution was added and a colour developed in proportion to the amount of leptin bound. The stop solution

changed the colour from blue to yellow, the intensity of which is measured at 450nm on a plate reader. The assay procedure was carried out according to the company's instructions and the concentration of leptin in the plasma samples were calculated from the standard curve.

2.5.5 Adiponectin assay

The adiponectin rat ELISA, ab108784 (abcam®) was used to detect its levels in plasma samples. This assay employs a quantitative sandwich enzyme immunoassay technique. Briefly, all reagents, standards (serially diluted) and samples were prepared according to the instruction booklet. Firstly, 50 µl of standards or samples were added to the 96 well plate (coated with a polyclonal antibody specific for rat adiponectin), with 1 hour incubation at room temperature, followed by addition of 50 µl biotin antibody (1 hour incubation), 50 µl streptavidin-peroxidase conjugate (30 minute incubation), 50 µl of chromagen substrate (10 minutes incubation) until a blue colour developed. A 5 times wash step with wash buffer was done before and after every addition, following the incubation of samples and standards. Lastly, 50 µl of stop solution was added to the wells. The absorbance at 450 nm was read with a plate reader and a standard curve generated to determine the unknown sample concentration, multiplied by the dilution factor (plasma samples were diluted 400x).

2.5.6 Insulin Assay

Fasting plasma was collected as described previously (see 2.5.1). The Coat-A-Count® Insulin radioimmunoassay was used for the quantitative measurement of plasma insulin levels. Briefly, four uncoated polypropylene tubes were labelled for total counts (T) and non-specific binding (NSB), in duplicate, to which 200 µl of zero calibrator A was added. Fourteen insulin antibody-coated tubes A-G (with 200 µl of calibrators (standards) A-G added to respective tubes) as well as 200 µl of plasma samples were added to prepared tubes (in duplicate).

^{125}I insulin (1 ml) was added to all tubes and incubated for 18-24 hours at room temperature. Following incubation, the tubes were decanted and all visible moisture removed. A gamma counter (Perkin Elmer WIZARD) was then used to count the radioacivity. The insulin levels of the samples were obtained from the standard curve. To assess insulin sensitivity/resistance, the homeostatic model assessment (HOMA-IR) index was calculated as follows:
(fasting glucose concentration (mmol/l) x fasting insulin concentration (µIU/ml)) divided by 22.5.

2.5.7 Lipid Assay

Lipid peroxidation, total cholesterol (TC), triglycerides (TG) and phospholipids (PL) were assayed for, using fasting plasma samples and enzymatic colorimetric kits (KAT Medicals, Calicom Trading (PTY), South Africa; WAKO Chemicals, Germany) by a Labsystems Multiskan MS analyzer (AEC Amersham Co., South Africa).

Plasma conjugate dienes (CD) and thiobarbituric acid reactive substances (TBARS) levels were determined spectrophotometrically by (i) initially mixing plasma samples with cyclohexane (Spectrosol) (Pryor and Castle, 1984; Esterbauer et al., 1989), followed by centrifugation at 14000 × g at 10°C, for 10 min. Thereafter, the absorbance of CD was measured at 234 nm using a GBC UV/VIS analyser (Wirsam Scientific and Precision Equipment, South Africa).

(ii) TBARS were measured according to the method of Jentzsch et al (1996). The samples (200 µl) were mixed with 25µl butylated hydroxytoluene (BHT) (Fluka Chemie, Switzerland) in ethanol (Merck Chemicals, South Africa) and orthophosphoric acid (Sigma Chemical Co) buffer at pH 3.6 and then vortexed for 10 seconds. Thereafter, 25 µL thiobarbituric acid (TBA) (Sigma) reagent was added to the mixture with subsequent vortexing, followed by a 45 min incubation (at 90ºC) in a water bath, after which the tubes

were placed on ice to stop the reaction.TBARS were extracted using n-butanol followed by addition of saturated NaCl and centrifugation at 12000 rpm for 1min. Lastly, absorbance was read at 532 nm using Labsystems Multiskan MS Analyser (AEC Amersham Co., South Africa). To determine LOOH, plasma was assayed in the presence of xylenol orange (3,3'-bis[N,N-di(carboxymethyl)-aminomethyl]-o-cresolsulfonephtalein (Sigma Chemical Co) and Fe2+ in the ferrous oxidation/xylenol orange (FOX) assay that was adapted to enhance the solubility of non-polar compounds by including chloroform. Absorbance of the resulting Fe3+-xylenol orange complex was measured at 560nm (Jiang et al., 1991; Jiang et al., 1992) using Labsystems Multiskan MS Analyser (AEC Amersham Co., RSA).

The final concentrations were then calculated using appropriate molar extinction, coefficients and standards.

2.6 DATA ANALYSIS

The data was analysed using GraphPad Prism software. All values were expressed as mean ± standard error of mean (SEM). The unpaired Student's t-test was used for comparison of the control and high-fat diet groups. In most cases a one way analysis of variance (ANOVA) was used for multiple comparisons along with the Bonferroni correction. A two way ANOVA was used where appropriate. A p-value (calculated probability) of less than 0.05 was regarded as significant.

CHAPTER 3

RESULTS

3.1 BIOMETRIC AND METABOLIC DATA

3.1.1 Characteristics of the 16 week high-fat diet model of obesity

Over a period of 16 weeks feeding of the high fat diet (HFD) to rats caused significant increases in body weight (30.5%), visceral fat (80.7%) and adiposity index (39.8%) (Table 3-1) compared to their age-matched controls (fig 3-1 A & B). Fasted blood glucose levels of the diet fed group (11.05±1.093 mmol/L, $p<0.005$), plasma insulin (47.66 ± 6.835 µlU/mL, $p<0.005$) and HOMA index (24.33 ± 4.634, $p<0.005$) were significantly higher than their respective controls (fig 3-2 A, B & C, respectively; Table 3-1).

Plasma leptin levels of the controls (5.060 ± 1.571 ng/mL) were significantly elevated by the diet to 10.84 ± 1.63 ng/mL, $p<0.05$ (fig 3-3 A). Compared to the controls, the HFD rats showed significantly lower adiponectin levels (HFD: 77.58 ± 3.79 ng/mL vs C: 109.2 ± 11.86 ng/mL, $p<0.05$) (fig 3-3 B; Table 3-1).

Significant changes in the lipid profile were seen in the high-fat group. Plasma free fatty acids (FFA) (0.261 ± 0.058 mM, $p<0.05$) (fig 3-4 A), triglyceride (1.320 ± 0.45 mmol/L, $p<0.05$) (fig 3-4 B) and phospholipid levels (3.15 ± 2.33 mmol/L, $p<0.05$) were significantly higher than those of their control counterparts (see Table 3-2).

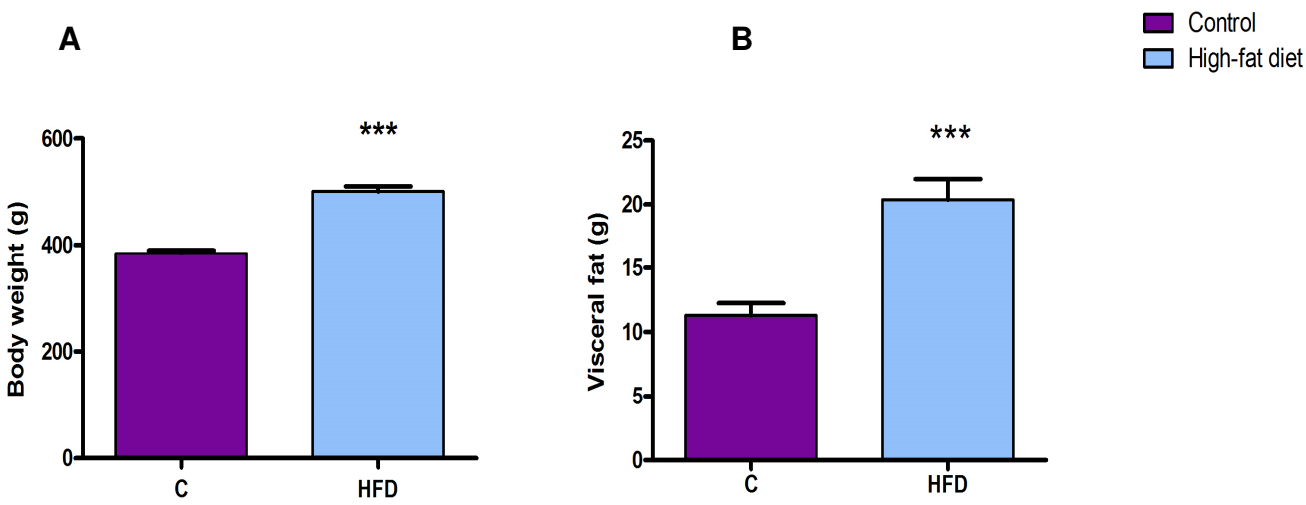

Figure 3-1 Impact of high-fat diet on (A) Body weight, **p<0.0001 (B) Visceral fat (n=16/group), ***p<0.001 vs C

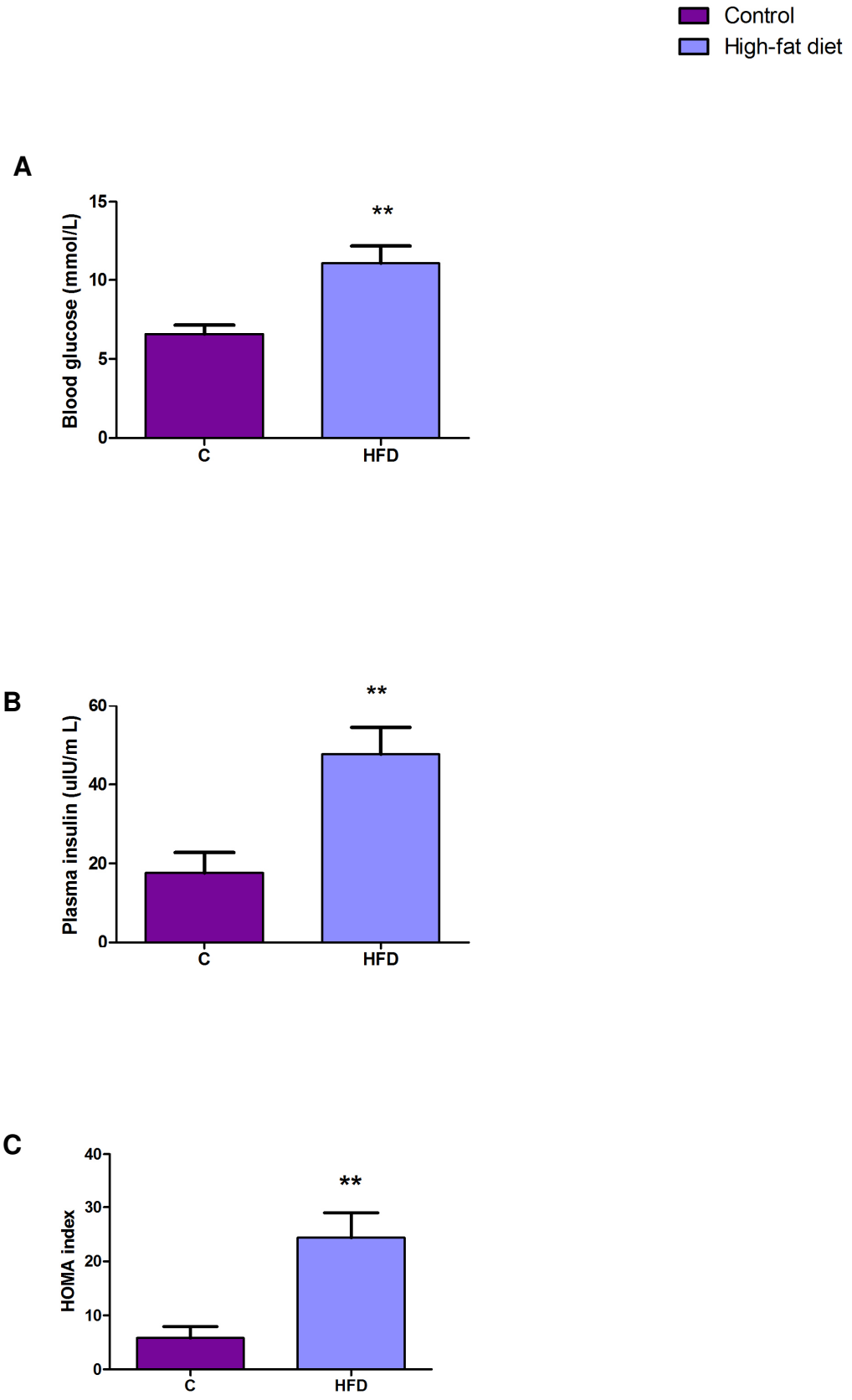

Figure 3-2 Effect of diet on (A) Fasted blood glucose, **p<0.01 (B) Plasma insulin, p<0.01 (C) HOMA index (n=8/group), **p<0.005 vs C

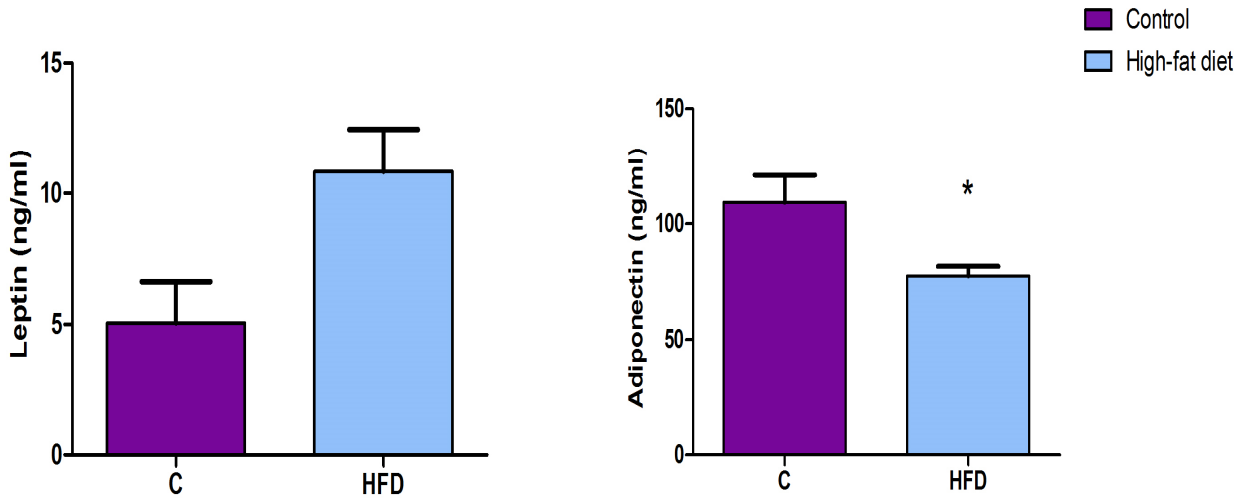

Figure 3-3 Effect of diet on (A) Plasma leptin (n=6-7) and (B) Plasma adiponectin (n=7), *p<0.05 vs C

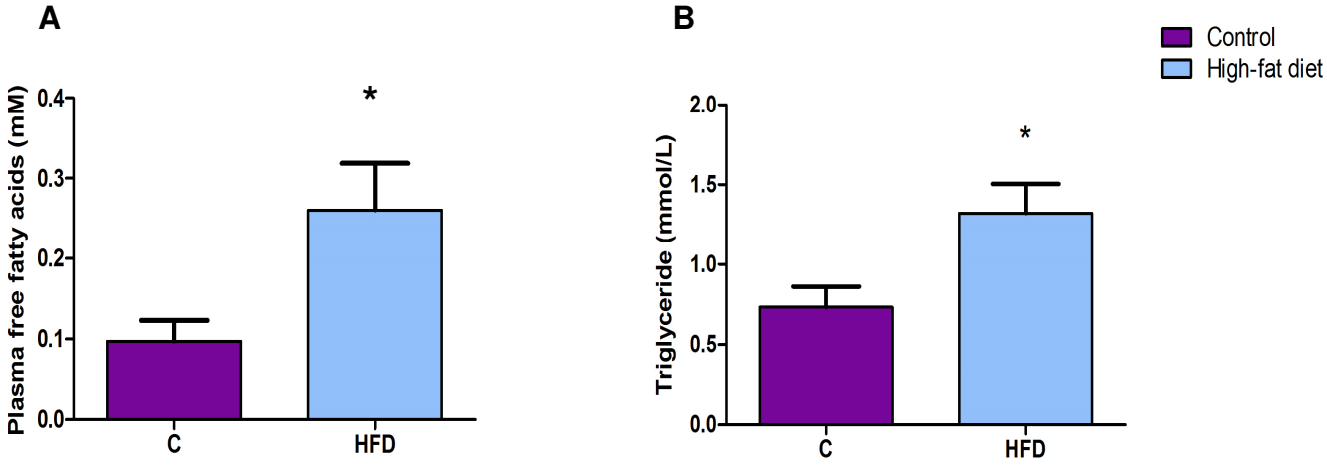

Figure 3-4 Effect of diet on (A) Plasma free fatty acids (n=8) and Triglyceride (n=6), *p<0.05 vs C

3.1.2 Effects of chronic melatonin intake on control and high-fat diet animals

Chronic melatonin consumption (16 weeks) significantly reduced body weight by 10.7% in control [342 ± 8(C+M) vs 384± 6g (C), p<0.05] and 22.8% in high-fat diet groups [387± 12 (HFD+M) vs 500± 9g (HFD), p<0.001] (fig 3-5 A). The intake of melatonin was also associated with reductions in visceral fat mass in both control (9.050 ± 0.8 vs 11.27 ± 0.99 g) and fat (15 ± 1.09 vs 20.37 ± 1.61 g, p<0.05) fed groups (fig 3-5 B). Melatonin significantly reduced blood glucose [11.05 ± 1.09 (HFD) vs 6.88 ± 0.55 mmol/L (HFD+M), p<0.005], HOMA index [24.33 ± 4.63 (HFD) vs 9.8 ± 2.43 (HFD+M)] (fig 3-6 A & C) and leptin [10.84 ± 1.63 (HFD) vs 5.86 ± 0.61 ng/mL (HFD+M), p<0.05] (fig 3-7 A) levels of rats receiving the high-fat diet, without having a significant effect on the controls. Melatonin had no significant effect plasma adiponectin (fig 3-7 B), free fatty acid (fig 3-8 A) and triglyceride (fig 3-8 B) levels in animals receiving standard rat chow or the high-fat diet. Although melatonin had no effect on plasma insulin levels of the control group, it caused a significant reduction in the plasma insulin levels of the HFD group (P=0.05, when using a two way ANOVA). Plasma CD and TBARS levels of both control and HFD rats were not affected by melatonin (Table 3-2).

Table 3-1 Biometric and metabolic parameters

Parameters	C	HFD	C+M	HFD+M	n
Body weight (g)	384 ± 6	501 ± 9***	342± 8 *	387± 12 ###	16
Visceral fat (g)	11.27 ± 0.99	20.37 ± 1.6 ***	9.05 ± 0.80	15 ± 1.09 #	16
Adiposity index	2.94 ± 0.25	4.11 ± 0.349 *	2.556 ± 0.21	3.87 ± 0.25	16
Blood glucose (mmol/L)	6.96 ± 0.58	11.05 ± 1.09 **	5.78 ± 0.3	6.9 ± 0.55 ##	8
Plasma insulin (ulU/mL)	17.6 ± 5.16	47.7 ± 6.835 **	17.51 ± 2.7	29.71 ± 5.43	8
HOMA index	5.82 ± 2.09	24.33 ± 4.634 **	4.38 ± 0.66	9.8 ± 2.43 #	8
Plasma leptin (ng/mL)	5.06 ± 1.57	10.84 ± 1.63 *	3.28 ± 0.55	5.86 ± 0.61 #	6-7
Plama adiponectin (ng/mL)	109.20 ± 11.86	77.58 ± 3.98 *	90.51 ± 4.83	67.00 ± 3.13	7

*p<0.05, **p<0.005, ***p<0.0001 vs C and #p<0.05, ## p<0.005 ###p<0.001vs HFD

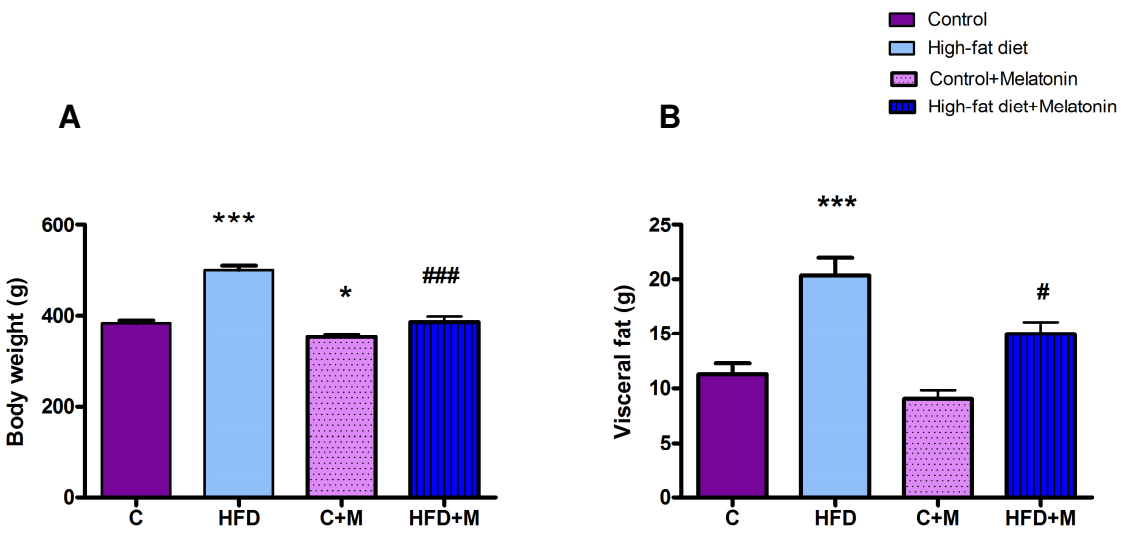

Figure 3-5 Effect of chronic melatonin treatment on (A) Body weight (B) Visceral fat. *p<0.05, ***p<0.0001 vs C and #p<0.05, ###p<0.001 vs HFD, (n=16/group)

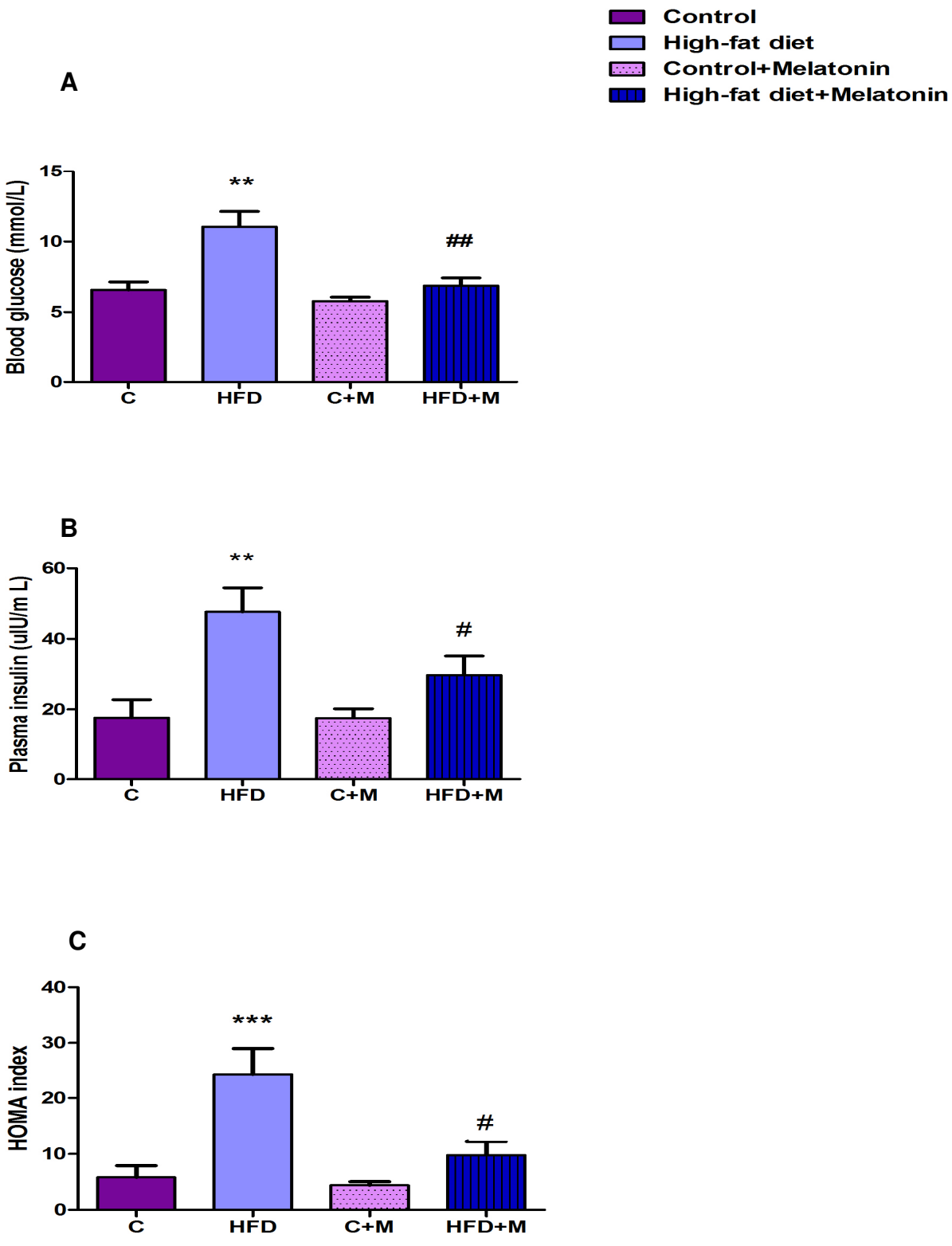

Figure 3-6 Effect of melatonin treatment on (A) Fasted Blood glucose, (B) Plasma insulin and (C) Homeostasis model assessment (HOMA) index. **, ***p<0.005 vs C, and # p<0.05, ##p<0.005 vs HFD, (n=8/group).

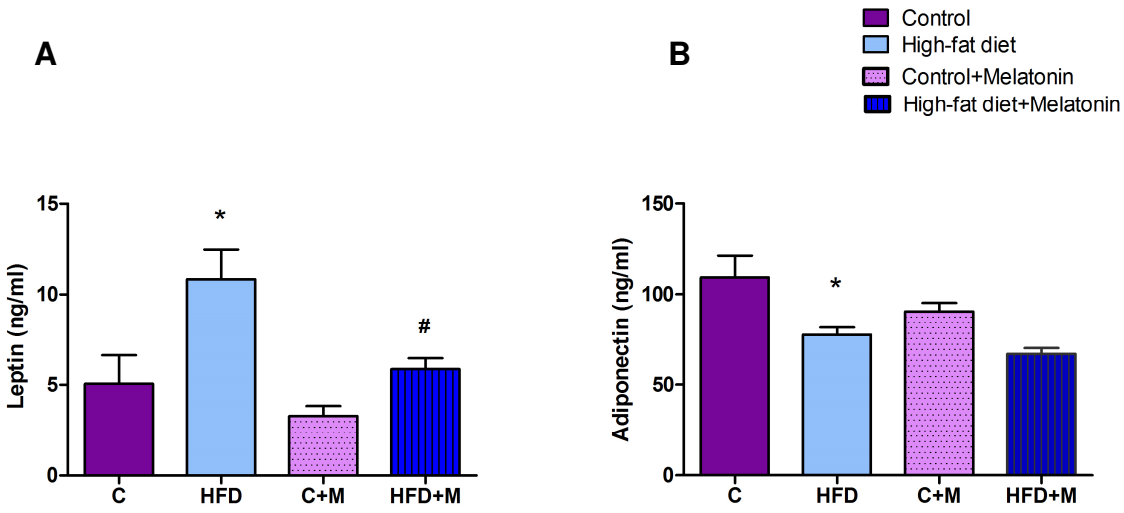

Figure 3-7 Effect of melatonin treatment on (A) Plasma leptin (n=6-7) and Plasma adiponectin (n=7), *p<0.05 vs C, #p<0.05 vs HFD

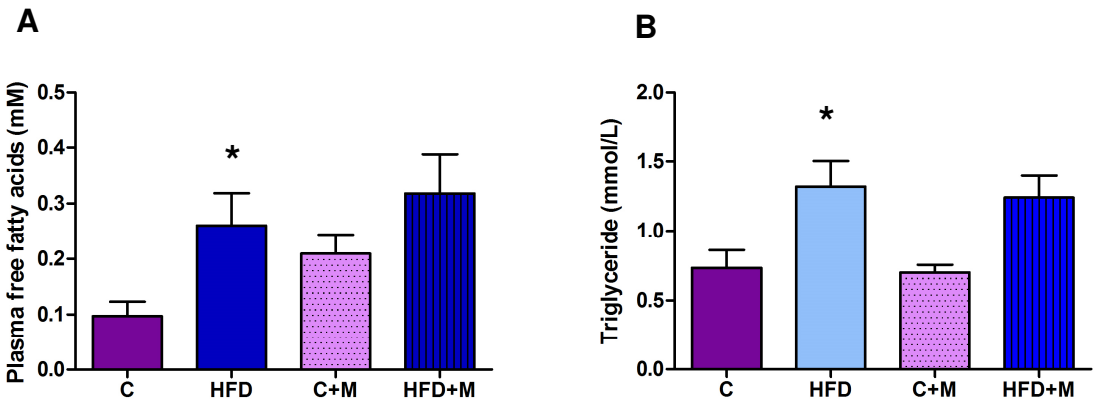

Figure 3-8 Effect of melatonin treatment on (A) Plasma free fatty acids (n=8) and Triglyceride (n=6), *p<0.05 vs C

Group	FFA (mM)	TG (mmol/L)	TC (mmol/L)	PL (mmol/L)	CD (µmol/L)	TBARS (µmol/L)	CD n (µmol/ mmol)	TBARS n (µmol/ mmol)
C	0.097±0.026	0.73±0.32	1.66±0.44	1.64±0.59	33.21±13.78	12.17±0.34	16.01±9.73	6.02±1.29
HFD	0.26±0.058*	1.32±0.45*	1.31±0.31	3.15±2.33	36.19±12.36	15.35±2.62	7.35±2.43	5.01±5.49
C+M	0.21±0.035	0.70±0.13	1.52±0.30	1.56±0.54	27.74±11.43	14.74±2.89	12.86±5.35	6.58±2.56
HFD+ M	0.318±0.07	1.24±0.39	1.48±0.48	2.39±1.25	30.74±12.13	13.54±1.41	8.99±3.39	3.18±0.84

Table 3-2 Impact of diet and treatment on plasma lipid profile: Plasma free fatty acids (FFA) (n=8), Total cholesterol (TC) (n=6), Triglyceride (TG) (n=6), Phospholipids (PL) (n=6), Conjugated dienes (CD) and CD (n) (n=6), TBARS and TBARS (n) (n=2), *$p<0.05$ vs C. CD (n) and TBARS (n): values were normalized.

3.2 EFFECT OF DIET AND TREATMENT ON MYOCARDIAL INFARCT SIZE AND FUNCTIONAL RECOVERY

3.2.1 REGIONAL ISCHAEMIA

To evaluate the effect of the diet and melatonin administration on myocardial functional recovery and infarct size, after a stabilization period of 30 min hearts were subjected to 35 minutes of regional ischaemia followed by 30 minutes of reperfusion, at which point functional recovery was evaluated. The hearts were then perfused for an additional 30 minutes after which time infarct size (total reperfusion time: 60 minutes) was determined.

The high-fat diet had no significant effect on myocardial infarct size when compared to those of rats on the control diet (fig 3-9). However, daily administration of melatonin significantly reduced the size of infarction in both control [% of area at risk: 20.59 ± 2.29 (C+M) vs 38.08 ± 2.77 (C) and high-fat diet groups [% of area at risk: 11.43 ± 2.94 (HFD+M) vs 38.06 ± 3.59 (HFD)] (fig 3-9 B). In addition, a significant difference between the control and HFD melatonin-treated groups were noted [20.59 ± 2.29 (C+M) vs 11.43 ± 2.94 (HFD+M)].

The area at risk was similar for all groups averaging at 45.18 ± 1.137% for C, 48.24 ± 2.7% for HFD, 45.89 ± 1.68% for C+M and 45.37 ± 1.68% for HFD+M. Additionally, fig 3-10 represents functional recovery [aortic output (A), cardiac output (B) and total work (C)] before ischaemia [A(i), B(i) and C (i)] and after reperfusion [A(ii), B(ii), C (ii)]. Evaluation of myocardial performance of hearts from control and HFD rats showed that the diet had no effect on any of the parameters of mechanical function when measured during perfusion under control conditions. Hearts subjected to 35 min regional ischaemia exhibited a profound effect on functional performance during reperfusion. In both control and HFD groups all parameters measured (except for heart rate where no significant changes were observed) were

significantly lower than their corresponding control values ($p<0.001$). Interestingly, no significant differences were seen between the two groups (Table 3-3 A). Similar observations were made when comparing mechanical performance of melatonin-treated control and HFD hearts when perfused under control conditions, before the onset of ischaemia. All parameters of mechanical function were similar as indicated in tables (3-3 A & B). The only difference was a reduction in aortic flow in control melatonin-treated hearts when compared to untreated controls. Hearts from melatonin-treated control and HFD rats showed the characteristic reduction in all parameters of function during reperfusion. However, no statistical difference was observed between any of the parameters in all four groups.

Apart from the percentages recoveries in heart rate and coronary flow in hearts from the HFD group, no significant differences were observed in the percentage recoveries of the other parameters between the four groups, similar to the observations made when the absolute values were compared.

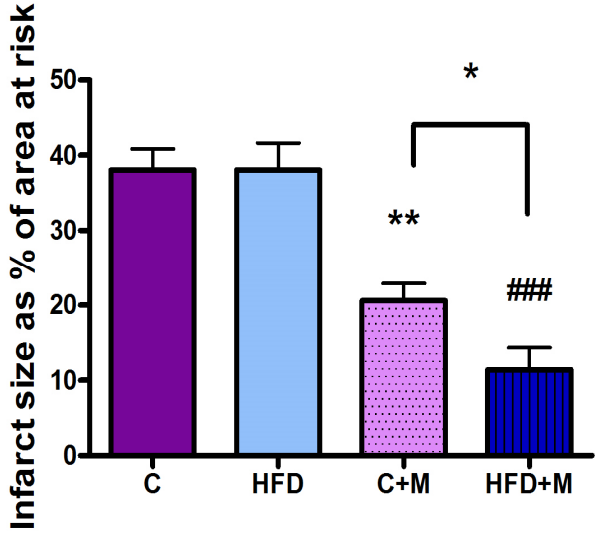

Figure 3-9 Effect of diet and melatonin treatment on myocardial infarct size.

**** p<0.01 vs C and ### p<0.001 vs HFD, *p<0.05 C+M vs HFD+M (n=8/group)**

Table 3-3 (A) Myocardial function before and after 35 min regional ischaemia

Time	Parameters	C	HFD	C+M	HFD+M
Stabilization after 30 minutes	**CF (ml/min)**	**16.56 ± 0.99**	**14.81 ± 1.08**	**12.38 ± 0.51**	**14.81 ± 1.34**
	AO (ml/min)	**42.75 ± 2.23**	**38.50 ± 2.20**	**38.00 ± 1.65**	**37.00 ± 2.54**
	CO (ml/min)	**59.31 ± 2.21**	**53.31 ± 2.70**	**50.38 ± 1.84#**	**51.81 ± 3.36**
	PSP (mmHg)	**96.50 ± 1.35**	**93.00 ± 0.95**	**91.25 ± 1.01**	**90.38 ± 0.91**
	H/R (beats/min)	**248 ± 8**	**247 ± 13.6**	**251 ± 10**	**279± 9.5**
	WT (mW)	**12.58 ± 0.57**	**10.99 ± 0.56**	**10.23 ± 0.47#**	**10.43 ± 0.76**
Reperfusion after 30 minutes	**CF (ml/min)**	**15.94 ± 1.39**	**8.25 ± 2.50**	**13.63 ± 0.91**	**10.81 ± 2.49**
	AO (ml/min)	**5.75 ± 1.62*****	**7.00 ± 4.84*****	**4.00 ± 1.60*****	**4.50 ± 2.80*****
	CO (ml/min)	**21.69 ± 2.84*****	**15.25 ± 6.56*****	**17.63 ± 2.32*****	**15.31 ± 4.34*****
	PSP (mmHg)	**82.38 ± 2.80*****	**47.38 ± 15.35***	**80.88 ± 2.00*****	**61.38 ± 13.47***
	H/R (beats/min)	**244 ± 8.8**	**164 ± 48**	**243± 9.7**	**205 ± 45**
	WT (mW)	**4.07 ± 0.62*****	**2.84 ± 1.33*****	**3.22 ± 0.47*****	**2.83 ± 0.83*****

Coronary flow (CF, ml/min), aortic output (AO, ml/min), cardiac output (CO, ml/min), systolic pressure (PSP, mmHg), heart rate (H/R, beats/min) and work total (WT, mWatts), *p<0.05, *** p<0.001 vs before; #p<0.05 vs control.

Table 3-3 (B) Percentage (%) recovery of hearts subjected to regional ischaemia (R.I)

Time	Parameters	C	HFD	C+M	HFD+M
% recovery of hearts subjected to regional ischaemia	**CF (ml/min)**	96.37 ± 5.673	55.82 ± 18.11	111.1 ± 7.95	82.98 ± 18.97
	AO (ml/min)	13.12 ± 3.934	15.49 ± 10.85	9.754 ± 3.883	12.31 ± 8.074
	CO (ml/min)	36.23 ± 4.348	26.40 ± 10.67	34.33 ± 3.653	31.70 ± 9.091
	PSP (mmHg)	85.25 ± 2.133	51.46 ± 16.61	88.57 ± 1.629	68.07 ± 14.91
	H/R (beats/min)	99.25 ± 5.422	66.35 ± 19.62	97.90 ± 4.687	75.07 ± 17.03
	WT (mW)	31.69 ± 4.254	23.70 ± 10.28	30.74 ± 3.662	29.14 ± 8.720

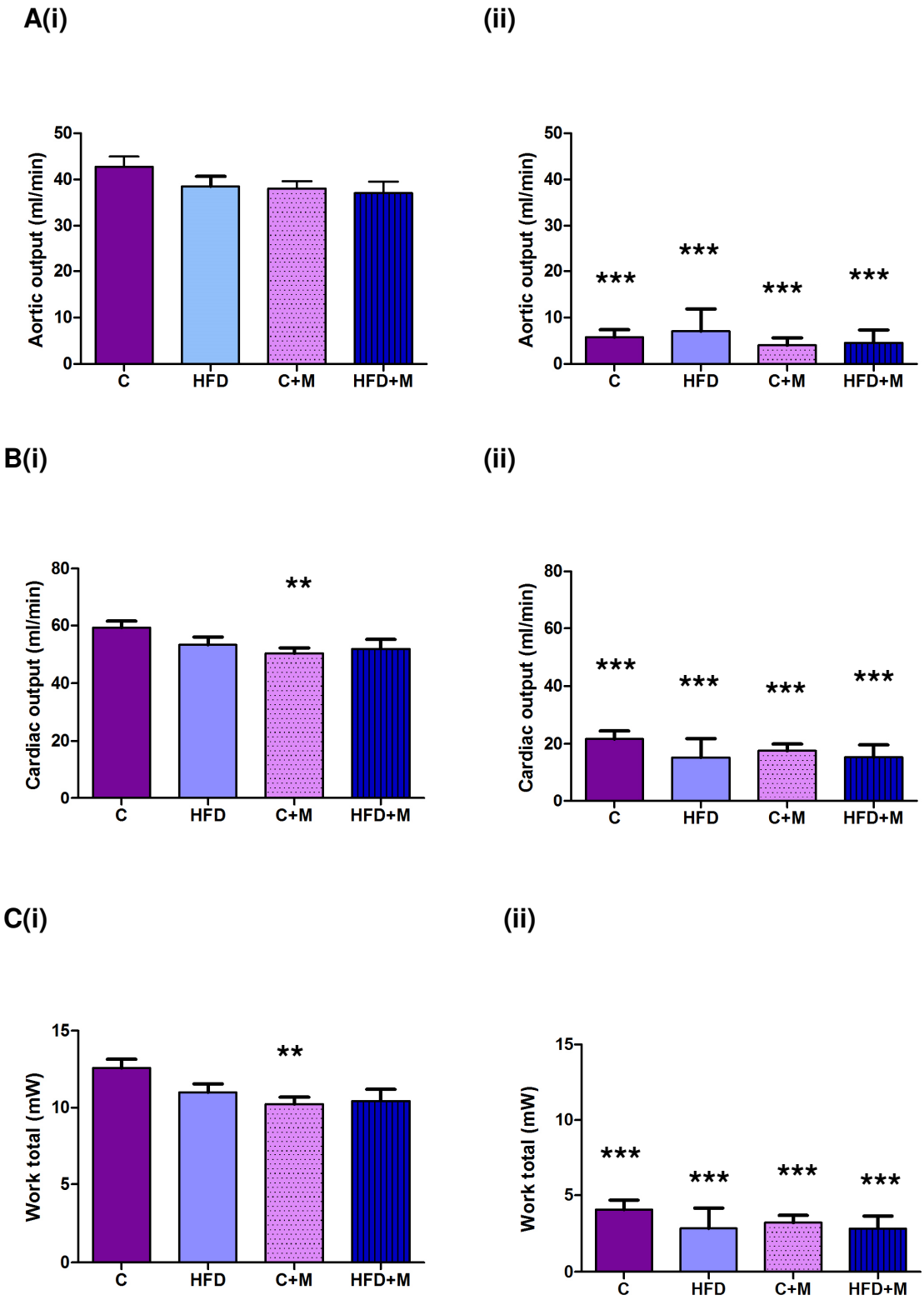

Figure 3-10 Effect of diet and melatonin administration on (A) Cardiac output, (B) Aortic output, (C) Work total (i) before and (ii) after myocardial ischaemia (n=8/group),p<0.05 vs C of the same parameter (aortic output), ***p<0.001 vs respective pre-ischaemic group (eg. Control before (i) vs Control after (ii))**

3.2.2 GLOBAL ISCHAEMIA

To further evaluate the effect of the high fat diet and melatonin treatment on the functional performance of the isolated perfused rat heart as well as its response to ischaemia/reperfusion injury, hearts were subjected to a 40 minute stabilization period (pre-ischaemia), followed by 20 minute global ischaemia and 30 minutes of reperfusion, during which time functional recovery was monitored. For the study of the signal transduction processes and Western blotting, a number of hearts were subjected to the same protocol, but freeze-clamped after 10 min reperfusion only for maximal activation of the kinases.

As was found in the previous study (Table 3-3), the high-fat diet had no significant effect on myocardial function during the control perfusion period before the onset of ischaemia: cardiac output (fig 3-11A), aortic output (fig 3-11B), total work (fig 3-11C), peak systolic pressure and heart rate, were similar in the control and HFD groups (Table 3-4 A & B). Exposure to 20 min global ischaemia, resulted in a significant reduction in aortic output, cardiac output and total work during reperfusion in both control and HFD groups. However, no difference between the response to ischaemia was observed. Melatonin treatment had no effect on the mechanical performance of control and HFD groups before as well as after ischaemia and no differences were observed between the four groups [fig 3-11 A(i), B(i) and C (i)]. However, a significant reduction in performance [fig 3-11 A(ii), B (ii)and C (ii)] during reperfusion was seen in all groups. Table 3-4 B shows the percentage recoveries of hearts during reperfusion after exposure to 20 min global ischaemia. As was the case in regional ischaemia, no significant differences were detected between hearts from control and HFD rats, while melatonin also did have a significant effect.

Time	Parameters	C	HFD	C+M	HFD+M
Stabilization after 40 minutes of perfusion	**CF (ml/min)**	**15.38 ± 1.663**	**14.88 ± 1.59**	**13.13 ± 1.28**	**14.13 ± 1.33**
	AO (ml/min)	**47.5 ± 4.99**	**44 ± 4.69**	**41 ± 2.65**	**40.50 ± 1.5**
	CO (ml/min)	**62.88 ± 6.15**	**58.88 ± 5.93**	**54.13 ± 3.73**	**54.63 ± 2.34**
	PSP (mmHg)	**95 ± 2.12**	**96 ± 2.12**	**92.25 ± 2.02**	**90.75 ± 0.75**
	H/R (b/min)	**273 ± 21**	**258 ± 3.5**	**273 ± 9**	**257 ± 13**
	TW (mW)	**13.34 ± 1.58**	**12.62 ± 1.52**	**11.64 ± 1.36**	**11.01 ± 0.51**
Reperfusion after 30 minutes	**CF (ml/min)**	**12.50 ± 0.84**	**12.25 ± 2.02**	**12.50 ± 1.54**	**9.75 ± 0.43**
	AO (ml/min)	**11 ± 4.34****	**9.5 ± 6.19****	**15.50 ± 5.12****	**10.50 ± 2.36****
	CO (ml/min)	**23.5 ± 4.73****	**21.75 ± 7.79****	**28 ± 6.15***	**20.25 ± 2.24*****
	PSP (mmHg)	**85 ± 1.683**	**82.25 ± 4.48**	**87.25 ± 2.29**	**85.75 ± 2.136**
	H/R (b/min)	**267 ± 25**	**248 ± 9.76**	**246 ± 6.59**	**224 ± 17.24**
	TW (mW)	**4.38 ± 1.05****	**4.18 ± 1.69****	**5.54 ± 1.30***	**3.874 ± 0.49*****

Table 3-4 (A) Myocardial function data: before and after 20 min global ischaemia

Coronary flow (CF, ml/min), aortic output (AO, ml/min), cardiac output (CO, ml/min), systolic pressure (PSP, mmHg), heart rate (H/R, beats/min) and total work (TW, mWatts), *p<0.05, **p<0.005, ***p<0.0001 vs respective pre-ischaemic group. No significant changes were observed in the same parameter for the different groups (eg. C vs HFD for aortic output).

Table 3-4 (B) Percentage (%) recovery of hearts subjected to global ischaemia (G.I)

Time	Parameters	C	HFD	C+M	HFD+M
% recovery of hearts subjected to global ischaemia	**CF (ml/min)**	82.71 ± 4.02	81.85 ± 7.198	94.90 ± 7.647	70.89 ± 7.061
	AO (ml/min)	21.09 ± 7.609	21.33 ± 12.89	36.83 ± 12.05	26.40 ± 6.169
	CO (ml/min)	36.41 ± 4.583	36.65 ± 11.29	50.98 ± 10.68	37.34 ± 4.544
	PSP (mmHg)	89.53 ± 1.558	85.58 ± 3.606	94.60 ± 1.840	94.53 ± 2.775
	H/R (beats/min)	97.66 ± 2.639	96.10 ± 3.333	90.12 ± 1.908	88.26 ± 9.058
	WT (mW)	31.73 ± 4.992	32.50 ± 11.00	47.01 ± 10.76	35.47 ± 4.711

Coronary flow (CF, ml/min), aortic output (AO, ml/min), cardiac output (CO, ml/min), systolic pressure (PSP, mmHg), heart rate (H/R, beats/min) and total work (TW, mWatts).

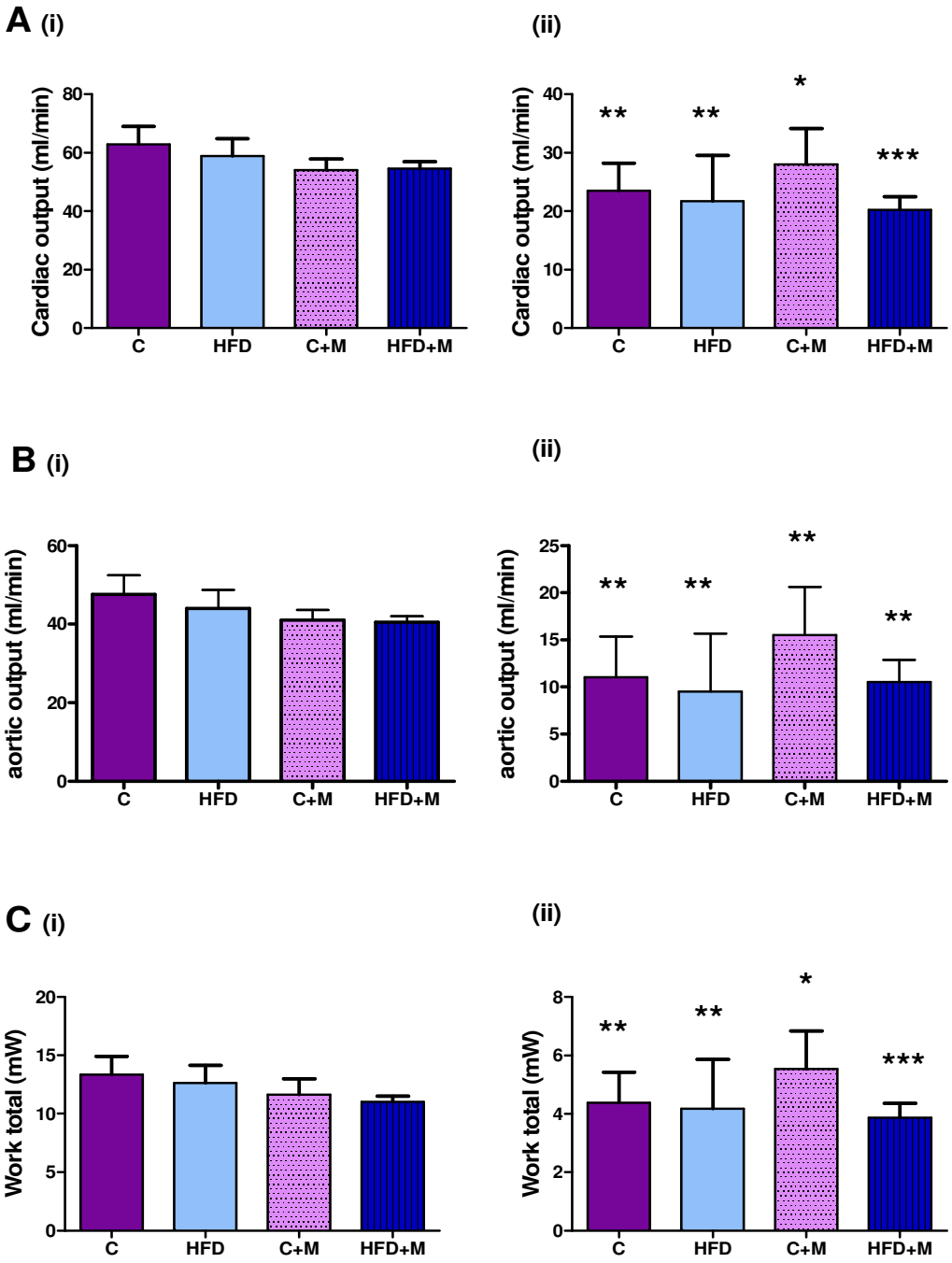

Figure 3-11 Effect of diet and melatonin administration on (A) Cardiac output, (B) Aortic output, (C) Work total (i) before and (ii) after myocardial ischaemia (n=4/group), *p<0.05, **p<0.005, ***p<0.0001 vs respective pre-ischaemic group. No significant changes were observed in the same parameter for the different groups (eg. C vs HFD for aortic output).

3.3 EFFECT OF DIET AND CHRONIC MELATONIN TREATMENT ON INTRACELLULAR SIGNALLING

As previously stated, the hearts that were freeze-clamped after 20 minutes of global ischaemia and 10 minutes of reperfusion were used for the evaluation of intracellular signalling. We investigated the effect of the diet and melatonin treatment on the following signalling pathways: the reperfusion injury salvage kinase (RISK) (PKB/Akt, ERK; baseline and reperfusion) and survivor activating factor enhancement (SAFE) (STAT3) pathways (reperfusion). Other kinases studied after 10 minutes reperfusion include: AMPK, JNK, UCP-3 and PGC1-α.

3.3.1 Reperfusion injury salvage kinases (RISK) pathway

3.3.1.1 Baseline PKB/Akt and ERK 42/44

The standard rat chow (control) and high-fat diet (HFD) had no significant effect on PKB/Akt. The HFD significantly increased ERK p44 expression and activation under baseline conditions while no effect on p42 was noted, compared to the controls. Melatonin treatment also had no effect on PKB/Akt or ERK 42/44 in both control and high-fat fed groups (figs 3-12 & 3-13, respectively).

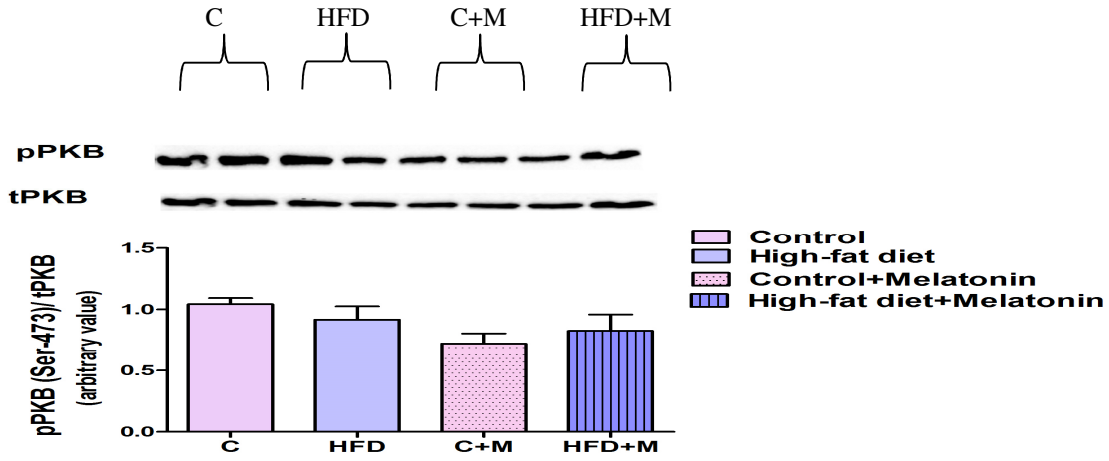

Figure 3-12 Baseline (non-perfused) phosphorylation of PKB/Akt (n=3/group)

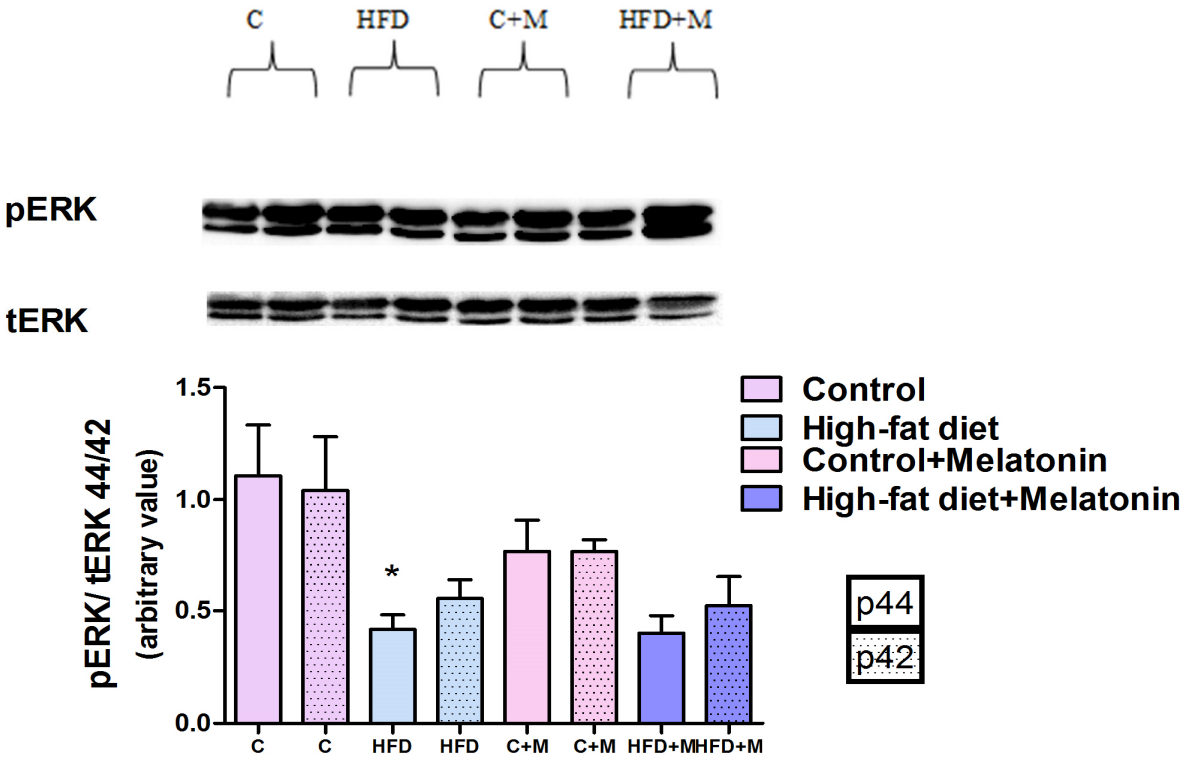

Figure 3-13 Baseline phosphorylation of ERK 44/42, p44 *p<0.05 vs control, (n=4/group)

3.3.2 PKB/Akt and ERK 44/42 following ischaemia/reperfusion

Following 10 minutes of reperfusion, no differences in expression and activation of PKB/Akt were observed between the untreated and melatonin treated control and high-fat diet groups (fig 3-14). Similar observations were made for ERK 42/44 (fig 3-15).

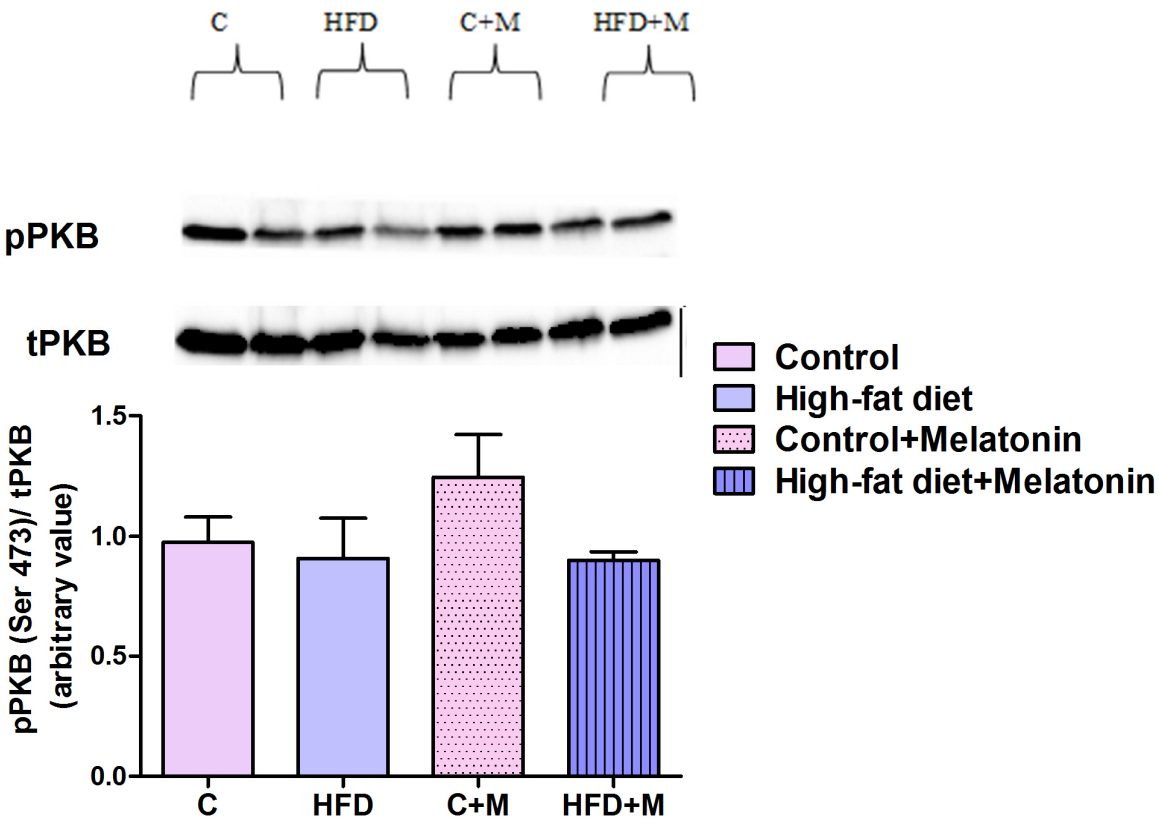

Figure 3-14 PKB/Akt phosphorylation after 10 minutes of reperfusion (n=3/group)

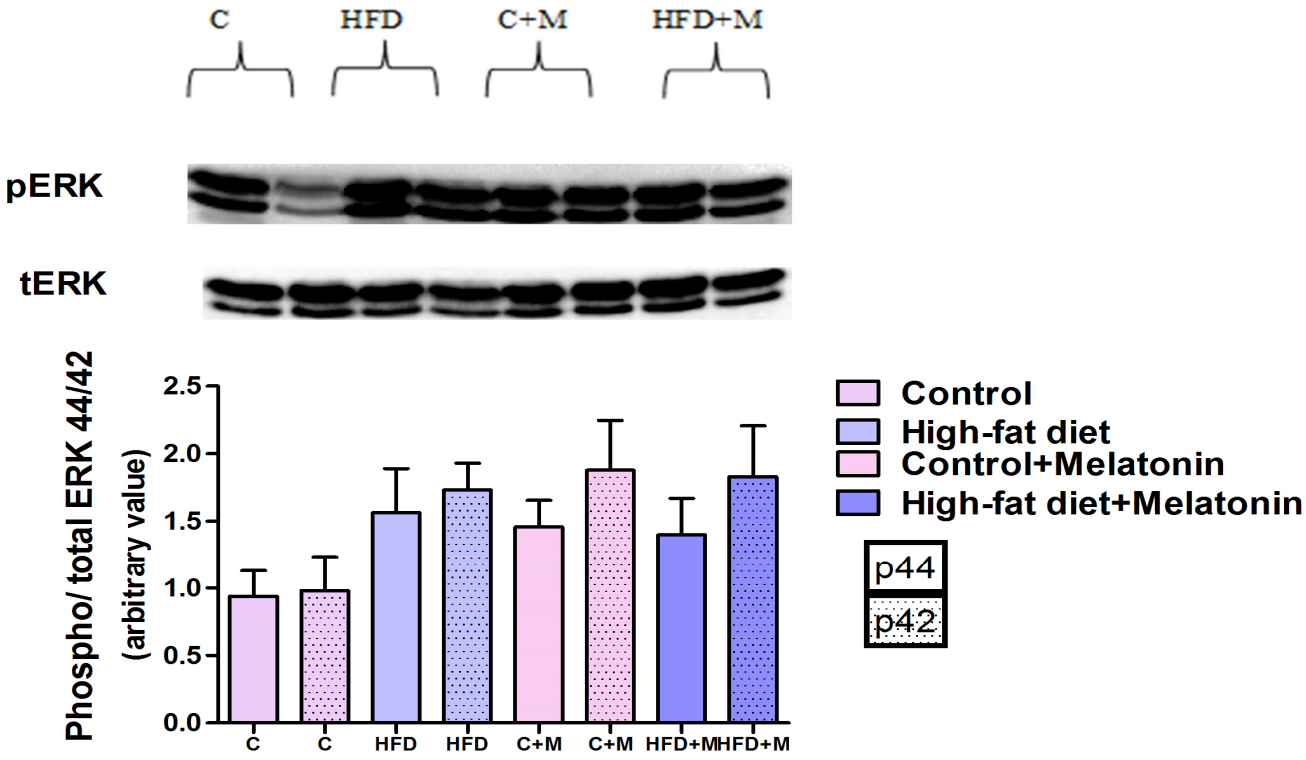

Figure 3-15 ERK 42/44 phosphorylation after 10 minutes of reperfusion (n=3/group)

3.3.3 Survivor activating factor enhancement (SAFE) pathway

3.3.3.1 STAT3 phosphorylation (tyr 705) following 10 minutes of reperfusion

The high-fat diet as well as chronic melatonin treatment did not affect the expression and phosphorylation patterns of both cytosolic and nuclear STAT3 (Tyrosine 705) after 10 min of reperfusion (figs 3-16 & 3-17).

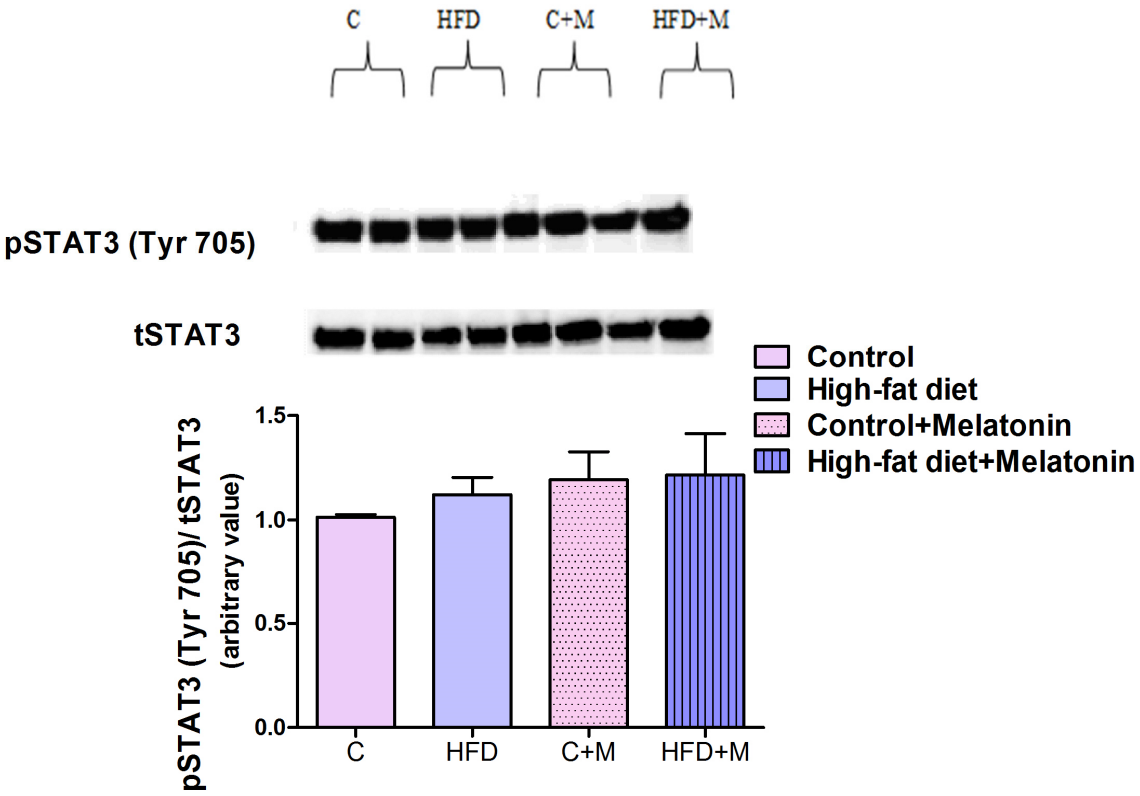

Figure 3-16 Cytosolic STAT-3 phosphorylation (Tyrosine 705) after 10 minutes reperfusion (n=3/group)

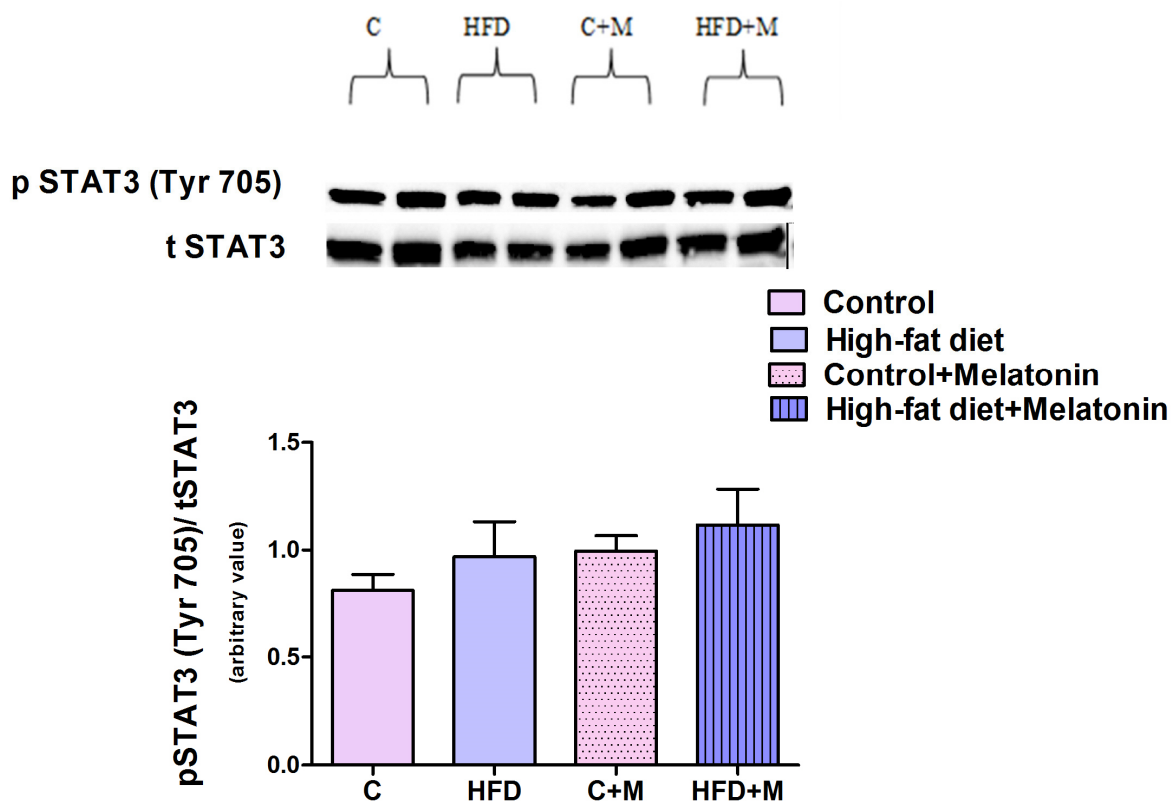

Figure 3-17 Nuclear STAT-3 phosphorylation (Tyr 705) after 10 minutes reperfusion (n=3/group)

3.3.3.2 STAT3 phosphorylation (ser 727) following 10 minutes of reperfusion

Both the high-fat diet and treatment with melatonin had no effect on cytosolic STAT3 expression and phosphorylation (Serine 727) after 10 minutes of reperfusion (figs 3-18). However, melatonin treatment of the HFD rats caused a reduction in the phosphorylation of

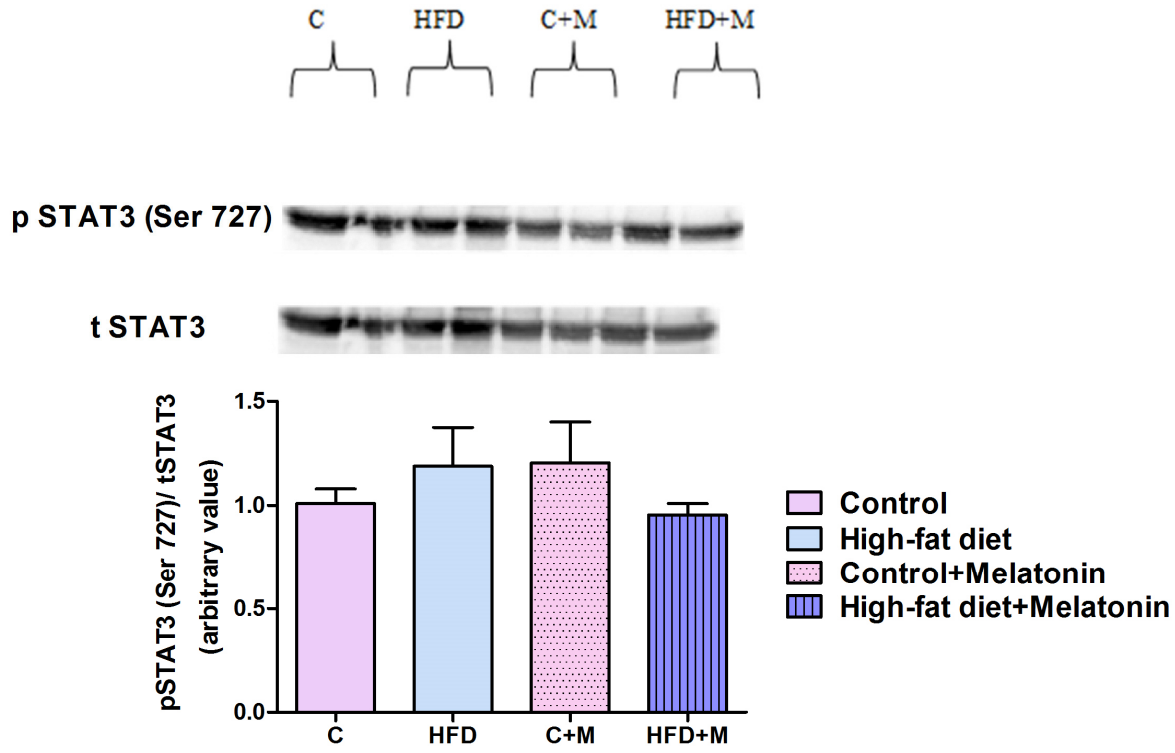

Figure 3-18 Cytosolic STAT-3 phosphorylation (Ser 727) after 10 minutes reperfusion (n=3/group)

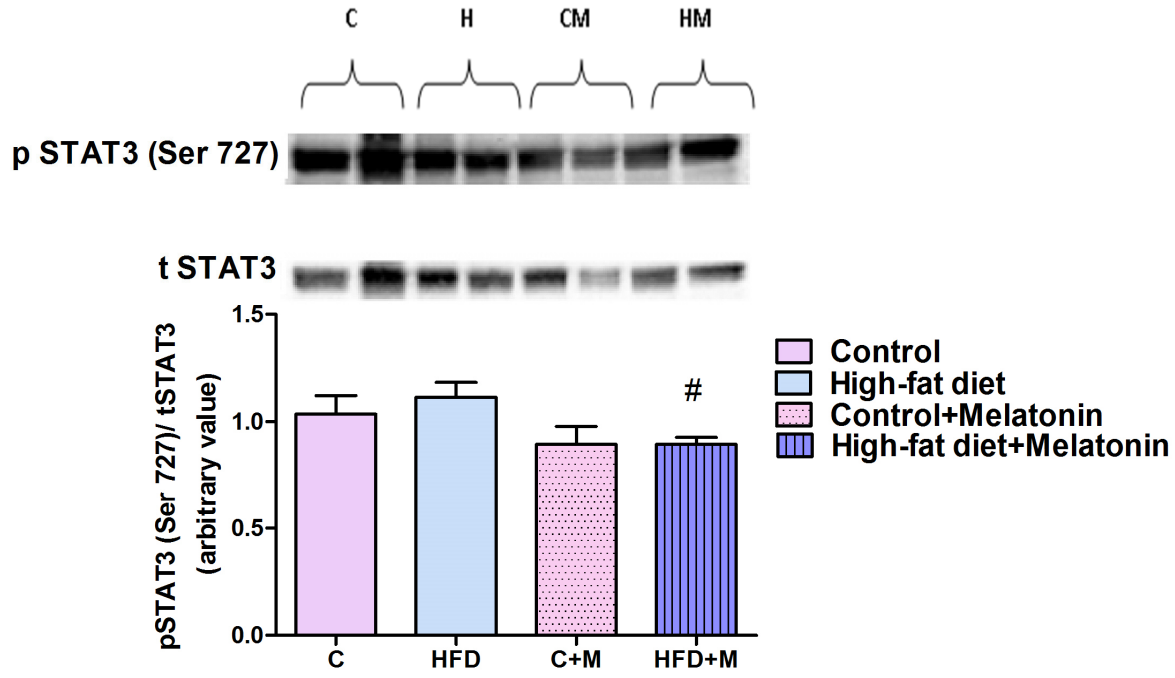

Figure 3-19 Nuclear STAT-3 phosphorylation (Ser 727) after 10 minutes reperfusion, #p<0.05 vs HFD, (n=4/group).

3.4 AMPK following reperfusion

The diet and melatonin treatment had no significant effect on the expression and phosphorylation of AMPK (fig 3-20) and JNK (fig 3-21) after 10 minutes of reperfusion.

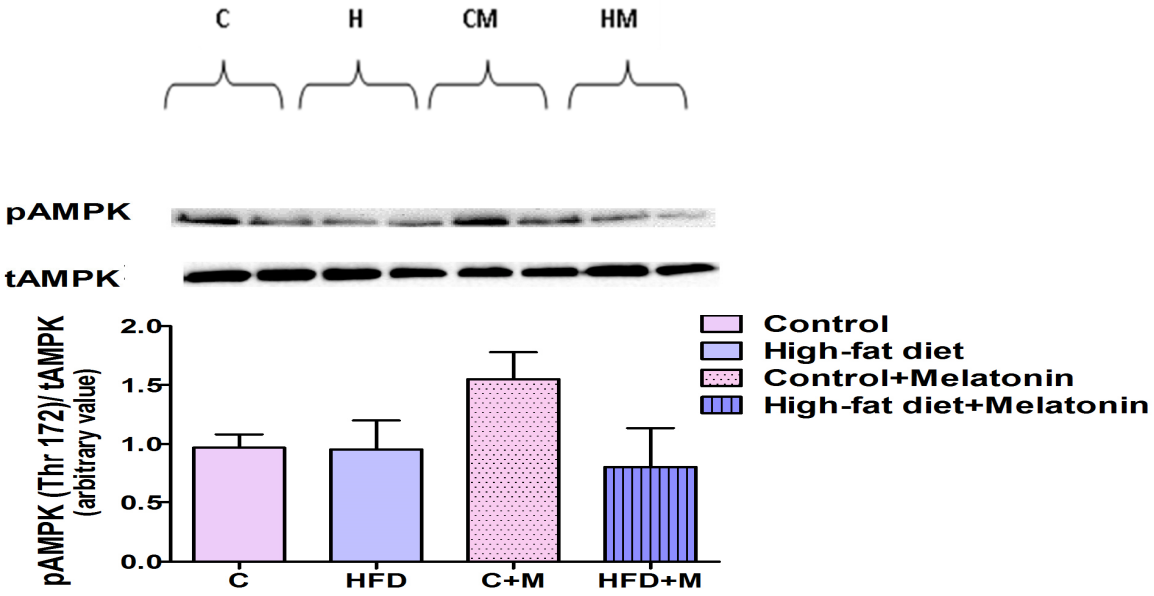

Figure 3-20 AMPK phosphorylation after 10 minutes reperfusion (n=4/group)

3.5 JNK following reperfusion

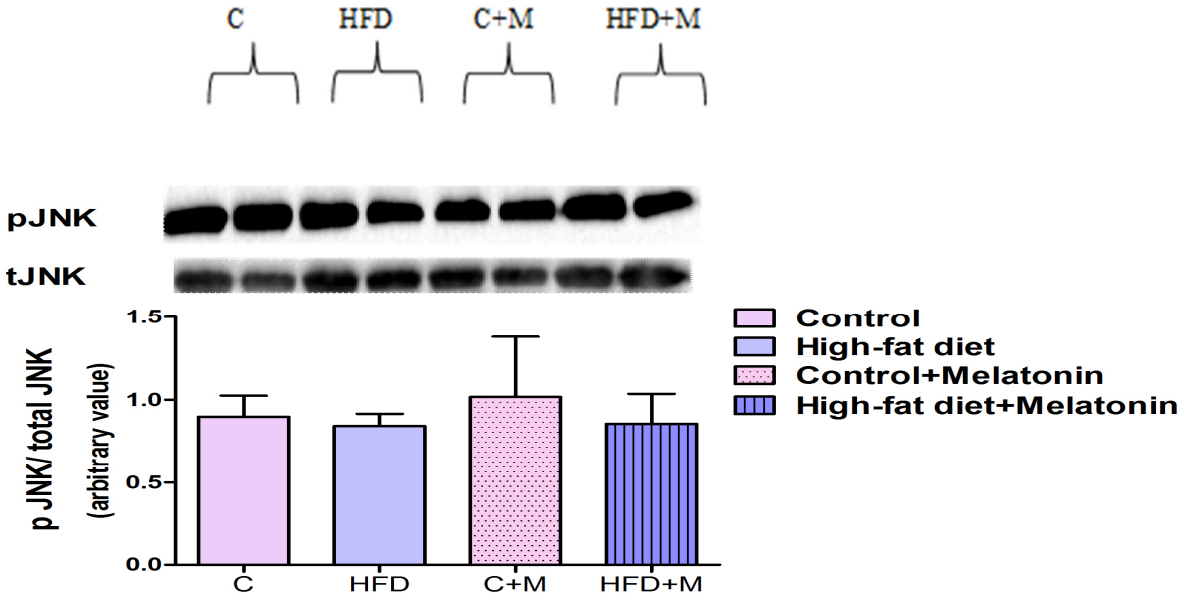

Figure 3-21 JNK phosphorylation after 10 minutes reperfusion (n=3/group)

3.6 UCP3 following reperfusion

The high-fat diet and melatonin treatment had no effect on UCP3 expression after 10 minutes of reperfusion (fig 3-22).

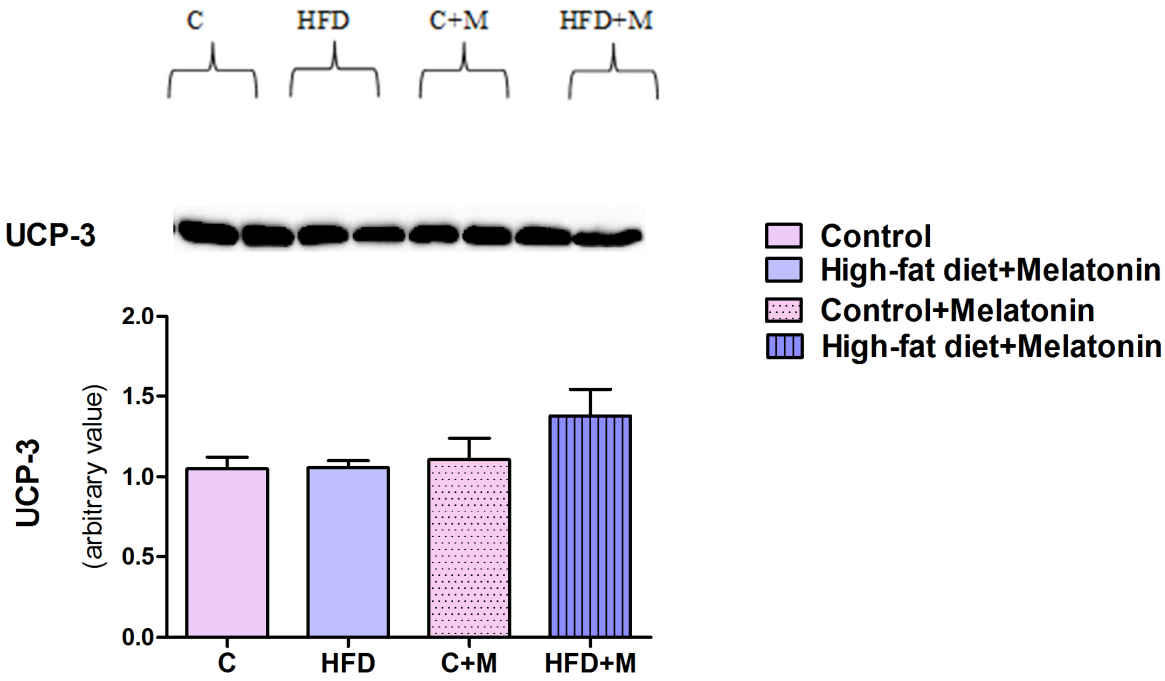

Figure 3-22 UCP-3 expression after 10 minutes reperfusion (n=3/group)

3.7 PGC1-α following reperfusion

The HFD as well as melatonin treatment had no effect on the expression of PGC-1α in the four groups studied.

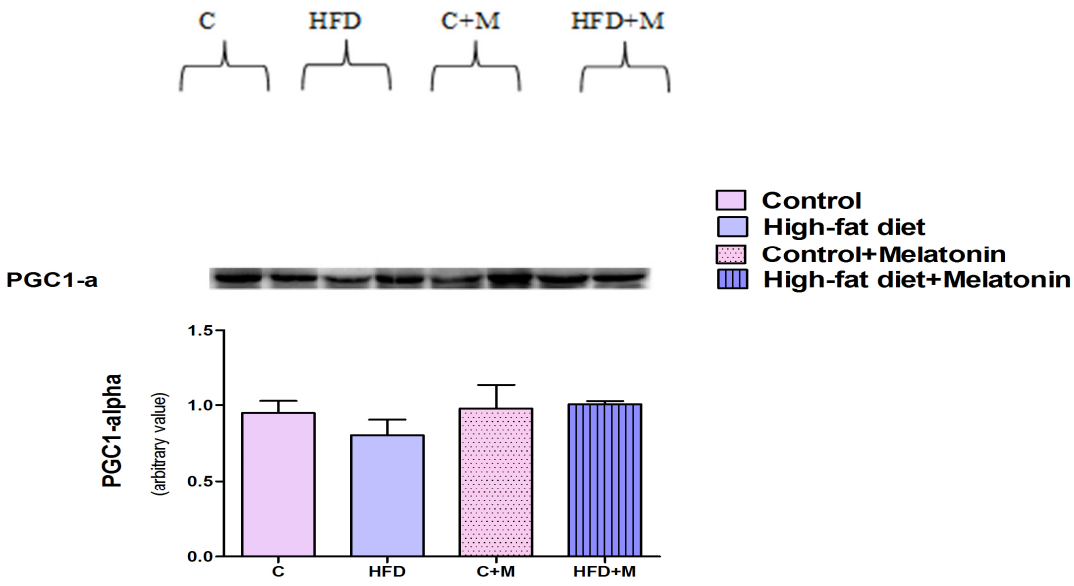

Figure 3-23 PGC1-α phosphorylation after 10 minutes reperfusion, (n=3/group)

CHAPTER 4

DISCUSSION

4.1 SUMMARY OF MOST SIGNIFICANT FINDINGS

The purpose of the present study was twofold: (i) to characterize a new model of obesity, the so-called high fat diet (HFD), induced by a modification of the well-established sucrose diet of Pickavance et al. (1999) and its effects on the response of the myocardium to ischaemia/reperfusion injury and concomitant intracellular signaling (ii) the effects of chronic administration of melatonin in the drinking water on the above parameters in control and HFD-induced obese rats.

The most significant observations were that addition of cooking fat to the sucrose-containing diet of Pickavance et al. (1999) caused significant increases in body weight and visceral adiposity, associated with insulin resistance and the well-established changes in circulating leptin and adiponectin, while having no effect on the response of the heart to ischaemia/reperfusion damage, when compared with their age-matched control counterparts. Chronic consumption of melatonin had profound effects on body weight gain, insulin resistance as well as the myocardial response to ischaemia/reperfusion injury in both control and HFD rats. In contrast, the patterns of intracellular signaling, baseline as well as during reperfusion after ischaemia, showed no significant changes, except for baseline ERK p44 activation of the HFD goup which differed significantly compared to the controls (fig 3-13) and nuclear STAT-3 phosphorylation (Ser 727) (fig 3-19).

4.2 THE EFFECTS OF HFD ON BIOMETRIC AND METABOLIC MARKERS

It is common knowledge that obesity results from an imbalance between energy consumption and expenditure (Sikaris *et al*., 2004; for a review see Fernandez-Sanchez *et al*., 2011). It is also well-established that obesity induced by a HFD is a major contributor to the development of heart disease and diabetes (Birse *et al*., 2010).

Our previous work on the sucrose diet-induced model of obesity (DIO) revealed that these animals are insulin resistant, but not diabetic (Nduhirabandi *et al.,* 2014; Salie *et al* 2014). However the increases in body weight were rather modest and varied significantly. It was therefore decided to develop a new high fat diet by adding cooking fat to the sucrose-containing diet and to evaluate its effects on the biometric and metabolic parameters of rats after a 16 week feeding period. The data obtained in the present study showed that animals fed the HFD gained significantly more body weight (fig 3-1 A) and visceral fat (fig 3-1 B) with a higher adiposity index (Table 3-1) due to energy over-consumption compared to age-matched controls. These findings are consistent with previous studies from our laboratory, using the DIO diet (Du Toit *et al*., 2008; Nduhirabandi *et al*., 2011 & 2014; Salie *et al*., 2014) and other models of diet-induced obesity (Puchalski *et al*., 2003; Hecker *et al*., 2012). However, the HFD over a period of 16 weeks appears to be more effective than the DIO diet in eliciting body weight gain over a period of 16 to 20 weeks (HFD, 30.5% (Table 3-1) vs ± DIO, 13% (Nduhirabandi *et al*., 2014; Salie *et al*., 2014) with little difference between the diets in visceral fat content gain.

Significantly elevated blood glucose, plasma insulin and HOMA index values (fig 3-2 A, B and C, respectively) were observed in the high fat fed animals when compared to the controls. As was also observed in the DIO model, these rats were insulin resistant, but not diabetic: although Du Toit *et al*. (2008) as well as Nduhirabandi *et al*. (2011) reported unchanged blood glucose levels in the 16 week DIO model, elevated blood glucose levels were observed after

16 (Salie *et al*. 2014) and after 20 weeks on the DIO diet (Nduhirabandi *et al*. 2014), in agreement with the observations made in the present study.

In addition to the observed increase in the HOMA index, the presence of insulin resistance induced by both DIO and HFD was confirmed by demonstration of a significant reduction in insulin-stimulated glucose uptake by cardiomyocytes isolated from these hearts (Huisamen *et al*., 2011; Flepisi *et al*., 2013; Nduhirabandi *et al*., submitted for publication).

Although both DIO and HFD induced obesity were not associated with diabetes, it should be kept in mind that these animals were on their respective diets for 16 weeks only and the possibility exists that they may have developed type 2 diabetes should the period of HFD were prolonged. Insulin resistance is considered to be an important link between obesity and type 2 diabetes (Kahn & Flier, 2000) with an increased risk for the development of cardiovascular disease (Wellen & Hotamisligil, 2005; Dunmore & Brown, 2013; Flehmig *et al*., 2014). However, thus far the question whether diet-induced obesity in experimental rats eventually leads to diabetes has not been addressed in the literature.

Several reviews have suggested that increased adiposity leads to adipose tissue dysfunction and altered adipose tissue secretions or adipokine pathways (Bastard *et al*., 2006; for a review see Cao, 2014). Given that adipokines can act as markers or predictors of obesity-related diseases and that the roles of leptin and adiponectin in the setting of obesity are well-established, we investigated the levels of these adipokines in both control and HFD rats. Our results showed that plasma leptin concentrations were significantly higher in the HFD group (fig 3-3 A). Leptin serves as an adiposity signal with levels directly proportional to adipose tissue mass. High leptin levels are known to be associated with obesity, insulin and leptin resistance accompanied by impaired actions of leptin (Lin *et al*., 2000; Yingzhong *et al*., 2006). On the other hand, plasma adiponectin concentrations were significantly lower in the HFD group (fig 3-3 B), in accordance with the reported negative correlation between body mass

index and plasma adiponectin levels (Yamauchi *et al* 2003; Ouchi *et al* 2006). It is also generally accepted that adiponectin acts as a protective factor against the development of insulin resistance and diabetes (Ouchi *et al* 2006). Thus, these findings for leptin and adiponectin in the HFD model are in agreement with the DIO model as reported by Nduhirabandi *et al*. (2011 & 2014), respectively. Similar findings for leptin were observed in other HFD studies by Koch *et al*., (2014); and for adiponectin by Younan *et al*. (2013).

4.2.1 HFD and oxidative stress

The evidence for oxidative stress in the HFD model of obesity was reviewed by Matsuzawa-Nagata *et al*. (2008); and has been associated with mitochondrial dysfunction (Bruce *et al*., 2009). Furthermore, oxidative stress has been implicated in several obesity related pathologies with an increased cardiovascular risk (Grattagliano *et al*., 2008).

The present study showed that the HFD elicited significant plasma lipid abnormalities as reflected by the higher plasma triglyceride (fig 3-4 B; Table 3-2), free fatty acid (fig 3-4 A; Table 3-2) and phospholipid levels (Table 3-2). Similar abnormalities were also seen in the DIO model (Du Toit, 2008; Nduhirabandi *et al.,* 2014). However, no evidence of lipid peroxidation was observed since the TBARS and CD levels were unchanged by the HFD diet (Table 3-2), which is in agreement with findings by Nduhirabandi et al. (2011). While elevated TBARS levels were observed in rats receiving short-tem melatonin administration (Nduhirabandi et al. (2014). It therefore seems that both HFD and DIO diets administered for a period of 16 weeks are not associated with overt lipid peroxidation. Although the DIO rats showed elevated TBARS levels (Nduhirabandi et al. (2014)), the differences disappeared when the data were normalized against total plasma triglyceride and phospholipid values.

4.2.2 High-fat diet, myocardial function and response to ischaemia/reperfusion damage

The negative impact of obesity on myocardial function has been attributed to metabolic derangements such as insulin and leptin resistance, dyslipidemia and impaired glucose homeostasis resulting in ROS accumulation associated with mitochondrial dysfunction and oxidative stress (Chess & Stanley, 2008; Kim *et al*., 2008).

Reports on the effects of obesity and insulin resistance on baseline myocardial function as well as I/R injury in experimental animals are controversial and subject to many confounding factors, for example the period of feeding, the age of the animals, the composition of the diet, to name but a few. In contrast to the reduction in baseline myocardial mechanical function reported in rats receiving the DIO diet for 16 weeks (Du Toit *et al*., 2008; Essop *et al*., 2009; Nduhirabandi *et al*., 2011), in the present study baseline function of hearts from rats fed a HFD did not differ from controls (Tables 3-3 & 3.4). Interestingly, du Toit and coworkers (Donner et al., 2013) could no longer detect any baseline contractile dysfunction when the period of the DIO diet was prolonged to 32 weeks. These workers also reported that a HFD (very similar in composition to ours) when given for 32 weeks had no deleterious effect on basal myocardial function whether evaluated either *in vivo* or *in vitro* (Wensley *et al*., 2013).

Indications are that changes in myocardial metabolic patterns may underlie some of the discrepancies in baseline myocardial function. For example, the mechanical dysfunction reported in rats fed a Western diet with high fat and reduced carbohydrates (Wilson et al., 2007) was attributed to impaired fatty acid oxidation. Indeed, decreased long chain fatty acid oxidation during reperfusion has been shown to impair postischaemic recovery in hearts from rats on high sucrose diet (Harmancey et al., 2013). On the other hand, others have reported that hearts from obese insulin resistant mice showed well-preserved function when perfused with palmitate plus insulin (Mazumder et al., 2004; Buchanan et al., 2005). Our results, showing no difference between the baseline function of the control and obese groups, obtained with glucose

as the only substrate ex vivo, were surprising in view of the plasma lipid abnormalities present in vivo in the diet group after 16 weeks (Tables 3-3,4), which may have predisposed hearts of obese rats toward fatty acid metabolism.

Similarly, no differences in functional recovery during reperfusion (Tables 3-3 & 3.4) or infarct size (fig 3-9A) after exposure to 35 min regional ischaemia were observed between control and HFD groups. In addition, the HFD had no effect on functional parameters during reperfusion following 20 min global ischaemia and values obtained were similar to those of the controls (figs 3-11 A(ii), B(ii) and C (ii), Table 3-4). These results suggest that hearts from obese HFD animals are not more susceptible to ischaemic damage than their corresponding controls (Tables 3-3 & 3-4) which is in agreement with studies by Thim *et al*. (2006) and Salie and coworkers (2014).

As mentioned above, the contribution of obesity and insulin resistance to I/R injury is complex and subject to many confounding factors (Miki *et al*., 2012; Whittington *et al*., 2012). Interpretation of the data is also complicated by reports of improved resistance to I/R in obesity (Donner *et al*., 2013; Salie *et al*., 2014). A reduction in infarct size was also observed in our laboratory after 16 weeks of a DIO diet (Huisamen and Fan, unpublished data). The above observations are in contrast with the results obtained by Du Toit and coworkers (2008) in 16 weeks fed DIO rats namely a significant increase in infarct size accompanied by a reduction in functional recovery during reperfusion after 35 min regional ischaemia. As yet, we do not have an explanation for these contradictory observations, but may be due to the different diet compositions. The lack of protection previously reported by Salie et al (2014) may be due to the difference in sample size (n=8 in the present study while Salie et al (2014) used n=5).

Despite evidence for a higher prevalence of obesity in patients with myocardial ischaemia, a number of recent publications suggested that obesity in humans with ischaemic heart disease is associated with reduced morbidity and mortality, the so-called "obesity paradox" (Chase *et al*., 2014; Clark *et al*., 2014). Our experimental results as well as those of others could indicate that this may also be true for obese animals. Unfortunately the mechanisms involved are unclear at this point in time.

4.3 Intracellular signaling

Expression of myocardial total and phosphorylated kinases was evaluated in baseline conditions as well as at 10 min reperfusion after 20min global ischaemia. The reason for choosing the latter model of ischaemia (rather than regional ischaemia) is that it yields a relatively large amount of tissue, while circumventing the problem of contaminating ischaemic tissue with normal tissue as may occur in regional ischaemia.

Similar baseline total and phosphorylated ERK p44/p42 and PKB were observed in hearts from control, HFD (Salie *et al*., 2014) and DIO (Nduhirabandi *et al*., 2014) rats, while unchanged total and phosphorylated STAT3 in both nuclear and cytosolic fractions were reported in DIO rat hearts when compared to the controls (Nduhirabandi *et al*., 2014). In the present study, similar observations were made: no changes in baseline total and phosphorylated PKB and ERK p42 were observed, however, phosphorylation of ERK p44 was seen in HFD rat hearts after 16 weeks on the diet (figs 3-12, 13). Interestingly, Wensley and coworkers (2013) found that feeding of a high fat obesogenic diet for 32 weeks resulted in a marked reduction in the activity of the RISK pathway. These observations suggest that the feeding

period may affect intracellular signaling pathways. However this possibility remains to be further investigated.

A number of differences in intracellular signaling emerged when activation of the RISK pathway was evaluated in hearts from control, DIO (Nduhirabandi et al., 2014) and HFD (Salie et al., 2014) rats after exposure to 20 min global ischaemia followed by 10 min of reperfusion. Nduhirabandi et al. (2011, 2014) could not detect any significant differences in ERK p44/p42 and PKB as well as GSK3β activation between control and DIO hearts during reperfusion. Although Salie *et al* (2014) found that the activation patterns of ERK p44/p42 and PKB were similar in control and DIO hearts, significantly less activation of both kinases occurred in hearts from the HFD rats. However, in the present study the expression and phosphorylation of ERK p44/p42, PKB and STAT3 during reperfusion after global ischaemia were similar in the HFD and control groups. Unfortunately Wensley and coworkers (2013) did not evaluate the RISK pathway during reperfusion of hearts from rats fed an obesogenic diet for 32 weeks. It is clear that the effects of diet composition on intracellular signaling need to be further investigated.

4.4 UCP3 and PGC-1α

In this study we also evaluated the respective roles of the uncoupling protein UCP3 and PGC-1-α in hearts from control and HFD rats. The uncoupling protein family has been reported to play an important regulatory role in mitochondrial function and free radical (ROS) generation (Mailloux & Harper, 2011). However, not much is known about these proteins in cardiovascular disease and results obtained are controversial (Akhmedov *et al*., 2015). In cardiomyocytes the mitochondria are a major source of reactive oxygen species. It is known that elevated ROS levels, as occurs during reperfusion, stimulate proton leaks mediated by

UCP3, leading subsequently to attenuation of ROS generation. The first evidence for a role for UCP3 in cardioprotection in experimental I/R was published recently (Ozcan *et al*., 2013) using knockout (UCP-/-) mice to demonstrate that ischaemic preconditioning was abolished in such mice. UCP2 and UCP3 have been shown to confer protection against ischaemia-reperfusion injury by others, but their role in the heart is yet to be established (Sack, 2006; Bodyak *et al*., 2007). UCP3 is closely associated with fatty acid metabolism, as its expression is downregulated when fatty acid oxidation is improved (Russell *et al*., 2003) and conversely, its levels are upregulated when the fatty acid supply exceeds oxidation as occurs upon a HFD (Weigle *et al*., 1998).

UCP3 has been shown to be reduced by 50% in skeletal muscle of obese type 2 diabetic patients (Krook *et al*., 1998; Schrauwen *et al*., 2001), while its expression is markedly increased in cardiac tissue of human heart failure patients (Murray *et al*., 2004). It has also recently been shown that UCP3 might affect cardiac energy efficiency under high fat conditions (Boudina *et al*., 2012). However, in the present study no significant differences in UCP3 expression were detected between hearts from control and HFD rats (fig 3-22). It should be kept in mind that these analyses were done at 10 min reperfusion and it is possible that different results could have been obtained if samples were taken at baseline. The role of UCP3 in HFD-induced obesity should be further investigated.

Peroxisome proliferator-activated receptor γ coactivator -1α (PCG-1α) is an inducible integrator of transcriptional circuits that regulate mitochondrial biogenesis and function. In the heart PGC1-α regulates the expression of genes encoding oxidative phosphorylation subunits and FFA metabolism proteins (Huss *et al*., 2004; Lehman *et al*., 2000). Thus this protein plays a central role in the regulation of fuel selection and mitochondrial ATP generating capacity and energy production in the heart. A recent study showed that hypoadiponectonemia, as is present

in obesity, impairs AMPK-PGC1α signaling resulting in dysfunctional mitochondrial biogenesis in neonatal rat ventricular myocytes (Yan *et al*., 2013). Thus the possibility exists that its expression and function may be altered in the presence of high circulating FFA concentrations and reduced plasma adiponectin levels as are present in rats fed a HFD for a period of 16 weeks. The preliminary data obtained in this study indicated that PGC1-alpha expression in hearts from HFD rats was similar to that of control hearts (fig 3-23). However, PGC1-α activation is modulated amongst others by acetylation at multiple acetylation sites which can occur via SIRT1 (Yan *et al* 2013). Thus evaluation of PGC1-α acetylation still needs to be done to allow more complete evaluation of this system in hearts from HFD rats.

4.5 EFFECT OF MELATONIN ON HFD-INDUCED OBESITY

There is increasing evidence for the use of antioxidants in treating obesity and its related pathologies, including cardiovascular disease (Ramos-Marquez *et al*., 2008). The potent free radical scavenging and anti-oxidant properties of melatonin have been discussed in detail in Chapter 1. In the present study we investigated the effects of melatonin on HFD-induced obesity and the heart. Melatonin was given at an oral dosage of 10mg/kg/day, which is higher than in our previous studies (4mg/kg/day, see Nduhirabandi 2011, 2014), but in agreement with several other studies in this regard (Shieh *et al*., 2009; Kitagawa *et al*., 2012).

Daily rhythms have been well implicated in metabolic homeostasis and disruption thereof has been associated with pathologies such as diabetes (Antunes *et al*., 2010; Kennaway *et al*., 2011), cancer and cardiovascular disease (for a review see Dominguez-Rodriguez *et al*., 2010). The pineal gland hormone, melatonin, is tightly controlled by the circadian clock and is released in a rhythmic fashion in blood (Reiter *et al*., 1991; for a review see Dominguez-

Rodriguez *et al*., 2010). Due to its circadian rhythmic secretion, melatonin is known to act as an 'internal synchronizer' with implications for timing of central (hypothalamus) and peripheral tissue (adipose tissue, skeletal muscle) functions thus mediating metabolic cycles necessary to maintain energy homeostasis, amongst many others (for a review see Cipolla-Neto *et al*., 2014).

Melatonin's involvement in metabolic regulation and weight-loss has been well studied (see Rasmussen *et al*., 1999; Wolden-Hanson *et al*., 2000; Prunet-Marcassus *et al*., 2003; Hussein *et al*., 2007; Rios-Lugo *et al*., 2010 and Nduhirabandi *et al*., 2011 & 2014). In a carefully controlled study Nduhirabandi showed that the daily consumption of melatonin had no effect on the food intake of both control and HFD rats (Nduhirabandi et al., 2014) and that this factor was thus not responsible for the characteristic weight loss. Also in the present study the presence of melatonin in the drinking water had no effect on the food intake in both the control and HFD groups. However, other studies did show a reduction in food intake (at 1 mg/kg Hussein *et al*., 2007; 20µg/ml Sanchez-Mateos *et al*., 2007), suggesting that melatonin's effects may perhaps depend on the type of diet, dosage and duration of administration.

Following the 16 week diet (standard rat chow or high-fat) and daily melatonin treatment (10 mg/kg/day) regime, our study revealed that the intake of melatonin prevented body weight gain in both control and HFD animals (fig 3-5A) compared to their untreated counterparts. In addition, the enlarged visceral fat mass due to the HFD was also significantly reduced with chronic melatonin consumption as seen in fig 3-5B. The oral intake of melatonin associated with body weight reduction was also evidenced in the DIO model (Nduhirabandi *et al*., 2011) and in studies by Wolden-Hanson *et al*. (2000) on rats receiving a normal laboratory rodent diet and Prunet-Marcassus *et al*. (2003) using rats on a high fat diet. The melatonin dosage does not appear to be important since a reduction in body weight was reported by using different

dosages of melatonin Rasmussen *et al*. (1999) (30-40 µg/kg), Wolden-Hanson *et al*. (2000) (14-35 µg/kg) and Nduhirabandi *et al*. (2011) (4-6 mg/kg/day).

The link between the pineal gland and body weight regulation was nicely demonstrated in a study employing pinealectomized rats, where the reduction in circulating levels of melatonin was associated with increased body weight gain (Prunet-Marcassus et al., 2003). Upon melatonin supplementation (30mg/kg/day for 3 weeks), the body weight gain was reduced in these rats (Prunet-Marcassus *et al*., 2003) confirming a role for endogenous melatonin in body weight regulation.

Although the anti-obesogenic effects of melatonin are not fully understood, it may act via its effects on brown adipose tissue. It is well-known that brown adipose tissue (BAT) functions to dissipate energy via heat production (Gesta *et al*., 2007; Zhou *et al*., 2014) and melatonin has been implicated in activation of brown adipose tissue and browning of white adipose tissue (Heldmaier *et al*., 1974; for a review see Cipolla-Neto *et al*., 2014), suggesting a thermogenic aspect whereby melatonin may induce weight loss (Jimenez-Aranda *et al*., 2013).

Melatonin is known to exert most of its actions via activation of membrane or nuclear receptors (Rodriguez *et al*., 2004; Wiesenberg 1998). In this regard, it has been proposed that it affects adipose tissue mass via the MT1/MT2 receptors present in adipose tissue, by sympathetic nervous system activation (Bartness *et al*., 2002) and activation of the uncoupling protein (UCP1) present in brown adipose tissue (for a review see Navarro-Alarcon *et al*., 2014). Determination of the effect of melatonin on adipose tissue UCP1 should give more insight into its effects on body weight regulation. In non-obese rats, the body weight reduction could be linked to the anti-ageing properties of the melatonin, maintaining the metabolic/physiologic parameters at youthful levels (Rasmussen *et al*., 1999; Wolden-Hanson *et al*., 2000).

4.5.1 Interplay between melatonin, leptin and insulin on body metabolism

Under physiological conditions, leptin (secreted by adipose tissue) and pancreatic insulin act in the hypothalamic centers as adiposity signals to relay information about peripheral fat stores and consequently control energy metabolism (Spanswick *et al*., 1997 & 2000; Alonso-Vale *et al*., 2005).

Several reports have highlighted a close relationship between melatonin and its capacity to influence insulin secretion as its MT1 and MT2 receptors are expressed in pancreatic islets (Peschke *et al*., 2013) suggesting that melatonin may play a role in the control of insulin production (Mulder *et al*., 2009). The relationship between melatonin and leptin is less well defined, however, it is known that both hormones display a circadian rhythm with peak values at night (Alonso-Vale *et al*., 2005) and since melatonin, via its receptors, act directly on adipocytes, it can possibly modulate leptin levels in this manner (Brydon *et al*., 2001).

Results from the present study show that melatonin significantly reduced plasma leptin levels (fig 3-7A) in the HFD animals compared to their untreated counterparts indicating that melatonin supplementation should have a significant effect on leptin signaling, regulation of body weight and metabolic processes in obesity. Similar observations were made in a HFD study by Rios-Lugo *et al*. (2010). In the present study melatonin significantly lowered plasma insulin levels, (fig 3-6 B) which is in agreement with other models of high-fat diet-induced obesity (Puchalski *et al*.2003; Hussein *et al*., 2007; Nduhirabandi *et al*., 2011 & 2014), control rats (Wolden-Hanson et al 2003) and in a type 2-diabetic model using Goto-Kakizaki rats (Peschke *et al*., 2010).

Interestingly, we found that treatment with exogenous melatonin counteracted the raised blood glucose levels (fig 3-6 A) caused by the HFD. Similar blood glucose lowering effects by melatonin were observed in studies using melatonin at 30 (Prunet-Marcassus *et al*., 2003), 1

(Hussein *et al*., 2007), 2.5, 5, 10 and 20 (Bibak *et al*., 2014) and 10 mg/kg/day (She *et al*., 2009; Bibak et al., 2014). Contrary to these findings, the blood glucose levels were not reduced in the DIO with melatonin administration at 4mg/kg/day (Nduhirabandi *et al*. 2011 & 2014).

Contrasting data exist for the effect of melatonin on glucose homeostasis in healthy animals possibly due to the absence of metabolic disorders, although studies suggest that it may increase glucose tolerance and insulin secretion in such animals (La Fleur *et al*., 2001) while improving insulin sensitivity and thus glucose uptake in obese and middle-aged rats (Larkin *et al*., 2001).

A role for melatonin receptors has also been implicated in this regard. Reports have shown that removal of the MT1 receptor resulted in impaired glucose metabolism and insulin resistance confirming that melatonin is associated with blood glucose regulation (Contreras-Alcantara *et al*., 2010). Sartori & coworkers (2009) further showed that glucose homeostasis was improved in insulin resistant rats when treated with melatonin. Furthermore, a reduction in melatonin levels (due to aging/night shift work leads to insulin resistance associated with chronodisruption and metabolic abnormalities as seen in obesity (for a review see Cipolla-Neto *et al*., 2014). Given that treatment with melatonin has marked beneficial effects on body weight regulation and metabolism (amongst others), it is suggested that melatonin treatment may re-establish timing of metabolic processes and reduce the aforementioned pathologies associated with obesity (for a review see Cipolla-Neto *et al*., 2014).

Finally, it has to be kept in mind that in most experimental studies on the effects of melatonin in experimental animals, melatonin is continuously present in the drinking water and may cause elevated circulating melatonin levels throughout the day, which is in contrast with the cyclic changes present in vivo. However, circulating melatonin levels have not been determined in the present study. Previous studies have shown that melatonin is more effective when given

shortly before dark (lights out) and that rats consumed more than 80% of their water intake during the dark (Wolden-Hanson *et al*., 2000; Prunet-Marcassus *et al* .,2003). It has also been shown, that using such an administration protocol, caused significant elevations in circulating melatonin levels during the night, while daytime levels were similar to those of untreated controls (Wolden-Hanson *et al*., 2000). In the present study melatonin was always given shortly before dark as suggested by Wolden-Hanson and coworkers (2000). It should also be taken into account that time of blood collection during melatonin treatment could affect plasma levels of insulin, glucose, adiponectin, cholesterol etc (Rios-Lugo et al., 2010). In the present study blood samples were collected early in the morning (~9h00).

4.5.2 Effect of melatonin on adiponectin

Diet-induced obesity is known to be associated with low circulating levels of adiponectin as evidenced in this study as well as other studies on obesity by Arita *et al*. (1999) and Nduhirabandi *et al*. (2014). Nduhirabandi and coworkers (2014) reported a significant increase in plasma adiponectin levels after melatonin treatment in the DIO animals. The association between melatonin administration and increases in plasma adiponectin levels was also demonstrated in streptozotocin-induced diabetes (de Oliveira *et al*., 2012) and in high fructose-induced metabolic syndrome in rats (Kitagawa *et al*., 2012). Interestingly, melatonin treatment of patients with non-alcoholic steatohepatitis, reversed the reduction in plasma adiponectin levels observed in these patients (Gonciarz *et al*., 2013). However, melatonin treatment in our study was not associated with increased adiponectin levels in either the control or HFD rats as shown in fig 3-7 B. The reason for this discrepancy is not clear: the dosage as well as period of melatonin treatment was sufficient to elicit changes in several parameters studied, so one has to assume that the high fat diet through a hitherto unknown mechanism, prevented the effects of melatonin on plasma adiponectin.

4.6 EFFECT OF MELATONIN ON THE LIPID PROFILE

The impact of obesity on lipid metabolism has been widely reviewed. Obesity is frequently associated with high plasma triglyceride and free fatty acid (FFA) levels (Dresner *et al*., 1999) which can stimulate insulin secretion. However prolonged exposure to these lipids result in impaired β-cell function associated with an insulin resistant state (Kashyap *et al*., 2003). In fact, it has been stated that the cornerstone affecting insulin insensitivity is the release of FFA. Increased release of FFA is observed in type 2 diabetes and in obesity and is associated with insulin resistance in both conditions (Kahn *et al*., 2006). Hyperlipidemia is also considered as a component of the metabolic syndrome or of a clustering of metabolic pathologies associated with increased cardiovascular and type 2 diabetes risk (Reaven, 2004).

Despite these marked lipid abnormalities in HFD rats, plasma analysis with regard to TBARS and conjugated dienes (CD) showed no evidence of lipid peroxidation caused by the diet (Table 3-2), which could explain why treatment with melatonin had no significant effect in this regard. On the other hand, Nduhirabandi *et al*. (2011 vs 2014) showed that the DIO model also did not affect TBARS and CD levels, while treatment with melatonin (4 mg/kg/day for 16 weeks) reduced the absolute TBARS levels. She *et al* 2009 using a rat model of high-fat/high sucrose diet-induced obesity, also showed that treatment with melatonin (4 mg/kg/day for 8 weeks) reduced oxidative stress as evidenced by lower malondialdehyde and increased superoxide dismutase levels. Melatonin-induced improved antioxidant status has also been reported in obese animals by She *et al*. (2009) and in studies on obese humans (Kozirog *et al*. 2011), possibly due to the hormone's powerful antioxidant properties (Bonnefont-Rousselot & Collin, 2010).

4.7 MELATONIN AND MYOCARDIAL ISCHAEMIA-REPERFUSION INJURY

4.7.1 Obesity and ischaemia-reperfusion injury

It is well-known that obesity and insulin resistance are considered as major risk factors for ischaemic heart disease (Jokinen, 2014) and may increase the susceptibility of the myocardium to ischaemia-reperfusion injury (Du Toit *et al*., 2008), increasing the risk for acute myocardial infarction (Kannel *et al*., 1965).

Despite the fact that numerous reports highlighted the increased incidence of ischaemic heart disease in obese individuals, a growing number of studies have described the opposite, namely a reduced morbidity and mortality in obese individuals, the so-called 'obesity paradox' (Chase *et al*., 2014; Clark *et al*., 2014). The injurious effects of ischaemia-reperfusion on the heart have been studied for decades both *in vivo* and *ex vivo*. It is characterized by, amongst others, cardiac contractile dysfunction, arrhythmias, irreversible myocyte damage and cell death (for a review see Opie *et al*., 2004).

It is well-documented that the damage encountered in the ischaemic-reperfused heart occurs partially as a result of hypoxia during a prolonged ischaemic insult. Although reperfusion is essential for recovery of damaged cells, it also has marked deleterious effects, the so-called reperfusion-injury. Reperfusion is associated with pH normalization, accumulation of free radicals (reactive oxygen/nitrogen species) and intracellular calcium (Maxwell *et al*., 1997; Bolli & Marban, 1999) followed by opening of the mitochondrial permeability transition pore (MPTP) (Hausenloy *et al*., 2009; for a review see Perrelli *et al*., 2011), which may lead to the death of some damaged cells.

Considering the facts that melatonin is associated with weight loss and improved metabolic abnormalities related to obesity, thus reducing the risk for cardiovascular disease, we investigated the effects of chronic administration of the hormone on the outcome of myocardial

ischaemia-reperfusion of hearts from healthy and obese animals (perfused in the absence of exogenous melatonin).

4.7.2 Effect of diet and melatonin treatment on myocardial infarct size and functional recovery

Amongst its many remarkable properties, melatonin, whether acutely or chronically administrated at supraphysiological concentrations, has been reported to have protective effects against the damage caused by ischaemia-reperfusion (for reviews see Reiter & Tan, 2003; Tengattini *et al*., 2008; Lochner et al., 2013).

In the present study, the effect of chronic melatonin administration (16 weeks) on myocardial functional parameters before 35 min coronary artery ligation (ie baseline) showed a significant though small effect on the cardiac output of the treated control animals (fig 3-10B i, Table 3-3). As for the functional parameters during reperfusion after 35 min coronary artery ligation, no significant differences were observed between the four groups. Thus melatonin treatment did not have a beneficial effect on the functional recovery of control or obese rat hearts, despite the significant reduction in infarct size. Similarly, functional recovery during reperfusion after 20 min global ischaemia, did not differ significantly between the groups regardless of melatonin pretreatment (figs 3-11, Table 3-4).

This dissociation between a reduction in infarct size and an improvement in functional recovery confirms previous observations made by Schulz *et al*. (2001), Lochner *et al*. (2003) and Genade *et al*. (2008) and is attributed to myocardial stunning during reperfusion (Lochner *et al*., 2003), which possibly over-rides the effects of melatonin on tissue damage (necrosis). However, in the DIO studies by Nduhirabandi *et al*. (2011 & 2014), melatonin was associated with both increased functional recovery as well as a reduction in infarct size of hearts from obese rats.

This could be attributed to the ability of melatonin to improve calcium homeostasis and /or its free radical scavenging properties (Salie *et al*., 2001).

Despite the lack of effect on functional recovery, chronic treatment with melatonin for 16 weeks reduces myocardial susceptibility to I/R damage in both control and HFD rats as evidenced by the reduction in infarct size in both groups. Measurement of infarct size is widely accepted as a more reliable indicator of tissue damage than functional recovery during reperfusion and is generally accepted to be the "golden standard" in the assessment of tissue damage (Genade *et al*., 2008).

As noted in previous studies, these hearts were perfused in the absence of exogenous melatonin and it must be concluded that chronic melatonin administration caused changes in the heart which persist upon removal and which are still present during the perfusion protocol. Previous studies have also shown that these cardioprotective effects persist for at least 4 days after withdrawal of melatonin (Lochner *et al*., 2006). We have also shown that these long-term protective effects are melatonin receptor dependent, since pretreatment of these animals with luzindole (a melatonin receptor blocker) abolished the beneficial effects (Lochner & Genade, unpublished data).

Besides the long–term effects, acute administration of melatonin to the perfusate of perfused rat hearts also demonstrated the cardioprotective effects of melatonin against I/R injury (Reiter & Tan 2003; Tengattini *et al*., 2008, Lamont *et al*., 2011; Lochner et al., 2013). As expected, Sahna *et al*. (2002) demonstrated that myocardial infarct size was significantly greater in untreated pinealectomized rats than those treated with melatonin.

The exact mechanism through which melatonin confers its cardioprotective effects is not known, although convincing evidence suggest that melatonin's effect in this regard can be attributed to its powerful antioxidant and free radical scavenging properties (Sahna *et al*., 2002;

Reiter & Tan, 2003; Genade *et al*., 2008; Tengattini *et al*., 2008). Acute melatonin administration was shown to protect the isolated ischaemic heart via its anti-adrenergic actions (via nitric oxide (NO)) with guanylyl cyclase and protein kinase C (PKC)) as important role players (Genade *et al*., 2008; Lochner *et al*., 2013). The melatonin receptors are also involved in these actions since the use of a melatonin receptor antagonist, luzindole, blocked the cardioprotective effects of the hormone (Lochner *et al* 2006).

4.8 MELATONIN AND CARDIOPROTECTION: INVOLVEMENT OF THE RISK AND SAFE PATHWAYS

Very little is known about events downstream of melatonin receptor stimulation after chronic treatment with the hormone. It was therefore decided to study of role of the RISK (Reperfusion Injury Salvage Kinase) pathway which entails investigation of the expression as well as activation of protein kinase B (PKB/Akt) and extracellular signal-regulated kinase (ERKp44/p42) in isolated hearts under baseline conditions (non-perfused) as well as during reperfusion after exposure to ischaemia.

We found that the expression of phosphorylated and total PKB/Akt and ERK p44/p42 as well as the phosphorylated/total ratios (fig 3-12,13) of both kinases under baseline conditions were unaffected by the 16 week HFD and melatonin treatment. Similar observations were made by Nduhirabandi *et al* in DIO treated hearts (2011).

Although there were exceptions, many studies have shown an association between activation of these pro-survival kinases and cardioprotection during reperfusion (Hausenloy & Yellon, 2007). In view of this, we sought to investigate the protein changes following 20 minutes of global ischaemia and 10 minutes reperfusion in hearts from these treated and untreated control and HFD animals.

The results showed that the HFD as well as melatonin treatment had no significant effect on the expression and activation of PKB/Akt (fig 3-14) and ERKp44/p42 (fig 3-15) during reperfusion when compared with the control hearts. This was a surprising finding, particularly in view of the marked reduction seen in infarct size of both groups (see fig 3-9B) but consistent in functional recovery after global ischaemia (fig 3-11). Several studies have shown that melatonin-induced cardioprotection is associated with PKB/Akt and ERKP44/P42 activation during early reperfusion. For example, Genade *et al*. (2008) showed that melatonin (50 µM) significantly activated the RISK pathway in non-obese animals, while reducing p38MAPK activation. Similar observations were also made by Lamont *et al*. (2011) using much lower concentrations of melatonin (75 ng/L). Using the DIO model of obesity, Nduhirabandi and coworkers (2011, 2014) confirmed the activation of both PKB/Akt and ERK p44/p42 as well as GSK-3β during reperfusion induced by short-term melatonin treatment. It is possible that the diet, the composition thereof as well as the period of treatment and dosage could affect the outcome of melatonin treatment on the RISK pathway.

It has been reported that under baseline conditions and after reperfusion, melatonin also has a significant effect on STAT3 phosphorylation (Nduhirabandi *et al*., 2014): it was shown that melatonin treatment was associated with increased phosphorylation of cytosolic (Tyr 705) as well nuclear STAT3 (Tyr 705) in both control and DIO groups after 10 minutes reperfusion. Melatonin-induced activation of STAT3 by low circulating concentrations has also been reported by Lamont *et al*. (2011) before ischaemia/reperfusion. However, such changes were not seen in the control and HFD treated hearts in the present study (figs 3-16 to 3-18) after reperfusion, except for nuclear STAT3 (Ser 727), where melatonin had a significant effect on the HFD group (fig 3-19).

Thus it is clear from the results obtained in the present study, that the very significant reduction in infarct size induced by melatonin in control and particularly the HFD group, is not necessarily associated with activation of RISK and SAFE signaling pathways. Although there is a wealth of experimental evidence supporting the causal role of these pathways in cardioprotection, there are also a number of studies where this association could not be demonstrated (see editorial by Heusch, 2009) raising the question whether RISK activation is truly mandatory for cardioprotection. More insight into the mechanisms should be obtained by using appropriate knockout models or pharmacological approaches. A possible contributing factor to the absence of activation of the RISK and SAFE pathways may be the small sample size – in most studies only three hearts were studied. A shorter perfusion period should also be considered, although previous studies in our laboratory showed that maximal activation of both pathways are usually seen at this time point (10 min), while n values of 3 are usually sufficient in the case of Western blotting (Lochner & Salie, unpublished observations).

The absence of activation of the RISK and SAFE pathways by melatonin in the present study now raises the question about the exact mechanism whereby melatonin exerts its cardioprotective effects which were particularly evident in the HFD group. Links have been proposed between the RISK and SAFE pathways and the mitochondrial permeability transition pore as final effector of cardioprotection. It has been suggested that these pathways are not mutually exclusive but potentially cooperative, converging on the mitochondrial permeability transition pore and inhibiting opening thereof (Boengler *et al*., 2010; Heusch *et al*., 2010). Indeed Petrosillo and coworkers (2009) presented convincing evidence that melatonin induced inhibition of the mitochondrial permeability transition pore, resulting in cardioprotection in control rats. The contribution of this pore to melatonin-induced cardioprotection in obesity remains to be determined.

4.8.1 AMPK

It is well-established that adenosine monophosphate-activated protein kinase (AMPK) is involved in cellular energy metabolism and activated by the increase in tissue AMP/ATP ratio (Gowans *et al*., 2013) as occurs in conditions of oxidative stress (Auciello *et al*., 2014). Given the fact that melatonin is associated with body weight regulation and energy metabolism (for a review see Cipolla-Neto *et al*., 2014), we investigated the effect of melatonin on post-ischaemic AMPK. Our results showed no effect of melatonin on AMPK expression or activation following 10 minutes reperfusion (fig 3-20), consistent with findings by Nduhirabandi *et al*. (2011). Interestingly, melatonin treatment also had no effect on AMPK activation in skeletal muscle both in vivo and in vitro (Teodoro et al 2014), while not affecting AMPK in HepG2 cells (Shieh et al 2009). It was concluded that it is very unlikely that melatonin confers cardioprotection via activation of AMPK during reperfusion.

4.8.2 c-Jun N-terminal kinase (JNK)

ERK, p38MAPK (not assessed in this study) and c-Jun N-terminal kinase (JNK) belong to the family of mitogen-activated protein kinases (MAPKs) that are involved in regulation of apoptosis in cardiomyocytes (Rose *et al*., 2010) and inflammation (Kyriakis *et al*., 1996). It has recently been suggested that activation of the stress kinase JNK is essential for PKB phosphorylation at the onset of reperfusion (Shao *et al*., 2006): activation of JNK phosphorylates PKB on Thr450, demonstrated to be a prerequisite for the phosphorylation of PKB at Thr308 and Ser473 to be fully active. Thus, these observations suggest that JNK activation during early reperfusion could be a prerequisite for cardioprotection.

However, despite the above convincing data (Muniyappa *et al*., 2007; Shao *et al*., 2006; Sato *et al*., 2000), the role of JNK activation in cell survival is not at all clear. For example, it has been reported that pharmacological inhibition of JNK activation during early reperfusion is cardioprotective, indicating that this kinase is pro-apoptotic (Ferrandi *et al*., 2004). JNK has been shown to phosphorylate the 14-3-3 scaffolding proteins, thereby releasing BAX to translocate to the mitochondria where it mediates release of cytochrome C and activates apoptosis (Dhanasekaran *et al*., 2008). However, this kinase is surprisingly under-researched in the phenomenon of ischaemia/reperfusion, particularly in the case of insulin resistant hearts. From our results no significant JNK activation in the presence of melatonin in both control and HFD groups (fig 3-21) was observed after a reperfusion period of 10 min, suggesting that this kinase is probably not involved in the cardioprotective actions of melatonin, although ideally sampling should have been done at an earlier reperfusion time-point.

4.8.3 UCP-3 and PGC1-α

In view of the increased circulating FFA concentrations in HFD rats, changes in myocardial UCP3 were expected. However, no difference was observed between the expression of UCP3 in the control and HFD untreated hearts; melatonin also had no effect on the expression of this protein (fig 3-22).

In the interpretation of these results, it should be borne in mind that these hearts were subjected to ischaemia and freeze-clamped during reperfusion in the absence of melatonin. It is possible that more marked effects would have been observed in the presence of melatonin in the perfusate. It is also possible that changes in UCP3 expression occurs at times other than 10 min reperfusion, for example during baseline conditions.

Melatonin's lipophilic properties allow it to act in cellular and subcellular compartments (Vural *et al*., 2001) and to maintain mitochondrial homeostasis by its effects on redox balance (Acuna Castroviejo *et al.*, 2011). The melatonin receptors present in the plasma membrane have been shown to be important in restoring mitochondrial function (Dragicevic *et al*., 2011). Melatonin has been reported to uncouple oxidative phosphorylation by activation of UCP2 and UCP3 thereby reducing ROS formation (Acuna-Castroviejo & Escames, unpublished data), which could contribute to the cardioprotection seen in hearts from the HFD melatonin-treated rats. It has become clear that, in view of melatonin's actions on the mitochondria, further studies are needed to clarify the role of UCP3 in melatonin-induced cardioprotection. Particularly, the effect of melatonin treatment should also be studied under baseline conditions.

Finally, the possibility that PGC1-α was involved in the cardioprotective actions of melatonin in the myocardium was also investigated. It has recently been shown that the beneficial effects of melatonin in improving cellular function and organism health directly or indirectly depend on activation of the sirtuin isoform, silent information regulator (SIRT) 1 (Cuesta S *et al.*, 2013). To identify whether the SIRT1/PGC1-α pathway mediates the observed mitochondrial protective effect of melatonin on hepatocytes, it was shown that blocking SIRT 1 activity reversed the protective effects of melatonin on cadmium mediated mitochondrial injury (Guo P *et al*., 2014). These data suggest that the effects of melatonin in promoting mitochondrial biogenesis and the amelioration of mitochondrial dysfunction are related to upregulation of the SIRT1 / PGC1-α pathway. Since the beneficial effects of melatonin could partially be abolished by luzindole, a melatonin receptor blocker, it was suggested that these receptors are involved in the SIRT 1/PGC1-α pathway. However in the present study, expression of PGC1-α (fig 3-23) was similar in all four groups studied, suggesting that further studies are required to determine its role in melatonin-induced cardioprotection.

CHAPTER 5

5.1 CONCLUSIONS

Obesity has long been associated with insulin resistance and increased cardiovascular risk. In view of this, many researchers have attempted to elucidate the underlying mechanisms and relationships underpinning obesity to facilitate management and potential treatment thereof. The present study demonstrated that the HFD significantly increased body weight and visceral fat mass as well as elevated blood glucose and insulin levels, indicative of insulin resistance. Alterations in the lipid profile and adipokine secretions (leptin, adiponectin) are in agreement with most of the results obtained in previous studies of diet-induced obesity thus the HFD proved to be a useful model of diet-induced obesity with a more pronounced impact on biometric and metabolic changes compared to the DIO model. However, the HFD did not exacerbate the effects of I/R injury, as evidenced by the fact that the infarct sizes and functional recovery during reperfusion were similar to those of rats receiving normal rat chow. Plasma analysis also showed no evidence of lipid peroxidation. Similarly, the HFD had no effect on activation of the RISK and SAFE pathways under baseline conditions as well as after 10min of reperfusion following ischaemia. Similarly no increased activation was seen for AMPK and JNK during reperfusion while levels of UCP3 and PGC1-alpha (α) were not affected during reperfusion either.

Since previous studies have shown melatonin to have beneficial effects in DIO, we investigated the effect of long-term oral administration on the HFD model. Melatonin treatment for 16 weeks was associated with reductions in body weight and visceral fat mass associated with lower blood glucose levels. Melatonin lowered plasma leptin levels in HFD-fed animals while having no effect on the leptin concentration of control animals. Melatonin did not affect plasma adiponectin levels or the lipid profile in both control and HFD rats. Intracellular signaling in

the presence of melatonin was similar in both the control and HFD groups for the RISK and SAFE pathway kinases.

Melatonin successfully reversed several of the changes due to obesity and significantly reduced myocardial infarct size. Unfortunately the experimental approach followed in this study did not shed more light on the mechanisms involved in melatonin-induced cardioprotection in obesity.

The study has a number of shortcomings which should be attended to: (i) the effect of melatonin on intracellular signaling should be evaluated under baseline conditions. Although this was done for PKB/Akt and ERK activation, the expression and activation of AMPK, JNK and STAT3, as well as the expression of UCP3 and PGC-1alpha should also have been studied under these conditions; (ii) the activation of the RISK and SAFE pathways should be studied in a larger series of animals, preferably at more and shorter reperfusion times.

Future studies should also attempt to elucidate the mechanism whereby melatonin treatment protects the hearts from obese animals, with particular focus on the mitochondria permeability transition pore. Since the rats with HFD-induced obesity did not show overt signs of lipid peroxidation, the other effects of melatonin on intracellular signaling should be considered, also the contribution of its profound anti-adrenergic effects.

However the anti-obesogenic and cardioprotective properties of melatonin were very significant indeed. Melatonin is a cheap drug with enormous clinical potential and may be of particular importance in a country such as the RSA with its high incidence of obesity and cardiovascular disease. Further studies, both clinical and experimental, are required to develop its full potential.

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